Antibody correlates of protection for Ebola virus infection: Effects of mutations within the viral glycoprotein on immune escape.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Kimberley Steeds.

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Contents

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Abstract

Ebola virus (EBOV) is an enveloped, single-stranded RNA virus that can cause Ebola virus disease (EVD), a highly lethal illness with up to 90% mortality. It is thought that EVD survivors are protected against subsequent infection with EBOV and that neutralising antibodies to the viral surface glycoprotein (GP) are potential correlates of protection. Serological studies are vital to assess neutralising antibodies targeted to GP; however handling of EBOV is limited to containment level 4 laboratories. Pseudotyped viruses can be used as alternatives to live infectious viruses that require high levels of bio-containment in serological and receptor binding and assays.

Neutralisation capacity can differ among pseudotyped virus platforms. The ability of EBOV GP pseudotyped lentivirus and vesicular stomatitis virus (VSV) systems to measure the neutralising ability of EVD convalescent plasma were compared. The results demonstrate that the sensitivity, specificity and correlation with live EBOV neutralisation are greater for the VSV-based pseudotyped virus system.

The extensive human-to-human transmission of EBOV observed during the 2013-2016 EVD epidemic in West Africa resulted in an accumulation of mutations within the EBOV genome. The current study undertook to assess how these might impact upon immune escape. Specifically, the influence of mutations within the EBOV GP on escape from neutralising antibodies derived from EVD convalescent individuals was assessed.

Site-directed mutagenesis was performed to introduce specific mutations that occurred during the 2013-2016 EVD outbreak into EBOV GP expression plasmids, which were subsequently used to generate a panel of mutant EBOV GP pseudotyped viruses. The effect of these mutations on neutralisation by polyclonal and monoclonal antibody (mAb) samples was assessed. Overall, the results suggest that multiple naturally occurring amino acid changes in EBOV GP do not have a significant impact on polyclonal neutralising antibodies derived from EVD convalescent volunteers or EBOV GP vaccinated individuals. However these mutations can result in reduced neutralisation by certain EBOV GP-specific mAbs. Specifically, a G74R mutation located in the receptor binding domain (RBD) of EBOV GP is associated with partial escape from neutralisation by a human anti-EBOV GP mAb, KZ52.

Sequencing studies and pseudotyped viruses represent an opportunity to study the possible impact of naturally occurring EBOV GP mutations on immune escape, which in turn has the potential to provide a better understanding of EVD vaccine efficacy and correlates of protection against EBOV.

Abbreviations

α	Alpha
β	Beta
Δ, δ	Delta
ε	Epsilon
γ	Gamma
μ	Mu
Ψ	Psi
μg	Microgram
μl	Microlitre
°	Degrees
°C	Degrees Celsius
%	Percentage
x *g*	Times gravity
Ad	Adenovirus
ADCC	Antibody-dependent cellular cytotoxicity
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BDBV	Bundibugyo virus
BGH	Bovine growth hormone
BHK	Baby hamster kidney
BN	Bavarian Nordic
BOMV	Bombali virus
bp	Base pairs
BSA	Bovine serum albumin

CA	California
Cat	Cathepsin
CCHF	Crimean-Congo haemorrhagic fever
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention or Complement-dependent cytotoxicity
cDNA	Complimentary DNA
CI	Confidence interval
CL	Containment level
CLEC	C-type lectin
cm^2	Square centimetre
CMV	Cytomegalovirus
CNS	Central nervous system
CO_2	Carbon dioxide
CoV	Coronavirus
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
DAP12	12-kDa DNAX-activating protein
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
DPBS	Dulbecco's phosphate buffered saline

DRC	Democratic Republic of the Congo
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EBOV	Ebola virus
ECACC	European Culture of Authenticated Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ETC	Ebola treatment centre
EVD	Ebola virus disease
Fab	Fragment antigen binding
FBS	Foetal bovine serum
Fc	Fragment crystallisable
FcR	Fc Receptor
FDA	Food and Drug Administration
FFU	Focus forming units
FRNT	Focus reduction neutralisation titration
g	Gram
GFP	Green fluorescent protein
GMT	Geometric mean titre
GP	Glycoprotein
HAVCR	Hepatitis A virus cellular receptor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HG	Hazard group

HIV	Human immunodeficiency virus
hMGL	Human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin
HR	Heptad repeat
Huh	Human hepatoma
HUVEC	Human umbilical vein endothelial cell
IC	Inhibitory concentration
ICAM	Intercellular adhesion molecule
IFL	Internal fusion loop
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IR	Intergenic region
kb	Kilobase
KCl	Potassium chloride
kDa	Kilo Dalton
l	Litre
L	Large (RNA-dependent RNA polymerase)
LB	Lysogeny broth
LLOV	Lloviu virus
LSECtin	Liver and lymph node sinusoidal endothelial cell C-type lectin
L-SIGN	Liver and lymph node-specific ICAM-3-grabbing non-integrin
LTR	Long tandem repeat
Luc	Luciferase
mAb	Monoclonal antibody
MAC	Membrane attack complex

MARV	Marburg virus
MCP	Monocyte chemoattractant protein
MD	Maryland
MEM	Minimum Essential Media
MERS	Middle East respiratory syndrome
$MgCL_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
ml	Millilitre
MLD	Mucin-like domain
MLV	Murine leukemia virus
mm	Millimetre
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger RNA
MVA	Modified Vaccinia Ankara
NaCl	Sodium chloride
NEAA	Non-Essential Amino Acids
NEB	New England Biolabs
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NHP	Non-human primate
NIBSC	National Institute for Biological Standards and Control
NK	Natural killer
nm	Nanometre

NO	Nitric Oxide
NP	Nucleoprotein
NPC1	Niemann-Pick C1
ORF	Open reading frame
PBS	Phosphate buffered saline
PD	Programmed death
PDB	Protein Data Bank
PEI	Polyethylenimine
PFU	Plaque forming units
PRNT	Plaque reduction neutralisation test
PRR	Pattern recognition receptor
PtdSer	Phosphatidylserine
RAVV	Ravn virus
RBD	Receptor binding domain
RESTV	Reston virus
RLU	Relative luminescence units
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
Rpm	Revolutions per minute
SARS	Severe acute respiratory syndrome
sGP	Secreted GP
SOC	Super Optimal broth with Catabolite repression
ssGP	Small secreted GP
SUDV	Sudan virus
SV40	Simian virus 40
Syk	Spleen tyrosine kinase

TACE	TNFα converting enzyme
TAE	Tris-acetate EDTA
TAFV	Taï Forest virus
TAM	Tyro3, Axl, Mer
$TCID_{50}$	50% tissue culture infectious dose
TCR	T cell receptor
TE	Tris-EDTA
TF	Tissue factor
TIM	T cell immunoglobulin and mucin domain
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand
trVLP	Transcription and replication competent VLP
UK	United Kingdom
USA	United States of America
VA	Virginia
VLP	Virus like particle
VP	Virion protein
VSV	Vesicular stomatitis virus
VSV-G	Vesicular stomatitis virus glycoprotein
WHO	World Health Organisation
WI	Wisconsin

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Chapter 1 Introduction

1.1 History of Ebola virus (EBOV)

1.1.1 Filovirus discovery

The family *Filoviridae*, classified within the order *Mononegavirales* which includes viruses with non-segmented, linear, single-stranded, negative-sense RNA genomes (Pringle *et al.*, 1991), contains three genera; *Marburgvirus*, *Ebolavirus* and *Cuevavirus* (Kuhn *et al.*, 2010). The genus *Marburgvirus* consists of one species, *Marburg marburgvirus*, which includes two viruses: Marburg virus (MARV) and Ravn virus (RAVV). The genus *Ebolavirus* is composed of six known species: *Sudan ebolavirus* (Sudan virus, SUDV), *Zaire ebolavirus* (Ebola virus, EBOV), *Reston ebolavirus* (Reston virus, RESTV), *Taï Forest ebolavirus* (Taï Forest virus, TAFV), *Bundibugyo ebolavirus* (Bundibugyo virus, BDBV) and *Bombali ebolavirus* (Bombali virus, BOMV), and the genus *Cuevavirus* contains one species, *Lloviu cuevavirus* (Lloviu virus, LLOV).

The first recorded outbreak of filovirus disease occurred in 1967, simultaneously in Marburg and Frankfurt, Germany, and Belgrade, former Yugoslavia (now Serbia), when laboratory workers developed severe haemorrhagic fever following contact with blood and tissues of African green monkeys (*Cercopithecus aethiops*) that had been imported from Uganda (Kissling *et al.*, 1968; Martini, 1973). A total of 31 people were infected, seven of which died. This new virus was named Marburg virus, after the city with the most cases (Brauburger *et al.*, 2012).

In 1976, two simultaneous outbreaks of viral haemorrhagic fever occurred in Nzara, southern Sudan (now the Republic of South Sudan) (Report of a WHO/International Study Team, 1978) and Yambuku, northern Zaire (now the Democratic Republic of the Congo, DRC) (Report of an International Commission, 1978), with case fatality rates of 53% and

88%, respectively. Viruses were isolated from patients of both outbreaks, and were shown to be morphologically similar but serologically distinct from MARV (Bowen *et al.*, 1977; Johnson *et al.*, 1977; Pattyn *et al.*, 1977). This virus was named Ebola virus, after a nearby small river in north-western DRC, to ensure that the Yambuku community was not stigmatised (Etymologia: Ebola, 2015; Breman *et al.*, 2016). These two outbreaks were later determined to be caused by two distinct species of Ebola virus, SUDV and EBOV (Cox *et al.*, 1983; Richman *et al.*, 1983). Since its discovery in 1976, EBOV has caused sporadic outbreaks, mainly across Central Africa (Table 1.1), and was responsible for the 2013-2016 Ebola virus disease (EVD) epidemic in West Africa (Baize *et al.*, 2014), which resulted in more than 28,600 cases and over 11,300 deaths. RESTV was identified in 1989 at a quarantine facility in Reston, Virginia (VA), United States of America (USA), in cynomolgus macaques (*Macaca fascicularis*) that had been imported from the Philippines (Jahrling *et al.*, 1990). RESTV causes lethal illness in some non-human primates (NHPs), but is non-pathogenic in humans and seroconversion has been observed in some individuals (Barrette *et al.*, 2009; CDC, 1990; Miranda *et al.*, 1999). In 1994, the forth ebolavirus species was discovered, when TAFV was isolated from an ethnologist who was infected while performing a necropsy on a dead chimpanzee found in the Taï Forest National Park, Ivory Coast (Le Guenno *et al.*, 1995). The patient survived, and thus far, this is the only observed human infection of TAFV (Formenty *et al.*, 1999). BDBV was discovered in 2007 in Uganda when it caused a large outbreak with a case fatality rate of 25% (Towner *et al.*, 2008). A list of human EVD outbreaks is shown in Table 1.1.

Recently, the complete genome of a new ebolavirus, BOMV was detected in samples collected from free-tailed bats in Sierra Leone between March and September 2016 (Goldstein *et al.*, 2018). This virus showed 55-59% nucleotide identity to other ebolaviruses and phylogenetic analyses showed that BOMV is sufficiently distinct to represent the prototype strain of a new species within the *Ebolavirus* genus.

Table 1.1 Outbreaks of human Ebola virus disease (EVD). In chronological order. Grouped according to Ebolavirus species. Data obtained from WHO (World Health Organization, 2018, 2019) and CDC (Centers for Disease Control and Prevention, 2017b).

Year	Country	Reported number of human cases	Reported number (%) of deaths among cases
Ebola virus			
1976	Zaire (DRC)	318	280 (88%)
1977	Zaire	1	1 (100%)
1994	Gabon	52	31 (60%)
1995	DRC (formally Zaire)	315	254 (81%)
1996	Gabon	31	21 (68%)
1996	Gabon	60	45 (75%)
1996	South Africa (ex-Gabon)	1	1 (100%)
2001-2002	Gabon	65	53 (82%)
2001-2002	Republic of the Congo	59	44 (75%)
2003	Republic of the Congo	143	128 (90%)
2003	Republic of the Congo	35	29 (83%)
2005	Republic of the Congo	12	10 (83%)
2007	DRC	264	187 (71%)
2008	DRC	32	14 (44%)
2014-2016	Multiple countries	28,646	11,323 (40%)
2014	DRC	66	49 (74%)
2017	DRC	8	4 (50%)
2018	DRC	54	33 (61%)
2018-present	DRC	991	614 (62%)-ongoing
Sudan virus			
1976	Sudan (South Sudan)	284	151 (53%)
1979	Sudan (South Sudan)	34	22 (65%)
2000	Uganda	425	224 (53%)
2004	Sudan (South Sudan)	17	7 (41%)
2011	Uganda	1	1 (100%)
2012	Uganda	24	17 (71%)
2012	Uganda	7	4 (57%)
Reston virus			
1990	USA	4 (asymptomatic)	0 (0%)
1989-1990	Philippines	3 (asymptomatic)	0 (0%)
2008	Philippines	6 (asymptomatic)	0 (0%)
Taï Forest virus			
1994	Cote d'Ivoire (Ivory Coast)	1	0 (0%)
Bundibugyo virus			
2007	Uganda	149	37 (25%)
2012	DRC	57	29 (51%)

Abbreviations: DRC, Democratic Republic of the Congo; USA, United States of America.

In 2011, a novel ebolavirus-like filovirus, LLOV, was detected in insectivorous bats (*Miniopterus schreibersii*) in Cueva del Lloviu, Spain (Negredo *et al.*, 2011), and has since been reported to be present within the same bat species in Hungary (Kemenesi *et al.*, 2018). LLOV is the first filovirus detected in Europe that was not imported from an endemic area in Africa.

1.1.2 Ecology and transmission

Human EBOV infection is thought to occur through zoonotic events, either from a natural animal reservoir or from an incidental animal host, such as chimpanzees, gorillas or forest duikers (antelope) (Groseth *et al.*, 2007) (Figure 1.1). The natural reservoir for EBOV remains unknown; however several species of bat have been implicated (Leroy *et al.*, 2009; Pourrut *et al.*, 2007). EBOV RNA and EBOV-specific antibodies have been detected in naturally infected wild bats, although live virus isolation has not been achieved (Leroy *et al.*, 2005; Pourrut *et al.*, 2009). Experimental infection of African bats has shown that these animals are capable of supporting EBOV replication and high viremia without showing clinical signs of disease (Swanepoel *et al.*, 1996). NHPs are not thought to be a reservoir host for EBOV, as they are susceptible to EBOV infection and are regarded as end hosts, however they may amplify the virus in nature, and some instances of human infection have been preceded by disease and mortality in wild NHP populations (Lahm *et al.*, 2007; Leroy *et al.*, 2004; Rouquet *et al.*, 2005). It has been hypothesised that the increase in frequency of EVD outbreaks in Africa (Table 1.1) is a result of increased contact between wildlife and humans (Jones *et al.*, 2008). Human encroachment into previously uninhabited areas, due to hunting (Leroy *et al.*, 2009) and deforestation (Rulli *et al.*, 2017) for example, can bring them into contact with EBOV infected animals. Furthermore, climate change and seasonal patterns (Ng and Cowling, 2014; Pinzon *et al.*, 2004; Schmidt *et al.*, 2017) can cause certain animals to modify their geographical and ecological distribution and bring them into closer proximity to human communities. Humans can become infected with EBOV by contact with

bodily fluids or droppings from an infected animal, or by handling contaminated fruit or bushmeat (Leroy *et al.*, 2009).

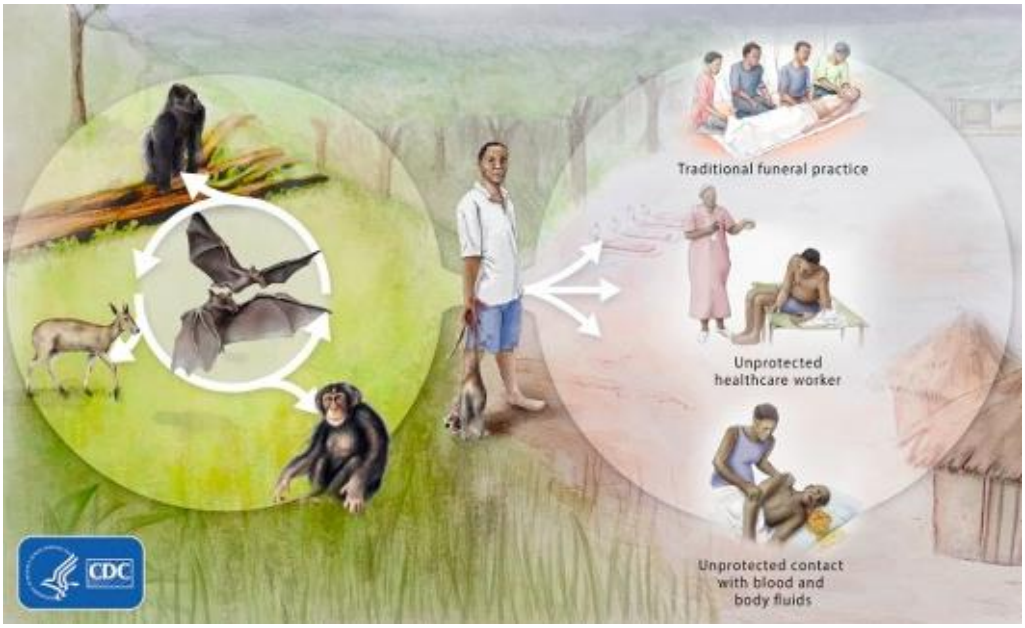


Figure 1.1: Ebola virus disease (EVD) ecology and transmission. EVD is a zoonosis. Bats have been implicated as a reservoir host for Ebola virus (EBOV). Animals, such as apes, monkeys and duikers, or humans can become infected with EBOV through contact with the reservoir host or another infected animal. Human-to-human transmission of EBOV can occur through the contact of bodily fluids from infected individuals or bodies of those who have died from EVD. Figure adapted from CDC (Centers for Disease Control and Prevention, 2017a).

The suspected index case of the 2013-2016 EBOV outbreak in West Africa was believed to be a two year old boy from Meliandou, a small village near Gueckedou in the Republic of Guinea, who died on 6 December 2013, and likely contracted the disease after exposure to an infected bat (Mari Saez *et al.*, 2015). However EBOV, as the causative agent of the epidemic, was not detected and reported until March 2014 (Baize *et al.*, 2014).

Following the initial transmission event from animal to human, the most likely human-to-human spread of EBOV is by direct or close contact of an individual with contaminated bodily fluids or fomites from an infected and symptomatic, or deceased patient of EVD

(Bausch *et al.*, 2007; Dietz *et al.*, 2015; Lawrence *et al.*, 2017) (Figure 1.1). In humans, EBOV has been found, either directly or via detection of viral RNA, in a range of bodily fluids including blood, urine, stool, saliva, sweat and tears, as well as breast milk and semen (Bausch *et al.*, 2007; Rodriguez *et al.*, 1999). Carers at home, family members and healthcare workers are at particular risk of infection (Dowell *et al.*, 1999; Roels *et al.*, 1999). Nosocomial transmission observed during the first recorded EVD outbreak was associated with contaminated needles (Report of an International Commission, 1978). Bodies of individuals that have died from EVD are a source of infectious virus at high levels (de La Vega *et al.*, 2015; Diallo *et al.*, 2016; Lanini *et al.*, 2015), and therefore funerals and burial practices can pose a risk for continued EBOV transmission (Brainard *et al.*, 2016; Tiffany *et al.*, 2017). Sexual transmission of EBOV also presents a risk, even when patients are no longer symptomatic (Mate *et al.*, 2015). Infectious EBOV can be detected in semen of survivors up to 16 months after convalescence (Deen *et al.*, 2017; Diallo *et al.*, 2016; Sissoko *et al.*, 2017a; Sow *et al.*, 2016; Uyeki *et al.*, 2016), and sexual transmission was linked to the initiation of new transmission chains during the 2013-1016 EBOV outbreak (Arias *et al.*, 2016; Blackley *et al.*, 2016). EBOV can persist in immune privileged sites such as the testes, central nervous system (CNS) and eyes, and persistent EBOV infection can lead to outbreak flare-ups or re-ignition, as well as relapses or complications in some affected individuals (Jacobs *et al.*, 2016; Lee and Nishiura, 2017; Varkey *et al.*, 2015).

Previous outbreaks of EBOV have been limited in size and geographical spread, typically involving a small number of people in remote forested areas. The extensive human-to-human transmission documented during the 2013-2016 EBOV epidemic in West Africa was thought to be due to societal factors such as poverty, urban density, population migration patterns, and poor public healthcare infrastructure (Chan, 2014; Spengler *et al.*, 2016; WHO Ebola Response Team, 2014). The first infections of the 2013-2016 EBOV outbreak occurred within a remote rural area of Guinea where no outbreaks of EBOV had previously

been identified. The lack of capacity in Guinea to detect EBOV and delay in identification allowed the virus to spread to bordering Liberia and Sierra Leone. These three countries had no past experience in recognising and managing EBOV outbreaks, were extremely resource-poor, and were recovering from the effects of years of civil instability, which included the collapse of government institutions and struggling basic healthcare infrastructures (Bausch and Schwarz, 2014). Inability to diagnose EVD, slow recognition of suspected cases, and absence of appropriate surveillance early in the outbreak severely hampered interruption of EBOV spread. Furthermore, high population mobility within each country and porous borders into neighbouring countries exacerbated widespread dissemination and transmission of EBOV.

1.1.3 Origin and evolutionary rate

The 2013-2016 EVD epidemic in West Africa was caused by a novel EBOV variant Makona, named after a river at the border of Guinea and Sierra Leone (Kuhn *et al.*, 2014). Molecular clock dating analyses of the sequenced EBOV Makona lineages suggest that the common ancestor be placed at the beginning of 2014 (Carroll *et al.*, 2015; Dudas and Rambaut, 2014; Gire *et al.*, 2014), which is in agreement with epidemiological investigations which placed the first case around late December 2013. These studies also suggest that EBOV Makona diverged from the other EBOV variants around 2004, and that all EBOV variants share a common ancestor around 1975 (Dudas and Rambaut, 2014; Gire *et al.*, 2014; Walsh *et al.*, 2005).

Various genomic sequencing studies performed in Guinea (Carroll *et al.*, 2015; Quick *et al.*, 2016; Simon-Loriere *et al.*, 2015), Sierra Leone (Arias *et al.*, 2016; Park *et al.*, 2015; Tong *et al.*, 2015) and Liberia (Hoenen *et al.*, 2016; Kugelman *et al.*, 2015c; Ladner *et al.*, 2015) during the epidemic identified local viral lineages and transmission patterns within each country, and provided key insights into EBOV evolution and molecular epidemiology (Dudas

et al., 2017; Holmes *et al.*, 2016). It was initially reported that mean evolutionary rate [defined here as the observed rate at which new variants arise and spread in the viral population (Holmes *et al.*, 2016)] of EBOV early in the epidemic was approximately twice as high as that from previous EBOV outbreaks (Gire *et al.*, 2014). However later studies reported lower rate estimates, which were more consistent with rates observed in previous EBOV outbreaks (Biek *et al.*, 2006; Carroll *et al.*, 2013; Walsh *et al.*, 2005). Evolutionary rates in RNA viruses can be dependent on the timescale over which they are measured. Higher rates can often be observed over short timescales, such as within disease outbreaks, because mildly deleterious mutations may not have yet been removed by purifying selection (Duchene *et al.*, 2014). This may explain why some estimates of evolutionary rate measured early in the 2013-2016 EVD epidemic appeared high. A list of evolutionary rates of EBOV reported during the 2013-2016 EVD epidemic in West Africa are shown in Table 1.2.

Table 1.2 Evolutionary rates of Ebola virus (EBOV) reported during the 2013-2016 Ebola virus disease (EVD) epidemic in West Africa.

Study	Evolutionary rate (substitutions per site per year)
(Gire *et al.*, 2014)	1.9×10^{-3}
(Hoenen *et al.*, 2015)	1.3×10^{-3}
(Park *et al.*, 2015)	1.1×10^{-3}
(Kugelman *et al.*, 2015c)	0.9×10^{-3}
(Simon-Loriere *et al.*, 2015)	0.9×10^{-3}
(Carroll *et al.*, 2015)	1.3×10^{-3}
(Tong *et al.*, 2015)	1.2×10^{-3}
(Quick *et al.*, 2016)	1.2×10^{-3}
(Hoenen *et al.*, 2016)	1.4×10^{-3}

1.1.4 Clinical manifestations

Generally, the abrupt onset of EVD follows an incubation period of two to 21 days, averaging four to 10 days, and is characterised by fever, chills, headache, malaise, fatigue and myalgia (muscle pain) (Bwaka *et al.*, 1999; Feldmann and Geisbert, 2011). The initial signs and symptoms of EBOV infection are non-specific and can resemble other more common diseases that are endemic to the area, such as Lassa fever, yellow fever and malaria (Boisen *et al.*, 2015). Therefore initial cases in an epidemic can often be misdiagnosed, leading to spread among family members and healthcare workers (Mahanty and Bray, 2004). Once identified, an EBOV outbreak can usually be controlled by effective patient isolation and barrier nursing practices. The subsequent signs and symptoms of EVD indicate multisystem involvement and include systemic, gastrointestinal (anorexia, nausea, vomiting, abdominal pain, diarrhoea), respiratory (cough), vascular, and neurologic (confusion) manifestations (Feldmann *et al.*, 2013). Haemorrhagic signs develop during the peak of illness, and a characteristic macropapular rash associated with varying degrees of erythema may develop by day five to seven of illness (Bwaka *et al.*, 1999; Feldmann and Geisbert, 2011). In fatal cases, clinical signs develop early during infection and death typically occurs six to 16 days after onset as a result of hypovolemic shock and multiple organ failure (Ksiazek *et al.*, 1999a). Non-fatal cases have fever for several days and improve typically around day six to 11 (Feldmann *et al.*, 2013).

Asymptomatic EBOV infection has been reported in close contacts of symptomatic EVD patients during follow up studies (Leroy *et al.*, 2001; Leroy *et al.*, 2000); however the frequency and impact of asymptomatic infection is unclear (Dean *et al.*, 2016; Glynn *et al.*, 2017; Mbala *et al.*, 2017). A case of possible EBOV transmission from an asymptomatic mother via breastfeeding to her nine month old child, who had died from EVD with an unknown epidemiological link, has been reported (Sissoko *et al.*, 2017b).

1.1.5 Disease pathogenesis

There is limited information regarding the pathogenesis of EVD in humans, and the majority of data has been obtained from animal studies. NHPs (cynomolgus and rhesus macaques) are considered the 'gold standard' animal model for EBOV (Bennett *et al.*, 2017; Geisbert *et al.*, 2015). They can be lethally infected with non-adapted human isolates of EBOV and the resulting pathology closely resembles that described in humans (Baseler *et al.*, 2017; Bente *et al.*, 2009). EBOV can enter the host through mucosal surfaces or breaks or abrasions in the skin. EBOV initially replicates in monocytes, macrophages and dendritic cells (DCs) at the site of infection (Geisbert *et al.*, 2003a; Geisbert *et al.*, 2003c), and is disseminated via these cells to regional lymph nodes through the lymphatics, and to the liver and spleen through the blood (Schnittler and Feldmann, 1998). EBOV infected monocytes, macrophages and DCs then migrate out of the spleen and lymph nodes to other cells and organs, such as the adrenal glands, thereby further disseminating the infection (Bray and Geisbert, 2005; Martines *et al.*, 2015). EBOV can cause extensive necrosis of hepatocytes leading to a decrease in the production of clotting factors and dysregulation of the coagulation cascade (Baseler *et al.*, 2017; Martines *et al.*, 2015), whereas infection and necrosis of adrenocortical cells may negatively affect blood pressure homeostasis, leading to haemorrhage (Geisbert *et al.*, 2003c).

EBOV infection triggers the production of several inflammatory mediators, including interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory proteins (MIPs), tumour necrosis factor alpha (TNFα), tissue factor (TF) and nitric oxide (NO) by macrophages and other cell types (Baize *et al.*, 1999; Baize *et al.*, 2002; Ebihara *et al.*, 2011; Geisbert *et al.*, 2003b; Hensley *et al.*, 2002; Villinger *et al.*, 1999). This causes a significant inflammatory response and lymphoid cell apoptosis (Geisbert *et al.*, 2000; Wauquier *et al.*, 2010), which leads to lymphopenia and suppression of an effective adaptive immune response, as well as vascular leakage and coagulation abnormalities.

Secreted chemokines can recruit more monocytes and macrophages to sites of infection, which act as new targets for viral infection. Inflammatory mediators released from virus infected cells can induce Fas and TNF related apoptosis inducing ligand (TRAIL) pathways (Baize *et al.*, 1999; Bradfute *et al.*, 2010; Gupta *et al.*, 2007; Hensley *et al.*, 2002), which can contribute to lymphocyte apoptosis and lack of an effective adaptive immune response.

EBOV and released inflammatory mediators can activate endothelial cells and induce endothelial cytotoxicity and permeability, leading to vascular leakage (Hensley *et al.*, 2002) and haemorrhagic manifestations. Pro-inflammatory cytokines can also upregulate expression of TF, resulting in over activation of the extrinsic pathway of coagulation and the development of disseminated intravascular coagulation (DIC) (Geisbert *et al.*, 2003b), a condition in which blood clots form throughout small blood vessels. These clots can reduce or block blood flow through the blood vessels, leading to organ damage. Additionally, increased blood concentrations of NO can also induce bystander lymphocyte apoptosis, tissue damage and loss of vascular integrity, which may contribute to hypotension and virus-induced shock (Feldmann and Geisbert, 2011; Sanchez *et al.*, 2004).

Inhibition of the type I interferon (IFN) response (Gupta *et al.*, 2001; Harcourt *et al.*, 1998, 1999), one of the major antiviral host defences, is another important aspect in the pathogenesis of EBOV (Kash *et al.*, 2006).

Whereas macrophages are activated by EBOV infection (Stroher *et al.*, 2001), DC maturation and function are impaired. EBOV infected DCs fail to secrete pro-inflammatory cytokines and do not upregulate expression of co-stimulatory molecules, leading to impairment in antigen presentation to T cells and induction of an adaptive immune response (Bosio *et al.*, 2003; Mahanty *et al.*, 2003). EBOV infected DCs may also promote T cell apoptosis via induction of Fas and TRAIL pathways (Bradfute *et al.*, 2010).

EBOV infection is associated with severe lymphopenia; however lymphocytes themselves are not infected by EBOV (Geisbert *et al.*, 2000; Iampietro *et al.*, 2017). EBOV has been shown to bind to and activate CD4+ T cells. Interaction of EBOV GP with toll-like receptor 4 (TLR4) on T cells triggers T cell death (Iampietro *et al.*, 2017), whereas interaction of viral membrane-associated phosphatidylserine (PtdSer) with T cell immunoglobulin (Ig) and mucin domain 1 (TIM-1 [also known as Hepatitis A virus cellular receptor 1 (HAVCR1)] on T cells induces cytokine production (Younan *et al.*, 2017). Additionally, interaction of EBOV GP with TLR4 on monocytes stimulates differentiation, which results in an increased susceptibility to EBOV infection (Iampietro *et al.*, 2017).

Therefore, EBOV is able to disrupt both the host innate and acquired immune responses, leading to uncontrolled viral replication and dissemination, and a strong and potentially deleterious inflammatory reaction that can lead to severe coagulopathy, multiple organ failure and shock (Figure 1.2).

Higher levels of viremia are often associated with fatal EVD cases compared to survivors, and are a strong indicator of disease outcome (de La Vega *et al.*, 2015; Faye *et al.*, 2015; Ksiazek *et al.*, 1999a; Lanini *et al.*, 2015; Schieffelin *et al.*, 2014).

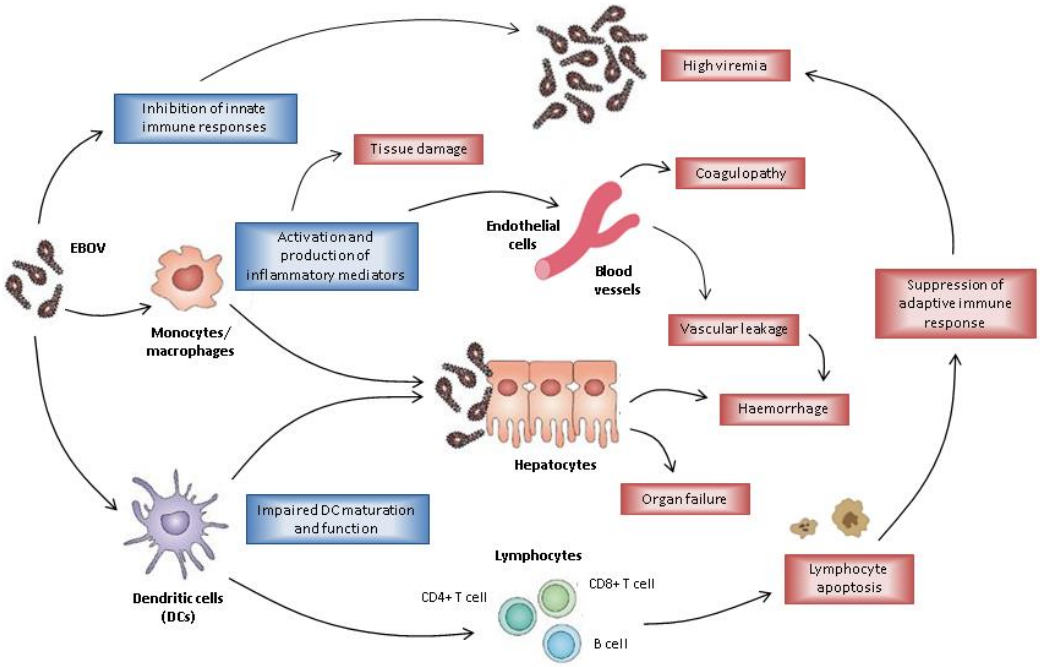


Figure 1.2: Model of Ebola virus (EBOV) pathogenesis. EBOV initially replicates in dendritic cells (DCs), monocytes and macrophages and is disseminated via these cells to various cells and organs, such as the liver. DC function is impaired leading to decreased antigen presentation and suppression of adaptive immunity. Macrophages are activated and produce inflammatory mediators, which can cause lymphocyte apoptosis, vascular leakage, coagulation abnormalities and tissue damage. EBOV also inhibits elements of the innate immune response enabling further viral replication and dissemination. Overall these events result in high viremia, shock and multiple organ failure. Figure adapted from (Mohamadzadeh *et al.*, 2007) and (Leroy *et al.*, 2011).

1.2 Virology

1.2.1 Genome structure and organisation

EBOV has a non-segmented, linear, negative-sense, single-stranded RNA genome of approximately 19 kb in length that encodes seven sequentially arranged open reading frames (ORFs), in the order: 3' (leader), nucleoprotein (NP), virion protein 35 (VP35) (polymerase cofactor), VP40 (major matrix protein), glycoprotein (GP), VP30 (minor nucleoprotein), VP24 (minor matrix protein), RNA-dependent RNA polymerase (L) and 5' (trailer) (Figure 1.3A). Each ORF is flanked by highly conserved transcription start (3' end) and stop (5' end) signals, which either overlap (VP35-VP40, GP-VP30 and VP24-L) or are separated by intergenic regions (IRs) (Sanchez *et al.*, 1993). There are extragenic sequences at the 3' (leader) and 5' (trailer) ends of the genome (Sanchez *et al.*, 1993), which contain promoters for replication and transcription (Volchkov *et al.*, 1999; Weik *et al.*, 2005).

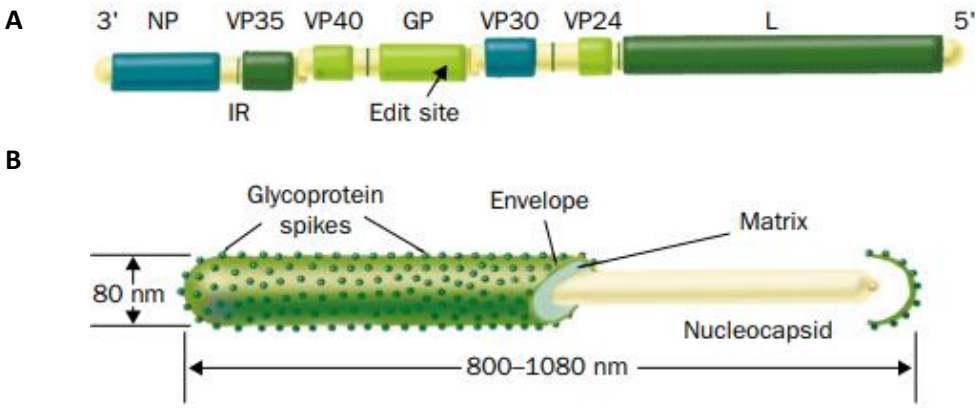


Figure 1.3: A) Organisation of Ebola virus (EBOV) genome and B) Schematic representation of EBOV particle. [Abbreviations: IR, intergenic region; GP, glycoprotein; NP, nucleoprotein; VP, virion protein; L, large protein (RNA-dependent RNA polymerase)]. Figure adapted from (Mahanty and Bray, 2004).

1.2.2 Virion structure and viral proteins

The Filoviridae family name is derived from *filum*, which is Latin for thread, because of the viruses' characteristic filamentous morphology (Kiley *et al.*, 1982). EBOV virions are filamentous in shape, but can also appear branched, or in U-shaped, 6-shaped, or circular conformations (Geisbert and Jahrling, 1995). Virions are approximately 800-1100 nanometres (nm) long and have a uniform diameter of 80 nm (Figure 1.3B). The single stranded RNA genome is encapsulated in a ribonucleoprotein (RNP) complex (the nucleocapsid) consisting of the NP, VP30, VP35 and L (Elliott *et al.*, 1985), which is approximately 50 nm in diameter and runs the length of the particle. The nucleocapsid is surrounded by a matrix layer consisting of VP40 and VP24, which is further surrounded by a lipid bilayer (envelope) derived from the host cell plasma membrane. The viral envelope is studded with membrane-anchored, homotrimeric GP spikes that project approximately 10 nm from the surface (Feldmann *et al.*, 2013).

VP30 is a transcription activator (Muhlberger *et al.*, 1999), and has also been implicated in supressing antiviral immunity through its antagonistic effect on the host cellular RNA interference (RNAi) pathway (Fabozzi *et al.*, 2011). VP35 is an essential cofactor in the viral polymerase complex that affects replication and transcription (Muhlberger *et al.*, 1999) and in addition, has an antagonist effect on the type I IFN pathway by binding virus generated double-stranded RNA (dsRNA) and by interfering with pathway kinases (Basler *et al.*, 2003; Basler *et al.*, 2000; Cardenas *et al.*, 2006; Hartman *et al.*, 2008). The major matrix protein VP40 is critical to the viral assembly and budding processes, as it initiates and drives envelopment of the nucleocapsid by the host plasma membrane (Noda *et al.*, 2002), and also contributes to regulation of genome replication and transcription (Hoenen *et al.*, 2010). VP24 has a minor matrix protein function (Han *et al.*, 2003) and, like VP35, has also been reported to antagonise the type I IFN signalling pathway (Halfmann *et al.*, 2011; Reid *et al.*, 2006). The surface GP is responsible for host cell attachment, fusion and entry

(Martinez *et al.*, 2013; Takada *et al.*, 1997; Wool-Lewis and Bates, 1998) and is also thought to play a role in pathogenesis and immune evasion (Cook and Lee, 2013; Ning *et al.*, 2017).

1.2.3 EBOV GPs

EBOV produces several forms of GP due to transcriptional editing and post-translational modifications (Cook and Lee, 2013; Feldmann *et al.*, 2001; Ning *et al.*, 2017).

Surface GP

The EBOV GP ORF contains an editing site of seven consecutive template uridine nucleotides (Sanchez *et al.*, 1996; Volchkov *et al.*, 1995). During transcription, slippage or stuttering of the viral polymerase at this site results in the introduction of an additional adenine nucleotide in approximately 25% of messenger RNA (mRNA) transcripts (Mehedi *et al.*, 2011). This insertion causes a frame-shift of the coding mRNA, leading to production of full length structural GP. The GP mRNA transcript is initially translated as a single precursor polypeptide, GP_0, that is cleaved by furin in the Golgi into two subunits (Volchkov *et al.*, 1998): a surface subunit, GP_1, and a membrane spanning subunit, GP_2, which remain linked by a single disulphide bond (Jeffers *et al.*, 2002). This heterodimer associates non-covalently with two other $GP_{1,2}$ heterodimers into a 450 kDa trimer to form the GP spike that projects from the virion surface (Lee *et al.*, 2008; Sanchez *et al.*, 1998). EBOV $GP_{1,2}$ is heavily glycosylated with both N- and O-linked glycans (Feldmann *et al.*, 1994).

The GP_1 subunit is responsible for mediating cellular attachment and contains the receptor binding domain (RBD). The GP_2 subunit is responsible for fusion of the viral and host cell membranes and contains a hydrophobic internal fusion loop (IFL), two heptad repeats (HR1 and HR2) and a transmembrane domain (Gallaher, 1996). GP_1 can be divided into three subdomains; base, head and glycan cap (Lee *et al.*, 2008). The base forms a semi-circular surface that clamps the IFL and HR1 region of GP_2 in its pre-fusion conformation. The head is located between the base and glycan cap, and contains residues required for receptor

binding (Brindley *et al.*, 2007; Kuhn *et al.*, 2006). The glycan cap is composed of a continuous polypeptide chain that contains six N-linked glycosylation sites and forms a dome over the GP_1 head (Lee *et al.*, 2008; Lennemann *et al.*, 2014). The GP_1 subunit also contains a highly glycosylated domain at the C-terminus, termed the mucin-like domain (MLD), which contains eight N-linked glycan sites and possibly up to 80 O-linked glycan sites (Jeffers *et al.*, 2002; Lennemann *et al.*, 2014).

The GP forms a three-lobed chalice-like structure, with the bowl of the chalice formed by the three GP_1 subunits and the base formed by the three GP_2 subunits (Figure 1.4A), which anchor the trimer to the viral membrane. The IFL and HR1 region of the GP_2 subunit wrap around the outside of the GP_1 trimer, and are thought to stabilise the structure (Lee *et al.*, 2008). The RBD is recessed in the bowl of the chalice and sits on top of the base domain with residues critical for binding facing up. The glycan cap is positioned on top and blocks access to the RBD. The MLD extends up and away from the viral membrane and globular GP_1 domains.

In addition to host cell attachment and fusion, EBOV surface $GP_{1,2}$ has an important role in the pathogenesis of EVD. Expression of $GP_{1,2}$ *in vitro* causes rounding and detachment of cultured adherent cells, and induces detachment of endothelial cells in blood vessel explants leading to increased vascular permeability (Chan *et al.*, 2000a; Francica *et al.*, 2009; Simmons *et al.*, 2002; Takada *et al.*, 2000; Yang *et al.*, 2000), which may contribute to the clinical manifestations of EVD, such as haemorrhage, shock and multiple organ failure.

A putative immunosuppressive domain (ISD) of EBOV $GP_{1,2}$ may also contribute to viral suppression of cell-mediated immunity, by inducing T cell dysfunction and apoptosis (Volchkov *et al.*, 1992; Yaddanapudi *et al.*, 2006).

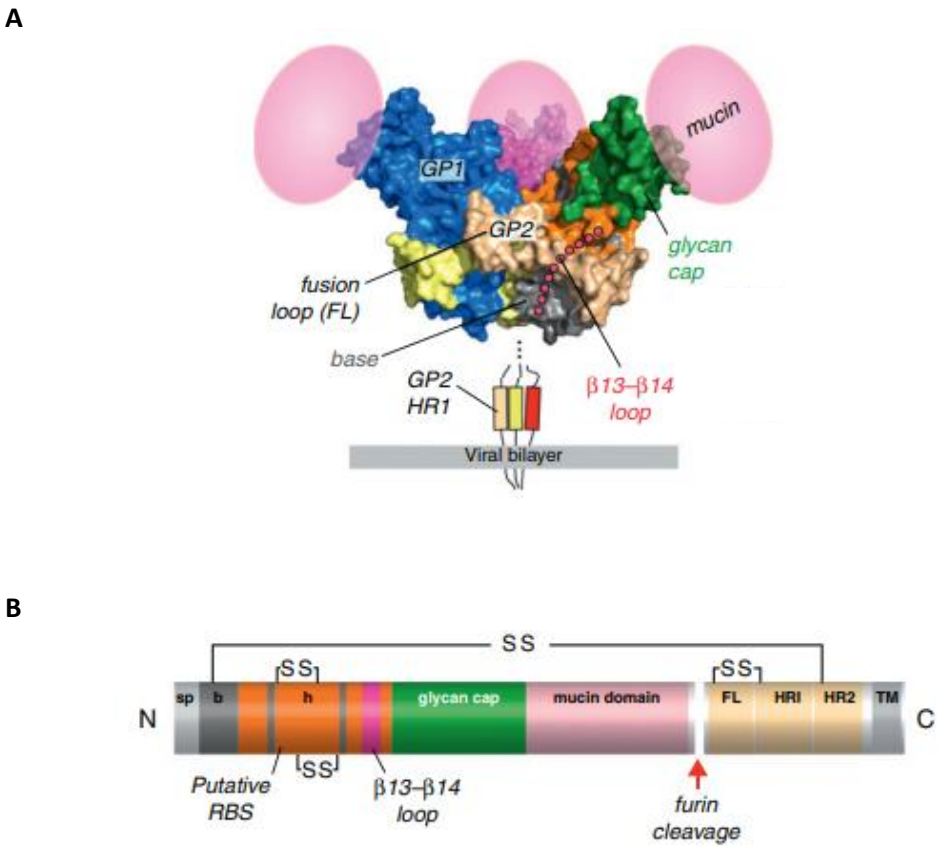


Figure 1.4: A) Schematic representation of Ebola virus (EBOV) surface glycoprotein ($GP_{1,2}$) trimer and B) linear organisation of GP sequence. (Abbreviations: N, amino terminus; SP, signal peptide; b, base; RBS, receptor binding site; h, head; FL, fusion loop; HR, heptad repeat; TM, transmembrane; C, carboxy terminus. SS indicates disulphide bonds). Figure adapted from (Miller and Chandran, 2012).

It has also been suggested that the MLD of $GP_{1,2}$ is involved in activation of the inflammatory response (Martinez *et al.*, 2007; Okumura *et al.*, 2010). $GP_{1,2}$ on virus like particles (VLPs) can activate DCs and macrophages and stimulate activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and elicit the production of inflammatory cytokines via the TLR4 signalling pathway (Wahl-Jensen *et al.*, 2005; Ye *et al.*, 2006). Interaction of EBOV GP with TLR4 on monocytes stimulates differentiation, whereas interaction of EBOV GP with TLR4 on T cells triggers cell death, demonstrating that $GP_{1,2}$ is able to directly subvert the host's immune response by increasing the susceptibility of monocytes to EBOV infection and triggering lymphopenia (Iampietro *et al.*, 2017). Liver and

lymph node sinusoidal endothelial cell C-type lectin (LSECtin; also known as CLEC4G) has also been shown to play an important role in $GP_{1,2}$-mediated inflammatory responses in human DCs, and acts as a pattern recognition receptor (PRR) for EBOV $GP_{1,2}$. Ebola $GP_{1,2}$ can trigger TNFα and IL-6 release by DCs through interaction with LSECtin, which initiates signalling via association with a 12-kDa DNAX-activating protein (DAP12) and induces spleen tyrosine kinase (Syk) activation (Zhao *et al.*, 2016). These effects are involved in the excessive and dysregulated inflammatory reactions elicited by EBOV infection and therefore contribute to EBOV pathogenicity.

EBOV $GP_{1,2}$ also functions in immune evasion. A number of neutralising antibodies have been generated against the MLD; however the MLD is not necessary for EBOV entry (Wilson *et al.*, 2000). The MLD masks antigenic epitopes of the core structure of $GP_{1,2}$, thereby blocking recognition by neutralising antibodies (Lennemann *et al.*, 2014). Furthermore, the MLD act as a 'glycan umbrella' and sterically shields epitopes and functions of host cellular surface proteins important in response to viral pathogens, such as major histocompatibility complex (MHC) class I molecules (Francica *et al.*, 2010; Reynard *et al.*, 2009).

It has been suggested that RNA editing of the GP gene may be an important mechanism utilised by EBOV to regulate $GP_{1,2}$ expression in order to optimise virus production and infectivity, while also evading the host immune response (Mohan *et al.*, 2015; Volchkova *et al.*, 2015).

Shed GP

Proteolytic cleavage of surface $GP_{1,2}$ at the membrane proximal external region by TNFα converting enzyme (TACE) results in release of a soluble trimeric form of GP, termed shed GP, from the surface of infected cells (Dolnik *et al.*, 2004). As shed GP is structurally similar to surface $GP_{1,2}$, it may function as a decoy antigen by binding to and sequestering neutralising antibodies directed against surface $GP_{1,2}$, thereby contributing to viral immune

evasion (Dolnik *et al.*, 2004). Additionally, shed GP has been shown to activate macrophages leading to secretion of inflammatory cytokines, and increases the permeability of human umbilical vein endothelial cell (HUVEC) monolayers (Escudero-Perez *et al.*, 2014). Therefore, shed GP may have an effect in immune dysregulation and damage of endothelial barriers.

Secreted GP (sGP)

The primary product of the EBOV GP ORF is a non-structural protein termed secreted glycoprotein (sGP) (Sanchez *et al.*, 1996; Volchkov *et al.*, 1995). Post-translational proteolytic cleavage of pre-sGP by furin results in the mature sGP and a 40 amino acid, heavily O-glycosylated, non-structural, secreted carboxy (C) terminal fragment termed delta (Δ) peptide (Volchkova *et al.*, 1999). Monomers of sGP are joined in a parallel orientation by two disulphide bonds to form a 110 kDa, mainly N-glycosylated homodimer that is secreted from infected cells (Barrientos *et al.*, 2004; Falzarano *et al.*, 2006; Sanchez *et al.*, 1998). As a result of transcriptional editing, $GP_{1,2}$ and sGP have the same amino (N) terminal 295 amino acids, but have distinct C terminal regions (Volchkov *et al.*, 1995). Therefore, similarly to shed GP, sGP may function as a decoy antigen by adsorbing antibodies raised against $GP_{1,2}$ (Ito *et al.*, 2001; Wilson *et al.*, 2000). Alternatively, sGP may mediate 'antigentic subversion' and actively re-direct the host immune response towards non-neutralising epitopes it shares with surface $GP_{1,2}$ (Mohan *et al.*, 2012).

Recent studies have shown that the EBOV Δ peptide is a viroporin; a small, hydrophobic, virus-encoded protein that is able to permeabilise cellular and viral membranes, and may play a role in the pathogenesis of EVD (He *et al.*, 2017; Pokhrel *et al.*, 2019). It has been suggested that the EBOV Δ peptide may affect the gastrointestinal tract by damaging cells after its release from infected cells, and that this activity may contribute to the severe gastrointestinal illness of EVD patients (Guha *et al.*, 2018; He *et al.*, 2017).

Small secreted GP (ssGP)

Deletion of one, or addition of two adenines at the editing site by the viral polymerase during transcription leads to the expression of another non-structural glycoprotein, termed small secreted glycoprotein (ssGP) (Mehedi *et al.*, 2011). ssGP is secreted as a 110 kDa, N-glycosylated homodimer that is held together by a single disulphide bond. ssGP shares the same N terminal 295 amino acids as and sGP and GP, but again, differs at the C terminus, however its role in viral infection remains unclear (Mehedi *et al.*, 2011).

1.2.4 EBOV life cycle

The first stage of any virus life cycle is attachment to the host cell surface (Figure 1.5). Proteins on the viral envelope interact with specific receptors on the host cell surface to mediate viral entry. Calcium-dependent (C-type) lectins (CLECs), such as dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing non-integrin (DC-SIGN; also known as cluster of differentiation (CD) 209), liver and lymph node-specific ICAM-3-grabbing non-integrin (L-SIGN; also known as CLEC4M), LSECtin and human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin (hMGL), are capable of interacting with N- and O-linked glycans on EBOV GP to facilitate virus entry into a number of different cell types (Alvarez *et al.*, 2002; Lennemann *et al.*, 2014; Powlesland *et al.*, 2008; Simmons *et al.*, 2003; Takada *et al.*, 2004). Members of the Tyro3, Axl, Mer (TAM) family of receptor tyrosine kinases have also been suggested to be involved in EBOV entry (Brindley *et al.*, 2011; Hunt *et al.*, 2011; Shimojima *et al.*, 2007; Shimojima *et al.*, 2006). TIM-1 and TIM-4 have been demonstrated to enhance EBOV entry into cells (Kondratowicz *et al.*, 2011; Moller-Tank *et al.*, 2013; Rhein *et al.*, 2016; Yuan *et al.*, 2015). TAM, TIM-1 and TIM-4 are thought to interact with PtdSer on the surface of the viral envelope. The host cell surface molecules described above are proposed to act as attachment factors, rather than specific entry receptors, to concentrate virions at the cell surface and promote subsequent

receptor attachment (Marzi *et al.*, 2007; Matsuno *et al.*, 2010). The ability of EBOV to utilise multiple attachment factors may contribute to its broad tissue tropism.

Following attachment to the host cell surface, EBOV virions are internalised into endosomes by macropinocytosis and trafficked to late endocytic compartments (Aleksandrowicz *et al.*, 2011; Mingo *et al.*, 2015; Nanbo *et al.*, 2010; Saeed *et al.*, 2010). Once inside the late endosome, the GP must be primed, and then triggered to induce fusion of the viral and host membranes, leading to release of the nucleocapsid into the cytoplasm (Harrison, 2008; White *et al.*, 2008). Priming of GP is mediated by the low pH dependent cysteine proteases cathepsin B (CatB) and cathepsin L (CatL) (Brindley *et al.*, 2007; Chandran *et al.*, 2005; Schornberg *et al.*, 2006), which remove the MLD and glycan cap from GP_1 to produce a 17 to 19 kDa protein (Dube *et al.*, 2009; Hood *et al.*, 2010). Proteolytic cleavage of GP exposes the RBD, enabling it to interact with Niemann-Pick C1 (NPC1) (Carette *et al.*, 2011; Cote *et al.*, 2011; Miller *et al.*, 2012), a cholesterol transporter present in late endosomal membranes. Following binding, NPC1 participates in triggering of the fusion activity of primed GP; however the signal required for fusion triggering remains unknown (Kuroda *et al.*, 2015).

In response to the fusion trigger, conformational changes, and possibly further proteolytic processing of primed GP (Brecher *et al.*, 2012; Mingo *et al.*, 2015; Wong *et al.*, 2010) expose the hydrophobic fusion loop in GP_2 (Gregory *et al.*, 2011), resulting in insertion of hydrophobic residues at the tip of the fusion loop into the endosomal membrane (Gregory *et al.*, 2014). Following insertion, unwinding of the GP_2 trimer causes refolding of the helical regions into an antiparallel, hairpin-like six-helix bundle between the HR 1 and 2 regions, pulling the viral and host membranes into proximity for fusion (Weissenhorn *et al.*, 1998), allowing subsequent release of the nucleocapsid into the cytoplasm.

Once inside the host cell cytoplasm, the negative-sense RNA genome undergoes transcription and replication. Transcription is initiated by binding of the polymerase complex to a single binding site located within the leader region of the negative-sense RNA genome, which is then transcribed into 5'-capped, 3'-polyadenylated monocistronic mRNAs as the polymerase complex recognises conserved start and stop sequences on the template (Muhlberger, 2007). The mRNAs are then translated into proteins. As the concentration of viral proteins increases, there is a switch from transcription to replication. The negative-sense genomic RNA template is copied into full length positive-sense copies of the viral RNA which, in turn, serve as templates for synthesis of full length negative-sense RNA genomes. Following replication, the newly synthesised genomes are assembled into new nucleocapsids. mRNAs encoding GP are translocated to the endoplasmic reticulum (ER) where GP is synthesised and processed. GP is further modified in the Golgi and transported to the plasma membrane in secretory vesicles (Hartlieb and Weissenhorn, 2006). The nucleocapsids assemble with the membrane associated proteins (VP24, VP40 and GP) at the plasma membrane, and the resultant virions bud from the cell surface (Noda *et al.*, 2006). A summary of the EBOV life cycle is shown in Figure 1.5.

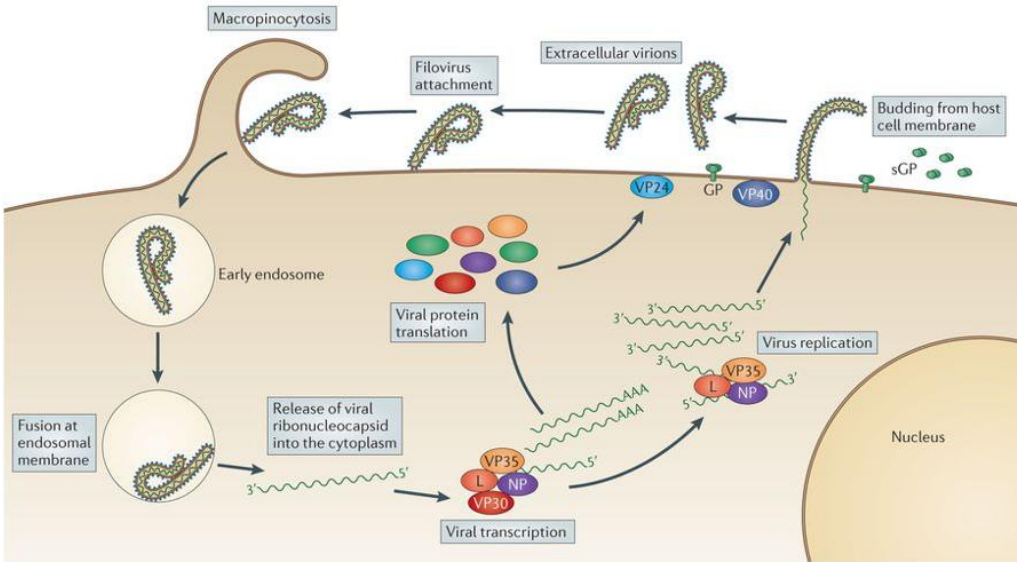


Figure 1.5: Ebola virus (EBOV) life cycle. The virion attaches to the host cell surface and is internalised into an early endosome by macropinocytosis. Upon acidification of the late endosome, the cellular proteases cathepsin B and cathepsin L cleave glycoprotein (GP), which allows it to interact with the host protein Niemann-Pick C1 (NPC1). GP then mediates fusion of the viral and the endosomal membranes, releasing the viral ribonucleocapsid into the cytoplasm, where the negative-strand RNA genome undergoes transcription and replication. Production of 5'-capped, 3'-polyadenylated mRNAs from individual viral genes, and translation of viral proteins occurs. Genome replication follows in which the genomic RNA template is copied into a full-length positive-sense copy, which serves as a template for the synthesis of additional negative-sense genomes. New nucleocapsids assemble with membrane associated proteins at the plasma membrane and resultant virions bud from the cell surface. (Abbreviations: NP, nucleoprotein; VP, virion protein; L, large protein (RNA-dependent RNA polymerase); sGP, secreted GP). Figure adapted from (Messaoudi *et al.*, 2015).

1.3 Immune response to EBOV

1.3.1 Innate immune response

The innate immune system is the host's first line of defence against infection. It consists of a network of non-specific cells and proteins that function to limit pathogen spread, and leads to initiation of the host adaptive immune response (Chaplin, 2010; Moser and Leo, 2010). Leukocytes (white blood cells) of the innate immune system include natural killer (NK) cells, mast cells, eosinophils, basophils and phagocytes, including macrophages, neutrophils and DCs. Proteins involved in the innate immune response include components of the complement system as well as cytokines and chemokines, which are cell signalling molecules that function to recruit more immune cells to the site of infection, induce inflammation, and assist in development of an effective adaptive immune response.

Comparison of cytokine and chemokine responses between human survivors and non-survivors of EVD can offer insights into innate immune responses that may contribute to survival from EBOV infection. In a study of blood samples obtained during five EVD outbreaks that occurred between 1996 and 2003 in Gabon and the Republic of the Congo, fatal outcome was associated with abnormal innate immune responses characterised by a 'cytokine storm', with hypersecretion of numerous pro-inflammatory cytokines, chemokines and growth factors (Wauquier *et al.*, 2010). Significant upregulation of pro-inflammatory cytokines and chemokines was also observed in fatal EVD cases during the 2013-2016 EBOV outbreak in West Africa (McElroy *et al.*, 2014; Ruibal *et al.*, 2016). Survivors from two EVD outbreaks in Gabon demonstrated a transient release of IL-1β, IL-6, TNFα, MIP-1α and MIP-1β in plasma early in disease (Baize *et al.*, 2002). Additionally, asymptomatic EBOV infections were also characterised by transiently high levels of IL-1β, IL-6, TNFα, MCP-1, MIP-1α and MIP-1β in plasma approximately one week following the first potential exposure to infectious material (Leroy *et al.*, 2000). These studies suggest

that survival from EBOV infection is associated with early, transient, and well-regulated inflammatory responses, which may help to control EBOV replication and induce an effective adaptive immune response.

1.3.2 Adaptive immune response

The adaptive immune response is responsible for eliminating pathogens from infected hosts, as well as preventing pathogen replication and spread, and can generate immunological memory (Bonilla and Oettgen, 2010). It consists of antibodies [immunoglobulins (Igs)], which are produced by B cells (lymphocytes that mature in the bone marrow), and T cells [lymphocytes that mature in the thymus and express a T cell receptor (TCR)].

There are two main classes of T cell, defined by the co-receptor they express; CD4 or CD8. Upon activation, CD8+ T cells differentiate into cytotoxic T lymphocytes (CTLs), which kill infected cells or tumour cells by inducing apoptosis. Whereas CD4+ T cells, upon activation, differentiate into T helper (Th) cells, which release cytokines that can stimulate further T cell function, macrophage activation or B cell antibody production. Th cells can be further subdivided into Th1, Th2, Th17 and regulatory T (Treg) cells, based on their cytokine secretion profiles and functions (Bonilla and Oettgen, 2010; Moser and Leo, 2010). Th1 cells are associated with control of intracellular pathogens and produce cytokines such as IL-12 and interferon gamma (IFNγ), whereas Th2 cells are important for protection against extracellular pathogens and helminths by producing cytokines such as IL-4, IL-5 and IL-13. Th17 cells are characterised by the production of IL-17 and are important for defence against extracellular pathogens, and have also been linked to autoimmunity (Bettelli *et al.*, 2008). Treg cells have a role in regulating and suppressing other immune cells by producing cytokines that have a suppressive function, such as IL-10 (Vignali *et al.*, 2008). CTLs can also secrete cytokines, primarily IFNγ and TNFα.

Upon binding antigen via their membrane bound cell surface receptor, B cells are activated and differentiate into antibody-secreting plasma cells or memory B cells (Moser and Leo, 2010). Antibodies are 'Y' shaped glycoproteins that consist of two identical heavy (H) polypeptide chains and two identical light (L) polypeptide chains, which are linked by disulphide bonds (Figure 1.5). The N terminal variable (V) domains of the heavy (V_H) and light (V_L) chains together make up the variable region of the antibody, and form the antigen binding sites, while the constant (C) domains of the heavy (C_H1, C_H2 and C_H3) and light (C_L) chains make up the constant region (Schroeder and Cavacini, 2010). Different parts of the antibody molecule are responsible for various functions. The Fab (Fragment antigen binding) fragments, which correspond to the two identical arms of the antibody molecule and contain the complete light chains paired with the V_H and C_H1 domains of the heavy chains, bind to antigen. The Fc (Fragment crystallisable) fragment, which corresponds to the paired C_H2 and C_H3 domains, interacts with effector molecules and cells via Fc receptors (FcRs), thereby mediating various immune functions. There are five major classes of Ig (IgM, IgD, IgG, IgA and IgE) defined by the structure of their heavy chain (μ, δ, γ, α and ε, respectively), which determines the effector function of the antibody molecule.

Immunity mediated by antibodies is known as humoral immunity. Antibodies can function by binding to and coating the surface of pathogens, thereby targeting them for destruction by phagocytes via Fc-FcR interactions. Fc-FcR interactions can also result in the death of pathogens, or pathogen infected cells via degranulation of effector cells, thereby inducing lysis or apoptosis of the target cells. This process is known as antibody-dependent cellular cytotoxicity (ADCC). Finally, antibodies can activate the complement cascade, resulting in destruction of the pathogen or infected cell by either direct lysis or phagocytosis. Antibody-antigen complexes can trigger the classical complement pathway by binding to the C1q subcomponent of C1 via the antibody Fc region. Following activation, as series of proteins are recruited to generate C3 convertase, C4b2a, which cleaves C3 into two

fragments; the large fragment, C3b, which remains attached to the surface of the microbial pathogen, and acts as an opsonin to enhance phagocytosis; and the small fragment, C3a (anaphylatoxin), which is released and mediates inflammation. Activated C3 can trigger the lytic pathway, which ultimately results in formation of a membrane attack complex (MAC) and target cell lysis. This process is known as complement-dependent cytotoxicity (CDC) In addition to influencing immune pathways, antibodies can also bind directly to toxins and viruses, thereby neutralising them by preventing interaction with host cells.

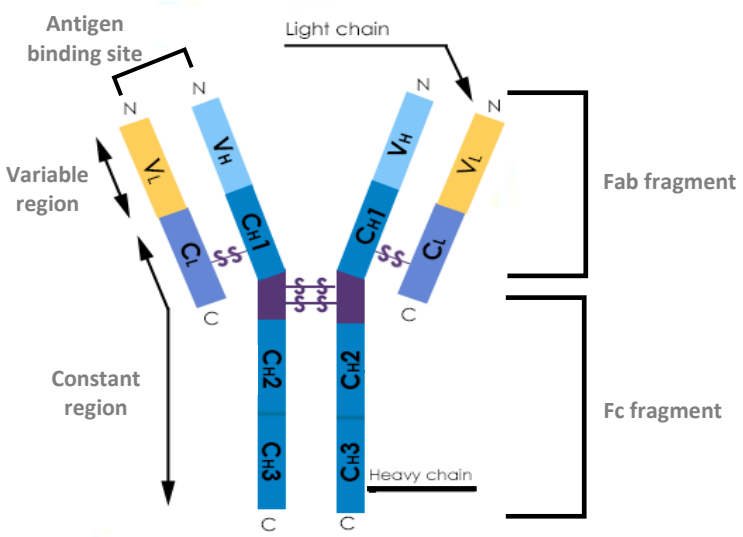


Figure 1.5: Schematic representation of immunoglobulin G (IgG) structure. Ig molecules are composed of two heavy (H) and two light (L) chains, linked by disulphide (S-S) bonds. Both chains have variable (V_H and V_L) and constant regions (C_H and C_L). (Abbreviations: N, amino terminus; C, carboxy terminus; Fab, Fragment antigen binding; Fc, Fragment crystallisable). Figure adapted from www.abcam.com.

There is evidence that an early and well-regulated specific adaptive immune response is required for survival from EBOV infection in humans (Prescott *et al.*, 2017; Wong *et al.*, 2014).

Humoral immune response

Comparison of immune responses between survivors and non-survivors during two EVD outbreaks in Gabon found that the early appearance of virus-specific IgM and IgG, followed by activation of CTLs at the time of antigen clearance from the blood, was associated with survival (Baize *et al.*, 1999). In contrast, fatal outcome was associated with impaired humoral responses, with no detectable virus-specific IgG. This was in addition to the early activation of T cells that were unable to control virus replication, followed by large decreases in T cells, possibly due to apoptosis. Asymptomatic individuals developed IgM responses at approximately 10 to 18 days following presumed exposure, and IgG responses approximately one week after the appearance of IgM (Leroy *et al.*, 2000).

Data from EVD patients evacuated to Europe following EBOV infection during the 2013-2016 outbreak in West Africa revealed robust EBOV-specific humoral responses during convalescence, with the generation of neutralising antibodies (Dahlke *et al.*, 2017b; Kreuels *et al.*, 2014; Luczkowiak *et al.*, 2016; Wolf *et al.*, 2015).

Cell-mediated immune response

IL-10 expression was upregulated early and transiently in asymptomatic EBOV patients, whereas in fatal EVD cases, IL-10 upregulation occurred later during infection (Baize *et al.*, 2002; Leroy *et al.*, 2001). IL-10 is an anti-inflammatory cytokine and can regulate immune responses by inhibiting Th1 cells and NK cells, by directly inhibiting INFγ production, and by stimulating the activity of anti-inflammatory Treg cells (Couper *et al.*, 2008). Therefore, upregulation of IL-10 early during infection could help control and downregulate the inflammatory response, whereas upregulation of IL-10 later in disease could contribute to the decrease in T cell responses observed late in lethal cases of EBOV infection (Baize *et al.*, 1999; Leroy *et al.*, 2001; Prescott *et al.*, 2017).

A study of four survivors from the 2013-2016 outbreak in West Africa showed robust T and B cell activation during the acute phase of EBOV infection in all four patients (McElroy *et al.*, 2015). However, these patients were treated at a hospital in the USA, and received substantial supportive care as well as experimental therapeutic interventions. Therefore it is unclear if any of these interventions impacted the course of disease, or modified the immune responses in these patients (McElroy *et al.*, 2015). High levels of activated CD8+ and CD4+ T cells were observed in two of the patients up to one month following discharge from hospital, suggesting persistence of viral antigen and ongoing T cell stimulation. Examination of lymphocyte dynamics during the convalescent phase of an EVD survivor who received only supportive therapy and no experimental drugs detected EBOV specific T cells and also demonstrated the persistence of T cell activation (Dahlke *et al.*, 2017b).

Another study of patients from the 2013-2016 EVD outbreak in Guinea demonstrated that expression of the inhibitory molecules cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD1) on peripheral blood CD4+ and CD8+ T cells was significantly upregulated in fatal EVD cases, and correlated with elevated inflammatory markers and high viremia (Ruibal *et al.*, 2016). Survivors displayed significantly lower expression of CTLA-4 and PD1, as well as lower inflammation, however overall T cell activation was similar for both survivors and non-survivors. CTLA-4 and PD1 are key regulators of T cell homeostasis, therefore it could be hypothesised that upregulation of CTLA-4 and PD1 on T cells in fatal EVD cases, caused by excess expression of pro-inflammatory mediators, leads to inhibition of T cell function and poor viral clearance. This study suggests that dysregulation of the T cell response may be a key component of EVD pathophysiology.

Long-term immunity

It is thought that EVD survivors are protected against subsequent infection with EBOV, and that both neutralising antibodies and T cell responses to the EBOV GP are involved. In an animal study, cynomolgus macaques that survived a previous EBOV challenge by receiving ZMAb, a cocktail of three EBOV GP-specific monoclonal antibodies (mAbs), beginning one or two days post-infection (Qiu *et al.*, 2012), were re-challenged 10 weeks after the initial challenge (Qiu *et al.*, 2013b). All NHPs survived re-challenge and showed no signs of disease, indicating that a robust immune response was generated during the initial EBOV challenge and treatment with ZMAb, which resulted in sustained protection against a second lethal exposure.

The duration of the immune response to EBOV in human EVD survivors is unknown. It is likely that virus-specific IgG response declines over time (Wauquier *et al.*, 2009), however various studies of survivors from previous EBOV outbreaks have found that specific IgG antibodies were still detectable in some individuals up to 11 years after infection (Corti *et al.*, 2016; Ksiazek *et al.*, 1999b; Wauquier *et al.*, 2009). A recent study of 14 survivors from the 1976 Yambuku EVD outbreak found that 12 of these survivors had detectable levels of anti-EBOV GP IgG 40 years after infection, and four of these displayed neutralising activity against live EBOV (Rimoin *et al.*, 2018).

There is little information regarding the memory T cell response following EBOV infection in humans. A recent study investigating the immune responses of EVD survivors of the 2013-2016 West African EBOV epidemic in Sierra Leone found that CD8+ T cells against the NP dominated the EBOV-specific responses, while only a minority of individuals had memory CD8+ T cell responses to the EBOV GP (Sakabe *et al.*, 2018).

1.3.3 Neutralising antibodies

Virus neutralisation by antibodies, as discussed here, is defined as the reduction of virus infectivity by the binding of antibodies to the viral particles (Klasse, 2014; Klasse and Sattentau, 2002). Neutralisation can be mediated by a number of different mechanisms, which are often classified according to which step in the viral replication cycle is blocked, these include; inhibition of virion attachment to target cells, inhibition of fusion of the virial membrane with the host membrane, inhibition of the entry of the genome of non-enveloped viruses into the cell cytoplasm, and inhibition of a function of the virion core through a signal transduced by an antibody (Dimmock, 1984; Mandel, 1978). The mechanism of neutralisation is determined by both the properties of the viral epitope and the antibody that binds to it. Therefore, as a virus has at least several unique epitopes on its surface, any one virus can be neutralised in several different ways. These are determined primarily by the specificity of the reacting antibody (Reading and Dimmock, 2007), and thus mechanisms of neutralisation are often analysed using mAbs. mAbs are produced by a single clone of B cells and are specific for a single epitope (Kohler and Milstein, 2005).

EBOV neutralising antibodies

Based on the entry process of EBOV into host cells, three mechanisms of neutralisation have been proposed (Saphire and Aman, 2016): inhibition of cathepsin-mediated cleavage, blockage of NPC1 binding, and interference of GP_2 structural rearrangements required for fusion of viral and host membranes. The different structural regions of EBOV $GP_{1,2}$ and examples of reported mAbs that bind to them are shown in Figure 1.6.

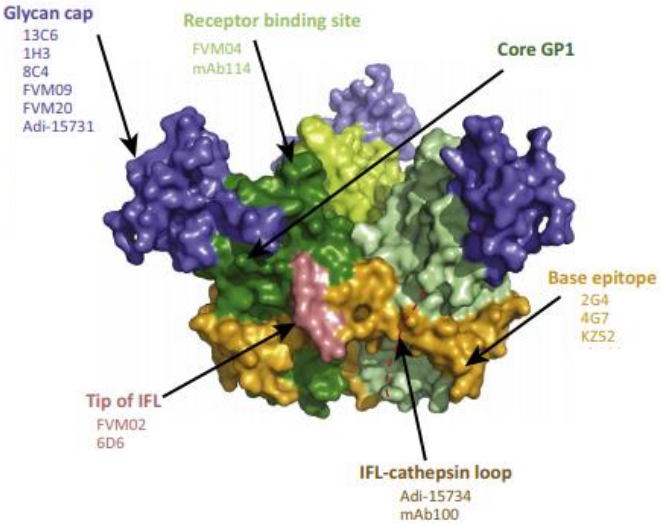


Figure 1.6: Schematic representation of Ebola virus (EBOV) surface glycoprotein ($GP_{1,2}$) trimer. Glycan cap is shown in shades of purple, GP_1 core in shades of green, GP_2 in orange, and tip of internal fusion loop (IFL) in pink. Red dotted line represents cathepsin cleavage loop. Examples of different monoclonal antibodies (mAbs) that bind to each region are listed. Figure adapted from (Saphire and Aman, 2016).

The first EBOV neutralising antibody, KZ52, was isolated from B cells of a human survivor of the 1995 Kikwit, DRC EVD outbreak (Maruyama *et al.*, 1999). KZ52 binds to residues within both GP_1 and GP_2 at the base of the GP trimer (Lee *et al.*, 2008), locking GP in its pre-fusion conformation and therefore preventing the conformational rearrangements required to drive membrane fusion (Aman, 2016). ZMAb is a cocktail of three EBOV GP-specific mAbs, 1H3, 2G4 and 4G7, that were previously generated from mice vaccinated with a vesicular stomatitis virus (VSV)-based EBOV vaccine (VSVΔG-EBOVGP) (Qiu *et al.*, 2011). Two of the ZMAb antibodies, 2G4 and 4G7, are neutralising and target a GP base epitope shared with KZ52, whereas the third antibody, 1H3, binds within the glycan cap and is non-neutralising *in vitro* (Audet *et al.*, 2014; Murin *et al.*, 2014). This suggests that Fc-mediated effector functions may play a role in protection against EBOV by non-neutralising antibodies (Gunn *et al.*, 2018).

mAb100 and mAb114 are two other mAbs isolated from a human survivor of the 1995 Kikwit EVD outbreak that neutralise EBOV (Corti *et al.*, 2016). mAb100 binds the GP base at a 60° angle relative to KZ52 and contacts residues near the tip of the GP_2 IFL as well as residues at the GP_1 N terminus, locking GP in its pre-fusion conformation (Misasi *et al.*, 2016). Part of the mAb100 epitope is within the cathepsin cleavage site, and therefore mAb100 may also neutralise EBOV by inhibiting cleavage. mAb114 binds to both the RBD and the glycan cap, with the glycan cap fraction not essential for binding (Misasi *et al.*, 2016). Therefore, mAb114 is able to remain attached to GP after cathepsin cleavage and blocks GP interaction with NPC1.

A number of additional neutralising epitopes within EBOV GP have also been identified. Antibodies against the IFL (Furuyama *et al.*, 2016; Wec *et al.*, 2017), against epitopes proximal to the viral membrane (termed 'stalk-binders') (Bornholdt *et al.*, 2016b; Flyak *et al.*, 2016), and that react to the region between the KZ52 epitope and the tip of the IFL (Bornholdt *et al.*, 2016b) have all been isolated. Several of these antibodies show cross-neutralising activity towards multiple ebolavirus species, suggesting that development of broadly neutralising immunotherapies and cross-protective vaccines might be achievable (Zhao *et al.*, 2017).

FVM02 and FVM04 are mAbs derived from cynomolgus macaques immunised repeatedly with a mixture of engineered GPs and VLPs for three different filovirus species (EBOV, SUDV and MARV) (Keck *et al.*, 2015). FVM02 is a pan-filovirus antibody that binds to the tip of the IFL with high affinity. FVM02 does not neutralise virus (EBOV and SUDV) *in vitro*, but provides significant protection from lethal EBOV challenge in mice, and therefore may function through Fc-mediated effector mechanisms *in vivo* (Schmaljohn and Lewis, 2016). FVM04 is a pan-ebolavirus mAb that binds to a conformational epitope within the core of GP_1, encompassing the tip of the RBD crest and the base of GP_1, and blocks NPC1 binding.

FVM04 effectively neutralises EBOV and SUDV *in vitro* and protects mice and guinea pigs from both EBOV and SUDV infection (Howell *et al.*, 2016). CA45 is another immunisation-elicited macaque antibody that protects rodents from EBOV and SUDV infection (Zhao *et al.*, 2017). It binds to a conserved epitope that includes residues within the IFL of GP_2 as well as the N terminus of GP_1, and neutralises EBOV, SUDV, BDBV and RESTV *in vitro*. CA45 appears to function by partially inhibiting cathepsin-mediated GP cleavage and, by blocking virus entry post-cleavage (Zhao *et al.*, 2017). It has recently been demonstrated that a cocktail of FVM04 and CA45 is able to protect NHPs against EBOV and SUDV infection when delivered four days post-infection (Brannan *et al.*, 2019).

Several glycan cap binders with cross-reactivity to multiple ebolavirus species and potent neutralising activity have also been reported (Bornholdt *et al.*, 2016b; Flyak *et al.*, 2016; Holtsberg *et al.*, 2015; Keck *et al.*, 2015). These antibodies also bind to sGP and the mechanism by which these antibodies neutralise is not clear.

A novel phenomenon of 'cooperative neutralisation' by mAb pairs has been described, whereby neutralisation by a weakly or moderately neutralising mAb is enhanced when they are paired with a specific non-neutralising mAb (Howell *et al.*, 2017). For example, the mAb FVM09 has almost no EBOV neutralising activity alone, however combination with the poorly neutralising antibody m8C4, was able to convert m8C4 into a potent neutraliser, and this cocktail fully protected EBOV infected mice. Binding of GP by FVM09 may cause a conformational change that leads to better access of m8C4 to its epitope (Howell *et al.*, 2017).

Measurement of neutralising antibodies

The plaque reduction neutralisation test (PRNT), first developed in the 1950s (Dulbecco *et al.*, 1956), is a traditional assay for the measurement of neutralising antibodies, and has been applied to a wide variety of viruses including EBOV (Maruyama *et al.*, 1999). Briefly,

samples are serially diluted and incubated with a standardised amount of virus to allow any antibodies in the sample to bind to the virus. The mixture is then added to a confluent monolayer of permissive cells and after a further short incubation period sufficient for virus to infect the cells, the sample suspension is replaced with a semi-solid overlay medium to prevent the virus from spreading to non-adjacent cells. Plates are then incubated, fixed, and stained. Virus infection is quantified indirectly by observing plaques of virus-induced cytopathic effect (CPE), which is measured in plaque forming units (PFU)/ml. The neutralising antibody titres are defined as the sample dilutions that result in a certain reduction [usually 50% ($PRNT_{50}$) or 90% ($PRNT_{90}$)] relative to the total number of plaques counted without antibody.

Neutralising antibody titres can also be determined by performing a focus reduction neutralisation test (FRNT), which is a variation of the PRNT, but instead of using cell lysis to detect plaque formation, utilise recombinant virus expressing a labelled protein or immunostaining of a specific viral antigen to detect infected host cells and infectious virus particles before an actual plaque is formed. Like the PRNT, host cell monolayers are infected with various dilutions of the sample-virus mix and overlaid with a semi-solid medium that restricts the spread of infectious virus, creating localised clusters (foci) of infected cells. Plates are subsequently probed with labelled antibodies against the viral antigen if necessary, and microscopy is used to count and quantify the number of foci; results are expressed as focus forming units (FFU)/ml. The neutralising antibody titre of a sample is determined as the dilution showing a reduction [e.g. 80% ($FRNT_{80}$)] in foci compared to control without antibody.

PRNT remains widely regarded as the ‘gold standard’ for the detection of neutralising antibodies for a number of viruses due to its high sensitivity and specificity, despite having limitations. The use of infectious virus often requires a high level of containment and

expert handling and manipulation, which require expensive facilities and highly trained staff. Furthermore, the assay format and time required for plaque development, which can take three to 14 days depending on the virus, makes it time consuming and restricts throughput.

Serological studies are fundamental to assess the neutralising ability of antibodies targeted to EBOV GP, however due to its severe pathogenicity, potential transmission from person-to-person contact, and lack of approved vaccines or antiviral treatments, EBOV is classified as a Hazard Group (HG) 4 pathogen, and handling of EBOV for clinical, diagnostic, or research-based purposes is limited to containment level (CL) 4 laboratories. High containment facilities are expensive and are not readily available, especially in countries and organisations with limited resources. Development of novel serological assays that utilise genetically modified recombinant or chimeric viruses with attenuated pathogenicity have enabled more widespread investigation of neutralising antibodies against highly pathogenic viruses including EBOV, for example during serosurveillance and vaccine or antiviral evaluation studies (Bentley *et al.*, 2015; Mather *et al.*, 2013).

1.4 Pseudotyped viruses

Pseudotyped viruses can be used as alternatives to live infectious virus in serological assays for the investigation of viral infection or vaccine seroconversion (Bentley *et al.*, 2015; King and Daly, 2014; Mather *et al.*, 2013). A pseudotyped virus is a replication-defective chimeric virion that consists of the structural and enzymatic core of one virus, bearing the envelope protein or glycoprotein of another, and encodes a quantifiable reporter gene. Transduction of target cells by a pseudotyped virus is dependent on the ability of the envelope glycoprotein to interact with receptors on the cell surface. If binding and transduction are successful, the genome is transferred from the pseudotyped virus to the target cell and the reporter gene expressed, resulting in a quantitative read out.

Pseudotyped viruses have been used for a variety of applications including the study of virus-host cell interactions, identification of potential virus entry inhibitors, and measurement of neutralising antibodies (King *et al.*, 2016; Li *et al.*, 2018; Temperton *et al.*, 2015). This avoids the use of native, pathogenic virus and the need for high bio-containment facilities, making pseudotyped viruses safer and less expensive alternatives. Furthermore, the range of reporter genes available, such as β-galactosidase, green fluorescent protein (GFP) or luciferase, provide a variety of cost and time benefits (Wright *et al.*, 2009), making the application of pseudotyped virus assays accessible to laboratories with differing resource levels.

Retroviruses, including lentiviruses and gammaretroviruses such as human immunodeficiency virus (HIV) and murine leukemia virus (MLV), respectively, have been used as cores for pseudotyped viruses, as well as rhabdoviruses, such as VSV. Pseudotyped virus systems have also been developed based on influenza virus (Powell *et al.*, 2012).

1.4.1 Retrovirus-based pseudotyped viruses

Retroviruses are able to incorporate foreign proteins, including host-derived proteins and envelope proteins of other viruses, into their envelope membrane (Landau *et al.*, 1991), and have been extensively used as cores for pseudotyped viruses (Temperton *et al.*, 2015). Retroviruses are single-stranded, positive-sense RNA viruses that replicate with a DNA intermediate through the process of reverse transcription. Their genome consists of two identical single-stranded RNA molecules within the virion. Following entry into the cytoplasm of a host cell, the viral RNA is converted into complementary DNA (cDNA) by the viral reverse transcriptase enzyme, and becomes integrated into the host genome as a provirus.

Pseudotyped retroviruses can be produced by co-transfection of producer cells using a three-plasmid system (Naldini *et al.*, 1996; Soneoka *et al.*, 1995) (Figure 1.7). The core plasmid encodes the *gag-pol* genes, responsible for the production and enzymatic processing of the core structural proteins, with the packaging signal psi (Ψ) omitted to prevent replication competence and remove the potential risk of pathogenic virus proliferation. The second plasmid encodes the envelope glycoprotein gene from the virus of interest, and the third plasmid encodes the chosen reporter gene and Ψ flanked by long tandem repeats (LTRs), which facilitate integration into the target cell genome. After transcription and translation of the imported genes, an RNA dimer of the reporter gene is packaged into the core. Pseudotyped virus capsids subsequently transit to the plasma membrane of the producer cell where they bud extracellularly; acquiring an envelope consisting of a lipid bilayer derived from the plasma membrane containing heterologous viral envelope proteins, and can be harvested in the culture supernatant.

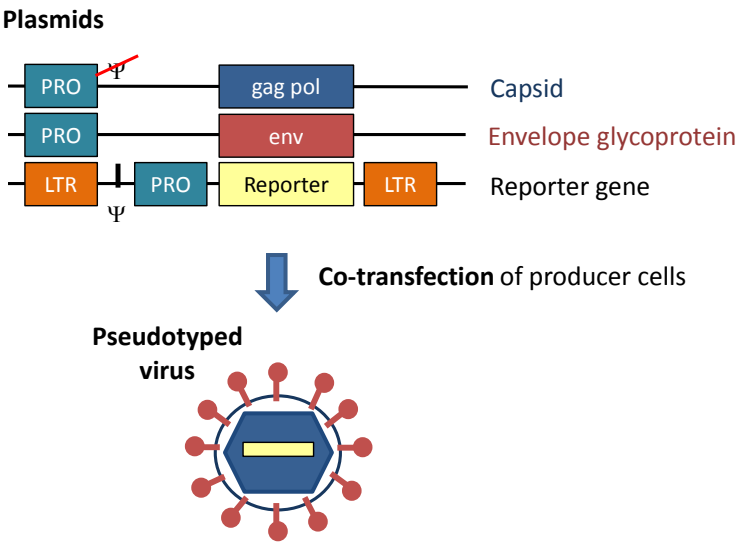


Figure 1.7: Schematic representation of three plasmid co-transfection system of pseudotyped virus production. Producer cells are transfected with plasmids encoding the *gag-pol* genes, the envelope glycoprotein gene, and the reporter gene. Following transfection and infection, pseudotyped viruses are harvested in the culture supernatant. (Abbreviations: PRO, promoter; LTR, long tandem repeat; Ψ, packaging signal psi). Figure adapted from (Bentley *et al.*, 2015).

The viral components are separated across multiple plasmids so that multiple recombination events would be needed to produce replication-competent virus. Also, as the genetic material packaged by the pseudotyped virus does not encode a viral envelope protein, the pseudotyped virus is capable of transducing susceptible target cells, but is unable to produce new virus progeny. The retrovirus- and lentivirus-based pseudotyped virus systems have undergone a number of developments to further improve safety, such as deletion of accessory genes and promoter sequences, and provision of required elements on a separate fourth plasmid (Dull *et al.*, 1998; Zufferey *et al.*, 1998; Zufferey *et al.*, 1997).

1.4.2 Rhabdovirus-based pseudotyped viruses

Rhabdoviruses, such as VSV, can also be used as pseudotyped virus cores (Takada *et al.*, 1997). VSV has a single-stranded, negative-sense RNA genome of approximately 11 kb in length that encodes five major viral proteins; nucleoprotein (N), phosphoprotein (P), matrix

protein (M), glycoprotein (G) and large protein (L). The G protein mediates both host cell binding and fusion with the endosomal membrane following endocytosis. The P and L proteins are subunits of the viral RNA-dependent RNA polymerase. VSV is able to incorporate heterologous transmembrane proteins into its viral membrane and has been shown to readily form pseudotypes when co-infected along with other enveloped viruses (Huang *et al.*, 1974). Pseudotyped VSVs can be generated by combining a recombinant VSV genome, in which the VSV-G gene has been deleted (rVSV-ΔG) and replaced with a reporter gene (represented here by a *), with an expression plasmid encoding the desired heterologous virus envelope protein (Whitt, 2010).

In order to recover rVSV-ΔG* from plasmids, cells are transfected with the rVSV-ΔG* genome plasmid along with plasmids individually encoding the VSV-G, N, P and L, all of which are under control of T7 promoters. T7 RNA polymerase is provided either by infection of the producer cells with a recombinant virus expressing the T7 RNA polymerase, or by using a cell line stably expressing the T7 RNA polymerase. Following transfection and incubation, the virus recovery supernatant is harvested and filtered to remove any T7 RNA polymerase helper virus. The primary recovery virus is then amplified by transfection of cells with a VSV-G expression plasmid followed by infection with the primary recovery virus, with subsequent plaque purification, to ensure homogeneity and that there is no residual T7 RNA polymerase helper virus that may have carried over during the initial rVSV-ΔG* amplification after filtering (Whitt, 2010). G-complemented rVSV-ΔG* (rVSV-ΔG*-VSV-G) plaque isolates are then amplified and working stocks generated and titrated, and used to produce heterologous pseudotypes as detailed below.

Producer cells are transfected with a heterologous viral envelope protein expression plasmid and are subsequently infected with rVSV-ΔG*-VSV-G at a high multiplicity of infection (MOI) to ensure every cell is infected. During budding, the rVSV-ΔG* acquires an

envelope consisting of a lipid bilayer derived from the plasma membrane containing heterologous viral envelope proteins (Figure 1.8). Pseudotyped VSVs are then harvested in the culture supernatant. Before use, the pseudotyped virus is treated with a VSV-G neutralising antibody to reduce background infection mediated by residual virus possessing VSV-G, which can be carried over during preparation (Whitt, 2010). As the genome of the resultant pseudotyped virus does not encode a viral envelope protein, the pseudotyped virus is capable of transducing susceptible target cells, but is unable to produce new virus progeny.

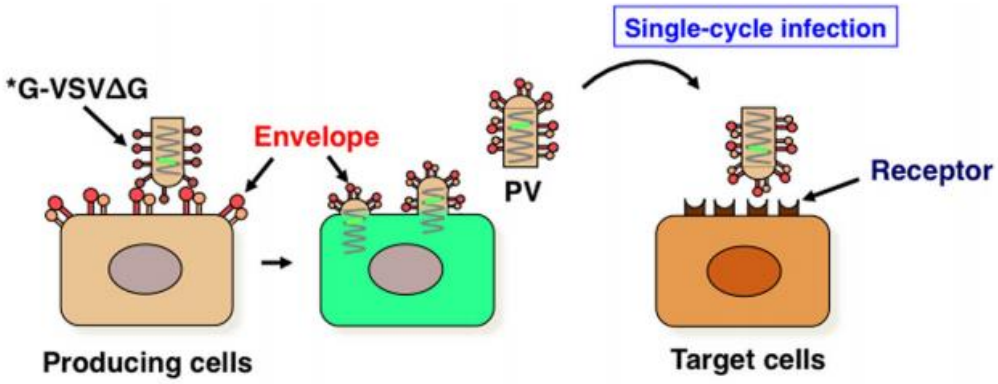


Figure 1.8: Schematic representation of pseudotyped vesicular stomatitis virus (VSV) production. Producer cells are transfected with an expression plasmid encoding a heterologous viral envelope gene, and are subsequently infected with a VSV-G-complemented pseudotyped VSV encoding a reporter gene (*G-VSVΔG). The resulting pseudotyped virus is able to transduce target cells but is unable to produce infectious progeny virus. (Abbreviations: PV, pseudotyped virus). Figure adapted from (Tani *et al.*, 2011).

Additional working stocks of rVSV-ΔG*-VSV-G virus can be generated by a similar method as that used to pseudotype heterologous envelope proteins onto rVSV-ΔG*, except that the cells are transfected using a VSV-G expression plasmid, and the infection is performed at a lower multiplicity to prevent accumulation of defective-interfering particles (Whitt, 2010).

1.4.3 Neutralisation of pseudotyped viruses

Pseudotyped viruses can be used in serological assays, to measure neutralising antibodies against the envelope glycoprotein coating the pseudotype (Figure 1.9). If target cell receptor binding and transduction by the pseudotyped virus are successful, the genome of the pseudotyped virus is transferred to the target cell and the reporter gene expressed. However, if neutralising antibodies to the surface glycoprotein are present, the pseudotyped virus does not bind to and transduce the target cell, and the reporter gene is not expressed. Neutralisation can then be quantified as a decrease in reporter gene expression relative to pseudotyped virus infection without antibody.

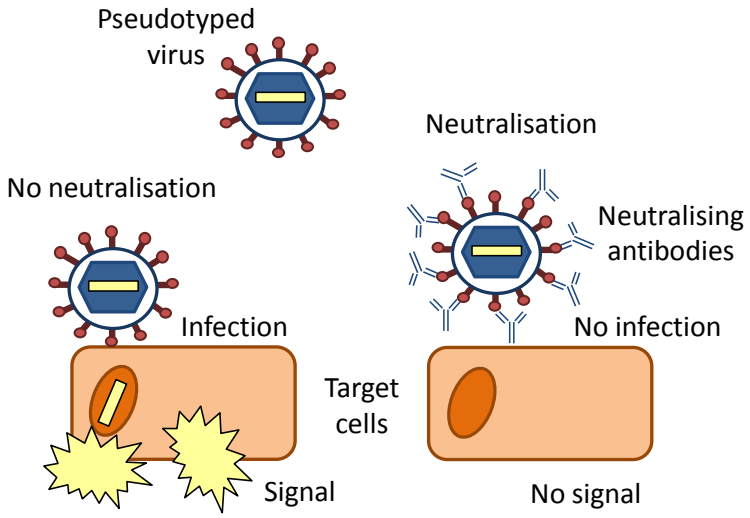


Figure 1.9: Schematic representation of pseudotyped virus neutralisation assay. Samples are serially diluted and incubated with a standardised amount of pseudotyped virus. In the absence of neutralising antibodies to the viral surface glycoprotein, the pseudotyped virus is able to bind to and transduce the target cell. The genome is transferred to the target cell and the reporter gene expressed. However, if neutralising antibodies are present, the pseudotyped virus does not bind to and transduce the target cell, and the reporter gene is not expressed. Neutralisation can then be quantified as a decrease in reporter gene expression relative to pseudotyped virus infection without antibody. Figure adapted from (Bentley *et al.*, 2015).

Pseudotyped viruses provide ideal and safe alternatives to live infectious virus, especially those that require a high level of bio-containment (Mather *et al.*, 2013). Pseudotyped virus neutralisation assays have been developed for a wide variety of viruses, including severe acute respiratory syndrome coronavirus (SARS-CoV), influenza and rabies virus, and have been shown to be highly sensitive and specific (Temperton *et al.*, 2007; Wright *et al.*, 2008). Furthermore, results correlated with those from the respective authentic virus neutralisation assays (Temperton *et al.*, 2005; Wright *et al.*, 2009). Pseudotyped virus neutralisation assays remove the need to handle native, pathogenic virus in high bio-containment facilities, making these assays safer, less expensive and more widely available. They are usually high throughput and often use smaller sample volumes compared to traditional assays. In addition, the flexibility and choice of reporter systems available for pseudotyped virus assays can make them quicker, cheaper, and easier to perform, such as automated, and less subjective assay readouts and data analysis.

1.4.4 Reverse genetics systems

Reverse genetics systems can be used to study the replication cycles of highly pathogenic RNA viruses, such as filoviruses, and to develop and assess novel antiviral therapies and mechanisms (Hoenen and Feldmann, 2014). Reverse genetics, as defined here, is the production and subsequent replication and transcription of virus RNA genomes, or truncated genome analogues (minigenomes), from cDNA (Hoenen *et al.*, 2011).

Minigenome systems have been used to study EBOV genome replication and transcription (Muhlberger *et al.*, 1999). In a minigenome, some or all of the EBOV ORFs have been removed and replaced with a reporter gene, which is flanked by the terminal non-coding regions (leader and trailer). The minigenome is expressed in mammalian cells (usually by transcription using T7 RNA polymerase) together with the viral proteins L, VP35, VP30 and NP. The minigenome is encapsidated by NP, and then replicated and transcribed by the

other nucleocapsid proteins using cis-acting signals localised in the leader and trailer, leading to reporter activity that reflects replication and transcription (Hoenen and Feldmann, 2014).

EBOV transcription and replication competent VLP (trVLP) systems have been established that allow the study of EBOV morphogenesis, budding, and entry, in addition to replication and transcription (Hoenen *et al.*, 2006; Hoenen *et al.*, 2014; Watanabe *et al.*, 2004). These systems can also be used to study antiviral agents with inhibitory effects against EBOV, as well as interactions between host proteins and EBOV (McCarthy *et al.*, 2016; Nelson *et al.*, 2016; Wang *et al.*, 2018; Yu *et al.*, 2018). EBOV trVLP systems are based on classical minigenome systems, but include expression of the other viral proteins VP24, VP40 and GP. The presence of VP40 leads to the formation of trVLPs, which bear GP on their surface, and contain a minigenome-containing nucleocapsid on the inside. These trVLPs can be used to infect naïve target cells, or target cells that have been pre-transfected with expression plasmids for L, VP35, VP30 and NP, to facilitate replication and transcription of minigenomes brought into the target cells within trVLPs. This results in reporter activity in target cells, which reflects the replication of the minigenomes in the producer cells, morphogenesis and budding of trVLPs, their entry into target cells, and, in the case of naive target cells, also primary transcription (i.e. transcription by viral proteins brought into target cells within trVLPs), or, in the case of pre-transfected target cells, also genome replication and secondary transcription (i.e. transcription by viral proteins produced in target cells) in target cells (Figure 1.10).

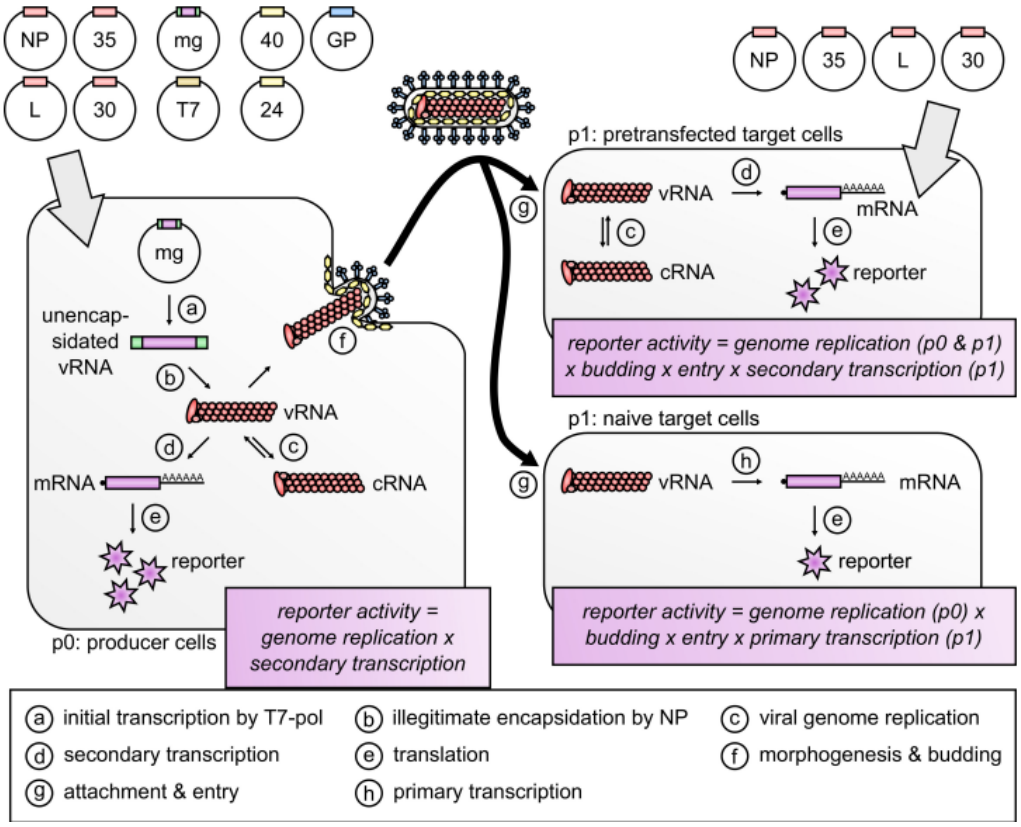


Figure 1.10: Schematic representation of trVLP assay with a monocistronic minigenome. Producer cells are transfected expression plasmids for the minigenome assay components (EBOV nucleocapsid proteins NP, VP35, VP30, L, a monocistronic minigenome and the T7 polymerase) as well as VP40, GP and VP24. This leads to the formation of trVLPs that incorporate minigenome-containing nucleocapsids (f). These trVLPs can then infect target cells (g), which are either pre-transfected with expression plasmids for NP, VP35, VP30, and L (top), resulting in replication and secondary transcription (d) leading to reporter expression (e), or naive target cells (bottom), resulting in primary transcription of the minigenomes (h), also leading to reporter expression (e). (Abbreviations: RV, recombinant virus). Figure adapted from (Hoenen *et al.*, 2014).

The monocistronic minigenome system described above can only mediate single cycle infection of target cells. Therefore, a tetracistronic minigenome system has been developed that, in addition to a reporter gene, also contains the genes encoding for VP40, GP and VP24 and can establish multicycle infection (Watt *et al.*, 2014) (Figure 1.11). This system leads to the production of trVLPs that can infect target cells; however VP40, GP, and VP24 are produced after viral genome transcription, rather than being overexpressed from

plasmids. As a result, the kinetics and expression levels of these proteins much more closely mimic those found during the viral lifecycle, and it is possible to continuously passage tetracistronic minigenome-containing trVLPs.

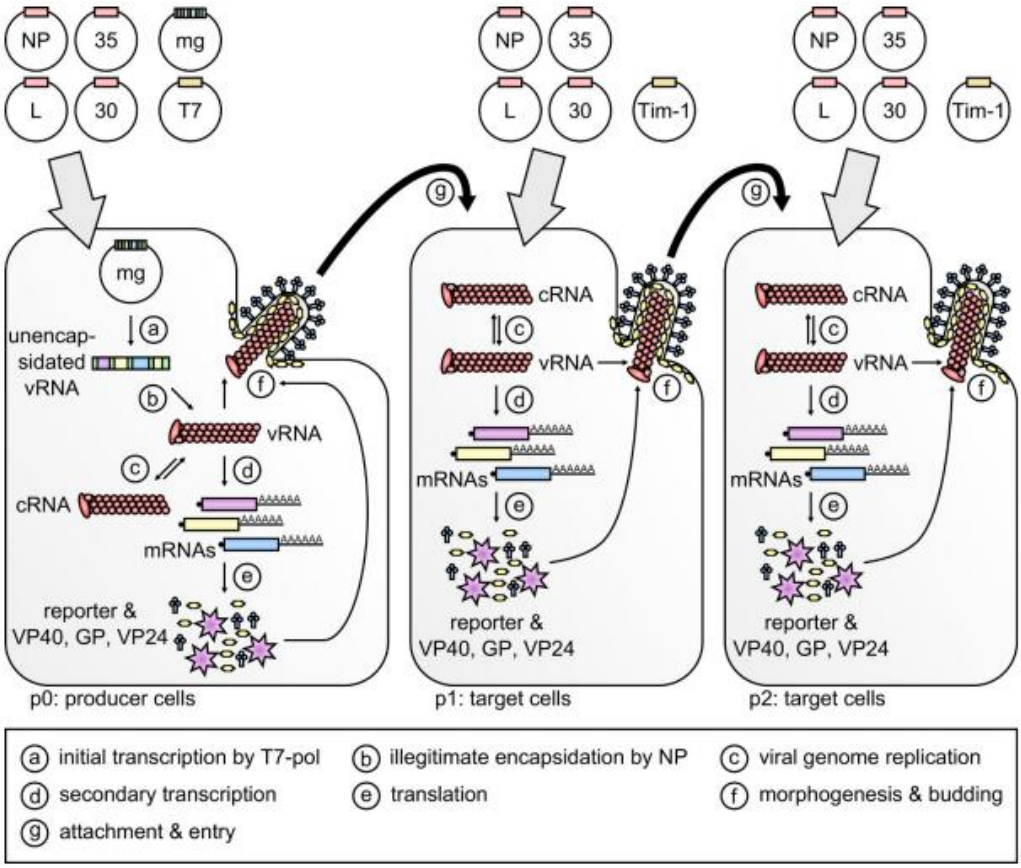


Figure 1.11: Schematic representation of trVLP assay with a tetracistronic minigenome. Producer cells are transfected with expression plasmids for EBOV nucleocapsid proteins (NP, VP35, VP30, L), a tetracistronic minigenome (mg) and the T7 polymerase. Initial transcription (a), encapsidation (b), genome replication (c) and transcription (d) as well as translation (e) occur as in a monocistronic minigenome assay. However, in addition to reporter mRNA, mRNAs for VP40, GP and VP24 are also transcribed from the tetracistronic minigenome, resulting in the formation of trVLPs (f). These trVLPs infect target cells that have been pre-transfected with expression plasmids for the nucleocapsid proteins NP, VP35, VP30 and L, as well as the cellular EBOV attachment factor Tim-1, resulting in genome replication and transcription, and production of trVLPs that can be used to infect fresh target cells. Figure adapted from (Hoenen *et al.*, 2014).

Recombinant virus systems, which encode a heterologous viral envelope gene instead of its own envelope gene in its genome, are also available by establishment of reverse genetics (Schnell *et al.*, 1996; Tani *et al.*, 2011) (Figure 1.12), and have been used to study EBOV entry into target cells, and evaluate anti-EBOV neutralising antibodies (Garbutt *et al.*, 2004; Konduru *et al.*, 2018; Lee *et al.*, 2017; Takada *et al.*, 2003; Wong *et al.*, 2010).

To generate recombinant virus, producer cells are infected with a VSV-G-complemented recombinant virus encoding a foreign envelope gene instead of VSV-G (rVSV-ΔG-Env-VSV-G). During budding, the rVSV-ΔG-Env acquires an envelope consisting of a lipid bilayer derived from the plasma membrane containing heterologous viral envelope proteins (Figure 1.12). Recombinant virus can then be harvested in the culture supernatant. As the genome of the resultant recombinant virus encodes a viral envelope protein, the recombinant virus is replication-competent, and is capable of infecting susceptible target cells and producing infectious progeny viruses.

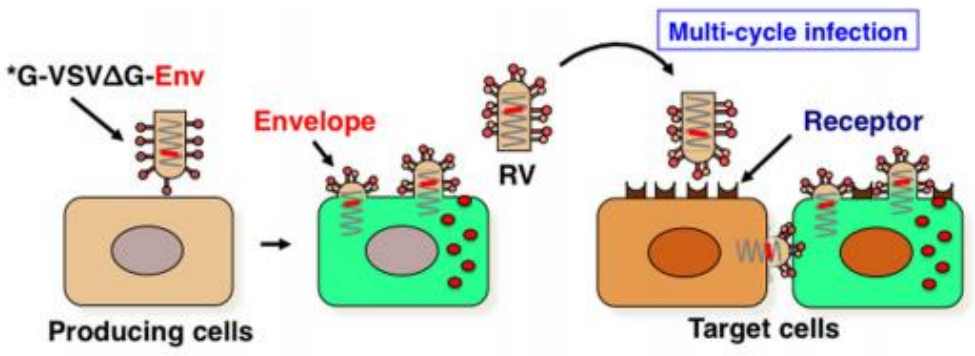


Figure 1.12: Schematic representation of recombinant vesicular stomatitis virus (VSV) production. Producer cells are infected with a VSV-G-complemented recombinant virus encoding a foreign envelope gene instead of VSV G (*G-VSVΔG-Env). The resulting recombinant virus is able to undergo a fully productive infection generating infectious progeny viruses that can be passaged into naïve cells. (Abbreviations: RV, recombinant virus). Figure adapted from (Tani *et al.*, 2011).

1.5 EVD treatment and prevention

Efforts to develop effective vaccines and therapeutics against EBOV began soon after its discovery in 1976; however, there are still no licenced treatments available for EVD. EBOV infection is managed with supportive therapy to maintain effective blood volume and electrolyte balance. The 2013-2016 EBOV outbreak in West Africa constituted a public health emergency of international concern, and highlighted the urgent need for medical countermeasures against EBOV. This outbreak greatly expedited the development and clinical evaluation of several promising therapies for EVD (Liu *et al.*, 2017; Mendoza *et al.*, 2016). These included small molecule inhibitors and immunotherapies, the latter of which are discussed below.

1.5.1 Convalescent therapy

Convalescent therapy is the treatment of an infectious disease by transferring blood products, which likely contain specific antibodies against the pathogen causing the disease, from convalescent donors to infected patients (Garraud *et al.*, 2016). During the 1995 EVD outbreak in Kikwit, eight people received whole blood transfusions from convalescent patients and seven survived (Mupapa *et al.*, 1999). However due to the small number of people treated, the possibility that patients had already developed antibodies, additional supportive care and lack of control subjects, the ability to draw conclusions regarding the efficacy of convalescent blood treatment was limited (Sadek *et al.*, 1999). Furthermore, transfusion of convalescent blood from NHP survivors of EVD into naïve animals shortly after EBOV challenge was not efficacious, casting further doubt on the therapeutic benefit of convalescent blood for treatment of EVD (Jahrling *et al.*, 2007). In addition, another NHP study assessing the protective efficacy of convalescent sera from rhesus macaques surviving EBOV challenge, showed that treatment of naïve NHPs with convalescent sera at the onset of viremia did not provide protection against lethal EBOV challenge (Mire *et al.*, 2016). However, treatment of three naïve NHPs at two, four and eight days post-EBOV

exposure with concentrated, polyclonal IgG antibody from vaccinated NHP survivors of a prior EBOV challenge prevented mortality in all animals (Dye *et al.*, 2012). Two of the three NHPs had no clinical signs of illness, while the third developed mild and delayed signs of disease prior to recovery. This study suggested a potential protective effect of antibody treatments for EVD.

During the 2013-2016 EVD outbreak in West Africa, the World Health Organisation (WHO) prioritised the evaluation of convalescent whole blood and plasma transfusion (World Health Organization, 2014b). Blood and plasma from EVD survivors are local and readily available sources of anti-EBOV antibodies that are specific to circulating strain, and can be used under circumstances of limited medical resources during EVD outbreaks. However establishment of large-scale transfusion programmes can be challenging during outbreak settings and within resource-poor areas. Donors must be clinically asymptomatic and have twice tested negative for EBOV RNA in two independent blood samples taken at least 48 hours apart. Donor testing is also performed to prevent transmission of blood-borne pathogens such as HIV, Hepatitis B virus (HBV), Hepatitis C virus (HCV) and syphilis, as well as blood typing to avoid ABO blood group reactions.

A non-randomised clinical trial conducted in Guinea to evaluate the safety and efficacy of convalescent plasma, found no significant difference in mortality between those treated with convalescent plasma and historical controls, suggesting limited efficacy of antibody therapy (van Griensven *et al.*, 2016b). However the levels of EBOV-specific neutralising antibodies in the convalescent plasma were unknown during the trial. Follow up data revealed that the dose of antibodies in the donations was low, and there was no significant correlation between antibody dose and mortality (van Griensven *et al.*, 2016a). Therefore, the amount of EBOV-specific antibodies transfused may have been insufficient to have an impact on EVD and larger antibody doses may be required to observe a clinical effect. In

addition, a study assessing the effectiveness of convalescent whole blood in the treatment of EVD patients in Sierra Leone also did not demonstrate a significant improvement in survival, although there was a significant decrease in viral load following the first 24 hours of treatment (Sahr *et al.*, 2017), suggesting that convalescent therapy alone may not be an effective treatment for EBOV infection.

1.5.2 Monoclonal antibodies (mAbs)

An alternative to the use of polyclonal antibody preparations for the treatment of infectious diseases is provided by the development of mAb therapies (Both *et al.*, 2013; Salazar *et al.*, 2017). mAbs can be manufactured in large scale and can be engineered to improve stability, safety, immunogenicity and therapeutic efficacy. However, they are expensive to produce and have relatively small markets, making them largely unavailable, especially to countries with low-income economies.

As described in section 1.3.3, the mAb KZ52 was isolated from B cells of a human survivor of the 1995 Kikwit EVD outbreak (Maruyama *et al.*, 1999). KZ52 was able to neutralise EBOV *in vitro* and protected rodents from EBOV infection (Parren *et al.*, 2002), however it was ineffective at inhibiting viral replication and preventing disease in NHPs (Oswald *et al.*, 2007), suggesting that neutralising activity alone may not always be sufficient for protection against EBOV (Gunn *et al.*, 2018). Reduced viral loads and partial protection of rhesus macaques were achieved with a cocktail of two human-mouse chimeric mAbs (ch133 and ch226) with strong neutralising activity against EBOV, administered intravenously at 24 hours before, and 24 and 72 hours after EBOV challenge (Marzi *et al.*, 2012). A cocktail (MB-003) of three human-mouse chimeric (c) or humanised (h) mAbs (c13C6, h13F6, and c6D8), manufactured in the tobacco plant, *Nicotiana benthamiana*, provided 67% protection in rhesus macaques when treatment was initiated one or two days after EBOV exposure, with no clinical indications of disease observed in survivors (Olinger *et*

al., 2012). Another cocktail (ZMAb) of three EBOV GP-specific mAbs (1H3, 2G4 and 4G7), previously generated from mice vaccinated with recombinant VSVΔG-EBOV GP, resulted in complete protection of cynomolgus macaques, with little viremia and no apparent side effects, when three doses were administered three days apart beginning one day after lethal infection with EBOV (Qiu *et al.*, 2012). These studies highlighted the importance of antibodies in controlling EBOV replication and supported the use of mAbs to treat EVD.

In order to develop an improved mAb cocktail that could be used as an EVD therapeutic in humans, the different components of MB-003 and ZMAb were tested in lethal EBOV challenge experiments in guinea pigs and NHPs (Qiu *et al.*, 2014). The individual murine antibodies in ZMAb were first chimerised with human constant regions and then produced in *Nicotiana benthamiana* before efficacy testing in animals. The optimised mAb combination was named ZMapp™ (Mapp Biopharmaceutical, Inc.) and consisted of c13C6 from MB-003 and c2G4 and c4G7 from ZMAb. ZMapp completely protected rhesus macaques when administered as late as five days post-EBOV infection (Qiu *et al.*, 2014).

During the 2013-2016 West African EBOV outbreak, the WHO declared that the use of experimental drugs for the humanitarian treatment of EVD patients was ethical (World Health Organization, 2014a). Several drugs and vaccines for EVD had shown promising results in the laboratory and in animal models, but had not yet been evaluated for safety and efficacy in humans. Ethical criteria guided the provision of such interventions and included, transparency about all aspects of care, fairness, informed consent, freedom of choice, confidentiality, respect for the person, preservation of dignity, involvement of the community and risk-benefit assessment. When unproven interventions were used to treat patients, there was a moral obligation to collect and share all scientifically relevant data generated. Researchers also had a moral duty to evaluate these interventions in the best possible clinical trials under the circumstances in order to establish the safety and efficacy

of the interventions, or to provide evidence to stop their use (World Health Organization, 2014a).

ZMAb and ZMapp were administered to human EVD patients under compassionate use protocols, however, as patients often also received additional treatments and supportive care measures, the significance of the effect of the mAb cocktail treatments could not be conclusively determined (Lyon *et al.*, 2014; Petrosillo *et al.*, 2015; Schibler *et al.*, 2015; Zeitlin *et al.*, 2016). In March 2015, a randomised controlled trial was launched to evaluate the efficacy of ZMapp (Davey *et al.*, 2016). Patients were randomised to receive either optimised standard of care or optimised standard of care plus three intravenous infusions of ZMapp three days apart (Dodd *et al.*, 2016). Although the estimated effect of ZMapp appeared to be beneficial, there was no statistically significant mortality benefit conferred by ZMapp. It is possible that efficacy was not demonstrated as the study was underpowered, or that the dosing schedule was not optimised.

As discussed in Section 1.3.3, mAb114 is a mAb isolated from a human survivor of the 1995 Kikwit EVD outbreak (Corti *et al.*, 2016). It binds to the glycan cap and inner chalice of GP, and remains associated with GP after proteolytic removal of the glycan cap, thereby inhibiting binding of cleaved GP to NPC1 (Misasi *et al.*, 2016). Rhesus macaques treated with three doses of mAb114 beginning one, or five days post-EBOV infection survived, suggesting that monotherapy with a single mAb may be a possible treatment option for EVD (Corti *et al.*, 2016). However, animals that began treatment one day post-EBOV infection still displayed transient viremia on days four to 15 post-challenge. In contrast, animals treated with three doses of a cocktail of mAb100 and mAb114, beginning one day post-EBOV infection, survived and exhibited no viremia, suggesting that use of mAb cocktails may be more protective. Furthermore, cocktails of more than one antibody reduce the possibility of treatment failure that may occur with genetic changes. Therefore

combination therapy is justifiable for the treatment of EVD; however it is not clear what combination of epitopes is best to target (Saphire and Aman, 2016).

1.5.3 Vaccines

A number of different vaccine platforms have been evaluated against EBOV including inactivated virus, DNA, protein subunits, VLPs and viral vectors. The majority of these vaccines express EBOV GP as the primary immunogen, as it is the only viral protein on the virion surface and mediates attachment and entry into host cells. Therefore EBOV GP is a key antigenic target for the development of vaccines against EVD, and has been shown to induce both antigen-specific cellular and humoral immune responses (Patel *et al.*, 2018; Qiu *et al.*, 2009). Additional EBOV proteins, such as NP, have also been shown to be immunogenic (Prehaud *et al.*, 1998; Sakabe *et al.*, 2018), and have been included in EBOV vaccine approaches (Bazhan *et al.*, 2019; Bounds *et al.*, 2017; Marzi *et al.*, 2015a). Several vaccines have been shown to protect NHPs from EBOV infection, however, prior to 2014, only a limited number of Phase I clinical trials had been conducted (Reynolds and Marzi, 2017). The 2013-2016 EBOV outbreak greatly expedited the development and clinical evaluation of the most promising vaccine candidates for EVD.

DNA vaccines

DNA vaccines are easy to produce and are considered safe; however they are poorly immunogenic in humans and often require multiple doses to achieve strong immune responses (Lu *et al.*, 2008). Several techniques can be applied to enhance the immunogenicity of DNA vaccines, such as the inclusion of adjuvants or optimisation of delivery method or immunisation regime, e.g. heterologous prime-boost (Ferraro *et al.*, 2011).

The first vaccine strategy to be 100% protective against lethal EBOV challenge in NHPs was a DNA prime vaccine in conjunction with a recombinant human adenovirus serotype 5

(Ad5) vector expressing EBOV GP boost (Sullivan *et al.*, 2000). Cynomolgus macaques were immunised with three doses of DNA encoding EBOV GP, NP and SUDV GP at four week intervals, followed by an Ad5-EBOV GP boost three months later. The NHPs were challenged with a lethal dose of EBOV three months after the final vaccination. All animals survived infection and displayed no clinical signs of disease or detectable viremia.

A Phase I clinical trial demonstrated that three doses of the DNA vaccine was safe and well tolerated in humans (Martin *et al.*, 2006). Antigen-specific antibody and T cell responses were induced, however multiple doses were required and responses declined rapidly.

Virus like particles (VLPs)

VLPs can be utilised as a non-replicating, protein subunit-based vaccine platform. VLPs have similar morphology to that of the authentic virus and are considered safe and immunogenic (Martins *et al.*, 2013; Warfield and Aman, 2011).

EBOV VLPs can be produced by expression of VP40 and GP in either mammalian or insect cells, and in some cases the NP is also included (Warfield and Aman, 2011). NHPs vaccinated three times at six week intervals with EBOV VLPs plus RIBI adjuvant elicited EBOV-specific humoral and cellular immune responses (Warfield *et al.*, 2007). Animals were completely protected from lethal EBOV challenge four weeks after the last vaccination, with no clinical signs or detectable viremia. NHPs vaccinated with two doses of EBOV- or SUDV-like particles plus QS-21 adjuvant were completely protected against lethal EBOV or SUDV challenge, respectively (Warfield *et al.*, 2015). Furthermore, the EBOV-like particles also provided cross-protection against TAFV, suggesting that development of a cross-protective VLP-based vaccine may be possible.

Non-replicating viral vectors

Several promising viral vector-based vaccine candidates expressing EBOV genes have been shown to protect NHPs from EBOV infection (Reynolds and Marzi, 2017).

Replication-deficient adenoviruses are commonly employed as viral vector platforms for the development of vaccines. A single immunisation of Ad5 expressing EBOV GP was able to protect cynomolgus macaques against lethal EBOV challenge four weeks later, and protection correlated with antigen-specific antibody responses (Sullivan *et al.*, 2006; Wong *et al.*, 2012). However, passive transfer of polyclonal antibodies from Ad5-EBOV GP-vaccinated to naïve NHPs induced only partial survival, suggesting a limited role for humoral immunity (Sullivan *et al.*, 2011). CD8+ T cell depletion resulted in abrogation of protective immunity, with only 20% survival following EBOV challenge, indicating that CD8+ T cells are important for Ad5-EBOV GP-induced immune protection against EBOV infection in NHPs. A single dose of an Ad5-vectored vaccine expressing the Makona EBOV GP elicited specific humoral and T cell immunity in NHPs, and conferred 100% protection when animals were challenged with EBOV four weeks after vaccination (Wu *et al.*, 2016).

Clinical trials have demonstrated that the Ad5-EBOV GP vaccine is safe and immunogenic in humans, however the short duration of antibody responses raised the possibility that a prime-boost strategy may be required to induce more durable immunity (Li *et al.*, 2017; Zhu *et al.*, 2015; Zhu *et al.*, 2017). Furthermore, there are concerns regarding pre-existing immunity to the Ad5 vector in humans, which may attenuate humoral and cellular immunogenicity (Ledgerwood *et al.*, 2010; Mast *et al.*, 2010), and therefore the protective efficacy of AdHu5-based vaccines (Kobinger *et al.*, 2006). This can be circumvented by using different Ad serotypes that exhibit lower seroprevalence in humans, such as Ad26 and Ad35, or by using chimpanzee Ad (ChAd) serotypes.

Vaccination of NHPs with Ad26-EBOV GP and SUDV GP followed by a boost with Ad35-EBOV GP and SUDV GP one month later induced antigen-specific B and T responses, and resulted in complete protection against lethal EBOV challenge four weeks after boost vaccination (Geisbert *et al.*, 2011).

The Ad26-EBOV GP vaccine together with a Modified Vaccinia Ankara (MVA) vector expressing filovirus antigens (EBOV GP, SUDV GP, MARV GP and TAFV NP) (MVA-BN-filo) was assessed in a Phase I clinical trial and demonstrated good safety and immunogenicity (Milligan *et al.*, 2016). Immune responses were observed following primary immunisation with Ad26-EBOV GP, and boosting with MVA-BN-Filo resulted in sustained elevation of EBOV GP-specific immunity, with humoral immune responses persisting for up to one year (Winslow *et al.*, 2017).

A single inoculation of a replication-deficient ChAd3 vector expressing EBOV GP provided complete protection of cynomolgus macaques from lethal EBOV infection five weeks after vaccination, which was associated strongly with antibody responses. However humoral and cellular responses declined over time and protection waned to 50% over ten months (Stanley *et al.*, 2014). Boosting of the ChAd3-EBOV GP vaccine with MVA expressing EBOV and SUDV GP eight weeks later was able to provide 100% protection from lethal EBOV infection ten months post-prime vaccination. This long-term protection was attributed to the generation of both effector and memory CD8+ T cells.

The ChAd3-based vaccine was accelerated for human Phase I clinical trials during the 2013-2016 EBOV epidemic in West Africa, either as a monovalent vaccine expressing EBOV GP only (De Santis *et al.*, 2016), or as a bivalent vaccine expressing the GPs of both EBOV and SUDV (Ledgerwood *et al.*, 2017). Both vaccines were shown to be safe and immunogenic in humans. For the bivalent strategy, antigen-specific antibody responses measured four weeks after vaccination were in the range reported to be associated with vaccine-induced protection in NHP challenge studies, and responses were sustained to week 48. However for the monovalent formulation, antibody titres were lower and started to decrease by six months post-vaccination. Boost of either vaccine with MVA-BN-filo was safe, and enhanced antigen-specific cellular and humoral immune responses compared to ChAd3 vaccination

alone, suggesting that a prime-boost strategy could confer long-term protection against EBOV infection (Ewer *et al.*, 2016; Tapia *et al.*, 2016).

Replication-competent viral vectors

Replicating viral vectors are highly immunogenic and elicit robust immune responses; however they carry the risk of recombination and reactogenicity (Ura *et al.*, 2014).

VSV naturally infects livestock and various animals, and can cause asymptomatic infection or mild, flu-like illness in humans. However human infection is very rare, resulting in very limited pre-existing immunity in the human population. The VSV surface glycoprotein (G) can be replaced with a heterologous viral surface protein, resulting in an attenuated replication-competent virus that can be used as a vaccine (Garbutt *et al.*, 2004; Lawson *et al.*, 1995; Roberts *et al.*, 1999).

A single dose of a VSV-EBOV GP vaccine was able to fully protect NHPs from lethal EBOV challenge four weeks post-vaccination (Jones *et al.*, 2005). The surviving animals were challenged with SUDV 234 days later, however only 25% survived, indicating a lack of cross-protection between ebolavirus species. A single-dose blended vaccine containing VSV-EBOV, VSV-SUDV and VSV-MARV GP protected NHPs from lethal EBOV, SUDV, TAFV and MARV challenge 28 days after vaccination, demonstrating the potential for a multivalent vaccine (Geisbert *et al.*, 2009). NHPs depleted of CD8+ T cells during vaccination survived subsequent EBOV challenge, suggesting a minimal role for CD8+ T cells in VSV-EBOV GP-mediated protection. Whereas animals depleted of CD4+ T cells during vaccination succumbed to infection and did not have a detectable anti-EBOV GP IgG response. In contrast, depletion of CD4+ T cells during challenge resulted in survival of the animals. These results demonstrated a minimal role for CD4+ T cells in VSV-EBOV GP-mediated protection against lethal EBOV infection, whereas the presence of EBOV GP-specific antibodies was required for survival, indicating that antibodies play a critical role in VSV-

EBOV GP-mediated protection against EBOV infection (Marzi *et al.*, 2013). In addition, the VSV-EBOV GP vaccine was able to completely protect NHPs from lethal EBOV challenge as early as seven days post-vaccination, suggesting that the vaccine is fast-acting and could be used for ring vaccination strategies during outbreaks (Marzi *et al.*, 2015b).

These preclinical studies supported the acceleration of VSV-EBOV GP clinical trials during the 2013-2016 West African EVD outbreak. Phase I trials demonstrated that the vaccine was safe and generally well tolerated in humans, although mild to moderate side effects and adverse reactions were observed in some participants (Agnandji *et al.*, 2017; Agnandji *et al.*, 2016; ElSherif *et al.*, 2017; Huttner *et al.*, 2015; Regules *et al.*, 2017). The vaccine was immunogenic and induced EBOV GP-specific antibodies, which were sustained up to two years after vaccination (Heppner *et al.*, 2017; Huttner *et al.*, 2018; Kennedy *et al.*, 2017).

There is little information regarding the cell-mediated immune response to VSV-EBOV GP vaccination in humans (Lai *et al.*, 2015), although studies have reported the generation of EBOV GP-specific T cells and cytokine networks following immunisation (Dahlke *et al.*, 2017a; Farooq *et al.*, 2016).

The VSV-EBOV GP vaccine was used in a ring vaccination Phase III efficacy trial in Guinea to assess the efficacy of the vaccine to protect against EVD, and therefore prevent EBOV transmission and control the epidemic (Henao-Restrepo *et al.*, 2017). The results indicated that VSV-EBOV GP is a safe and fast-acting vaccine, and showed 100% efficacy after 10 days of vaccination with no new cases of EVD occurring among immediately vaccinated contacts and contacts of contacts. This study demonstrated the feasibility of using the VSV-EBOV GP vaccine in a ring vaccination design to help control outbreaks. However, there are still some concerns regarding the safety of VSV-EBOV GP, especially in vulnerable populations such as children and young adults, pregnant women or immunocompromised individuals (Agnandji *et al.*, 2017). In March 2016, ring vaccination with the VSV-EBOV GP vaccine was

introduced in Guinea in response to a reported flare up of EVD, suggesting that ring vaccination can be rapidly and effectively implemented as part of the response to EVD outbreaks (Gsell *et al.*, 2017). Subsequently, the VSV-EBOV GP vaccine was used in 2018 in the DRC to vaccinate high risk populations against EVD in affected areas, and has also been used to vaccinate over 60,000 individuals during the ongoing EBOV outbreak in the DRC (World Health Organization, 2019).

1.5.4 Correlates of protection

In 2002, the US Food and Drug Administration (FDA) established the 'animal rule' for regulatory approval of drugs and biological products when human efficacy studies are not ethical and field trials to study effectiveness are not feasible (Food and Drug Administration, 2015). The animal rule allows data from animal studies to provide substantial evidence of effectiveness when the following criteria are met:

1. There is a reasonably well-understood pathophysiological mechanism of the toxicity of the substance and its prevention or substantial reduction by the product;
2. The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterised animal model for predicting the response in humans;
3. The animal study endpoint is clearly related to the desired benefit in humans, generally the enhancement of survival or prevention of major morbidity; and
4. The data or information on the kinetics and pharmacodynamics of the product or other relevant data or information, in animals and humans, allows selection of an effective dose in humans.

NHPs are considered the 'gold standard' animal model for EBOV (Bennett *et al.*, 2017; Geisbert *et al.*, 2015), as they can be lethally infected with non-adapted human isolates of

EBOV and the resulting disease pathogenesis closely resembles that observed in humans (Bente *et al.*, 2009; Feldmann and Geisbert, 2011).

Bridging of animal protection data to humans is a key aspect of the demonstration of efficacy using animal data. An immune correlate can be used to bridge the gap between animal efficacy studies and human immunogenicity trials. A correlate, as defined here, is an immune response that is responsible for and statistically interrelated with protection (Plotkin, 2010). However the human correlates of protection for EBOV are currently unclear (Bradfute and Bavari, 2011). Studies of EVD survivors have identified qualitative associations between immune end points and survival; however they have not identified predictive markers of protective immunity. Furthermore, immunity that contributes to survival during natural infection may not be the same as immunity that can protect following active or passive immunisation.

The protective effect of antibodies against EVD had been unclear based on early passive transfer studies in animals and humans (Zeitlin *et al.*, 2016), particularly polyclonal and convalescent blood product preparations (discussed in Section 1.5.1). However NHP studies using anti-EBOV mAbs have highlighted the importance of antibodies in controlling EBOV infection (discussed in Section 1.5.2). Although it is not clear what antibody mechanism (i.e. neutralisation of Fc-mediated effector functions) is associated with protection (Gunn *et al.*, 2018; Saphire *et al.*, 2018).

Several vaccines have been shown to be effective against EBOV challenge in NHP studies and have been evaluated for safety and immunogenicity in clinical trials (discussed in Section 1.5.3). However is unknown how well correlates of protection in NHPs apply to humans. Furthermore, it is likely that correlates of protection induced by vaccination are different depending on the vaccine platform used, and are possibly related to underlying immune mechanisms of virus clearance (Sullivan *et al.*, 2009). EBOV GP-specific antibodies

have been shown to be associated with protection of NHPs after vaccination with VSV-EBOV GP vaccine (Marzi *et al.*, 2013), whereas CD8+ T cells may be more important for Ad5-EBOV GP-induced immune protection against EBOV in NHPs (Sullivan *et al.*, 2011). In addition, variations in methodology used for assessment of immunogenicity, and the absence of standard assays introduce uncertainty into comparisons of different vaccine platforms and clinical trials.

1.5.5 Immune escape

Typically, RNA viruses have high spontaneous mutation rates due to error-prone RNA-dependent RNA polymerases (Alfson *et al.*, 2015; Holland *et al.*, 1982). During virus infection and transmission from person-to-person, EBOV may be predicted to evolve and changes within the genome be selected. During the 2013-2016 EBOV outbreak, sequencing studies were performed to assess mutation rates and to support molecular epidemiology (Carroll *et al.*, 2015; Gire *et al.*, 2014; Park *et al.*, 2015; Quick *et al.*, 2016; Simon-Loriere *et al.*, 2015; Tong *et al.*, 2015). These results and other data revealed mutations in the genetic sequence encoding EBOV GP. Preliminary studies into this topic suggest that a mutation encoding a valine substitution for alanine at residue 82 of the EBOV GP that appeared early during the 2013-2016 epidemic, is associated with increased infectivity of human cells (Diehl *et al.*, 2016; Dietzel *et al.*, 2017; Kurosaki *et al.*, 2018; Ueda *et al.*, 2017; Urbanowicz *et al.*, 2016b), potentially by reducing the threshold for activation of GP_2 (Wang *et al.*, 2017). However no significant differences were found in disease progression, pathogenicity or virus shedding of EBOV Makona isolates derived from different stages of the epidemic in rhesus macaques (Marzi *et al.*, 2018).

As mentioned above, EBOV GP is a target for novel EBOV vaccines and immunotherapies. Therefore mutations in EBOV GP may have important implications for anti-GP-based interventions, i.e. changes in the EBOV GP may affect the ability of antibodies to bind,

thereby leading to the emergence of escape mutants (Kugelman *et al.*, 2015a; Kugelman *et al.*, 2015b; Miller *et al.*, 2016). Investigation of how changes in EBOV GP might affect antibody neutralisation is the subject of this study.

1.6 Hypothesis

The following hypothesis was investigated:

- Naturally acquired mutations in EBOV GP can result in escape from neutralising antibodies derived from EVD convalescent volunteers, EBOV GP vaccinated individuals, and EBOV GP-specific mAbs.

To test the above hypothesis, specific objectives for the study were to:

1. Optimise an EBOV GP pseudotyped virus system and compare neutralisation by EVD survivor plasma with live EBOV.
2. Identify mutations that arose in the EBOV GP during the 2013-2016 EBOV epidemic that may have an impact on immune escape.
3. Generate EBOV GP mutant pseudotyped viruses and assess escape from neutralisation by anti-EBOV GP polyclonal and mAb samples.

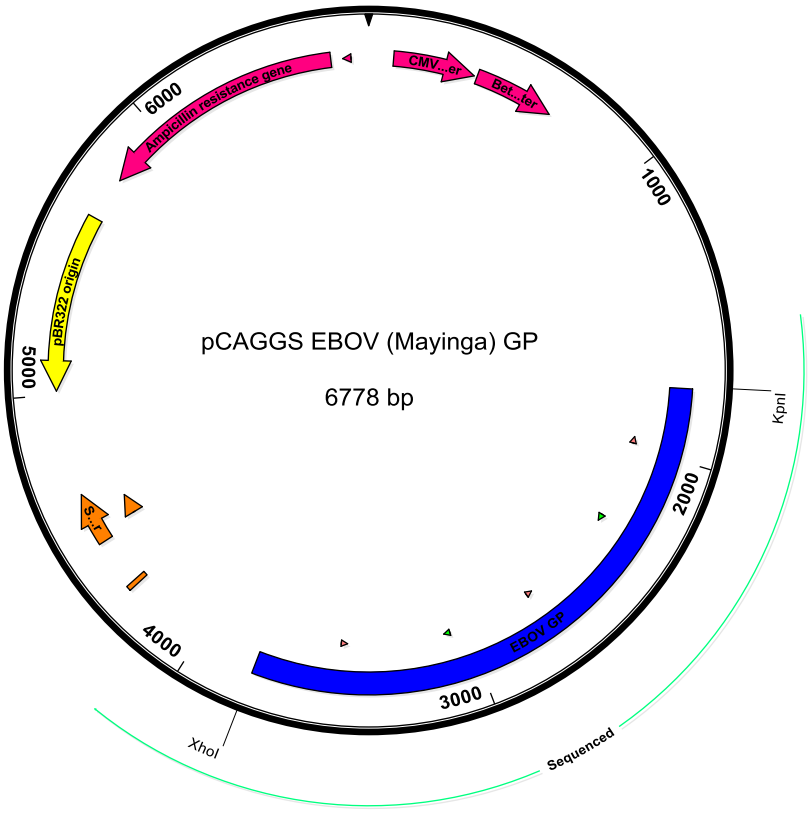
Chapter 2 Materials and Methods

2.1 Plasmids for generation of pseudotyped viruses

2.1.1 Plasmids

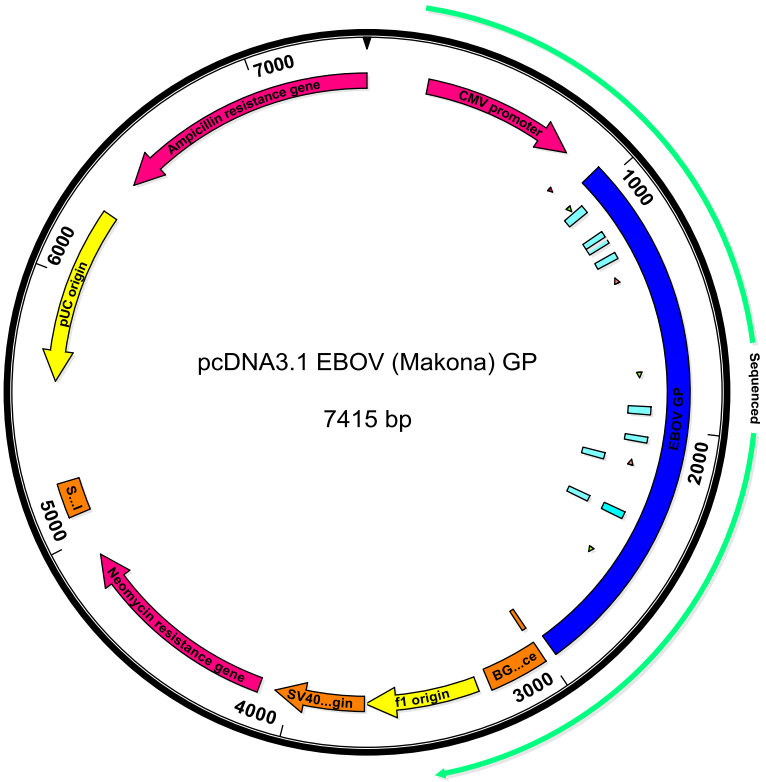
The HIV-1 *gag-pol* plasmid p8.91 (Zufferey *et al.*, 1997), the firefly luciferase reporter construct pCSFLW (Wright *et al.*, 2008), and a pCAGGS EBOV (Mayinga) GP (GenBank accession number NC_002549) expression construct (Figure 2.1A) were kind gifts from Edward Wright [University of Westminster, London, United Kingdom (UK)]. A pcDNA3.1 expression plasmid (ThermoFisher Scientific, Paisley, UK) for codon optimised EBOV (Makona) GP [GenBank accession number KJ660348 (Baize *et al.*, 2014)] (Figure 2.1B) was kindly provided by Georgios Pollakis (University of Liverpool, Liverpool, UK). A VSV-G expression plasmid was obtained from Masayuki Shimojima (National Institute of Infectious Diseases, Tokyo, Japan).

A



Feature	Plasmid bases
CMV enhancer	87 – 371
Beta-actin promoter	382 – 662
EBOV GP	1757 – 3787
M13 reverse primer	4281 – 4297
SV40 promoter	4465 – 4644
SV40 origin	4500 – 4577
pBR322 origin	5013 – 5632
Ampicillin resistance gene	5787 – 6647
AmpR promoter	6689 – 6717

B



Feature	Plasmid bases
CMV promoter	232 – 819
T7 promoter/priming site	863 – 882
EBOV GP	944 – 2971
BGH reverse priming site	3009 – 3026
BGH polyadenylation sequence	3015 – 3239
f1 origin	3285 – 3713
SV40 early promoter and origin	3718 – 4061
Neomycin resistance gene	4123 – 4917
SV40 early polyadenylation signal	5091 – 5221
pUC origin	5604 – 6274
Ampicillin resistance gene	6419 – 7415

Figure 2.1: A) pCAGGS EBOV (Mayinga) and B) pcDNA3.1 EBOV (Makona) GP expression vectors were sequenced across the ORF to obtain continuous data for one or both strands. The sequenced region is indicated by a green arrow. The positions of primer sites utilised in the sequencing and site-directed mutagenesis processes are also highlighted. The plasmid maps were generated in SeqBuilder DNA Lasergene 11 (Madison, WI, USA).

2.1.2 *E. coli* transformation

One Shot® TOP10 competent cells (Invitrogen, Paisley, UK) were transformed with plasmid DNA according to the manufacturer's instructions. Briefly, 1 or 5 µl of plasmid DNA was added directly to the vial of competent cells, mixed by tapping gently, and incubated on ice for 30 minutes. The cells were incubated for exactly 30 seconds in a 42°C water bath then placed on ice. 250 µl of pre-warmed Super Optimal broth with Catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each vial and the cells were shaken at 37°C for exactly 1 hour at 225 rpm in a shaking incubator. The transformation mix was diluted in SOC medium and spread on Luria Bertani (LB) agar (Lennox) (15 g/l agar, 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) (Sigma-Aldrich, Gillingham, UK) containing 100 µg/ml ampicillin (Sigma-Aldrich). The plates were inverted and incubated at 37°C overnight.

2.1.3 *E. coli* culture

E. coli clones were amplified in liquid culture by transferring a single colony from a freshly streaked selective agar plate to 5 ml LB Lennox medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 2.2 g/l inert binding agents) (Sigma-Aldrich) containing 100 µg/ml ampicillin. Cultures were incubated for approximately 8 hours at 37°C with vigorous shaking (approximately 250 rpm). Cultures were diluted 1/500 into 100 ml (high-copy plasmids) selective LB Lennox medium and grown at 37°C for 12 to 16 hours with vigorous shaking (approximately 250 rpm). The bacterial cells were harvested by centrifugation at 3000 x *g* for 30 minutes at 4°C and the supernatant discarded.

2.1.4 Plasmid DNA purification

Plasmid DNA was extracted from transformed *E. coli* using EndoFree® Plasmid Maxi or QIAprep® plasmid Miniprep kits (both Qiagen, Manchester, UK) as per the manufacturer's instructions. Briefly, neutralised bacterial lysates were cleared by centrifugation and

loaded onto anion-exchange tips, where plasmid DNA selectively binds under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low-molecular-weight impurities were then removed by a medium-salt wash, and ultrapure plasmid DNA was eluted in high-salt buffer. The DNA was concentrated and desalted by isopropanol precipitation and collected by centrifugation. Plasmid DNA was dissolved in endotoxin-free Buffer Tris-EDTA (TE) or nuclease-free water and the concentration determined by spectrophotometry (Nanodrop 2000; ThermoFisher Scientific).

2.1.5 Restriction enzyme digest

Restriction enzymes (New England Biolabs (NEB), Hitchin, UK) were used according to the manufacturer's instructions. Briefly, 1 unit of enzyme (1 µl) was added to 1 µg DNA (plasmid). Each reaction included the appropriate NEB buffer, with or without bovine serum albumin (BSA) as prescribed and was made up to the required volume with nuclease-free water. The reaction was incubated at 37°C for at least 1 hour. For digestion with two enzymes, a double-digest was performed provided enzyme conditions were compatible.

2.1.6 Separation of DNA fragments by agarose gel electrophoresis

Horizontal gels were prepared by dissolving agarose (1%) in Tris-acetate ethylenediaminetetraacetic acid (TAE) buffer. Sybr® Safe DNA gel stain (ThermoFisher Scientific) was added so that DNA fragments were visible under ultra-violet light for image capture (UVIdoc). Samples of DNA were loaded into each gel using gel loading dye and were run in parallel with 1 kb DNA ladder (ThermoFisher Scientific). Electrophoresis was performed at 100 V for up to 1 hour.

2.1.7 Site-directed mutagenesis

Site-directed mutagenesis was carried out on plasmid pcDNA3.1 EBOV (Makona) GP using QuikChange Lightning Site-Directed Mutagenesis kits (Agilent Technologies, Stockport, UK) as per the manufacturer's instructions. Briefly, 125 ng forward and reverse primers were

added. Primers (Table 2.1) were designed using the manufacturer's online primer design tool. They were required to be 25-45 base pairs in length with base pair changes at or near the centre of the sequence. Template dsDNA was present at between 10 and 100 ng. 5 µl 10x reaction buffer, 1 µl dNTP mix and 1.5 µl QuikSolution reagent were added. The reaction volume was made up to 50 µl using nuclease-free water and 1 µl of QuikChange Lightning Enzyme was added.

Table 2.1 Primers designed for site-directed mutagenesis. Base pair changes were made to plasmid pcDNA3.1 EBOV (Makona) GP in order to introduce the desired amino acid substitution. Mutations are shown in red bold letters.

Amino acid change	Primer name	Primer sequence (5' to 3')
A82V	C245T_C246G	F CCCCATCTCTTGGT**CA**CGCTGGGCACATCG
		R CGATGTGCCCAGCG**TG**ACCAAGAGATGGGG
G74R	G220C_C222G	F GGTGGCCAC**C**C**G**ATTGCCCTCGAGGTTC
		R GAACCTCGAGGGCAAT**C**G**G**GTGGCCACC
R29K	C85A_G86A	F GGGGGATGCTGAAGGTC**TT**CTGGAACAGGATGATGA
		R TCATCATCCTGTTCCAG**AA**GACCTTCAGCATCCCCC
I371L	A1111C_C1113G	F GAGGGCTGGTGCT**C**A**G**GGTGGCCAGGGTG
		R CACCCTGGCCACC**C**T**G**AGCACCAGCCCTC
G480S	G1438A	F GTTGGTGATCAGGC**T**CAGCTTGCCAGAGC
		R GCTCTGGCAAGCTG**A**GCCTGATCACCAAC
G480D	G1439A	F GTGTTGGTGATCAGG**T**CCAGCTTGCCAGAGC
		R GCTCTGGCAAGCTGG**A**CCTGATCACCAACAC
P330S	C988A_C989G	F GGTGGTGTTTGTCTCG**CT**GTCGCTGCTGGTTCTG
		R CAGAACCAGCAGCGAC**AG**CGAGACAAACACCACC
N107D	A319G	F CAGGTTGTAGCAGT**C**CTCGGCCCACTCGC
		R GCGAGTGGGCCGAG**G**ACTGCTACAACCTG
H407Y	C1219T	F GCCCGTCTGTGGT**A**CTGTCCCACTTGG
		R CCAAGTGGGACAG**T**ACCACAGACGGGC

Initial denaturation was performed at 95°C for 2 minutes. Cycling conditions were 18 cycles of 95°C for 20 seconds, 60°C for 10 seconds and 68°C for 30 seconds per kilobase (kb) of plasmid length. A final extension step of 68°C for 5 minutes was performed before cooling the reaction to 4°C. 2 µl of *Dpn* I restriction enzyme was added directly to each amplification reaction, mixed gently and thoroughly, and incubated immediately at 37°C for 5 minutes to digest the parental supercoiled dsDNA. Mutated DNA was then transformed into *E. coli* XL10-Gold ultracompetent cells.

2.1.8 DNA sequencing

Sanger sequencing of DNA samples was performed using an external sequencing service (GENEWIZ, Takeley, UK). Plasmid DNA was sent at, or above, the minimum concentration and volume required. Where universal primers were not available, sequences for custom synthesised primers (Table 2.2) were provided. Primers were designed to bind to the template sequence at approximately 700 base pair intervals, were typically 20-30 nucleotides long and had a guanine-cytosine (GC)-content of between 40 and 60%. Sequences with secondary structure [examined using an online tool (http://biotools.nubic.northwestern.edu/OligoCalc.html)] were avoided.

Table 2.2 Custom primers designed for DNA sequencing of Ebola virus (EBOV) Makona and Mayinga glycoprotein (GP). Forward (F) and reverse (R) primers were designed to bind to the template sequence at approximately 700 base pair intervals.

Primer name		Primer sequence (5' to 3')
EBOV Makona GP		
1	F	ATCGATTCAAGCGGACCAGCTTC
2	F	GAAAGTTAACCCCGAGATCGACAC
3	F	ATCTACACCGAGGGCCTGATGCA
4	R	GGACACCTTGTGCACGTATCTGCAT
5	R	TCTGTGGTGCTGTCCCACTTGGGT
EBOV Mayinga GP		
6	F	GGTGTCGTTGCATTTCTGATACTGCCCC
7	F	CGAGCAAGAGCACTGACTTCCTGGA
8	R	GCCACTCCATTCCCTTCGAGATTCAG
9	R	CGCCGGACTCTGACCACTGATGTTT
10	R	TGCGTAGCTCAGTTGTGGCTCTCAG

2.2 Cells for pseudotyped virus assays

2.2.1 Cell culture

Cell line Human embryonic kidney (HEK) 293T/17 (CRL-11268 American Type Culture Collection (ATCC), Teddington, UK) was maintained in Dulbecco's Modified Eagle Medium (DMEM), high glucose, with L-glutamine (Gibco®, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), heat inactivated (Sigma-Aldrich). Cells were maintained in static tissue culture flasks, incubated at 37°C, 5% CO_2 and 95% humidity. Cells were passaged every 2-3 days. Briefly, the cell monolayer was washed with phosphate buffered saline (PBS) without calcium or magnesium (Gibco®) using a volume equivalent to half the volume of culture medium. Trypsin-EDTA (0.05%) (Gibco®) was pipetted onto the washed cell monolayer using 1 ml per 25 cm^2 of surface area. The culture flask was rotated to cover the monolayer with trypsin and incubated at 37°C, 5% CO_2 and 95% humidity for 2 to 5 minutes. The

culture flask was examined to ensure that cells were detached, and the side of the culture flasks was gently tapped to release any remaining attached cells if necessary. The cells were resuspended in a small volume of fresh serum-containing medium to inactivate the trypsin. The required volume of cells was transferred to a fresh culture flask containing pre-warmed medium up to the appropriate volume. No more than a total of 50 passages were performed.

Cell lines Vero C1008 [Vero 76, clone E6, Vero E6 (85020206 European Culture of Authenticated Cell Cultures (ECACC), Salisbury, UK) and Huh-7 (Arvind Patel, University of Glasgow, UK) cells were maintained as described above. Cells were passaged every 2-4 days.

HeLa cells (ECACC 93021013) were cultured as described above in Minimum Essential Media (MEM) + GlutaMAX (Life Technologies, Paisley, UK) supplemented with 10% FBS and 1x MEM Non-Essential Amino Acids (NEAA) solution (Life Technologies). Cells were passaged every 2-3 days.

2.3 Generation of pseudotyped viruses

2.3.1 Production of EBOV GP pseudotyped lentiviruses by transfection

The generation of lentiviral pseudotyped viruses was performed as detailed previously (Mather *et al.*, 2014; Temperton *et al.*, 2007; Wright *et al.*, 2008). 24 hours prior to transfection, approximately 8 x 10^5 293T/17 cells were seeded into sterile, 6 well cell culture plates (Corning, Ewloe, UK) and incubated at 37°C, 5% CO_2 and 95% humidity until 60-80% confluence. The medium was replaced with fresh medium. The HIV *gag-pol* plasmid, p8.91, and the firefly luciferase reporter construct, pCSFLW, were transfected simultaneously with the EBOV (Mayinga) GP expression vector at a ratio of 0.6:0.9:0.6 µg

(core:reporter:envelope) using 10 µl of 1 µg/ml polyethylenimine (PEI) (408727 Sigma-Aldrich) per 1 µg DNA in Opti-MEM® medium (Gibco®). Following overnight transfection, the cells were incubated with fresh medium and incubated at 37°C, 5% CO_2. Pseudotyped virus supernatants were harvested at 48 and 72 hours post-transfection, passed through a 0.45 µm pore filter (Millex®, Millipore, Watford, UK) and stored at -80°C.

2.3.2 Production of EBOV GP pseudotyped VSV-ΔG by transfection and infection

EBOV GP pseudotyped viruses were prepared using rVSV-ΔG with luciferase reporter (rVSV-ΔG-Luc) by a method similar to that described previously (Whitt, 2010). 24 hours prior to transfection, approximately 2.4 x 10^6 293T/17 cells were seeded into sterile, 100 mm cell culture dishes (Corning) and incubated at 37°C, 5% CO_2 and 95% humidity until 60-80% confluence. The medium was replaced with fresh medium (DMEM, 10% FBS). The cells were transfected with the EBOV GP expression vectors using *Trans*IT®-LT1 Transfection Reagent (Mirus Bio, Madison, Wisconsin (WI), USA) as per the manufacturer's instructions. Briefly, the *Trans*IT®-LT1 Reagent was warmed to room temperature, and vortexed gently before use. For each 100 mm cell culture dish, 45 µl *Trans*IT®-LT1 Reagent was added to 600 µl Opti-MEM® medium in a sterile tube and incubated for 5 minutes at room temperature. 15 µg plasmid DNA was added to 600 µl Opti-MEM® medium in a separate sterile tube. The diluted DNA mixture was the added to the diluted *Trans*IT®-LT1 Reagent, pipetted gently to mix completely, and incubated at room temperature for 15 to 30 minutes. The *Trans*IT®-LT1 Reagent:DNA complex was added drop-wise to different areas of the cell layer and the culture dish was gently rocked back-and-forth and from side-to-side to evenly distribute the *Trans*IT®-LT1 Reagent:DNA complex. Following overnight transfection, the medium was removed and the cells were infected with rVSV-ΔG-Luc-VSV-G virus at a MOI of 5 in Opti-MEM® medium and incubated at 37°C, 5% CO_2. After 2 hours, the inoculum was removed, cells were washed twice with Dulbecco's phosphate buffered saline (DPBS) (Gibco®), and fresh medium was added. Pseudotyped virus supernatants

were harvested at 18-24 hours post-infection, clarified twice by centrifugation at 200 x *g* for 5 minutes at 10°C and stored at -80°C. Prior to use, the pseudotyped viruses were incubated with anti-VSV-G hybridoma cell culture supernatant (Masayuki Shimojima, National Institute of Infectious Diseases, Tokyo, Japan) at a 1:125 dilution for 1 hour at 37°C.

2.3.3 Expansion of rVSV-ΔG-Luc-VSV-G virus stock by transfection and infection

rVSV-ΔG-Luc-VSV-G virus stock was amplified by a method similar to that described above to generate EBOV GP pseudotyped VSV (Section 2.3.2), except that the virus was pseudotyped with VSV-G, and the infection was performed at a lower MOI, to prevent accumulation of defective-interfering particles (Whitt, 2010). 293T/17 cells were seeded and transfected with a VSV-G expression plasmid as described in Section 2.3.2. Following overnight transfection, the medium was removed and the cells were infected with rVSV-ΔG-Luc-VSV-G virus at an MOI of 0.1 in Opti-MEM® medium and incubated at 37°C, 5% CO_2. After 1-2 hours, the inoculum was removed, cells were washed twice with DPBS, and fresh medium was added. rVSV-ΔG-Luc-VSV-G virus supernatants were harvested and stored as described in Section 2.3.2.

2.4 Titration of pseudotyped viruses

2.4.1 Pseudotyped lentivirus titration by luciferase assay

Five-fold serial dilutions of pseudotyped virus at a starting dilution of 1:5 were prepared in quadruplicate in Opti-MEM® medium at a final volume of 100 µl/well in 96 well solid white flat bottom polystyrene TC-treated microplates (Corning). 100 µl of approximately 2 x 10^4 293T/17, Huh-7 or Vero E6 cells, or 1 x 10^4 HeLa cells were then added to each well and incubated at 37°C, 5% CO_2 for 48 hours. The medium was removed and 50 µl of a 50:50

mix of Bright-Glo™ luciferase assay reagent (Promega, Southampton, UK):fresh medium was added to each well, and incubated for at least 2 minutes at room temperature to allow complete cell lysis. Luminescence was measured within 5 minutes following lysis using a Glomax®-Multi+ detection system luminometer (Promega), and relative luminescence units per ml (RLU/ml) were determined.

2.4.2 Pseudotyped VSV-ΔG titration by luciferase assay

24 hours prior to transduction, approximately 2.5 x 10^4 293T/17 or 1 x 10^4 Huh-7, HeLa cells or Vero E6 cells were seeded in 96 well solid white flat bottom polystyrene TC-treated microplates and incubated at 37°C, 5% CO_2 and 95% humidity. The medium was removed and two-fold serial dilutions of pseudotyped virus in Opti-MEM® medium, starting with neat pseudotyped virus were added to each well in quadruplicate at a final volume of 100 µl/well. After 24 hours, a chemiluminescent readout was taken as described above (Section 2.4.1). The negative cut-off was set at 2.5 times the average of the cells only control wells. 50% tissue culture infectious dose ($TCID_{50}$)/ml values were determined using the Reed-Muench method (Reed and Muench, 1938).

2.4.3 rVSV-ΔG-Luc-VSV-G virus titration by plaque assay

24 hours prior to transfection, approximately 3.0 x 10^5 Vero E6 cells were seeded into 12 well plates (ThermoFisher Scientific) and incubated at 37°C, 5% CO_2 and 95% humidity. The medium was replaced with fresh medium. The cells were transfected with a pVSV-G expression plasmid using *Trans*IT®-LT1 Transfection Reagent (Mirus Bio) as per the manufacturer's instructions as described above (Section 2.3.2). At 5-6 hours post-transfection, the medium was removed and the cells were inoculated with 200 µl/well of a ten-fold dilution series of rVSV-ΔG-Luc-VSV-G virus in Opti-MEM® medium at a starting dilution of 1:1000 in duplicate, and incubated at 37°C, 5% CO_2. After 1 hour, the inoculum was removed and cells were washed twice with Opti-MEM® medium. Approximately 1 ml

of overly medium (1% again prepared in DMEM, 5% FBS) was added to each well and incubated at 37°C, 5% CO_2 for 48 hours. Agar plugs were removed and cells were fixed in 20% formaldehyde DPBS for at least 1 hour. Cells were then stained with crystal violet. Plates were washed with tap water, air dried, and plaques were counted.

The rVSV-ΔG-Luc-VSV-G virus was also assayed for luciferase activity in 293T/17 cells at 24 hours post-infection as described above (Section 2.4.2), using a five-fold serial dilution series of pseudotyped virus at a starting dilution of 1:25.

2.5 Neutralisation of pseudotyped viruses

2.5.1 Human plasma samples

Plasma samples from EVD survivors of the 2013-2016 EBOV outbreak, recruited 3 to 14 months post-infection from two regions of Guinea (Gueckedou and Coyah), and from negative control blood donors in the UK and Guinea (Table 2.3) were heat inactivated at 56°C for 30 minutes. The samples were obtained from a pre-existing biobank, for which live EBOV neutralisation (Agnandji *et al.*, 2016) data were available (Thomas Strecker, Philipps University of Marburg, Germany) in link-anonymised format. The biobank was established by Horizon 2020 EU research initiative 'EVIDENT' under appropriate ethical approval from the Guinean National Ethics Committee.

Table 2.3 Ebola virus disease (EVD) survivor and negative plasma samples tested in EBOV GP pseudotyped virus neutralisation assays. Plasma samples from EVD survivors of the 2013-2016 EBOV outbreak, recruited 3 to 14 months post-infection from two regions of Guinea (Gueckedou and Coyah), were obtained from a pre-existing biobank. Live EBOV (Mayinga) neutralisation data were available for each sample.

Sample	Sex	Age (years)	Date discharged from ETC	Live EBOV (Mayinga) neutralisation (GMT)
Gueckedou – Survivors				
G001	-	-	15-Apr-14	181
G002	-	-	03-May-14	4
G003	-	-	01-Jul-14	91
G004	M	62	10-Oct-14	76
G005	F	48	08-Sep-14	128
G006	M	26	01-Jul-14	4
G007	F	38	08-Oct-14	362
G008	F	35	08-Sep-14	609
G009	F	45	17-Sep-14	32
G010	M	37	29-May-14	76
G011	F	26	08-May-14	645
G013	F	28?	13-Sep-14	108
G014	M	40	05-Jun-14	181
G015	M	28	05-Jun-14	128
G016	M	-	08-Jun-14	38
G017	M	38	27-May-14	724
G018	F	40	30-Dec-14	54
G019	M	-	17-Dec-14	38
G020	F	27	31-Dec-14	45
G021	F	48	07-Jun-14	215
G022	F	19	04-Jul-14	6
G023	F	30	03-May-14	38
G024	F	30	17-Jun-14	76
G025	F	44	13-Jun-14	91
G026	M	45	13-Nov-14	45
G027	M	48	26-Jul-14	38
G028	F	22	22-Nov-14	54
G029	F	20	11-Aug-14	32
G030	M	44	18-May-14	23
G031	M	45	26-Apr-14	54
G032	M	35	08-Jun-14	10
G033	F	30	16-Nov-14	54
G035	M	45	29-May-14	215
G036	F	40	20-Apr-14	512

G037	F	17	09-Apr-14	609
G038	F	30	04-Apr-14	45
G039	F	27	21-Jun-14	431
G040	F	24	12-Apr-14	45
G041	M	22	02-Jul-14	861
G042	M	17	-	181
G043	F	28	27-Jun-14	76
G044	M	49	15-Apr-14	76
G045	M	38	03-Sep-14	108
G046	M	48	18-Sep-14	861
G047	F	22	08-Oct-14	76
G048	M	18	29-Aug-14	724
G049	F	43	10-Sep-14	152
G051	M	36	-	724
G052	F	30	03-Dec-14	32
Coyah - Survivors				
CS001	F	26	14-Dec-14	1218
CS002	F	27	27-Nov-14	152
CS003	M	40	25-Oct-14	54
CS004	F	22	19-Mar-15	91
CS005	F	-	23-Oct-14	108
CS011	M	-	25-Dec-14	13
CS012	M	-	22-Oct-14	91
CS013	F	-	29-Oct-14	861
CS014	F	-	22-Oct-14	152
CS015	F	-	12-Dec-14	45
CS021	F	-	02-Dec-14	19
CS022	F	-	14-Dec-14	91
CS025	F	-	26-Oct-14	76
CS031	M	-	09-Feb-15	54
CS032	F	-	08-Oct-14	181
CS033	F	-	19-Jan-15	54
CS034	F	-	20-Oct-14	152
CS041	F	-	02-Dec-14	108
CS042	F	-	10-Dec-14	64
CS043	F	-	06-Dec-14	152
CS044	F	-	19-Jan-15	256
CS045	M	-	05-Sep-14	128
CS048	F	-	20-Nov-14	38
CS050	F	-	06-Dec-14	108
CS051	M	19	27-Mar-15	128
CS052	M	28	27-Mar-15	304
CS053	M	-	22-Apr-15	256
CS054	M	-	12-Dec-14	861
CS055	M	-	12-Dec-14	38

CS056	F	-	31-Dec-14	4
CS059	F	-	22-Dec-14	362
CS061	M	-	02-Jan-15	304
CS062	M	-	25-Dec-14	181
CS063	M	-	25-Dec-14	91
CS064	M	-	19-Jan-15	4
CS066	M	-	31-Dec-14	76
CS067	F	32	28-Feb-15	45
CS070	F	-	03-Jan-15	54
CS071	F	-	27-Feb-15	38
CS072	M	-	13-Dec-14	362
CS073	M	-	07-Dec-14	91
CS074	M	-	22-Oct-14	91
CS075	F	-	11-Dec-14	76
CS076	F	-	13-Dec-14	128
CS077	M	-	06-Dec-14	45
CS078	M	-	13-Dec-14	32
CS079	F	35	08-Mar-15	76
CS080	F	-	16-Dec-14	91
CS081	M	-	24-Oct-14	54
CS082	F	-	08-Dec-14	32
CS083	F	65	--/06/2015	4
CS084	F	40	29-May-15	38
CS085	F	-	-	27
CS087	M	-	08-Oct-14	64
CS089	M	-	06-Mar-15	54
CS090	M	-	19-Apr-15	362
CS091	M	-	05-Feb-15	76
CS092	M	-	27-Dec-14	362
CS093	F	-	-	11
CS094	F	-	14-Dec-14	54
CS099	-	-	-	76
CS100	F	35	08-Mar-15	76
CS101	F	40	25-Feb-15	91
CS102	M	-	21-Dec-14	45
CS103	F	-	11-Nov-14	54
CS104	F	-	08-Mar-15	45
CS105	F	-	03-Jan-15	152
CS106	M	-	17-Oct-14	609
CS107	M	-	24-Nov-14	23
CS108	M	-	31-Dec-14	45
CS109	M	-	27-Dec-14	32
CS113	F	37	03-Apr-15	108

Gueckedou – Negatives				
GC025	M	22	N/A	4
GC026	M	36	N/A	5
GC027	M	19	N/A	4
GC028	M	24	N/A	5
G050	M		N/A	4
Coyah - Negatives				
CN001	M	-	N/A	4
CN002	-	-	N/A	4
CN003	-	-	N/A	4
CN004	M	-	N/A	4
CN005	M	-	N/A	4

Abbreviations: ETC, Ebola treatment centre; GMT, Geometric mean titre; - , data unknown.

2.5.2 Pseudotyped lentivirus neutralisation assay

Two- or three-fold serial dilutions of plasma samples at a starting dilution of 1:5 or 1:10, respectively, were prepared in duplicate in Opti-MEM® medium at a final volume of 50 µl/well in 96 well solid white flat bottom polystyrene TC-treated microplates and incubated with 50 µl of a standardised RLU per well of pseudotyped virus [as calculated from the titration assay (Section 2.4.1)] prepared in Opti-MEM® medium for 1 hour at 37°C. 100 µl of approximately 2 x 10^5 293T/17 cells were then added to each well and incubated for 48 hours at 37°C, 5% CO_2, prior to taking a chemiluminescent readout as described in Section 2.4.1. Infectivity was calculated using the formula: Percentage (%) infectivity = [(RLU with sample)/(RLU without sample)] x 100.

2.5.3 Pseudotyped VSV-ΔG neutralisation assay

24 hours prior to neutralisation, approximately 1 x 10^4 Vero E6 cells were seeded in 96 well solid white flat bottom polystyrene TC-treated microplates and incubated at 37°C, 5% CO_2 and 95% humidity. Two-fold serial dilutions of plasma samples at a starting dilution of 1:10 were prepared in duplicate in Opti-MEM® medium at a final volume of 120 µl/well were prepared in 96 well microplates and incubated with 120 µl of a standardised RLU per well of

pseudotyped virus [as calculated from the titration assay (Section 2.4.2)] prepared in Opti-MEM® medium for 1 hour at 37°C. The medium was removed from the cells, 50 µl of the plasma-pseudotyped virus mixtures were added to each well in quadruplicate at incubated at 37°C, 5% CO_2. After 1 hour, 50 µl of fresh medium was added to each well. Luminescence was measured after 24 hours (Section 2.4.1) and infectivity was calculated as described above (Section 2.5.2).

2.5.4 Statistical analysis

50% inhibitory concentration (IC_{50}) with 95% confidence interval (95% CI) of pseudotyped virus neutralisation were estimated by model of nonlinear regression fit with settings for log (inhibitor) vs. normalised response curves using GraphPad Prism v5 (San Diego, California (CA), USA).

Statistical comparison between two unpaired groups was performed using the Mann-Whitney test (GraphPad Prism v5). Comparison between multiple unpaired groups was achieved using one-way Analysis of Variance (ANOVA) (GraphPad Prism v5). Multiple comparisons after ANOVA was examined by post hoc Tukey's multiple comparison test (GraphPad Prism v5). Multiple matched or paired groups were compared using the Friedman test (GraphPad Prism v5).

Correlation between two variables was quantified using Spearman nonparametric correlation (GraphPad Prism v5).

Chapter 3 Assessment of an EBOV GP pseudotyped lentivirus neutralisation assay

3.1 Introduction

In order to test the study hypothesis that naturally occurring mutations in EBOV GP can result in escape from neutralising antibodies derived from EVD convalescent volunteers and EBOV GP vaccinated individuals (see Section 1.6), an EBOV GP pseudotyped virus neutralisation assay needed to be used. EBOV is classified as a HG4 biological agent, and for research-based purposes must be handled within CL4 facilities. These are widely limited and require highly trained staff, and expensive and specialised equipment. Furthermore, genetic manipulation and potential 'gain of function' experiments involving pathogens with pandemic potential are complex, time-consuming and costly, and are sometimes considered controversial (Fears and ter Meulen, 2015; Kilianski *et al.*, 2016). As discussed in Chapter 1.4, pseudotyped viruses can be used in experiments as safer alternatives to live infectious viruses that require a high level of bio-containment. Retrovirus-based EBOV GP pseudotyped viruses have previously been used to investigate EBOV tropism, host cell attachment factors and mechanisms of entry (Alvarez *et al.*, 2002; Chan *et al.*, 2000b; Wool-Lewis and Bates, 1998). They have also been utilised to undertake genetic manipulation of EBOV GP in order to identify residues important for binding and entry (Brindley *et al.*, 2007; Kuhn *et al.*, 2006; Manicassamy *et al.*, 2005), and determination of epitope residues for therapeutic mAbs (Davidson *et al.*, 2015). For the current study, EBOV GP pseudotyped lentiviruses were initially generated using a HIV-1 *gag-pol* packaging and luciferase reporter construct pNL4.3.Luc.R-E- (Connor *et al.*, 1995; He *et al.*, 1995). However, luminescence generated following infection of 293T/17 target cells was very low, which could have been due unreliability of EBOV GP incorporation and/or particle assembly (Urbanowicz *et al.*, 2016a). Furthermore, the assay was non-specific, as the pseudotyped viruses were able to

be neutralised by EBOV negative plasma samples. Therefore, an alternative HIV-1-based pseudotyped virus system was selected for the current study on the basis that the required plasmids and methodology were available for use (Long *et al.*, 2015; Wright *et al.*, 2008). Lentivirus-based EBOV GP pseudotyped virus neutralisation assays have been used to study immune responses to EBOV infection and vaccination (Ewer *et al.*, 2016; Luczkowiak *et al.*, 2016; Rimoin *et al.*, 2018; Sullivan *et al.*, 2006), as well as for evaluation of immunotherapeutics (Corti *et al.*, 2016; Zhang *et al.*, 2016). Before the EBOV GP pseudotyped virus neutralisation assay could be used to investigate the effects of EBOV GP mutations, its suitability needed to be assessed.

3.1.1 Generation and quantification of pseudotyped lentiviruses

Pseudotyped lentiviruses can be produced by co-transfection of producer cells using a three-plasmid system (Naldini *et al.*, 1996) (Figure 1.7). The first plasmid encodes the *gag-pol* genes, responsible for the production and enzymatic processing of the core structural proteins. The second plasmid encodes the envelope glycoprotein gene from the virus of interest, and the third plasmid encodes the chosen reporter gene, which becomes packaged into the core. The plasmids available for the current study were a HIV-1 *gag-pol* plasmid (p8.91), a firefly luciferase reporter construct (pCSFLW) and a pCAGGS EBOV (Mayinga) GP expression construct (Section 2.1.1). Following transfection of 293T cells with the appropriate plasmids, cells are incubated for 2-3 days and pseudotyped viruses harvested. The pseudotyped virus supernatants are clarified by filtration and/or centrifugation and stored at -80°C.

Pseudotyped viruses can be quantified by measuring reporter gene, e.g. luciferase, expression in susceptible target cells. Serial dilutions of the pseudotyped virus are prepared prior to the addition of permissive cells. After 2 days incubation, the transduced

cells are lysed and luciferase substrate added. Luminescence is measured and RLU/ml calculated according to the dilution factor.

3.1.2 Neutralisation of pseudotyped viruses

Pseudotyped viruses can be used to measure neutralising antibodies against the envelope glycoprotein coating the pseudotype (Mather *et al.*, 2013) (Figure 1.9). Samples are serially diluted and incubated with a standardised amount of pseudotyped virus for 1 hour at 37°C. Target cells are then added and incubated for 2 days, prior to measuring reporter gene expression as described above. Neutralisation can then be quantified as a decrease in reporter gene expression relative to pseudotyped virus infection without antibody.

3.1.3 Use of pseudotyped viruses as alternatives for infectious virus

As previously mentioned, pseudotyped viruses can be used as surrogates in receptor binding and serological assays instead of live infectious virus. However, important processes in the assembly and maturation of the envelope protein in the native, wild-type virus may be different in the generation of a pseudotyped virus. Furthermore, the density of envelope protein on the surface of a pseudotyped virus may not be the same as that found on the respective live virus. It is therefore important to carefully compare results obtained with assays using pseudotyped viruses with those from assays using authentic, live virus. Method optimisation and standardisation, to ensure accuracy and reproducibility, as well as establishment of reference material of known neutralising antibody titres, are integral to maximising the use of alternative assays for the detection of neutralising antibodies against highly pathogenic viruses such as EBOV (Mather *et al.*, 2013). Accordingly, aims for EBOV GP pseudotyped lentivirus construction, and assessment for suitability for use were as described below.

3.2 Chapter aims

The overall objective for this Chapter was to assess the suitability of an EBOV GP pseudotyped lentivirus system to measure the neutralising ability of EVD convalescent plasma. Specific objectives were to:

- Propagate and confirm identity of the required plasmids.
- Generate and quantify stocks of EBOV GP pseudotyped lentivirus.
- Optimise EBOV GP pseudotyped lentivirus neutralisation assay methodology.
- Assess neutralisation of EBOV GP pseudotyped lentivirus by EVD survivor plasma.
- Compare neutralisation results of EBOV GP pseudotyped lentivirus with live EBOV.

3.3 Results

3.3.1 Preparation of plasmids for production of EBOV GP pseudotyped lentivirus

The HIV-1 *gag-pol* plasmid p8.91, the firefly luciferase reporter construct pCSFLW, and the pCAGGS EBOV (Mayinga) GP expression construct were propagated as described in Section 2.1. After purification using Endofree® Plasmid Maxi kits, plasmid identity was confirmed via analytical restriction enzyme digest (Section 2.1.5) and separation by agarose gel electrophoresis (Section 2.1.6). Fragments of the expected sizes were observed for all digests (Figure 3.1). In addition, the pCAGGS EBOV (Mayinga) GP expression construct was sequenced (Section 2.1.8) to confirm integrity of the entire ORF. The plasmid map, annotated to show the restriction enzyme sites and sequenced region, is presented in Figure 2.1A. The plasmids were then used to generate EBOV (Mayinga) GP pseudotyped virus as described in Section 2.3.1.

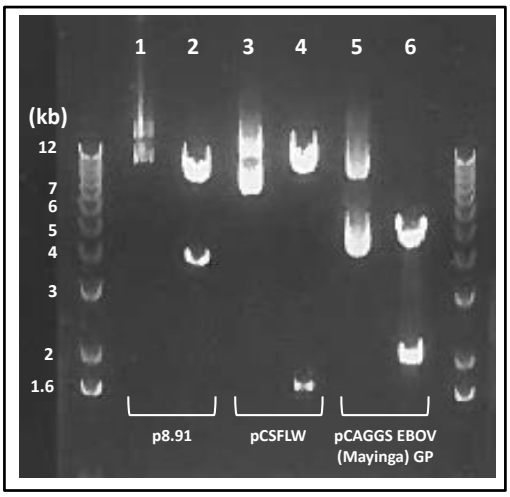


Figure 3.1: Analytical digest of p8.91 with BglII (#2), pCSFLW with BamHI and NotI (#4), and pCAGGS EBOV (Mayinga) GP with KpnI and XhoII (#6). Purified plasmid DNA was subject to restriction enzyme digest. Fragments of the expected sizes were observed for all digests. Undigested plasmids were included as controls (#1, #3 and #5).

3.3.2 Cell tropism of EBOV GP pseudotyped lentivirus

EBOV (Mayinga) GP pseudotyped virus was generated by transfection of 293T/17 cells using p8.91, pCSFLW and pCAGGS EBOV (Mayinga) GP as described in Section 2.3.1. The pseudotyped virus was used to infect 293T/17, Huh-7, HeLa and Vero E6 cells as described in Section 2.4.1 and RLUs/ml were determined. A pseudotyped virus bearing the VSV-G protein was used as a positive control, and cells only controls were used to determine background levels of luminescence. All cell lines tested were permissive to infection by EBOV GP pseudotyped virus, demonstrating the broad tissue range conferred by EBOV GP, although differences in infectivity were observed (Figure 3.2). Highest luminescence values were observed in 293T/17 cells (8×10^7 RLU/ml), followed by Huh-7 cells (5×10^7 RLU/ml). RLUs/ml generated by infection of 293T/17 cells were approximately 2, 33 and 196 times greater than those produced by infection of Huh-7, HeLa and Vero E6 cells, respectively. This variation may reflect a general defect in viral entry in these cells, or a problem in

integration or expression of the HIV-1 genome (Wool-Lewis and Bates, 1998). The relatively low level of transduction exhibited by Vero E6 cells might reflect the poor ability of HIV to replicate in many non-human primate (NHP) cells (Besnier *et al.*, 2002). Based on this result, the 293T/17 target cell line was selected for use in all subsequent neutralisation assays.

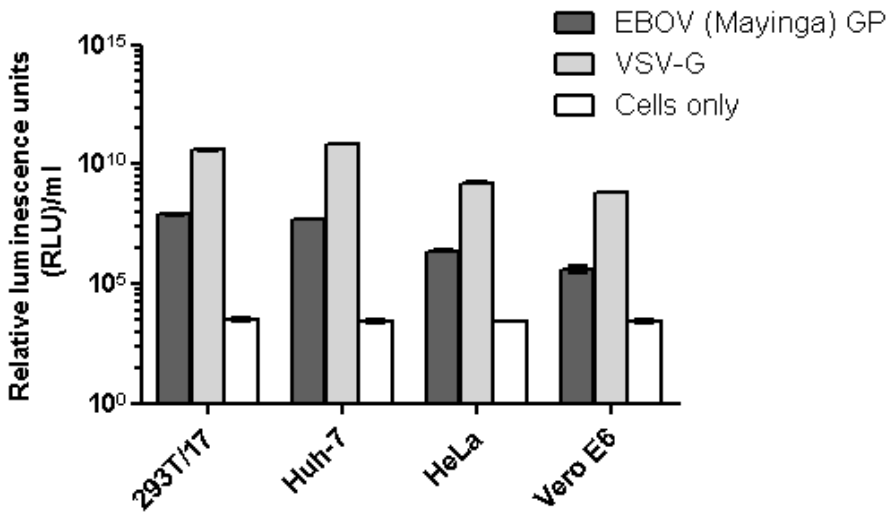


Figure 3.2: Infection of different cell lines with EBOV (Mayinga) GP and VSV-G pseudotyped viruses. Pseudotyped viruses were serially diluted and incubated with $2x10^4$ 293T/17, Huh-7 or Vero E6 cells, or $1x10^4$ HeLa cells per well. Luminescence was detected after 48 hours and relative luminescence units (RLUs)/ml were calculated. Error bars are 1 standard error above and below the mean, n=4. Background luminescence is represented by cells only controls.

3.3.3 Assessment of EBOV GP pseudotyped lentivirus input for neutralisation

To assess the effects of differing amounts of pseudotyped virus input on the neutralisation assay results, plasma samples from a UK negative control individual (PN004) and from a Guinean EVD survivor donor (CS001) were screened against three different amounts (2.2×10^4, 8.6×10^4 and 2.4×10^5 RLU/well) of EBOV (Mayinga) GP pseudotyped virus (Section 2.5.2). Percentage infectivity was determined relative to infectivity of 293T/17 cells by

EBOV GP pseudotyped virus alone (Figure 3.3A), and IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves as described in Section 2.5.4 (Figure 3.3B). Plasma from EVD survivor CS001 displayed neutralising activity against all amounts of EBOV GP pseudotyped virus tested (Figure 3.3A). Negative plasma (PN004) displayed no or little inhibitory effect against EBOV GP pseudotyped virus, however IC_{50} values increased gradually with decreasing amounts of pseudotyped virus input (Figure 3.3B). There was better separation of the positive and negative responses when at least 8.6×10^4 RLU/well was used (Figure 3.3B). Lower pseudotyped virus input resulted in larger variability and less curve fitting, as supported by decreasing R^2 values (Figure 3.3C). Therefore a pseudotyped virus input of at least 8.6×10^4 RLU/well, with a target input of 2.0×10^5 RLU/well, was used in subsequent neutralisation assays.

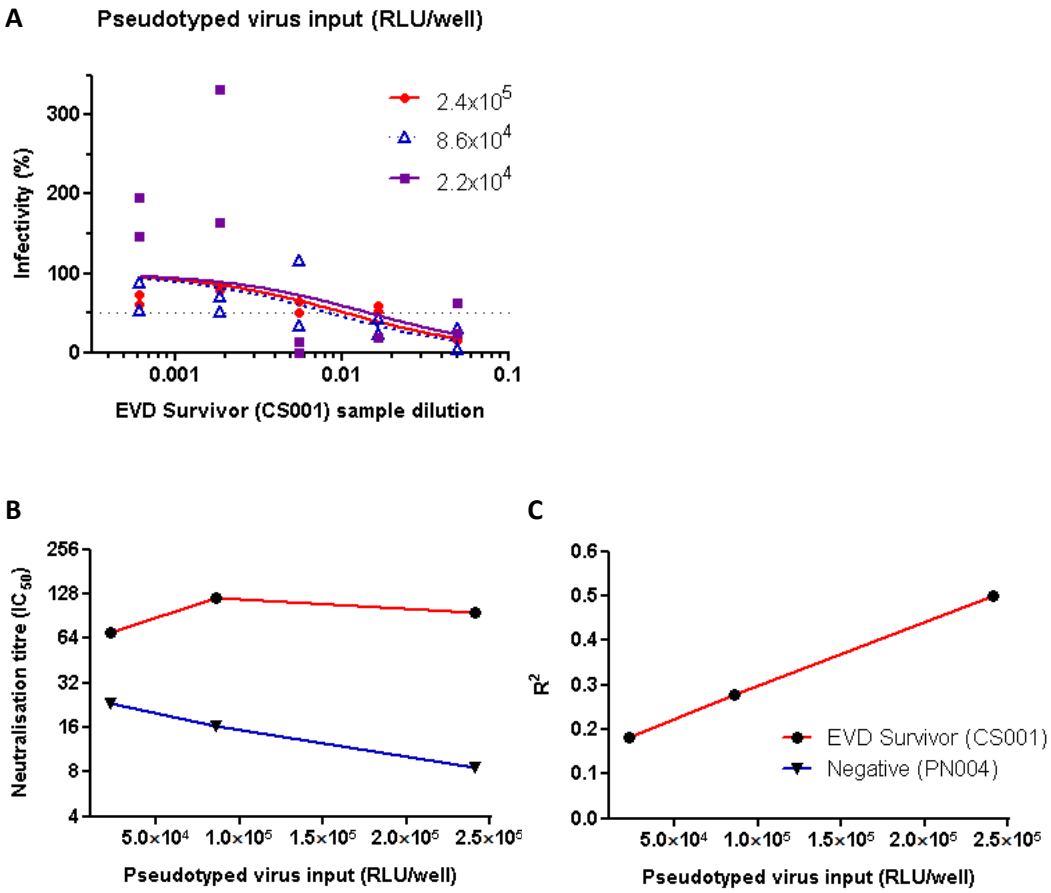


Figure 3.3: Effect of EBOV (Mayinga) GP pseudotyped virus input on neutralisation by EVD survivor (CS001) plasma. Different amounts of pseudotyped virus were incubated with dilutions of heat inactivated plasma samples at 37°C for 1 hour before the addition of 293T/17 cells. Luminescence was detected after 48 hours and A) percentage infectivity was calculated. Dotted line represents 50% infectivity. B) The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves, and C) the R^2 values of dose-response curves were determined.

3.3.4 Neutralisation of EBOV GP pseudotyped lentivirus by EVD survivor plasma

Neutralisation of EBOV (Mayinga) GP pseudotyped virus by plasma samples collected from 119 EVD survivors of the 2013-2016 EBOV outbreak 3 to 14 months post-infection, and 10 negative control donors from Guinea (Section 2.5.1) was evaluated (Section 2.5.2) over the course of seven assays. In order to control for any differences due to variability in pseudotyped virus production and/or titration, the same batch of pseudotyped virus was

used for all assays. Assay controls and reproducibility are discussed in Section 3.3.5. The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves as described in Section 2.5.4. The results are presented in Figure 3.4. Dose-response curves were unable to be fitted for 21 (20 EVD survivor and one negative) out of the 129 samples tested due to lack of neutralisation, and therefore IC_{50} values could not be calculated for these samples. Although there appeared to be little difference in neutralisation titres between the EVD survivor and negative plasma samples, there was a statistically significant difference between the two groups (Mann-Whitney, $p = 0.0023$).

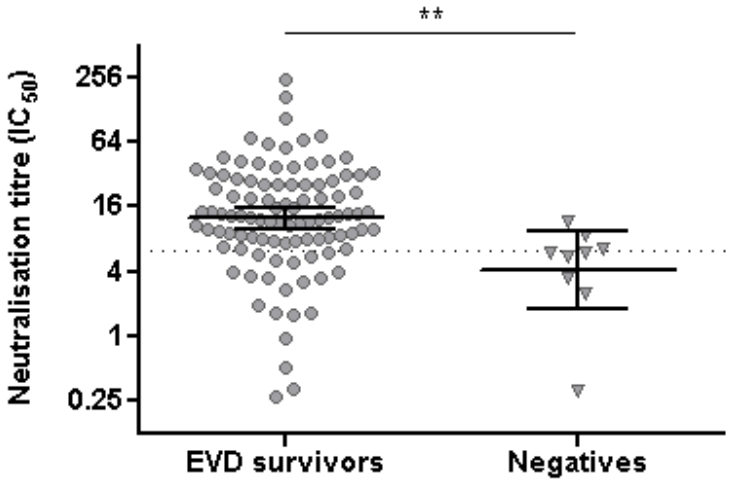


Figure 3.4: Neutralisation of EBOV (Mayinga) GP pseudotyped virus by EVD survivor and negative plasma samples. Pseudotyped virus was incubated with dilutions of heat inactivated plasma samples at 37°C for 1 hour before the addition of 293T/17 cells. Luminescence was detected after 48 hours and percentage infectivity was calculated. The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves. Data are shown for individuals, and the geometric mean with 95% CI. Dotted line represents background level of pseudotyped virus neutralisation (6.28) and is equal to UK negative control plasma mean plus two standard deviations. Statistically significant difference is highlighted (**$p < 0.05$; Mann-Whitney).

To further validate the EBOV GP pseudotyped virus neutralisation assay, a human anti-EBOV GP mAb, KZ52 (IBT Bioservices, Rockville, Maryland (MD), USA), and anti-EBOV plasma, human WHO reference reagent (Code 15/220 National Institute of Biological Standards and Control (NIBSC), Potters Bar, UK) were tested (Section 2.5.2). KZ52 is an antibody isolated from a human survivor of the 1995 outbreak in Kikwit that neutralises EBOV *in vitro* and recognises a conformational epitope at the base of the GP (Lee *et al.*, 2008; Maruyama *et al.*, 1999; Parren *et al.*, 2002). Anti-EBOV plasma, human WHO reference reagent has been established by the WHO Expert Committee on Biological Standardization (ECBS) for use in serology assays (World Health Organization & WHO Expert Committee on Biological Standardization, 2015), and the source material is plasma obtained from a single donor recovered from EVD. The anti-EBOV plasma, human WHO reference reagent was able to neutralise the EBOV GP pseudotyped virus (Figure 3.5A), however human anti-EBOV GP mAb, KZ52 was not (Figure 3.5B). This suggests that the conformation or density of EBOV GP on the pseudotyped virus may differ from that on live virus.

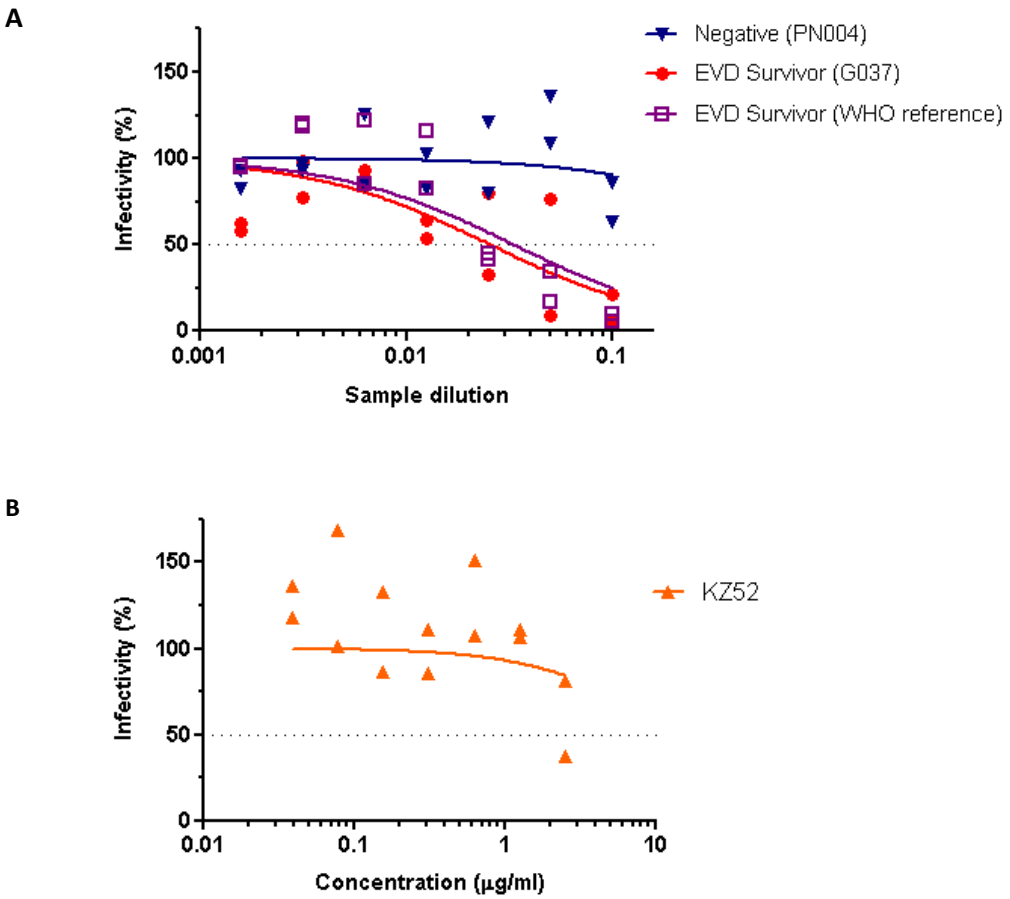


Figure 3.5: Neutralisation of EBOV (Mayinga) GP pseudotyped virus by A) EVD survivor and negative control (PN004) plasma samples and B) human anti-EBOV GP mAb, KZ52. Pseudotyped virus was incubated with dilutions of heat inactivated plasma samples at 37°C for 1 hour before the addition of 293T/17 cells. Luminescence was detected after 48 hours and percentage infectivity was calculated. Dotted lines represent 50% infectivity.

3.3.5 Reproducibility and correlation with live EBOV neutralisation

Plasma from Guinean EVD survivor G037, and a UK negative control donor PN004, were included in each neutralisation assay to serve as a positive and negative control, respectively. Neutralisation of EBOV (Mayinga) GP pseudotyped virus by plasma from EVD survivor G037 was fairly consistent (Figure 3.6); IC_{50} values ranged from 39.11 to 121.68, with an average of 69.17. The average IC_{50} value of PN004 from all the assays plus two standard deviations was used to determine background neutralisation (IC_{50} of 6.28).

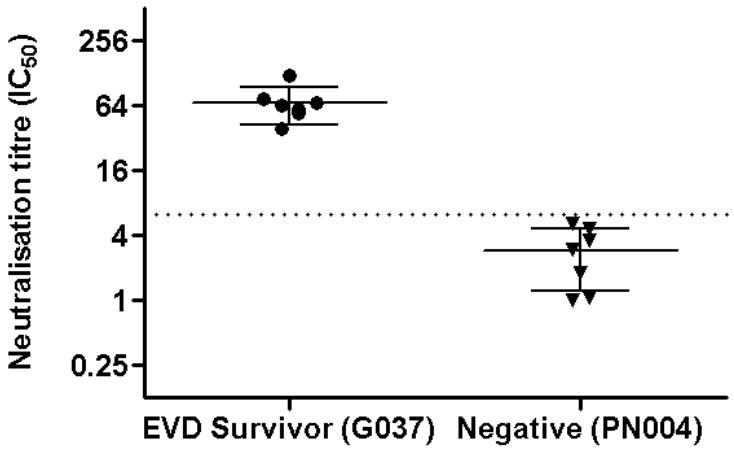


Figure 3.6: Reproducibility of neutralisation of EBOV (Mayinga) GP pseudotyped virus by positive (G037) and negative (PN004) control plasma samples. Pseudotyped virus was incubated with dilutions of heat inactivated plasma samples at 37°C for 1 hour before the addition of 293T/17 cells. Luminescence was detected after 48 hours and percentage infectivity was calculated. The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves. Data are shown for individuals and the mean with standard deviation, n=7. Dotted line represents background level of pseudotyped virus neutralisation (6.28) and is equal to negative control plasma mean plus two standard deviations.

Out of the 119 EVD survivor samples tested, 20 were deemed below the background limit of the assay (Figure 3.4). Three of the 10 Guinean negative plasma samples tested were above the background level of neutralisation. The reason for this non-specific neutralisation is unknown; however it could be due to interference by specific antibodies to related viruses or pre-existing immunity (Boisen *et al.*, 2015; O'Hearn *et al.*, 2016; Schoepp *et al.*, 2014), although this is unlikely as these samples were negative in a live EBOV neutralisation assay and also in an EBOV enzyme linked immunosorbent assay (ELISA) (data not shown).

For each sample tested, pre-existing data from a live EBOV neutralisation assay were available (Section 2.5.1). There was a positive correlation (r_s = 0.52) when IC_{50} values of EBOV GP pseudotyped virus neutralisation were compared with geometric mean titre

(GMT) values for live EBOV neutralisation using the nonparametric Spearman correlation coefficient (Figure 3.7), and this was statistically significant ($p < 0.0001$).

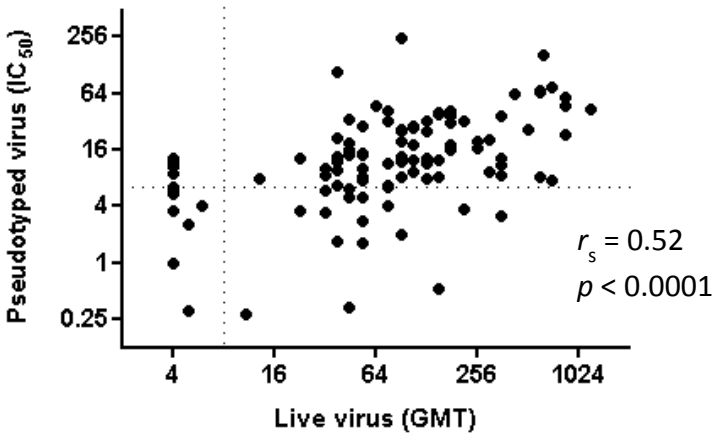


Figure 3.7: Correlation of EBOV GP pseudotyped virus (IC_{50}) and live EBOV (GMT) neutralisation using the nonparametric Spearman correlation coefficient, n=108. Dotted lines represent background level of neutralisation. Background level of pseudotyped virus neutralisation (6.28) is equal to negative control plasma mean plus two standard deviations. Seropositivity in the live EBOV neutralisation assay is defined by a GMT > 8.

3.4 Discussion

In this Chapter, the suitability of an EBOV GP pseudotyped lentivirus system to measure the neutralising ability of EVD convalescent plasma was assessed. The pseudotyped virus system used was selected based on the plasmids and methodology that were available at the onset of the study (Long *et al.*, 2015; Wright *et al.*, 2008). Furthermore, this system had been applied during a Phase I clinical study to measure neutralising antibodies following EBOV vaccination (Ewer *et al.*, 2016). A number of lentivirus-based EBOV GP pseudotyped virus neutralisation assays have been used to investigate immune responses to EBOV infection and vaccination (Luczkowiak *et al.*, 2016; Rimoin *et al.*, 2018; Sullivan *et al.*, 2006),

as well as for evaluation of mAb therapies (Corti *et al.*, 2016; Davidson *et al.*, 2015; Zhang *et al.*, 2016).

3.4.1 Generation and quantification of EBOV GP pseudotyped lentivirus

EBOV (Mayinga) GP pseudotyped virus was successfully generated by transfection using plasmid DNA encoding EBOV GP, along with HIV-1 *gag-pol* packaging, and luciferase reporter constructs. The pseudotyped virus was titrated using a range of target cell lines and quantified by measuring luminescence. Previous studies have shown that EBOV GP has a broad host range, infecting cells derived from a wide variety of species and tissues (Chan *et al.*, 2000b; Wool-Lewis and Bates, 1998). In this study, all cell lines tested (293T/17, Huh-7, HeLa and Vero E6) were permissive to infection by EBOV GP pseudotyped virus, although differences in luminescence were observed. This may reflect a general defect in viral entry in different cells, or a problem in integration or expression of the HIV-1 genome (Wool-Lewis and Bates, 1998). A relatively lower level of transduction was exhibited by Vero E6 cells, which might be due to an intrinsic restriction factor, TRIM5α, which restricts retroviral infection by specifically recognising the HIV-1 capsid and promoting its rapid, premature disassembly (Stremlau *et al.*, 2006). Highest luminescence values were obtained following infection of 293T/17 cells, which have previously been reported to be permissive to EBOV infection, and have been used as target cells in pseudotyped virus assays (Corti *et al.*, 2016; Davidson *et al.*, 2015; Kuhn *et al.*, 2006; Manicassamy *et al.*, 2005; Wool-Lewis and Bates, 1998). Therefore this cell line was selected for use in subsequent neutralisation assays.

3.4.2 Neutralisation of EBOV GP pseudotyped lentivirus by EVD survivor plasma

Pseudotyped virus neutralisation assays have been developed for a variety of pathogens, including rabies (Nie *et al.*, 2017; Wright *et al.*, 2008), Middle East respiratory syndrome coronavirus (MERS-CoV) (Zhao *et al.*, 2013), chikungunya virus (Kishishita *et al.*, 2013; Salvador *et al.*, 2009) and influenza (H5N1) (Garcia *et al.*, 2010; Wang *et al.*, 2010). Samples

from vaccinated individuals and/or from patients recovered from the disease of interest are often used in the optimisation of these assays to assess experimental parameters that can affect assay performance, and to ensure accuracy and reproducibility. The lentivirus-based EBOV GP pseudotyped virus generated for the current study was used to assess the neutralising activity of plasma from EVD survivors of the 2013-2016 EBOV outbreak and a UK negative control donor. Decreasing quantities of pseudotyped virus led to more variable and unreliable results, and therefore a pseudotyped virus input of at least 8.6×10^4 RLU/well was used in subsequent neutralisation assays. Plasma from a UK negative control donor displayed little or no neutralising activity against EBOV GP pseudotyped virus and was used to determine a background level of neutralisation for the assay. The EBOV GP pseudotyped lentivirus neutralisation assay was able to measure neutralising antibodies in plasma from EVD convalescent patients and was reproducible.

3.4.3 Suitability of EBOV GP pseudotyped lentivirus neutralisation assay for future use

As mentioned previously, pseudotyped viruses can be used in serological assays as alternatives to live infectious virus. Pseudotyped virus assays used to profile neutralising antibody responses against SARS-CoV (Temperton *et al.*, 2005), influenza (H5N1 and H7N9) (Alberini *et al.*, 2009; Qiu *et al.*, 2013a; Temperton *et al.*, 2007; Wang *et al.*, 2010), rabies (Nie *et al.*, 2017; Wright *et al.*, 2009; Wright *et al.*, 2008) and chikungunya virus (Kishishita *et al.*, 2013), for example, found that results correlated with, or were more sensitive than those from conventional replication-competent or live virus assays. A Phase I EBOV vaccine study found that neutralising antibody titres in a live EBOV and an EBOV pseudotyped virus assay correlated positively with each other (r_s = 0.57, p = 0.001) (Ewer *et al.*, 2016). However, a comparison of a range of assays for antibody to EBOV (Wilkinson *et al.*, 2017), raised questions of the reliability and relevance of neutralisation of the lentiviral pseudotypes evaluated. This was both in relation to specificity, as a number of negative samples were scored positive, and poor quantitative correlation with the wild-type

neutralisation assays. The EBOV GP pseudotyped lentivirus neutralisation assay assessed for the current study correlated positively with a live EBOV neutralisation assay. However, the discriminatory power of the assay with regards to differing antibody titres appeared to be low. Some of the samples tested, which showed neutralising activity against live EBOV, did not display neutralisation against the pseudotyped virus and vice versa, therefore raising questions on the sensitivity and specificity of the pseudotyped virus assay. In the current study, human embryonic kidney (293T/17) cells were used for the pseudotyped virus neutralisation assays, whereas African green monkey kidney (Vero) cells were used in the live EBOV assay, and therefore this could account for some of the differences in results observed between the two assays. Furthermore, a human anti-EBOV GP mAb, KZ52, did not display neutralisation in the pseudotyped virus neutralisation assay, suggesting that the EBOV GP on the pseudotyped virus may not be folded correctly. KZ52 has been shown previously to neutralise live EBOV and EBOV pseudotyped viruses (Davidson *et al.*, 2015; Dias *et al.*, 2011; Luczkowiak *et al.*, 2016; Maruyama *et al.*, 1999; Shedlock *et al.*, 2010). Therefore, the apparent poor sensitivity of the current assay may explain the lack of neutralisation displayed by KZ52, and neutralisation may be observed using a higher concentration.

There are several differences between the EBOV GP pseudotyped virus and the live EBOV neutralisation assays that could affect their results (Saphire *et al.*, 2018). Firstly, the round, spherical shape of EBOV GP pseudotyped lentivirus compared to the filamentous shape of authentic EBOV could affect their susceptibility to neutralisation. Also, the density of GP on the surface of the pseudotyped virus may not be the same as that found on live EBOV and may result in the loss or masking of quaternary epitopes (Mather *et al.*, 2013; Nelson *et al.*, 2008). Furthermore, processes in the assembly and maturation of GP in live EBOV, such as trimer formation and glycosylation, may be different in the generation of EBOV pseudotyped virus resulting in different targets and/or conformational epitopes when using

whole live EBOV as opposed to EBOV GP alone in a pseudotyped virus. The presence of sGP in the live EBOV assay compared to no sGP in the EBOV GP pseudotyped virus assays could also have an effect on neutralisation. In the live EBOV assay, sGP could reduce neutralisation of circulating virus by sGP-cross-reactive antibodies, however in the current study, weaker relative neutralisation was observed in the pseudotyped virus assay. Therefore, other components, such as cell debris or free GP generated during pseudotyprd virus production by transfection could interfere with neutralisation in the pseudotyped virus assay. Finally, detection of infected cells via measurement of luminescence in the EBOV GP pseudotyped virus neutralisation assay compared to plaque formation in the live EBOV neutralisation assay could affect neutralisation readout.

3.5 Conclusions

The EBOV GP pseudotyped lentivirus neutralisation assay was able to detect neutralising antibodies in plasma from EVD survivors and correlated positively with a live EBOV neutralisation assay. However, the ability of the pseudotyped virus neutralisation assay to differentiate between neutralising activity appeared to be limited. In order to study the effects of EBOV GP mutations on neutralising antibodies, a sensitive and specific EBOV GP pseudotyped virus assay is needed. Therefore, a VSV-based pseudotyped virus system was investigated to determine if this was a more suitable platform than HIV-1 to measure the neutralising ability of EVD convalescent plasma, and is described in Chapter 4.

Chapter 4 Assessment of an EBOV GP pseudotyped VSV neutralisation assay

4.1 Introduction

Like retroviruses, rhabdoviruses, such as VSV, can also be used as pseudotyped virus cores (Takada *et al.*, 1997), and have been used to study pathogenic viruses that require high level bio-containment facilities, including SARS-CoV (Fukushi *et al.*, 2005), influenza (Zimmer *et al.*, 2014) and Crimean-Congo haemorrhagic fever (CCHF) (Suda *et al.*, 2016). However establishment of a VSV-based pseudotyped system is slightly more complex than retrovirus-based, and requires stocks of VSV-G protein pseudotyped rVSV, in which the VSV-G gene has been deleted and replaced with a reporter gene, to be generated and quantified (Whitt, 2010). VSV-based pseudotyped viruses have previously been used to investigate EBOV tropism, host cell attachment factors and mechanisms of virus entry (Chandran *et al.*, 2005; Dube *et al.*, 2009; Hoffmann *et al.*, 2017; Ito *et al.*, 1999; Kondratowicz *et al.*, 2011; Kuroda *et al.*, 2015), as well as for measurement of neutralising antibodies to EBOV (Ito *et al.*, 2001; Takada *et al.*, 2003).

4.1.1 Generation and quantification of pseudotyped VSVs

Pseudotyped VSVs can be produced by combining an rVSV genome, in which the VSV-G gene has been deleted and replaced with a reporter gene, represented here by a *, (rVSV-ΔG*), with an expression plasmid encoding the envelope protein from the virus of interest (Whitt, 2010) (Figure 1.8). Following transfection of 293T [or baby hamster kidney (BHK-21)] cells with the appropriate viral envelope protein expression plasmid, cells are incubated for 1 day and infected with rVSV-ΔG* that has previously been pseudotyped with VSV-G protein (rVSV-ΔG*-VSV-G) at an MOI of approximately 3-5, to ensure every cell is infected, for 1-2 hours. Pseudotyped viruses are then harvested at 18-24 hours post-infection. The pseudotyped virus supernatants are clarified by centrifugation and stored at

-80^{o}C. Before use, the pseudotyped virus is treated with a VSV-G neutralising antibody to reduce background infection mediated by residual virus possessing VSV-G, which can be carried over during the preparation of the pseudotyped virus. Additional working stocks of rVSV-ΔG*-VSV-G virus can be generated by a similar method as that used to pseudotype heterologous envelope proteins onto rVSV-ΔG*, except that the cells are transfected using a VSV-G expression plasmid, and the infection is performed at a lower multiplicity to prevent accumulation of defective-interfering particles (Whitt, 2010). rVSV-ΔG*-VSV-G virus can be titrated by plaque assay on Vero E6 cells by pre-transfection with pVSV-G expression plasmid, followed by infection with serial dilutions of rVSV-ΔG*-VSV-G virus for 1 hour. After 2 days incubation under a semi-solid overlay medium, cells are fixed and stained, plaques are counted, and PFUs/ml calculated according to the dilution factor. rVSV-ΔG*-VSV-G virus can also be titrated and quantified by measuring reporter gene expression in susceptible target cells as detailed below.

Like pseudotyped lentiviruses, pseudotyped VSVs can be quantified by measuring reporter gene, e.g. luciferase, expression in target cells. Serial dilutions of the pseudotyped virus are prepared and added to permissive cell monolayers. After 1 day incubation, the transduced cells are lysed and luciferase substrate added. Luminescence is measured and RLU/ml calculated according to the dilution factor. An alternative method for quantifying pseudotyped viral particles is to titrate for determination of $TCID_{50}$. This is the concentration of virus required to produce a CPE in 50% of tissue cultured cells inoculated, and can be determined using the Reed-Muench method (Reed and Muench, 1938).

4.1.2 Use of pseudotyped VSVs as alternatives for infectious virus

Pseudotyped VSV neutralisation assays have been developed for a variety of pathogens, and comparative serology studies have shown that results from conventional assays using live, native virus correlate well with those obtained by pseudotyped virus neutralisation assays (Fukushi *et al.*, 2006; Logan *et al.*, 2016). VSV-based pseudotyped viruses have previously been used in neutralisation assays to assess EBOV vaccines (Agnandji *et al.*, 2017; Agnandji *et al.*, 2016; Huttner *et al.*, 2018; Huttner *et al.*, 2015; Regules *et al.*, 2017) and antibody-based therapies (Bornholdt *et al.*, 2016a; Holtsberg *et al.*, 2015; Howell *et al.*, 2017; Howell *et al.*, 2016; Keck *et al.*, 2015; Takada *et al.*, 2007; Zhao *et al.*, 2017). Before the EBOV GP pseudotyped VSV neutralisation could be used to study the effects of EBOV GP mutations, its suitability to study the properties of neutralising antibodies derived from EVD convalescent volunteers, needed to be assessed. Accordingly, aims for this Chapter were as follows.

4.2 Chapter aims

The overall objective for this Chapter was to assess the suitability of an EBOV GP pseudotyped VSV system to measure the neutralising ability of EVD convalescent plasma. Specific objectives were to:

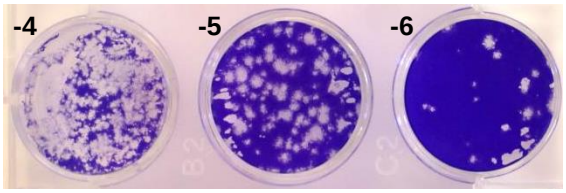
- Amplify and titrate working stock of rVSV-ΔG-Luc-VSV-G virus.
- Generate and quantify stocks of EBOV GP pseudotyped VSV.
- Optimise EBOV GP pseudotyped VSV neutralisation assay methodology.
- Assess neutralisation of EBOV GP pseudotyped VSV by EVD survivor plasma.
- Compare neutralisation results of EBOV GP pseudotyped VSV with EBOV GP pseudotyped HIV-1 and live EBOV.

4.3 Results

4.3.1 rVSV-ΔG-Luc-VSV-G virus expansion and titration

A working stock of rVSV-ΔG-Luc-VSV-G virus was prepared by transfection of 293T/17 cells using pVSV-G followed by infection with rVSV-ΔG-Luc-VSV-G virus, as described in Section 2.3.3. The rVSV-ΔG-Luc-VSV-G virus was titrated by plaque assay on Vero E6 cells by pre-transfection with pVSV-G expression plasmid, followed by infection with rVSV-ΔG-Luc-VSV-G virus for 1 hour (Section 2.4.3). Plaque assays were fixed and stained 48 hours post-infection. The titre of the rVSV-ΔG-Luc-VSV-G virus stock was determined as $1.75x10^8$ pfu/ml (Figure 4.1A). The rVSV-ΔG-Luc-VSV-G virus was also assayed for luciferase activity in 293T/17 cells at 24 hours post-infection, as described in Section 2.4.2. The titration of luciferase activity showed that the dilution corresponding to 95% of the maximum value was equal to the dilution needed for MOI 5 (Figure 4.1B). The rVSV-ΔG-Luc-VSV-G virus was then used to generate EBOV (Mayinga) GP pseudotyped virus as described in Section 2.3.2.

A



B

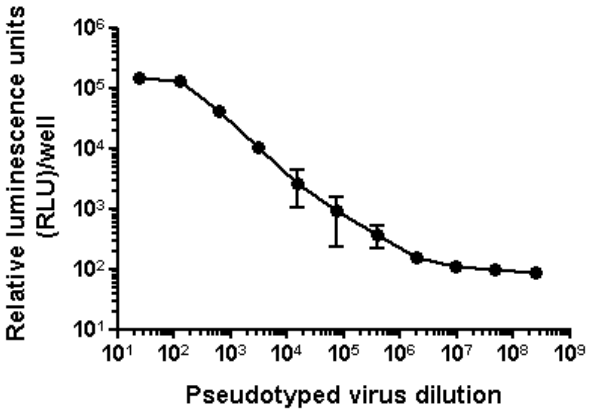


Figure 4.1: rVSV-ΔG-Luc-VSV-G titration. A) Vero E6 cells, pre-transfected with pVSV-G expression plasmid for 5-6 hours, were infected with serial dilutions of pseudotyped virus for 1 hour. After 2 days incubation under a semi-solid overlay medium, cells were fixed and stained. B) 293T/17 cells were infected with serial dilutions of pseudotyped virus for 24 hours and luminescence (RLU/well) was detected. Error bars are 1 standard error above and below the mean, n=4.

4.3.2 Cell tropism of EBOV GP pseudotyped VSV

EBOV (Mayinga) GP pseudotyped VSV was generated by transfection of 293T/17 cells using pCAGGS EBOV (Mayinga) GP, and subsequent infection with rVSV-ΔG-Luc-VSV-G virus as described in Section 2.3.2. The pseudotyped virus was titrated on 293T/17, Huh-7, HeLa and Vero E6 cell monolayers, luminescence was measured, and $TCID_{50}$/ml values were calculated as described in Section 2.4.2. These cell lines were permissive to infection, although differences in luminescence were observed, with highest pseudotyped virus titres being obtained in Vero E6 cells (Figure 4.2). The $TCID_{50}$/ml values generated by infection of Vero E6 cells were approximately 1.5, 22 and 30 times greater than those produced by infection of 293T/17, Huh-7 and HeLa cells, respectively (Figure 4.2B). Based on these

results, the Vero E6 target cell line was selected for use in all subsequent neutralisation assays.

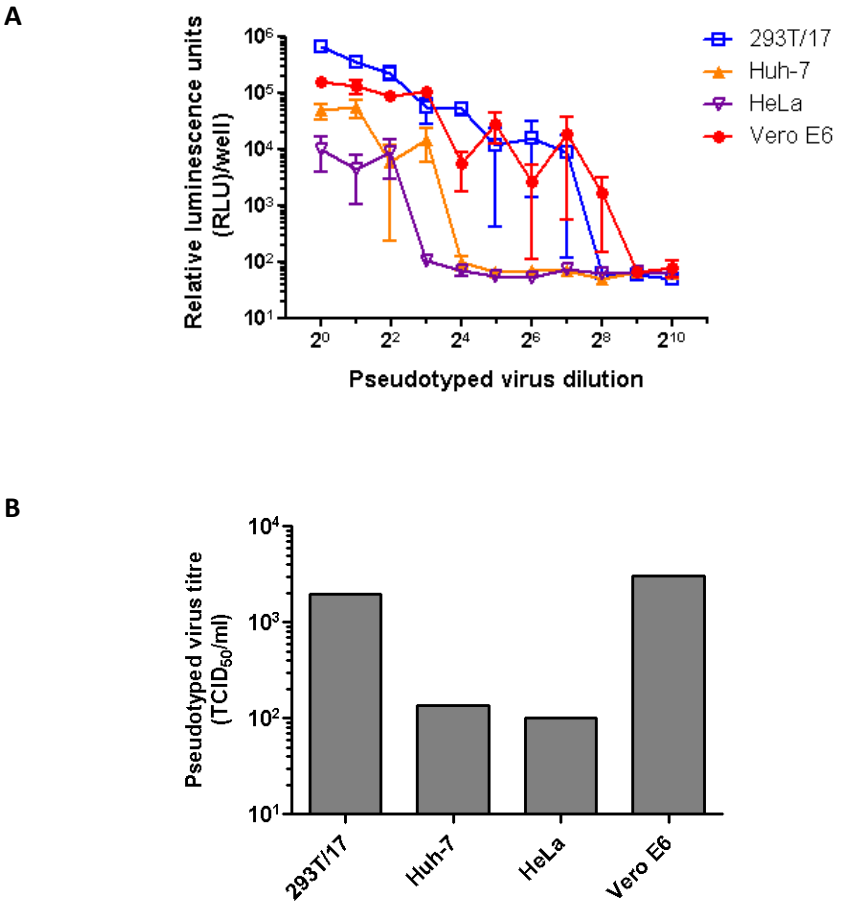


Figure 4.2: Infection of different cell lines with EBOV (Mayinga) GP pseudotyped virus. $2x10^4$ 293T/17, Huh-7 or Vero E6 cells, or $1x10^4$ HeLa cells per well were infected for 24 hours with serial dilutions of pseudotyped virus. A) Luminescence (RLU/well) was detected (error bars are 1 standard error above and below the mean, n=4), and B) Pseudotyped virus titres ($TCID_{50}$/ml) were calculated.

4.3.3 Assessment of EBOV GP pseudotyped VSV input on neutralisation

To assess the effects of differing amounts of pseudotyped virus input on the neutralisation assay results, human anti-EBOV GP mAb KZ52 was screened against four different dilutions of EBOV GP (Mayinga) pseudotyped virus (Section 2.5.3). KZ52 was selected for the current study as it was commercially available, and there was accompanying information regarding its neutralisation activity against EBOV GP pseudotyped VSV expressing luciferase. Percentage infectivity was determined relative to infectivity of Vero E6 cells by EBOV GP pseudotyped virus alone, and IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves (Section 2.5.4). KZ52 neutralised all dilutions of EBOV GP pseudotyped virus tested (Figure 4.3) and IC_{50} values decreased with decreasing amounts of pseudotyped virus input (Figure 4.3B). When using neat, or a 1:2 dilution of pseudotyped virus, IC_{50} of virus neutralisation were similar to that expected according to the manufacturer's product data sheet (0.06 µg/ml). Therefore a dilution of EBOV GP (Mayinga) pseudotyped VSV that corresponded to approximately 3.0×10^4 RLU/well was used in subsequent neutralisation assays.

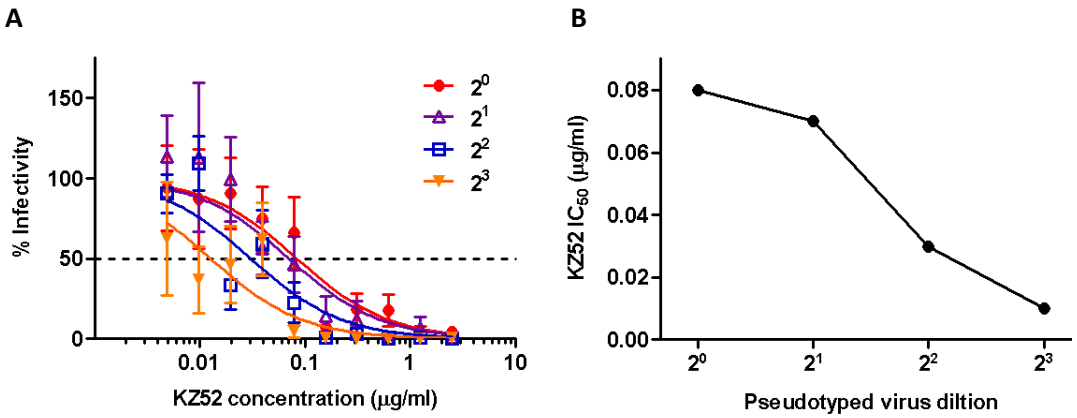


Figure 4.3: Neutralisation of different amounts of EBOV (Mayinga) GP pseudotyped VSV by anti-EBOV GP mAb, KZ52. A) Different dilutions of pseudotyped virus were incubated with dilutions of KZ52 at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and percentage infectivity was calculated. Error bars are 1 standard error above and below the mean, n=4. B) IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves.

4.3.4 Neutralisation of EBOV GP pseudotyped VSV by control samples

To further validate the EBOV GP pseudotyped virus neutralisation assay, KZ52, and plasma from a Guinean EVD survivor (G037) and from a UK negative control donor (PN004) were tested in a number of independent assays, and IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves (Section 2.5.4). KZ52 and EVD survivor G037 plasma were able to neutralise the EBOV GP pseudotyped virus (Figure 4.4). Neutralisation of EBOV GP pseudotyped virus by KZ52 was very reproducible; IC_{50} values ranged from 0.06 to 0.11 µg/ml, with an average of 0.07 µg/ml. Neutralisation of EBOV GP pseudotyped virus by EVD survivor G037 plasma was less reproducible; IC_{50} values ranged from 202 to 2117, with an average of 887. The negative plasma displayed no neutralisation against EBOV GP pseudotyped virus and dose-response curves were unable to be fitted. Therefore an IC_{50} value of 20, which was the lowest dilution of sample tested in the assay, was assigned as the background level of

neutralisation for the assay. Anti-EBOV plasma, human WHO reference reagent was also tested in the assay, and was able to neutralise the EBOV GP pseudotyped virus, with an IC_{50} of 923 (Figure 4.4).

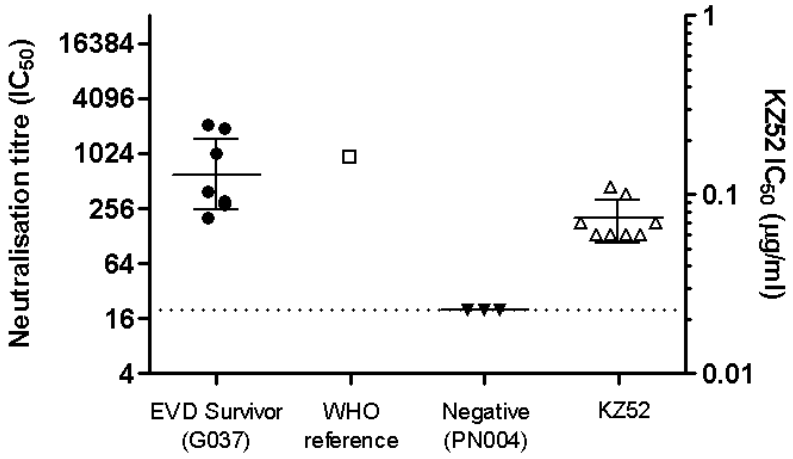


Figure 4.4: Reproducibility of neutralisation of EBOV (Mayinga) GP pseudotyped virus by positive (G037 and WHO) and negative (PN004) control plasma samples and human anti-EBOV GP mAb, KZ52. Pseudotyped virus was incubated with dilutions of samples at 37°C for 1 hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and percentage infectivity was calculated. The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves. Data are shown for individuals and the geometric mean with 95% CI. Dotted line represents background level of pseudotyped virus neutralisation and is equal to the lowest dilution of sample tested in the assay (1/20).

4.3.5 Neutralisation of EBOV GP pseudotyped VSV by EVD survivor plasma

Neutralisation of EBOV (Mayinga) GP pseudotyped virus by plasma samples collected from 30 EVD survivors of the 2013-2016 EBOV outbreak 3 to 14 months post-infection (Table 4.1), and 10 negative control donors from Guinea (Section 2.5.1) was evaluated as described in Section 2.5.3. Due to time constraints and limited amount of EBOV (Mayinga) GP pseudotyped virus available, a smaller number of EVD survivor plasma samples was tested in the EBOV GP pseudotyped VSV neutralisation assay than was tested in the HIV-1

based assay (Section 3.3.4). Samples covering a range of EBOV neutralising ability were selected based on their performance in a live EBOV neutralisation assay (Table 4.1).

Table 4.1 Ebola virus disease (EVD) survivor samples tested in the EBOV GP pseudotyped virus neutralisation assay. Plasma samples from EVD survivors of the 2013-2016 EBOV outbreak were obtained from a pre-existing biobank. Live EBOV (Mayinga) neutralisation data were available for each sample.

Sample	Live EBOV (Mayinga) neutralisation (GMT)
G041	861
G048	724
G011	645
G037	609
G036	512
CS090	362
CS053	256
G021	215
G035	215
G001	181
G014	181
G005	128
G045	108
G013	108
G025	91
G024	76
G044	76
G033	54
G028	54
G031	54
G018	54
G026	45
G038	45
G040	45
G020	45
G027	38
G019	38
CS084	38
G030	23
G022	6

The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves as described in Section 2.5.4 and results are presented in Figure 4.5. All but one of the EVD survivor plasma samples displayed neutralising activity against the EBOV GP pseudotyped virus. A dose-response curve was unable to be fitted for this sample, and therefore an IC_{50} value of 20 was assigned to it. Dose-response curves were unable to be fitted for seven out of the 10 negative samples tested due to lack of neutralisation, and therefore IC_{50} values of 20 were assigned to these samples. There was a statistically significant difference between the two groups (Mann-Whitney, $p < 0.0001$).

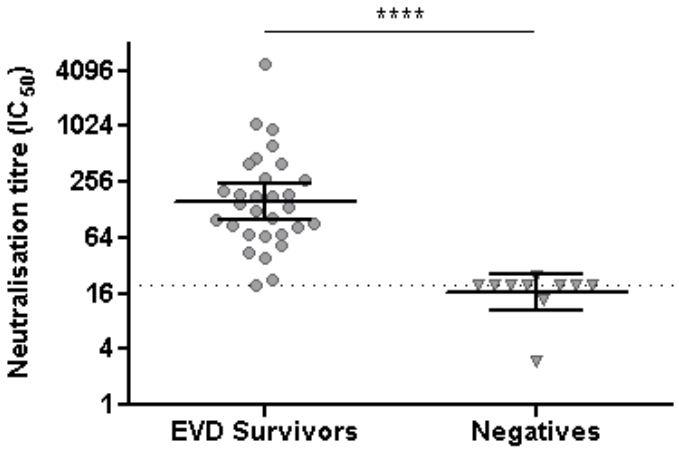


Figure 4.5: Neutralisation of EBOV (Mayinga) GP pseudotyped VSV by EVD survivor and negative plasma samples. Pseudotyped virus was incubated with dilutions of heat inactivated plasma samples at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and percentage infectivity was calculated. The IC_{50} of pseudotyped virus neutralisation were estimated by model of nonlinear regression dose-response curves. Data are shown for individuals and the geometric mean with 95% CI. Dotted line represents background level of pseudotyped virus neutralisation and is equal to the lowest dilution of sample tested in the assay (1/20). Statistically significant difference is highlighted (****$p < 0.0001$; Mann-Whitney).

4.3.6 Correlation with live EBOV neutralisation

For each sample tested, pre-existing data from a live EBOV neutralisation assay were available (Section 2.5.1). There was a positive correlation (r_s = 0.86) when IC_{50} values of EBOV GP pseudotyped virus neutralisation were compared with GMT values for the live EBOV neutralisation assay, using the nonparametric Spearman correlation coefficient (Figure 4.6), and this was statistically significant ($p < 0.0001$).

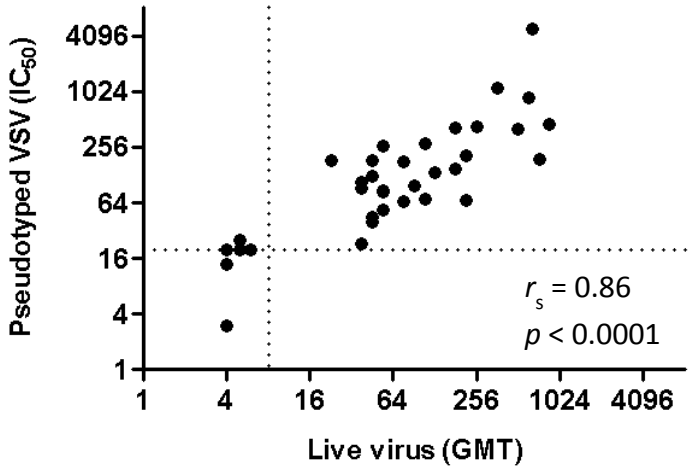


Figure 4.6: Correlation of EBOV GP pseudotyped VSV (IC_{50}) and live EBOV (GMT) neutralisation using the nonparametric Spearman correlation coefficient, n=40. Dotted lines represent background level of neutralisation. Background level of pseudotyped VSV neutralisation is equal to the lowest dilution of sample tested in the assay (1/20). Seropositivity in the live EBOV neutralisation assay is defined by a GMT > 8.

4.3.7 Comparison with EBOV GP pseudotyped lentivirus neutralisation

In order to directly compare the lentivirus- (assessed in Chapter 3) and VSV-based pseudotyped virus systems, EBOV GP pseudotyped HIV-1 neutralisation results from the same 30 EVD survivor and 10 negative plasma samples that were tested in the EBOV GP pseudotyped VSV neutralisation assay were assessed. In the HIV-1 based assay, dose-response curves were unable to be fitted for three of the 30 EVD survivor samples, and six

of the samples were deemed below the background level of neutralisation. In contrast, a dose-response curve was unable to be fitted for only one of the EVD survivor samples tested in the VSV-based neutralisation assay, and this sample was also negative in the live EBOV neutralisation assay. In the HIV-1 based assay, three of the 10 negative plasma samples tested were above the background level of neutralisation, whereas only one of the negative samples tested was above the background level of neutralisation in the VSV-based assay.

When IC_{50} values of EBOV GP pseudotyped HIV-1 neutralisation of the 30 EVD survivor and 10 negative plasma samples were compared with GMT values for the live EBOV neutralisation assay, there was a positive correlation (r_s = 0.57) (Figure 4.7), and this was statistically significant (p = 0.0002).

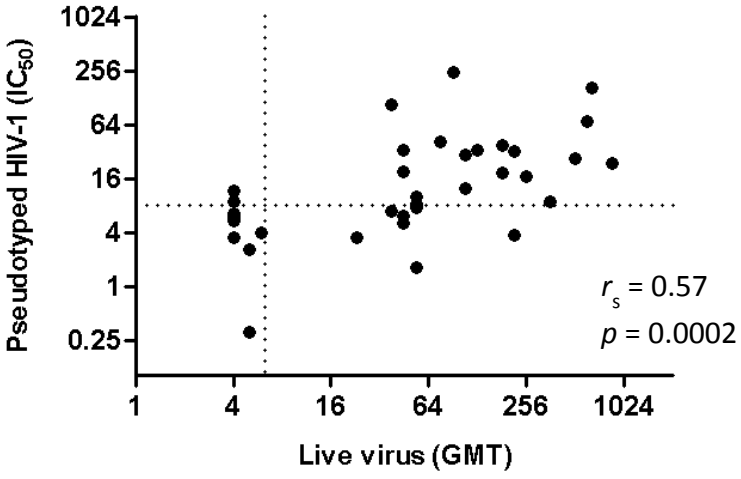


Figure 4.7: Correlation of EBOV GP pseudotyped HIV-1 (IC_{50}) and live EBOV (GMT) neutralisation using the nonparametric Spearman correlation coefficient, n=36. Dotted lines represent background level of neutralisation. Background level of pseudotyped HIV-1 neutralisation (6.28) is equal to negative control plasma mean plus two standard deviations. Seropositivity in the live EBOV neutralisation assay is defined by a GMT > 8.

4.4 Discussion

As discussed in Section 1.4, pseudotyped viruses can be used as alternatives to infectious virus to measure neutralising antibodies to the envelope glycoprotein. Lentiviruses and rhabdoviruses can provide cores for pseudotyped viruses (Page *et al.*, 1990; Takada *et al.*, 1997), and have been used to study neutralising antibodies against EBOV (Sullivan *et al.*, 2006; Takada *et al.*, 2003). A previous comparison of a range of assays for antibody to EBOV found that the assays evaluated based on VSV gave better correlation with live EBOV neutralisation than those based on lentiviruses (Wilkinson *et al.*, 2017). The suitability of an EBOV GP pseudotyped lentivirus assay to measure neutralising antibodies derived from EVD convalescent volunteers was assessed in Chapter 3. However the ability of the assay to differentiate between neutralising activity appeared to be limited, and concerns were raised regarding the specificity and sensitivity of the assay, especially as some of the negative samples displayed neutralising activity, and a human anti-EBOV GP mAb was unable to neutralise the EBOV GP pseudotyped HIV-1. Therefore in this Chapter, a VSV-based pseudotyped virus system was investigated, to determine if this was a more suitable platform than HIV-1 to study the effects of EBOV GP mutations on antibody neutralisation.

4.4.1 Production and titration of rVSV-ΔG-Luc-VSV-G virus

Establishment of VSV-based pseudotyped virus systems is slightly more complex than lentivirus-based pseudotyped virus systems, and requires stocks of VSV-G protein pseudotyped rVSV, in which the VSV-G gene has been deleted and replaced with a reporter gene, represented here by a *, (rVSV-ΔG*-VSV-G), to be generated and quantified (Whitt, 2010). rVSV-ΔG-Luc-VSV-G virus was successfully prepared by transfection of 293T/17 cells using pVSV-G and subsequent infection with rVSV-ΔG-Luc-VSV-G virus. The rVSV-ΔG-Luc-VSV-G virus was titrated by plaque assay on Vero E6 cells, and was also assayed for luciferase activity in 293T/17 cells. Titration of luciferase activity showed that the dilution

corresponding to 95% of the maximum value was equal to the dilution needed for MOI 5, which was subsequently used to produce EBOV (Mayinga) GP pseudotyped virus.

4.4.2 Generation and quantification of EBOV GP pseudotyped VSV

EBOV (Mayinga) GP pseudotyped VSV was successfully produced by transfection of 293T/17 cells with plasmid DNA encoding EBOV GP, followed by infection with rVSV-ΔG-Luc-VSV-G virus. The EBOV GP pseudotyped virus was quantified by measuring luminescence in a range of target cell lines and $TCID_{50}$ values were calculated. All cell lines tested (293T/17, Huh-7, HeLa and Vero E6) were permissive to infection by EBOV GP pseudotyped virus, demonstrating the broad tissue range conferred by EBOV GP (Ito *et al.*, 2001). Highest titres were obtained in Vero E6 cells, which have previously been reported to be highly permissive to EBOV infection, and are commonly used as target cells in live EBOV, as well as EBOV pseudotyped VSV assays (Bornholdt *et al.*, 2016a; Chandran *et al.*, 2005; Holtsberg *et al.*, 2015; Howell *et al.*, 2016; Kondratowicz *et al.*, 2011; Maruyama *et al.*, 1999; Takada *et al.*, 2007). Therefore this cell line was selected for use in subsequent neutralisation assays.

4.4.3 Optimisation of EBOV GP pseudotyped VSV neutralisation assay

In order to ensure accuracy and reproducibility of neutralisation results, samples from vaccinated individuals and/or from patients recovered from the disease of interest are often used in optimisation experiments to evaluate assay parameters that could affect assay performance. To assess the effects of differing amounts of pseudotyped virus input on neutralisation, human anti-EBOV GP mAb KZ52 was screened against different amounts of the EBOV GP (Mayinga) pseudotyped VSV. KZ52 neutralised all dilutions of EBOV GP pseudotyped virus tested, providing evidence for correct folding of the EBOV GP on the pseudotyped virus. This was in contrast to EBOV GP pseudotyped HIV-1, which was not neutralised by KZ52 (Figure 3.5B). The IC_{50} of pseudotyped virus neutralisation values decreased with decreasing amounts of pseudotyped virus input, therefore a pseudotyped

virus input of approximately 3.0 x 10^4 RLU/well was used in subsequent neutralisation assays. The variability in neutralisation observed between different amounts of pseudotyped virus input highlights the importance of including standards or reference material with a known activity or potency when comparing neutralising activity, allowing calibration of results (Temperton and Page, 2015).

KZ52 was tested in the EBOV GP pseudotyped VSV neutralisation assay on a number of independent occasions and results were highly reproducible. Anti-EBOV plasma, human WHO reference reagent was also able to neutralise the EBOV GP pseudotyped virus, and could be used to calibrate secondary reference material, which would improve comparability and reliability of results (Wilkinson *et al.*, 2017). Plasma from a UK negative control donor displayed no neutralising activity against EBOV GP pseudotyped VSV, and therefore the lowest dilution of sample tested in the assay was assigned as the background level of neutralisation for the assay. This was in contrast to the HIV-1-based neutralisation assay, in which a low level of neutralisation was observed by the UK negative control plasma (Section 3.3.5), therefore the background level of neutralisation appears to be lower in the EBOV GP pseudotyped VSV assay.

4.4.4 Neutralisation of EBOV GP pseudotyped VSV by EVD survivor and negative plasma

The EBOV GP pseudotyped VSV was used to assess the neutralising activity of plasma from 30 EVD survivors of the 2013-2016 EBOV outbreak and 10 negative control donors. The EBOV GP pseudotyped VSV neutralisation assay was able to measure neutralising antibodies in plasma from EVD convalescent patients and results correlated positively with a live EBOV neutralisation assay. Plasma from the negative control donors displayed little or no neutralising activity against EBOV GP pseudotyped VSV. Therefore, the VSV-based neutralisation assay performed better than the lentivirus-based assay, both in relation to specificity and correlation with the live EBOV neutralisation assay (Section 3.3.5). In the

current study, human embryonic kidney (293T/17) cells were used for the lentivirus-based neutralisation assay, whereas African green monkey kidney (Vero) cells were used in the VSV-based assay, which are also used in the live EBOV assay, and therefore this could account for the better performance of the VSV-based assay in relation to live EBOV neutralisation. Also, the lentivirus- and VSV-based pseudotyped virus systems assed in the current study utilise different transfection methods, which could have implications on pseudotyped virus production and neutralisation results. Processes in the assembly and maturation of GP, such as trimer formation and glycosylation, may differ between the transfection procedures, resulting in different targets and/or conformational epitopes and therefore neutralisation. Other components, such as cell debris or free GP generated during transfection could also interfere with neutralisation.

4.4.5 Selection of EBOV GP pseudotyped virus assay for future use

A head-to-head comparison of neutralisation results from the EBOV GP pseudotyped lentivirus (HIV-1) and VSV assays of the same 30 EVD survivor and 10 negative plasma samples was undertaken. For the HIV-1-based assay, dose-response curves were unable to be fitted for three of the EVD survivor samples, and six of the samples were deemed below the background level of neutralisation. In contrast, a dose-response curve was unable to be fitted for only one of the samples in the VSV-based assay, and this sample was also negative in the live EBOV neutralisation assay, suggesting that the sensitivity and discriminatory power of the VSV-based assay are greater. In the HIV-1 based assay, three of the negative plasma samples tested were above the background level of neutralisation, whereas only one of the negative samples was above the background level of neutralisation in the VSV-based assay. This suggests that the level of non-specific background neutralisation is greater in the HIV-1-based assay.

There was a statistically significant positive correlation when GMT values for a live EBOV neutralisation assay were compared with IC_{50} values of EBOV GP pseudotyped VSV neutralisation, but not with EBOV GP pseudotyped HIV-1 neutralisation. This outcome was in agreement with a previous comparison of a range of assays for antibody to EBOV, which found that the assays evaluated based on VSV gave better correlation with live EBOV neutralisation than those based on lentiviruses (Wilkinson *et al.*, 2017).

As mentioned in Section 3.4.3, there are several differences between EBOV GP pseudotyped virus and live EBOV neutralisation assays that could affect their results, including the shape of the virions and density of GP on the viral surfaces, the presence or absence of sGP, and the method used to detect infected cells (Saphire *et al.*, 2018). Furthermore, EBOV neutralisation assays have previously been shown to yield variable results (Saphire *et al.*, 2018; Wilkinson *et al.*, 2017), and therefore a single assay alone may not be enough to determine neutralisation. However, a pseudotyped virus could be used as an initial qualitative, rather than quantitative, screen for escape mutants before live virus is used to investigate the effects of EBOV GP mutations on antibody neutralisation.

4.5 Conclusions

The EBOV GP pseudotyped VSV neutralisation assay was reproducible and correlated positively with a live EBOV neutralisation assay. The sensitivity, specificity and ability of the VSV-based pseudotyped virus neutralisation assay to differentiate between neutralising activity appeared to be greater than the lentivirus-based system. Correlation between live EBOV and EBOV GP pseudotyped virus neutralisation was best for the VSV-based assay when a head-to-head comparison with the lentivirus-based assay was carried out. Therefore, the VSV-based neutralisation assay was subsequently selected to test the hypothesis of the study (described in Section 1.6) that naturally occurring mutations in

EBOV GP can result in escape from neutralising antibodies derived from EVD convalescent volunteers and EBOV GP vaccinated individuals, and is discussed in Chapter 5.

Chapter 5 Production and neutralisation of mutant EBOV GP pseudotyped viruses

5.1 Introduction

Neutralising antibodies are important components of a protective immune response against many viral pathogens (Plotkin, 2010). Some viruses, such as HIV and HCV for example, are able to escape neutralising antibody responses that arise during infection of individual hosts, by mutation of the viral neutralising determinants (Corti and Lanzavecchia, 2013; Fofana *et al.*, 2012; Hangartner *et al.*, 2006; Richman *et al.*, 2003; von Hahn *et al.*, 2007; Wei *et al.*, 2003).

The 2013-2016 EVD epidemic in West Africa, which gave rise to the EBOV Makona variant, was characterised by extensive human-to-human transmission, which resulted in an accumulation of mutations within the EBOV genome (Gire *et al.*, 2014). During the outbreak, sequencing studies were performed to assess mutation rates and to support molecular epidemiology studies, revealing mutations in the genetic sequence encoding EBOV GP (Carroll *et al.*, 2015; Gire *et al.*, 2014; Park *et al.*, 2015; Quick *et al.*, 2016; Simon-Loriere *et al.*, 2015; Tong *et al.*, 2015). Studies into this topic suggest that a single mutation encoding a valine substitution for alanine at residue 82 of the EBOV GP that arose early during the 2013-2016 epidemic, is associated with increased infectivity of human cells (Diehl *et al.*, 2016; Dietzel *et al.*, 2017; Kurosaki *et al.*, 2018; Ueda *et al.*, 2017; Urbanowicz *et al.*, 2016b; Wang *et al.*, 2017). Mutations in the EBOV GP gene may also have important implications for anti-EBOV GP-based therapeutics and vaccines, i.e. changes in the EBOV GP may affect the ability of antibodies to bind, thereby leading to the emergence of escape mutants (Carroll *et al.*, 2015; Kugelman *et al.*, 2015a; Kugelman *et al.*, 2015b; Miller *et al.*, 2016). A useful application for pseudotyped viruses, therefore, is to investigate how

changes in EBOV GP might affect host cell receptor interaction and antibody binding and neutralisation.

5.1.1 Generation of mutant EBOV GP pseudotyped viruses

In order to test the study hypothesis, that naturally occurring mutations in EBOV GP can result in escape from neutralising antibodies, mutant EBOV GP pseudotyped viruses needed to be designed and produced. The VSV-based pseudotyped virus system used for the current study was optimised in Chapter 4. Site-directed mutagenesis can be used to introduce specific mutations into the EBOV GP gene and generate a panel of mutant EBOV GP expression plasmids, which can subsequently be used to generate mutant EBOV GP pseudotyped VSVs, as described in Section 4.1.1. It is only following satisfactory preparation and characterisation of pseudotyped viruses that their relative properties can be effectively evaluated and compared. The aims for this Chapter, described below, were implemented accordingly.

5.2 Chapter aims

The overall objective for this Chapter was to select and produce a panel of mutant EBOV GP pseudotyped viruses, based on mutations that occurred during the 2013-2016 EVD outbreak, and to investigate the potential for immune escape as per the study hypothesis outlined in Section 1.6. Specific objectives for this Chapter were to:

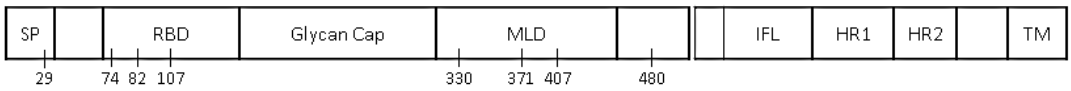
- Identify a set of mutations within the EBOV (Makona) GP that arose during the 2013-2016 EBOV outbreak in West Africa.
- Introduce selected mutations into an expression plasmid using site-directed mutagenesis.
- Generate and quantify stocks of mutant EBOV GP pseudotyped viruses.

- Test a panel of mutant EBOV GP pseudotyped viruses in neutralisation assays with plasma from EVD survivors, EBOV GP vaccinated individuals and neutralising anti-EBOV GP mAbs to assess the effect on immune escape.

5.3 Results

5.3.1 Selection of EBOV GP mutations

The initial EBOV GP sequence used in the current study was based on EBOV (Makona) isolate GenBank accession number KJ660348 (Baize *et al.*, 2014), collected in Guinea in March 2014, and is referred to here as the 'ancestral' EBOV GP variant. Using bioinformatics analysis of 1457 published EBOV genome sequences from the 2013-2016 EVD outbreak, a number of non-synonymous mutations within the EBOV GP were identified (Section 2.1.7, Table 2.1). Three variants of particular interest, as they contain multiple amino acid changes, are shown in Figure 5.1. EBOV GP sequences containing multiple amino acid changes were selected for investigation, as it was thought that this would increase the possibility of observing any differences in neutralisation, especially by polyclonal antibody samples.



EBOV GP variant	29	74	82	107	330	371	407	480
Ancestral (KJ660348)	R	G	A	N	P	I	H	G
N107D, P330S, G480D	R	G	A	**D**	**S**	I	H	**D**
R29K, A82V, I371L, G480S	**K**	G	**V**	N	P	**L**	H	**S**
G74R, P330S, H407Y	R	**R**	A	N	**S**	I	**Y**	G

Figure 5.1: Schematic representation of amino acid combinations in EBOV GP. Amino acid changes identified in the EBOV (Makona) GP during the 2013-2016 EBOV outbreak in West Africa are shown in red and bold. A, Alanine; D, Aspartic acid; G, Glycine; H, Histidine;, I, Isoleucine; K, Lysine; L, Leucine; N, Asparagine; P, Proline; R, Arginine; S, Serine; V, Valine; Y, Tyrosine. SP, signal peptide; RBD, receptor binding domain; MLD, mucin-like domain; IFL, internal fusion loop; HR, heptad repeat; TM, transmembrane. Figure adapted from (Urbanowicz *et al.*, 2016b).

Further analysis of the genome sequences of EBOV from the 2013-2016 EVD outbreak was carried out to determine approximately when each of the EBOV GP variants was first observed. An EBOV isolate containing the N107D, P330S and G480D GP mutations (GenBank accession number KR534528) was sampled in Guinea in September 2014 (Simon-Loriere *et al.*, 2015), and a variant containing the R29K, A82V, I317L and G480S GP mutations (GenBank accession number KU296622) was identified in Sierra Leone in January 2015 (Arias *et al.*, 2016). An EBOV isolate containing a G47R GP mutation (GenBank accession number KR817241) was sampled in Liberia in July 2014 (Carroll *et al.*, 2015), although this sequence also contained an A82V GP mutation. An EBOV variant containing P330S and H407Y GP mutations (GenBank accession number LT630494) was detected in Guinea in March 2015 (Quick *et al.*, 2016), however this sequence was incomplete and the

amino acid at position 74 of the GP was undetermined, therefore it is unknown if a G74R, P330S and H407Y triple GP mutation variant naturally occurred.

5.3.2 Mutation of EBOV GP expression vector

The starting expression vector used in the current study was a pcDNA3.1 expression plasmid for codon optimised EBOV (Makona) GP [GenBank accession number KJ660348 (Baize *et al.*, 2014)]. Site mutagenesis primers were designed accordingly and the desired mutations introduced sequentially into the EBOV GP sequence (Section 2.1.7, Table 2.1). Plasmid DNA isolated from putative clones using QIAprep® plasmid Miniprep kits was subjected to sequencing (Section 2.1.8) across the manipulated area to confirm that the desired mutation(s) had been successfully introduced. A plasmid map, annotated to show the sequenced region, is presented in Figure 2.1B. Once the expression constructs were confirmed to be correct, they were propagated as described in Section 2.1. After purification using Endofree® Plasmid Maxi kits, plasmid identity was confirmed again via sequencing (Section 2.1.8). The plasmids were then used to generate mutant EBOV GP pseudotyped viruses as described in Section 2.3.2.

5.3.3 Production and titration of EBOV GP mutant pseudotyped viruses

Mutant EBOV GP pseudotyped VSVs were generated by transfection of 293T/17 cells using the appropriate pcDNA3.1 mutant EBOV GP expression plasmid and subsequent infection with rVSV-ΔG-Luc-VSV-G virus as described in Section 2.3.2. The pseudotyped viruses were titrated on Vero E6 cell monolayers, luminescence was measured, and $TCID_{50}$/ml values were calculated as described in Section 2.4.2. A pseudotyped virus bearing the VSV-G protein was used as a positive control, and a pseudotyped virus with no envelope protein was used to determine background level of luminescence. Differences in luminescence generated by the mutant EBOV GP pseudotyped VSVs were observed (Figure 5.2), with highest pseudotyped virus titres being obtained by mutant GP N107D, P330S, G480D,

closely followed by ancestral GP and then mutant GP R29K, A82V, I317L, G480S (Figure 5.2B). The $TCID_{50}$/ml of mutant GP N107D, P330S, G480D pseudotyped virus was approximately 1.5, 2.5 and 10 times greater than that of ancestral GP, GP R29K, A82V, I317L, G480S and GP G74R, P330S, H407Y pseudotyped viruses, respectively. Based on the titration results, a target pseudotyped virus input of approximately 2 x 10^4 RLU/well was selected for use in all subsequent neutralisation assays.

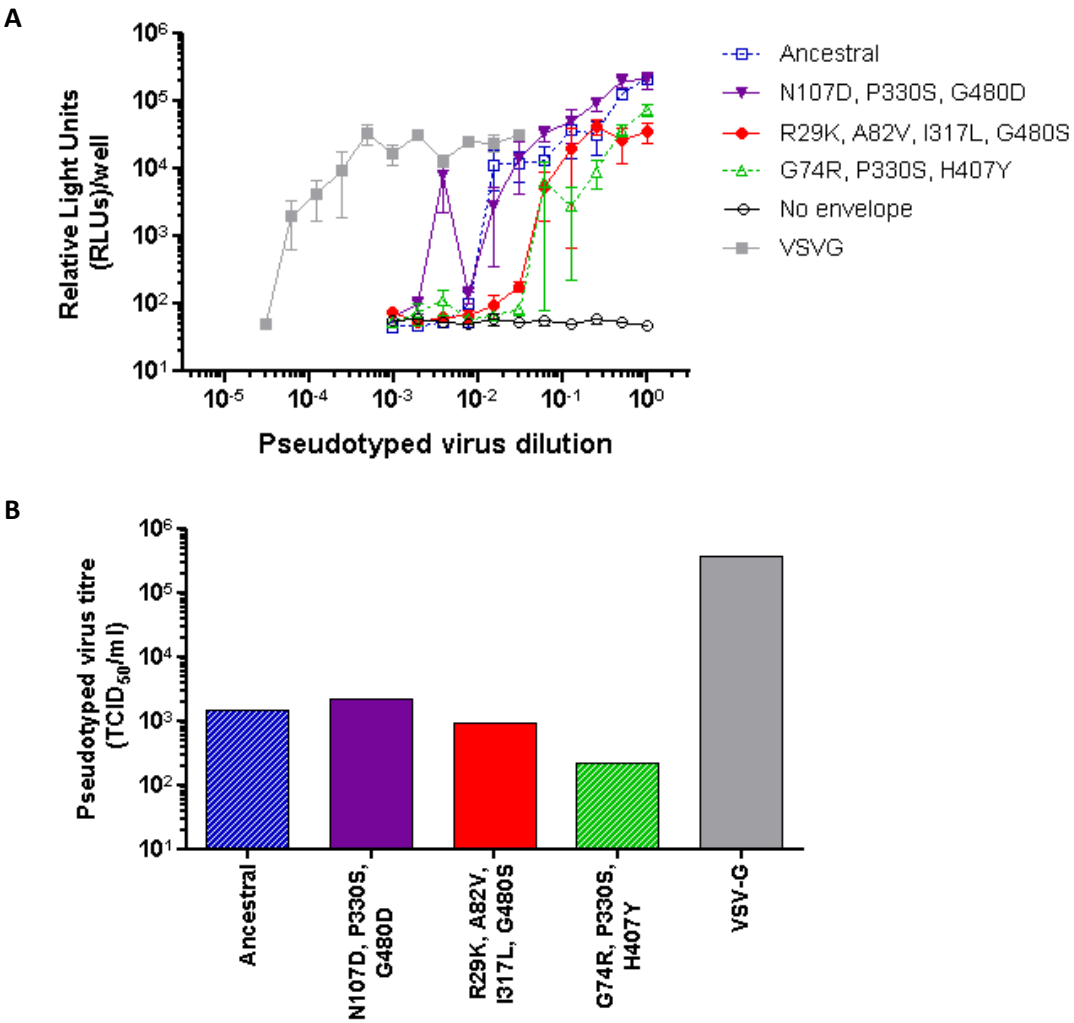


Figure 5.2: Quantification of mutant EBOV GP and VSV-G pseudotyped viruses. Vero E6 cell monolayers were infected for 24 hours with serial dilutions of pseudotyped viruses. A) Luminescence (RLU/ml) was detected (error bars are 1 standard error above and below the mean, n=4), and B) $TCID_{50}$/ml were calculated.

5.3.4 Neutralisation of mutant EBOV GP pseudotyped viruses by control samples

Neutralisation of mutant EBOV GP pseudotyped viruses by plasma samples

The mutant EBOV GP pseudotyped viruses were used in neutralisation assays with anti-EBOV plasma, human WHO reference reagent, and plasma from a Guinean negative control donor (GC027) as described in Section 2.5.3. Plasma from an individual who had previously been vaccinated with two candidate EBOV vaccines (ChAd3 and VSV-EBOV GP) was also tested. Results are presented in Figure 5.3. Plasma from the negative control donor displayed no neutralising activity against any of the mutant EBOV GP pseudotyped viruses, whereas anti-EBOV plasma, human WHO reference reagent, and plasma from the EBOV vaccinated individual were able to neutralise all of the mutant EBOV GP pseudotyped viruses tested.

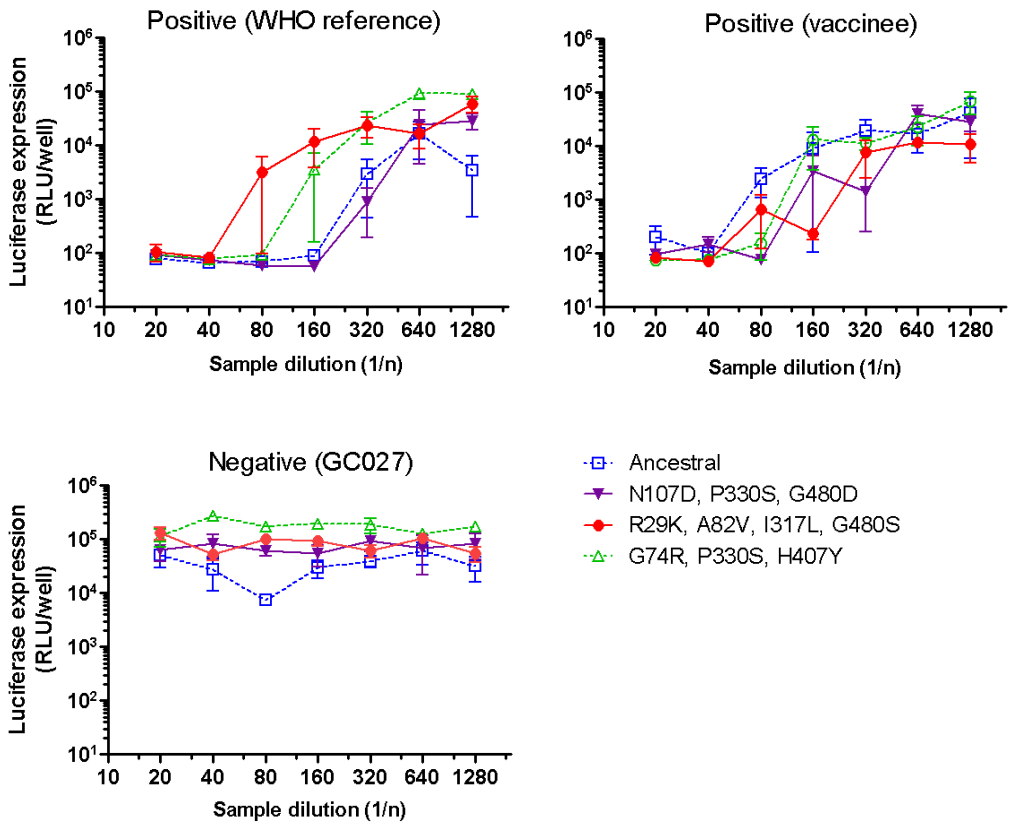


Figure 5.3: Neutralisation of mutant EBOV GP pseudotyped viruses by positive (WHO reference and vaccinee) and negative (GC027) control plasma samples. Pseudotyped viruses were incubated with dilutions of heat inactivated plasma samples at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours. Error bars are 1 standard error above and below the mean, n=4.

For each sample and mutant EBOV GP pseudotyped virus tested, the 90% inhibitory concentration (IC_{90}) of pseudotyped virus neutralisation was determined using an alteration of the Reed-Muench method (Reed and Muench, 1938). Results are presented in Figure 5.4. For samples that did not display neutralising activity, an IC_{90} value of 20, which was the lowest dilution of sample tested in the assay, was assigned.

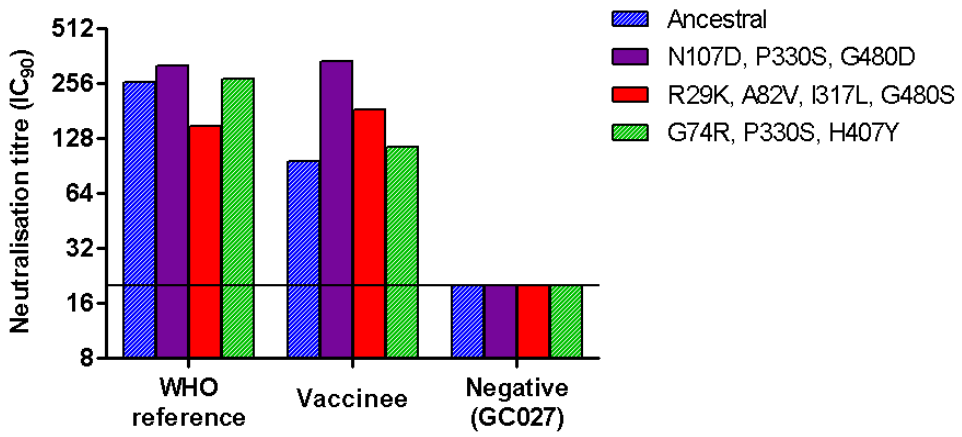


Figure 5.4: Neutralisation of mutant EBOV GP pseudotyped viruses by positive (WHO reference and vaccinee) and negative (GC027) control plasma samples. Pseudotyped viruses were incubated with dilutions of heat inactivated plasma samples at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and IC_{90} of pseudotyped virus neutralisation were determined. Horizontal line represents background level of pseudotyped virus neutralisation and is equal to the lowest dilution of sample tested in the assay (1/20).

Neutralisation of mutant EBOV GP pseudotyped viruses by anti-EBOV GP mAb

To further evaluate the effects of these mutations, the mutant EBOV GP pseudotyped viruses were used in neutralisation assays with human anti-EBOV GP mAb KZ52 as described in Section 2.5.3. KZ52 was able to neutralise the ancestral GP, mutant GP N107D, P330S, G480D and GP R29K, A82V, I317L, G480S pseudotyped viruses, however mutant GP G74R, P330S, H407Y pseudotyped virus was able to escape neutralisation by mAb KZ52 (Figure 5.5A). For each mutant EBOV GP pseudotyped virus tested, the IC_{90} of pseudotyped virus neutralisation by KZ52 was determined as described above, and results are presented in Figure 5.5B. KZ52 was unable to neutralise mutant GP G74R, P330S, H407Y pseudotyped virus, and therefore an IC_{90} of 1.25 µg/ml, which was the highest concentration of KZ52 tested in the assay, was assigned.

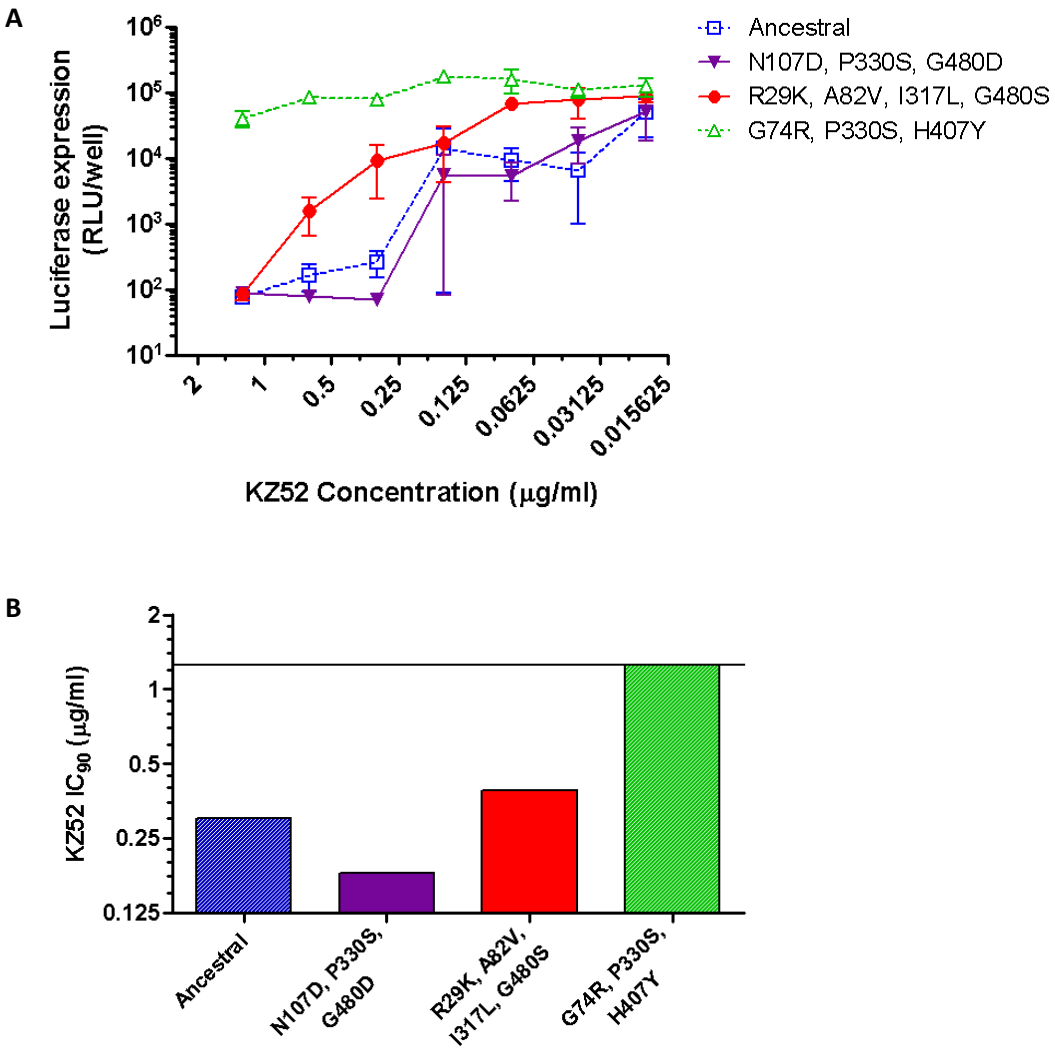
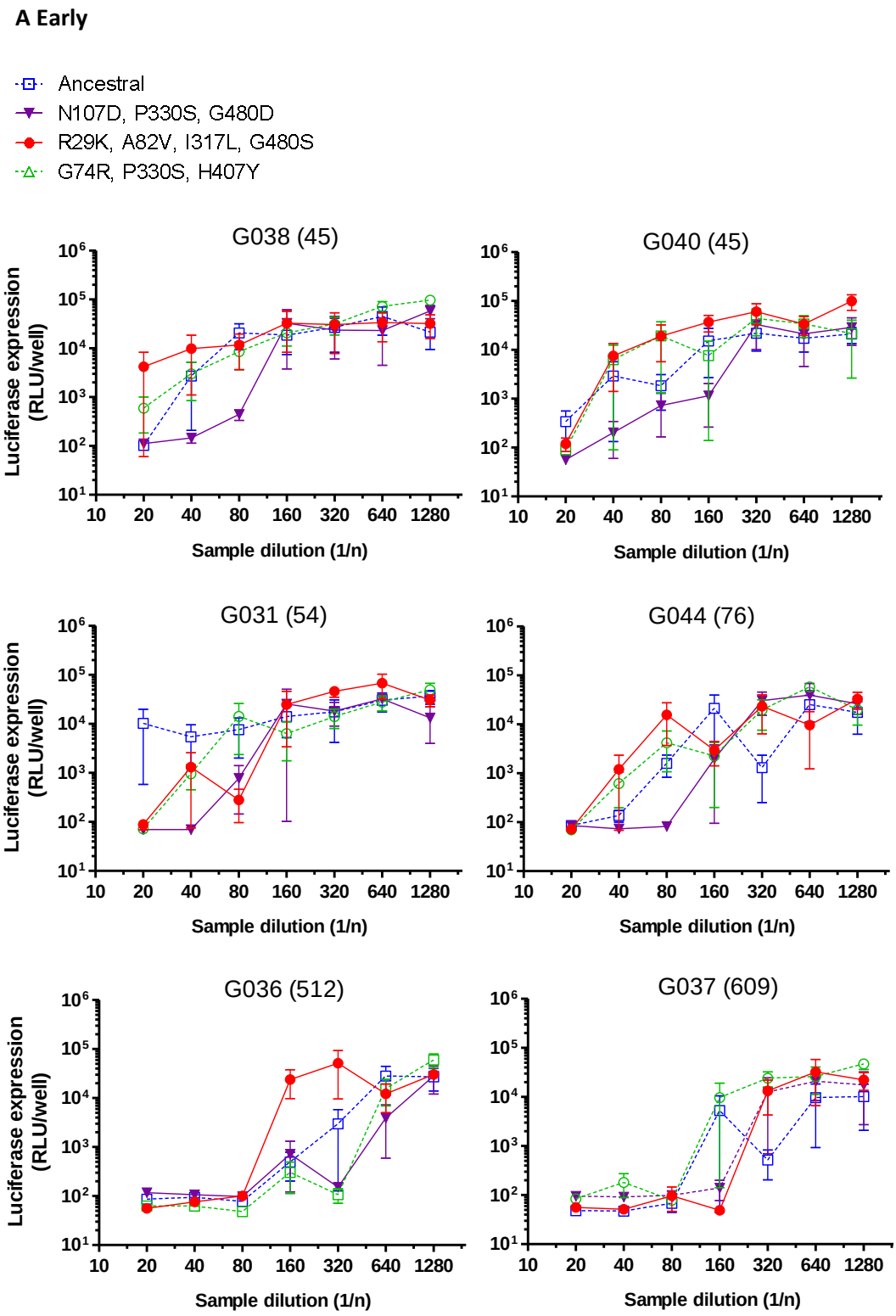


Figure 5.5: Neutralisation of mutant EBOV GP pseudotyped viruses by human anti-EBOV GP mAb, KZ52. Pseudotyped viruses were incubated with dilutions of KZ52 at 37°C for one hour before being added to Vero E6 cell monolayers. A) Luminescence was detected after 24 hours (error bars are 1 standard error above and below the mean, n=4), and B) IC_{90} of pseudotyped virus neutralisation were determined. Horizontal line represents background level of pseudotyped virus neutralisation and is equal to the highest concentration of KZ52 tested in the assay (1.25 µg/ml).

5.3.5 Neutralisation of mutant EBOV GP pseudotyped viruses by EVD survivor plasma

Neutralisation of the mutant EBOV GP pseudotyped viruses by plasma samples collected from 12 EVD survivors of the 2013-2016 EBOV outbreak 3 to 14 months post-infection (Section 2.5.1) was evaluated as described in Section 2.5.3. Results for each sample and

pseudotyped virus tested are represented graphically as luminescence against sample dilution in Figure 5.6.



B Late

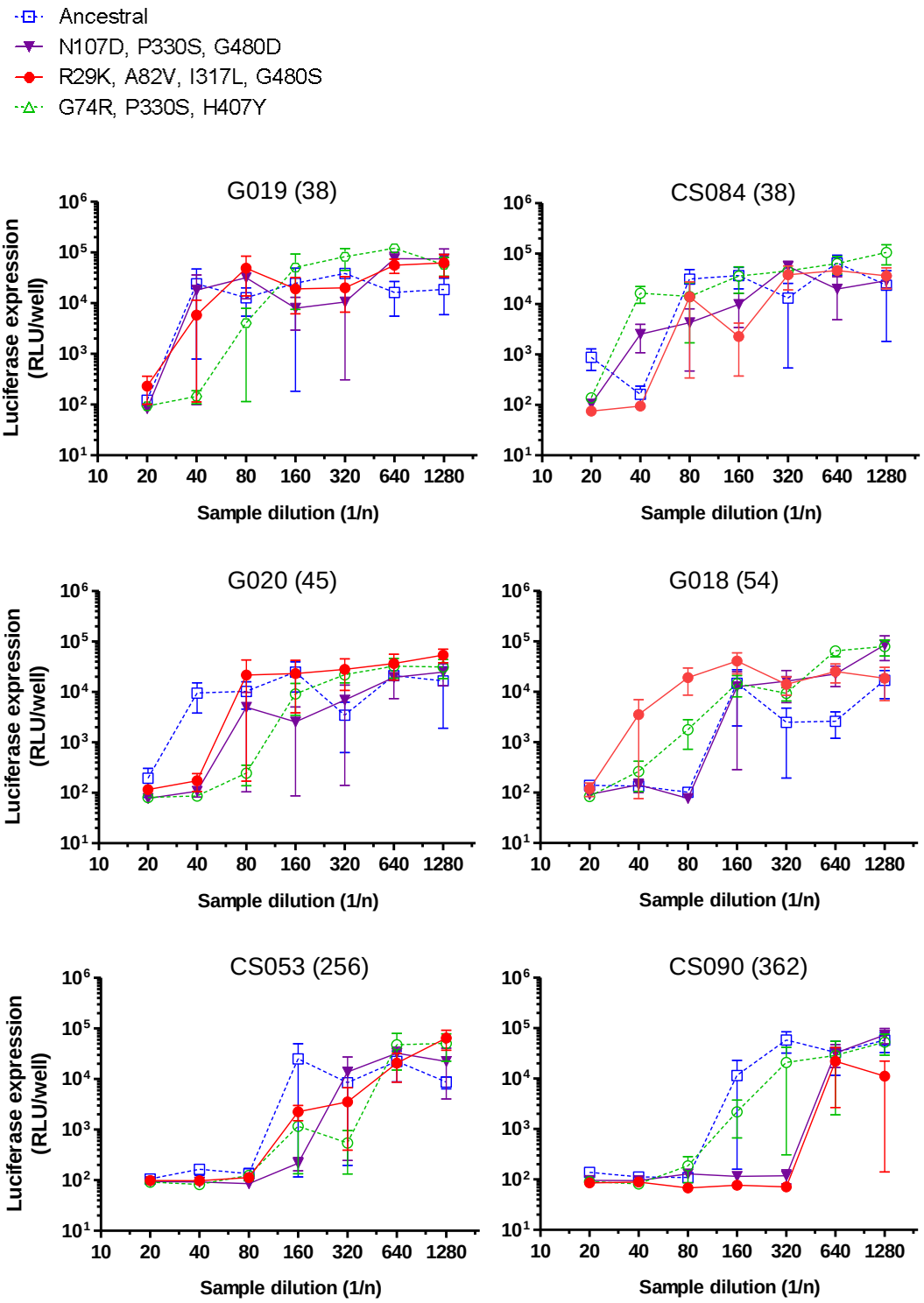


Figure 5.8: Neutralisation of mutant EBOV GP pseudotyped viruses by plasma samples from EVD survivors infected A) early or B) later on during the 2013-2016 EBOV outbreak. Pseudotyped viruses were incubated with dilutions of heat inactivated plasma samples at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours. Error bars are 1 standard error above and below the mean, n=4. Live EBOV (Mayinga) neutralisation results (geometric mean titre) are shown in brackets.

The samples were divided into two groups, depending on if the donor was infected early or later on during the outbreak, which was determined based on the date the donor was discharged from the Ebola treatment centre (ETC) (Table 5.1). Live EBOV neutralisation results for each sample are also shown in Table 5.1, although it should be noted that the EBOV strain used in the live neutralisation assay is Mayinga, not Makona.

Table 5.1 Ebola virus disease (EVD) survivor plasma samples tested in the EBOV GP pseudotyped virus neutralisation assay. Plasma samples from EVD survivors infected early or later on during the 2013-2016 EBOV outbreak were obtained from a pre-existing biobank. Live EBOV (Mayinga) neutralisation data were available for each sample.

Sample		Date discharged from ETC	Live EBOV (Mayinga) neutralisation (GMT)
G038	Early	04-Apr-14	45
G037		09-Apr-14	609
G040		12-Apr-14	45
G044		15-Apr-14	76
G036		20-Apr-14	512
G031		26-Apr-14	54
G019	Late	17-Dec-14	38
G018		30-Dec-14	54
G020		31-Dec-14	45
CS090		19-Apr-15	362
CS053		22-Apr-15	256
CS084		29-May-15	38

Abbreviations: ETC, Ebola treatment centre; GMT, Geometric mean titre.

The IC_{90} of pseudotyped virus neutralisation by each sample was determined as previously described (Section 5.3.4), and results are presented in Figure 5.7. Although differences in IC_{90} values were observed, the EVD survivor plasma samples tested were still generally able to neutralise the mutant EBOV GP pseudotyped viruses. Samples that did not display sufficient neutralising activity for an IC_{90} value to be able to be calculated were, G038 against mutant GP R29K, A82V, I317L, G480S pseudotyped virus, G040 against mutant GP

N107D, P330S, G480D pseudotyped virus and G019 against ancestral GP pseudotyped virus, and therefore IC_{90} values of 20 were assigned to these.

As mentioned above, although the EVD survivor plasma samples were still able to neutralise the mutant EBOV GP pseudotyped viruses, differences in IC_{90} values were observed (Figure 5.7). For example, plasma from EVD survivors G038, G044, G036 and G019, which were infected earlier on during the 2013-2016 EVD outbreak, displayed lower neutralising activity against pseudotyped virus bearing the R29K, A82V, I317L and G480S mutations in GP, which were identified later on in the outbreak, compared to pseudotyped viruses bearing GPs that were identified earlier during the outbreak. Whereas plasma from EVD survivors G019, G020, CS090 and CS053, which were infected later on during the outbreak, displayed lower neutralising activity against pseudotyped virus bearing the ancestral (early) GP compared to pseudotyped viruses bearing GPs that were identified later on in the outbreak. However no statistically significant differences in neutralisation between the mutant EBOV GP pseudotyped viruses were detected for either the early or late EVD survivor plasma samples (Friedman test, $p = 0.9396$ and $p = 0.0882$, respectively). The mean results are presented in Figure 5.8.

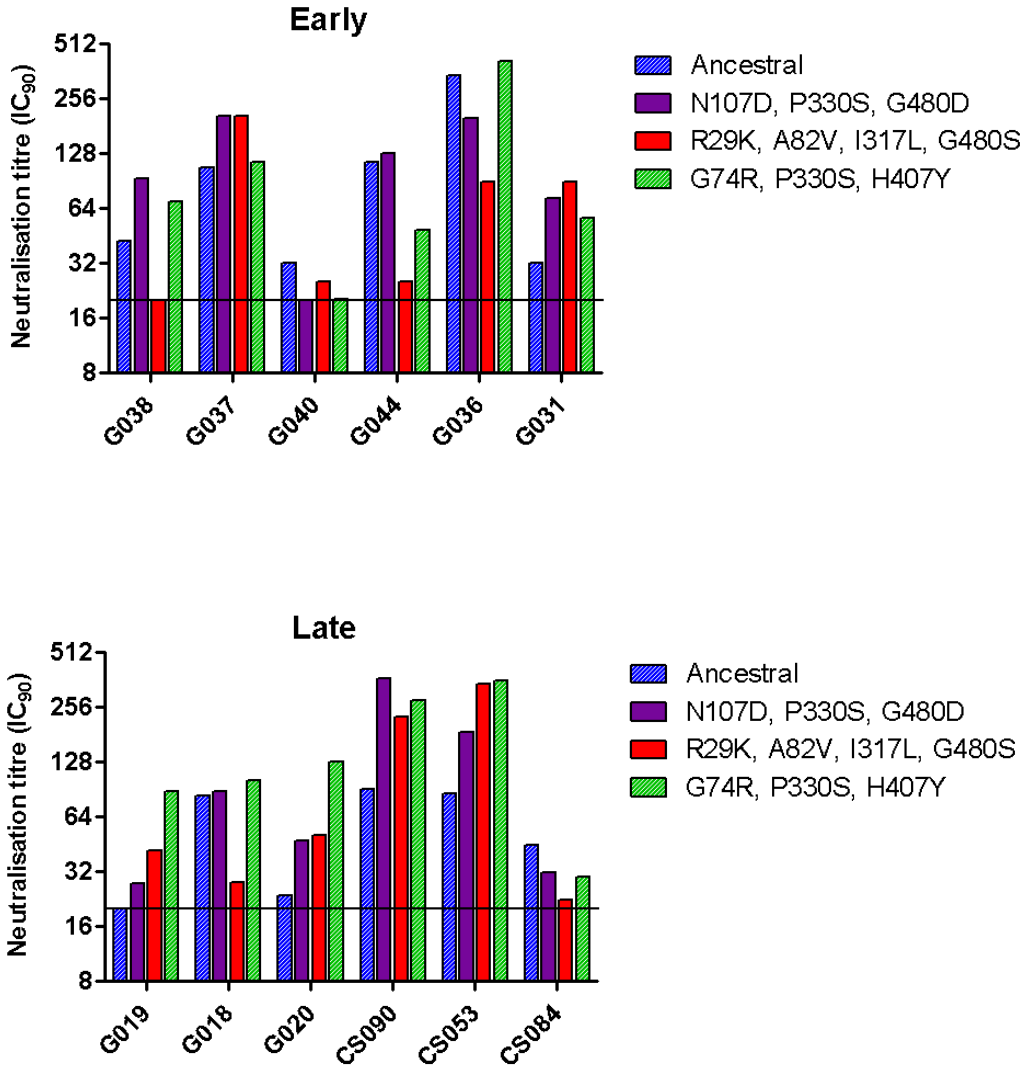


Figure 5.7: Neutralisation of mutant EBOV GP pseudotyped viruses by plasma samples from EVD survivors infected early or later on during the 2013-2016 EBOV outbreak. Pseudotyped viruses were incubated with dilutions of heat inactivated plasma samples at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and IC_{90} of pseudotyped virus neutralisation were determined. Horizontal line represents background level of pseudotyped virus neutralisation and is equal to the lowest dilution of sample tested in the assay (1/20).

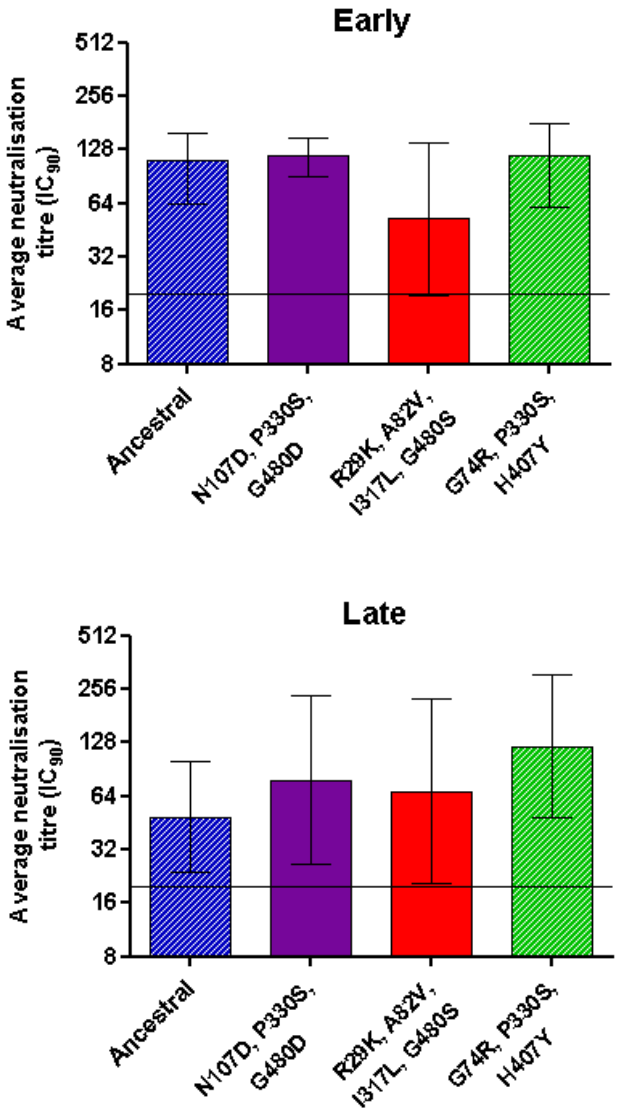


Figure 5.8: Neutralisation of mutant EBOV GP pseudotyped viruses by plasma samples from EVD survivors infected early or later on during the 2013-2016 EBOV outbreak. Pseudotyped viruses were incubated with dilutions of heat inactivated plasma samples at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and IC_{90} of pseudotyped virus neutralisation were determined. Geometric means with 95% CIs are shown, n=6. Horizontal line represents background level of pseudotyped virus neutralisation and is equal to the lowest dilution of sample tested in the assay (1/20).

5.3.6 Neutralisation of mutant EBOV GP pseudotyped viruses by anti-EBOV GP mAb

Anti-EBOV GP mAb KZ52, at a single concentration of 1.25 µg/ml, was included in each neutralisation assay to serve as a positive control. KZ52 consistently neutralised ancestral GP, mutant GP N107D, P330S, G480D and GP R29K, A82V, I317L, G480S pseudotyped viruses, however mutant GP G74R, P330S, H407Y pseudotyped virus was only partially neutralised by KZ52 (Figure 5.9). This confirmed that the escape of mutant GP G74R, P330S, H407Y pseudotyped virus from neutralisation by KZ52 previously observed (Figure 5.5) was reproducible. Analysis of the data by one-way ANOVA revealed statistically significant differences between the groups (p = 0.0021). The difference between individual groups was further examined by post hoc Tukey's multiple comparison test using GraphPad Prism v5. Statistically significant differences are highlighted in Figure 5.9.

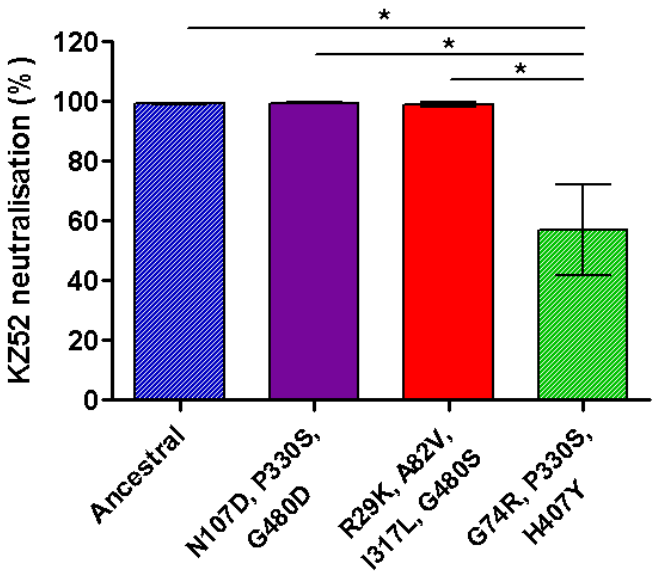


Figure 5.9: Neutralisation of mutant EBOV GP pseudotyped viruses by human anti-EBOV GP mAb, KZ52. Pseudotyped viruses were incubated with KZ52 (1.25 µg/ml) at 37°C for one hour before being added to Vero E6 cell monolayers. Luminescence was detected after 24 hours and percentage neutralisation was calculated. Error bars are 1 standard error above and below the mean, n=6. The difference between groups was examined by one-way ANOVA and by post hoc Tukey's multiple comparison test. Statistically significant differences are highlighted (*p < 0.05).

In order to elucidate the possible mechanism of escape of mutant GP G74R, P330S, H407Y pseudotyped virus from neutralisation by KZ52, the amino acid positions were mapped onto a published structure of EBOV GP in complex with KZ52 [Protein Data Bank (PDB) identifier 3CSY (Lee *et al.*, 2008)] using the molecular-graphics programme CCP4MG (McNicholas *et al.*, 2011). Amino acid position 74 is located in the RBD of EBOV GP, whereas positions 330 and 407 are located in the MLD (Figure 5.1). The MLD had been deleted from the GP, as it is refractory to crystallisation (Lee *et al.*, 2008), and therefore amino acid positions 330 and 407 could not be mapped. By observing the EBOV GP/KZ52 structure, it can be seen that amino acid position 74 of EBOV GP is located near the interface of EBOV GP and KZ52 (Figure 5.10). Amino acid positions 82 and 107 of EBOV GP were also mapped onto the EBOV GP/KZ52 structure and are displayed in Figure 5.10.

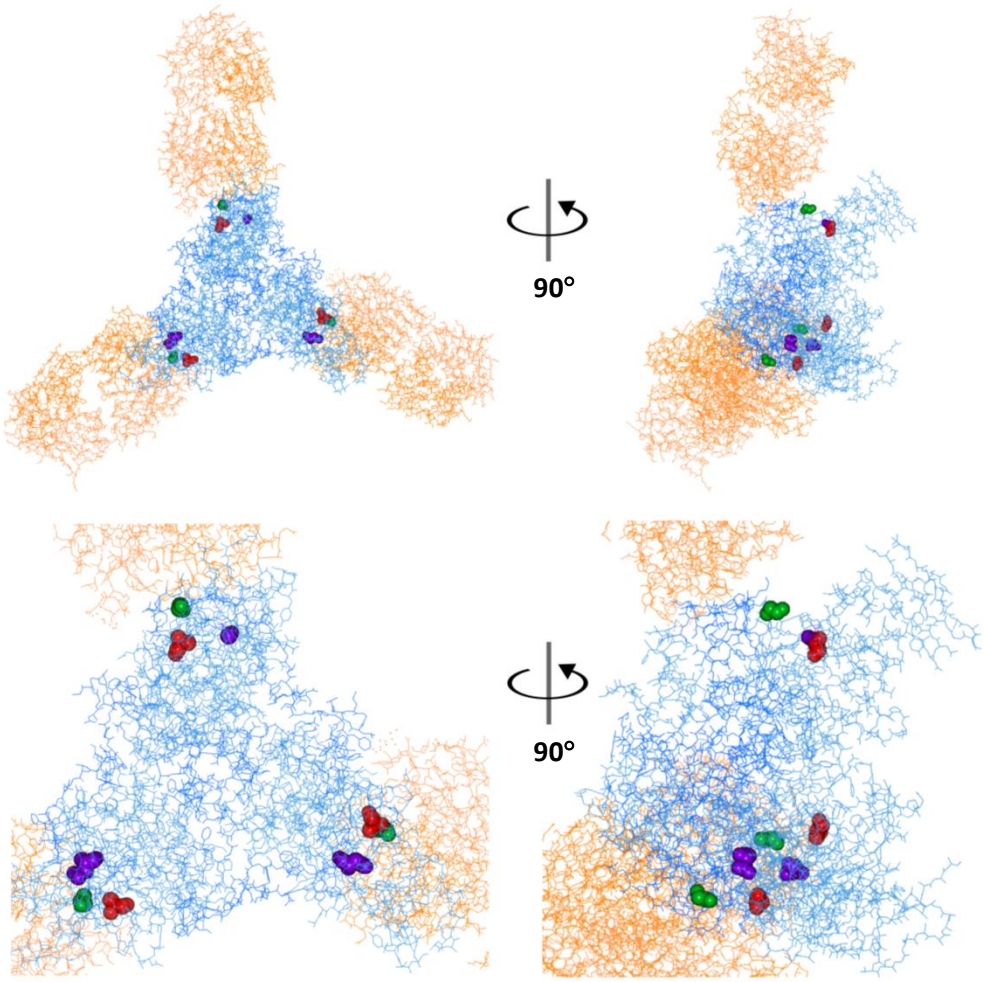


Figure 5.10: Structure of Ebola virus (EBOV) surface glycoprotein ($GP_{1,2}$) trimer in complex with neutralising antibody KZ52 [PDB identifier 3CSY (Lee *et al.*, 2008)]. $GP_{1,2}$ are shown in blue, and heavy and light chains of KZ52 are shown in orange. Green, red and purple spheres indicate amino acid residues 74, 82 and 107 of EBOV GP, respectively. Figure created by Dr Gillian Slack using CCP4MG.

5.4 Discussion

The primary objective for this Chapter was to compare neutralisation of mutant EBOV GP pseudotyped viruses by EVD survivor plasma, with a view to investigating the potential for immune escape of naturally occurring EBOV GP mutants. The impact of amino acid changes in EBOV GP that arose during the 2013-2016 EVD epidemic on neutralisation of EBOV GP pseudotyped viruses by EVD survivor and EBOV GP vaccinee plasma, as well as a neutralising anti-EBOV GP mAb was investigated.

5.4.1 Selection and production of EBOV GP mutant pseudotyped viruses

Published EBOV genome sequences were compared with an early EBOV (Makona) isolate [GenBank accession number KJ660348 (Baize *et al.*, 2014)], referred to here as the 'ancestral' EBOV GP variant, to identify a series of mutations that occurred within the EBOV GP during the 2013-2016 EVD outbreak. Three EBOV variants of particular interest, as they contained multiple amino acid changes in the GP, were selected for further investigation (Figure 5.1). Site-directed mutagenesis was then applied to introduce the desired mutations into the EBOV GP sequence to generate a panel of mutant EBOV GP expression vectors. The plasmids were sequenced to confirm that the modification(s) had been made successfully. This was considered an essential prerequisite to the use of the plasmids in the generation of mutant EBOV GP pseudotyped viruses. Long term stocks of transformed *E. coli* were deposited for each plasmid. The extent of the sequenced regions for the mutant EBOV GP panel of plasmids is shown in Figure 2.1B.

Mutant EBOV GP pseudotyped VSVs were generated by transfection of 293T/17 cells with plasmid DNA encoding mutated EBOV GP, followed by infection with rVSV-ΔG-Luc-VSV-G virus. After each transfection and infection, the EBOV GP pseudotyped viruses were harvested and quantified by measuring luminescence in Vero E6 cells. Differences in luminescence were observed depending on the pseudotyped virus used for infection;

however these differences may be due technical variability in the production and titration of the pseudotyped viruses, rather than due to the mutations, and therefore repeat experiments are required to confirm the validity of these results. Similar studies utilising EBOV GP pseudotyped viruses have found that an A82V mutation in EBOV GP is associated with increased infectivity of Vero cells (Diehl *et al.*, 2016; Kurosaki *et al.*, 2018). Although, no significant differences were found in disease progression, pathogenicity or virus shedding of EBOV Makona isolates derived from different stages of the epidemic in rhesus macaques (Marzi *et al.*, 2018). In the current study, pseudotyped VSV bearing EBOV GP containing an A82V mutation did not displayed increased infectivity of Vero E6 cells compared to pseudotyped VSV bearing ancestral (A82) EBOV GP.

The panel of mutant EBOV GP pseudotyped viruses were subsequently analysed in neutralisation assays with plasma from EVD survivors, to assess the effect on immune escape. Based on the titration results, a target pseudotyped virus input of approximately 2 x 10^4 RLU/well was selected for use.

5.4.2 Neutralisation of mutant EBOV GP pseudotyped viruses by EVD survivor plasma

It is thought that EVD survivors are protected against subsequent infection with EBOV, and that neutralising antibodies to the EBOV surface GP are involved. However, mutations in EBOV GP may affect the ability of antibodies from convalescent patients to protect against new variants of the virus. It could therefore be hypothesised that plasma from individuals infected early on during on outbreak may be less effective at neutralising viral variants that arise later in an outbreak. Plasma from EVD survivors of the 2013-2016 EBOV epidemic that had been infected early (during April 2014), or later on (between December 2014 and June 2015) during the outbreak, was evaluated for neutralisation capacity of ancestral and mutant EBOV GP pseudotyped viruses. Generally, the EVD survivor plasma was still able to neutralise the ancestral and mutant EBOV GP pseudotyped viruses, however differences in

neutralisation were observed. Three samples did not display sufficient neutralising activity for IC_{90} values to be able to be calculated. These samples displayed relatively low levels of neutralisation against live EBOV (Table 5.1), therefore the lower sensitivity and high variability of the pseudotyped virus neutralisation assay may explain the lack of neutralisation observed by these samples, and neutralisation may be achieved if a lower dilution of sample is used.

In the current study, plasma from four out of nine EVD survivors that were infected earlier on during the 2013-2016 outbreak displayed lower neutralising activity against pseudotyped VSV bearing EBOV GP containing R29K, A82V, I317L and G480S mutations, which were identified later on in the outbreak, compared to pseudotyped VSVs bearing EBOV GPs that were identified earlier during the outbreak. Whereas plasma from four out of eight EVD survivors that were infected later on during the outbreak, displayed lower neutralising activity against pseudotyped VSV bearing the ancestral (early) GP compared to pseudotyped VSVs bearing EBOV GPs that were identified later on in the outbreak. Although no statistically significant differences in neutralisation between the mutant EBOV GP pseudotyped viruses were observed. These results suggest that mutations in EBOV GP could affect the level of neutralisation of antibodies derived from EVD convalescent volunteers; however the mutations evaluated did not result in complete loss of neutralisation. Although this is not surprising considering the polyclonal nature of antibodies in plasma from EVD survivors, and number of neutralising epitopes within EBOV GP. These differences could also partially be due to general variability and low sensitivity of the neutralisation assay, rather than effects of the mutations, and therefore repeat experiments should be performed as the current observations have been made on the basis of one study. Another limitation of this study is that it is not known which variant of EBOV each individual was infected with, and also the small number of samples tested.

5.4.3 Neutralisation of mutant EBOV GP pseudotyped viruses by EBOV GP vaccine plasma

As discussed in Section 1.5.3, EBOV GP is a key antigenic target for the development of vaccines against EVD. Neutralisation of ancestral and mutant EBOV GP pseudotyped viruses by plasma from an individual who had previously been vaccinated with two candidate EBOV vaccines (ChAd3 and VSV-EBOV GP) was assessed, to determine potential immune escape resulting from multiple GP amino acid changes from the vaccine GP antigen. The VSV-EBOV vaccine candidate is a live attenuated recombinant virus consisting of the VSV strain Indiana, with the GP of the EBOV Kikwit 1995 strain replacing the gene for the VSV-G, whereas the ChAd3-EBOV GP vaccine consists of a recombinant replication deficient ChAd3 vector expressing GP from the EBOV Mayinga strain. Amino acid differences in the GP of EBOV Mayinga, Kikwit and Makona strains are shown in Table 5.2. The GP sequences of EBOV Mayinga and Kikwit differ by 10 amino acids. The GP sequences of EBOV Mayinga and Kikwit differ by 19 and 18 amino acids, respectively, compared to EBOV Makona. The vaccinee plasma was able to neutralise the ancestral and mutant EBOV (Makona) GP pseudotyped viruses, suggesting that vaccines containing GP from previous EBOV strains are able to induce neutralising antibodies against variants of the EBOV Makona strain that arose during the 2013-2016 EVD epidemic in West Africa. Although additional samples from EBOV GP vaccinated individuals should be tested as this observation was made on the basis of one vaccinee sample only.

In summary, mutation of EBOV GP did not lead to escape of neutralisation of EBOV GP pseudotyped viruses by EVD survivor or EBOV GP vaccinee plasma. This is likely due to the presence of multiple neutralising epitopes within the GP of EBOV, which are targeted by multiple antibodies generated as part of the hosts' polyclonal humoral immune response to infection or vaccination.

Table 5.2 Amino acid differences in the GP of different EBOV strains.

Subunit	Domain	Position	Mayinga (NC_002549)	Kikwit (AY354458)	Makona (KJ660348)
GP_1	Base	47	Aspartic acid (D)	Glutamic acid (E)	Aspartic acid (D)
	Glycan cap	262	Threonine (T)	Threonine (T)	Alanine (A)
		310	Valine (V)	Alanine (A)	Alanine (A)
	MLD	314	Glycine (G)	Arginine (R)	Glycine (G)
		315	Alanine (A)	Alanine (A)	Proline (P)
		331	Glycine (G)	Glycine (G)	Glutamic acid (E)
		336	Threonine (T)	Threonine (T)	Asparagine (N)
		359	Glutamic acid (E)	Glutamic acid (E)	Lysine (K)
		377	Serine (S)	Proline (P)	Proline (P)
		378	Leucine (L)	Proline (P)	Proline (P)
		382	Proline (P)	Proline (P)	Threonine (T)
		405	Glutamic acid (E)	Glutamic acid (E)	Glycine (G)
		411	Threonine (T)	Threonine (T)	Alanine (A)
		422	Serine (S)	Proline (P)	Proline (P)
		430	Proline (P)	Proline (P)	Leucine (L)
		440	Serine (S)	Glycine (G)	Serine (S)
		441	Threonine (T)	Threonine (T)	Alanine (A)
		443	Phenylalanine (F)	Leucine (L)	Serine (S)
		446	Proline (P)	Proline (P)	Leucine (L)
		455	Histidine (H)	Histidine (H)	Tyrosine (Y)
GP_2	Furin cleavage site	499	Threonine (T)	Alanine (A)	Threonine (T)
	Pre-IFL	503	Alanine (A)	Alanine (A)	Valine (V)
	IFL	544	Isoleucine (I)	Threonine (T)	Threonine (T)

Abbreviations: MLD, Mucin-like domain; IFL, Internal fusion loop.

5.4.4 Neutralisation of mutant EBOV GP pseudotyped viruses by anti-EBOV GP mAb

Various preclinical studies have demonstrated that mAbs can be effective EVD treatments, however a potential limitation is that epitope mutations might reduce efficacy (Moekotte *et al.*, 2016). Furthermore, EBOV escape variants have previously been discovered in infected NHPs after treatment with the mAb cocktail MB-003, suggesting that EBOV could evolve to become resistant to sequence-based candidate therapeutics (Kugelman *et al.*, 2015a; Kugelman *et al.*, 2015b).

Ancestral and mutant EBOV GP pseudotyped viruses were evaluated for their neutralisation by human anti-EBOV GP mAb, KZ52. KZ52 is an antibody isolated from a human survivor of the 1995 outbreak in Kikwit. KZ52 binds to residues within both GP_1 and GP_2 at the base of the GP trimer, locking GP in its pre-fusion conformation and thereby preventing the conformational rearrangements required to drive membrane fusion. KZ52 recognises residues 42-43 at the N terminus of GP_1, and 505-514 and 549-556 at the N terminus of GP_2 (Lee *et al.*, 2008). Alanine-scanning mutagenesis (shotgun mutagenesis) has been used to determine the specific epitope residues for KZ52, and results were validated by neutralisation escape (Davidson *et al.*, 2015). In the current study, mutant EBOV GP G74R, P330S, H407Y pseudotyped virus was able to partially escape neutralisation by mAb KZ52. Amino acid positions 330 and 407 are located in the MLD, which is removed during priming of GP in late endosomes (see Section 1.2.4), whereas amino acid position 74 is located in the RBD, suggesting that the G74R mutation may be responsible for reduced neutralisation by KZ52. Amino acid position 74 was mapped onto a published structure of EBOV GP in complex with KZ52 and was shown to be located near the interface of EBOV GP and KZ52. Although the G74R substitution is located outside of the KZ52-specific epitope, this mutation may affect the KZ52 epitope conformation by changing electric charge around the epitope (Kajihara *et al.*, 2013). Interestingly, in the current study, mutant EBOV GP G74R, P330S, H407Y pseudotyped virus displayed lower luminescence following infection of Vero

E6 cells compared to the other EBOV GP pseudotyped viruses tested (Figure 5.2), and therefore the G74R mutation may impose a fitness cost on the virus (Zhao *et al.*, 2006). However, this mutation did not affect neutralisation by EVD survivor plasma in this study, and therefore further investigation should be performed to determine the impact of this mutation on viral infectivity and fitness.

A recent study evaluating binding of anti-EBOV GP mAbs to V82 and A82 variants of EBOV (Makona) GP expressed in 293T cells by flow cytometry, found that KZ52 exhibited more that 50% reduction in binding to V82 compared to A82 EBOV GP variants (Brannan *et al.*, 2019). In the current study, KZ52 did not display decreased neutralisation of pseudotyped VSV bearing EBOV GP containing an A82V mutation, therefore the A82V mutation could reduce KZ52 binding, but without affecting neutralisation. The mutations evaluated in the current study could be applied in various binding assays, to assess their effects on antibody binding, as well as host cell receptor binding.

5.5 Conclusions

A set of mutations that occurred within the EBOV GP during the 2013-2016 EVD outbreak was identified, and site-directed mutagenesis was successfully applied to generate the expression plasmids required. Subsequently, they were used to generate mutant EBOV GP pseudotyped viruses, which were then used to test the hypothesis of the study (described in Section 1.6). Thus, the impact of mutations in EBOV GP on the neutralising ability of antibodies derived from EVD convalescent volunteers was investigated.

The results presented here have examined the effect of mutations in EBOV GP on antibody immune escape. They support the view that multiple naturally occurring mutations in EBOV GP do not result in escape from neutralising polyclonal antibodies derived from EVD

convalescent volunteers or EBOV GP vaccinated individuals. This suggests the presence of multiple neutralising epitopes within EBOV GP that enable the GP sequence of EBOV strains from over 20 years ago to induce antibodies that are able to neutralise newly emerging EBOV strains with up to 20 amino acid changes in their GP. However, some of these mutations can result in reduced neutralisation by certain EBOV GP-specific mAbs. This reiterates the importance of using antibody cocktail treatments targeting multiple distinct neutralising epitopes, rather than monotherapy with a single neutralising mAb, to reduce the possibility of escape from neutralisation due to mutations in EBOV GP. These results also highlight the importance of rapidly sequencing viral isolates from patients to be treated, to ensure that the selected mAb therapy is likely to be effective (Vaughan *et al.*, 2018).

The current study has developed tools with which to expedite future work. Mutant EBOV GP expression plasmids have been generated and are available for pdeudotyped virus production and further assessment of effects of EBOV GP mutations on antibody binding and neutralisation. This has the potential to provide a better understanding of the correlates of protection against EBOV.

Chapter 6 General Discussion

It is thought that EVD survivors are protected against subsequent infection with EBOV, and that neutralising antibodies to the EBOV surface GP are potential correlates of protection (Saphire *et al.*, 2018; Warfield *et al.*, 2018). Due to its severe pathogenicity, potential transmission from person-to-person contact, and lack of approved vaccines or antiviral treatments, handling of EBOV for research-based purposes is limited to CL4 laboratories. Pseudotyped viruses can be used in serological assays as alternatives to live infectious viruses that require high levels of bio-containment, to investigate host cell receptor interaction and antibody binding and neutralisation.

Immune responses by an infected host exert strong selection pressures on pathogens. The prolonged transmission of EBOV in humans during the 2013-2016 EVD outbreak in West Africa resulted in an accumulation of mutations within the EBOV genome, which may impact on the efficacy of vaccines and immunotherapeutics. A useful application for pseudotyped viruses, therefore, is to investigate how amino acid changes in EBOV GP might affect neutralisation by antibodies. The current study investigated the potential for naturally acquired mutations in EBOV GP to result in escape from neutralising antibodies derived from EVD convalescent volunteers and EBOV GP vaccinated individuals, as well as EBOV GP-specific neutralising mAbs.

6.1 Generation and neutralisation of mutant EBOV GP pseudotyped viruses

6.1.1. EBOV GP pseudotyped virus neutralisation assays

Pseudotyped viruses are replication-defective chimeric virions that consist of the structural and enzymatic core of one virus, bearing the envelope protein or glycoprotein of another, and encode a quantifiable reporter gene. As discussed in Section 1.4, retroviruses and rhabdoviruses are commonly used as cores for pseudotyped viruses. In the current study, EBOV GP pseudotyped lentivirus and VSV systems were assessed for their suitability to measure the neutralising activity of EVD convalescent plasma (Chapters 3 and 4, respectively). The VSV-based pseudotyped virus neutralisation assay was applied in the current study, as its sensitivity, specificity and ability to differentiate between neutralising activity appeared to be greater than the lentivirus-based system (see section 4.3.7). Importantly, correlation between live EBOV and EBOV GP pseudotyped virus neutralisation was significantly greater for the VSV-based assay.

6.1.2 Generation of mutant EBOV GP pseudotyped viruses

As demonstrated in Chapter 5 (see Section 5.3), the envelope protein of a pseudotyped virus can be modified by mutating the expression plasmid used to generate the pseudotyped virus. Pseudotyped viruses have previously been used to study the effects of mutations within the EBOV genome on host cell tropism and infectivity. In the current study, bioinformatics analysis of publically available EBOV genome sequences was used to identify non-synonymous mutations that occurred within the EBOV GP during the 2013-2016 EVD outbreak. Three variants that contained multiple amino acid changes were selected to test the hypothesis of the study described in Section 1.6.

6.2 The study hypothesis

The current study sought to address whether naturally acquired mutations in EBOV GP could result in escape from neutralisation by anti-EBOV GP polyclonal and mAbs.

6.2.1 Anti-EBOV GP polyclonal antibodies

Mutations in EBOV GP may affect the ability of antibodies from convalescent patients or vaccinated individuals to protect against new variants of the virus. It was shown that mutations in EBOV GP did not result in significant escape in neutralisation of EBOV GP pseudotyped viruses by EVD survivor or EBOV GP vaccinee plasma. This is likely due to the presence of multiple neutralising epitopes within the EBOV GP, which are targeted by multiple antibodies generated as part of the hosts' polyclonal humoral immune response to infection or vaccination. This also suggests that the presence of multiple neutralising epitopes in EBOV GP enable the GP sequence of EBOV strains from over 20 years ago to induce antibodies that are able to neutralise newly emerging EBOV strains with up to 20 amino acid changes in their GP.

6.2.2 Anti-EBOV GP neutralising mAbs

Results of the current study, suggest that a G74R mutation in EBOV GP is able to result in escape from neutralising mAb, KZ52. KZ52 was isolated from B cells of a human survivor of the 1995 Kikwit EVD outbreak (Maruyama *et al.*, 1999) and protected rodents from EBOV infection (Parren *et al.*, 2002), however it was ineffective at inhibiting viral replication and preventing disease in NHPs (Oswald *et al.*, 2007), suggesting that monotherapy with this single neutralising mAb may not be a suitable treatment for EVD. Furthermore, epitope mutations may result in reduced efficacy of mAb therapies. EBOV escape variants have previously been discovered in infected NHPs after treatment with the mAb cocktail MB-003, suggesting that EBOV could evolve to become resistant to sequence-based candidate therapeutics (Kugelman *et al.*, 2015a; Kugelman *et al.*, 2015b). These findings reiterate the

importance of using antibody cocktail treatments targeting multiple distinct neutralising epitopes rather than monotherapy with a single neutralising mAb, to reduce the possibility of treatment failure due to genetic changes. The current study further highlights the potential of EBOV GP evolutionary mutations to evade mAb therapeutics, and supports the role of real-time sequencing during outbreaks to predict the efficacy of antibody-based treatments.

6.3 Future work

6.3.1 Alternative pseudotyped virus platforms

During the course of assessing the suitability of an EBOV GP pseudotyped virus assay to measure the neutralising ability of EVD convalescent plasma, it was apparent that pseudotyped virus neutralisation results are often variable and correlate poorly with live EBOV neutralisation (Wilkinson *et al.*, 2017). In addition, live EBOV neutralisation results could vary also. Therefore, alternative pseudotyped virus systems could be investigated to determine a more suitable platform. An EBOV pseudotyped virus (E-S-FLU) based on a non-replicating influenza virus (S-FLU) has been described (Xiao *et al.*, 2018) and replication-competent VSVs pseudotyped with EBOV GP have also been used to measure anti-EBOV neutralising antibodies (Wec *et al.*, 2016). However, it also became apparent that neutralisation capacity can differ among assays (Saphire *et al.*, 2018). Therefore, assessment of the impact of GP mutations on neutralisation using a single assay may not be optimal. Nevertheless, the EBOV GP pseudotyped VSV assay could be used as an initial qualitative screening assay, or as part of a panel of pseudotyped virus neutralisation assays, for identification of escape mutants before further investigation is carried out using infectious viral variants.

6.3.2 Additional EBOV GP mutations

Future studies could continue to utilise the EBOV GP pseudotyped virus system to characterise the effect of each of the mutations assessed during the current study individually, as well as the effects of additional mutations. Especially G74R, to ascertain whether this single mutation alone is responsible for mutant GP G74R, P330S, H407Y pseudotyped virus escape from neutralisation by KZ52. An extended panel of tools has been made available for this purpose by the current project, as relevant primers for each individual mutation are available (Table 2.1), and a selection of mutant EBOV GP expression plasmids have already been synthesised. Primer design and site-directed mutagenesis could be applied to generate additional mutant EBOV GP expression plasmids as required. Alternatively, custom synthesis of plasmids containing multiple mutations could also be utilised as far as is economically possible. Protein structural analysis could then be further investigated to predict the impact of amino acid changes on EBOV GP structure and antibody binding.

6.3.3 Additional antibody samples

The mutant EBOV GP pseudotyped viruses evaluated in the current study should be used in further studies to characterise the effect of these amino acid changes in EBOV GP on neutralisation by other EBOV GP-specific mAbs. In particular, it would be interesting to test mAbs that recognise a similar epitope to KZ52, such as 4G7 and 2G4 (Murin *et al.*, 2014), for their neutralising ability of mutant GP G74R, P330S, H407Y pseudotyped virus, which, in the current study, was able to escape from neutralisation by KZ52. 4G7 and 2G4 are components of the ZMAb and ZMapp antibody cocktails, which were administered to EVD patients under compassionate use protocols during the 2013-2016 EBOV outbreak. Therefore, it would also be interesting to test these anti-EBOV therapeutics against the mutant EBOV GP pseudotyped viruses generated in the current study.

Additional plasma or serum samples from EBOV GP vaccinated individuals should also be tested in neutralising assays using these mutant EBOV GP pseudotyped viruses, to confirm that vaccines containing GP from previous EBOV strains are able to induce neutralising antibodies against new variants of EBOV.

6.4 Conclusions

Since its discovery in 1976, EBOV has caused sporadic outbreaks across Central Africa and was responsible for the 2013-2016 EVD epidemic in West Africa, which was the largest EBOV outbreak on record and resulted in more than 28,600 cases and over 11,300 deaths. This outbreak constituted a public health emergency of international concern, and highlighted the urgent need for vaccines and therapeutics against EBOV. It is thought that EVD survivors are protected against subsequent infection with EBOV, and that neutralising antibodies, as well as T cell responses, to the EBOV surface GP are involved. The vast number of EVD survivors from the 2013-2016 EBOV outbreak has provided an opportunity to study the human immune response to EBOV infection.

The extensive human-to-human transmission of EBOV that occurred during the 2013-2016 EVD epidemic resulted in an accumulation of mutations within the EBOV genome. This raised concerns that EBOV could adapt to better infect and transmit between humans, and could also evolve to become resistant to sequence-based candidate therapeutics. Results of the current study suggest that multiple naturally occurring amino acid changes in EBOV GP do not have a significant impact on polyclonal neutralising antibodies derived from EVD convalescent volunteers or EBOV GP vaccinated individuals, however these changes can result in reduced neutralisation by certain EBOV GP-specific mAbs.

The current study demonstrated the potential value of real-time sequencing analyses and pseudotyped viruses to evaluate the potential impact of EBOV GP mutations on neutralising antibody immune escape. EBOV still poses a major public health threat, as evidenced by the current ongoing EVD outbreak in the DRC. Therefore these types of studies are more pertinent than ever, and have the potential to provide a better understanding of EVD vaccine and therapeutic efficacy, and correlates of protection against EBOV.

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