

Development of viral vectors for passive immunisation against influenza

**Thesis submitted in partial fulfilment of the requirements for the degree
of Doctor of Philosophy**



Tiong Kit Tan

Gene Medicine Research Group

Radcliffe Department of Medicine

and

Wolfson College

University of Oxford

Supervisors: Prof Stephen C. Hyde and Prof Deborah R. Gill

Trinity Term, 2017

Development of viral vectors for passive immunisation against influenza

Tiong Kit Tan

Radcliffe Department of Medicine and Wolfson College, University of Oxford

Thesis submitted for the degree of Doctor of Philosophy, Trinity Term, 2017

Abstract

Seasonal influenza is a major global health threat causing more than 500,000 deaths annually. Pandemic influenza can arise through antigenic shift, or when novel influenza strains escape from animal reservoirs, and can affect a large geographical area leading to a large number of deaths. Neither prior infection, nor current vaccines, provide lasting protection due to the rapid antigenic evolution of influenza virus. The discovery of broadly neutralising antibodies (bnAbs) against influenza has opened the possibility of passive immunisation, which could provide protection against multiple influenza strains. Passive immunisation by parenteral administration of purified antibody is challenging, hindered by high antibody production costs and the short half-life of antibody in the circulation leading to relatively transient protection; however the use of viral vectors to deliver bnAbs can begin to address these limitations. Studies presented in this thesis investigated the feasibility of a novel, lung-targeting lentiviral vector pseudotyped with the fusion (F) and haemagglutinin-neuraminidase (HN) coat proteins from Sendai virus (rSIV.F/HN) as a gene transfer agent for bnAb expression. Recombinant SIV.F/HN vector expressing

a secreted reporter protein *Gaussia* luciferase showed expression in the mouse lung lumen 1 month after lung administration. A single dose of rSIV.F/HN encoding novel anti-influenza bnAb (T1-3B) in a single Open Reading Frame directed expression of T1-3B into the lung lumen and systemic circulation, and conferred partial protection against lethal influenza challenge. Intramuscular (IM) delivery of a recombinant adeno-associated virus vector (rAAV2/8), previously reported to be effective against influenza challenge in mice, directed levels of T1-3B that conferred complete protection against influenza challenge. Daily repeat administrations of rSIV.F/HN did not improve protection against influenza challenge, but the ability of this vector to be repeatedly administered without loss of efficacy may be an advantage in potential clinical applications. Engineering of the bnAb, into the IgA1 backbone greatly enhanced the potency of several bnAbs and might improve influenza protection mediated by rSIV.F/HN. In conclusion, prophylaxis provided by gene transfer during an influenza pandemic might be feasible, providing long-lasting, more cost-effective and time-responsive protection than traditional vaccines or parenteral administration of therapeutic antibody.

Acknowledgement

Foremost, I would like to express my deepest appreciation to my supervisors, Prof Steve Hyde and Prof Deborah Gill for willing to take me as a student in the lab and nurture me. Their immense knowledge, patience, guidance, unlimited support and encouragement have helped me to get through my DPhil and maturing as a scientist.

I would also like to thank the past and current members of the Gene Medicine Research Group: Dr Ian Pringle, Dr Lee Davies, Dr Stephanie Sumner-Jones, Dr Cathy Oliveira, Dr Rebekka Harding-Smith, Dr Jean-François Gélinas, Dr Natasha Davies, Agata Antepowicz, Joost van Haasteren, Rosie Munday, Barnabas Williams, Rachel Ashworth, Veronika Hartleb, Tina Garland, Karen Bamford and Charlotte Rush for their help, support, companies, scientific advice, cakes, BBQs, B52s, nights out and gossips. Thank you for making Gene Medicine not just a workplace, but a family.

Another important person that I would like to thank is Prof Alain Townsend who has guided and taught me so much throughout my DPhil. Words can never be enough to express my appreciation to Alain. It has been a pleasure to learn from and work with Alain who is one of the smartest, kindest yet humble scientists and mentors that I have ever met. Thanks for making me a better scientist and person. Also thanks to Pramila for helping me a lot throughout my DPhil, all the scientific discussions and being such a good friend. Thanks to Lisa, Julie, Fran and Holly for their help and support too.

To my parents and family, thank you for the unconditional support and love. Also thanks to my friends back home in Malaysia whom I grew up with for all the incredible memories. To my friends in Oxford: Chris, Estelle, Paige, Jonathan, Sanaz, Ash, Laura, Lin, Aileen, Nick, Marlene, Jenny and the rest of my Wolfson family. Thank you for the companies, potlucks and nights out. You guys have made my life in Oxford enjoyable and amazing.

Lastly, I would like to thank the examiners of my vivas, Prof Vincenzo Cerundolo, Prof Len Seymour, Prof Sir Andrew McMichael and Prof Wendy Barclay for the useful advice and critics on my project.

My DPhil was funded by the King's Scholarship from the Malaysian government as well as support from the Nuffield Division of Clinical Laboratory Sciences. Part of the studies were supported by the RDM Pump Priming grant.

Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented were obtained by myself and contributions made by others have been acknowledged within the text and legends. All sources of published or unpublished information have been specifically acknowledged by means of a reference or personal communication notation, respectively.

List of Abbreviations

AAT	alpha-1-antitrypsin
AADC	aromatic amino acid decarboxylase
ABCD1	adenosine triphosphate (ATP)-binding cassette, subfamily D
Ad	adenovirus
ADCC	Antibody Dependent Cell-mediated Cytotoxicity
ADA-SCID	adenosine deaminase deficiency form of SCID
ALI	air-liquid interface
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	analysis of variance
ASC	antibody-secreting plasma cells
BALF	broncho-alveolar lavage fluid
bp	base pairs
BSA	Bovine Serum Albumin
bnAb	broadly neutralising antibody
CA	capsid
CAG	modified chicken β -actin promoter with human cytomegalovirus immediate early enhancer
CAR	Chimeric Antigen Receptor
CASI	CMV enhancer and chicken β -actin promoter and novel intron containing elements of the ubiquitin C enhancer/promoter
Cap	capsid protein
cDNA	complementary DNA
CF	cystic fibrosis
CFTR	CF transmembrane conductance regulator
CH	constant heavy region
CL	constant light region
CLL	chronic lymphocytic leukemia
CpG	cytidine-guanosine dinucleotide
CMV	human cytomegalovirus immediate early enhancer and promoter
cPPT	central polypurine tract
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
dsRNA	double-stranded RNA
EC ₅₀	50% effective concentration
eGFP	enhanced green fluorescent protein
EGFP _{Lux}	EGFP Lux fusion protein
EGFP	epidermal growth factor receptor
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immunosorbent assay
ELF	epithelial lining fluid
Env	envelope glycoprotein of HIV
F2A	foot-and-mouth disease virus 2A self-processing sequence adjacent to a 4-amino acid spacer and a furin cleavage site
F/HN	Sendai virus fusion and haemagglutinin-neuraminidase
FGFR	fibroblast growth factor receptor
FIV	feline immunodeficiency virus
FMDV	foot-and-mouth disease virus
FreeStyle	FreeStyle 293 Expression Medium
GDP	gross domestic products
GFP	green fluorescent protein

GLux	<i>Gaussia</i> luciferase
GP	glycoprotein
H	heavy chain
HA	haemagglutinin
HCC	hepatocellular carcinoma
hCEF	CpG-free CMV/elongation factor 1 alpha
HEK 293	Human Embryonic Kidney 293 cell line
HGFR	heparan sulphate proteoglycan
HIV	human immunodeficiency virus
HIV-1	HIV type 1
HIV-2	HIV type 2
HPAI	High Pathogenic Avian Influenza
IA	immunoadhesin
IAV	Influenza A virus
Ig	immunoglobulin
IM	intramuscular
IN	intranasal/ integrase
IFN	interferon
IP	intraperitoneal
IRES	internal ribosome entry site
ITR	inverted terminal repeats
L	light chain
LAIV	live-attenuated influenza vaccine
LB	lysogeny broth
LTR	long terminal repeat
Lux	firefly luciferase
LV	lentivirus
mAb	monoclonal antibody
M1	matrix 1
M2	matrix 2
MA	matrix
MDCK	Madin Darby Canine Kidney
MLD	metachromatic leukodystrophy
MN	microneutralisation
miRNA	microRNA
mRNA	messenger RNA
NA	neuraminidase
NEP	nuclear export protein
NP	nucleoprotein
NS	non-structural
ORF	open-reading frame
P	influenza viral polymerases
PCR	polymerase chain reaction
pDNA	plasmid DNA
polyA	polyadenylation
PA	acidic polymerase
PBS	primer binding site/ phosphate buffered saline
PB1	basic polymerase 1
PB2	basic polymerase 2
PDGRF	platelet-derived growth factor receptor
PEI	polyethylenimine
P:I	particle to infectivity ratio
polybrene	hexadimethrine bromide
ppm	particle per million
PPT	polypurine tract

QIV	quadrivalent influenza vaccine
RA	rheumatoid arthritis
RT	room temperature
rAAV	recombinant AAV
rAd	recombinant Adenovirus
rcf	relative centrifugal force
Rep	replication protein
rHIV	recombinant HIV
RLU	relative light unit
rLV	recombinant lentiviral vector
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
RRE	Rev response element
RSV	Rous sarcoma virus promoter
RT	reverse transcriptase
RT-PCR	RT-quantitative PCR
rSIV	recombinant SIV
S-Flu	signal sequence deleted influenza virus
S-eGFP	S-Flu expressing eGFP
S-Lux	S-Flu expressing flux
SA	sialic acid
scAAV	self-complementary AAV
SCID	severe combined immunodeficiency
SCID-X1	X-linked SCID
SD	standard deviation
SEM	standard error of the mean
SERCA2a	sarcoplasmic reticulum calcium ATPase 2a
snRNA	small nuclear RNA
SIAT	sialyltransferase
SIN	self-inactivating
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
soGLux	codon-optimised CpG-depleted GLux
SU	surface envelope protein
SV40	simian virus 40
TCID ₅₀	50% tissue culture infectious dose
TIV	trivalent influenza vaccine
TM	transmembrane envelope protein
TNF	tumour necrosis factor
tRNA	transfer RNA
UK	United Kingdom
UKCFGTC	UK CF Gene Therapy Consortium
USA	United States of America
VAI RNA	adenovirus virus-associated RNA I
VGM	virus growth media
VP	viral proteins
VH	heavy variable region
VL	light variable region
vRNP	viral RNP
VSV	vesicular stomatitis virus
VSV-G	VSV G glycoprotein
v/v	volume/volume
WAS	wiskott-aldrich syndrome
WHO	World Health Organisation

WPRE	posttranscriptional regulatory element of woodchuck hepatitis virus
w/v	weight/volume
X-ALD	X-linked adrenoleukodystrophy
Δ U3	deletion of the U3 region
ψ	packaging signal
κ	kappa
λ	lambda
γ	gamma

Table of Contents

Abstract.....	I
Acknowledgement.....	III
Declaration	V
List of Abbreviations	VI
List of Figures.....	XIV
List of Tables	XVII
Chapter 1 Introduction	1
1.1 The influenza virus.....	1
1.1.1 Influenza virology.....	1
1.1.2 The genome structure of influenza virus	1
1.1.3 Antigenic evolution of influenza virus.....	7
1.1.4 The life cycle of influenza virus	9
1.1.5 Influenza- the disease	9
1.2 Treatment and prevention of influenza infections.....	13
1.2.1 Antiviral drugs	13
1.2.2 Vaccination	14
1.2.2.1 Active immunisation.....	14
1.2.2.2 Passive immunisation	17
1.3 Vector-mediated passive immunisation against infectious diseases	23
1.3.1 Vector-mediated passive immunisation against influenza.....	27
1.4 Adeno-associated virus	27
1.4.1 Structure of the adeno-associated virus genome	28
1.4.2 Recombinant adeno-associated viral vector.....	30
1.5 Retrovirus and Lentivirus	36
1.5.1 Retrovirus	36
1.5.2 Lentivirus	37
1.5.3 The lentiviral vector	37
1.5.4 Application of lentiviral vectors in gene therapy.....	46
1.5.5 The development of recombinant lentiviral vector pseudotyping	46
1.6 Aims	48
Chapter 2 Materials and Methods	49
2.1 Materials	49
2.1.1. Reagents and solutions	49
2.1.2 Mammalian cells.....	50
2.1.2.1 Cell culture conditions for the suspension HEK 293T cell line	50
2.1.2.2 Cell culture conditions for the adherent HEK 293T cell line.....	50
2.1.2.3 Cell culture conditions for the MDCK-SIAT1 cell line.....	51
2.1.3 Plasmids.....	51
2.2 Methods.....	55
2.2.1 Centrifugation	55
2.2.2 Microscopy	55
2.2.3 Molecular cloning techniques.....	56

2.2.3.1 Gel electrophoresis.....	56
2.2.3.2 Restriction enzyme digests.....	56
2.2.3.3 Gel extraction of DNA fragments.....	57
2.2.3.4 Ligation of DNA fragments.....	57
2.2.3.5 Bacterial transformation of plasmid DNA.....	57
2.2.3.6 Inoculation of bacterial colonies into growth media.....	58
2.2.3.7 Purification of plasmid DNA from transformed bacteria.....	58
2.2.3.8 Spectrophotometric quantification of DNA concentration in solution.....	58
2.2.3.9 Polymerase Chain Reaction	59
2.2.3.10 Site-directed mutagenesis	59
2.2.3.11 Gibson cloning.....	60
2.2.4 Polyethylenimine (PEI) transfection of plasmid DNA in mammalian cells	61
2.2.5 Viral vectors production.....	62
2.2.5.1 Large-scale lentiviral vector production.....	62
2.2.5.1.1 Determination of Lentiviral titre	64
2.2.5.2 Production of recombinant adeno-associated virus vector.....	67
2.2.5.2.1 Determination of recombinant adeno-associated virus vector titre	70
2.2.5.3 Functional validation of rSIV.F/HN and rAAV	71
2.2.6 <i>In vitro</i> assays	71
2.2.6.1 <i>Gaussia</i> Luciferase (GLux) Assay	71
2.2.6.2 Anti-Human IgG Enzyme-linked Immunosorbent Assay.....	72
2.2.6.3 Anti-Influenza Haemagglutinin Enzyme-linked Immunosorbent Assay.....	72
2.2.6.4 Microneutralisation assay	73
2.2.6.5 Urea assay.....	75
2.2.7 Production of IgG1 and monomeric IgA1.....	75
2.2.7.1 Purification of IgG1 and monomeric IgA1.....	76
2.2.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE)	77
2.2.9 Animal studies.....	81
2.2.9.1 Mouse strains.....	81
2.2.9.2 Mouse anaesthesia.....	81
2.2.9.3 Delivery to the mouse lung via intranasal instillation	83
2.2.9.4 Delivery to the mouse muscle via injection	83
2.2.9.5 Delivery to the mouse peritoneal cavity via injection.....	83
2.2.9.6 Sampling of bronchoalveolar lavage fluid (BALF)	84
2.2.9.7 Sampling of serum	84
2.2.9.8 Harvesting of lung tissue and preparation of lung lysate.....	84
2.2.10 Statistical analyses.....	85

Chapter 3 Generation of rSIV.F/HN and rAAV vectors for passive immunisation against influenza infection.	86
3.1 Introduction.....	86
3.1.1 Selection of recombinant lentivirus and recombinant adeno-associated virus vectors.....	86
3.1.2 Selection of anti-influenza broadly neutralising antibodies	87
3.1.3 Aim	88

3.2 Results.....	91
3.2.1 Design of a single-Open Reading Frame (ORF) transgene for antibody expression from vector genome plasmids	91
3.2.2 Construction of rSIV and rAAV genome plasmids	92
3.2.3 Functional validation of rSIV and rAAV genome plasmids.....	93
3.2.4 Functional assessment of antibodies expressed as single-ORF and dual-ORF configurations.....	94
3.2.5 Investigation of the effect of deletion of C-terminal lysines on the bioactivity of broadly neutralising antibodies	100
3.2.6 Assessment of viral vector function in cell culture	108
3.3 Discussion.....	113
Chapter 4 Lung antibody production mediated by rSIV.F/HN	126
4.1 Introduction	126
4.1.1 Antibody production in non-haematopoietic cells.....	126
4.1.2 Selection of secreted reporter protein	126
4.1.3 Aim	127
4.2 Results.....	127
4.2.1 Expression of secretory reporter protein <i>Gaussia</i> luciferase (GLux) following intramuscular delivery of rAAV2/8 in mice	127
4.2.2 Expression of bnAb following intramuscular delivery of rAAV2/8 in mice.....	129
4.2.3 Expression of secreted reporter protein GLux following delivery of rSIV.F/HN to the lungs of mice.....	134
4.2.4 Expression of bnAb T1-3B following delivery of rSIV.F/HN to the mouse lung.....	135
4.2.5 Determination of the dilution of murine epithelial lining fluid (ELF) in broncho-alveolar lavage fluid (BALF)	141
4.3 Discussion.....	142
4.3.1 Lung and muscle bnAb expression mediated by viral vectors.....	142
4.3.2 The expression route of GLux and bnAb following delivery of viral vectors	144
4.3.3 Signal sequence and expression of ectopic proteins in the lung epithelia.....	148
4.3.4 Conclusion.....	149
Chapter 5 Single and repeat administration of rSIV.F/HN for passive immunisation against influenza in a mouse model.	151
5.1 Introduction.....	151
5.1.1 Mouse model for influenza challenge study.....	151
5.1.2 Aim	152
5.2 Results.....	152
5.2.1 Confirmation of complete protection against influenza virus challenge in mouse model mediated by rAAV2/8.....	152
5.2.2 Protection mediated by a single dose of rSIV.F/HN against 1,000 and 10,000 TCID ₅₀ influenza challenge in mice.....	153
5.2.3 Repeat administration of rSIV.F/HN to improve protection against influenza challenge in mice	158
5.3 Discussion.....	164

5.3.1 Production of monoclonal antibody in the lung epithelia for influenza protection mediated by rSIV.F/HN	164
5.3.2 Relevant animal models for influenza challenge studies	171
5.3.3 Conclusion.....	173
Chapter 6 Enhancement of anti-influenza broadly neutralising antibodies activity via antibody engineering	174
6.1 Introduction	174
6.1.1 Human immunoglobulins (Igs)	174
6.1.2 Structure of human IgA	176
6.1.3 Enhancement of bioactivity of bnAb in IgA backbone	176
6.1.4 Aim	180
6.2 Results.....	180
6.2.1 Generation of recombinant anti-influenza HA IgA1	180
6.2.2 Comparison of neutralising and binding activity of recombinant IgA1 and their IgG1 equivalents	181
6.2.2.1 Enhancement of neutralising activity in HA stem-targeting recombinant IgA1	181
6.2.2.2 Enhancement only observed in H5 and H7 strains on all recombinant IgA1 tested	182
6.2.2.3 Decrease in neutralising activity of HA head-targeting recombinant IgA1	182
6.2.2.4 No difference in binding affinity against HA was observed between IgA1 and their IgG1 equivalents	183
6.2.3.1 Generation of IgG1 with the IgA1 hinge region (IgGA)	189
6.2.3.2 Comparison of neutralising activity of the hybrid IgGA with recombinant IgA1 and their IgG1 equivalents	190
6.3 Discussion.....	195
6.3.1 Enhancement of neutralising activity and cross-reactivity in HA-stem targeting IgA1	195
6.3.2 Enhancement in neutralising activity in H5 and H7 strains	201
6.3.3 Decreased neutralising activity in HA-head targeting IgA1	203
6.3.4 Conclusion.....	204
Chapter 7 Discussion.....	206
7.1 Viral vectors for passive immunisation against influenza.....	206
7.2 Potential issues for the translation of rAAV2/8 and rSIV.F/HN approaches into the clinic	210
7.3 Potential viral vector related toxicity	213
7.4 Future directions.....	216
7.5 Conclusions	218
References	219
Appendix I	255

List of Figures

Figure 1.1 Structure of influenza A virus	3
Figure 1.2 Schematic of the influenza virus life cycle.....	12
Figure 1.3 A timeline of influenza pandemics and medical advances in the past century.	16
Figure 1.4 Structure of the adeno-associated virus genome.....	29
Figure 1.5 Plasmids needed to generate recombinant adeno-associated virus vector.	35
Figure 1.6 Prototypic retrovirus particle.....	38
Figure 1.7 Plasmids required for first, second and third generation lentiviral vectors.	44
Figure 3.1 Schematic representation of the genome plasmid cDNA configuration. ...	89
Figure 3.2 EGFP-positive cells following transfection of pAAV2.CAS1.EGFP Lux plasmid	96
Figure 3.3 Levels of GLux activity following pAAV2.CAS1.soGLux transfection in cell culture.....	97
Figure 3.4 Supernatant binding titre (A) and neutralizing titre (B) produced by viral genome plasmids <i>in vitro</i>	99
Figure 3.5 SDS-PAGE of antibodies expressed from single-ORF and dual-ORF constructs	102
Figure 3.6 Binding and neutralising titre of single-ORF antibodies compared to their dual-ORF equivalents.....	105
Figure 3.7 Schematic to illustrate the dual-ORF and single-ORF IgG cDNA constructs	107
Figure 3.8 Schematic illustrating possible IgG isoforms after C-terminal lysine clipping	109
Figure 3.10 Purity of rAAV vector preparations assessed by SDS-PAGE	115

Figure 3.11 Levels of GLux activity present in the supernatant of HEK 293T cells transduced with rAAV2/8.CASI.GLux.	116
Figure 3.12 Expression of EGFP-positive cells following transduction of rAAV2/8.CASI.EGFP Lux vector in cell culture	117
Figure 3.13 Human IgG present in the supernatant of HEK 293T cells transduced with rSIV.F/HN vectors.....	118
Figure 3.14 Human IgG present in the supernatant of HEK 293T cells transduced by rAAV2/8 vectors.....	119
Figure 3.15 Schematic of possible mechanism of the expression of bnAb from single-ORF cDNA.....	124
Figure 4.1 Expression of GLux following IM delivery of rAAV2/8.CASI.GLux in mice	131
Figure 4.2 Expression of T1-3B following IM delivery of rAAV2/8.CASI.T13B in mice	133
Figure 4.3 Expression of GLux following intranasal delivery of rSIV.F/HN.hCEF.soGLux in mice	137
Figure 4.4 Expression of T1-3B following intranasal delivery of rSIV.F/HN.hCEF.T13B in mice	139
Figure 4.5 Proposed expression and distribution routes of IgG and GLux following rSIV.F/HN IN delivery or rAAV2/8 IM delivery	147
Figure 5.1 Effect of rAAV2/8.CASI.T13B on protecting mice against 10,000 TCID ₅₀ influenza challenge.....	155
Figure 5.2 Effect of intranasal delivery of rSIV.F/HN.hCEF.T13B on weight loss in mice	157
Figure 5.3 Effect of rSIV.F/HN.hCEF.T13B on protecting mice against 10,000 TCID ₅₀ influenza challenge.....	160
Figure 5.4 Effect of rSIV.F/HN.hCEF.T13B on protecting mice against 1,000 TCID ₅₀ influenza challenge.....	162

Figure 5.5 Human IgG levels in the ELF following single and repeat intranasal administration of rSIV.F/HN.hCEF.T13B	168
Figure 5.6 Effect of repeat administration of rSIV.F/HN.hCEF.T13B in protecting mice against 1,000 TCID ₅₀ influenza challenge.	170
Figure 6.1 Schematic representation of the structure of a typical human immunoglobulin (based on human IgG1) and the antigen binding domain	175
Figure 6.2 Schematic representation of the various forms of human IgA	177
Figure 6.3 Crystallography models of intact human IgG1 and human IgA1	179
Figure 6.4 Visualisation of recombinant IgA1 using SDS-PAGE.....	185
Figure 6.5 <i>In vitro</i> neutralisation titre (EC ₅₀) of IgA1 and IgG1 equivalents against various influenza strains	187
Figure 6.6 Binding activity of IgA1 compared with the respective IgG1 equivalent.	192
Figure 6.7 Schematic of the IgGA construct generated in this study.	193
Figure 6.8 Visualisation of MEDI8852 IgGA using SDS-PAGE.....	194
Figure 6.9 Neutralising titre of IgGA compared to the IgG1 and IgA1 equivalents..	198

List of Tables

Table 1.1 Characteristics, advantages and disadvantages of currently available influenza vaccines	18
Table 1.2 Summary of influenza broadly neutralising antibodies (bnAbs)	21
Table 1.3 Summary of viral vectors used to express monoclonal antibodies against infectious diseases	25
Table 1.4 Recombinant adeno-associated virus vector (rAAV) serotypes, cellular receptors and tissue tropism	31
Table 1.5 Summary of gene therapy clinical trials using adeno-associated virus vector (rAAV).....	39
Table 1.6 Summary of gene therapy clinical trials using lentiviral vector (rLV)	41
Table 2.1 Plasmids used in this study	52
Table 2.2 Ratio calculation of plasmids for rAAV production	69
Table 2.3 Preparation of iodixanol step-solutions for four iodixanol gradients	69
Table 2.4 Details of MDCK-HA, wild type influenza, reassortant influenza and S-eGFP used in this study.....	79
Table 2.5 Details of IgG1 and monomeric IgA1 produced in this study	82
Table 3.1 Characteristics of broadly neutralising influenza haemagglutinin-specific monoclonal antibodies used in this study	90
Table 3.2 Control SIV and AAV genome plasmids constructed	95
Table 3.3 SIV and AAV genome plasmids constructed.....	95
Table 3.4 rSIV.F/HN produced in this study	114
Table 3.5 rAAV2/8 vectors generated in this study	114
Table 4.1 Summary of T1-3B expression levels mediated by rSIV.F/HN and rAAV2/8 in mice on day 28 post vector delivery	140
Table 4.2 Mean dilution factor of broncho-alveolar lavage fluid based on urea concentration	143

Table 6.1 IgG1 and monomeric IgA1 produced and investigated in this study	184
Table 6.2 Summary of the neutralising titre (EC_{50}) of IgA1 compared with their IgG1 equivalents	188

Chapter 1 Introduction

1.1 The influenza virus

1.1.1 Influenza virology

Influenza viruses (from the family *Orthomyxoviridae*) are enveloped, negative-sense, single-stranded RNA viruses with segmented genomes. There are five genera, including Influenza A, influenzavirus B, influenzavirus C, Thogotovirus and Isavirus (infectious salmon anaemia virus) (Taubenberger and Kash, 2010). The influenza virus genera differ in host range and pathogenicity and diverged evolutionarily at least several thousand years ago (Suzuki and Nei, 2002). Among all the subtypes, Influenza A virus (IAV) is the most virulent in humans and has caused major outbreaks and pandemics. Wild aquatic birds are the major reservoir of IAV, where it causes predominantly asymptomatic gastrointestinal tract infection (Webster et al., 1992). Based on the antigenic variation of the haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins, to date, there are 16 known subtypes of HA and nine known subtypes of NA (Laver et al., 1984, Fouchier et al., 2005). However, only H1N1, H2N2, H3N2, H5N1, H7N7 and H9N2 subtypes have been isolated from human (Claas et al., 1998, Guan et al., 1999, Fouchier et al., 2004).

1.1.2 The genome structure of influenza virus

The genome of the IAV contains eight segments in the form of ribonucleoprotein (RNP), which are numbered in order of decreasing length (Figure 1.1).

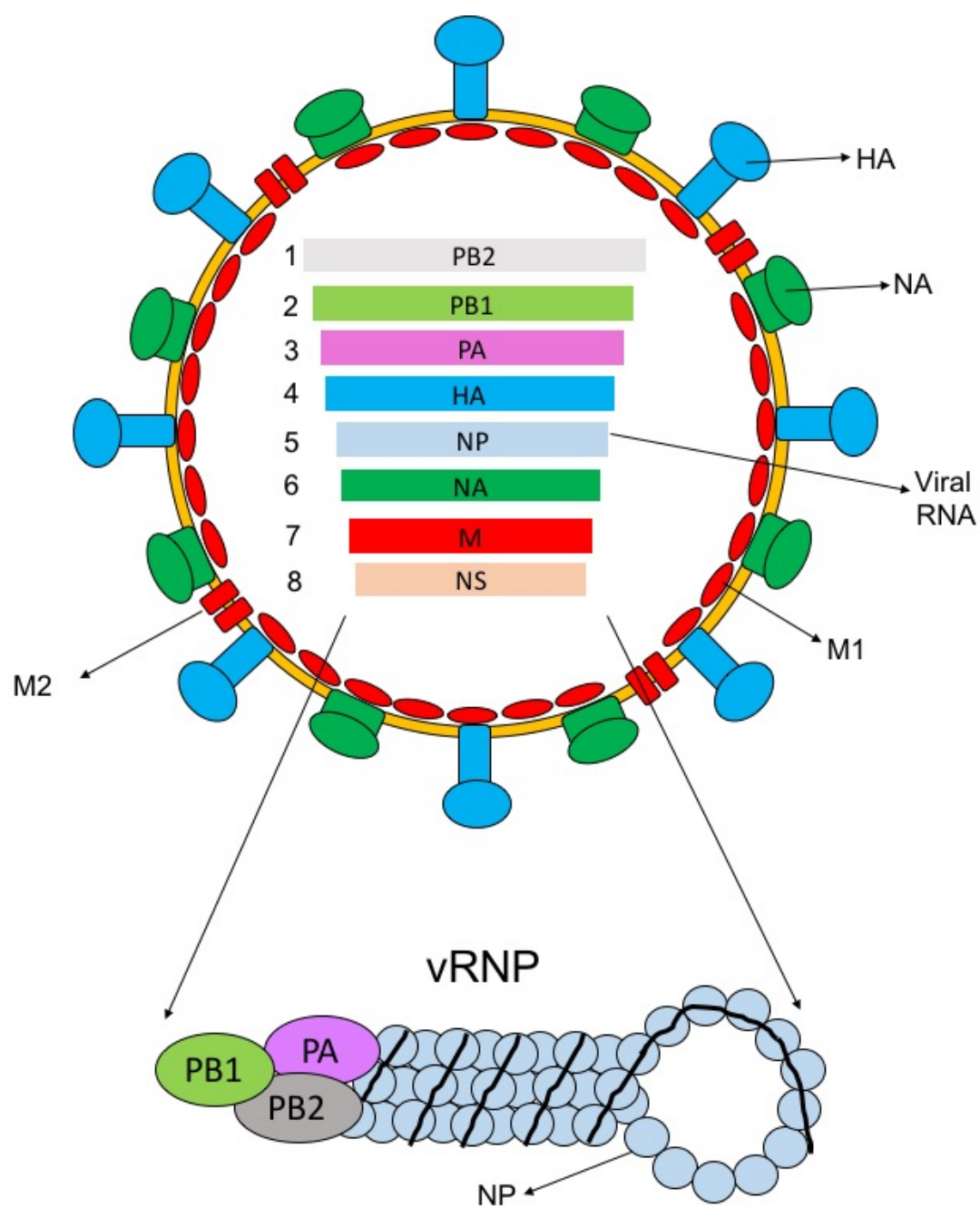


Figure 1.1 Structure of influenza A virus. The genome of influenza A virus is composed of eight genomic negative-sense single-stranded RNA segments. Each segment contains a coding region that encodes at least one protein, and short 5' and 3' flanking sequences. Three segments encode core proteins that form the virus polymerase complex: (1) basic polymerase 2 (PB2), (2) basic polymerase 1 (PB1) and (3) acidic polymerase (PA). Two segments encode for surface glycoproteins (4) haemagglutinin (HA) which is involved receptor binding and viral entry and (6) neuraminidase (NA) which involved in viral budding. Segment five encodes for the (5) nucleoprotein (NP) which binds to viral RNAs. Segment seven encodes for two proteins; (7) Matrix 1 (M1) which forms the viral capsid and Matrix 2 (M2) which acts an ion channel on the viral membrane. Segment eight encodes for two proteins; (8) non-structural protein 1 (NS1) which affects cellular RNA transport, splicing and translation and nuclear export protein (NEP) which mediates the export of ribonucleoprotein (RNPs) from the host nucleus. A mature influenza virus is composed of the nucleocapsid, and the membrane envelope acquired from host cells which contain the HA, NA and M2 proteins supported by a layer of M1 proteins underneath. The rod-shaped, double-helical viral ribonucleoprotein (vRNP) (bottom diagram) forms the genome of the influenza virus. Each RNP contains a NP bound-viral RNA and heterotrimeric viral polymerases (PA, PB1 and PB2). The function of each viral component is discussed in detail in Section 1.1.

Segments one, two and three of the viral RNAs (vRNAs) encode Basic Polymerase Protein 2 (PB2), Basic Polymerase Protein 1 (PB1) and Acidic Polymerase (PA) respectively, which make up the influenza viral polymerase (P complex). The P complex is a heterotrimer of subunits PB1, PB2 and PA with a mass of around 250 kDA as part of the vRNPs (Figure 1.1) (Area et al., 2004, Coloma et al., 2009). The P complex carries out mRNA transcription and replication (Das et al., 2010). The P complex possesses helicase, ATP-binding activities and acts as RNA polymerases for viral transcription and replication (de la Luna et al., 1989). It also contains nuclear localisation signal that aids the transportation of vRNAs into the nucleus of host cells for viral transcription and replication (Mukaigawa and Nayak, 1991, Perales et al., 1996). Segment two and three also encode several other viral proteins known as PB1-F2 (Chen et al., 2001), PB1-N40 (Wise et al., 2009), PA-X (Jagger et al., 2012), PA-N155 and PA-N182 (Muramoto et al., 2013).

Segment four encodes for the haemagglutinin (HA) which is responsible for the binding of viral particles to sialic acid receptors on the cell surface (Weis et al., 1988). 16 subtypes of HA have been identified in IAV and they can be divided into two phylogenetic groups. The first group (Group 1) consists of H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 subtypes and the second group (Group 2) consists of H3, H4, H7, H10, H14 and H15 subtypes (Russell et al., 2006).

The HA molecule is a homotrimer that forms spikes on the viral lipid membrane with two structurally distinct regions: a stem, consisting of a triple-stranded coil of alpha-helices, and a globular head of antiparallel beta-sheet, positioned on

top of the stem (Wilson et al., 1981). The HA precursor, HA0, is cleaved by serine proteases in the lung into HA1 and HA2 at position 329. Thus post-translational modification is necessary for the conformational change required for fusion of viral and host membranes, and virus infectivity. The HA₂ portion mediates the fusion of virus envelope with cell membranes, while the HA₁ protein contains the sialic acid receptor binding and antigenic sites (reviewed in Steinhauer DA, 1999).

The nucleoprotein (NP) is encoded by segment five. NP encapsidates the vRNA and forms vRNP together with the other influenza polymerases. NP folds into a crescent shape with a head and a body domain (Das et al., 2010). NP contains a RNA-binding domain which binds the viral RNA in a nonspecific pattern (Kobayashi et al., 1994, Albo et al., 1995). NP interacts with the viral polymerase subunits and regulates viral RNA replication (Huang et al., 1990, Perales and Ortin, 1997). NP is also involved in the nuclear export and import of vRNPs (Mena et al., 1999, Robb et al., 2011).

Segment six encodes for the neuraminidase (NA), a homotetramer that resembles a mushroom shape. Each influenza virus contains around 50 NA tetramers that form clusters on the viral surface (Harris et al., 2006). Nine subtypes of NA have been identified in IAV and they can be divided into two phylogenetic groups. The first group (Group 1) consists of N1, N4, N5 and N8 subtypes and the second group (Group 2) consists of N2, N3, N6, N7 and N9 subtypes (Russell et al., 2006). NA comprises of four domains, an N-terminal cytoplasmic domain, a hydrophobic transmembrane domain, a thin stalk

domain and a globular head domain (Air, 2012). The head domain contains the enzyme active site (possess exosialidase activity) and calcium binding domain which stabilises the enzyme structure at low pH (Suzuki et al., 2004). The enzymatic reaction of NA helps to release newly formed progeny virions from the host cell membranes during budding. NA also helps during viral entry. Before reaching the target epithelia in the in airways, IAVs have to penetrate the lungs mucus layer, a defensive layer of highly glycosylated mucins rich in terminal sialic acid (SA) which act as decoys to trap IAVs. NA allows IAVs to penetrate the mucus by cleaving these sialylated decoys (Cone, 2009, Duez et al., 2009, Fahy and Dickey, 2010, Cohen et al., 2013)

Segment seven encodes two proteins: M1 which is a collinear transcription product of segment seven and M2 which is produced by the spliced mRNA of segment seven. The M1 protein comprises an N-terminal domain and a C-terminal domain and contains a nuclear localisation signal (Ito et al., 1991). The M1 protein forms a layer underneath the cell membrane to separate the vRNPs from the viral membrane and stabilise the HA and NA on the viral membrane (Figure 1.1) (Ruigrok et al., 1989, Zhang et al., 2012). The M1 protein has several functions including inhibiting viral transcription by binding non-specifically to RNA, regulating vRNP nuclear transport and inhibiting vRNP nuclear import (Martin and Helenius, 1991, Bui et al., 1996, Watanabe et al., 1996). M2 is a type III transmembrane protein that forms a homotetramer, whose transmembrane domains form a proton-selective ion channel for pH regulation (Holsinger and Lamb, 1991, Pinto et al., 1992). In the endosome of infected cells, M2 allows the acidification of the interior of the viral particle which

then allows the vRNPs to dissociate from M1 proteins facilitating nuclear import (Martin and Helenius, 1991, Bui et al., 1996). During the later stage in the viral life cycle, M2 maintains the high pH in the Golgi apparatus to stabilise the native conformation of the newly synthesised HA for intracellular transport and viral assembly (Takeuchi and Lamb, 1994).

NS1 is on a collinear mRNA derived from segment eight, which upon splicing results in the synthesis of the nuclear export protein mRNA (NEP, previously termed NS2) (Inglis et al., 1979, Lamb and Choppin, 1979). NS1 protein consists of two functional domains: a N-terminal RNA-binding domain and a C-terminal effector domain (Hatada and Fukuda, 1992, Qian et al., 1995, Chien et al., 2004). NS1 exists as a homodimer, with both the RNA-binding and effector domains contributing to multimerisation (Nemeroff et al., 1995). NS1 is a multifunctioning protein. The RNA-binding domain is able to bind to RIG-I, thus prevent the activation of interferon response within host cells (Hale et al., 2008). The RNA-binding domain also bind to dsRNA, polyA and U6 snRNA which blocks the host protein translation and allows the translation of viral proteins (Lu et al., 1995). NS2 was originally known as one of the non-structural protein. The NS2 protein was later on renamed as the nuclear export protein (NEP) when its role in mediating the export of vRNPs from the host cell nucleus was discovered (O'Neill et al., 1998).

1.1.3 Antigenic evolution of influenza virus

Influenza undergoes two types of antigenic evolution; antigenic drift and antigenic shift. Antigenic drift is the gradual evolution of influenza strains due to

accumulation of mutations on the antigenic sites (Both et al., 1983). Antigenic drift is a subtle process, involving point mutations during viral replication within antibody-binding sites in the HA and NA proteins (Boni et al., 2004, Finkenstadt et al., 2005, Koelle et al., 2006). Antigenic drift occurs partly due to the error-prone RNA dependent RNA polymerase (RdRP) which has no proof-reading ability and in response to the selection pressure to evade human immunity (Scholtissek, 1995, Plotkin et al., 2002, Smith et al., 2004, Koelle et al., 2006, Chambers et al., 2015, Huang et al., 2015a). Antigenic drift is subtle and does not normally change the conformation of the HA or NA but these mutations sometimes cause changes to the HA and NA such that the binding of host antibodies is affected, allowing the drifted strains to infect more rapidly among the population (Webby and Webster, 2001).

Antigenic shift is the process by which two different influenza viruses exchange and reassort intact genome segments. Antigenic shift can happen due to the segmented nature of the viral genome and occurs when two or more distinct influenza strain infect the same host cell. In a cell co-infected by two distinct influenza strains (each carries eight genome segments), 256 different progeny genotypes from the assortment of the viral segments are possible to be generated from antigenic shift. Hence, antigenic shift has a great potential to generate significant viral diversity and often a novel pandemic influenza strain. Successful antigenic shift, generating a novel strain with enhanced pandemic potential is estimated to occur about three times in a century (Potter, 2001). Swine possess both α -2,3 and α -2,6 sialic acid containing receptors on their respiratory surface which make them susceptible to both avian influenza and

human influenza viruses (Rogers and Paulson, 1983, Rogers and D'Souza, 1989). Hence, swine serves as a good mixing vessel for the reassortment of viral genomes between human and avian influenza viruses when they both co-infect the same cell (Ito et al., 1998). This theory was first proposed in 1985 based on the observation that human influenza viruses do not transmit easily to birds and vice versa but much easier to swine (Scholtissek, 1990, Scholtissek, 1996). In fact, swine has been shown to transmit reassorted influenza viruses to humans (Olsen et al., 2006, Robinson et al., 2007, Killian et al., 2013).

1.1.4 The life cycle of influenza virus

The life cycle of influenza is presented in Figure 1.2. The preference of influenza binding to host cell depends on the nature of the glycosidic linkage between the terminal SA and the penultimate galactose residue on the receptor (Weis et al., 1988). Human influenza preferentially recognises SA linked to galactose by α -2,6 linkages, whereas avian influenza prefer SA linked to galactose via α -2,3 linkages (Rogers and Paulson, 1983, Suzuki, 1994, Matrosovich et al., 1997).

1.1.5 Influenza - the disease

Influenza viruses cause acute respiratory infection in humans and they are among the most common causes of human respiratory disease and associated with high morbidity and mortality (Wright, 2007). Influenza viruses cause seasonal influenza which leads to three to five million severe cases and about 250,000 to 500,000 deaths worldwide annually (Thompson et al., 2003, WHO,

2016). Influenza viruses lead to seasonal influenza which affects the human population annually and pandemic influenza when novel influenza strains emerge from animal reservoir due to antigenic shift. An influenza pandemic is an epidemic of an influenza that spreads on a worldwide scale in different geographical regions. In the past century, pandemic influenza has occurred every eight to 41 years and up to 50% of the population can be infected in a single pandemic year (Beveridge, 1977, Simonsen, 1999). Four pandemic outbreaks of influenza occurred in the past century; in 1918, 1957, 1968 and 2009 (Figure 1.3).

Although no human to human transmission has been reported to date, two influenza subtypes known to be circulating in birds (H5N1 and H7N9) possess risk for a potential future pandemic. High Pathogenic Avian Influenza (HPAI) H5N1 was initially found to be endemic mainly in birds where it is highly contagious and deadly for poultry. Since 2003, HPAI H5N1 has been repeatedly crossing the species barrier and infecting humans. To date, there have been 859 cases of human HPAI H5N1 infections with a mortality rate of about 53% (WHO, 2017b). The increased number of human zoonotic infections of HPAI H5N1, has triggered intense public health interest and concern for a future pandemic (Wright, 2007). The first human case of H7N9 avian influenza virus infection was detected in China in 2013. At the time of writing, there have been 1,554 cases of human infections with a mortality rate of 39% (WHO, 2017a). So far, human-to-human transmission of H7N9 has been limited (Hu et al., 2014). Mutations, or reassortment with circulating human viruses, might enable HPAI H5N1 and H7N9 to efficiently infect and transmit among humans.

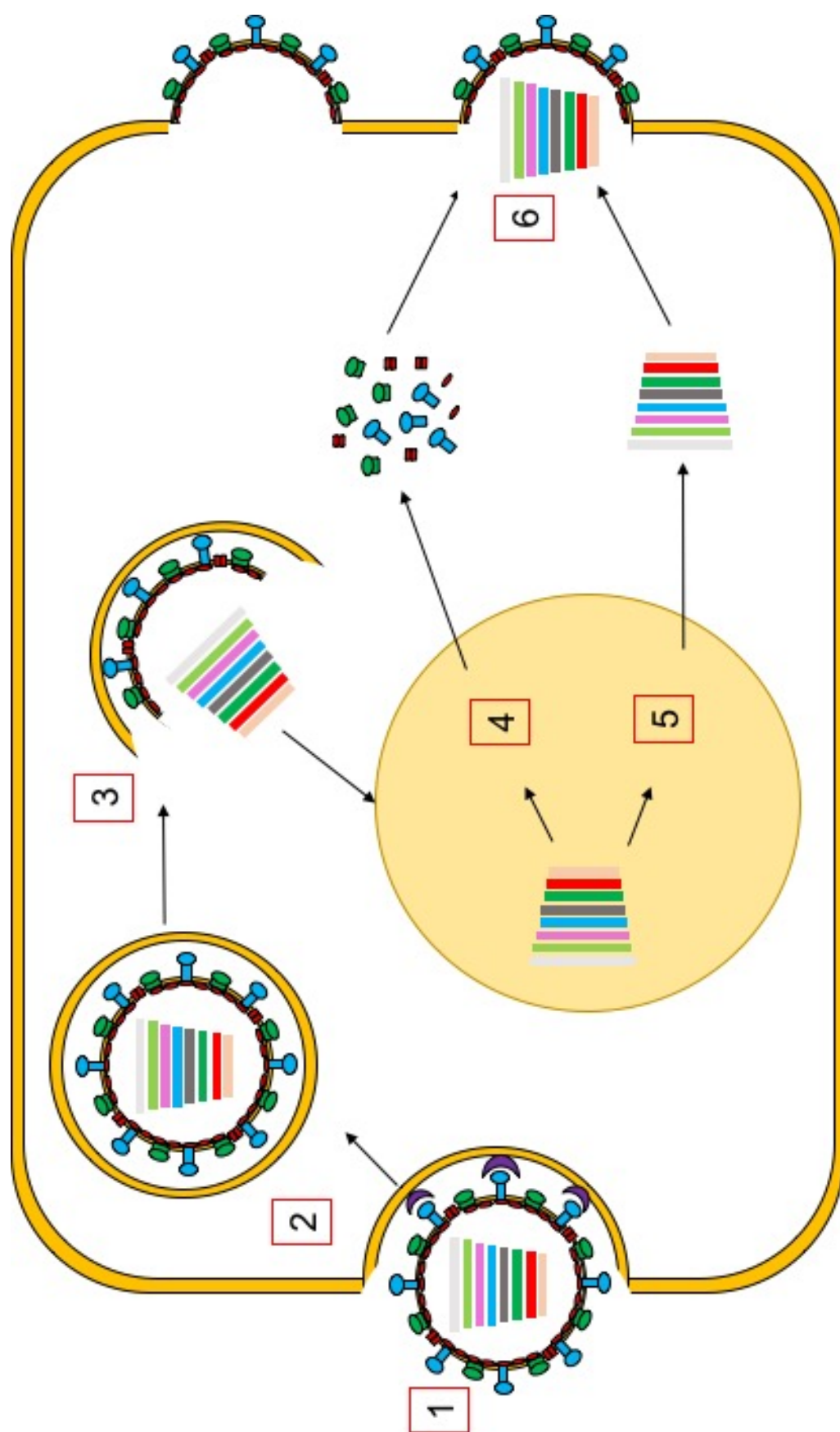


Figure 1.2 Schematic of the influenza virus life cycle. 1. Attachment: Influenza infection starts by attaching to sialylated host receptors (α -2,3 or α -2,6 sialic acid) on the cellular surface. Viral entry is mediated by endocytosis. 2. Fusion: In the host cell, endosomal acidification activates conformational changes in HA, which leads to fusion between the viral and endosomal membrane. 3. Uncoating: The viral M2 ion concurrently promotes acidification of the virion core which leads to dissociation of the M1 proteins from the viral genome, vRNPs are released from the endosome into the host nucleus. 4. Transcription: The negative-sense vRNAs are converted into two positive-sense vRNAs: Complementary RNA (cRNA) and, through a cap-snatching mechanism, mRNA. The cRNA acts as a template for the production of negative-sense vRNAs which are packaged into new virions. The mRNAs are transported to the cytoplasm and translated into viral proteins by cellular ribosomes. Newly translated viral proteins are transported to the nucleus (PB1, PB2, PA, M1 and NEP) for viral genome replication or the plasma membrane (HA, NA and M2) for packaging. 5. Replication: The viral RNA dependent RNA polymerase (RdRP) initiates RNA synthesis internally on viral RNA and form vRNP together with NP, PB1, PB2 and PA. vRNPs are then transported to the cytoplasm via the help of NEP and M1 proteins. 6. Assembly and budding: Newly exported vRNPs are trafficked to the plasma membrane and incorporated into progeny virus particle containing HA, NA, M2 and M1 and newly formed virions bud off to infect new cells.

Since humans are lacking immunity against HPAI H5N1 and H7N9, these viruses possess significant pandemic threat (CDC, 2017).

1.2 Treatment and prevention of influenza infections

1.2.1 Antiviral drugs

Currently, the only approved treatment for influenza are antivirals, of which there are two major classes: the M2 inhibitors and the NA inhibitors. The M2 inhibitors block the M2 proton channel, preventing fusion of the virus in the endosome and the subsequent migration of the vRNPs into the nucleus. The NA inhibitors block NA thus preventing the release of newly formed progeny virions from infected cells. The M2 inhibitors, amantadine and rimantadine, discovered in 1964, only act on IAV as other influenza viruses such as influenza B lack the M2 ion channels. Its use has been limited due to the emergence of a high number of M2 inhibitor-resistant influenza strains. For instance, pandemic H1N1 (A/California/7/2009), most of the seasonal H3N2 viruses, and H5N1 strains circulating in Southeast Asia have become resistant to the M2 inhibitors (Bright et al., 2006, Cheung et al., 2006, Monto, 2008, Saito et al., 2008, Gubareva et al., 2010). There are currently two licensed NA inhibitors globally, Zanamivir (Ralenza) and oseltamivir (Tamiflu). Zanamivir is administered via oral inhalation and concentrated in the lungs and oropharynx (Moscona, 2005). Zanamivir has poor oral bioavailability and this has limited its use in the clinic as oral inhalation of zanamivir has been associated with adverse events such as cough, fatal bronchospasm and decreased pulmonary function and is not available for intubated patients (Ison, 2010). Oseltamivir was later developed based on the knowledge and structure from zanamivir. There

are two other NA inhibitors; peramivir and laninamivir (Inavir) that have been developed and licensed in Japan and are undergoing clinical trials in some other countries (Babu et al., 2000, Koyama et al., 2010). Resistance to NA inhibitors has been reported. For oseltamivir, during the initial use of the drug in the clinic, the incidence of resistance in young children was reported to be up to 18% (Kiso et al., 2004). Higher resistance levels were also observed in immunocompromised patients and humans infected with H5N1 (de Jong et al., 2005, Le et al., 2005, Baz et al., 2006, Ison et al., 2006). Compared to oseltamivir, resistance to zanamivir is relatively rare (Thorlund et al., 2011), however cases of zanamivir resistance were reported in China among patients infected with H7N9 (Hu et al., 2013). Apart from that, there are several antivirals targeting the viral polymerases currently being tested in clinical trials (Koszalka et al., 2017) .

1.2.2 Vaccination

1.2.2.1 Active immunisation

Vaccination against influenza is the most cost-effective method to prevent infections (Osterhaus et al., 2011). Currently licensed influenza vaccines focus mainly on eliciting humoral immunity against the viral HA protein to prevent viral entry and require annual evaluation and reformulation to keep pace with the antigenic evolution of circulating influenza strains. Selection of vaccine strains is based on the global surveillance for circulating influenza strains, data collected by the WHO Global Influenza Surveillance Network (GISN)

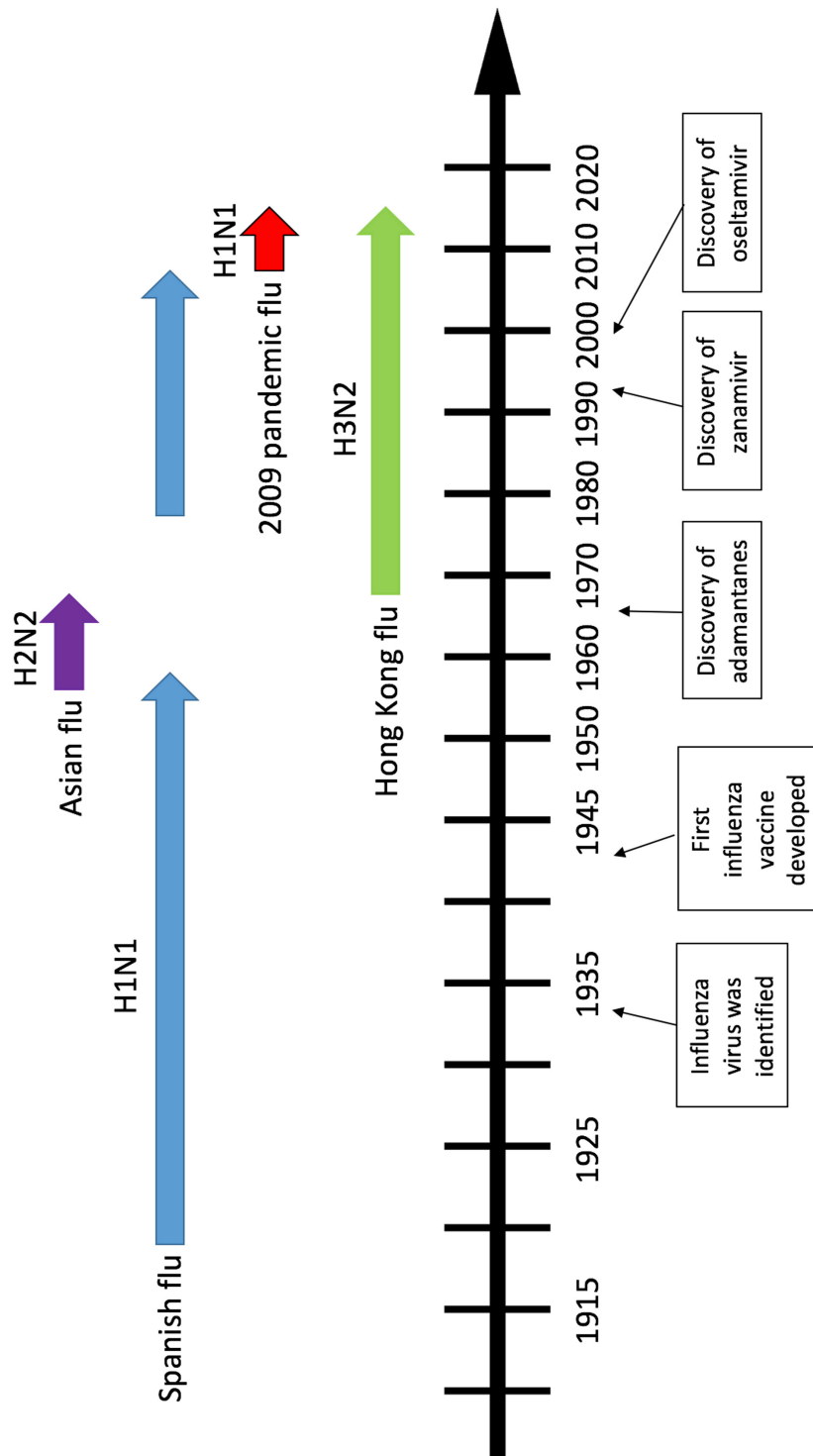


Figure 1.3 A timeline of influenza pandemics and medical advances in the past century. The timeline of influenza pandemics shows that the first recorded influenza pandemic occurred in 1918 (Spanish flu, H1N1), claiming 50 million lives worldwide. H1N1 remained epidemic after that but was replaced by H2N2 in 1957 by the Asian flu (H2N2) which remained in the human populations until 1968 pandemic where it was in turn replaced by the Hong Kong flu, H3N2 which has remained epidemic to the current day. H1N1 re-emerged in 1977 but it did not trigger a pandemic nor replace H3N2 but instead co-circulated with H3N2 until 2009 when it was replaced by the triple-reassorted H1N1 the cause of the 2009 pandemic flu. The 2009 pandemic H1N1 and H3N2 remain circulating in the human populations today causing seasonal influenza. Influenza virus was only identified in 1933, around 15 years after the Spanish flu pandemic (Smith et al., 1995). The first influenza vaccine was developed and used for vaccination in 1936 (Smorodintseff AA, 1937). Neuraminidase inhibitor, zanamivir and oseltamivir were discovered in 1993 and 1997 respectively. Details of influenza pandemics and medical advances are discussed in Section 1.1.5 and 1.2.

(Cox et al., 1994). The WHO consultation on the composition of influenza vaccine is held twice a year to analyse the antigenic and genetic data collected by the WHO GISN, to assess the need for an update of the vaccine. For the northern hemisphere, the recommendation is made in February each year to allow time for the vaccines to be manufactured for vaccination in October and November. The process takes around 6-7 months from selection of specific strains for inclusion in the vaccine to the production, packaging and delivery (Gerdil, 2003). The majority of the licensed influenza vaccines are produced in embryonated eggs, a method first used in 1937 (Doherty, 2008). Currently licensed influenza vaccines are either trivalent or quadrivalent. Trivalent influenza vaccine (TIV) contains three influenza strains: two influenza A (H1N1 and H3N2) and one influenza B (Victoria or the Yamagata lineages). Quadrivalent influenza vaccine (QIV), consisting of two influenza A (H1N1 and H3N2) and two influenza B (Victoria and Yamagata lineages) strains, entered the market in 2012 (Belshe, 2010, Ambrose and Levin, 2012). Characteristics, advantages and disadvantages of currently licensed vaccines are summarised in Table 1.1.

1.2.2.2 Passive immunisation

Passive immunisation, by means of delivering sera from recovered patients or pathogen-specific monoclonal antibody (mAb), offers an alternative to conventional vaccines. The first successful use of passive immunisation was the passive treatment for diphtheria using sera in the late 19th century (Silverstein, 1989). Several other successful uses of passive immunisation have been reported, including the use for the prevention of tetanus, auto-

Table 1.1 Characteristics, advantages and disadvantages of currently available influenza vaccines

Vaccine format	Type of immunity	Indications	Vaccine content	Products	Disadvantages	Advantages
<u>Inactivated</u> Trivalent inactivated vaccine (TIV) Quadrivalent inactivated vaccine (QIV)	Predominantly humoral (strain specific)	For individuals of 6 months and older	Split virion or subunit vaccine contains purified HA proteins Contains 15ug (IM) or 9µg (ID) of each purified HA protein. * 60 µg of each purified HA for the elderly (>65 years old)	<u>QIV</u> Fluarix FluLaval Fluzone <u>TIV (eggs based)</u> Afluria Fluvirin Fluzone <u>TIV (MDCK-derived)</u> Flucelvax <u>*TIV (high dose for >65 years old)</u> Fluzone High-Dose	Strain specific immunity Dependence on eggs for production Lengthy production time Annual vaccination Selection of vaccine strains is based on prediction Require healthy immune system for maximum efficacy	Low reactivity QIV protects against both influenza B lineages
<u>Live attenuated (LAIV)</u> Trivalent	- Humoral - Cellular - Mucosal	Mucosal administration	Live viruses with temperature-sensitive and attenuating	FluMist FluMist Quadrivalent	Dependence on eggs for production	Mucosal administration

Quadrivalent	(More cross-reactive than inactivated vaccine)	For healthy individuals between the ages of 2 and 49 years old	mutations (egg based).		Lengthy production time Selection of vaccine strains is based on prediction Require healthy immune system for maximum efficacy	Higher and broader response in children.
<u>Recombinant</u>	Predominantly humoral (strain specific)	For adults aged 18 to 49 years old. For those who are allergic to eggs.	Recombinant trivalent HA vaccine with HA proteins (135 µg each) that are expressed in insect cells (ExpressSF+) expressed from baculovirus vector.	FluBlok	Strain specific immunity Annual vaccination Selection of vaccine strains is based on prediction Require healthy immune system for maximum efficacy	Rapid production No infectious virus during production No antigenic changes during production or passaging Does not rely on egg supply

Source of the data: (Belshe et al., 2007, CDC, 2013, Wong and Webby, 2013, CDC, 2014, Krammer and Palese, 2015)

inflammatory disorders and snake toxins (Keller and Stiehm, 2000). Passive immunisation with mAb has been replacing sera with the advancement in isolation and characterisation of mAb from animals and human (Brekke and Loset, 2003). Neutralising antibodies alone have been shown to prevent the spread of many diseases within populations and the role of antibodies against infections is best exemplified by the passive transfer of maternal antibodies to neonates (Zinkernagel, 2001, Chames et al., 2009, Plotkin, 2010). For a long time, the conventional view was that passive immunisation against influenza was not feasible due to the mAb being strain-specific along with the high variability of viral glycoproteins and the capacity of influenza to undergo antigenic evolution to escape from the antibody response. This view was challenged following the isolation of antibodies that can neutralise multiple influenza strains by targeting the conserved epitope on the HA stalk (reviewed in (Corti and Lanzavecchia, 2013)). These antibodies, namely broadly neutralising antibodies (bnAb) raised the possibility of using passive immunisation to provide broad protection against multiple influenza strains. Many bnAbs against influenza have been isolated, each with different HA specificity and mechanism of action (Table 1.2). In details, these bnAbs could neutralise influenza viruses via locking the HA trimer in a pre-fusion form and prevent conformational change in the endosome (Ekiert et al., 2009, Sui et al., 2009, Brandenburg et al., 2013), sterically blocks access of serine proteases to cleave HA0 into functioning HA1 and HA2 subunits (Ekiert et al., 2009, Brandenburg et al., 2013) and also retain newly formed HA on infected host cells' surface to inhibit viral budding (Tan et al., 2014). Apart from the ability to neutralise influenza viruses, these bnAbs could also confer protection via

Table 1.2 Summary of influenza broadly neutralising antibodies (bnAbs)

Antibody	Specificity	VH gene usage	Mechanism (if known)	Isolation method	Reference
C179	Group 1 HA stem	Humanised murine	-	Hybridoma	(Okuno et al., 1993)
CR6261	Group 1 HA stem	1-69	Stabilises pre-fusion state of HA and prevents membrane fusion	Phage display	(Throsby et al., 2008)
F10	Group 1 HA stem	1-69	Inhibits membrane fusion	Phage display	(Sui et al., 2009)
D005	H1N1 and H5N1	-	-	Single-cell sorting and RT-PCR	(Beerli et al., 2009)
TCN-032	H1N1 and H5N1	4-59	-	B cell proliferation	(Grande et al., 2010)
FI6, FI6v3	Group 1 and 2 HA stem	3-30	Inhibits membrane fusion and HA ₀ cleavage	Single-plasma cell culture	(Corti et al., 2011)
CR0820	Group 2 HA stem	1-18	Inhibits membrane fusion and HA ₀ cleavage	Lentiviral B cell immortalisation	(Ekiert et al., 2011)

CH65	H1N1 strains (HA head)	1-2	Reduction of the amounts of viral particles released into the supernatant	Single-cell RT-PCR from plasma cells	(Whittle et al., 2011)
C05	Group 1 and 2 HA	3-23	-	Phage display	(Ekiert et al., 2012)
CR9114	Group 1, 2 and B HA stem	1-69	Blocks the HA pH-induced fusion	Phage display	(Dreyfus et al., 2012)
D1-8	H3 strains (HA head)	-	-	ASC	(Benjamin et al., 2014)
12D1	H3 strains (HA stem)	Humanised murine	-	Hybridoma	(Wang et al., 2010)
S139/1	Group 1 and 2 HA head	Humanised murine	Inhibit binding to sialic acid receptor	Hybridoma	(Yoshida et al., 2009)
MEDI8852	Group 1 and 2 HA stem	6-1	Blocks the HA pH-induced fusion	Isolation from memory B cells & ex vivo affinity maturation	(Kallewaard et al., 2016)

VH: heavy chain V genes; HA: haemagglutinin; ASC: antibody-secreting plasma cells; RT-PCR: reverse-transcription polymerase chain reaction

antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (Terajima et al., 2011, Jegaskanda et al., 2013, DiLillo et al., 2014). Indeed, several of these bnAbs have shown promising results in clinical trials (Nachbagauer and Krammer, 2017).

While passive immunisation by means of mAb delivery has many advantages over conventional vaccines, there are several drawbacks. Firstly, mAbs have a relatively short half-life *in vivo*; all of the currently licensed mAbs have a half-life ranging from 2 days to 1 month which necessitates repeated and frequent dosing for therapeutic benefit (Keizer et al., 2010). In addition, most mAbs are needed in high concentration to achieve clinical efficacy, resulting in a high cost for a therapeutic dose (Pescovitz, 2006, Chames et al., 2009). Furthermore, mAbs require cold-chain storage and the administration of mAbs is complicated requiring trained medical personnel (Keizer et al., 2010).

1.3 Vector-mediated passive immunisation against infectious diseases

To circumvent some of the limitations of passive immunisation, one strategy has been to utilise a vector for the delivery of a transgene encoding relevant antibodies. Such vectors can deliver antibody transgene into target cells where they direct the expression of mAbs into the systemic circulation and/or the local environment. Many vectors, including the non-viral and viral vectors previously developed for gene therapy of genetic diseases, have been evaluated for this approach and some have shown therapeutic benefits in animal models. (Table 1.3). This strategy was first tested using naked (non-complexed) plasmid DNA encoding mAb delivered via electroporation, an approach that successfully

yielded production of mAb, in mice and sheep (Tjelle et al., 2004). These experiments demonstrated proof-of-principle for using vectors to mediate rapid expression of mAb although the transient and sub-therapeutic level of mAb mediated by plasmid DNA limits its clinical potential.

Further studies have been carried out with recombinant viral vectors including recombinant Adenovirus (rAd), recombinant adeno-associated virus (rAAV) and recombinant lentivirus (rLV) to express mAb against various infectious diseases (Table 1.4). Recombinant lentiviral vectors have the ability to transduce both dividing and non-dividing cells and when integrated into the host genome can direct persistent transgene expression. They have been used to transduce haematopoietic stem cells (HSC) for expression of a HIV bnAb b12 *in vitro* (Luo et al., 2009) and were used to direct expression of a HIV bnAb 2G12 in mice following *ex vivo* transduction of B cells (Joseph et al., 2010). However, all studies using lentiviral vectors currently utilised *ex vivo* transduction, no *in vivo* transduction study has been reported.

Another widely-used viral vector is rAAV, which has also been studied for mAb delivery *in vivo*. Due to AAV packaging restraints (see Section 1.4), early studies utilised smaller antibody variants such as single-chain variable fragment (scFV) or immunoadhesins (Wuertzer et al., 2008, Wang et al., 2009, Patel et al., 2014). The limited packaging capacity (around 4.68 kb) was circumvented by using the 2A self-processing sequence from the foot and mouth disease (HFMD) virus adjacent to a furin cleavage site (F2A) to facilitate expression of full length mAb comprising both heavy and light IgG chains from

Table 1.3 Summary of viral vectors used to express monoclonal antibodies against infectious diseases

Target diseases	Vector	Ab type	Ab origin	Ab isotype	Transgene promoter	Animal model(s)	Vector dose	Delivery route	Ab	Peak serum concentration (ug/ml)	Quantification methods	References
Influenza	rAAV2/8	Full-length	Human	IgG1	CASI	BALB/c, NSG, ferrets	1×10^{11} GC 5×10^{12} GC	IM	F10 CR6261	200 (mice) 10-100 (ferrets)	Anti-IgG ELISA (Bethyl)	(Balazs et al., 2013)
	rAAV2/9	IA	Human	IgG1	CAG	BALB/c, ferrets, rhesus macaques	1×10^{11} GC 1×10^{12} GC 1×10^{13} GC	IN	FI6	2 (BALF; mice)	Western blot	(Limberis et al., 2013)
	Ad5	Single-domain	-	-	CMV	BALB/c	1×10^8 PFU 1×10^7 PFU	IN	aHA-7	-	-	(Tutykhina et al., 2013)
HCV	rAAV2/9	Full-length	Human	IgG1	CASI	Rosa26-Fluc, FNRR humanised mice	1×10^{11} GC	IM	AR3A AR3B AR4A	1000-3000	In house ELISA	(de Jong et al., 2014)
Malaria	rAAV2/8	Full-length	Human	IgG1	CASI	C57BL/6NCr	1×10^{11} GC	IM	2A10 2C11	>1000; 100-500	In house ELISA	(Deal et al., 2014)
Anthrax	Ad5 rAAVrh.10	Full-length	Mouse	IgG1	CMV	C57BL/6	1×10^{11} PFU 1×10^{11} GC	IV Intrapleural	14B7-1H	-	-	(De et al., 2008)
RSV	Ad5 rAAVrh.10	Full-length	Mouse	IgG1	CMV	BALB/c	5×10^{10} GC 1×10^{11} GC	IV Intrapleural	Palivizumab	-	-	(Skaricic et al., 2008)
Prion	rAAV2/9	scFv	Human	-	-	CD1	1.4×10^{12} GC	Intracranial	D10	-	-	(Moda et al., 2012)
	rAAV2/2	scFv	Human	-	CMV	C57BI/6	9×10^9 GC	Intracranial	D10 3:3 6:4 6:6	-	-	(Wuertzer et al., 2008)
HIV	pDNA	Fab	Mouse	IgG1	CMV	BALB/c	25 ug	IM	VRC01	3.3	In house ELISA	(Muthumani et al., 2013)
	Lentivirus	Full-length	Human	IgG1	-	NSG humanised	1000 MOI	Ex vivo B cell	b12	1	Anti-IgG ELISA (Bethyl)	(Luo et al., 2009)
	Lentivirus	Full-length	Human	IgG1	hPGK	NSG humanised	100 ng p24	Ex vivo B cell	2G12	40 ng/ml	In house ELISA	(Joseph et al., 2010)

	rAAV2/2	Full-length	Human	IgG1	CMV, EF1a	Rag-1	5×10^{11} GC	IM	b12	8	Bindazyme Human Ig Subclass Enzyme Immunoassay Kit	(Lewis et al., 2002)
	scAAV2/2	IA	Rhesus	IgG2	CMV	Rhesus macaques	2×10^{13} GC	IM	4L6 5L7 b12 2G12 2F5 VRC01 4E10	400-500 200-300 100 >250 20 >250 20	In house ELISA	(Johnson et al., 2009)
	rAAV2/8	Full-length	Human	IgG1	CAS1	NSG humanised	1×10^{11} GC	IM	B12 VRC01 VRC07 3BNC117 12A12 VRC-PG04 VRC07G54W NIH45-46G54W PGT121 PGT128 PG9	115 100 130 24 23 30 74 40 256 50 390	Anti-IgG ELISA (Bethyl)	(Balazs et al., 2012)
	rAAV2/8	Full-length	Human	IgG1	CAS1	NSG and BLT humanised	1×10^{11} GC	IM	10-1074 3BNC117	20 300	In house ELISA	(Balazs et al., 2014)
	rAAV2/8	Full-length	Human	IgG1	Human thyroglobulin	NSG humanised	2.5×10^{11} GC 2×10^{11} GC	IV	eCD4-Ig	75-180	In house ELISA	(Horwitz et al., 2013)
	scAAV2/1	Full-length	Rhesus	IgG2	CMV	Rhesus macaques	2.5×10^{13} particles	IM	3BNC117 PGT121 10-1074 NIH45-46	-	In house ELISA	(Gardner et al., 2015)

a single-ORF with a size that can be accommodated into rAAV and yielded high levels of antibody expression (Fang et al., 2005a, Fang et al., 2007) (Figure 3.1)

1.3.1 Vector-mediated passive immunisation against influenza

With regards to influenza infection, several mAb expressing viral vectors have achieved therapeutic benefits in animal models. Ad5, rAAV2/8 and rAAV2/9 have shown protection against influenza challenge in animal models (Tutykhina et al., 2013). Although both rAAV and rAd appear to be equally protective against influenza challenge, rAAV offers more persistent mAb transgene expression and a safer clinical profile. rAAV has been used in the clinic for gene therapy with no adverse effects were reported for up to 6 years (Gaudet et al., 2016). This data shows the safety and efficacy of rAAV to direct long-term clinical benefit. Another feature of rAAV is that it can be lyophilised and remains stable at room temperature for months with negligible loss of function (Croyle et al., 2001). This feature is important when such a drug is to be distributed across areas where frozen storages may not be available during a disease outbreak. For these reasons, rAAV2/8 delivered via intramuscular injection for passive immunisation against influenza was chosen as a benchmark in this study. (see Table 1.4 for the summary of viral vectors used for passive immunisation against infectious diseases).

1.4 Adeno-associated virus

Adeno-associated virus (AAV) was discovered in 1965, as a subunit contaminant of adenovirus preparations in the laboratory of Bob Atchison

(Atchison et al., 1965, Hoggan et al., 1966) and is a small (25 nm), non-pathogenic virus that has not been associated with any disease. AAV belongs to the family *Parvoviridae* and is placed in the genus *Dependovirus* because it is a replication deficient virus that depends on functions provided by a helper virus (adenovirus or herpes virus) for productive replication in mammalian cells (Atchison et al., 1965). However, in the absence of helper virus, AAV can establish a latent infection by either integrating into a specific site in the chromosome 19q13.4 or persisting in episomal form (Wu et al., 2006a). Interestingly, AAV is the only mammalian DNA virus capable of site-specific integration (Daya and Berns, 2008).

1.4.1 Structure of the adeno-associated virus genome

The AAV genome is a 4,680-nucleotide linear, single-stranded DNA of either positive or negative sense containing two open reading frames (ORFs); the *rep* gene and the *cap* gene, flanked by two inverted terminal repeats (ITRs) (Berns and Rose, 1970, Wang et al., 2014). The ITR is a 145 base pair (bp) palindromic sequence which can fold upon itself to maximize base pairing and form a T-shaped hairpin structure which plays a crucial role in genome replication and packaging (Carter et al., 1976, Gao et al., 2002a). In detail, the self-priming ITR hairpins allow primase-independent synthesis of the second DNA strand by itself serving as the template for the synthesis of the second complementary strand. The *rep* and *cap* genes encode seven proteins needed for the replication and packaging of the virus (Figure 1.4).

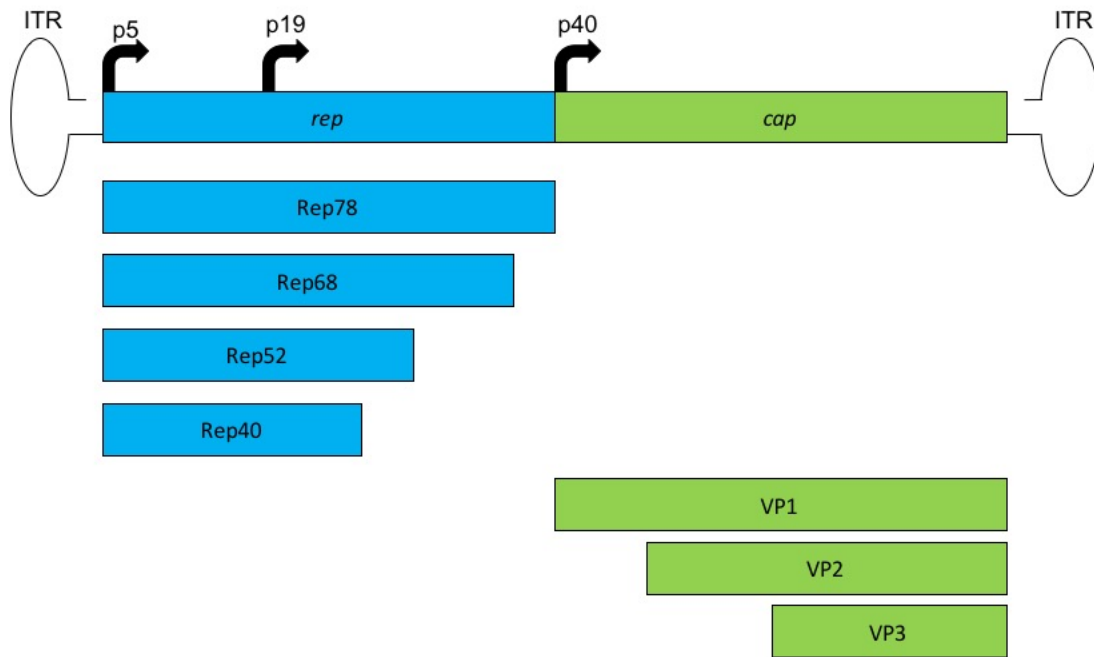


Figure 1.4 Structure of the adeno-associated virus genome. Schematic representation of the genome of the AAV. The AAV genome consists of the *rep* and the *cap* genes, flanked by two inverted terminal repeats (ITRs); by utilising two transcription initiation sites and alternative splicing, the left ORF (*rep* gene; blue) produces four Rep proteins (Rep78, Rep68, Rep52 and Rep40) driven by two promoters p5 and p19 (Jay et al., 1981). These *trans*-acting Rep proteins are important in viral replication, genome packaging, site-specific integration as well as gene expression regulation. The right ORF contains the *cap* gene (green), which produces three viral capsid proteins (VP1, VP2, and VP3) using the transcript of p40 promoter and also by alternative splicing. These viral proteins (VP1, VP2 and VP3 in 1:1:10 molar ratio) assemble into an icosahedral structure to form the AAV capsid which is involved in binding, internalization and trafficking of virus within target cells (Daya and Berns, 2008).

The AAV life cycle can be divided into two stages upon successful infection - a lytic stage and a lysogenic stage. The lytic cycle occurs in the presence of helper virus, as mentioned above, where AAV undergoes genome replication, gene expression and virion production (Daya and Berns, 2008). In the absence of helper virus, AAV enters the lysogenic stage in which there is limited replication and repressed gene expression; at this stage the AAV genome establishes latency by integrating into human chromosome 19 (Daya and Berns, 2008).

1.4.2 Recombinant adeno-associated viral vector

The first rAAV vector was produced in 1984, generated by removing 95% of the viral genome (Samulski et al., 1982). At that time, recombinant AAV was produced by transfecting vector and packaging constructs into cells infected by adenovirus (Muzyczka, 1992). Later on, advancement in technology allowed the production of recombinant AAV with the absence of wild type virus (adenovirus or herpesvirus) and all the genes encoding proteins (E1a, E1b, E2a, E4 and VA RNA) needed for rAAV production were contained and provided in a helper plasmid (Ferrari et al., 1997, Daya and Berns, 2008). Each of the essential viral proteins play a specific role in AAV production, for instance, E1A is involved in transcriptional activation, E1B and E4 are involved in mRNA maturation and VA and E2A are needed in translation enhancement (Ferrari et al., 1996). With this method, rAAV can be generated by transfecting producer cells with genome plasmid, packaging plasmid and helper plasmid without the need of helper virus (see Figure 1.5). Currently, AAV vectors are produced by triple transfection of HEK 293T cells

Table 1.4 Recombinant adeno-associated virus vector (rAAV) serotypes, cellular receptors and tissue tropism

AAV serotype	Primary receptor(s)	Co-receptor(s)	Tissue tropism	Reference(s)
rAAV1	$\alpha 2,3/\alpha 2,6$ N-linked sialic acid	-	Skeletal muscle, central nervous system, retina, pancreas	(Chao et al., 2000, Wang et al., 2003, Loiler et al., 2005, Wu et al., 2006b, Lebherz et al., 2008, Ng et al., 2010)
rAAV2	HSPG	FGFR, HGFR, laminin R, integrin $\alpha V\beta 5/\alpha 5\beta 1$	Vascular smooth muscle cell, skeletal muscle, central nervous system, liver, kidney	(Koeberl et al., 1997, Bartlett et al., 1998, Summerford and Samulski, 1998, Qing et al., 1999, Richter et al., 2000, Manno et al., 2003, Takeda et al., 2004, Kashiwakura et al., 2005, Akache et al., 2006, Asokan et al., 2006, Kurzeder et al., 2007)
rAAV3	HSPG	HGFR, laminin R	Hepatocarcinoma, skeletal muscle	(Chao et al., 2000, Rabinowitz et al., 2002, Akache et al., 2006, Blackburn et al., 2006, Glushakova et al., 2009, Ling et al., 2010)
rAAV4	$\alpha 2,3$ O-linked sialic acid	-	central nervous system, retina	(Davidson et al., 2000, Kaludov et al., 2001, Weber et al., 2003)
rAAV5	$\alpha 2,3$ N-linked sialic acid	PDGFR	Skeletal muscle, central nervous system, retina, lung	(Chao et al., 2000, Davidson et al., 2000, Kaludov et al., 2001, Di Pasquale et al., 2003, Seiler et al., 2006, Lebherz et al., 2008)
rAAV6	$\alpha 2,3/\alpha 2,6$ N-linked sialic acid	EGFR	Skeletal muscle, heart, lung	(Halbert et al., 2001, Blankinship et al., 2004, Gregorevic et al., 2004, Wu et al.,

				2006b, Ng et al., 2010, Weller et al., 2010, Zincarelli et al., 2010)
rAAV7	-	-	Skeletal muscle, central nervous system, retina	(Gao et al., 2002b, Allocca et al., 2007, Taymans et al., 2007)
rAAV8	-	Laminin R	Liver, skeletal muscle, central nervous system, retina, pancreas, heart	(Gao et al., 2002b, Wang et al., 2005, Akache et al., 2006, Allocca et al., 2007, Taymans et al., 2007)
rAAV9	N-linked galactose	Laminin R	Liver, skeletal muscle, brain, lung, pancreas, heart	(Akache et al., 2006, Inagaki et al., 2006, Bostick et al., 2007, Vandendriessche et al., 2007, Foust et al., 2009, Yue et al., 2011)
rAAV10	-	-	-	(Mori et al., 2004)
rAAVrh.10	-	-	-	(Gao et al., 2004)
rAAV11	-	-	-	(Mori et al., 2004)
rAAV12	-	-	-	(Schmidt et al., 2008)
rAAVrh32.33	-	-	-	(Calcedo et al., 2009)

rAAV: recombinant adeno-associated viral vector; HSPG: heparan sulfate proteoglycan; FGFR: fibroblast growth factor receptor; HGFR: hepatocyte growth factor receptor; EGFR: epidermal growth factor receptor; PDGRF: platelet-derived growth factor receptor

with *cis*-genome plasmid, adenoviral helper plasmid and a chimeric packaging plasmid in which the AAV2 *rep* gene is linked with *cap* genes of different AAV serotypes (Gao et al., 2002a). There are more than 12 natural serotypes and many variants of AAV have been isolated or generated (Mori et al., 2004, Salganik et al., 2015). By combining different *Cap* genes with the AAV 2 *rep* gene, many different rAAV vectors have been generated, such as AAV2/8 and AAV2/9. These different types of rAAV can result in different vector tropisms, which is useful for providing a selection of vectors to target different cell types. The tropism and receptors for different AAV serotype are summarised in Table 1.4. Several problems have been tackled to improve the efficiency of single stranded AAV (ssAAV) vectors. For example, the immediate-early step, also known as the second-strand synthesis (see Section 1.4.1 above), was found to be rate-limiting in the replication of ssAAV (Ferrari et al., 1996). To overcome this, research has been carried out to find ways to bypass the second-strand synthesis step which later led to the development of self-complementary rAAV (scAAV). The scAAV vector, sometimes known as double-stranded rAAV (dsAAV), is made by reducing the vector construct size by about half, to around 2,500 bp including two 145 bp ITRs (Wang et al., 2007). The advantage of scAAV is that it can bypass the rate-limiting second-strand synthesis step, thus increasing the AAV replication and genome expression in infected cells. The disadvantage is that the packaging capacity of scAAV is greatly reduced compared with ssAAV (Wang et al., 2007), restricted to incorporation of transgenes no greater than 2,350 bp.

Recombinant AAV fulfils several key criteria as a viral vector for gene therapy, including: the ability to bind and enter target cells, the successful transfer to the nucleus, the ability of the gene to be expressed in the nucleus for a sustained period and the lack of reported toxicity (Daya and Berns, 2008). Owing to the fact that the ITRs are the only sequences required *in cis* while *rep* and *cap* genes can be delivered *in trans* (Xiao et al., 1997), many methods have been established for the production of rAAV based on this important feature of AAV. Furthermore, rAAV results in high transduction efficiencies ranging from 25 to several hundred vector genome-containing particles (VGP) per transducing unit, depending on the cell type (McCarty, 2008). Recombinant AAV vectors used for gene therapy have been mostly based on AAV2 (Daya and Berns, 2008), which was the first AAV isolate to be developed into recombinant vectors for transgene delivery (Wu et al., 2006a). The addition of alternative capsid variants to the basic rAAV2 vector has generated a wide variety of AAV serotypes (such as rAAV2/8 and rAAV2/9) with potential tropisms for a range of target tissues.

As of 2016, rAAV vectors have been used in nearly 200 clinical trials worldwide. Examples of gene therapy applications where rAAV has been evaluated in clinical trials are summarised in Table 1.5. In general, the rAAV vector is considered a safe vector for gene therapy as it has not been associated with any disease and the integrations of wild type AAV appears not to occur at random sites in the genome. Several studies have reported a link between rAAV infection and the development of hepatocellular carcinoma (HCC) in rodent (Donsante et al., 2001, Donsante et al., 2007). However, contrary

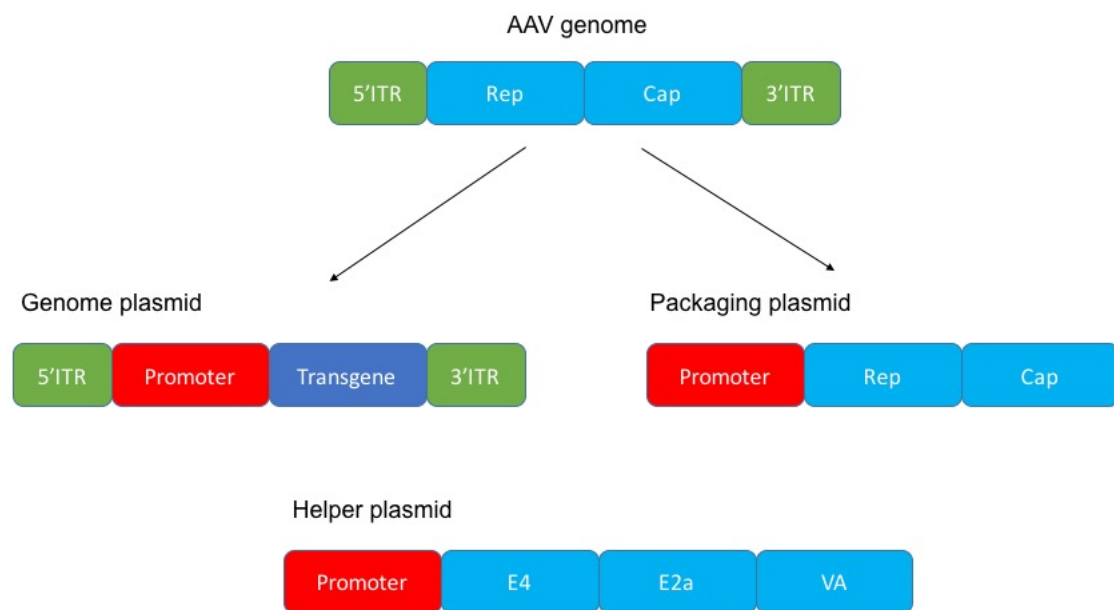


Figure 1.5 Plasmids needed to generate recombinant adeno-associated virus vector. Schematic representation of the plasmids required to produce recombinant adeno-associated virus (rAAV) vector. The AAV genome consists of two genes, flanked by two inverted terminal repeats (ITRs); the Rep gene which encodes four Rep proteins (Rep78, Rep68, Rep52 and Rep40) needed for viral replication and the Cap gene which encodes the capsid proteins (VP1, VP2 and VP3). The elements to produce rAAV are supplied in three different plasmids: (1) the genome plasmid which contains the transgene and a promoter flanked by the 5' and 3' ITRs; (2) the packaging plasmid, containing the Rep and Cap gene of the selected AAV serotype; (3) the helper plasmid, which contains genes from adenovirus (E4, E2a and VA) that mediate AAV replication. Another adenoviral gene needed in AAV replication, E1, is supplied by the HEK 293T cells.

findings defying this link have also been reported, indeed, no evidence for HCC formation in dogs and non-human primates receiving rAAV vectors (Niemeyer et al., 2009, Kaeppel et al., 2013b). Interestingly, one further study has highlighted that the formation of HCC is highly influenced by vector dose, enhancer/promoter selection and the timing of gene delivery (Chandler et al., 2015).producer cells (HEK 293T) (not shown in the figure). Additional details are provided in Section 1.4.2.

In conclusion, rAAV is a popular vector for gene therapy and became the first gene therapy vector licenced for gene therapy in the European Union (Yla-Herttuala, 2012). Thus, rAAV vectors have gained an excellent track record in many pre-clinical gene-therapy studies and it has a great potential to be used in many clinical settings in the future.

1.5 Retrovirus and Lentivirus

1.5.1 Retrovirus

Retroviruses belong to the *Retroviridae* family and are 80-100 nM diameter, enveloped viruses. The virus was named based on the fact that the viral genome requires the reverse transcriptase enzyme (Baltimore, 1970, Temin and Mizutani, 1970) to convert the two genome copies of single-stranded positive sense ribonucleic acid (RNA) into DNA in the infected host cells. Retroviruses can be divided into several subfamilies such as gammaretroviruses, spumaviruses and lentiviruses (discussed next).

1.5.2 Lentivirus

Lentivirus (LV) is a genus of the *Retroviridae* family characterised by a long incubation period prior to causing disease (Figure 1.6). Lentiviruses are a family of enveloped, single-stranded RNA virus that integrate into the host genome. They are further classified into five serogroups. The viral genome is around 9 to 11 kb pairs long and present in its integrated proviral DNA form, is flanked by two long terminal repeats (LTRs). Each of the LTRs consists of three regions; U3, R and U5. The U3 region contains a promoter that drives the expression of the viral genome. The U3 or R regions contain a polyadenylation (pA) signal which is involved in the cleavage and polyadenylation at the end of the 3' R region. There are some other important regions such as the packaging signal (Ψ) (Lever et al., 1989), splice donor and splice acceptor sites and the polypurine tract (PPT) (Charneau et al., 1992). Examples of LV include Equine Infectious Anaemia Virus (EIAV), Human Immunodeficiency Virus type 1 (HIV-1), HIV type 2 (HIV-2), Simian Immunodeficiency Virus (SIV) and Feline Immunodeficiency Virus (FIV).

1.5.3 The lentiviral vector

Recombinant lentiviral vectors (rLV) have also gained popularity in the gene therapy field, due to several advantages over other viral vectors, including the ability to accommodate larger transgenes compared with AAV, to transduce non-dividing cells and to establish long-term transgene expression (Weinberg et al., 1991, Bukrinsky et al., 1993). Recombinant LV vectors have been developed for gene therapy based on: Equine Infectious Anaemia Virus (EIAV) (Olsen, 1998), Feline immunodeficiency Virus (FIV) (Poeschla et al., 1998b),

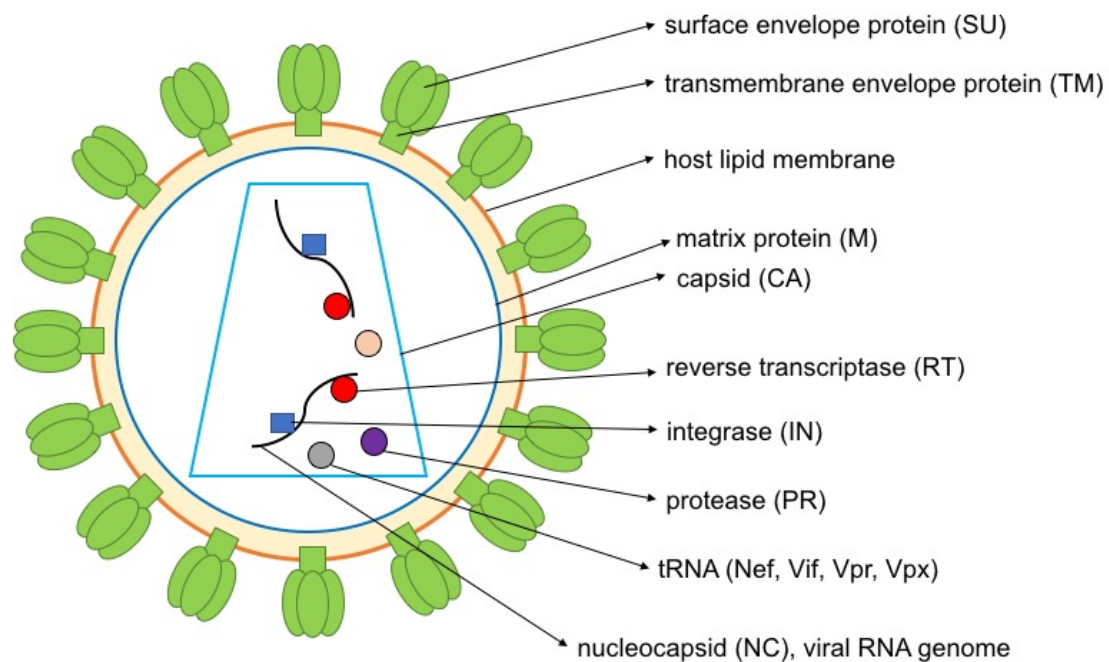


Figure 1.6 Prototypic retrovirus particle. Schematic representation of the prototypic retroviral particle shows the viral RNA surrounded by the internal structure made up of nucleocapsid (NC), capsid (CA) and matrix (MA) proteins. The internal structure is protected by lipid membrane derived from host cells contains the envelope glycoproteins (surface envelope protein (SU) and transmembrane envelope protein (TM)). The viral particle also contains enzymes reverse transcriptase (RT), integrase (IN), protease (PR) and a transfer RNA (tRNA). In lentiviral particles, there are also regulatory and accessory proteins such as Nef, Vif, Vpr and Vpx (in the case of Human Immunodeficiency Virus (HIV)).

Table 1.5 Summary of gene therapy clinical trials using adeno-associated virus vector (rAAV)

Vector type	Disease	Transgene	Clinical phase	Route of administration	References
rAAV2	Haemophilia B	Factor IX	Phase I/II	Intramuscular	(Manno et al., 2003)
rAAV8			Phase I/II	Intravenous	(Nathwani et al., 2011)
rAAV2	RA	TNF receptor-immunoglobulin Fc fusion	Phase I/II	Intraarticular	(Mease et al., 2009, Mease et al., 2010)
rAAV2	CF	CFTR	Phase I/II/IIb	Aerosol	(Aitken et al., 2001, Moss et al., 2004, Moss et al., 2007)
rAAV1	LPL deficiency	LPL	Phase I/II/III	Intramuscular	(Stroes et al., 2008, Gaudet et al., 2010)
rAAV2	Leber's congenital amaurosis	RPE65	Phase I/II	Subretinal	(Maguire et al., 2008, Maguire et al., 2009a, Jacobson et al., 2012, Bainbridge et al., 2015)
rAAV2	Choroideremia	REP1	Phase I/II	Subretinal	(MacLaren et al., 2014)
rAAV2	Canavan's disease	Aspartoacylase	Phase I/II	Intracranial	(Janson et al., 2002, McPhee et al., 2006, Leone et al., 2012)
rAAV1/2 chimera	Muscular dystrophy	Microdystrophin	Phase I	Intramuscular	(Bowles et al., 2012)
rAAV2		Alpha-Sacroglycan	Phase I	Intramuscular	(Mendell et al., 2009, Mendell et al., 2010)
rAAV1		Follistatin	Phase I/II	Intramuscular	(Mendell et al., 2015)

rAAV2	AAT deficiency	AAT	Phase I/II	Intramuscular	(Brantly et al., 2006)
rAAV1			Phase I/II	Intramuscular	(Brantly et al., 2009, Flotte et al., 2011)
rAAV1	Severe heart failure	SERCA2a	Phase I/II	Coronary artery infusion	(Jaski et al., 2009, Jessup et al., 2011)

rAAV: recombinant adeno-associated viral vector; RA: rheumatoid arthritis; TNF: tumour necrosis factor; Fc: fragment crystallisable from immunoglobulin; CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator; LPL: lipoprotein lipase; RPE65: retinal pigment epithelium-specific protein 65 kDA; REP1: Rab 1 escort protein; AAT: alpha-1-antitrypsin; SERCA2a: sarcoplasmic reticulum calcium ATPase 2a; ARSA: Arylsulfatase A.

Ongoing and clinical trials prior to 2001 were not included in this table

Table 1.6 Summary of gene therapy clinical trials using lentiviral vector (rLV)

Vector type	Disease	Transgene	Clinical phase	Route of administration	References
rHIV	β -thalassemia	β -globin	Phase I/II	<i>Ex vivo</i> (HSCs)	(Cavazzana-Calvo et al., 2010)
rHIV	X-ALD	ABCD1	Phase I	<i>Ex vivo</i> (HSCs)	(Cartier and Aubourg, 2010)
rHIV	WAS	WAS	Phase I/II	<i>Ex vivo</i> (HSCs)	(Aiuti et al., 2013)
rHIV	MLD	ARSA	Phase I/II	<i>Ex vivo</i> (HSCs)	(Biffi et al., 2013)
rHIV	CLL	CAR	Phase I	<i>Ex vivo</i> (T cells)	(Porter et al., 2011)
rEIAV	Parkinson	Tyrosine hydroxylase, AADC and cyclohydrolase 1	Phase I/II	Intracranial	(Palfi et al., 2014)

rHIV: recombinant human immunodeficiency virus vector; rEIAV: recombinant equine anemia infectious virus vector; CLL: chronic lymphocytic leukemia; CAR: chimeric antigen receptor; AADC: aromatic amino acid decarboxylase; WAS: wiskott-aldrich syndrome; MLD: metachromatic leukodystrophy; X-ALD: X-linked adrenoleukodystrophy; ABCD1: adenosine triphosphate (ATP)-binding cassette, subfamily D.

Ongoing and clinical trials prior to 2010 were not included in this table.

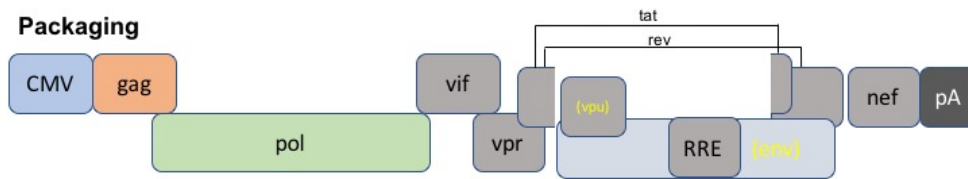
HIV-1 (Naldini et al., 1996), HIV-2 (Poeschla et al., 1998a) and Simian immunodeficiency Virus (SIV) (Nakajima et al., 2000, Negre et al., 2000).

For first generation rLV vectors, the different elements of the viral genome were distributed between different plasmids (Naldini et al., 1996), such that a three-plasmid system was developed for rLV production following transient transfection into mammalian producer cells (see Figure 1.7). The packaging plasmid consists of the proviral DNA, with the long-terminal repeats (LTR) substituted by human cytomegalovirus immediate early enhancer and promoter (CMVIE) (Foecking and Hofstetter, 1986) to drive expression of all viral proteins needed for rLV production. This plasmid, however, is deficient for the expression of the viral envelope and accessory protein vpu. The genome plasmid, carries the transgene of interest, driven by an internal promoter and the elements needed for packaging, reverse transcription and integration. The third (pseudotype) plasmid contains the transgene encoding heterologous envelope protein(s) to replace the HIV-1 Env protein.

In the second generation rLV (Zufferey et al., 1997), a three-plasmid similar to the first generation rLV was employed except that env and the genes encoding the accessory proteins (nef, vif, vpr and vpu) have been removed from the production (also see Figure 1.7). Removal of the four accessory proteins improve the safety of the rLV in such that if replication-competent rLVs are generated, they would be devoid of all virulent factors.

First generation lentiviral vector plasmids

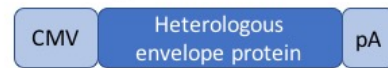
Packaging



Genome

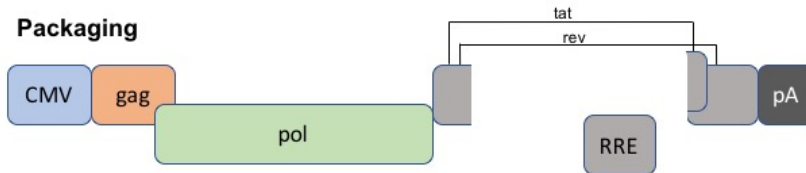


Pseudotype



Second generation lentiviral vector plasmids

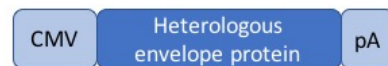
Packaging



Genome

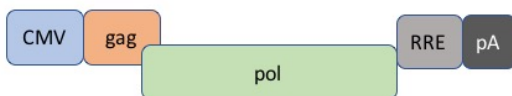


Pseudotype



Third generation lentiviral vector plasmids

Packaging



Rev



Genome



Pseudotype

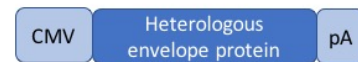


Figure 1.7 Plasmids required for first, second and third generation lentiviral vectors. The first generation vectors (Naldini et al., 1996) are produced from a three-plasmid system containing the different elements of the viral genome: (1) packaging plasmid, which contains the proviral DNA but defective for Env and Vpu (in yellow texts); (2) genome plasmid, containing the transgene, packaging elements and a transgene promoter; (3) pseudotype plasmid, containing surface glycoprotein from different viruses. The second generation (Zufferey et al., 1997) is similar to the first generation but with env and the virulence genes *nef*, *vif*, *vpr* and *vpu* deleted from the packaging plasmid. In the third generation, a constitutive promoter (eg, CMV or the RSV promoter) is included to replace the U3 region in the 5' LTR allowing the removal of tat gene and rev is provided in a separate plasmid. Third generation lentiviral vector is made self-activating (SIN) via the deletion of some key nucleotides (Δ U3) in the 3' LTR of the genome plasmid (Miyoshi et al., 1998, Zufferey et al., 1998). The post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) is included to enhance gene expression (Zufferey et al., 1999). Additional details are provided in Section 1.5

To further improve clinical safety, the third generation rLV was developed (Dull et al., 1998, Kim et al., 1998). In third generation rLV, a chimeric 5' LTR was introduced by replacing the U3 region with a heterologous constitutive promoter, typically CMV or the Rous Sarcoma Virus (RSV) promoter (Gorman et al., 1982). The Tat gene, which is needed for the expression of transgene via the transactivation of the 5' LTR, was removed since the transgene can now be driven by the heterologous promoter. The rev gene which is needed for rLV packaging is supplied in a separate plasmid. In term of safety, third generation vectors are self-inactivating (SIN), via deletion of the promoter- enhancer region of the 3' LTR (Δ U3), which contains elements such as transcription factor binding sites and the TATA box needed to regulate the LTR (Miyoshi et al., 1998, Zufferey et al., 1998). The proviral 5' LTR is copied from the Δ U3 LTR during reverse transcription. This eliminates the 5' LTR promoter activity without affecting the transgene expression due to the inclusion of the heterologous internal promoter. The post-transcriptional regulatory element of woodchuck hepatitis virus (WPRE) was included to enhance transgene expression (Zufferey et al., 1999). The SIN rLV has an improved safety profile because it prevents the downstream transcription from the 3' LTR into the genomic DNA, which can lead to transactivation of downstream endogenous oncogenes (Herman and Coffin, 1987). Risk of generating replication-competent rLV is further reduced by reducing the chance of homologous recombination between vector and packaging cassettes via separation of these elements in separate plasmids.

1.5.4 Application of lentiviral vectors in gene therapy

Recombinant lentiviral vector has shown benefits for gene therapy in animal models and most of the use of rLV in the clinical phase has been so far restricted to *ex vivo* applications. Clinical trials using lentiviral vectors is summarised in Table 1.6.

1.5.5 The development of recombinant lentiviral vector pseudotyping

Recombinant lentiviral vector is commonly pseudotyped with the glycoprotein (G) from vesicular stomatitis virus (VSV) (LV-VSV-G) for broad tropism and stability (Akkina 1996, Naldini, 1996b, Reiser 1996). Several other glycoproteins have been used in rLV pseudotyping to alter their tropism such as glycoproteins from filovirus (Medina et al., 2003), lyssavirus glycoproteins (Badrane et al., 2001), lymphocytic choriomeningitis virus glycoproteins and alphavirus glycoproteins (Kang et al., 2002).

The UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC) has been developing non-viral and viral vectors for treatment of cystic fibrosis (CF) lung disease. A non-viral gene transfer approach using plasmid DNA expressing the CFTR cDNA complexed with cationic liposome GL67A has shown some clinical benefit in a phase IIb clinical trial (Alton et al., 2015). With a view to improving efficiency, however, the group are also developing a Lentiviral approach. The common rLV pseudotype based on VSV-G has no natural lung tropism because its receptor, heparan sulphate proteoglycan, is located at the basolateral surface of the lung epithelia and is not accessible from the lung lumen (Guibinga et al., 2002, Gill and Hyde, 2014). For efficient lung transduction, the

basolateral receptors can be accessible via the opening of tight junctions between the airway epithelial cells, with adjuncts such as lysophosphatidylcholine (LPC) and ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), which need to be administered prior to rLV administration. This use of 'tight junction openers' clinically could be problematic in CF patients whose lungs have a high bacterial burden, possibly risking systemic infections.

An alternative approach to improving transduction of the lung epithelium has been to pseudotype rLV with glycoproteins from viruses with a natural tropism for airway cells. Several glycoproteins have been used to pseudotype rLV to improve uptake into the airway epithelium including glycoproteins from filovirus, surface glycoproteins (HA, NA and M2) from influenza virus (McKay et al., 2006), baculovirus GP64 surface protein (Sinn et al., 2008a) and Sendai virus fusion (F) and haemagglutinin (HN) proteins (Mitomo et al., 2010b).

Lentiviral vectors based on Simian Immunodeficiency Virus (SIV) pseudotyped with the F and HN proteins from Sendai virus (rSIV.F/HN) have shown efficient lung tropism and high and persistent levels of transgene expression following lung delivery in mice (Kobayashi et al., 2003a). A single dose of the F/HN pseudotyped LV leads to robust and persistent transgene expression up to 2 years (the lifetime) of the mouse model (Mitomo et al., 2010b, Griesenbach et al., 2012a). Furthermore, daily and monthly repeat administration of this vector has been shown to be feasible, without toxicity and accompanying loss of transgene expression (Griesenbach et al., 2012b). The mechanism by which

this vector can be repeatedly administered without loss of efficacy is so far unknown, but illustrates how rLV may be unique amongst viral vectors. This vector has also been shown to efficiently transduce human air-liquid interface (ALI) cultures *in vitro* and human lung slices *ex vivo* (Griesenbach et al., 2012b). A regulatory-compliant version of this vector (rSIV.F/HN) has been developed for clinical use (Alton et al., 2017) will be evaluated in CF patients for the treatment of CF lung disease.

1.6 Aims

Many viral vectors have been studied in a non-clinical setting to provide confirmation of passive immunisation (Table 1.5 and 1.6). None, however, has made it to the clinic, and no direct comparison of viral vector platforms for this purpose has been reported so far. The main aim of this study, therefore, is to evaluate the feasibility of two widely-used viral gene delivery platforms (based on AAV and LV) for delivery of novel bnAbs isolated from vaccinated donors to the lung, for passive immunisation against influenza. In particular, rLV has not been previously tested for influenza protection in this way. In addition to IN delivery of rSIV.F/HN to the lung, IM delivery of the rAAV2/8 vector will be used as a benchmark, since this strategy has previously shown to be effective in providing long-term protection in mice (Balazs et al., 2013).

Chapter 2 Materials and Methods

2.1 Materials

2.1.1. Reagents and solutions

All chemicals were supplied by Sigma-Aldrich (Gillingham, UK) unless otherwise stated. All solutions were prepared with Milli-Q reverse osmosis ultrapure water (Merck, Feltham, UK), nuclease-free water (Promega, Southampton, UK), endotoxin-free water for injection (WFI) (B. Brain Medical Ltd., Sheffield, UK) or absolute ethanol (VWR, Lutterworth, UK). Where needed, solutions were sterilised by autoclaving for 15 to 30 minutes (min) at 121°C, or by filtration through a 0.22 µm surfactant-free cellulose acetate filter (Thermo Fisher Scientific, Cramlington, UK). Solutions were stored at room temperature (RT) unless otherwise stated. Where concentrations of solutions are indicated by percentages, they refer to weight: volume (w/v) ratios for solids at RT and volume: volume (v/v) ratios for liquids at RT.

Lysogeny broth (LB) was prepared using 1% (w/v) Tryptone (Ameresco, Solon, OH, United States of America (USA)), 1% (w/v) Sodium chloride (Sigma), 0.5% (w/v) granulated yeast extract (Merck) in 4 L Milli-Q water. LB agar was prepared by additional of Select Agar (Life Technologies, Paisley, UK) into the LB. Both LB and LB agar solutions were sterilised by autoclaving for 15 to 30 min at 121°C. Kanamycin (50 mg/mL, filter sterilised and stored at 4°C) (Sigma) or Ampicillin (100 mg/mL, filter sterilised and stored at 4°C) (Sigma) was added to a final concentration of 50 µg/mL or 100 µg/mL respectively before use.

2.1.2 Mammalian cells

2.1.2.1 Cell culture conditions for the suspension HEK 293T cell line

Cells of the Human Embryonic Kidney 293T/17 cell line (referred to as HEK 293T cells (Graham et al., 1977, Rio et al., 1985, Dubridge et al., 1987, Lin et al., 2014), previously adapted to suspension growth using sequential serum reduction (gift from Lee Davies) were used. HEK 293T cultures were grown in polycarbonate Erlenmeyer flasks (Corning) maintained on an orbital platform with an orbital diameter of 1.9 cm (Thermo Fisher Scientific) in an 8% CO₂ humidified agitating incubator (135 revolutions per minute (rpm)) (Thermo Fisher Scientific) at 37°C in FreeStyle 293 Expression medium (FreeStyle) (Life Technologies) without any serum or antibiotics. Cell growth and count was determined using 0.2% trypan blue dye and a Neubauer haemocytometer (Sigma). Cells were passaged twice a week by diluting to 3.5E5 live cells per mL media.

2.1.2.2 Cell culture conditions for the adherent HEK 293T cell line

For production of rAAV vector and certain experiments as mentioned in the text, adherent HEK 293T cells were used. Adherent HEK 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) (2mM L-glutamine (Life Technologies), 10% foetal bovine serum (FBS) (Life Technologies), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies)) (D10). Cells were passaged to the required concentration twice a week and received fresh medium during each passage.

2.1.2.3 Cell culture conditions for the MDCK-SIAT1 cell line

Cells based on the Madin-Darby Canine Kidney Epithelial (MDCK) cell line were used, specifically MDCK-SIAT1 with overexpression of human alpha-2,6 sialic acid via stable transfection with the cDNA of human alpha2,6-sialyltransferase 1 (SIAT1) (Matrosovich et al., 2003). The MDCK-SIAT1 cells were maintained in D10 media, passaged to required concentration, receiving fresh medium during each passage.

2.1.3 Plasmids

Broadly neutralising Abs (bnAbs) (IgG1) used in this study are expressed from a single-ORF cDNA construct (Section 1.3). These constructs were designed from the variable heavy (VH) and variable light (VL) sequences of the respective antibody (Alain Townsend, personal communication) (Appendix I) using the MacVector software (MacVector Inc., Apex, USA). Single-ORF bnAb constructs were codon-optimised and synthesised as cDNA by GeneArt (Invitrogen, Paisley, UK) and then sub-cloned into SIV or AAV genome plasmids using the methods described below. Construction of SIV and AAV bnAb genome plasmids are discussed in Section 3.3. Accessory plasmids needed for rSIV.F/HN and rAAV2/8 production are described in Table 2.1. Plasmids required to produce dual-ORF IgG1 and monomeric IgA1 antibodies

Table 2.1 Plasmids used in this study

Plasmid	Promoter	Transgene	Source
Genome plasmids for rSIV.F/HN production in the studies			
pSIV.hCEF.soGLux (pGM358)	hCEF	soGLux	Mary Connolly
pSIV.hCEF.T13B (pGM443)	hCEF	Single-ORF T1-3B	This study
pSIV.CMV.T13B (pGM444)	CMV	Single-ORF T1-3B	This study
pSIV.hCEF.21D85A (pGM445)	hCEF	Single-ORF 21-D8-5A	This study
pSIV.CMV.21D85A (pGM446)	CMV	Single-ORF 21-D8-5A	This study
pSIV.hCEF.F10 (pGM447)	hCEF	Single-ORF F10	This study
pSIV.CMV.T13B (pGM448)	CMV	Single-ORF T1-3B	This study
Genome plasmids for rAAV2/8 production in the studies			
pAAV2.CASI.soGLux (pGM453)	CASI	soGLux	This study
rAAV2.CASI.EGFPLux (pGM452)	CASI	EGFPLux	This study
pAAV2.CASI.F10 (pGM449)	CASI	Single-ORF F10	Derivative of (Balazs et al., 2013)
pAAV2.CASI.T13B (pGM450)	CASI	Single-ORF T1-3B	This study
pAAV2.CASI.21D85A (pGM451)	CASI	Single-ORF 21-D8-5A	This study
Accessory plasmids for rSIV.F/HN production in the studies			
pCMV.SIV.GagPol (pGM297)	CMV	<i>Gag</i> and <i>pol</i> genes from SIV	Addgene
pRSV.Rev (pGM299)	RSV	<i>Rev</i> gene from SIV	Addgene
pCAG.SeV.Fct4 (pGM301)	CAG	Gene for Sendai virus F envelope protein	Derivative of (Kobayashi et al., 2003b)

pCAG.SeV.EnvctHN (pGM303)	CAG	Gene for Sendai virus HN envelope protein	Derivative of (Kobayashi et al., 2003b)
Accessory plasmids for rAAV production in the studies			
pAAV2/8		<i>Rep</i> gene from AAV serotype 2 and <i>Cap</i> gene from AAV serotype 8	UPenn Vector Core
pAdDeltaF6		<i>E2a</i> , <i>E4</i> and <i>VARNA</i> genes from adenovirus	UPenn Vector Core
Plasmids for IgG1 production in the studies			
pCMV.T13B.GH	CMV	T1-3B IgG1 heavy chain	Alain Townsend
pCMV.T13B.GHΔK	CMV	T1-3B IgG1 heavy chain without C-terminal lysine	This study
pCMV.T13B.κ	CMV	T1-3B kappa (κ) light chain	Alain Townsend
pCMV.21D85A.GH	CMV	21-D8-5A IgG1 heavy chain	Alain Townsend
pCMV.21D85A.GHΔK	CMV	21-D8-5A IgG1 heavy chain without C-terminal lysine	This study
pCMV.21D85A.λ	CMV	21-D8-5A lambda (λ) light chain	Alain Townsend
pCMV.T25D.GH	CMV	T2-5D IgG1 heavy chain	Alain Townsend
pCMV.T25D.λ	CMV	T2-5D κ light chain	Alain Townsend
pCMV.T25A.GH	CMV	T2-5A IgG1 heavy chain	Alain Townsend
pCMV.T25A.κ	CMV	T2-5A κ light chain	Alain Townsend
pCMV.Q11C11.GH	CMV	Q11-C11 IgG1 heavy chain	Alain Townsend
pCMV.Q11C11.κ	CMV	Q11-C11 κ light chain	Alain Townsend
pCMV.212C.GH	CMV	2-12C IgG1 heavy chain	Alain Townsend
pCMV.212C.κ	CMV	2-12C κ light chain	Alain Townsend
pCMV.T34B.GH	CMV	T3-4B IgG1 heavy chain	Alain Townsend
pCMV.T34B.κ	CMV	T3-4B κ light chain	Alain Townsend
pCMV.T26A.GH	CMV	T2-6A IgG1 heavy chain	Alain Townsend
pCMV.T26A.λ	CMV	T2-6A λ light chain	Alain Townsend
pCMV.Z3B2.GH	CMV	Z3-B2 IgG1 heavy chain	Alain Townsend
pCMV.Z3B2.GAH	CMV	Z3-B2 IgG1 heavy chain with IgA1 hinge region	This study
pCMV.Z3B2.λ	CMV	Z3-B2 λ light chain	Alain Townsend
pCMV.MEDI8852.GH	CMV	MEDI8852 IgG1 heavy chain	(Kallewaard et al., 2016)
pCMV.MEDI8852.GAH	CMV	MEDI8852 IgG1 heavy chain with IgA1 hinge region	This study

pCMV.MEDI8852.κ	CMV	MEDI8852 κ light chain	(Kallewaard et al., 2016)
Plasmids used for monomeric IgA1 production in the studies			
pCMV.T13B.AH	CMV	T1-3B IgA1 heavy chain	This study
pCMV.21D85A.AH	CMV	21-D8-5A IgA1 heavy chain	This study
pCMV.T25D.AH	CMV	T2-5D IgA1 heavy chain	This study
pCMV.T25A.AH	CMV	T2-5A IgA1 heavy chain	This study
pCMV.Q11C11.AH	CMV	Q11-C11 IgA1 heavy chain	This study
pCMV.212C.AH	CMV	2-12C IgA1 heavy chain	This study
pCMV.T34B.AH	CMV	T3-4B IgA1 heavy chain	This study
pCMV.T26A.AH	CMV	T2-6A IgA1 heavy chain	This study
pCMV.Z3B2.AH	CMV	Z3-B2 IgA1 heavy chain	This study
pCMV.MEDI8852.AH	CMV	MEDI8852 IgA1 heavy chain	This study

rAAV: recombinant adeno-associated virus; hCEF: a combination of human CMV enhancer and human elongation factor 1 alpha promoter (Hyde et al., 2008); CAG: modified chicken β -actin promoter with human cytomegalovirus immediate early enhancer (Miyazaki et al., 1989); CMV: cytomegalovirus enhancer/promoter; CASI (Balazs et al., 2013); a combination of the CMV enhancer and chicken β -actin promoter and novel intron containing elements of the ubiquitin C enhancer/promoter; SIV: simian immunodeficiency virus; soGLux: codon-optimised CpG-depleted *Gaussia* luciferase. EGFPLux: enhanced green fluorescence protein fused to firefly luciferase at its N-terminal

in this study are described in Tables 2.1. The amino acid sequence of these antibodies can be found in Appendix I.

2.2 Methods

2.2.1 Centrifugation

Centrifugation was carried out using the Micro Star 17 microcentrifuge with the Ch.500034PP rotor (VWR) or 5415D microcentrifuge with the F45-36-8 rotor (Eppendorf, Westbury, New York, USA) for microtubes (Corning, Flintshire, UK), the J2-HS centrifuge with the JA-10 or JA-20 rotor (Beckman, High Wycombe, UK) for centrifuge tubes (Starstedt, Leicester, UK) and pots (Beckman), or the Sorvall Legend RT with swing bucket for conical tubes (Corning). Centrifugation using the J2-HS centrifuge was performed at 4°C while other centrifugations were performed at RT.

2.2.2 Microscopy

Microscope slides were visualised using an Optiphot-2 optical microscope (Nikon, Kingston upon Thames, UK). Plates and flasks were visualised with a Leitz DM IRBE inverted optical microscope (Leica Microsystems, Milton Keynes, UK) with a Lumen 200 Fluorescence Illumination System (Prior Scientific, Rockland, MA, USA) for fluorescence microscopy. Microscopy images were captured using an iPhone 6 smartphone at indicated magnification (Apple Inc, CA, USA).

2.2.3 Molecular cloning techniques

2.2.3.1 Gel electrophoresis

DNA was size-fractionated by agarose gel electrophoresis. Gels consisted of 0.7% agarose (Life Technologies), 500 ng/mL ethidium bromide (Sigma) in 1x Tris-acetate-EDTA (TAE) buffer (40 mM Tris(hydroxymethyl)aminomethane (Tris)-acetate and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.3). Agarose was dissolved by heating in a microwave for 1.5 to 2 min, then allowed to cool to hand-hot, with the 100 μ L ethidium bromide or 7 μ L SYBRgreen (Invitrogen) added before pouring into a mould. Prior to electrophoresis, DNA samples were mixed with 1 x Gel Loading Dye (NEB) in a final volume of 10 to 20 μ L, then electrophoresed at 10 to 15 V/cm for 1 to 2 hours. To allow DNA fragment size estimation, DNA standards, 500 ng of a 100 bp DNA ladder (range 100 bp to 1517 bp) (NEB), one kb DNA ladder (range 500 bp to 10 kb), or supercoiled DNA ladder (range 2 to 10 kb) (NEB), were included on each gel. Following electrophoresis, DNA bands were visualised under UV (312 nm wavelength), using GeneFlash Gel Documentation System (Syngene, Cambridge, UK) and images were captured and saved.

2.2.3.2 Restriction enzyme digests

All restriction enzymes digests were performed using enzymes purchased from New England Biolabs Ltd. (NEB), according to the manufacturer's instructions. Generally, a 20 μ L reaction volume containing 2.5 μ g of plasmid DNA was incubated for 2 hours at the recommended temperature, using not more than 10% final enzyme concentration.

2.2.3.3 Gel extraction of DNA fragments

Digested DNA was mixed with 1 x Gel Loading Dye (NEB) and electrophoresed on a 0.7% agarose gel containing 100 μ L ethidium bromide or 7 μ L SYBRgreen (Invitrogen), alongside a 1 kb or 100 bp ladder (NEB). Bands were visualised with long-wavelength UV light (366 nm), using a UVL-56 hand-held gel illuminator (UVP Inc., California, USA). The QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) was used for fragment purification according to the manufacturer's instructions. The DNA fragments were eluted into 30 μ L nuclease-free water and stored at -20°C.

2.2.3.4 Ligation of DNA fragments

Purified DNA fragments were ligated using T4 DNA ligase and associated buffers (NEB) according to manufacturer's instructions. Generally, 1 μ L of T4 DNA ligase (400 U) was mixed with 100 ng vector DNA and 700 ng insert DNA in a final volume of 10 μ L and incubated for at least 1 hour, and up to 18 hours, at 18°C and stored at -20 °C until needed.

2.2.3.5 Bacterial transformation of plasmid DNA

All plasmid DNA constructs were transformed into *E.coli* Stbl3 chemically competent cells (Invitrogen) according to the manufacturer's instructions, unless otherwise stated. Various dilutions of transformed cells were spread on LB agar plates containing 50 μ g/mL kanamycin or 100 μ g/mL ampicillin to isolate a single colony for subsequent amplification and analysis.

2.2.3.6 Inoculation of bacterial colonies into growth media

A single transformed *E.coli* colony, grown overnight on selective LB agar plates was inoculated into either 5 mL or 400 mL LB containing 50 µg/mL kanamycin or 100 µg/mL ampicillin. Cultures were incubated overnight in an orbital shaking incubator with an orbital diameter of 2.54 cm (Innova 4300, New Brunswick Scientific, Edison, NJ, USA) at 175 rpm, at 34°C.

2.2.3.7 Purification of plasmid DNA from transformed bacteria

Small-scale (approximately 10 to 15 µg) preparations of plasmid DNA were purified from 5 mL cultures using the Wizard Plus SV MiniPrep DNA Purification System (Promega) according to manufacturer's instructions. The DNA was eluted in 100 µL nuclease-free water and stored at -20 °C. Large-scale (approximately 1.5 to 2 mg) preparations of plasmid DNA were purified from 400 mL cultures using the EndoFree Plasmid Mega Kit (Qiagen) according to manufacturer's instructions. Final plasmid DNA pellets were typically resuspended in 500 µL of WFI and stored at -20°C.

2.2.3.8 Spectrophotometric quantification of DNA concentration in solution

DNA concentration was measured spectrophotometrically using a NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific) (range 0.002 to 15 mg/mL), using the relationship between the absorption of UV light by DNA at 260 nm

and the DNA concentration, with an optical density at 260 nm (OD_{260}) of 1.0 for a 50 $\mu\text{g/mL}$ solution of dsDNA. Each 2 μL sample was read in duplicate or triplicate, and the average was used to determine the final concentration. The purity of the preparation was measured by calculation of the OD_{260}/OD_{280} ratio, rejecting preparations with values of < 1.8 , which may be indicative of protein contamination.

2.2.3.9 Polymerase Chain Reaction

Phusion HotStart II DNA Polymerase (Thermo Fisher Scientific) and primers (Sigma) as indicated were used for the PCR, which was performed in an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific) under conditions indicated. PCR products were resolved using agarose gel electrophoresis, purified and quantified as described above.

2.2.3.10 Site-directed mutagenesis

Site-directed mutagenesis was employed to remove the C-terminal lysine-encoding nucleotides from pCMV.T13B.GH and pCMV.21D85A.GH (Section 3.2.5). Site-directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Stockport, UK). Primers needed to create the mutagenesis were designed using the Agilent website, and were synthesised as oligos (Sigma) (GM1041: TCCCTGTCTCCGGGTTGAAGCTT and GM1042: GGCCAAGCTTCAACCCGGAGACA). PCR was performed using the Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific) under the following

conditions: 1x QCLM buffer (Agilent Technologies), 100 ng of plasmid template, 11 pmole of primers and 10 μ mol of dNTP mix. PCR cycle was as following: 1 min at 95°C and 30 cycles at 95°C for 1 min, 55°C for 1 min and 65°C for 12 min. PCR products were digested with *DpnI* (10 U/ μ L) (Agilent Technologies) for 1 hour at 37°C to remove the parental strand. Mutagenised plasmids were transformed into XL-10-Gold Competent cells (Agilent Technologies) according to the manufacturer's instruction. Plasmids were then purified as described above.

2.2.3.11 Gibson cloning

To generate plasmids expressing the human IgA1 heavy chain from the IgG1 heavy chain expression plasmids (pCMV.GH) (Table 2.1). Gibson assembly was chosen as it provides seamless and digestion-free cloning in a one-step isothermal reaction via assembly of overlapping DNA fragments (Gibson et al., 2009). Human IgA1 heavy constant region (IgA1C_H) (GenBank ID: AJ294729.1) was synthesised as codon-optimised cDNA fragment by GeneArt (Invitrogen). Overlapping regions (20 to 25 nucleotides) compatible with pCMV.GH were added to the 5' and 3' end of the IgA1C_H cDNA fragment using PCR with the following primers (GM1047: GTCACCGTCTCCTCAGCGTC and GM1048: TCCGCCTCAGAAGGTA CTCA) under the following conditions: 1x buffer HF (Life Technologies), 30 ng/ μ L of pDNA template, 0.5 U Phusion polymerase (Life Technologies), 1 μ M of each primer and 200 μ M of dNTP mix (Life Technologies). The PCR profile consists of an initial denaturation step at 98°C for 15 min, 40 cycles of denaturation (98°C for 10 s), primers annealing (66°C for 30 s) and extension (72°C for 30 s) followed by a final extension step at

72°C for 7 min. PCR products were purified as described above. The IgG1 constant region in the pCMV.GH was removed via restriction digest (*Sall-KpnI*) and linearised fragments were purified as described above. Gibson assembly was performed using the Gibson Assembly Cloning Kit (NEB) using 50 ng/μL of pCMV.GH and 150 ng/μL of IgA1C_H with the Gibson Assembly Master Mix (NEB) in a 20 μL reaction. Reaction was carried out using Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific) at 50°C for 1 hour. Assembled plasmid was transformed and amplified as described above.

2.2.4 Polyethylenimine (PEI) transfection of plasmid DNA in mammalian cells

Transient transfection of pDNA in HEK 293T cells using PEI-based transfection agent was used in this study. Plasmid DNA were transfected into suspension HEK 293T cells using 25 kDa branched polyethylemine (PEI) (4.3 μg/μL) (Sigma) at the N:P ratio of 15:1. For rAAV2/8 production, 25 kDa linear PEI (1 mg/mL) (Polysciences Inc, Hirschberg an der Bergstrasse, Germany) was used, the ratio of pDNA was described in Table 2.2. For monoclonal antibodies and rSIV.F/HN production, a total of 0.33 μg pDNA per 10⁶ cells were transfected using PEIpro (PolyPlus, Illkirch-Graffenstaden, France) at a volume:mass ratio of 2:1. Plasmid DNA and PEI or PEIpro were allowed to form complexes for 20 min at RT before adding to HEK 293T cells.

2.2.5 Viral vectors production

2.2.5.1 Large-scale lentiviral vector production

Recombinant lentiviral (rLV) vectors were produced by transient transfection of suspension-cultured HEK 293T cells, according to previously established conditions (Lee Davies, personal communication) using a mixture of five plasmids at a total of 0.33µg plasmid DNA per 10⁶ cells using PEIpro (PolyPlus) at a volume:mass ratio of 2:1. Recombinant SIV-based lentiviral vectors were produced using the respective genome plasmid (see Table 3.2 and 3.3) and four accessory plasmids (Table 2.1) in a mass ratio of genome plasmid:pCMV.SIV.GagPol:pRSV.Rev:pCAG.SeV.Fct4:pCAG.SeV.EnvctHN at 20:9:6:6:6.

HEK 293T cells were cultured in single-use 10 L (5 L working volume) WAVE Cellbag bioreactors (GE Healthcare) mounted on a WAVE 20/50 rocking platform (GE Healthcare). The Cellbag was inflated with 5% CO₂, primed with 2 L FreeStyle media containing 2.5 ml/L 10% Pluronic P-68 (Life Technologies), seeded with HEK 293T cells to 0.8E6 cells/mL and incubated at 37°C, rocking at 20 rpm with an 8° rocking angle for 48 hours. pH of the growth media was automatically maintained at 7.2 by controlling the concentration of CO₂ in the headspace and/or 7% sodium bicarbonate (Sigma). Subsequently cells were transiently transfected using PEIpro (PolyPlus), using the five-plasmid mixture described above based on the cell counts in the cell bag. At approximately 18 hours post-transfection, 3 L of FreeStyle media, 7.5 mL of 10 % Pluronic P-68 (Life Technologies) and sufficient sodium butyrate (Sigma), a known enhancer

of transfection efficiency and transgene expression (Gorman et al., 1983), to achieve a concentration of 5 mM in the resultant 5 L culture was added.

Cells were also supplemented with 5 ml/L of 45 % glucose (Sigma) immediately prior to and 48 hours post-transfection. Two separate additions of Benzonase (5 U/L) (Merck) were added to remove nucleic acid contaminants; 48 hours post transfection and again 1 hour before harvest. Magnesium chloride was added to 1 mM 1 hour before harvest. At 72 hours post- transfection, the cell supernatant was filtered through a chain comprising a GE Ultra 5µm filter (GE Healthcare, Little Chalfont, UK) and a AcroPak 0.8/0.45µm filter (Pall, Portsmouth, UK) and the filtrate was stored at -80°C.

Viral supernatant (1.5 L) was thawed and the Sendai F protein activated by incubation (30 min, 37°C) with 0.05x TrypLEselect recombinant trypsin analogue (Life Technologies). Activated virus was then cooled to 4°C and concentrated / purified using a combination of anion exchange chromatography (AEX) and tangential flow filtration (TFF).

Initial virus capture was performed using an Akta Pure 150L chromatography station (GE Healthcare) in conjunction with a 5 mL Mustang Q XT5 quaternary ammonium AEX membrane (Pall). Viral supernatant was loaded onto the membrane at a flow rate of 25 mL/min, washed with low-salt buffer (150mM NaCl, 25 mM HEPES pH 7.5) and eluted in high salt buffer (1M NaCl, 25 mM HEPES pH 7.5). Virus containing fractions were identified by UV absorbance peaks and diluted to a final volume of 100 mL in FreeStyle media (4°C) to

maintain virus viability. Isolated virus was then concentrated and purified via TFF using a KRIII TFF system (Spectrum Europe, Breda, The Netherlands). Initially, 100 mL of virus in FreeStyle media was concentrated via ultrafiltration to 10 mL using a C02-E500-05N MicroKros TFF module (Spectrum Europe) consisting of a 20 cm² surface area modified polyethylsulfone membrane with a 500 kDa molecular weight cut-offs (MWCO). Concentrated virus was then diafiltered against 10 diavolumes (100 mL) of FreeStyle media before a final ultrafiltration volume reduction step yielding approximately 1.5 mL of final viral product. Purified virus was split into appropriate aliquots and stored at -80°C.

2.2.5.1.1 Determination of Lentiviral titre

2.2.5.1.1.1 Viral particle titre (VP/mL)

Physical lentiviral particle titre (VP/ml) was determined by quantitative reverse transcriptase PCR (qRT-PCR) using the Applied Biosystems 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific) as described (Alton et al., 2017). Viral RNA from the Lentivirus produced as described above was extracted using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's instructions. During viral RNA extraction, residual DNA was removed from the viral preparations by incubation with an excess of DNase (Life Technologies). The viral RNA was quantified in triplicate using one-step

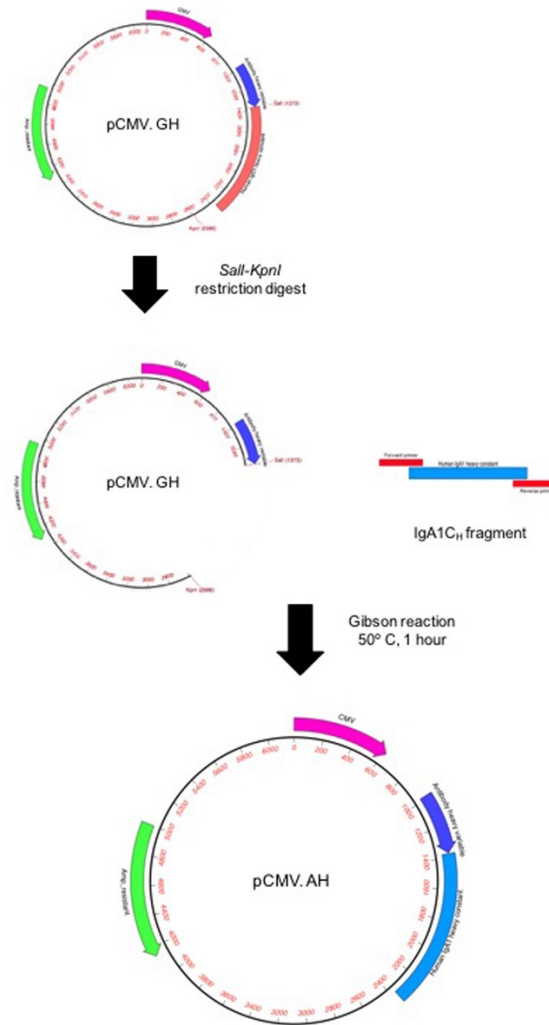


Figure 3.7: Schematic to illustrate the generation of pCMV.AH using Gibson cloning. The diagram shows the Gibson cloning used to generate pCMV.AH (Table 2.1). IgG1 heavy constant region was removed from pCMV.GH using *Sall-KpnI* restriction digest. Human IgA1 heavy constant region (IgA1C_H) (GenBank ID: AJ294729.1) was synthesised as codon-optimised cDNA fragment by GeneArt (Invitogen). Overlapping regions (20 to 25 nucleotides) compatible with pCMV.GH were added to the 5' and 3' ends of the IgA1C_H cDNA fragment using PCR and the products were used to assemble pCMV.AH using Gibson cloning. The assembled pCMV.AH was transformed and amplified (not shown in diagram).

TaqMan qRT-PCR using QuantiTect (Qiagen) against a standard curve of RNA mimics containing the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) sequence (gift from Stephanie Sumner-Jones). The primers used were as following: Forward: TGGCGTGGTGTGCACTGT; Reverse: CCCGGAAAGGAGCTGACA; Probe: FAM-TTGCTGACGCAACCCCCCACTGG-TAMRA. The PCR profile contained an initial reverse transcription step at 50°C for 30 min and initial denaturation step at 95°C for 15 min followed by 40 cycles of 94°C for 15 seconds (s) and 60°C for 1 min. Data generated was used to determine the viral particles per mL (VP/mL) assuming each lentiviral particle contains 2 copies of viral RNA. This assay was also used to measure vector-specific RNA expression in lung lysates (Section 2.10.8) following transduction. Vector-specific RNA expression was normalised against murine CFTR mRNA levels. Murine CFTR mRNA levels were quantified using the same method as described above using the following set of primers: Forward: AGCCAGCTTTATCTCCAAACTCTTC; Reverse: GCTGTCTGTACCCTTTCCTCAAA; Probe: VIC-TCAGCTGGGAGCACAGC.

2.2.5.1.1.2 Functional titre (TTU/mL)

The functional titre of lentiviral vectors was determined based on the genomic integration of WPRE DNA sequence. HEK 293F suspension cells were transduced with dilutions of purified lentiviral vectors in FreeStyle media in the presence of eight µg/mL of polybrene. Cells were incubated for 72 hours before genomic DNA was extracted using the QIAamp blood DNA kit (Qiagen). The integrated WPRE DNA was quantified against a standard curve of DNA mimics containing the WPRE sequence (gift from Stephanie Sumner-Jones) using the

following primers: Forward: TGGCGTGGTGTGCACTGT; Reverse: CCCGGAAAGGAGCTGACA; Probe: FAM-TTGCTGACGCAACCCCCCACTGG-TAMRA in triplicate using TaqMan FAST Universal Master Mix (Life Technologies), normalised against total ng DNA determined using Nanodrop (Section 2.4.8). The PCR profile used was as following: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Functional transducing units per mL of virus supernatant (TTU/mL) was determined from the slope of a linear regression curve generated from the dilution series using Prism 7 software (GraphPad Software, Inc., CA, USA). Lentiviral production and quantification was performed with the help of Lee Davies and Ian Pringle.

2.2.5.2 Production of recombinant adeno-associated virus vector

The production of rAAV production was performed as described (Silva et al., 2015). Briefly, 1.7E7 HEK 293T cells were seeded in a 175 cm² tissue culture flask (Corning) with D10 supplemented with 4.5 g/L glucose. After 72 hours, or when the cells were 80% confluent, each flask was then used to seed one 1,720 cm² surface area HYPERFlask (Corning); two HYPERFlasks were used for each rAAV preparation. The HEK 293T cells, seeded in HYPERflasks (Corning), were transfected with 500 µg of the genome plasmid pAdDeltaF6 (Table 3.3), and pAAV2/8 (calculation of plasmids ratio described in Table 2.2) using PEI (Section 2.2.4) in DMEM (4.5 g/L glucose, 2mM L-glutamine, 2% FBS, 100 U/mL penicillin and 100 ug/mL streptomycin). After 72 hours, cells were pelleted by centrifugation at 1,400 rcf for 10 min. Cell pellets were resuspended in lysis

buffer (1M Tris(hydroxymethyl)aminomethane, 150mM NaCl) and pre-dissolved EDTA-free protease inhibitor cocktail (Roche, Welwyn Garden City, UK) was added to the manufacturers recommended concentration. Cell pellets were then underwent four freeze-thaw cycles (freeze for 1 hour at -80°C and thaw at 37°C waterbath for 30 min). Benzonase (Merck) was added to the cell lysates for a final concentration of 50 U/mL followed by incubation at 37°C for 30 min. Cell lysates were then centrifuged at 3,700 rcf for 20 min.

Virus was purified by iodixanol gradient fractionation. Iodixanol step-gradient solutions were prepared according to the recipe in Table 2.3 with 60% iodixanol (OptiPrep, Sigma), 5x PBS-MK (5x PBS, 12.5 mM MgCl₂, 12.5 mM KCl), 5M NaCl, Milli-Q water and 0.5% phenol red (Sigma).

The 15%, 25%, 40% and 60% iodixanol solutions (volumes stated in Table 2.3) were layered in order underlaid in 32.4 mL OptiSeal tubes (Beckman) using a Pasteur pipette (Appleton Woods Ltd, Birmingham, UK). In detailed, 7.2 mL of 15% was laid at the bottom of the tube, followed by laying of 4.8 mL of 25% iodixanol fraction below the 15% fraction, 4 mL of 40% below the 25% fraction and 4 mL of 60% below the 40% fraction. The benzonase-treated cell lysate (around 30 mL) was layered on top of the final 15% fraction in three tubes and any remaining volume in the tube filled with lysis buffer. After centrifugation (~500,000 rcf for 1.5 hours) in the Optima XPN-80 Ultracentrifuge (Beckman) with the 70Ti rotor (Beckman), the tube was punctured at the junction between the 25% and 40% iodixanol steps using an 18-gauge (G) needle (Becton Dickinson) and the 40% step was collected. The collected fraction was concentrated and dialysed into PBS using Amicon Ultra-15 100K MWCO centrifugal devices

Table 2.2 Ratio calculation of plasmids for rAAV production

Plasmid	Size(kb)	Amount per transfection (µg)
pAdDeltaF6	15.4	15.4 x R
pAAV2/8	7.3	7.3 x R
pTransgene	T	T x R
Total	K	500µg
Ratio (R) mass/kb	500/K	

rAAV: recombinant adeno-associated virus; pAdDeltaF6: helper plasmid containing the *E2a*, *E4*, and *VARNA* genes from adenovirus; pAAV2/8: plasmid containing the *Rep* gene from AAV serotype 2 and *Cap* gene from AAV serotype 8; pTransgene: plasmid containing transgene flanked by the AAV serotype 2's ITRs

Table 2.3 Preparation of iodixanol step-solutions for four iodixanol gradients

Step	60% iodixanol	5M NaCl	5X PBS-MK	H ₂ O	Phenol red
15%	8 mL	6.4 mL	6.4 mL	11.2 mL	-
25%	10 mL	-	4.8 mL	9.2 mL	40µl
40%	13.4 mL	-	4 mL	2.6 mL	-
60%	20 mL	-	-	-	40µl

NaCl: sodium chloride; PBS-MK: phosphate buffer saline containing sodium chloride, magnesium chloride and potassium chloride

(Merck Millipore, UK). Purified rAAV was aliquoted and stored at -80°C .

2.2.5.2.1 Determination of recombinant adeno-associated virus vector titre

For physical rAAV particle titre, the number of DNase-resistant Genome Copies (DRGC/ ml) was determined by qPCR using the Applied Biosystems 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific). Purified rAAV was diluted 1:10 in Milli-Q water and treated with 2U of DNase I (Life Technologies) in 1x DNase I buffer (Life Technologies) in a final volume of 10 μL for 20 min at 37°C to remove any residual pDNA or unpackaged viral DNA. Samples were then diluted 1:10 with Milli-Q water and incubated at 100°C for 10 min to denature the viral capsids and release the viral DNA. Samples were further diluted 1:10 in Milli-Q water and the viral DNA was quantified in triplicate using Taqman Fast Universal PCR Master Mix (Life Technologies) against a standard curve of DNA mimics containing the WPRE sequence (gift from Stephanie Sumner-Jones). The primers used were as follows: Forward: TGGCGTGGTGTGCACTGT; Reverse: CCCGGAAAGGAGCTGACA; Probe: FAM-TTGCTGACGCAACCCCCACTGG-TAMRA. The qPCR was performed according to the following conditions: 50°C for 2 min followed by 40 cycles of 95°C for 10 s, 95°C for and 60°C for 1 min. Amplification data generated were used to determine the DRGC/ mL.

2.2.5.3 Functional validation of rSIV.F/HN and rAAV

Adherent HEK 293T cells were used to validate the transgene expression directed by rSIV.F/HN and rAAV2/8 vectors produced. Approximately 5E5 HEK 293T were seeded in a 12-well plate (Corning) in 1 mL of D10 and incubated at 37°C for 24 hours. Media was then replaced with 1 mL of fresh D10. The rAAV (1E10 GC) or rSIV.F/HN (1E7 TTU) were added to each well and allowed to infect for 6 days. Supernatants were removed and expression of the transgene (GLux reporter or IgG) was quantified as described in Section 2.8 and 2.9.1, respectively. For wells infected with rAAV2/8.CAS1.EGFP_{Lux}, the presence of EGFP-positive cells was determined 72 hours post-infection using a fluorescence microscope.

2.2.6 *In vitro* assays

2.2.6.1 *Gaussia* Luciferase (GLux) Assay

The activity of *Gaussia* luciferase (GLux) in culture supernatant, serum and broncho-alveolar lavage fluid (BALF) was determined by measuring the light produced by the reaction catalysed by GLux in the presence of coelenterazine (BioLux® *Gaussia* Luciferase Assay Kit, NEB) using the TD20/20 luminometer (Promega). A volume of 50 µL of GLux assay substrate, diluted in GLux assay buffer, was added to each 20 µL of serum (diluted 1 in 10 in TBS based on estimation done previously (Ian Pringle, personal communication) and BALF (assayed neat). After 2 s, light output in arbitrary relative light units (RLU) was measured over 10 s. All samples were read in duplicate.

2.2.6.2 Anti-Human IgG Enzyme-linked Immunosorbent Assay

Anti-Human IgG Enzyme-Linked Immunosorbent Assay (ELISA) was performed using Human IgG ELISA kit (Bethyl Laboratories, Cambridge, UK) according to the manufacturer's instructions. In brief, flat-bottomed 96-well plates (Corning) were coated with goat anti-human IgG-Fc antibody at 10 µg/mL. Plates were washed with TBS wash buffer (50mM Tris-Cl and 150 mM NaCl, 0.05% Tween 20, adjusted to pH 8.0) and blocked with blocking buffer (TBS, 1% BSA) overnight at 4°C. Samples diluted in sample diluent (TBS, 0.1% BSA, 0.05% Tween 20) were added to the plates. Serum was diluted 1:20 or 1:10 (depending on the volume of serum) and BALF samples were analysed without dilution. After the 1-hour incubation, plates were washed with TBS wash buffer, and HRP-conjugated goat anti-human IgG antibody (1: 200, 000) was added and incubated at RT for 1 hour. Plates were then washed with TBS wash buffer and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to the dried wells for 15 min in the dark. The colour reaction was stopped with the addition of 0.18 M H₂SO₄ stop solution. Plates were read at 450 nm (OD₄₅₀) using SpectraMAX 250 spectrophotometer plate reader (Molecular Devices, Wokingham, UK). The concentration of human IgG in the samples were determined using a standard curve generated using Human Reference Serum (Bethyl) (range from 7.5 to 500ng/mL).

2.2.6.3 Anti-Influenza Haemagglutinin Enzyme-linked Immunosorbent Assay

Anti-influenza haemagglutinin ELISA was performed as described previously (Powell et al., 2012a) with slight modifications. Flat-bottomed 96-well plates

(Corning) were coated with the H5 antigen (A/Vietnam/1203/2004) at 2 µg/mL in 1x PBS containing 0.02% sodium azide (PBS/azide) and blocked with 5% skimmed milk overnight. Alternatively, flat-bottom 96-well plates (Corning) were coated with 3E4 MDCK cells expressing HA in DMEM and incubated overnight at 37°C, 5% CO₂ (details of HA expressing MDCK cells used are described in Table 2.4). Samples were added to the plates at various dilutions, starting from 1:2. After the 1-hour incubation, plates were washed with PBS/azide and blocked with PBS containing 0.1% bovine serum albumin (BSA) (1x PBS/BSA), and horseradish peroxidase (HRP)-conjugated Rabbit Anti-human IgG antibody (Dako, Ely, UK) was then added at a 1:1,600 dilution in PBS. For the experiment in Chapter 6 to compare binding activity of IgG and IgA, HRP-conjugated Anti-Human Kappa Light Chain (Abcam) or HRP-conjugated Anti-Human Lambda Light Chain secondary antibodies (Abcam) was added at a 1:2,000 dilution in PBS/BSA. Plates were then washed with PBS, and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to the dried wells for 5 min in the dark. The colour reaction was stopped with the addition of 1M H₂SO₄ stop solution. Plates were read at 450 nm (OD₄₅₀) using ClarioStar plate reader (BMG Labtech, Ortenberg, Germany). Titres were calculated as fold-dilution of samples that resulted in 50% binding to the plateau positive signal (EC₅₀).

2.2.6.4 Microneutralisation assay

Microneutralisation (MN) assays were performed using recombinant influenza viruses expressing EGFP (S-eGFP)(Fang et al., 2005b, Powell et al., 2012a), or using wild type or reassortant influenza virus (gift from Alain Townsend) (see Table 2.4 for details of S-Flu, wild type and reassortant influenza strains) and

MDCK-SIAT1 cells (Section 2.2.3). On a flat-bottom 96-well tissue culture dish (Corning), supernatant or purified antibody were serially diluted 1:2, with PBS. Between 2 and 4 haemagglutination units (HAU) of influenza virus (determined previously by Alain Townsend) diluted in virus growth media (VGM) (DMEM/ 0.1% BSA/ 10 mM HEPES (Life Technologies)) was then added to each well. The mixtures of diluted supernatant and virus were incubated for 2 hours (5% CO₂, 37°C). After the incubation, 3E4 MDCK-SIAT1 cells in VGM were added to each well. A virus-only control and a cells-only control were included for each plate. Plates were incubated for 24 hours (5% CO₂, 37°C). Post-incubation, 10% formalin was added to each well and plates were incubated at 4°C for 30 min. For MN assays using S-eGFP, formalin was removed and plate was washed 3 times with 200 µL PBS. Wells were then filled with 100 µL and GFP intensity was quantified using a ClarioStar plate reader. For MN assays using wild type influenza virus, 50 µL permeabilisation buffer (1x PBS/ 20 mM glycine, 0.5% Triton) was added to the wells for 20 min at RT. The plate was then washed with PBS and mouse anti-influenza A nucleoprotein (NP) antibody (0.3 µg/ mL) (Abcam, Cambridge, UK) was added. After incubation for 1 hour at RT, plates were washed 3 times with 200 µL PBS and goat anti-mouse IgG HRP conjugated antibody (Dako) was added at 1:800 dilution and incubated for 1 hour at RT. Plates were then washed 3 times with 200 µL, and 50 µL TMB substrate was added to the wells for 5 min in the dark. The colour reaction was stopped with the addition of 50 µL of 1M H₂SO₄ (VWR) stop solution. Plates were read at 450 nm (OD₄₅₀) using a ClarioStar plate reader (BMG Labtech) and titres reported as fold-dilution of samples that resulted in 50% reduction of the GFP signal (Figure 2.1) or NP expression (EC₅₀).

2.2.6.5 Urea assay

Urea Assay kit (Abcam) was used to determine the urea concentration in the serum and matched BALF samples to determine the dilution of BALF. The assay was performed according to the manufacturer's instructions. Murine serum (diluted 1:150) and BALF samples (diluted 1:10) were analysed on a flat-bottom 96-well plate (Corning). A reaction mix consisting of OxiRed probe, enzyme mix, developer and converter enzyme was added to each well. Plate was then incubated at 37°C in dark. Plates were read at 570 nm (OD₅₇₀) using SpectraMax i3x Multi-Mode Detection Platform (Molecular Devices, Wokingham, UK). The concentration of urea in the samples was determined using a standard curve generated using the urea standard supplied in the kit (Abcam) (range from 0 to 5 nM/well urea). The fold-difference of the urea concentration in the BALF and its matched serum sample was used to determine the fold dilution of the BALF sample.

2.2.7 Production of IgG1 and monomeric IgA1

IgG1 and monomeric IgA1 were produced by transient transfection of suspension cultured HEK 293T cells (Section 2.2.1) with plasmids expressing the IgG1 or IgA1 heavy and light chains (Tables 2.3 and 2.4) at an equal ratio at 0.33 µg pDNA per 10⁶ cells using PEIpro (PolyPlus) at a volume:mass ratio of 2:1. Production of single-ORF IgG1 was performed using AAV genome plasmids expressing the indicated bnAb (Table 3.3). Sodium butyrate (5mM) and 150 mL of FreeStyle media were added 24 hours post-transfection. At 6 days post-transfection, the suspension cells were centrifuged at 1,400 rcf for 5

min and the supernatant was filtered through 0.45 µm filter (Pall) and the filtrate stored at 4°C.

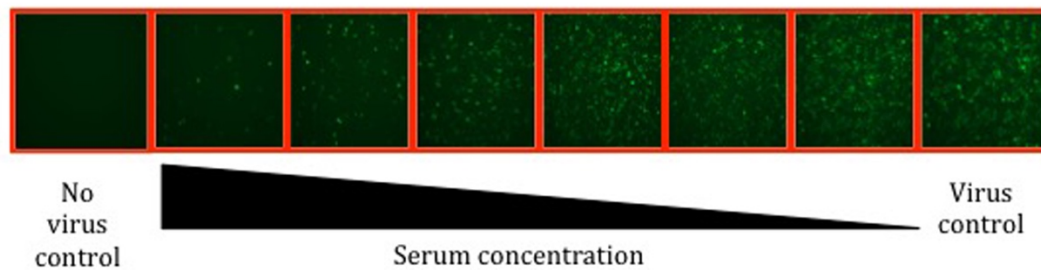
2.2.7.1 Purification of IgG1 and monomeric IgA1

For IgG1 purification, protein A-Sepharose (Sigma) was allowed to swell in 1 mL Tris-buffered saline (TBS) (50mM Tris-Cl, 150 mM NaCl, adjusted to pH 8.6) in a washed column (Bio-Rad). Supernatant collected above was run through the column and the column was washed with 50 to 100 mL of TBS buffer. The IgG1 was eluted with 1 mL of citrate buffer (0.1M sodium citrate, 0.1M citric acid) and filtrate was collected into several fraction tubes containing 300 µL Tris-Cl buffer. For monomeric IgA1 purification, 1mL of peptide M/agarose (Invivogen, CA, USA) was packed into a wash column (Bio-Rad) and equilibrated with 10mL of TBS buffer (pH 7.2). Supernatant was allowed to run through the column, which was then washed with TBS buffer (pH 7.2). Monomeric IgA1 was eluted using 1mL glycine buffer (0.2 M glycine (Sigma), pH 2.7) into several fraction tubes containing 20 µL 2M Tris-Cl (pH 10.0). The protein concentration in the collected fraction tubes was measured at OD₂₈₀, fractions containing a high protein concentration were pooled, concentrated and buffer exchanged into PBS (final volume around 250 to 500 µL) using Amicon Ultra-15 30K MWCO (Merck Millipore) and stored at 4°C. The concentration of IgG was calculated using the mass extinction coefficient of 1.37 at OD₂₈₀ for a 0.1% (1 mg/ mL) IgG1 solution. The concentration of monomeric IgA1 was calculated in a similar way with the mass extinction coefficient of 1.32.

2.2.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One-dimensional SDS-PAGE was performed using precast 4-12% gradient Bolt Bis-Tris Plus acrylamide gels (Life Technologies) according to the manufacturer's protocol. For IgG and IgA studies, affinity purified antibodies were resolved under reducing and non-reducing conditions. Antibody (2.5 µg) was added to Laemmli buffer (Laemmli, 1970) pre-mixed with 2-betamercaptoethanol (5%) (reducing condition) or Laemmli buffer without any reducing agent (non-reducing condition). Samples were heated at 95°C for 8 min before loading onto the gel. Following electrophoresis (200 V), gels were stained for 1 hour with 50mL Quick Coomassie Blue (Generon, Maidenhead, UK) followed by two de-staining washes (30 min each, 50 mL) using Milli-Q water according to the manufacturer's instructions. To determine the purity of rAAV preparations, 10^{10} DRGC of rAAV vector was diluted in 9 µL of 5x Protein Loading Buffer (National Diagnostics) in a total volume of 36 µL and incubated at 95°C for 10 min. Incubated samples were resolved on a pre-cast 4-12% Bolt Bis-Tris Plus acrylamide gel (Life Technologies) as described above. The purity of rAAV preparations was determined as a relative percentage of the viral capsid proteins (VP1, VP2 and VP3) to other protein contaminants using ImageJ software (Schindelin et al., 2015).

(A)



(B)

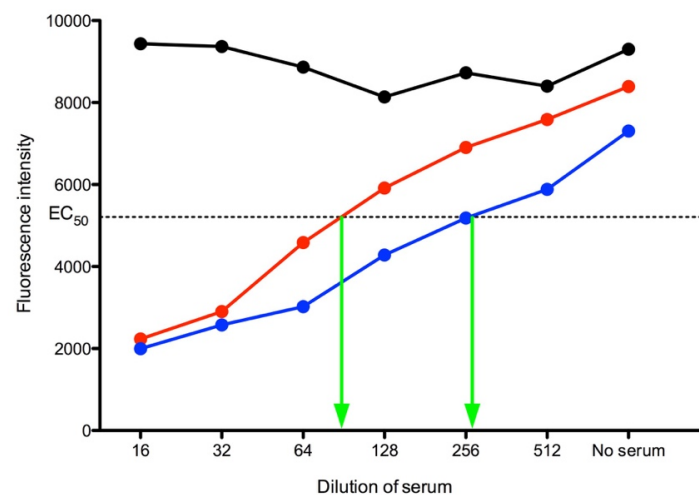


Figure 2.1 Determination of EC₅₀ values from *in vitro* microneutralisation

(MN) assay. (A) Microscopy images of the *in vitro* microneutralisation assay in which MDCK-SIAT1 cells are infected with pseudotyped-influenza virus carrying EGFP in the HA segment expressing HA or ZEBOV glycoprotein (viewed at magnification of 100x) (Section 2.2.2). When performed in the presence of titration of sera with neutralising activity, EGFP expression is reduced in proportion to the decline in infected cells. B) Sample data from three samples demonstrating no neutralising activity (black), medium (blue) and high neutralising activity (red). X-axis represents the fold-dilution of serum. Data from each sample are fit, using a nonlinear function and the fold-dilution, to result in 50% of GFP signal reduction to determine the EC₅₀ value (green arrow)

Table 2.4 Details of MDCK-HA, wild type influenza, reassortant influenza and S-eGFP used in this study

Origin of HA/EBOV GP glycoprotein	Origin of NA glycoprotein	MDCK-HA	S-eGFP/wild type influenza/reassortant influenza	Source
A/PuertoRico/8/1934 (H1)	A/PuertoRico/8/1934 (N1)	MDCK-H1(PR8)	S-eGFP/H1/PR8	Alain Townsend
			A/PR/8/1934 (Cambridge)	
A/California/7/2009 (H1)	A/California/7/2009 (N1)	MDCK-H1 (California)	S-eGFP/H1/California	Alain Townsend
			A/California/7/2009 (X179A) (5:3 reassortant of A/PR/8/1934 with the HA, NA and PB1 segments of A/California/7/2009 (H1N1))	
A/Belgium/4G0500/2014 (H1)	A/California/7/2009 (N1)	-	S-eGFP/H1/Belgium	Alain Townsend
A/Victoria/361/2011 (H3)	A/Victoria/361/2011 (N2)	MDCK-H3 (Victoria)	S-eGFP/H3/Victoria	Alain Townsend
	A/Victoria/361/2011 (N2)		A/Victoria/361/2011 (X217) (6:2 reassortant of A/PR/8/1934 with the HA and NA segments of A/Victoria/361/2011 (H3N2))	

A/Switzerland/97515293/2 013 (H3)	A/Victoria/361/2011 (N2)	-	S-eGFP/H3/SW	Alain Townsend
A/HongKong/5738/2014 (H3)	A/Victoria/361/2011 (N2)	-	S-eGFP/H3/HK	Alain Townsend
A/Vietnam/1203/2004 (H5)	A/PuertoRico/8/1934 (N1)	MDCK-H5 (Vietnam)	S-eGFP/H5/Vietnam	Alain Townsend
A/Anhui/1/2013 (H7N9)	A/PuertoRico/8/1934 (N1)	-	S-eGFP/H7/Anhui	Alain Townsend
A/Shanghai/1/2013 (H7)	A/PuertoRico/8/1934 (N1)	MDCK-H7 (Shanghai)	S-eGFP/H7/Shanghai	Alain Townsend
A/Netherlands/219/2003 (H7)	A/PuertoRico/8/1934 (N1)	-	S-eGFP/H7/ Netherlands	Alain Townsend
A/NewYork/107/2003 (H7)	A/PuertoRico/8/1934 (N1)	-	S-eGFP/H7/NY	Alain Townsend

HA: haemagglutinin; NA: neuraminidase; MDCK-HA; Madin-Darby canine kidney epithelial cells stably expressing influenza haemagglutinin; S-eGFP: recombinant influenza virus with EGFP gene flanking by the 3' and 5' untranslated region of the original influenza haemagglutinin (HA) genome; reassortant influenza virus: replication competent influenza virus made from reassorting A/PR/8 genome with the HA and NA from desired strains.

2.2.9 Animal studies

All procedures involving laboratory mice were carried out at the Biomedical Services Unit (BMS) (University of Oxford, John Radcliffe Hospital, Oxford, UK). The author, or other licensed members of the Gene Medicine or Molecular Immunology groups supervised or assisted the author in performing procedures. All procedures were carried out under UK Home Office approved project and personal licenses for performing experiments on animals under the terms of the Animals (Scientific Procedures) Act 1986 (ASPA 1986). Mice were housed in accordance with the UK Home Office ethical and welfare guidelines and fed on standard chow and water *ad libitum*.

2.2.9.1 Mouse strains

Female BALB/c mice aged 5 to 7 weeks at point of procedure were purchased from the BMS or Envigo RMS UK (Loughborough, UK), and were allowed to acclimatise for at least 7 days prior to procedures being performed.

2.2.9.2 Mouse anaesthesia

For delivery to the lung via intranasal instillation (Section 2.10.3) and intramuscular injection (Section 2.10.4), mice were anaesthetised using inhalation of Isoflurane (University of Oxford Vet Services, Oxford, UK) until a balanced state of anaesthesia was achieved, as determined by pedal withdrawal, tail pinch and depth of breathing.

Table 2.5 Details of IgG1 and monomeric IgA1 produced in this study

Antibody name	VH gene usage	Antibody class	HA/ EBOV GP specificity	Source
T1-3B	VH1-69	IgG1	HA stem cross-reactive H1 & H5	Alain Townsend
		IgA1		This study
21-D8-5A	VH4-39	IgG1	HA head cross-reactive H1 & H5	Alain Townsend
		IgA1		This study
Q11-C11	VH4-61	IgG1	HA head specific H3 (A/Victoria/361/2011)	Alain Townsend
		IgA1		This study
T2-6A	VH3-7	IgG1	HA head specific H1 (A/California/7/2009)	Alain Townsend
		IgA1		This study
MEDI8852	VH6-1	IgG1	Stem cross-reactive H1, H2, H3, H5 & H7 (pan-influenza)	This study
		IgA1		This study
Z3-B2	VH3-53	IgG1	HA stem cross-reactive H1, H3 and H7	Alain Townsend
		IgA1		This study
T3-4B	VH3-15	IgG1	HA head cross-reactive H1	Alain Townsend
		IgA1		This study
T3-5D	VH3-23	IgG1	HA head cross-reactive H1, H3 & H5	Alain Townsend
		IgA1		This study
T2-5D	VH3-15	IgG1	HA head cross-reactive H1	Alain Townsend
		IgA1		This study
T2-5A	VH1-69	IgG1	HA stem cross-reactive H1 & H5	Alain Townsend
		IgA1		This study
2-12C	VH5-51	IgG1	HA head specific H1 (A/California/7/2009)	Alain Townsend
		IgA1		This study

VH: variable region of the antibody heavy chain; HA: haemagglutinin

2.2.9.3 Delivery to the mouse lung via intranasal instillation

A volume of 50 μ L or 100 μ L of rLV vector (diluted in FreeStyle medium) was delivered to the lung via intranasal instillation. In brief, the anaesthetised mouse was held vertically with mouth closed. A single, continuous droplet was maintained during topical application of the 50 μ L or 100 μ L virus to the nose (using a Gilson P200 pipette (Gilson Inc., Middleton, W1, USA), with the liquid being drawn into the nasal cavity and lungs as the mouse inhaled for breathing. For influenza challenge study, wild type influenza virus (H1N1 A/PR/8/1934) (gifts from Alain Townsend) diluted in 50 μ L VGM was delivered via intranasal instillation in an identical manner.

2.2.9.4 Delivery to the mouse muscle via injection

A volume of 40 μ L of rAAV vector (in 1x PBS) was delivered to the muscle via injection. In brief, fur on one of the thighs of a mouse was removed and the mouse was anaesthetised (Section 2.9.2). The thigh was cleaned with 70% ethanol and an rAAV containing 30-gauge (30G) needle (B.Braun Medical Ltd, Sheffield, UK) was inserted at 90° and the content was injected into the gastrocnemius muscle.

2.2.9.5 Delivery to the mouse peritoneal cavity via injection

A volume of 500 μ L of affinity purified antibody (diluted in 1x PBS) was delivered to the peritoneal cavity via injection. Mouse was restrained using the scruff method, the ventral side of the mouse was exposed and the head was tilted down at a slight angle. The abdomen of the mouse was then cleaned with 70% ethanol. The needle, with bevel facing up was inserted at 30° into the lower right quadrant of the mouse abdomen.

2.2.9.6 Sampling of bronchoalveolar lavage fluid (BALF)

Mice were euthanised by cervical dislocation prior to BALF sampling. The trachea was exposed by blunt dissection. A small tear was made in the trachea and a cannula (0.75 mm, Harvard Apparatus, Kent, UK), attached to a 21G (Becton Dickinson), was inserted into the trachea and advanced until the end was immediately above the carina. A 1 mL syringe, attached to the needle, was used to first infuse the lung with BALF solution (PBS, 1 mg/mL BSA, 50 µmol/L EDTA) and then collect the infused fluid by gentle aspiration. This was repeated twice; the collected fluid (3 x 1 mL for GLux studies or 1 x 1 mL for bnAb studies) was pooled for each mouse and stored at -80°C until further processing.

2.2.9.7 Sampling of serum

Mice were restrained throughout the procedure by placing the mouse in a mouse restrainer exposing its tail. A small nick was made on the vein at the distal part of the tail using a 21G needle (Becton Dickinson). Blood (not more than 15% of total blood volume over 28 days) was collected using Microvette[®] CB 300 tube (Starstedt). The collected blood was then allowed to clot at 4°C overnight before centrifugation for 10 min at 10,000 rcf. Serum was transferred into a separate Eppendorf tube. Serum was stored at -80°C until further analysis.

2.2.9.8 Harvesting of lung tissue and preparation of lung lysate.

Mice were euthanised by cervical dislocation and the lungs were dissected and removed. The tissue was rinsed in 10 mL PBS until most of blood on the surface of the lung was removed, then immediately immersed in 300 µL 1x Reporter Lysis Buffer

(RLB) (Promega) and placed on ice. Lungs were homogenised using FastPrep™ Lysing Matrix D tube (MP Biomedicals, Santa Ana, California, USA) and processed using the FastPrep Instrument (MP Biomedicals) for 45 s at a speed setting of 4.0 m/s. The homogenates were frozen at -80°C for at least 15 min. Upon thawing to RT, the homogenates were mixed for 10 s and transferred to QIAshredder columns (Qiagen) and centrifuged at 9,300 rcf for 2 min. The lysates were frozen at -80°C for at least 30 min, then thawed to RT and centrifuged for 10 min at 9,300 rcf. The supernatant was transferred to a clean microcentrifuge tube and stored at -80°C for later analysis.

2.2.10 Statistical analyses.

Statistically significant difference between groups was determined by parametric tests (unpaired t-test and one-way analysis of variance (ANOVA)) or non-parametric tests (Mann-Whitney U test and Kruskal-Wallis H test) as appropriate using Prism 7 (GraphPad, USA) followed when appropriate by post-hoc tests using Dunn's corrections. Whenever appropriate, data were log-transformed prior to statistical analysis to assure normality of data and equivalence of variance. A calculated p value of > 0.05 was deemed non-significant (indicated with 'ns'). Calculated p values of < 0.05, <0.01 or < 0.001 were deemed significant difference (indicated with single (*), double (**), or triple asterisks (***), respectively). Error bars in figures represent the standard error of the mean (SEM).

Chapter 3 Generation of rSIV.F/HN and rAAV vectors for passive immunisation against influenza infection.

3.1 Introduction

3.1.1 Selection of recombinant lentivirus and recombinant adeno-associated virus vectors

Both rLV and rAAV vectors were considered for delivery of novel bnAbs. Lentiviral vectors have been widely used in non-clinical and clinical gene therapy (Section 1.5) and several have been pseudotyped for efficient airway delivery (Section 1.5.5). The SIV-based lentiviral vector pseudotyped with Sendai virus's fusion (F) and haemagglutinin-neuraminidase (HN) surface glycoproteins (section 1.5.5) has been shown to express reporter transgene in the lung for the lifetime of a mouse after IN delivery (Griesenbach et al., 2012b), without the need for the addition of tight-junction openers. Furthermore, a regulator-compliant version of this vector (rSIV.F/HN) has been shown to efficiently transduce (approximately 15%) mouse lung cells (Alton et al., 2017). On the basis of these characteristics, rSIV.F/HN was selected as the preferred rLV vector to evaluate for passive immunisation against influenza. This rLV is third generation (Section 1.5.3; Figure 1.6) with a good safety profile (Dull et al., 1998). The elements for rSIV.F/HN construction are distributed amongst five plasmids: (i) the genome plasmid, which includes the antibody or reporter protein transgene flanked by the SIV LTRs; (ii-iii) two packaging plasmids providing SIV *gag*, *pol* and *rev* specific to the vector-type; and (iv-v) two packaging plasmids supplying the Sendai F and HN pseudotyping proteins (Table 2.1).

Many AAV serotypes are available (Section 1.4.2); for this study rAAV2/8 vector was selected as it has been shown to direct protective levels of bnAb expression against influenza in mice following a single IM injection (Balazs et al., 2013). The elements for rAAV2/8 are distributed between three plasmids: (i) the genome plasmid which contains the antibody or reporter protein transgenes flanked by the AAV ITRs; (ii) a packaging plasmid providing AAV2 Rep and AAV8 Cap; and (iii) a helper plasmid providing Adenovirus *E2A*, *E4* and *VA RNAs* gene products that are needed in AAV genome replication (Samulski and Shenk, 1988, Carter, 1992, Ward et al., 1998) (Table 2.1). For details of plasmids used in this study see Table 2.1.

Production of both rSIV.F/HN and rAAV2/8 is initiated by transient co-transfection of their constituent plasmids, five for rSIV.F/HN and three for rAAV2/8 (Table 2.1) into a mammalian producer cell line (HEK 293T cells) (Section 2.2.5.1 & Section 2.2.5.2 respectively).

3.1.2 Selection of anti-influenza broadly neutralising antibodies

Three anti-influenza antibodies (F10, T1-3B and 21-D8-5A) were selected for this study (Section 1.2.2.2). The F10 antibody has been shown to provide protection against multiple influenza strains in mice mediated by rAAV2/8 following IM delivery (Balazs et al., 2013). Antibody T1-3B was selected as it targets the conserved stem region of the HA to neutralise multiple influenza strains and has shown protection in mice against influenza challenge when given as purified antibody prophylactically (Alain Townsend, personal communication). Antibody 21-D8-5A is a HA head-targeting antibody which is less cross-reactive than T1-3B, but is more potent (lower EC₅₀) than T1-3B and has shown protection in mice when administered as purified

antibody post-influenza challenge (Alain Townsend, personal communication). Characteristics of these antibodies are summarised in Table 3.1.

3.1.3 Aim

This chapter describes the construction of rLV and rAAV vectors expressing reporter protein, or anti-influenza single-ORF antibody transgenes, together with the confirmation of their function in cell culture.

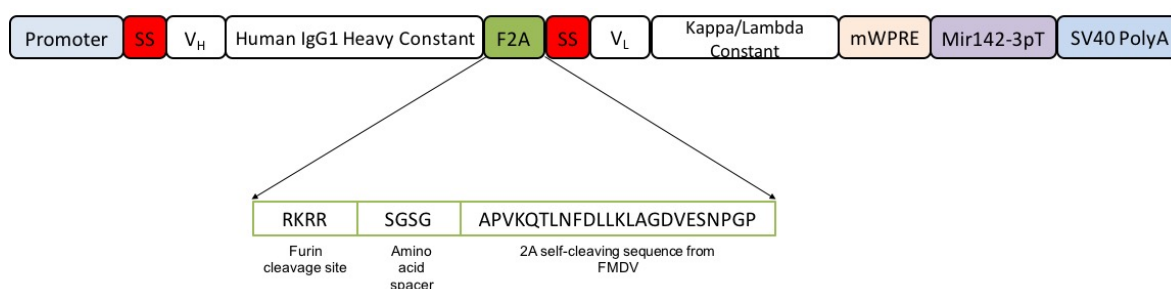


Figure 3.1 Schematic representation of the genome plasmid cDNA configuration. The diagram illustrates the genome plasmid configuration used in all rAAV and rLV vectors expressing antibodies from single-ORF described in this study. Antibody heavy and light chains, consisting of V_H (heavy variable region) and V_L (light variable region), and either a *kappa* or *lambda* constant region, are fused by F2A (furin/2A; green). Each chain is prefixed with a SS (signal sequence; red), which is required for translocation and secretion of the antibody. The components of the F2A cleavage site are shown: foot-and-mouth disease virus (FMDV) 2A self-processing sequence adjacent to a 4-amino acid spacer and a furin cleavage site. Modified WPRE (mWPRE; peach) was included to enhance transgene expression. A mir-142-3p target sequence (Mir142-3pT; purple) was included to reduce transgene directed immunogenicity. SV40 PolyA (SV40 PolyA; light blue) was included as transcription terminators. The genome plasmid configuration is discussed in detail in Section 3.2.2.

Table 3.1 Characteristics of broadly neutralising influenza haemagglutinin-specific monoclonal antibodies used in this study

Antibody	Haemagglutinin interaction site	VH gene usage	Extent of subtype reactivity	Reference
T1-3B	Stem cross-reactive	VH1-69	pH1, H1, H5	Alain Townsend (personal communication); (Huang et al., 2015a)
21-D8-5A	Head cross-reactive	VH3-15	H1, H5	Alain Townsend (personal communication)
F10	Stem cross-reactive	VH1-69	pH1, H1, H2, H5	(Sui et al., 2009)

pH1: haemagglutinin from 2009 pandemic H1N1 influenza (A/California/7/2009); VH: gene encoding the variable region of the antibody heavy chain

3.2 Results

3.2.1 Design of a single-Open Reading Frame (ORF) transgene for antibody expression from vector genome plasmids

Recombinant AAV vector genomes have stringent length limitations (Hermonat et al., 1997) resulting in restrictions on packaging capacity. To overcome this challenge, antibody transgenes can be synthesised as small, single-ORF cDNAs, which can be accommodated by the limited packaging capacity of AAV (Section 1.3; Figure 3.1). The single-ORF cDNA comprises antibody heavy and light chain sequences fused by the F2A sequence, consisting of a cellular protease furin adjacent to a 2A self-cleaving sequence from the foot-and-mouth disease virus (FMDV) (F2A) (Fang et al., 2007).

The single-ORF strategy was used to design cDNAs expressing the selected antibodies (F10, T1-3B and 21-D8-5A; Table 3.1) for both rAAV and rSIV vectors used in this study. A 4-amino acid peptide linker (SGSG), the inclusion of which is found to increase transgene expression, was incorporated between the furin and 2A sequences in all single-ORF antibody constructs used in this study (Yang et al., 2008, Fuchs et al., 2016) (Figure 3.1). Codon-optimised, single-ORF constructs for the T1-3B and 21-D8-5A antibodies isolated from vaccinated donors (Alain Townsend, personal communication), were designed from the original heavy and light chain sequences (Appendix I). Both heavy and light chain antibody sequences were prefixed with human growth hormone (HGH) signal sequences (Balazs et al., 2013). The DNA sequence of the two HGH signal sequences in the single-ORF cDNAs were differentially constrained, whilst maintaining identical protein coding, to minimise the possibility of deleterious homologous recombination (Balazs et al., 2013). The single-

ORF antibody cDNA constructs were designed using MacVector software and synthesised by GeneArt (LifeTechnologies) (Section 2.1.3).

3.2.2 Construction of rSIV and rAAV genome plasmids

Once the single-ORF cDNAs had been synthesised, they were sub-cloned into genome plasmids of both rAAV and rSIV (Section 2.2.3.2, 2.2.3.3, and 2.2.3.4), which were then transformed, amplified and purified (Section 2.2.3.5, 2.2.3.6 and 2.2.3.7) and confirmed using restriction enzymes *NheI* and *PstI* (Section 2.2.3.2). Transgenes in the AAV genome plasmids were driven by the CASI promoter (a combination of the CMV enhancer and chicken β -actin promoter and novel intron containing elements of the ubiquitin C enhancer/promoter; (Balazs et al., 2013)). The CASI promoter was selected as it was previously shown to direct high and persistent transgene expression in the murine muscle (Balazs et al., 2013). Three AAV2 genome plasmids were generated: pAAV2.CASI.T13B, pAAV2.CASI.21D85A and pAAV2.CASI.F10 (Table 3.3). For rSIV genome plasmids, transgenes were driven by either the widely used CMV promoter, or the hCEF promoter (a combination of human CMV enhancer and human elongation factor 1 alpha promoter (Hyde et al., 2008)). The hCEF promoter was selected based on its ability to drive high and persistent transgene expression in ALI cultures and the murine lung compared to other promoters such as CMV and CAG (Alton et al., 2017). Altogether, six SIV genome plasmids were constructed, containing single-ORF cDNAs for each of the three antibodies: pSIV.hCEF.T13B, pSIV.hCEF.21D85A, pSIV.hCEF.F10, pSIV.CMV.T13B, pSIV.CMV.21D85A and pSIV.CMV.F10 (Tables 3.3) as described in the methods in Section 2.2.3. A micro RNA (mir142-3p) target sequence was included in all genome plasmids to reduce transgene-directed immunogenicity (Brown et al., 2006). Genome

plasmids were confirmed by digestion with restriction enzymes *NheI* and *PstI*. Using a similar approach, rSIV and rAAV genome plasmids, expressing the GLux and EGFP-Lux reporter transgenes were also constructed (Table 3.2).

3.2.3 Functional validation of rSIV and rAAV genome plasmids

To confirm expression of functional reporter proteins and antibodies from the genome plasmids, HEK 293T cells were transfected with each of the individual rSIV or rAAV genome plasmids (Table 3.2) as described in Section 2.2.4. For pAAV2.CAS1.EGFP-Lux, the presence of EGFP-positive cells was observed under the fluorescence microscope at 72 hours post-transfection (Section 2.2.2); EGFP-positive cells were observed in cells transfected with pAAV2.CAS1.EGFP-Lux but not in the non-transfected control (Figure 3.2). For pAAV2.CAS1.soGLux, the presence of GLux activity in the culture supernatant at 72 hours post-transfection was compared (Section 2.2.6.1) with the non-transfected control (Figure 3.3) and confirmed the function of pAAV2.CAS1.soGLux in cell culture. The ability of pSIV.hCEF.soGLux to express GLux was previously confirmed (Lee Davies, personal communication).

For genome plasmids expressing T1-3B, 21-D8-5A or F10 antibodies, cell culture supernatant was collected 72 hours post-transfection and anti-HA ELISA (Section 2.2.6.3) and microneutralisation assays (MN) (Section 2.2.6.4) were performed to determine the functional titre of the expressed antibody in the supernatant. Figure 3.4 shows that all genome plasmids can express functional antibody in terms of HA-binding and neutralising influenza infection in cell culture. Purified H5 protein and recombinant H5 influenza (S-eGFP/H5/Vietnam) (Table 2.4) were used in the assays

because all three antibodies tested were known to bind and neutralise this influenza strain (Alain Townsend, personal communication).

3.2.4 Functional assessment of antibodies expressed as single-ORF and dual-ORF configurations

Before proceeding, it was important to investigate whether the single-ORF antibody expression approach, including the F2A cleavage site, had affected the function of the expressed antibodies. Therefore, the binding and neutralising titre of the affinity-purified antibodies (Section 2.2.6.3 and 2.2.6.4) expressed from the single-ORF constructs were compared with equivalent antibodies produced from two separate ORFs (namely the dual-ORF approach). Single-ORF T1-3B and 21-D8-5A mAb are produced by transfecting pAAV2.CASI.T13B and pAAV2.CASI.21D85A respectively into suspension HEK 293T cells followed by purification using Sepharose A columns (Section 2.2.7.1). For their dual-ORF equivalents, plasmids encoding the heavy and light chains (pCMV.T13B.GH and pCMV.T13B.k or pCMV.21D85A.GH and pCMV.21D85A.l) (Table 2.1; gifts from Alain Townsend) were used to transfect suspension HEK 293T cells and purified using the same methods as above (Section 2.2.7.1). No difference was observed between T1-3B or 21-D8-5A antibodies expressed from the single-ORF and their respective dual-ORF equivalents visualised by SDS-PAGE (Figure 3.5). This suggests that the single-ORF constructs can successfully assemble a full IgG1 molecule that is similar to the dual-ORF equivalent.

Inspection of the ELISA results (Figure 3.6), shows that for T1-3B, there was no difference in antibody produced from the single-ORF cDNA compared with the dual-

Table 3.2 Control SIV and AAV genome plasmids constructed

Plasmid name	rSIV/rAAV	Transgene promoter	Transgene
pSIV.hCEF.soGLux (pGM358)	rSIV	hCEF	sogLux
pAAV2.CASl.soGLux (pGM453)	rAAV	CASl	sogLux
rAAV2.CASl.EGFP Lux (pGM452)	rAAV	CASl	EGFP Lux

Table 3.3 SIV and AAV genome plasmids constructed

Plasmid name	rSIV/rAAV	Transgene promoter	Transgene
pSIV.hCEF.T13B (pGM443)	SIV	hCEF	T1-3B
pSIV.CMV.T13B (pGM444)	SIV	CMV	T1-3B
pSIV.hCEF.21D85A (pGM445)	SIV	hCEF	21-D8-5A
pSIV.CMV.21D85A (pGM446)	SIV	CMV	21-D8-5A
pSIV.hCEF.F10 (pGM447)	SIV	hCEF	F10
pSIV.CMV.T13B (pGM448)	SIV	CMV	T1-3B
pAAV2.CASl.F10 (pGM449)	AAV	CASl	F10
pAAV2.CASl.T13B (pGM450)	AAV	CASl	T1-3B
pAAV2.CASl.21D85A (pGM451)	AAV	CASl	21-D8-5A

AAV: adeno-associated virus; hCEF: a combination of human CMV enhancer and human elongation factor 1 alpha promoter; CMV: cytomegalovirus enhancer/promoter; CASl; a combination of the CMV enhancer and chicken β -actin promoter and novel intron containing elements of the ubiquitin C enhancer/promoter; SIV: simian immunodeficiency virus; soGLux: codon-optimised CpG-depleted *Gaussia* luciferase. EGFP Lux: enhanced green fluorescence protein fused to firefly luciferase at its N-terminal

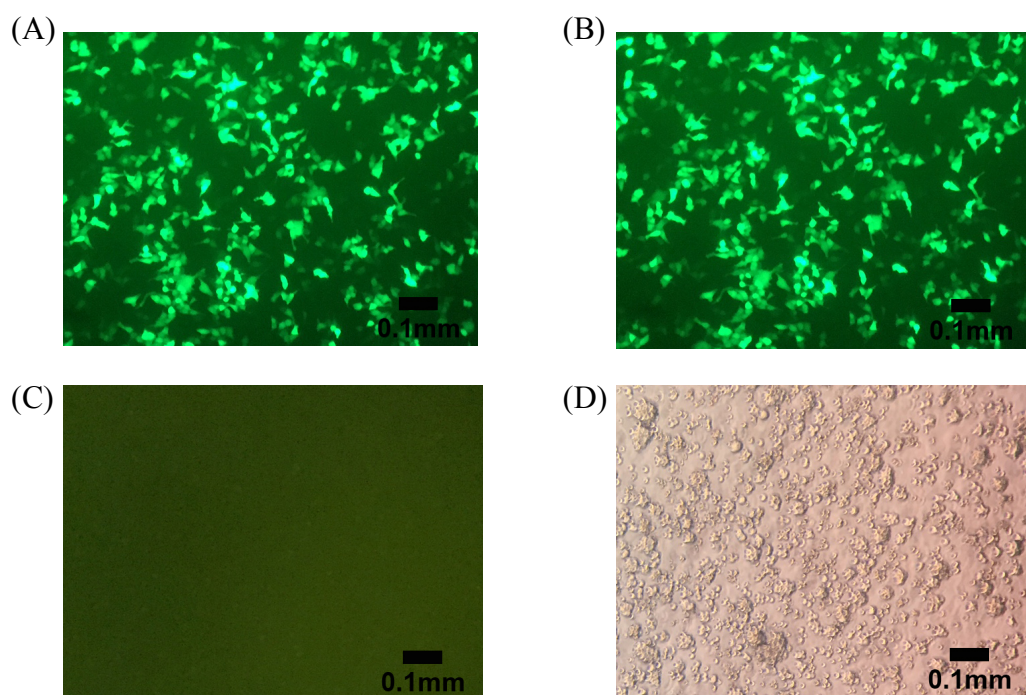


Figure 3.2 EGFP-positive cells following transfection of pAAV2.CAS1.EGFP Lux plasmid.

Expression of EGFP in HEK 293T cells was observed 48 hours after transfection of plasmid pAAV2.CAS1.EGFP Lux (pGM452; Table 3.2) (1E6 cells/mL in 2 mL FreeStyle media, 3 μ g plasmid and 6 μ g of 25 kDa branched PEI; n=3; Section 2.2.4) using a fluorescence microscope (viewed at 100x magnification) (Section 2.2.2). (A) and (B) show two of the triplicate wells of cells transfected with pAAV2.CAS1.EGFP Lux containing EGFP-positive cells. Non-transfected cells viewed under (C) fluorescence and (D) bright field microscopes show no EGFP-positive cells.

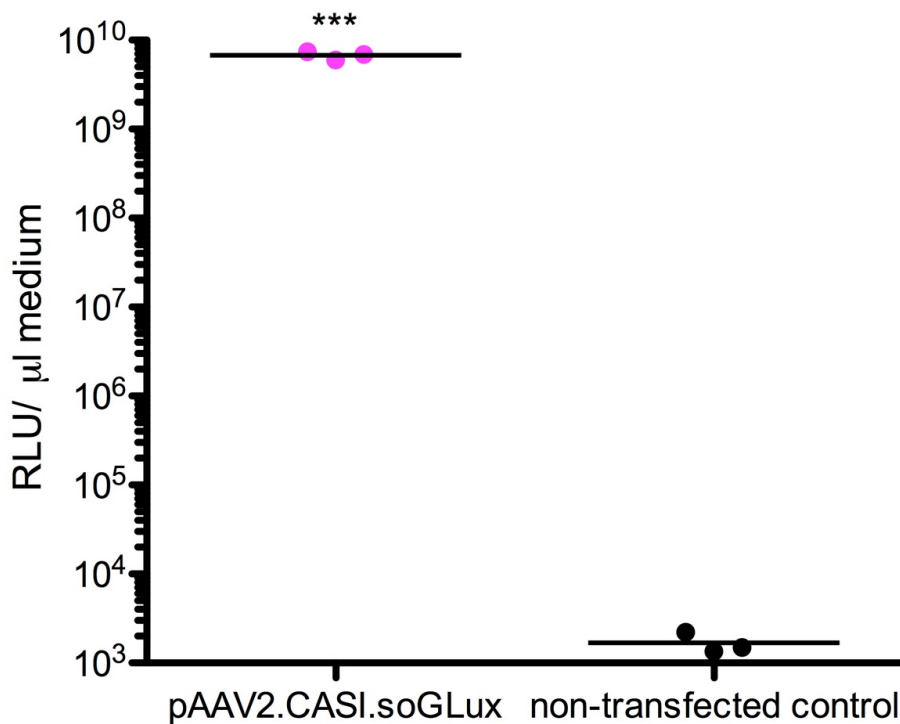
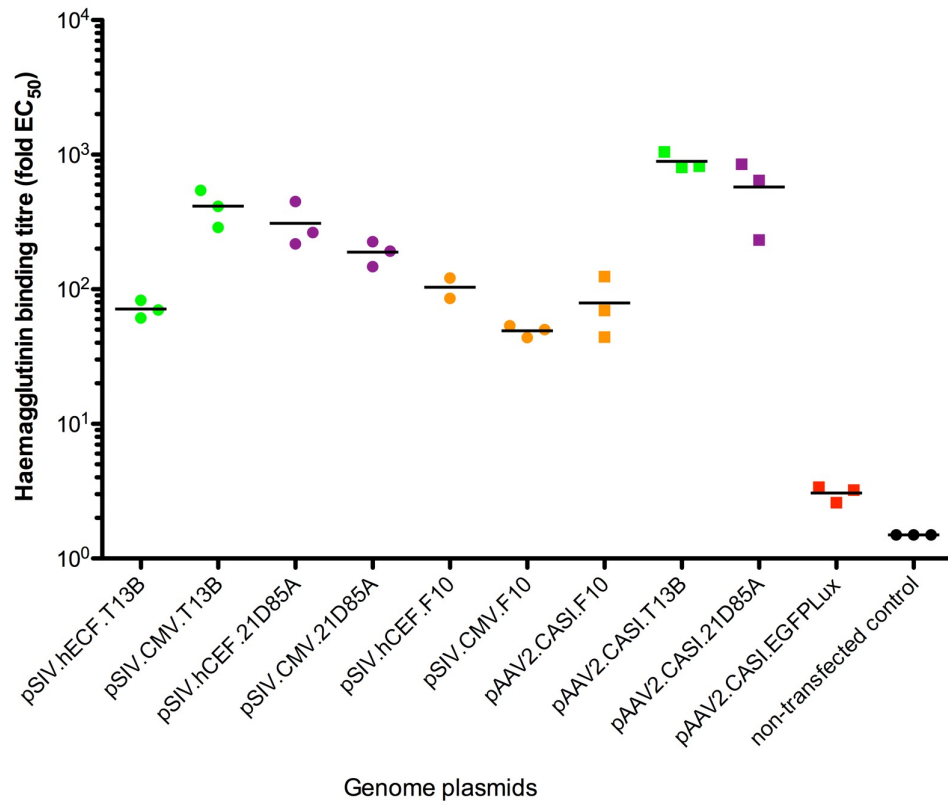


Figure 3.3 Levels of GLux activity following pAAV2.CASl.soGLux transfection in cell culture. Expression of GLux reported as relative lights unit (RLU) in culture supernatant following transfection of the genome plasmid pAAV2.CASl.soGLux in HEK 293T cells (1E6 cells/mL in 2mL FreeStyle media, 3 μ g plasmid and 6 μ g of 25 kDa branched PEI; n=3; Section 2.2.4) was measured 72 hours post-transfection (Section 2.2.6.1). Each data point represents one well of cells on a six-well plate obtained from duplicate readings on the assays. Horizontal bars represent group means. Statistical significance was determined by unpaired t-test. A calculated p value of <0.001 was deemed a significant difference and indicated with triple asterisk (***) on the graph.

(A)



(B)

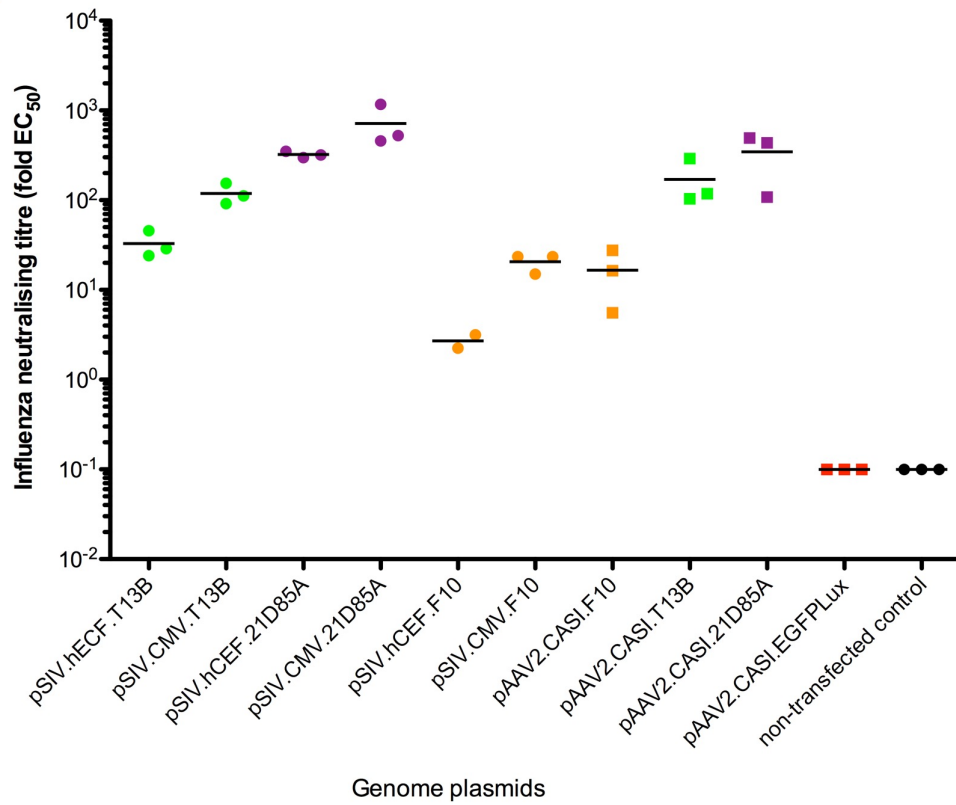
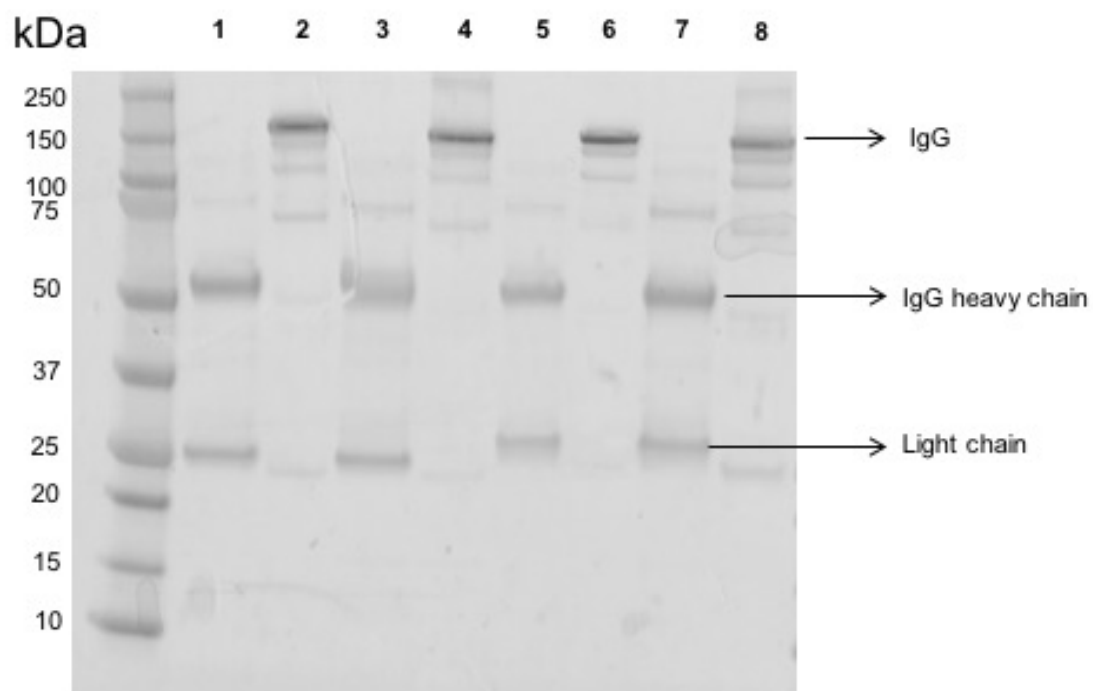


Figure 3.4 Supernatant binding titre (A) and neutralizing titre (B) produced by viral genome plasmids *in vitro*. Following transfection of HEK 293T cells (1E6 cells/mL in 2mL FreeStyle media) with respective genome plasmids (3 µg plasmid and 6 µg of 25 kDa branched PEI; n=2 for pSIV.hCEF.F10, otherwise n=3; Section 2.2.4), expression of functional antibody in the culture supernatant was tested against purified haemagglutinin (H5) and S-eGFP/H5/Vietnam (Table 2.4). Binding titre (A) and neutralising titre (B) expressed in fold EC₅₀ in supernatant was measured 72 hours post-transfection as described in Sections 2.2.6.3 and 2.2.6.4. Each data point represents one well of cells on a six-well plate obtained from duplicate readings on the assays. Horizontal bars represent group means. Value plotted at 1.5-fold EC₅₀ in A indicates no binding activity detected. Value plotted at 0.1-fold EC₅₀ in B indicates no neutralising activity detected.

ORF approach, in terms of binding to all HA tested (Figure 3.6A). However, for 21-D8-5A, the single-ORF antibody, but not the dual-ORF equivalent, shows binding activity towards H1 (A/California/07/2009) (Figure 3.6 B). The results also show that T1-3B antibody produced from the single-ORF cDNA was more potent in terms of neutralising activity with an approximately three-fold lower EC_{50} value compared with the dual-ORF T1-3B antibody, against all influenza strains tested (Figure 3.6 A). However, there was no difference in neutralisation activity observed for 21-D8-5A against all influenza strains tested (Figure 3.6 B). The overall results reveal a difference in bioactivity between the single-ORF antibody and the dual-ORF equivalent, which has not been reported previously.

3.2.5 Investigation of the effect of deletion of C-terminal lysines on the bioactivity of broadly neutralising antibodies

The C-terminal of all IgG1 heavy chains is conserved, with the terminal amino acid sequence SLSLSPGK. The C-terminal lysine is often removed by carboxypeptidases (Cp) during production in cell culture via a process called C-terminal lysine clipping (Fricker and Herbert, 1988, Harris, 1995, Dick et al., 2008). The degree of C-terminal lysine clipping is thought to be related to the difference in the expression of Cp in different producer cell lines, with retention of the C-terminal lysine residue being as high as 50% when IgG is produced in B cell hybridoma, compared with as little as 5% when expressed in a Chinese Hamster Ovary (CHO) cell line (Dick et al., 2008). The degree of C-terminal lysine variation in antibody produced in the HEK 293T cells used here has not been reported previously.

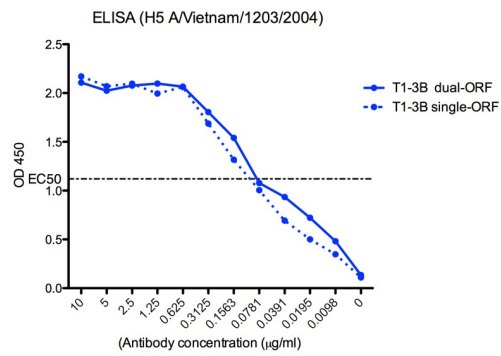


Lane	Antibody	SDS-PAGE condition
1	T1-3B dual-ORF	Reducing
2		Non-reducing
3	T1-3B single-ORF	Reducing
4		Non-reducing
5	21-D8-5A dual-ORF	Reducing
6		Non-reducing
7	21-D8-5A single-ORF	Reducing
8		Non-reducing

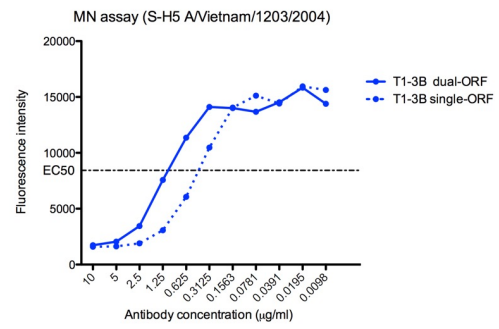
Figure 3.5 SDS-PAGE of antibodies expressed from single-ORF and dual-ORF constructs. Single-ORF antibodies, and their dual-ORF equivalents were prepared from HEK 293T cells transfected with (1,2) pCMV.T13B.H and pCMV.T13B.L, (3,4) pAAV2.CASI.T13B, (5,6) pCMV.21D85A.H and pCMV.21D85A.L, and (7,8) pAAV2.CASI.21D85A (Table 2.1). Antibodies were purified using Sepharose A column (2.5 µg per lane; Section 2.2.7.1), and separated using SDS-PAGE under reducing (1,3,5,7) and non-reducing (2,4,6,8) conditions and detected by staining with Quick Coomassie Stain (Generon) (Section 2.2.8). The positions of the bands containing the full IgG molecule, heavy chain and light chain are indicated. Protein markers (Bio-Rad) ranging from 10 to 250 kDa were included.

(A)

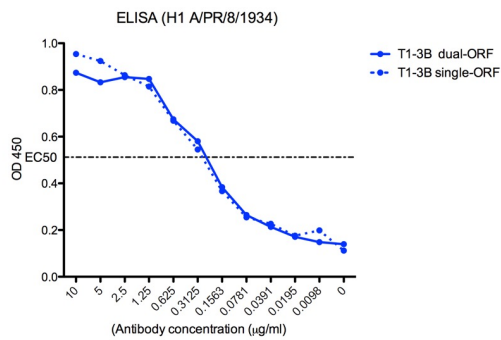
i



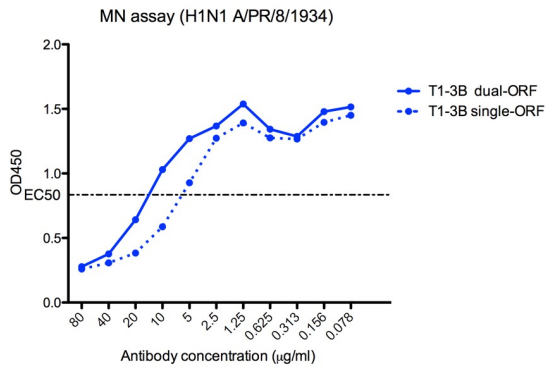
ii



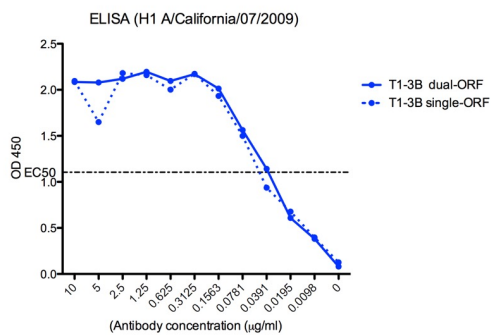
iii



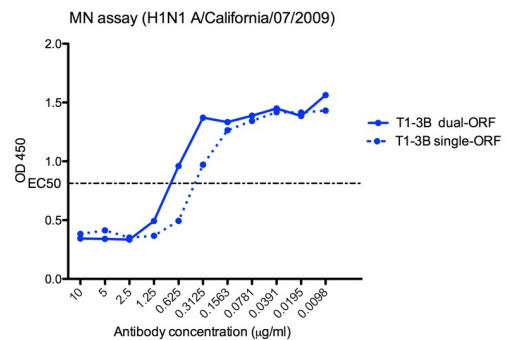
iv



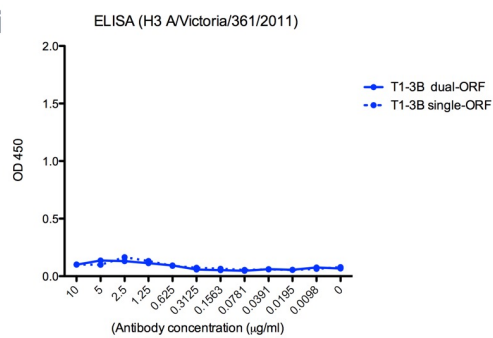
v



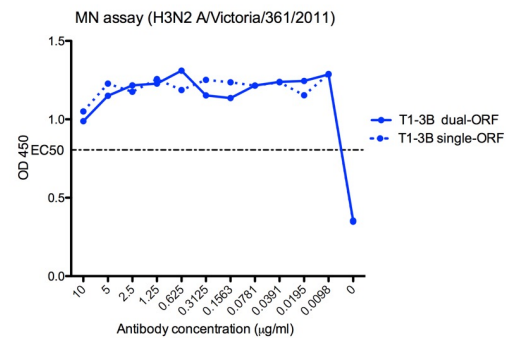
vi



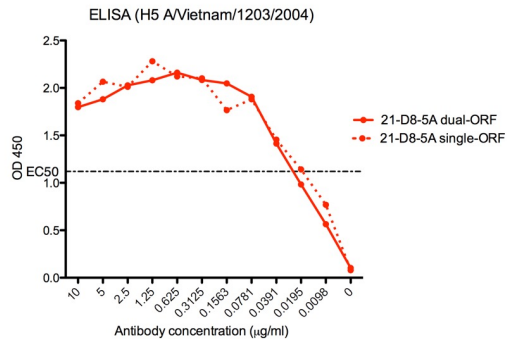
vii



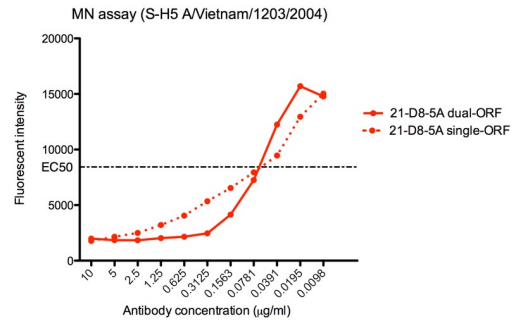
viii



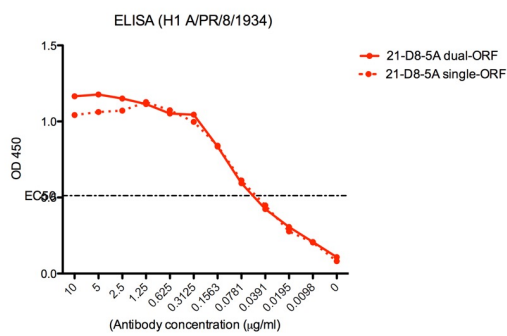
(B) i



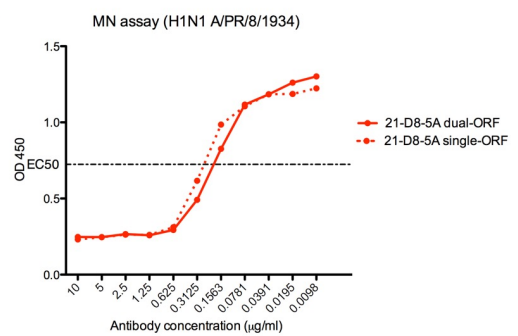
ii



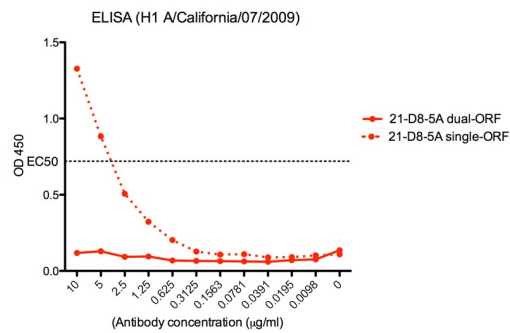
iii



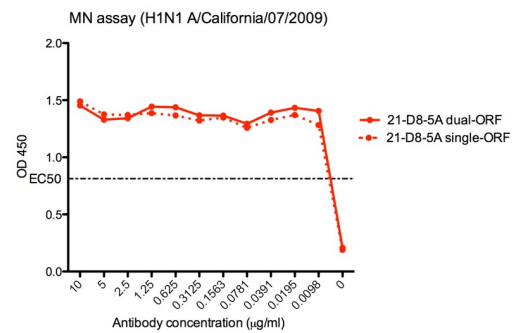
iv



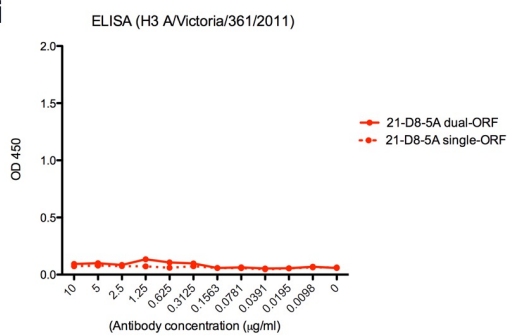
v



vi



vii



viii

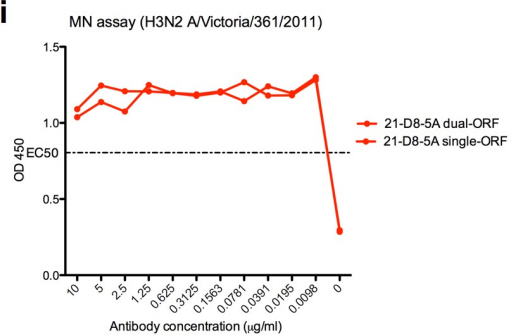


Figure 3.6 Binding and neutralising titre of single-ORF antibodies compared to their dual-ORF equivalents. Single-ORF antibody T1-3B (A) and 21-D8-5A (B) and their dual-ORF equivalents, prepared from transfection of pAAV2.CASI.T13B, pAAV2.CASI.21D85A, pCMV.T13B.GH and pCMV.T13B.κ, pCMV.21D85A.GH and pCMV.21D85A.λ (Table 2.1) into HEK 293T cells following purification using Sepharose A column (Section 2.2.7.1) were compared. Binding and neutralising titre were determined using anti-HA ELISA (Section 2.2.6.3) against various purified HA proteins (Table 2.4) and microneutralisation (MN) assay (Section 2.2.6.4) against various recombinant influenza strains (Table 2.4). Each data point represents the mean of duplicate readings. The EC₅₀ was calculated as the concentration of antibody that results in half-maximal response. In the MN assay, infection was expressed as OD450 (influenza NP expression) when wild-type influenza was used and fluorescence intensity when S-Flu (Table 2.4) was used.

C-terminal lysine clipping has the potential to result in three possible IgG isoforms, because the C-terminal lysine can be present on both the heavy chains of the IgG (K2), on either one of the heavy chains (K1), or missing in both of the heavy chains (K0) (Figure 3.8). In the single-ORF IgG cDNA construct, to prevent heterogeneity at the last amino acid of the heavy chain (with or without C-terminal lysine), the lysine-encoding nucleotides in the C-terminus of the heavy chain were removed from the cDNAs. This produced a final amino acid sequence of SLSLSPG followed by a furin cleavage site RKRR which has been shown to result in complete cleavage at the furin site with expression of homogeneous antibody product *in vitro* (Fang et al., 2007) (Figure 3.7).

Although the actual rate of C-terminal lysine clipping in the IgG production system used here, with HEK 293T cells, was not determined, one can speculate that the final IgG preparations could contain a heterogeneous mixture of IgG isoforms as described above. To investigate the effect of potential heterogeneity of C-terminal lysine on the bioactivity of T1-3B and 21-D8-5A IgG, lysine-encoding nucleotides were specifically removed from the heavy chain cDNA of the dual-ORF construct using site-directed mutagenesis (Section 2.2.3.10). Versions of T1-3B and 21-D8-5A dual-ORF IgG without the C-terminal lysine (namely T1-3B Δ K and 21-D8-5A Δ K) were produced, affinity purified (Section 2.2.7.1) and compared with the original T1-3B and 21-D8-5A dual-ORF antibodies, using HA-ELISA and MN assays (Figure 3.9). The results show that complete lysine removal does not affect the binding and neutralising activity of the dual-ORF antibodies, therefore, the enhanced binding and neutralising activity of T1-3B and 21-D8-5A single-ORF antibodies, seen in Figure 3.6, was not due to the absence of the C-terminal lysine.

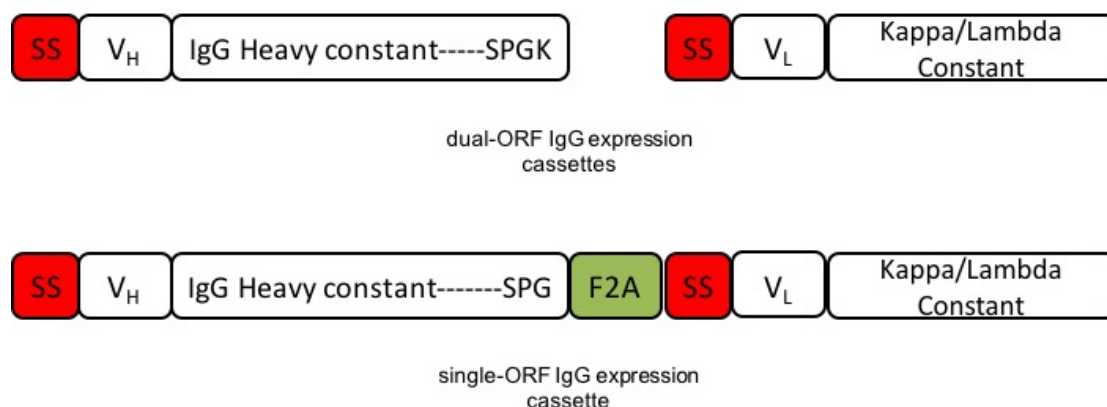


Figure 3.7 Schematic to illustrate the dual-ORF and single-ORF IgG cDNA constructs.

The diagram shows elements within the expression cassettes of the dual-ORF IgG and the single-ORF IgG, along with their respective C-terminal amino acid sequences, SPGK and SPG, on the heavy chain. In the single-ORF cDNA construct, nucleotides encoding the C-terminal lysine (K) were specifically removed to prevent IgG heterogeneity of C-terminal lysine variants, whereas the nucleotide encoding the C-terminal lysine (K) is intact on the dual-ORF cDNA construct.

3.2.6 Assessment of viral vector function in cell culture

Having validated the function of all viral genome plasmids constructed above, three rSIV.F/HN (Table 3.4) and five rAAV2/8 (Table 3.5) viral vectors were produced at large scale, purified and titrated (Section 2.2.5).

Recombinant LVs in this study were produced using a serum-free media, suspension approach in a WAVE bioreactor (5 L culture) (Section 2.2.5.1). Crude virus preparations were then purified using a two-stage purification strategy (Section 2.2.5.1), resulting in highly concentrated rLV in FreeStyle media. Recombinant AAVs in this study were produced in adherent cells cultured in HYPERflasks (~1.1 L culture) (Section 2.2.5.2). Crude virus preparations were purified using a two-stage purification strategy (Section 2.2.5.2), resulting in highly concentrated rAAV in PBS.

The purity of rAAV vector can affect their transduction efficiency (Ayuso et al., 2010). Protein bands corresponding to the viral capsids proteins, VP1, VP2, and VP3 can be visualised on SDS-PAGE (Section 2.2.8) and their relative intensity can be assessed along with contaminating cellular proteins. All rAAV preparations generated in this study were analysed using SDS-PAGE and the purity was quantified using ImageJ software (Abramoff et al., 2004). Results from the SDS-PAGE indicated that rAAV preparations produced have a purity of >90% (Figure 3.10).

To validate the function of the viral vectors produced, HEK 293T cells were transduced with each of the rSIV.F/HN or rAAV viral vectors (Table 3.4 and 3.5) using methods in Section 2.2.5.3. For vector rAAV2/8.CAS1.GLux, the GLux reporter

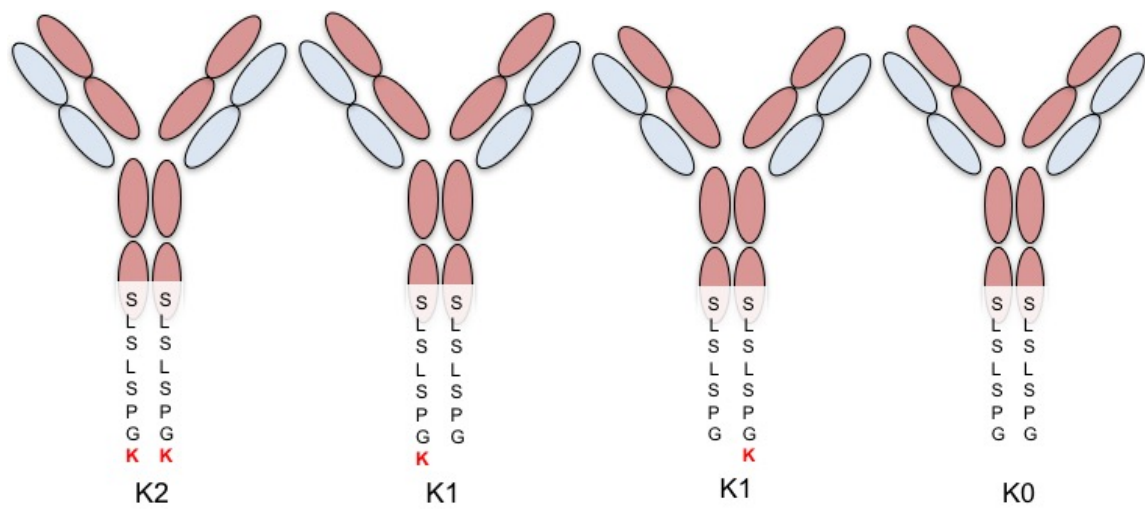
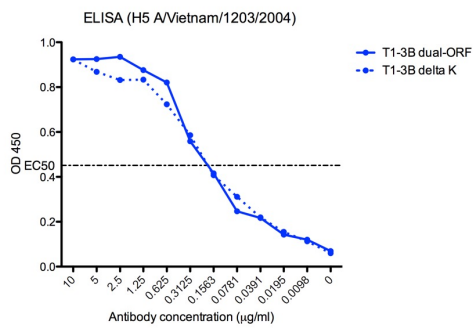


Figure 3.8 Schematic illustrating possible IgG isoforms after C-terminal lysine clipping.

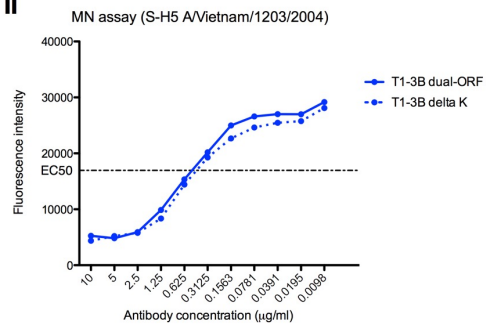
The diagram shows the three possible IgG isoforms with C-terminal lysine variants, due to complete or incomplete C-terminal lysine clipping. The C-terminal lysine can potentially be retained on both the heavy chains of the IgG (K2), on either one of the heavy chains (K1), or completely absent in both heavy chains (K0). Red represents the heavy chains; blue represents the light chains of the IgG.

(A)

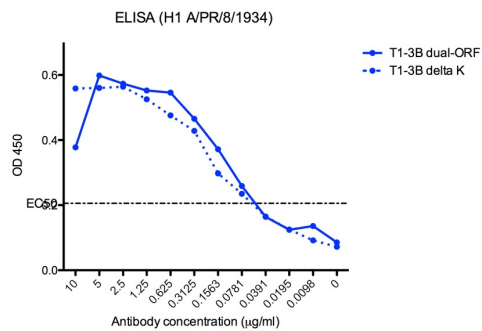
i



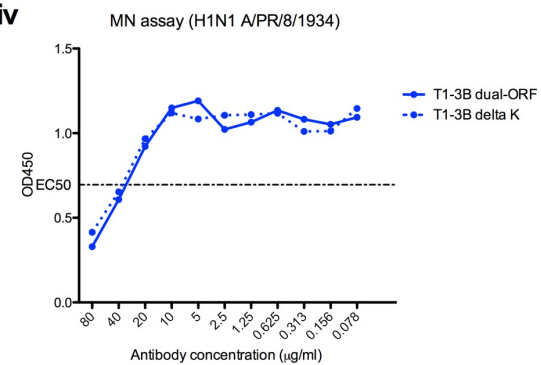
ii



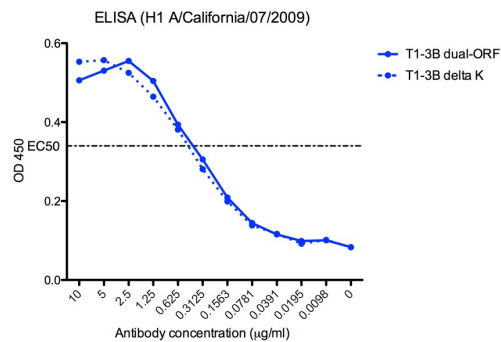
iii



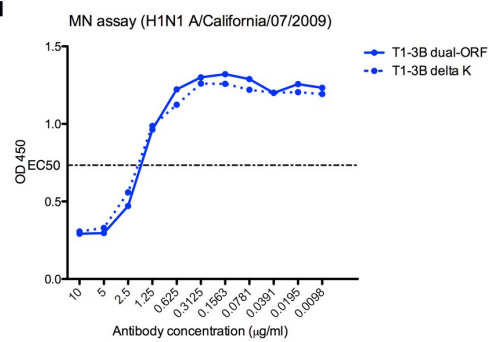
iv



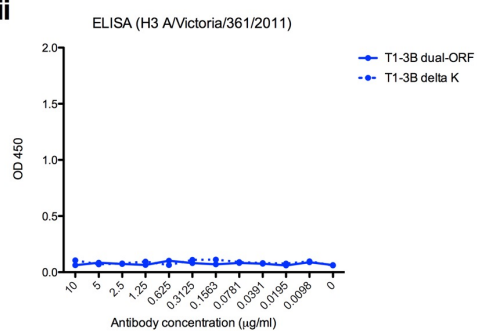
v



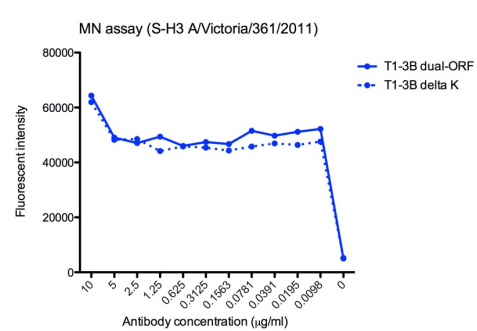
vi



vii

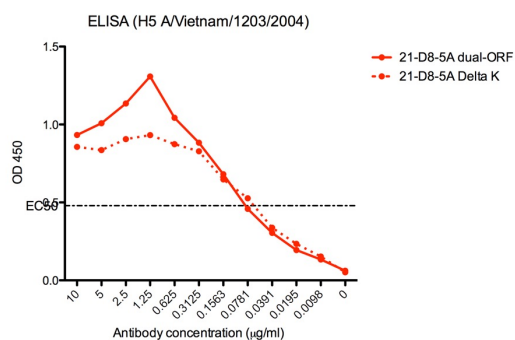


viii

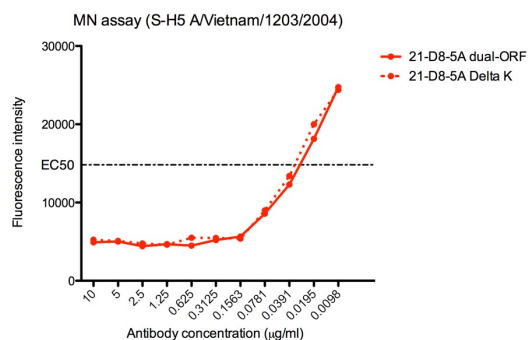


(B)

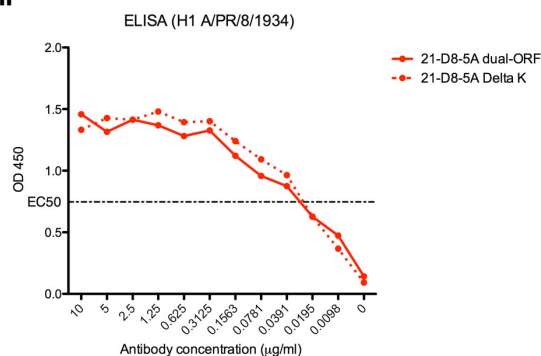
i



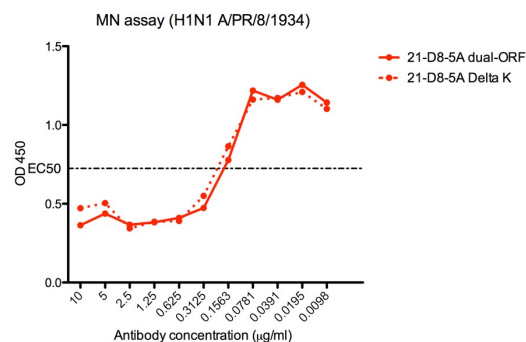
ii



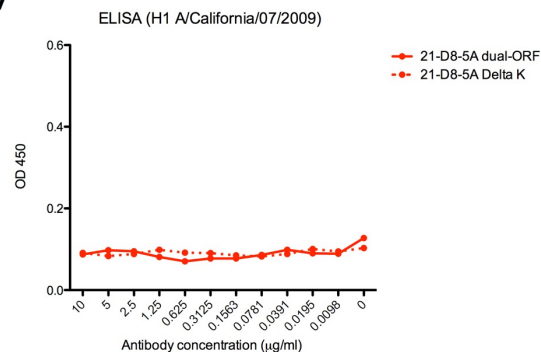
iii



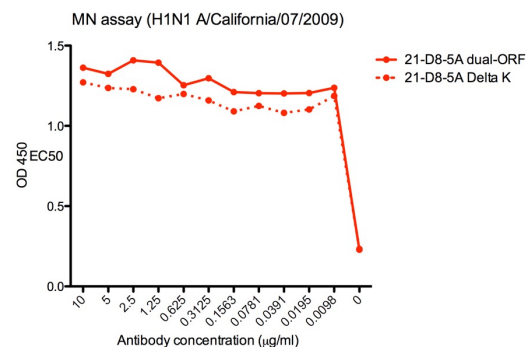
iv



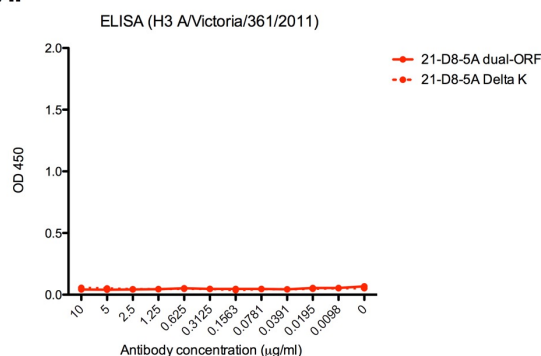
v



vi



vii



viii

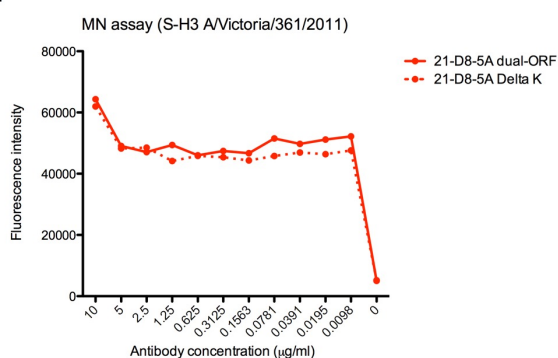


Figure 3.9 Binding and neutralising titre of IgGs with the C-terminal lysines deleted compared with their dual-ORF equivalents. C-terminal lysine-deleted IgG T1-3B Δ K (A) and 21-D8-5A Δ K (B) and the dual-ORF equivalents were prepared from transfection of pCMV.T13B.GH Δ K and pCMV.T13B. κ , pCMV.21D85A.GH Δ K and pCMV.21D85A. λ , pCMV.T13B.GH and pCMV.T13B. κ , pCMV.21D85A.GH and pCMV.21D85A. λ (Table 2.1) into HEK 293T cells, followed by purification using Sepharose A column (Section 2.2.7.1). Binding and neutralising titres were determined using anti-HA ELISA (Section 2.2.6.3) against various purified HA proteins (Table 2.4) and microneutralisation (MN) assay (Section 2.2.6.4) against various recombinant influenza strains (Table 2.4). Each data point represents the mean of duplicate readings. The EC₅₀ was calculated as the concentration of antibody that results in half-maximal response. In the MN assay, infection was expressed as OD450 (influenza NP expression) when wild-type influenza was used and fluorescence intensity when S-Flu (Table 2.4) was used.

the GLux reporter activity in the culture supernatant was tested (Section 2.2.6.1) and Figure 3.11 shows that the transduced cells express significantly higher Glux activity compared with the non-transduced control. For rAAV2/8.CASI.EGFP_{Lux}, the presence of EGFP-positive cells was monitored with fluorescence microscopy to confirm transduction and transgene expression (Figure 3.12). For vectors expressing bnAbs, cell culture supernatant was collected 6 days post-transduction and anti-human IgG ELISA (Section 2.2.6.2) was used to determine the levels of expressed human IgG in the supernatant. Anti-human IgG ELISA, instead of antigen-specific ELISA, was used to facilitate comparison between viral vectors expressing human IgG with different specificity, and for future comparisons. Figures 3.13 and 3.14 show that all the rSIV.F/HN and rAAV2/8 vectors transduced HEK 293T and expressed around 100 to 300 ng/mL of human IgG in cell culture. The results confirmed the function of virus preparations generated.

3.3 Discussion

The aim of the studies presented in this chapter was to generate new recombinant LV and AAV vectors expressing antibodies, and to confirm their function in cell culture, alongside control vectors expressing reporter genes.

In non-clinical settings, rAAV vectors are normally purified using a continuous caesium chloride (CsCl) density gradient or an iodixanol step density gradient followed by downstream concentration (Zolotukhin et al., 1999, Grimm et al., 2005). Here, an iodixanol gradient followed by ultracentrifugation downstream processing was chosen for purifying all the rAAV vectors used in this study, because of its

Table 3.4 rSIV.F/HN produced in this study

Vector	Vector type	Promoter	Transgene	Genome plasmid	Titre (TTU/ml)
rSIV.F/HN.hCEF.T13B (vGM152)	SIV	hCEF	T1-3B	pSIV.hCEF.T13B (pGM443)	1.08x10 ⁹ or 6.58x10 ⁹
rSIV.F/HN.hCEF.21D85A (vGM150)	SIV	hCEF	21-D8-5A	pSIV.hCEF.21D85A (pGM445)	4.72x10 ⁹
rSIV.F/HN.hCEF.F10 (vGM151)	SIV	hCEF	F10	pSIV. hCEF.F10 (pGM447)	3.08x10 ⁹

Table 3.5 rAAV2/8 vectors generated in this study

Vector	AAV serotype	Promoter	Transgene	Genome plasmid	Titre (DRGC/mL)
rAAV2/8.CASI.F10	2/8	CASI	F10	pAAV2.CASI.F10 (pGM449)	1.20x10 ¹³
rAAV2/8.CASI.T13B	2/8	CASI	T1-3B	pAAV2.CASI.T13B (pGM450)	1.84x10 ¹³
rAAV2/8.CASI.21D85A	2/8	CASI	21-D8-5A	pAAV2.CASI.21D85A (pGM451)	1.36x10 ¹³
rAAV2/8.CASI.EGFPLux	2/8	CASI	EGFPLux	pAAV2.CASI.EGFPLux (pGM452)	2.74x10 ¹²
rAAV2/8.CASI.soGLux	2/8	CASI	soGLux	pAAV2.CASI.soGLux (pGM453)	2.07x10 ¹³

rAAV2/8: recombinant adeno-associated virus serotype 2 packaged in serotype 8 capsid;

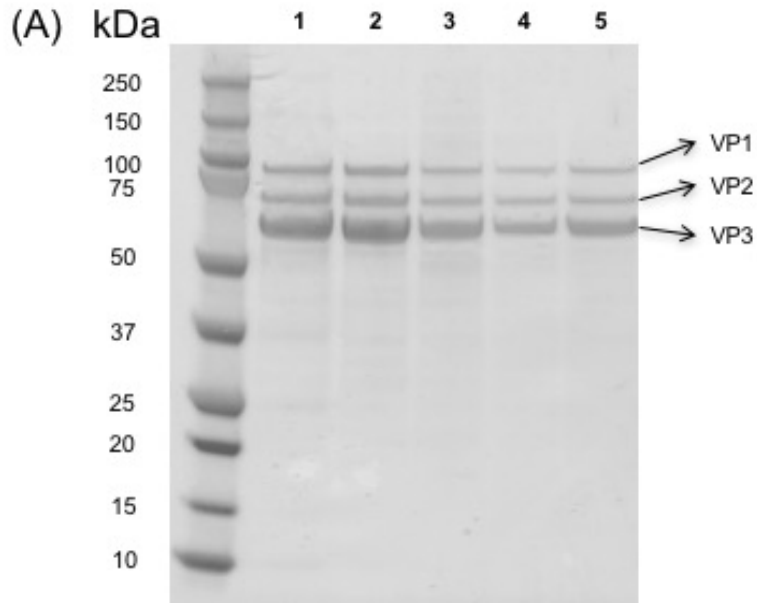
hCEF: a combination of human CMV enhancer and human elongation factor 1 alpha promoter;

CASI; a combination of the CMV enhancer and chicken β -actin promoter and novel intron containing elements of the ubiquitin C enhancer/promoter; SIV: simian immunodeficiency virus;

soGLux: codon-optimised CpG-depleted *Gaussia* luciferase; EGFPLux: enhanced green

fluorescence protein fused to firefly luciferase at its N-terminal; DRGC (DNase-resistant

Genome Copies)



(B)

	Percent of total protein (%)				
Lane	1	2	3	4	5
rAAV2/8	T1-3B	21-D8-5A	F10	GLux	EGFPLux
VP1	20.54	21.42	16.49	18.05	18.19
VP2	15.43	18.20	18.20	20.73	18.13
VP3	54.10	56.63	59.58	58.27	61.41
Impurities	9.92	3.75	5.72	2.95	2.28
Purity	90.10	96.25	94.28	97.05	97.72

Figure 3.10 Purity of rAAV vector preparations assessed by SDS-PAGE. Recombinant AAV preparations were boiled to disintegrate the viral capsid (Section 2.2.8) and were then (A) separated by SDS-PAGE (1E10 DRGC per lane) and visualised with Quick Coomassie Stain (Generon) (Sections 2.2.8). The position of the AAV capsids protein VP1, VP2, VP3 are indicated. The intensity of the protein bands was quantified using ImageJ (Section 2.2.8). (B) The table shows the calculated purity of rAAV vector preparations using this approach.

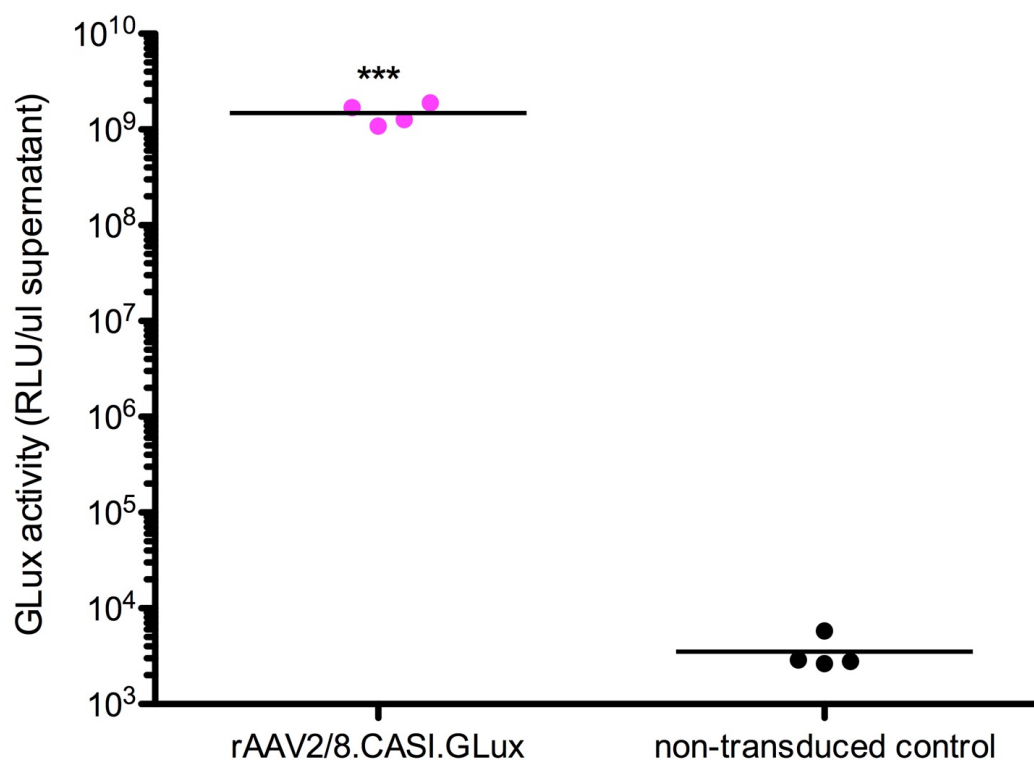


Figure 3.11 Levels of GLux activity present in the supernatant of HEK 293T cells transduced with rAAV2/8.CAS1.GLux. HEK 293T cells (0.5×10^6 cells/mL in 1mL D10 media) were transduced with rAAV2/8.CAS1.GLux (1×10^{10} DRGC rAAV2/8; n=4; Section 2.2.5.3) and GLux activity was measured in culture supernatant 6 days post-transduction (Section 2.2.6.1). Each data point represents one well of cells on a 12-well plate obtained from duplicate readings on the assays. Horizontal bars represent group means. Statistical significance was determined by unpaired t-test. A calculated p value of <0.001 was deemed a significant difference and indicated with triple asterisk (***) on the graph.

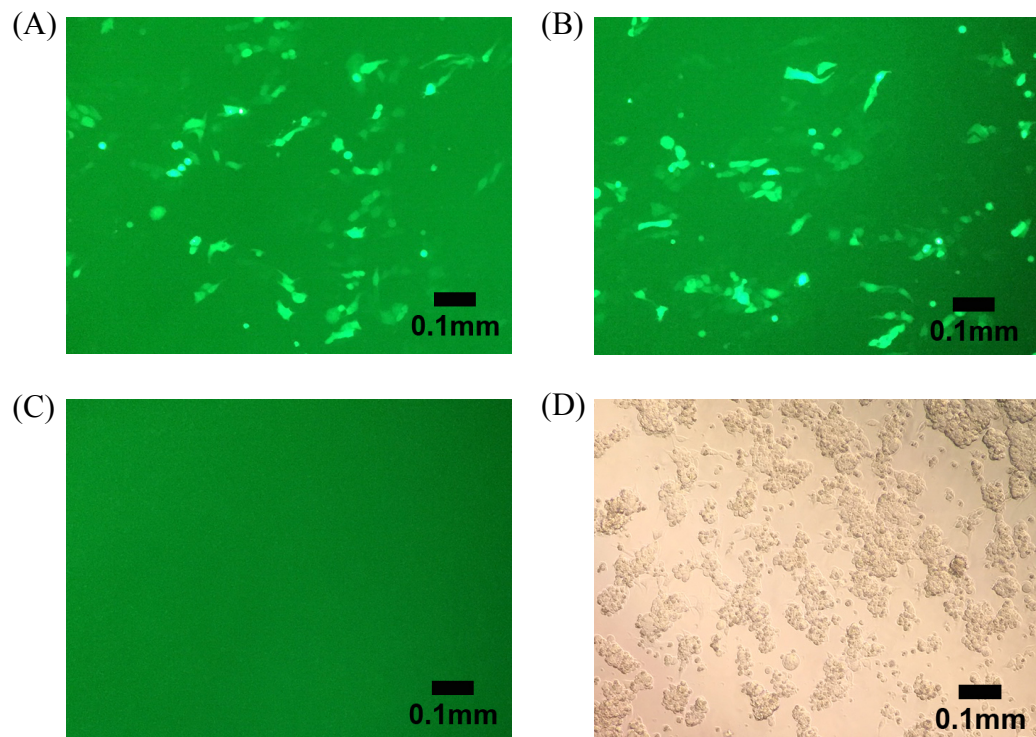


Figure 3.12 Expression of EGFP-positive cells following transduction of rAAV2/8.CASI.EGFP Lux vector in cell culture. HEK 293T cells (0.5×10^6 cells/mL in 1mL D10 media) were transduced with rAAV2/8.CASI.EGFP Lux (1×10^{10} DRGC rAAV2/8; $n=4$; Section 2.2.5.3) in a 12-well plate and EGFP expression observed 6 days post-transduction using a fluorescence microscope (viewed at 100x magnification) (Section 2.2.2). (A) and (B) are two of the four wells transduced by rAAV2/8.CASI.EGFP Lux. A representative image of control, non-transduced cells visualised under (C) fluorescence and (D) bright field microscopy shows the absence of EGFP-positive cells.

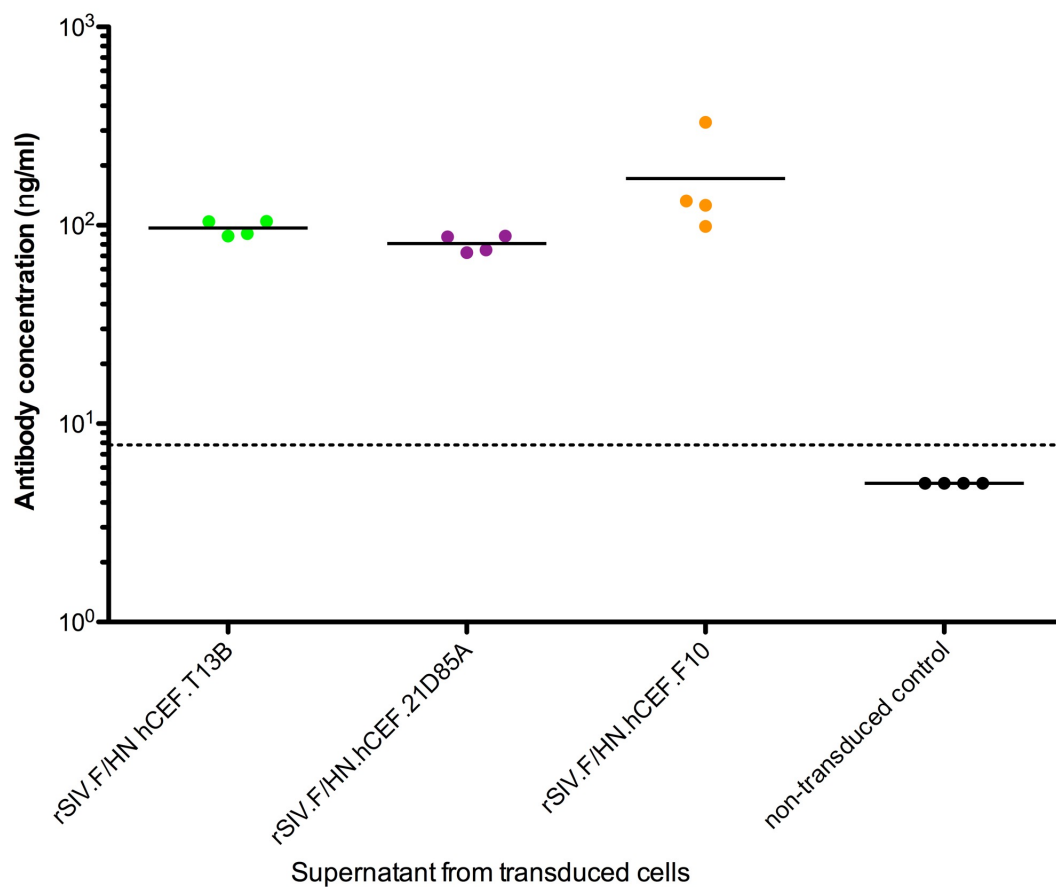


Figure 3.13 Human IgG present in the supernatant of HEK 293T cells transduced with rSIV.F/HN vectors. HEK 293T cells (0.5×10^6 cells/mL in 1mL D10 media) were transduced with the rSIV.F/HN vectors indicated (1×10^7 TU rSIV.F/HN; $n=4$; Section 2.2.5.3) and human IgG was measured in the cell culture supernatant 6 days post-transduction (Section 2.2.6.2). Each data point represents one well of cells on a 12-well plate obtained from duplicate readings on the assays. Horizontal bars represent group means. Dotted line denotes the lowest detection limit of the assay. Black dots (group mean of 5 ng/mL) indicated no detectable human IgG in the supernatant from non-transduced, control cells.

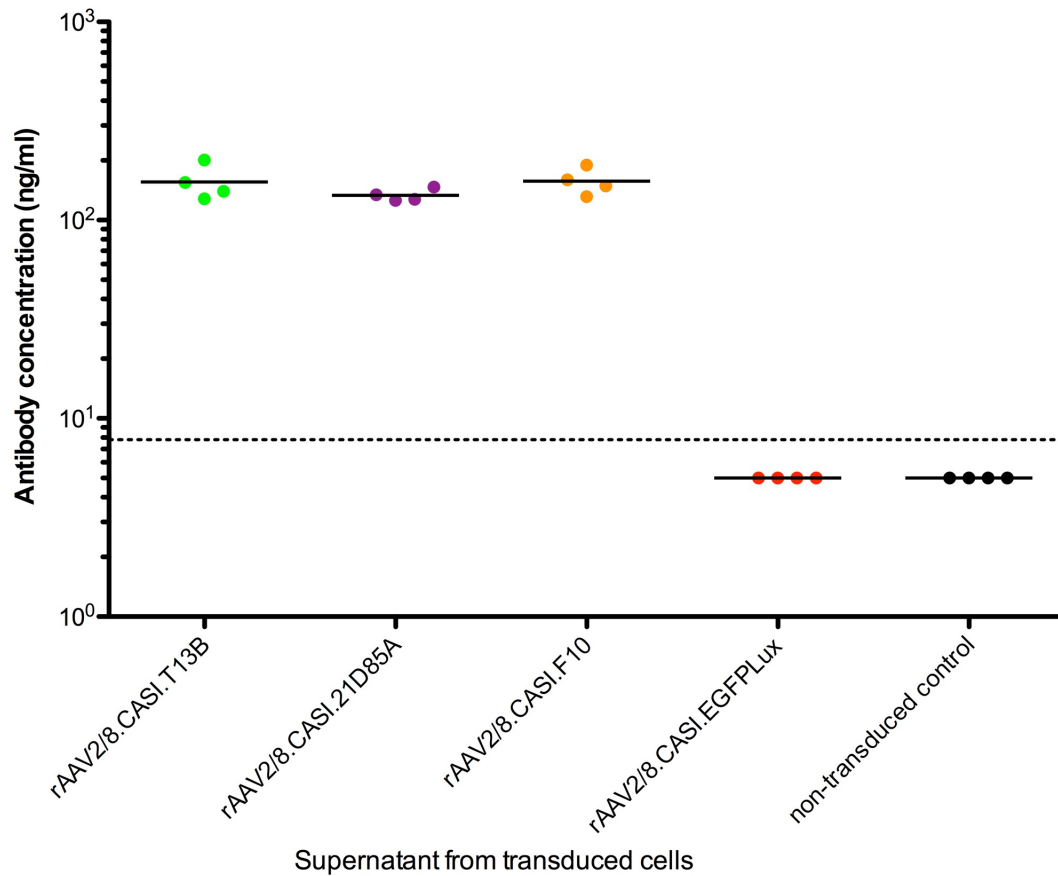


Figure 3.14 Human IgG present in the supernatant of HEK 293T cells transduced by rAAV2/8 vectors. HEK 293T cells (0.5E6 cells/mL in 1mL D10 media) were transduced with the rAAV2/8 vectors indicated (1E10 DRGC rAAV2/8; n=4; Section 2.2.5.3) and human IgG in culture supernatant was measured 6 days post-transduction (Section 2.2.6.2). Each data point represents one well of cells on a 12-well plate obtained from duplicate readings on the assays. Horizontal bars represent group means. Dotted line denotes the lowest detection limit of the assay. Black dots (group mean of 5 ng/mL) indicated no detectable human IgG in the supernatant from non-transduced control cells.

simplicity and time-saving compared with the CsCl density gradient. It has also been reported that this strategy is the most effective purification method to remove protein contaminants, and maintain infectivity with high rAAV vector recovery compared with CsCl gradients (Strobel et al., 2015); and resulted in rAAV preparations with > 90% purity (Figure 3.10). Highly pure preparations are important for *in vivo* studies to prevent unwanted immune reactions due to impurities from the viral preparations. Purification using an iodixanol gradient, however, often results in a high proportion (~20%) of empty capsids (missing the genome) compared with purification using CsCl gradients (<1%). The ratio of fully encapsidated rAAV to empty rAAV particles was not determined in this study. The rSIV.F/HN preparations used in this study were produced using European Pharmacopoeia-compliant raw materials and plasmid constructs. This resulted in highly pure rSIV.F/HN vectors, which have >99.9% host cell protein removal and are of a comparable standard to virus produced for clinical use by Contract Manufacturing Organisations (CMOs) (Steve Hyde, personal communication). Furthermore, rSIV.F/HN produced using this approach had been used successfully in animal studies with no product-related toxicity (Alton et al., 2017).

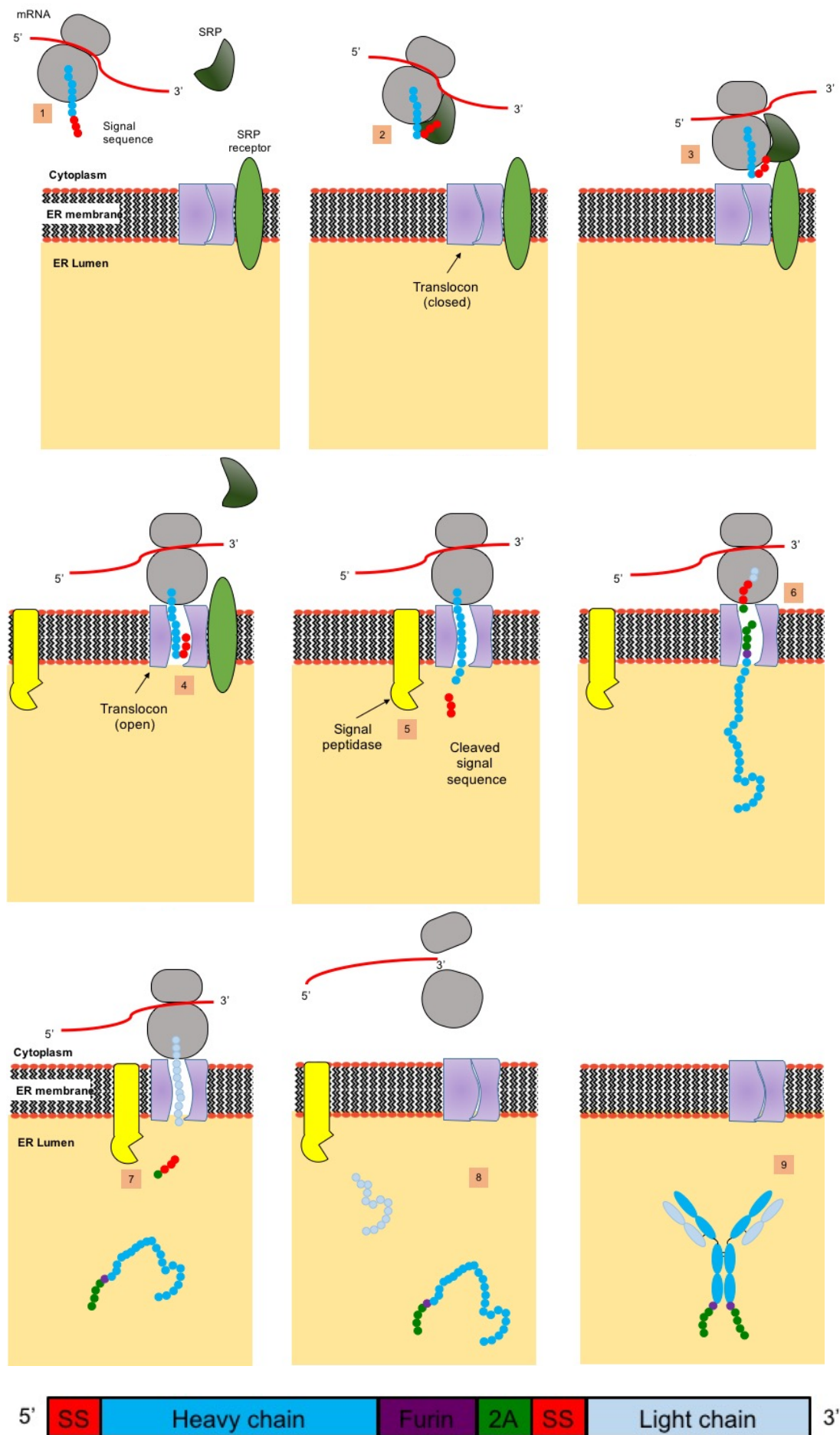
The bnAbs generated in this study are expressed from a single-ORF construct (Section 3.2.1) to facilitate packaging of the genomes into rAAV vectors, which have limited packaging capacity, making it difficult to package bnAb expressed from a dual-ORF construct. Results from SDS-PAGE suggests that single-ORF constructs lead to the assembly of a full IgG molecule that is similar to that produced using the dual-ORF approach (Figure 3.5). Functional assays show that single-ORF bnABs T1-3B and 21-D8-5A have enhanced bioactivity compared with their dual-ORF equivalents (Section 3.2.4 and Figure 3.6). This enhancement has not been previously reported. There are

two possible hypotheses for the enhancement of the single-ORF bnAbs. Firstly, it is possible that the complete absence of a C-terminal lysine in the single-ORF bnAbs, as described in Section 3.2.5, could be responsible for the bioactivity enhancement. The effect of C-terminal lysine on antigen binding or neutralisation has not been reported before. It has been shown, however, that complete clipping of C-terminal lysines is required for maximal complement activation, whereas partial removal of C-terminal lysine compromises complement activation (Bremer et al., 2015). This is because the positively charged C-terminal lysines lead to charge repulsion of IgG molecules, preventing the formation of IgG hexamer which is needed for complement activation (Bremer et al., 2015). It was speculated that the absence of negatively charged C-terminal lysine on IgG prevents charge repulsion between two IgG molecules leading to the enhancement of neutralising activity. However, no enhancement was observed when the dual-ORF bnAbs were expressed from cDNA without C-terminal lysine (Figure 3.9). Another observation, that supports the argument that C-terminal lysine was not the cause of the enhancement of T1-3B single-ORF antibody, was that the enhancement of neutralising activity was only observed with T1-3B, and not with 21-D8-5A. Enhancement of neutralisation might be expected to also be observed with single-ORF 21-D8-5A if the complete absence of C-terminal lysine was the cause.

The second hypothesis was that the enhancement of neutralisation could be due to difference in antibody structure as a result of the remnant of F2A sequence during antibody folding in the ER. As illustrated in Figure 3.15, the F2A cleavage occurs in several stages and in different organelles. In the ER, while the heavy and light chain polypeptides were formed and being folded into antibody, part of the F2A remnant

sequence is still present and attached to the C-terminal of the heavy chains. These remnant sequences are being cleaved off later in the Golgi network. It could be speculated that the presence of the F2A remnant affects the antibody folding, which might be responsible for the functional enhancement of single-ORF bnAbs. The results show that the enhancement of neutralisation was only observed with T1-3B which is a haemagglutinin stem-binding antibody. Whether the single-ORF construct enhances all haemagglutinin stem-binding antibodies, or whether this is antibody-dependent needs further exploration.

In summary, the data in this chapter suggest that the viral vectors generated here are functional and that the single-ORF bnAb construct expresses antibody that is comparable to the dual-ORF equivalent. Although cell culture experiments represent a good model to confirm gene expression from viral vectors, they rarely predict efficacy in animal models. The high purity of the virus preparations generated here facilitates the testing of these vectors in animal models described in the next chapters.



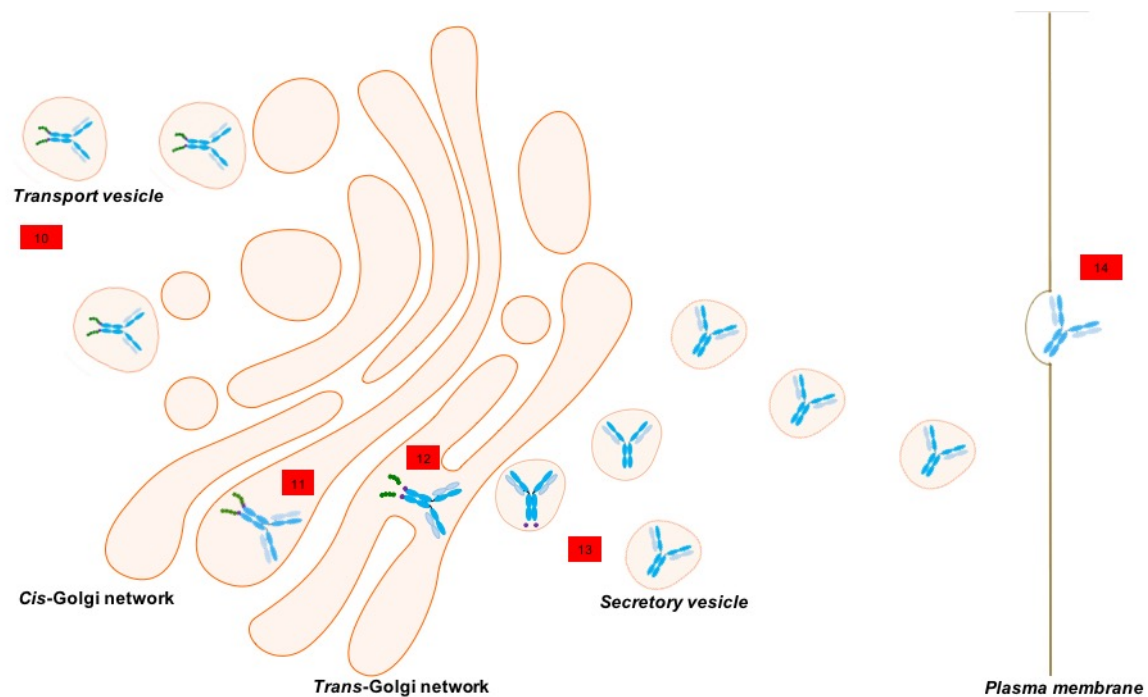


Figure 3.15 Schematic of possible mechanism of the expression of bnAb from single-ORF cDNA. 1. Following transduction of viral vectors into the host cells, single-ORF bnAb cDNA is transcribed into mRNA in the nucleus, mRNA enters cytoplasm and attaches to ribosome and translation occurs. 2. Once the signal sequence emerges from the ribosome, it is bound by a signal-recognition protein (SRP) which halts translation (Walter et al., 1981). 3. The SRP delivers the ribosome/nascent polypeptide complex to the SRP receptor in the ER membrane (Gilmore et al., 1982). 4. The signal sequence is inserted into the translocon and protein translation continues (Gorlich et al., 1992) 5. As the polypeptide chain elongates, it passes through the translocon channel into the lumen of the ER and the signal peptide is cleaved off by signal peptidase and rapidly degraded (Blobel and Dobberstein, 1975a, Blobel and Dobberstein, 1975b). 6. When the ribosomal Peptidyl Transferase Centre (PTC)

recognizes the 2A sequence, “ribosomal skipping” occurs where translation is skipped between the last two amino acid on the 2A sequence (glycine and proline). The first polypeptide (heavy chain) is released into the lumen and the translation resumes at proline followed by the second polypeptide (light chain) (Donnelly et al., 2001a, Donnelly et al., 2001b). 7. Signal sequence together with the proline at its’ N-terminal from the newly translated light chain is being cleaved off as it passes through the translocon channel into the lumen. 8. Unfolded heavy and light chains polypeptide undergo folding co-translationally into fully folded antibody. 9. Fully folded antibody with remnant of F2A cleavage site attaches to the tip of the constant heavy chain. 10. Newly synthesised antibodies with the F2A remnant are packaged into transport vesicles that bud from the ER and fuse together to form new *cis*-Golgi cisternae. 11. *Cis*-Golgi cisternae, with its antibody content, physically moves from the *cis* to *trans*-Golgi network. 12. In the trans-Golgi network, furin (proprotein convertase) cleaves off the furin cleavage site (RKRR↓) leaving RKRR attached to the C-terminal of the heavy chain (Nakayama, 1997). 13. Antibodies with the RKRR attached to the C-terminal of the heavy chains are packaged into the secretory vesicle to the plasma membrane. Decreasing pH in the secretory vesicles activate metallocarboxypeptidases which cleave off C-terminal basic amino acid residues (↓RKRR) from the heavy chain (Fricker and Snyder, 1982, Docherty and Hutton, 1983, Hook and Loh, 1984). 14. Secretory vesicles carrying antibodies free of F2A remnant fused with plasma membrane and secrete the antibodies extracellularly.

Chapter 4 Lung antibody production mediated by rSIV.F/HN

4.1 Introduction

4.1.1 Antibody production in non-haematopoietic cells

Antibodies are produced naturally by B lymphocytes as part of the humoral immune response, upon encounters with pathogens, but can also be produced ectopically from other cells using gene transfer vectors. Previous studies using this approach are discussed in Section 1.3, where vectors, both non-viral and viral, can deliver antibody transgenes into target cells to direct the expression of mAbs into the systemic circulation and/or the local environment. In Chapter 3, the construction of rSIV and rAAV vectors expressing reporter protein, and anti-influenza single-ORF antibody transgenes, was described along with confirmation of function in cell culture.

4.1.2 Selection of secreted reporter protein

Reporter assays can serve as a good marker, or proxy, to help understand the transgene expression profile mediated by gene transfer vectors. In order to study the expression profile of secreted proteins, such as antibodies, a number of commonly used secretory reporter proteins are available. The secreted embryonic alkaline phosphatase (SEAP) (Berger et al., 1988, Bao et al., 2000) has been used to study gene expression following IM delivery of plasmids in mice (Abruzzese et al., 1999). More recently, secreted luciferases have gained popularity as reporter proteins due to their high sensitivity, lack of toxicity and smaller size. Secreted luciferase from the marine organism *Gaussia princeps* (Wurdinger et al., 2008), commonly known as GLux, converts the substrate coelenterazine into a bioluminescent signal and has been

used in various cell-based and *in vivo* applications (Maguire et al., 2009b, Tannous, 2009). GLux offers several advantages; expression of GLux in the extracellular media is linear to cell number, growth and proliferation (Tannous et al., 2005) and is thermostable and pH resistant. Compared with SEAP, GLux is 20,000-fold more sensitive *in vitro* (Badr et al., 2007) and at least 1,000-fold more sensitive *in vivo* such that blood samples can be analysed directly without the need of further processing (Wurdinger et al., 2008, Tannous, 2009). More importantly, GLux does not display toxic effects in mammalian cells (Tannous et al., 2005), which makes GLux a good reporter protein for long-term animal studies. In the airways, GLux was shown to be a sensitive reporter protein for monitoring gene expression following administration of non-viral vector into murine lungs (Griesenbach et al., 2011). Therefore, GLux was selected as the reporter protein in this study to understand and compare secreted transgene protein expression following rSIV.F/HN and rAAV2/8 delivery.

4.1.3 Aim

The aim of the studies described in this chapter was to study the feasibility of using the lung to produce antibody mediated by rSIV.F/HN and compare with the antibody expression profile produced by rAAV2/8 delivered to the muscle.

4.2 Results

4.2.1 Expression of secretory reporter protein *Gaussia* luciferase (GLux) following intramuscular delivery of rAAV2/8 in mice

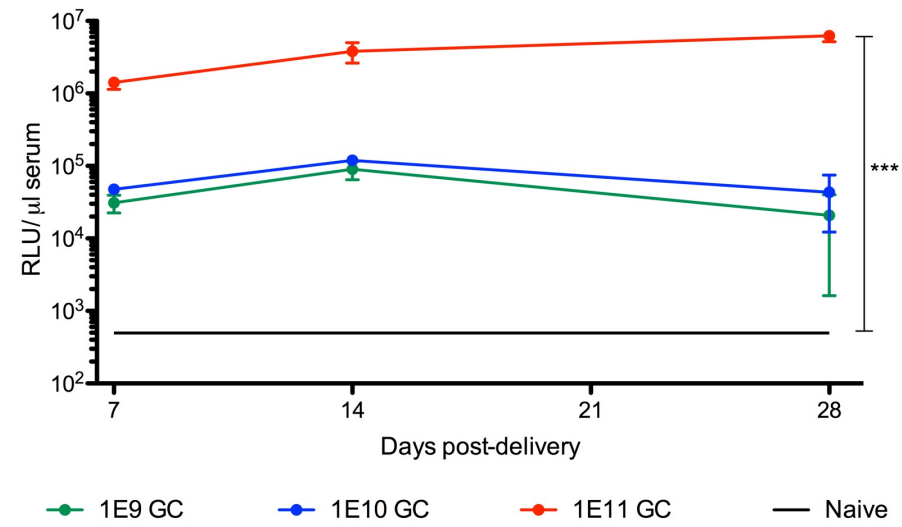
Studies have been reported in which rAAV2/8 expressing the F10 bnAb can result in full protection against lethal influenza challenge following IM injection in mice (Balazs

et al., 2013). Recombinant AAV2/8 (administered IM) was therefore selected as the benchmark vector for bnAb expression in this study, with which to compare lung delivery of rSIV.F/HN. It has also been shown, however, that antibody expression can be specific to a selected antibody (Balazs et al., 2013); therefore, a study using secreted reporter protein GLux was performed initially to facilitate comparison between the two viral vectors. Mice were dosed with 1E9, 1E10 or 1E11 GC of rAAV2/8.CAS1.soGLux (Section 3.2.6) (Table 3.5) via IM injection (Section 2.2.9.4). Serum was collected on day 7, 14 and 28 post-delivery and GLux activity was determined (Section 2.2.6.1). Mice were culled at the end of the experiment (day 28) and BALF was obtained to determine the GLux activity (Section 2.2.6.1). Dosing of rAAV2/8.CAS1.soGLux led to significant levels of GLux activity in the serum at all time-points assessed, as early as day 7, which persisted throughout the course of the experiment (Figure 4.1 A). There was no difference in GLux activity between mice receiving 1E9 and 1E10 GC, and GLux activity in mice receiving 1E11 GC was significantly higher than naïve mice ($p < 0.001$), around 10 to 20-fold higher than the 1E9 and 1E10 GC groups at all time-points assessed (Figure 4.1 A). GLux activity can be detected in the ELF (Section 2.2.6.5) at around 1.2 to 7-fold lower than the GLux activity in the serum (Figure 4.1 B & C). These data indicate the GLux expression profile following a single dose of rAAV2/8 delivered via IM administration, with persistent high levels expression in the systemic circulation, and lower but detectable levels in the lung lumen (ELF). *Gaussia* luciferase activity in the ELF was corrected from the BALF samples based on the dilution factor as determined using urea assay (Section 2.2.6.2). The conversion of BALF to ELF is discussed in detail in Section 4.2.5.

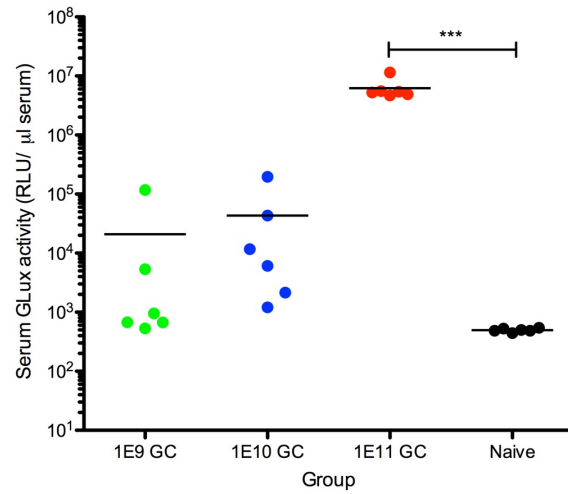
4.2.2 Expression of bnAb following intramuscular delivery of rAAV2/8 in mice.

Recombinant AAV2/8.CASI.T13B was produced to study the expression of bnAb *in vivo* (Section 3.2.6 & Table 3.5) and mice were dosed with 1E9, 1E10 or 1E11 GC of the vector (Section 2.2.5.2) via IM injection (Section 2.2.9.4). Serum was collected on day 7, 14 and 28 post-delivery and the human IgG concentration (corresponding to T1-3B levels) was determined (Section 2.2.6.2). Mice were culled at the end of the experiment (day 28) and BALF was obtained to measure the human IgG levels in the lung lumen (Section 2.2.6.2). Administration of 1E9, 1E10 or 1E11 GC of rAAV2/8.CASI.T13B led to the expression of high levels of T1-3B in the serum at all time-points assessed, as early as 7 days post-administration, which also persisted throughout the course of the experiment (Figure 4.2). All treatment groups expressed significantly higher levels of human IgG than the naïve mice (1E9 GC, $p < 0.05$; 1E10 GC, $p < 0.05$; 1E11 GC, $p < 0.001$) (Figure 4.3). Serum T1-3B levels in the treatment groups ranged from 1.3 to 160 $\mu\text{g/mL}$, with a trend for a dose-related increase in T1-3B expression. This confirms the ability of rAAV2/8 to direct expression of T1-3B bnAb from the muscle, at serum levels similar to those reported for the F10 bnAb, between 100 and 200 $\mu\text{g/mL}$ (Balazs et al., 2013). Importantly, at these high serum levels, F10 bnAb offered full protection against a lethal dose of influenza challenge in mice (Balazs et al., 2013). Human IgG was also detected in the ELF at around 10 to 30-fold lower than human IgG levels in the serum, ranging from 0.09 to 4.8 $\mu\text{g/mL}$.

(A)



(B)



(C)

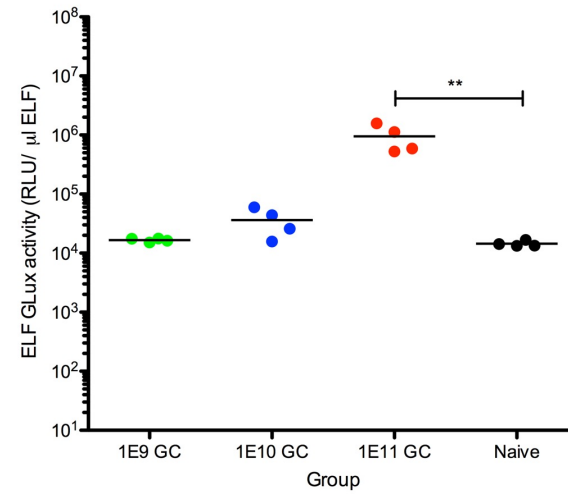


Figure 4.1 Expression of GLux following IM delivery of rAAV2/8.CAS1.GLux in mice.

Mice (female BALB/c, 5 to 7 weeks old) were dosed IM with rAAV2/8.CAS1.soGLux (1E9 GC (green), 1E10 GC (blue) or 1E11 GC (red) per mouse, diluted in PBS) and compared with naïve mice (black) (n=6 for serum samples; n=4 for Epithelial Lining Fluid (ELF) samples; Section 2.2.9.4) (Table 3.5). Serum was collected at the indicated time-points and mice were culled at day 28 post-delivery to obtain BALF (Sections 2.9.7 and 2.9.6). The GLux activity was measured in Relative Light Units (RLU) in the serum and the ELF (Section 2.2.6.1). Data in (A) are presented as group mean and standard errors of the mean (SEM). The GLux activity on day 28 in the serum (B) and ELF (C) is shown; each dot represents one animal, with horizontal bars representing group means. Statistical significance was determined by Kruskal-Wallis test followed by Dunn's post-hoc test. A calculated p value of <0.01 or <0.001 was deemed a significant difference and indicated with double asterisks (**) or triple asterisks (***) on the chart.

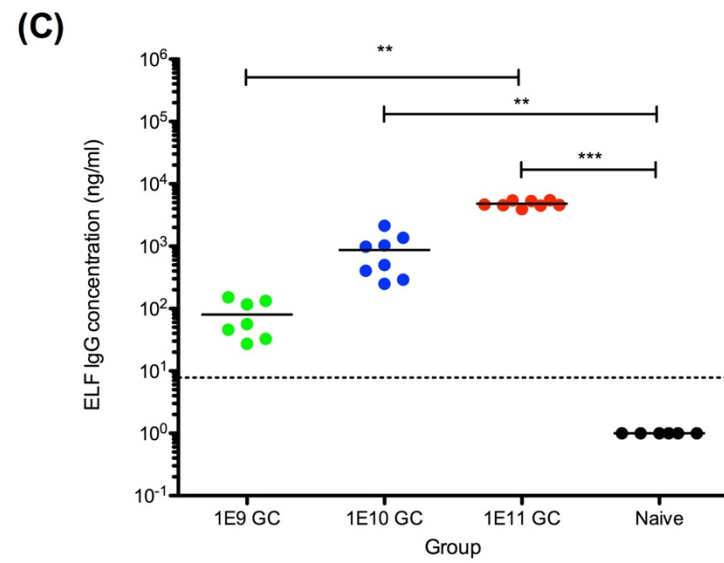
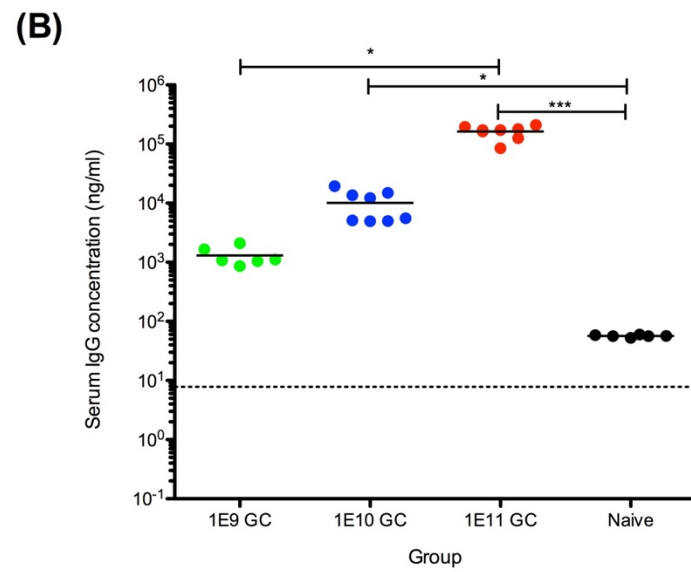
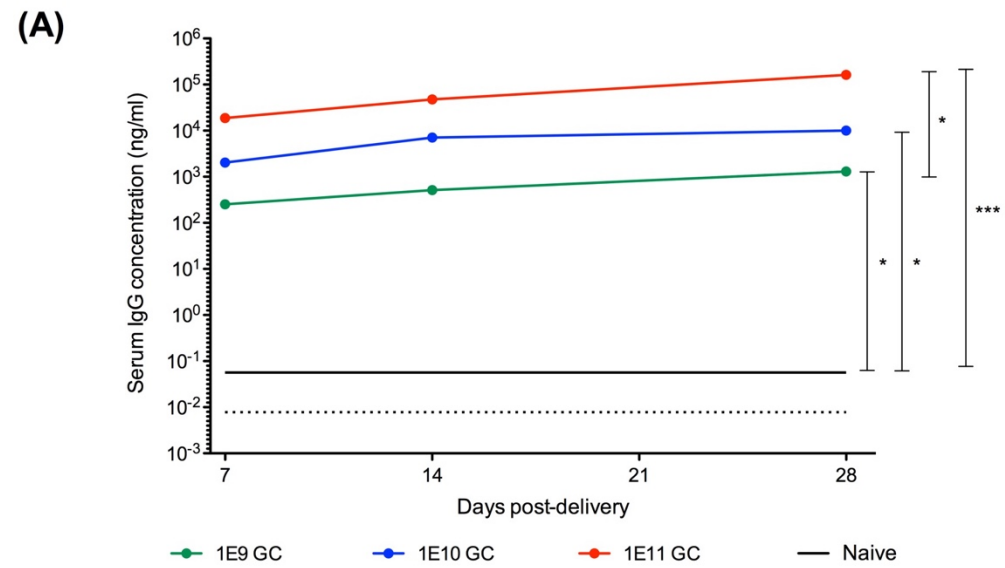


Figure 4.2 Expression of T1-3B following IM delivery of rAAV2/8.CASI.T13B in mice.

Mice (female BALB/c, 5 to 7 weeks old) were dosed IM with rAAV2/8.CASI.T13B (1E9 GC (green), 1E10 GC (blue) or 1E11 GC (red) per mouse, diluted in PBS) and compared with naïve mice (black) (n=8; Section 2.2.9.4) (Table 3.5). Serum was collected at the indicated time-points and mice were culled at day 28 post-delivery to obtain BALF (Section 2.2.9.7 and 2.2.9.6). Human IgG levels in the serum and the ELF were determined (Section 2.2.6.2). Data in (A) are presented as group mean and standard errors of the mean (SEM). The T1-3B levels on day 28 in the serum (B) and ELF (C), are shown. Each dot in (B) and (C) represents one animal, with horizontal bars representing group means. Dotted lines in the graphs represent the lowest detection limit of the assays; values below this line are extrapolated from the curve fit. Values plotted at 1 ng/mL in (C) indicated no detectable human IgG. A calculated p value of <0.05, <0.01 or <0.001 was deemed a significant difference and indicated with a single asterisk (*), double asterisks (**) or triple asterisks (***) on the graph.

(Figure 4.2 C). All treatment groups expressed significantly higher human IgG levels in the ELF than naïve mice (1E9 GC, $p<0.01$; 1E10 GC, $p<0.01$; 1E11 GC, $p<0.001$).

4.2.3 Expression of secreted reporter protein GLux following delivery of rSIV.F/HN to the lungs of mice.

The rSIV.F/HN vector system has not previously been used for the expression of antibody in mice. As a proof-of-principle study, a time-course experiment was performed to determine the dose, kinetics and duration of transgene expression using secretory reporter protein, GLux. Mice were dosed with 1E7 or 1E8 TU of rSIV.F/HN.hCEF.soGLux (Section 2.2.9.3) via intranasal instillation (IN) (Section 2.2.9.3) and GLux activity measured (Section 2.2.6.1) in serum collected on day 7, 14 and 28 post-delivery. Mice were culled at the end of the experiment (day 28) and BALF was collected to determine the GLux activity in the lung lumen (Section 2.2.9.6). Nasal instillation of rSIV.F/HN.hCEF.soGLux led to significant levels of GLux activity in the serum at all time-points assessed, from as early as 7 days post-administration, and persisting for the duration of the experiment (Figure 4.3 A). Mice receiving rSIV.F/HN.hCEF.soGLux at both doses expressed significantly higher GLux activity than the naïve control ($p<0.05$) (Figure 4.3 A). Although not statistically significant, GLux activity in mice receiving 1E8 TU showed a general trend of about three-fold higher than in mice receiving 1E7 TU. A substantial amount of GLux activity, which was about 100,000-fold (5-log) higher than in the serum, was detected in the ELF on day 28 (Figure 4.2 B). There was no apparent difference in GLux activity between the 1E7 TU and 1E8 TU treatment groups in the ELF. Overall, the results indicated that high levels of GLux can be found locally within the lung lumen (ELF), with lower but detectable levels distally in the systemic circulation (serum). Importantly, the general

expression profiles obtained demonstrated the proof-of-principle for the use of rSIV.F/HN to express antibody locally into the ELF and systemically into the circulation following lung delivery.

4.2.4 Expression of bnAb T1-3B following delivery of rSIV.F/HN to the mouse lung.

After confirming GLux expression mediated by rSIV.F/HN in the lung, concentrated rSIV.F/HN.hCEF.T13B expressing T1-3B bnAb (Section 3.2.6; Table 3.4) was produced (Section 2.2.5.1) and mice were dosed with 6E5, 6E6 or 5E7 TU via IN (Section 2.2.9.3). Serum was collected at day 7, 14 and 28 post-delivery and T1-3B expression was measured (Sections 2.2.9.7 and 2.2.6.2). At the end of the experiment (day 28), mice were culled and BALF was collected to determine the T1-3B levels in the lung lumen (Section 2.2.9.6). Nasal instillation of 6E6 and 5E7 TU of rSIV.F/HN.hCEF.T13B led to expression of significant levels of T1-3B in the serum at all time-points assessed, as early as 7 days post-administration, which persisted for the duration of the experiment (Figure 4.4 A). Serum T1-3B in mice receiving 6E5 TU was only detected from day 14 onwards. Serum T1-3B expression in the treatment groups showed an initial increment, reaching a plateau on day 14, ranging from 160 to 350 ng/mL. Only mice receiving the highest dose (5E7 TU) of rSIV.F/HN.hCEF.T13B expressed T1-3B levels significantly higher than the serum naïve control ($p<0.001$) (Figure 4.4 A). Higher levels of T1-3B, ranging from 340 to 2,300 ng/mL, approximately 2 to 10-fold higher than the serum T1-3B levels, were detected in the ELF on day 28 (Figure 4.2 B). All treatment groups expressed significantly higher T1-3B than the naïve mice (6E5 TU, $p<0.05$; 6E6 TU, $p<0.01$,

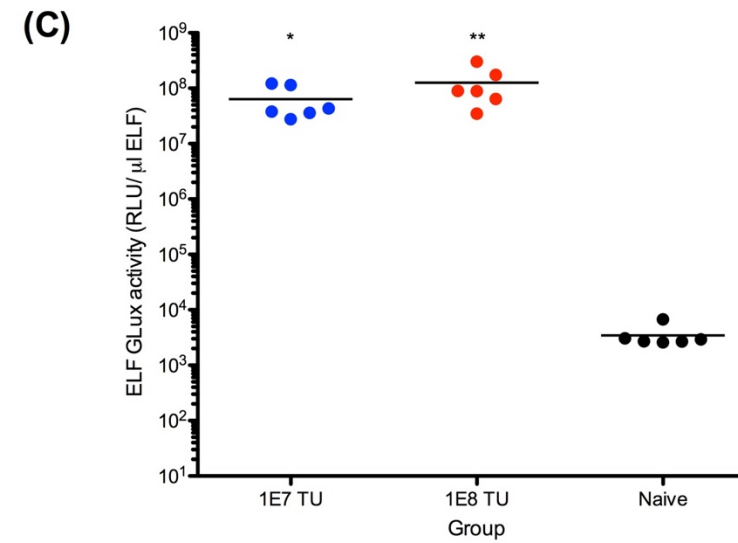
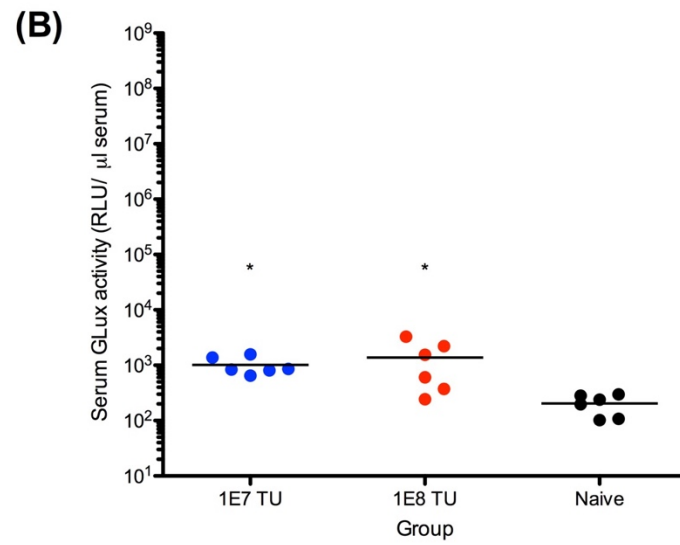
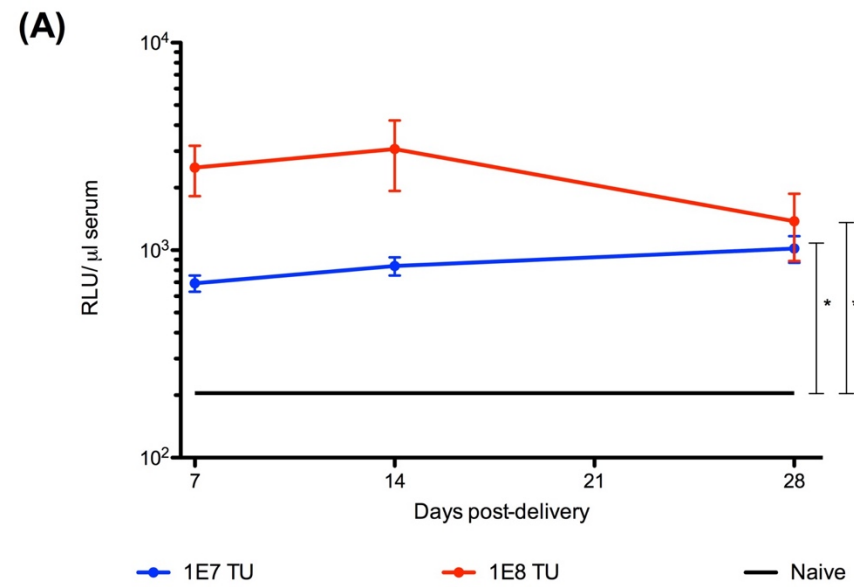
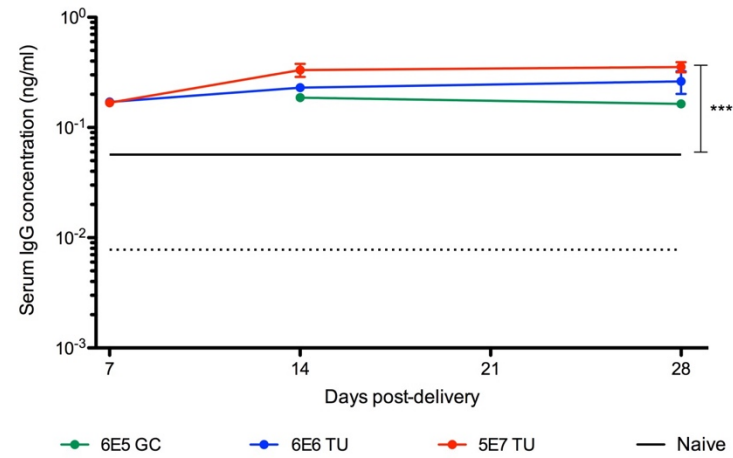
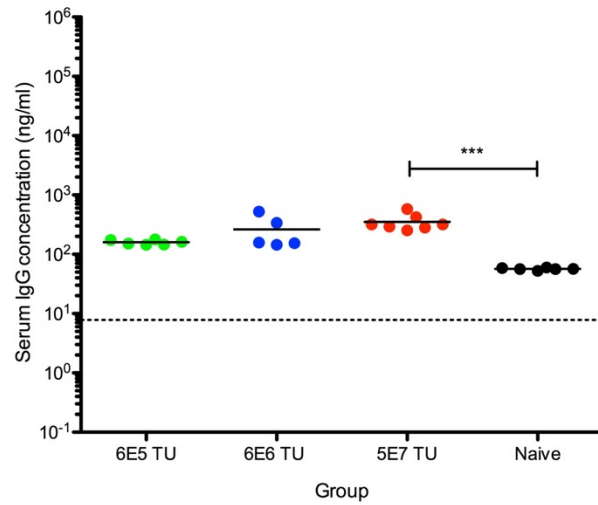


Figure 4.3 Expression of GLux following intranasal delivery of rSIV.F/HN.hCEF.soGLux in mice. Mice (female BALB/c, 5 to 7 weeks of age) were dosed via IN instillation with rSIV.F/HN.hCEF.soGLux (1E7 TU (blue), 1E8 TU (red) per mouse, diluted in FreeStyle media; n=6; Section 2.2.9.3) (Table 3.4) or remained naïve (black). Serum was collected at the indicated time-points and mice were culled on day 28 post-delivery to obtain BALF (Sections 2.2.9.7 and 2.2.9.6). GLux activity in serum and the ELF was measured (Section 2.2.6.1). Data in (A) are presented as group mean and standard errors of the mean (SEM). GLux activity on day 28 in the serum (B) and ELF (C) are shown. Each dot in (B) and (C) represents one animal; horizontal bars represent group means. Statistical significance was determined by Kruskal-Wallis test followed by Dunn's post-hoc test. A calculated p value of <0.05 or <0.01 was deemed a significant difference and indicated with a single asterisk (*) or double asterisks (**) on the graph.

(A)



(B)



(C)

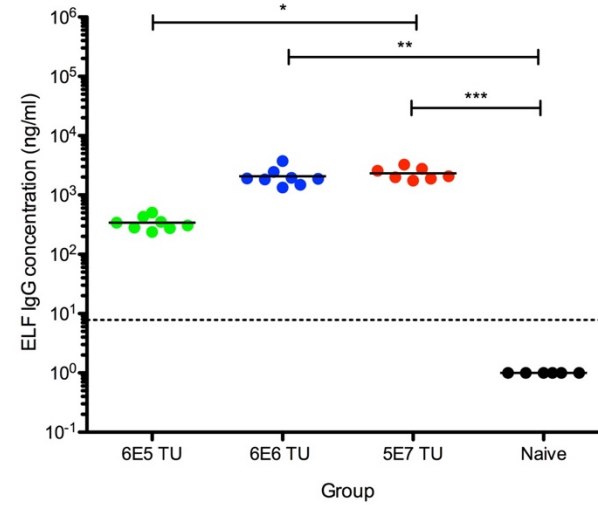


Figure 4.4 Expression of T1-3B following intranasal delivery of rSIV.F/HN.hCEF.T13B in mice. Mice (female BALB/c, 5 to 7 weeks of age) were dosed via IN instillation with rSIV.F/HN.hCEF.T13B (6E5 TU (green), 6E6 TU (blue), 5E7 TU (red) per mice, diluted in FreeStyle media; n=8; Section 2.2.9.3) (Table 3.4) or remained naïve (black). Serum was collected at the indicated time-points and mice were culled on day 28 post-delivery to collect BALF (Section 2.2.9.7 and 2.2.9.6). Human IgG levels in the serum and the ELF were determined (Section 2.2.6.2). Data in (A) are presented as group mean and standard errors of the mean (SEM). The T1-3B levels on day 28 in the serum (B) and ELF (C) are shown. Each dot in (B) and (C) represents one animal; horizontal bars represent group means. Dotted lines in the graphs represent the lowest detection limit of the assays and values below this line are extrapolated from the curve fit. Values plotted at 1 ng/mL in C indicated no detectable human IgG. A calculated p value of <0.05, <0.01 or <0.001 was deemed a significant difference and indicated with a single asterisk (*), double asterisks (**) or triple asterisks (***) on the graph.

Table 4.1 Summary of T1-3B expression levels mediated by rSIV.F/HN and rAAV2/8 in mice on day 28 post vector delivery

rSIV.F/HN			rAAV2/8		
Dose (TU)	Levels of T1-3B (ng/mL (SEM))		Dose (GC)	Levels of T1-3B (ng/mL (SEM))	
	Serum	ELF		Serum	ELF
6E5	161.45 (5.5)	341.6 (36.04)	1E9	1,309.66 (192.11)	80.4 (19.5)
6E6	231.1 (56.38)	2,067.6 (302)	1E10	9,406.26 (2164.8)	869.7 (264.4)
5E7	354.4 (42.63)	2,032.7 (393.4)	1E11	162,873 (16314)	4,799.28 (215.4)

ELF: epithelial lining fluid; SEM: standard error of the mean; TU: transducing unit, GC: genome copies; bnAb: broadly neutralising antibody (T1-3B)

5E7 TU, $p < 0.001$). Interestingly, there was no apparent difference in the level of T1-3B expression between the treatment groups in the serum or the ELF.

4.2.5 Determination of the dilution of murine epithelial lining fluid (ELF) in broncho-alveolar lavage fluid (BALF)

The lung epithelia are the main site of entry for influenza virus in the lung, and therefore understanding the level of neutralising bnAb present in the ELF is an important indicator of first line protection for influenza infection. Broncho-alveolar lavage (BAL), and the subsequent collection of lavage fluid, is routinely used to sample lung cells and to evaluate expression of transgene proteins secreted into the murine lung (Griesenbach et al., 2011, Davies et al., 2012). However, BALF collection can be very variable, which makes it difficult to reliably assess levels of secreted protein present in the lung lumen. In addition, BAL dilutes the ELF hugely, depending on the volume used for the BAL procedure. Therefore, to more reliably measure transgene protein expressed in the ELF, it is necessary to calculate the degree of dilution of ELF when performing BAL; this should more accurately reflect the local concentration in the lung lumen and its therapeutic relevance.

The most commonly used method for determining the dilution factor of BALF is to perform a urea assay (Rennard, Basset, & al, 1986). Urea is a small organic molecule that readily diffuses throughout the body compartments, and is thus present at a constant concentration in all body compartments (Baughman, 2007). By measuring the concentration of urea in serum and the BALF sample of the same mouse, the degree to which the ELF has been diluted can be determined and used to calculate the actual protein concentration in the ELF.

Serum and BALF collected from mice (Sections 2.2.9.7 and 2.2.9.6, respectively) and the urea dilution factor of the BALF were used to determine the dilution factor for the GLux (3 mL BALF collected; Section 2.2.9.6) and bnAb studies (1 mL BALF collected; Section 2.2.9.6). The average dilution factor of the BALF samples in the GLux studies was 46-fold whereas for the bnAb studies, the dilution factor was 28-fold (Table 4.1). These values were used to correct the protein levels in the BALF samples in the respective studies.

4.3 Discussion

4.3.1 Lung and muscle bnAb expression mediated by viral vectors

Delivery of both rSIV.F/HN and rAAV2/8 resulted in persistent transgene expression *in vivo*. The data show that GLux expression persisted for at least 1 month following a single administration of rSIV.F/HN.hCEF.soGLux (Figure 4.3) and rAAV2/8.CAS1.soGLux (day 28; Figure 4.1). Furthermore, in independent experiments, administration of rAAV2/8.CAS1.soGLux and rSIV.F/HN.hCEF.soGLux resulted in GLux expression for at least 1 year (Agata Antepowicz; personal communication). In the serum, both GLux and IgG expression following rAAV2/8 IM delivery showed a trend for dose-related increments (Figures 4.1 and 4.2). However, for rSIV.F/HN delivered to the lung, both GLux and IgG expression seem to plateau at a higher dose (Figures 4.3 and 4.4). Although an apparent decline in GLux expression may be observed between day 14 and 28 in mice receiving the highest dose (1E8 TU) of rSIV.F/HN.hCEF.GLux (Figure 4.3 A), and a similar decline was seen in independent experiments (Agata Antepowicz, personal communication), these trends were not statistically significant.

Table 4.2 Mean dilution factor of broncho-alveolar lavage fluid based on urea concentration

(A) *Gaussia* luciferase studies

	Serum (nM)	BALF (nM)	Dilution factor
N =	10	10	-
Mean	526.94	12.47	46.32
SD	91.52	3.79	17.09

(B) Antibody studies

	Serum (nM)	BALF (nM)	Dilution factor
N =	10	10	-
Mean	483.97	18.85	28.0
SD	119.65	5.79	10.42

BALF: broncho-alveolar lavage fluid; SD: standard deviation

Any potential decline in expression is unlikely to be due to an immune response, because if this were the case, one might expect the GLux expression to be eliminated altogether. One concern is that the very high levels of GLux expression in the lung epithelia (around five orders of magnitude above background; Figure 4.3), might ultimately exceed the cells' capacity for protein production and result in toxicity. It is known, for example, that the protein-folding capacity of the endoplasmic reticulum (ER) can be exceeded, leading to accumulation of unfolded and misfolded proteins triggering the unfolded protein response (UPR) due to increased ER stress (Schroder and Kaufman, 2005). The ER stress response, in turn, results in attempts to restore cell homeostasis to preserve the lung epithelia, but if all attempts to restore homeostasis fail, apoptosis or autophagy can ensue (reviewed in (Hetz, 2012)).

4.3.2 The expression route of GLux and bnAb following delivery of viral vectors

Since rSIV.F/HN and rAAV2/8 were administered via different routes, the transgene proteins are distributed via different pathways. The proposed expression and distribution routes of IgG and GLux following rAAV2/8 IM or rSIV.F/HN IN delivery are illustrated in Figure 4.5. For rAAV2/8 administered IM, transgene proteins expressed in the muscle cells are secreted into the systemic circulation, then transported into the capillaries of the lung and cross the lung epithelia into the lung lumen (detected in the ELF). Both GLux and IgG are transported across the lung epithelia, but via different pathways. The partitioning of GLux is not well understood, but one study has proposed that movement of GLux across epithelia is mediated via non-specific pinocytosis (El-Amouri et al., 2013). On the other hand, transcytosis of human IgG across lung epithelial cells is receptor-specific; human IgG enters cells via pinocytosis or

endocytosis, then binds to the rodent neonatal-Fc receptor (FcRn) (Ober et al., 2001) present on the endosomal membrane (Tesar et al., 2006) at acidic pH (pH <6.5). The IgG is then either transcytosed across to the apical membrane and released from the FcRN receptor into the pH-neutral lung lumen, or recycled basolaterally into the blood circulation (Raghavan et al., 1995, Ober et al., 2001). Interestingly, despite these possible differences, the secreted portion between the lung lumen and serum of GLux and IgG following IM delivery of rAAV2/8 is similar (Figures 4.1 B & C and 4.2 B & C).

For rSIV.F/HN, transgene proteins are produced in the polarised lung epithelia, and subsequently detected in the lung lumen (ELF) and serum. Since both GLux and human IgG are ectopic proteins in the lung epithelia, their secretion preference/mechanism is unknown. Mediated by FcRN, transportation of IgG in polarised cells occurs bidirectionally (Dickinson et al., 1999, Guo et al., 2016). The FcRN can bind free IgG in the lung lumen and transport it basolaterally into the blood circulation across the lung epithelia. IgG detected in the serum can therefore be a result of direct secretion from the lung epithelia via the basolateral secretion pathway or FcRN-mediated retro-transportation of IgG from the lung lumen into the serum across the polarised lung epithelia, or via both pathways. However, it is not known whether GLux in the serum was a result of direct basolateral secretion from the lung epithelia, or a result of diffusion from the lung lumen.

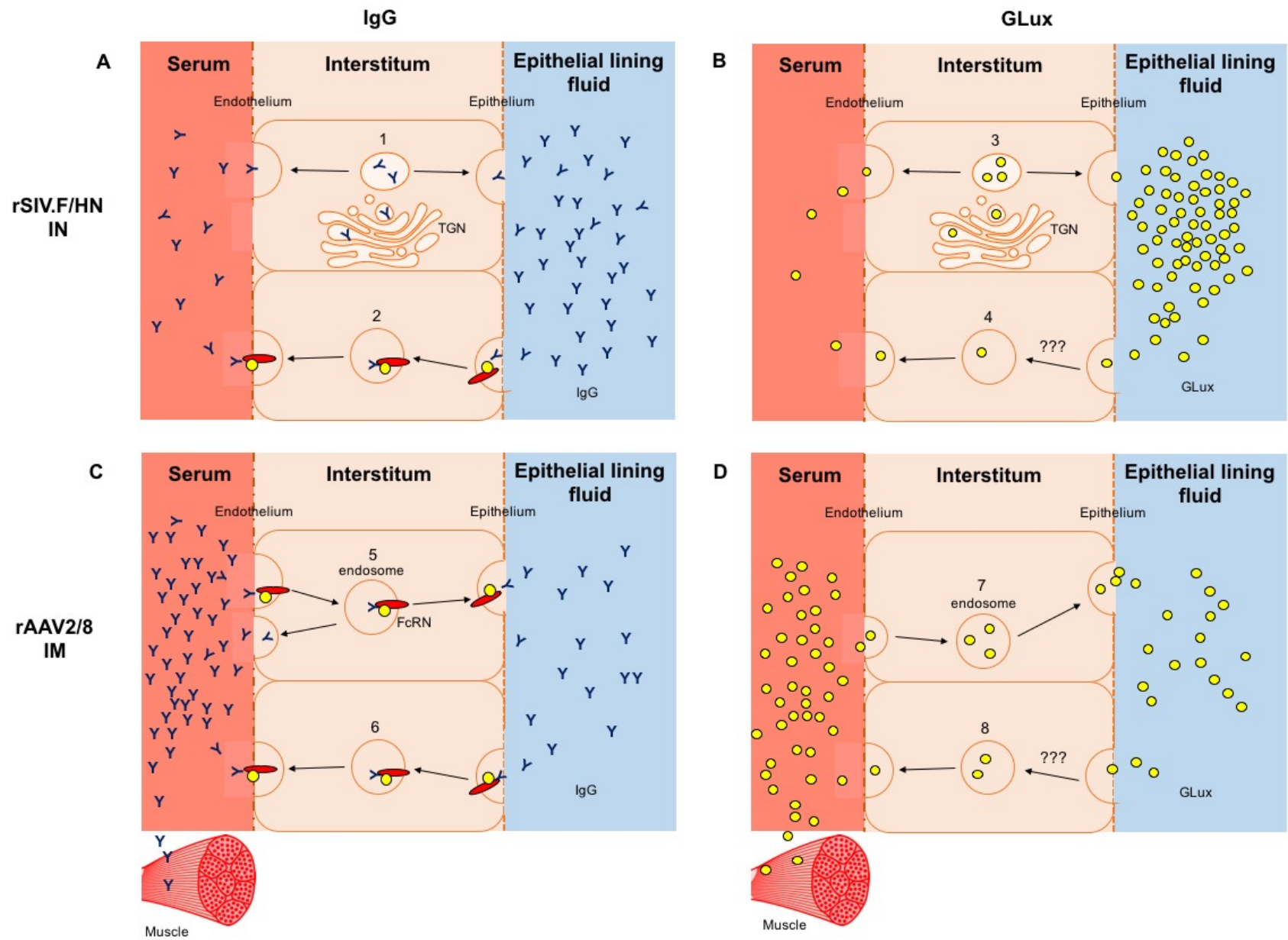


Figure 4.5 Proposed expression and distribution routes of IgG and GLux following rSIV.F/HN IN delivery or rAAV2/8 IM delivery. Recombinant SIV.F/HN transduces the lung epithelia to produce IgG (A) or GLux (B). Fully form IgG (1) or GLux (3) in the lung epithelia are being sorted into apical or basolateral secretory pathway in the *trans* Golgi network (TGN). (2) Transport of IgG across polarised cells is bidirectional (Dickinson et al., 1999, Guo et al., 2016), IgG can be transported from the apical surface of a polarised cell into the basolateral compartment mediated by FcRN (Dickinson et al., 1999, Guo et al., 2016). GLux detected in the serum could be the result of basolateral expression from the lung epithelia (3) or the result of retro-transportation from the lung lumen into the serum (4). Recombinant AAV2/8 transduces the muscle cells to produce IgG (C) or GLux (D) which is secreted into the serum and distributed in the systemic circulation. (5) IgG is taken up into the lung epithelia via pinocytosis or endocytosis. In the acidic endosome, IgG binds to FcRN and is transcytosed across to the apical surface, or recycled basolaterally into the serum. (6) Since IgG transportation in polarised cells may occur bidirectionally, IgG in the lung lumen can be retro-transported into the serum mediated by FcRN. (7) GLux is taken up into the lung epithelia via pinocytosis and transcytosed across the epithelia into the lung lumen. (8) It is unknown if GLux can be retro-transported from the apical compartment into the basolateral compartment across the polarised epithelia. The proposed mechanisms do not take account of uptake preference of IgG and GLux from serum into other tissue compartments that might affect the levels of IgG and GLux in the serum.

The uptake and retention of serum GLux and IgG into different compartments or organs, which might affect their levels in the serum is unclear. The difference in secretion/ transportation pathway and the secreted portion between IgG and GLux in the serum and lung lumen (Figures 4.3 B & C and 4.4 B & C) indicate that GLux might serve as a qualitative, but not quantitative, reporter protein to study antibody expression mediated by lung gene transfer vectors.

4.3.3 Signal sequence and expression of ectopic proteins in the lung epithelia

One possible reason for the difference in expression of GLux (Figure 4.3) and IgG (Figure 4.4) from the rSIV.F/HN transduced lung epithelia is the use of different signal sequences, required to direct newly synthesised proteins into the ER lumen for secretion (illustrated in Figure 3.15). Signal sequences (typically 20 to 30 amino acids in length) consist of a basic N domain, a hydrophobic H domain and a polar C domain, and this tripartite structure is shared between eukaryotes and prokaryotes (Hegde and Bernstein, 2006). The translocation of secretory proteins into the ER lumen, guided by the signal sequence, has been identified as a bottleneck in secretory protein expression (Zhang et al., 2005, Knappskog et al., 2007, Kober et al., 2013). Signal sequences are highly interchangeable between different species (Knappskog et al., 2007), which allows the use of non-native signal peptide to increase protein secretion. In this study, the single-ORF IgG construct was prefixed with the HGH signal sequence (Section 3.2.1) whereas the GLux was prefixed with the native GLux signal sequence. Since both GLux and IgG are ectopic proteins in the lung epithelia, use of different signal sequence could influence their

expression. Additionally, it is unknown if the same signal sequence would function equally well in the different lung epithelial cell types transduced. However, it is highly unlikely that signal sequences influence the directional secretion preference of protein as the sorting process of protein into apical and basolateral transport vesicles occurs at the TGN (reviewed in (Anitei and Hoflack, 2011)). It may be worth investigating the use of different signal sequences, such as the native GLux signal sequence, to increase IgG expression into the lung lumen.

4.3.4 Conclusion

Comparison of the two vector delivery strategies at their respective maximum doses, showed that rSIV.F/HN resulted in approximately 350 ng/mL of T1-3B in the serum, which was about 600-fold lower than rAAV2/8 (100 to 200 µg/mL), and 2.3 µg/mL of T1-3B in the ELF, which was around two-fold lower than rAAV2/8 (4.8 µg/mL) (Table 4.1). The respiratory tract is the target for influenza virus infection and it is generally accepted that antibody in the lung lumen plays an important role in preventing influenza infection. While the *in vivo* minimum protective serum concentration of T1-3B against lethal influenza challenge is unknown, the EC₅₀ of T1-3B against wild type A/PR/8/34 H1N1 influenza virus in the *in vitro* MN assay is around 1 µg/mL (Alain Townsend, personal communication). The T1-3B levels in the ELF following IN delivery of rSIV.F/HN is around two-fold higher than the *in vitro* EC₅₀, thus, a degree of protection against influenza is expected. The studies presented in this chapter may be the first to demonstrate that rSIV.F/HN can be used to transduce the lung epithelia for production of bnAb, both systemically and locally into the lung lumen. These

findings support the initiation of influenza challenge studies to determine the protection mediated by rSIV.F/HN; and this work is discussed in Chapter 5. Although specifically focused on expressing anti-influenza antibodies as part of a passive immunisation strategy, these findings also support the possibility of using the rSIV.F/HN vector to express antibodies against other infectious diseases, particularly those infecting the respiratory system.

Chapter 5 Single and repeat administration of rSIV.F/HN for passive immunisation against influenza in a mouse model.

5.1 Introduction

5.1.1 Mouse model for influenza challenge study

In Chapter 4, the ability of rSIV.F/HN to direct expression of IgG, both locally and systemically, following lung delivery in a mouse model was demonstrated and these promising results supported further experiments to test for protection against challenge with influenza. One of the most commonly used methods in non-clinical research on influenza vaccines and antiviral drugs is to study potential efficacy in animal models of influenza challenge. Among the many available animal models, mice are the most widely used largely for reasons of simplicity and lower costs, compared with larger animal models. Although mice are not naturally susceptible to infection by human influenza strains, several mouse-adapted human influenza viruses have been developed; some early human isolates, such as H1N1 A/PuertoRico/8/1934 (A/PR/8) and H1N1 A/Wisconsin/1933 (Smith et al., 1995), have become the prototype laboratory influenza strains of choice in the mouse model (Bouvier and Lowen, 2010). A number of distinct lineages of A/PR/8 H1N1 with different levels of virulence in mice have been used; the A/PR/8 strain of the Cambridge lineage (used in this study) is generally more virulent than those of the Mount Sinai lineage (Grimm

et al., 2007). In this study, BALB/c mice and A/PR/8 (Cambridge) (Table 2.4) were used to assess protection by antibody expressed from rSIV.F/HN.

5.1.2 Aim

The aim of the studies in this chapter was to investigate protection against influenza in a mouse model, following lung delivery of rSIV.F/HN expressing bnAb.

5.2 Results

5.2.1 Confirmation of complete protection against influenza virus challenge in mouse model mediated by rAAV2/8.

Recombinant AAV2/8 vector (dose 1E11 GC) encoding a previously published bnAb F10 has been shown to confer complete protection in mice against 10 LD₅₀ of A/PR/8 (lineage unknown) following IM delivery (Balazs et al., 2013). The efficacy of the novel bnAb T1-3B used here (Table 2.5) was first tested using the benchmark vector, rAAV2/8.CASI. Recombinant AAV2/8.CASI.T13B virus was generated (Section 2.2.5.2) and the antibody expression profile following IM delivery was determined as described earlier (Section 4.2.2). Mice were given doses of 1E9, 1E10 or 1E11 GC of rAAV2/8.CASI.T13B via IM injection (Section 2.2.9.4) and were then challenged with 10,000 TCID₅₀ A/PR/8 (Table 2.4; equivalent to 150 LD₅₀) 28 days after rAAV2/8 delivery. Body weight was monitored up to 14 days and mice that succumbed to $\geq 20\%$ weight loss were humanely sacrificed. A group of mice that remained naïve was also included as a negative control. All naïve mice, and all those receiving 1E9 GC

of rAAV2/8.CASI.T13B, succumbed to $\geq 20\%$ weight loss by day 5 post-influenza challenge (Figure 5.1). Approximately 20% of mice receiving the mid dose of 1×10^{10} GC of rAAV2/8.CASI.T13B survived, whereas 1×10^{11} GC of rAAV2/8.CASI.T13B prevented weight loss and yielded complete protection of all mice from influenza challenge ($p < 0.01$; log-rank test) (Figure 5.1). The results showed that complete protection against lethal influenza challenge in mice was achieved with the novel bnAb T1-3B, using the benchmark rAAV2/8 vector. In fact, here, an approximately 15-fold higher influenza challenge dose was used compared with the influenza challenge dose used in the study by Balazs *et al* (10 LD_{50}) (Balazs *et al.*, 2013), demonstrating that the T1-3B bnAb is at least as good as, if not better than, the published F10 bnAb in providing protection against influenza when delivered using rAAV2/8.

5.2.2 Protection mediated by a single dose of rSIV.F/HN against 1,000 and 10,000 TCID₅₀ influenza challenge in mice.

Recombinant rSIV.F/HN.hCEF.T13B was generated (Section 2.2.5.1) and bnAb expression was validated *in vitro* (Section 3.2.6) and *in vivo* (Section 4.2.4). Mice were given doses of 6×10^5 , 6×10^6 and 5×10^7 TU of rSIV.F/HN.hCEF.T13B diluted in FreeStyle via nasal instillation (Section 2.2.9.3). Since weight loss was the primary readout following a lethal dose of influenza challenge, the effect of IN delivery of rSIV.F/HN on mouse weight was first measured. Body weight was monitored for 7 days following IN delivery of rSIV.F/HN at doses 6×10^5 , 6×10^6 and 5×10^7 TU; and it was confirmed that there was no weight loss or apparent distress in the mice (Figure 5.2).

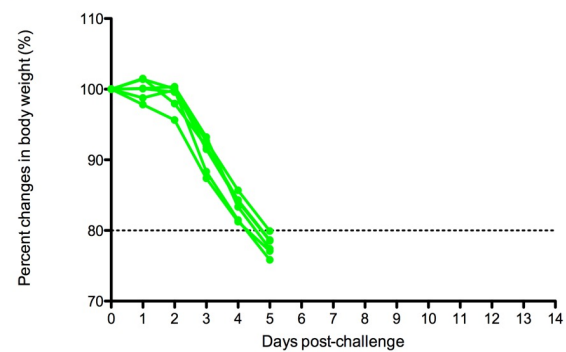
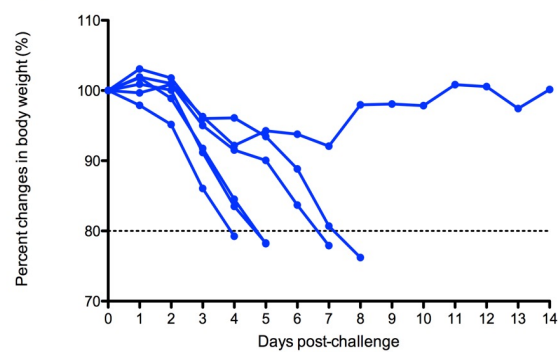
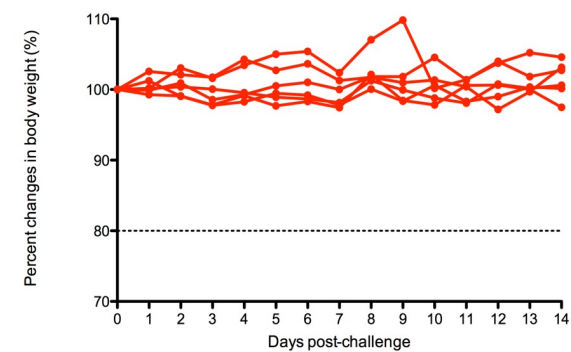
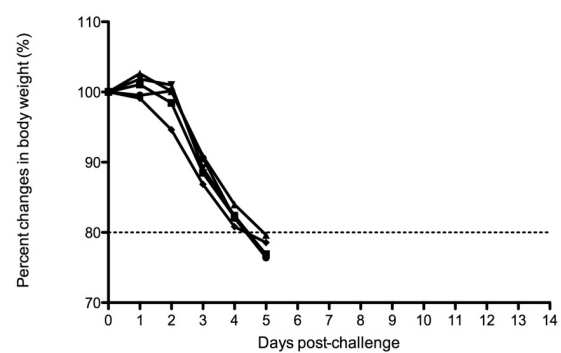
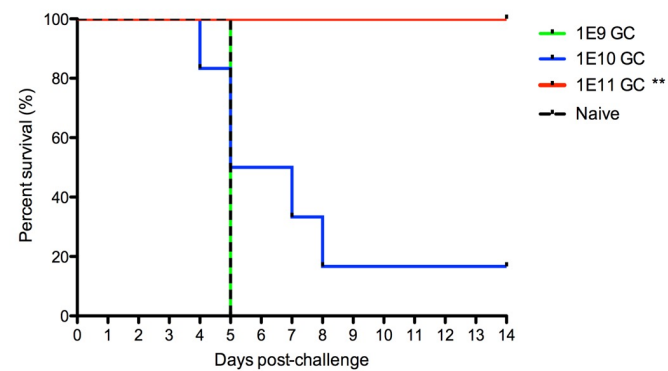
A**B****C****D****E**

Figure 5.1 Effect of rAAV2/8.CASI.T13B on protecting mice against 10,000 TCID₅₀ influenza challenge. Mice (female BALB/c, 5 to 7 weeks) received rAAV2/8.CASI.T13B (Table 3.5), 4 weeks prior to intranasal infection with 10,000 TCID₅₀ (150 LD₅₀) of A/PR/8/1934 H1N1, doses of 1E9 (green), 1E10 (blue) or 1E11 (red) GC per mouse in PBS or remained naïve (black) (n=5 for naïve group and n=6 for the other groups; Section 2.2.9.4). The weight loss of each mouse was monitored for the indicated time period (A to D) and when the percentage change in body weight was $\geq 20\%$ of the pre-challenge value the mice were euthanised, as indicated in the Kaplan-Maier plot (E). Each line in A to D represents the body weight changes of individual mice in the group. Statistical significance was determined by log-rank test. A calculated p value of <0.01 was deemed a significant difference and indicated with a double asterisk (**).

The same groups of mice were then challenged with 10,000 TCID₅₀ (150 LD₅₀) of A/PR/8 28 days after rSIV.F/HN delivery and body weight was monitored for up to 14 days; mice that succumbed to $\geq 20\%$ weight loss were humanely sacrificed. A group of mice receiving affinity-purified T1-3B bnAb (10 mg/kg) via IP injection (Section 2.2.9.5) 24 hours pre-challenge was included as a control. All naïve mice succumbed to $\geq 20\%$ weight loss by day 5 post-influenza challenge and were humanely sacrificed. Similarly, all mice in the 6E5 and 6E6 TU dose groups succumbed to 20% weight loss and were humanely sacrificed. In contrast, 20% (one out of five) of the mice in the high dose (5E7 TU) group survived (Figure 5.3). Although complete protection was not achieved, all mice receiving rSIV.F/HN.hCEF.T13B experienced a delayed onset of weight loss and showed significantly prolonged survival compared with naïve mice (6E5 TU, $p < 0.05$; 6E6 TU, $p < 0.01$; 5E7 TU, $p < 0.05$; log-rank test).

In order to study the extent of protection mediated by rSIV.F/HN.hCEF.T13B, a similar challenge experiment was carried out with a 10-fold lower influenza challenge dose (1,000 TCID₅₀, 15 LD₅₀). All naïve mice succumbed to $\geq 20\%$ weight loss by day 6 post-influenza challenge (Figure 5.4). Doses of 6E5 and 5E7 TU of rSIV.F/HN.hCEF.T13B conferred 20% and 50% protection, respectively, whereas, surprisingly, none of the mice in the 6E6 TU group survived (Figure 5.4). Although complete protection was not achieved, all mice receiving rSIV.F/HN.hCEF.T13B experienced a delayed onset of weight loss and significantly prolonged survival compared with naïve mice (6E5 TU, $p < 0.001$; 6E6 TU, $p < 0.01$; 5E7 TU, $p < 0.001$; log-rank test). Overall, these

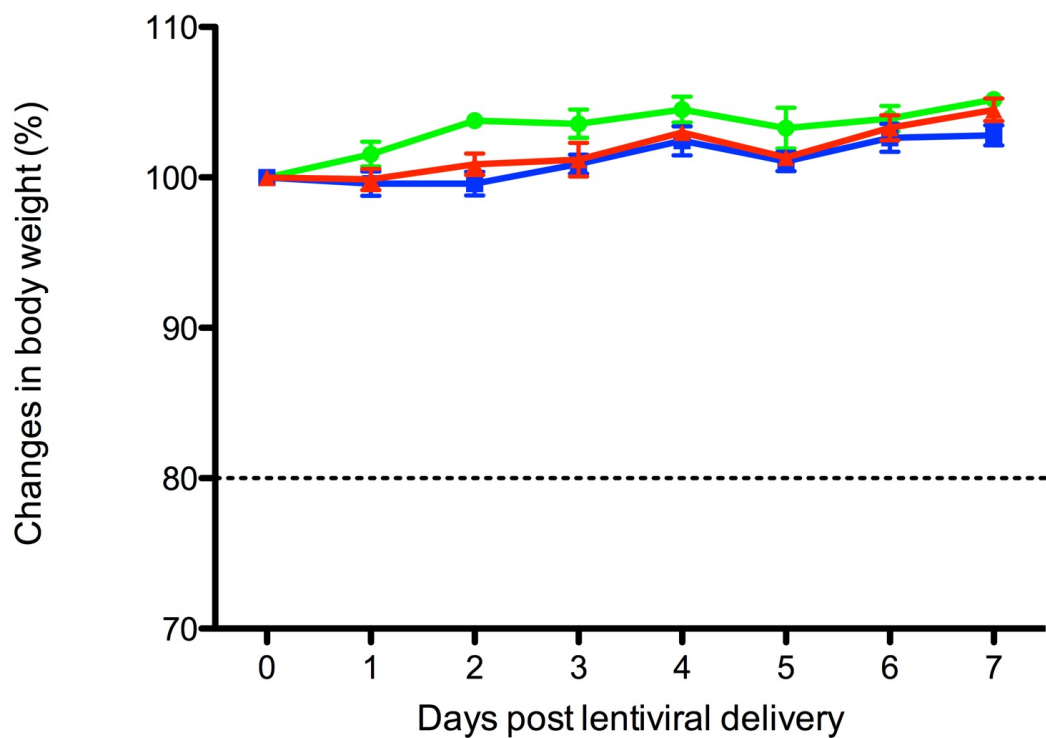


Figure 5.2 Effect of intranasal delivery of rSIV.F/HN.hCEF.T13B on weight loss in mice. Mice (female BALB/c, 5 to 7 weeks, n=6 per group) were dosed intranasally with rSIV.F/HN.hCEF.T13B (6E5 (green), 6E6 (blue) or 5E7 (red) TU per mouse) (Table 3.4) diluted in FreeStyle or remained naïve to treatment (black); Section 2.2.9.3). The body weight of each mouse was monitored for the indicated time period and data are presented as the average body weight change in each group. Error bars represent the standard error of the mean (SEM).

data demonstrated that lung delivery of rSIV.F/HN.hCEF.T13B can provide passive immunity against influenza infection (up to 50% protection when given as 5E7 TU), although at a lower level than observed with rAAV2/8 dosed IM (Section 5.2.1).

5.2.3 Repeat administration of rSIV.F/HN to improve protection against influenza challenge in mice

It has been previously shown that daily repeated IN administration of rSIV.F/HN in mice can lead to an additive, dose-related increase in transgene expression levels, and that repeated monthly administration was feasible without loss of gene expression (Mitomo et al., 2010a, Griesenbach et al., 2012b). In order to improve the expression of bnAb, and determine if this could lead to improved protection against influenza, a strategy of daily repeated administration was employed. Mice were dosed with a single or ten daily doses of rSIV.F/HN.hCEF.T13B as summarised in Table 5.1. Six mice from each group (two from the 1E8 TU x 10 dose group) were culled on day 28 following the final administration of rSIV.F/HN.hCEF.T13B to obtain BALF (Section 2.2.9.6) and the bnAb levels in the BALF were measured (Section 2.2.6.1). A further six mice from each group were challenged with 1,000 TCID₅₀ (15 LD₅₀) of A/PR/8 (Section 2.2.9.3) and body weights were monitored for up to 14 days; mice that succumbed to ≥20% weight loss were humanely sacrificed. A group of mice that received no rSIV.F/HN (naïve) was also included.

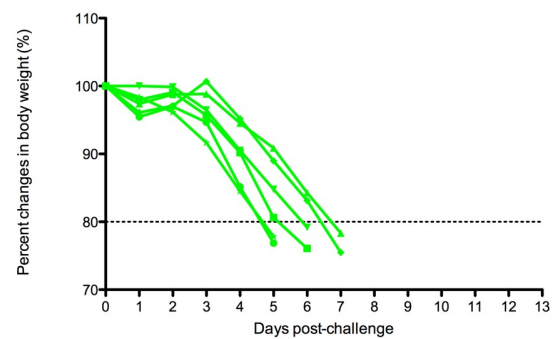
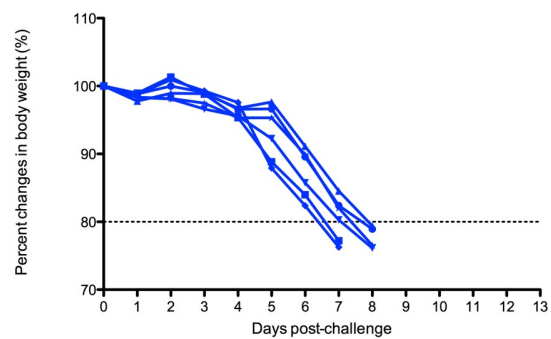
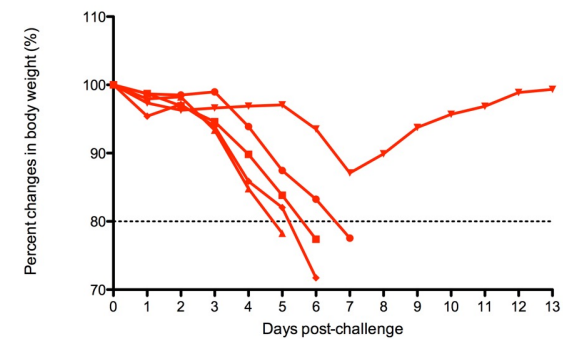
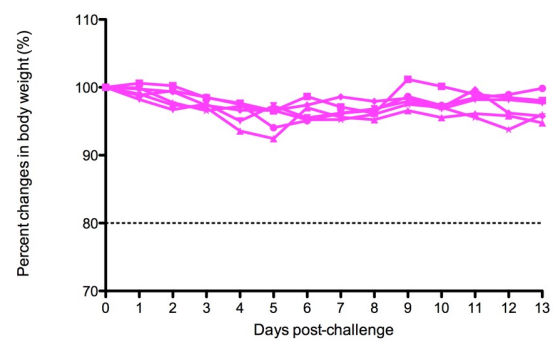
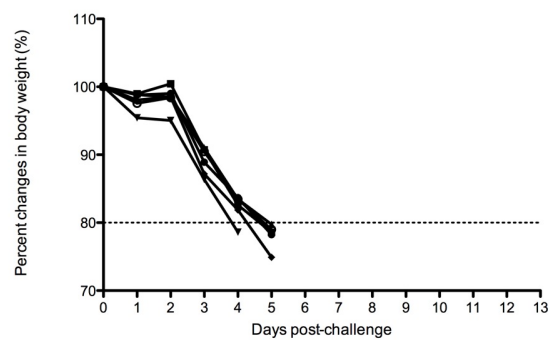
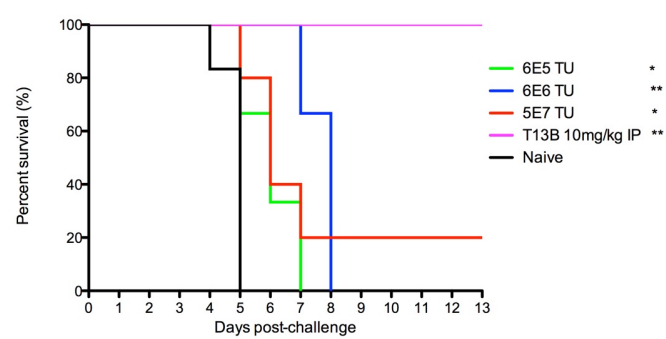
A**B****C****D****E****F**

Figure 5.3 Effect of rSIV.F/HN.hCEF.T13B on protecting mice against 10,000 TCID₅₀ influenza challenge. Mice (female BALB/c, 5 to 7 weeks) received rSIV.F/HN.hCEF.T13B (Table 3.4), 4 weeks prior to intranasal infection with 10,000 TCID₅₀ (150 LD₅₀) of A/PR/8/1934 H1N1, doses of 6E5 (green), 6E6 (blue) or 5E7 (red) TU per mouse in FreeStyle, or remained naïve (black) (n=5 for 5E7 TU group and n=6 for the other groups; Section 2.2.9.3). The weight loss of each mouse was monitored for the indicated time period (A to E) and when the percentage change in body weight was $\geq 20\%$ than the pre-challenge value the mice were euthanised, as indicated in the Kaplan-Maier plots (F). Each line in A to E represents the body weight changes of individual mice in the group. Statistical significance was determined by log-rank test. A calculated p value of <0.05 or <0.01 was deemed a significant difference and indicated with single asterisk (*), or double asterisks (**) respectively.

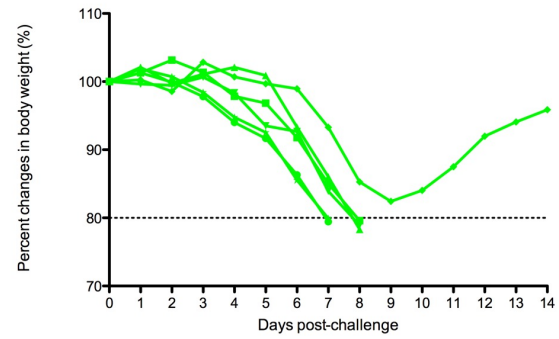
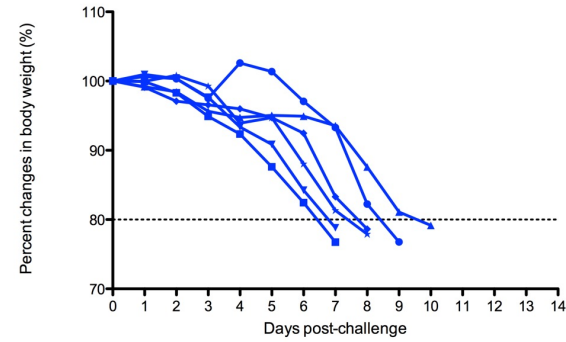
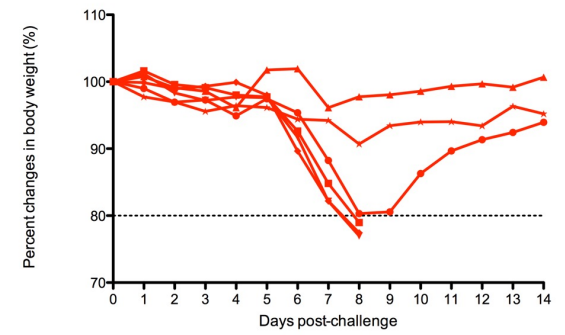
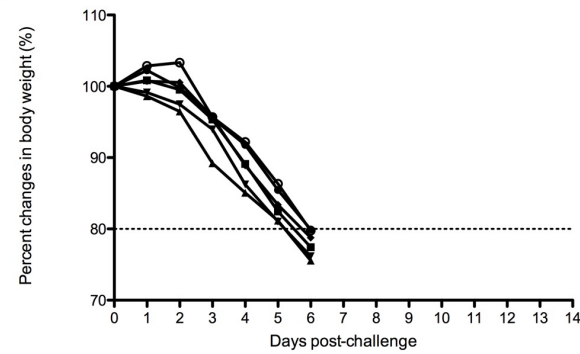
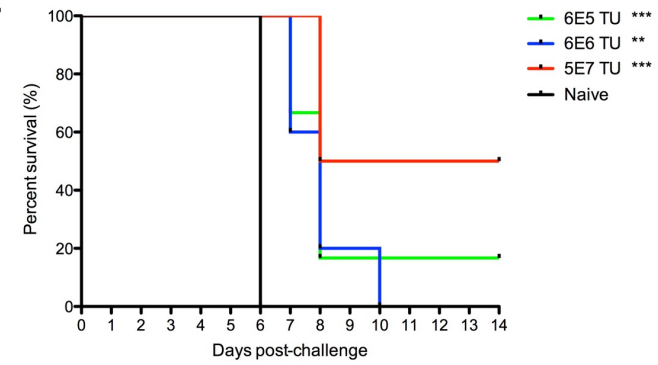
A**B****C****D****E**

Figure 5.4 Effect of rSIV.F/HN.hCEF.T13B on protecting mice against 1,000 TCID₅₀ influenza challenge. Mice (female BALB/c, 5 to 7 weeks) received rSIV.F/HN.hCEF.T13B (Table 3.4), 4 weeks prior to intranasal infection with 1,000 TCID₅₀ (15 LD₅₀) of A/PR/8/1934 H1N1, doses of (6E5 (green), 6E6 (blue) or 5E7 (red) TU per mouse in FreeStyle and naïve (black); n=6; Section 2.2.9.3). The weight loss of each mouse was monitored for the indicated time period (A to D) and mice were euthanised when the weight loss $\geq 20\%$ than the pre-challenge body weight indicated in the Kaplan-Maier plot (E). Each line in A to D represents the body weight changes of individual mice in the group. Statistical significance was determined by log-rank test. A calculated p value of <0.01 or <0.001 was deemed a significant difference and indicated with double asterisks (**) or triple asterisks (***) respectively.

Daily repeat dosing of 1E6 TU resulted in a significant increase in bnAb expression ($p<0.01$) (Figure 5.5) and higher survival compared with a single 1E6 TU dose (Figure 5.6; survival: 1E6 TU, 0%; 1E6 TU x 10 doses, 16.6%) (Figure 5.6). However, there was no significant difference in bnAb levels between the single dose and repeat dosing groups for both 1E7 TU and 1E8 TU doses (Figure 5.5). Surprisingly, bnAb levels appeared to be lower in the 1E8 repeat dose group compared with the 1E8 single dose group; statistical analysis was not performed due to the small sample size ($n=2$) in the 1E8 TU x 10 doses group (Figure 5.5). When comparing groups of mice receiving the same total dose, the bnAb levels in mice receiving 1E7 and 1E8 TU via a single dose appeared to show a trend for higher bnAb compared with mice receiving the equivalent dose via repeat dosing, but this was not statistically significant (Figure 5.5 (mean $\pm$ SEM): 1E7 TU, 890.2 ng/mL $\pm$ 148; 1E6 TU x 10, 764.1 ng/mL $\pm$ 155.7; 1E8 TU, 2,618 ng/mL $\pm$ 472; 1E7 TU x 10, 1,286.6 ng/mL $\pm$ 116).

Although complete influenza protection was not achieved, groups of mice receiving 1E7 TU, 1E8 TU, 1E6 TU x 10 doses and 1E8 TU x 10 doses of rSIV.F/HN showed significantly better survival compared with naïve (Figure 5.6: 1E7 TU, $p<0.001$; 1E8 TU, $p<0.01$, 1E6 TU x 10 doses, $p<0.01$; 1E8 TU x 10 doses, $p<0.01$; log-rank test). Considering groups of mice that received the same total dose, mice receiving 1E7 and 1E8 TU via a single dose appeared to show better survival compared with mice receiving an equivalent dose via repeat dosing (Figure 5.6 (survival): 1E7 TU, 33.3%; 1E6 TU x 10 doses, 16.7%) (Figure 5.6 (survival): 1E8 TU, 33.3%; 1E7 TU x 10 doses, 0%). Repeated

administration of a lower dose (1E6 TU) appeared to confer partial protection (Figure 5.6: 16.67%) against lethal influenza challenge, while repeat dosing 1E7 TU or 1E8 TU did not confer protection (0%) in mice against 1,000 TCID₅₀ influenza challenge. Overall, a single dose of 1E7 TU and 1E8 TU provided the best protection in mice against 1,000 TCID₅₀ influenza challenge (Figure 5.6: survival, 33.3%).

5.3 Discussion

5.3.1 Production of monoclonal antibody in the lung epithelia for influenza protection mediated by rSIV.F/HN

The aim of this chapter was to investigate the efficacy of rSIV.F/HN as a lung gene transfer vector to express bnAb and provide passive immunisation against influenza in a mouse model. Although complete protection was not achieved, mice receiving rSIV.F/HN.hCEF.T13B experienced a delayed onset of weight loss and showed significantly improved survival compared with naïve mice upon challenge with both 1,000 TCID₅₀ (Figure 5.3) and 10,000 TCID₅₀ (Figure 5.4) of influenza. These results show that rSIV.F/HN could potentially be used to provide passive immunisation against influenza, and perhaps other respiratory infectious diseases.

Further investigations would need to focus on developing strategies to allow rSIV.F/HN dosing to provide complete protection against influenza infection in the mouse model as achieved using rAAV2/8 in the muscle.

Repeated administration of viral vectors, especially with rAd vectors and rAAV, is hindered by the strong immune responses to the viral vector delivered. A previous study showed that IN delivery of GP64-pseudotyped feline immunodeficiency virus to the mouse nasal mucosa resulted in only minimal immune responses and that repeat administration is feasible (Sinn et al., 2008b). It has also been shown that rSIV.F/HN can be repeat administered daily to the lung to increase reporter transgene (Lux) expression and monthly without losing transgene expression (Griesenbach et al., 2012b). Although the underlying mechanism of how rSIV.F/HN escapes the immune response is unknown, it has been shown that when given as five or ten daily doses, rSIV.F/HN (1E6 TU per dose) generated transgene expression that was significantly higher than in mice receiving a single total dose of 1E6 TU (Griesenbach et al., 2012c). Here, a similar increment was observed in mice receiving ten daily doses of 1E6 TU rSIV.F/HN.hCEF.T13B compared with a single 1E6 TU dose but, surprisingly, the increment was not seen in mice receiving repeat dosing with a higher dose of rSIV.F/HN (1E7 or 1E8 TU) (Figure 5.6). Whether repeat administration of rSIV.FHN leads to increase in the percentage of lung epithelia expressing the bnAb, or an increase in the number of bnAb transgene copies/cell, requires further investigation. Understanding this might provide more insight into why repeat administering a higher dose (1E7 TU or 1E8 TU) of rSIV.F/HN does not lead to an increase in bnAb expression.

In terms of protection against influenza, the data show that when considering the same total dose (1E7 and 1E8 TU), mice receiving the dose in a single

administration (Figure 5.6 B & C) appeared to experience a slower onset of weight loss, and better survival, compared with mice receiving the equivalent dose in ten daily administrations (Figure 5.6 D & E). The reason for this is unclear. One might speculate that because mice receiving ten repeated doses were also subjected to ten daily sessions of inhalation anaesthesia, this might increase their susceptibility to influenza infections. Published studies show that isoflurane anaesthetic as used here (Section 2.2.9.2) can lead to increased serum corticosterone in BALB/c mice that lasts for several hours before returning to baseline (Jacobsen et al., 2012). Corticosterone is the main effector hormone in the stress response of mice, and acute and chronic elevations of corticosterone are associated with immunological dysfunction (Padgett and Glaser, 2003). Aside from this, the high volume of fluid delivered to the mouse lung following repeat administration might also increase the susceptibility to influenza infection. All mice in the repeat administration groups received a total of 500 μ L of fluid IN, over the course of 10 days, whereas mice in the single dose group received only 50 μ L of fluid. Although IN delivery of rSIV.F/HN did not lead to any visible signs of distress in the mice, cellular or molecular changes, for example changes in the cytokine profile, have not been investigated in lung epithelia transduced with rSIV.F/HN. A previous study, however, found that intravenous (IV) delivery of a rLV vector (resulting in transduction mainly in the liver and spleen) could activate an interferon-alpha/beta (IFN $\alpha\beta$) response in BALB/c mice that lasted for several days, the induction of which was independent of the surface glycoprotein pseudotype of the rLV used (Brown et al., 2007). High IFN $\alpha\beta$ levels have been associated with increased lung damage, morbidity and mortality in some

Table 5.1 Dosing regimen used for rSIV.F/HN.hCEF.T13B repeat administration studies

Group/ Day	1	2	3	4	5	6	7	8	9	10	Total dose
1E6 TU x 10	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E6	1E7
1E7 TU x 10	1E7	1E7	1E7	1E7	1E7	1E7	1E7	1E7	1E7	1E7	1E8
1E8 TU x 10	1E8	1E8	1E8	1E8	1E8	1E8	1E8	1E8	1E8	1E8	1E9
1E6 TU	-	-	-	-	-	-	-	-	-	1E6	1E6
1E7 TU	-	-	-	-	-	-	-	-	-	1E7	1E7
1E8 TU	-	-	-	-	-	-	-	-	-	1E8	1E8
Naive	-	-	-	-	-	-	-	-	-	-	-

TU: transducing unit

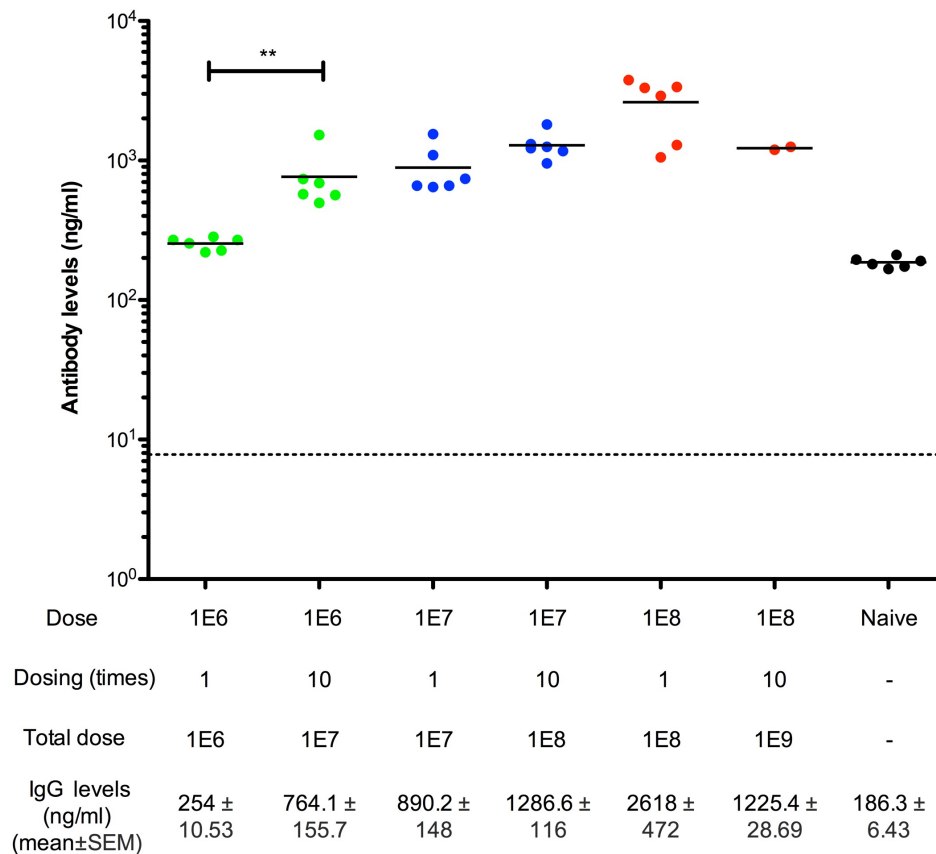


Figure 5.5 Human IgG levels in the ELF following single and repeat intranasal administration of rSIV.F/HN.hCEF.T13B. Expression of human IgG in the ELF following single, or ten daily repeated, IN administrations of 1E6 (green), 1E7 (blue) or 1E8 (red) TU of rSIV.F/HN.hCEF.T13B (Table 3.4) in mice (female BALB/c, 5 to 7 weeks, n=2 for 1E8 TU x 10 doses group, n=6 for other groups; Section 2.2.9.3). Mice were humanely culled at day 28 following rSIV.F/HN administration and BALF was collected. Human IgG in the ELF was measured (Section 2.2.6.1). Horizontal bars represent group means. Statistical significance was determined by Mann-Whitney test to compare single and repeat dosing groups. The dotted line represents the lowest detection limit of the assays with values below this line extrapolated from the curve fit. A calculated p value of <0.01 was deemed significant and indicated with double asterisks (**).

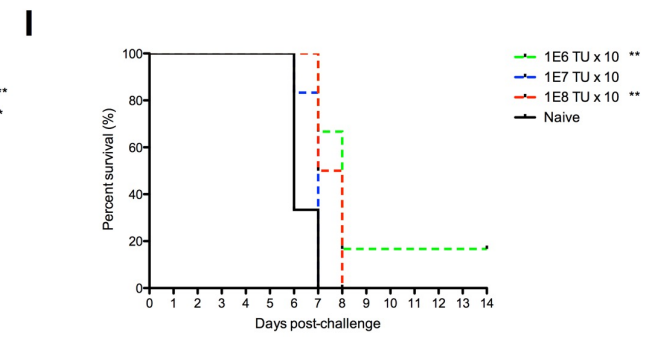
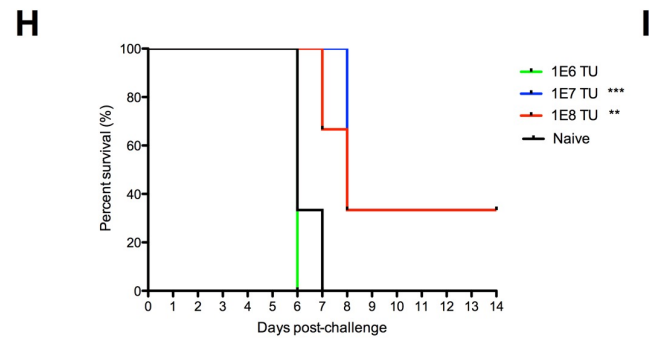
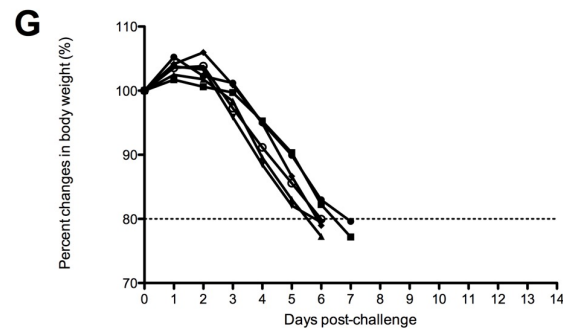
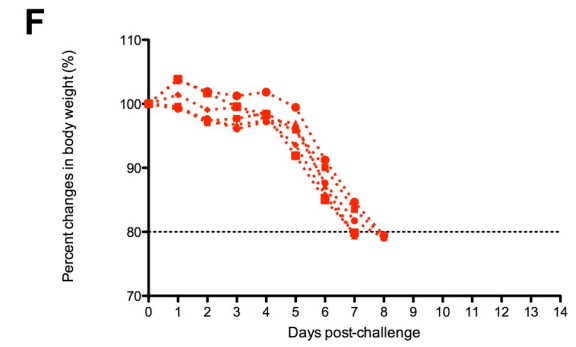
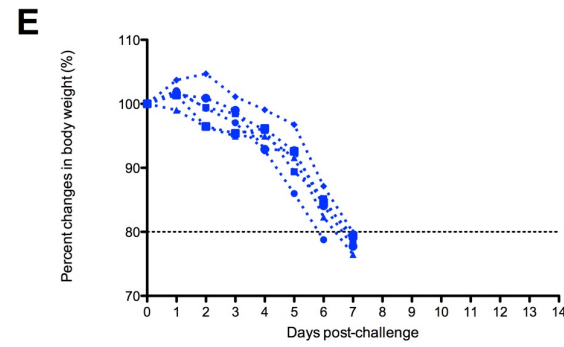
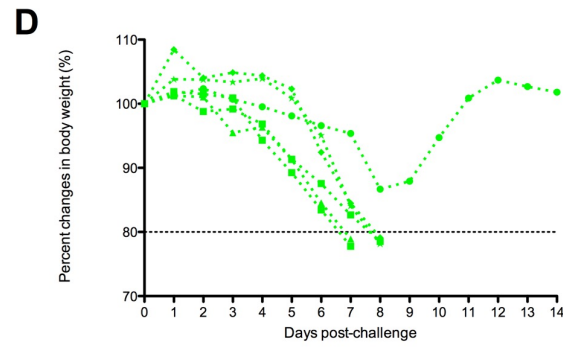
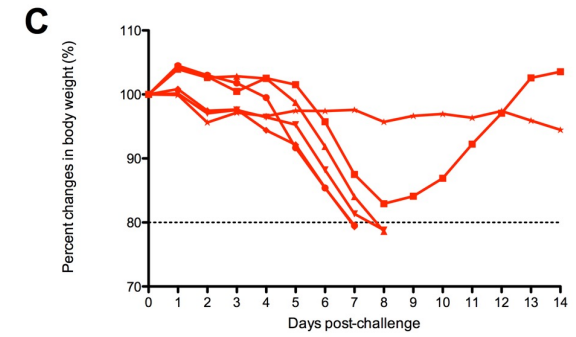
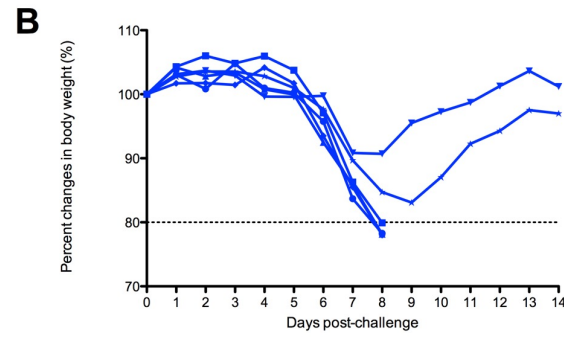
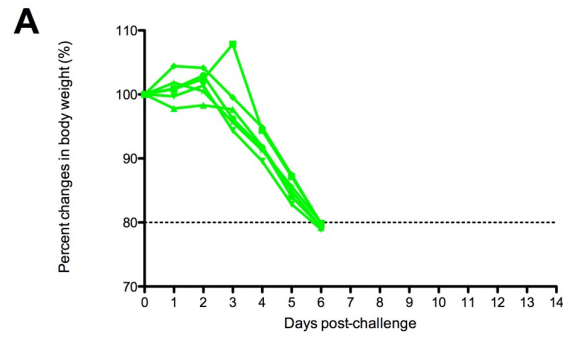


Figure 5.6 Effect of repeat administration of rSIV.F/HN.hCEF.T13B in protecting mice against 1,000 TCID₅₀ influenza challenge. Mice (female BALB/c, 5 to 7 weeks) received a single or ten daily doses of rSIV.F/HN.hCEF.T13B, 4 weeks prior to intranasal infection with 1,000 TCID₅₀ (15 LD₅₀) of A/PR/8/1934 H1N1. A single dose of 1E6 (full green line), 1E7 (full blue line) or 1E8 (full red line) TU or ten daily doses of 1E6 (dotted green line), 1E7 (dotted blue line) or 1E8 (dotted red line) TU per mouse in FreeStyle was administered. A group of mice also remained naïve (black; n=6; Section 2.2.9.3). The weight loss of each mouse was monitored for the indicated time period (A to G) and mice were euthanised at a weight loss of $\geq 20\%$ compared with the pre-challenge body weight was observed, as indicated in the Kaplan-Maier graphs (H & I). Each line in A to G represents the body weight changes of individual mice in the group. Statistical significance was determined by log-rank test. A calculated p value of <0.01 or <0.001 was deemed a significant difference and indicated with double asterisks (**) or triple asterisks (***) respectively.

strains of influenza-infected mice (Davidson et al., 2014). Although the previous study (Brown et al., 2007) showed that the IFN $\alpha\beta$ response resolves several days after rLV delivery, it is unknown if repeat administration, and/or the high dose/volume of viral vector, could affect the kinetics of the IFN $\alpha\beta$ response. Whether or not transduction of lung epithelia by rSIV.F/HN leads to increased expression of IFN $\alpha\beta$ in the lung, and whether this might affect subsequent resistance to influenza challenge, would require further investigation. Taken together, however, the reduced survival of mice receiving the higher dose of rSIV.F/HN via repeated administration could be due to a combination of the factors described above.

5.3.2 Relevant animal models for influenza challenge studies

The mouse model used in this study was relatively simple and inexpensive, however, it is known that the mouse is not the best model for influenza infection for several reasons. For instance, unlike humans, mice develop hypothermia instead of hyperthermia in response to influenza infection (Yang and Evans, 1961). In addition to the anatomy and histology of the murine respiratory tract being very different from humans, the distribution of the viral receptor (SA) in the respiratory tract of mice is also different (Ibricevic et al., 2006). In mice, viral replication and tissue damage is often concentrated in the lower, rather than the upper, respiratory tract and this has been linked to the higher severity of infection than what normally occurs in human hosts (Hatta et al., 2007). The ferret, on the other hand, is a more reliable model for influenza study. First described in 1933, and used widely in influenza research (Smith et al., 1995), ferrets are susceptible to most human influenza virus strains without prior adaptation to the species and present symptoms similar to influenza-infected humans such as nasal discharge and fever. The SA distribution in the ferret airways is also

similar to human airways (van Riel et al., 2007, Xu et al., 2010). Since ferrets efficiently transmit human influenza viruses, this model (via direct contact or aerosol transmission) can be used to study the efficacy of viral vectors mediating immunisation to prevent transmission of human viruses, such as occur in influenza pandemics and seasonal influenza, whereas the mouse model does not to serve as a suitable model for influenza virus transmission (Lowen et al., 2006). There are several drawbacks of using ferrets, however, such as the high cost and the more stringent husbandry requirements, which make it less accessible to most research groups. Another noteworthy point of the studies described here is that a very high dose of influenza (1,000 or 10,000 TCID₅₀; Section 5.2.2) was used in the influenza challenge experiments, which was several logs higher than the dose required to initiate influenza infection in human via aerosol inhalation (0.6 to 3 TCID₅₀) (Alford et al., 1966). The median lethal dose (LD₅₀) of A/PR/8 (lineage unknown) in BALB/c was 100 plaque-forming units (PFU) (Quan et al., 2008) and as little as 7 TCID₅₀ of A/PR/8 (Cambridge) was sufficient to lead to 20% weight loss in BALB/c (Powell et al., 2012b). In the mouse model, the primary readout following a lethal dose of influenza challenge is weight loss and death (euthanasia at a humane endpoint, approximating $\geq 20\%$ weight loss according to the ASPA 1986). There are also several secondary parameters to assess influenza virus pathogenicity or vaccine efficacy, for instance, lung viral titres, increase in lung weight and increase in 1-acid glycoprotein in cases where non-lethal influenza strains or non-lethal doses of influenza were used (Sidwell and Smee, 2000). The influenza challenge dose used in the studies described in this chapter was selected based on the published dose for evaluating influenza vaccines and viral vector-mediated immunoprophylaxis against influenza (Quan et al., 2010, Powell et al., 2012b, Balazs et al., 2013, Limberis et al., 2013, Kamlangdee et al., 2014). It was

found that a low-dose IN challenge in the ferret model results in slower viral replication, but higher virus shedding, which more closely resembles the disease parameters of natural influenza infection in humans (Marriott et al., 2014). This means that the failure of rSIV.F/HN to provide complete protection in mice from a lethal dose does not necessarily mean that it would not protect humans from natural influenza infections. In fact, using a high-dose challenge model might obscure the real therapeutic effect of antiviral drugs or vaccines (Marriott et al., 2014). A high-dose challenge mice model might be more relevant for the study of influenza treatments, than for vaccines or prophylaxis. It is therefore worth considering the low-dose challenge ferret model in the future studies to evaluate the efficacy of rSIV.F/HN as a prophylaxis against influenza infection.

5.3.3 Conclusion

In conclusion, the results show that rSIV.F/HN is a potential platform to mediate passive immunisation against influenza virus and perhaps also other infectious diseases. However, optimisation of this platform would be required to improve its efficacy compared with the rAAV2/8 IM delivery approach. Further efforts have focused on optimising the antibodies to improve rSIV.F/HN as a platform for passive immunisation against influenza. This work is discussed in detail in Chapter 6.

Chapter 6 Enhancement of anti-influenza broadly neutralising antibodies activity via antibody engineering

6.1 Introduction

In Chapter 5, efforts were made to enhance the protective effects of rSIV.F/HN expressing antibody against influenza infection by daily repeat administration, but such an approach was not successful (Figure 5.6). In this chapter, studies on the bnAb were performed in an attempt to improve their effectiveness against influenza. All of the bnAbs against influenza investigated in this study were of the IgG1 isotype (Section 3.1.2). To date, IgG (especially IgG1) predominates antibody-based therapeutics in the market (Shepard et al., 2017). The potency of other antibody isotypes as a treatment for influenza, especially IgA which is the main antibody isotype at the mucosal surface (Macpherson et al., 2008), has yet to be explored.

6.1.1 Human immunoglobulins (Igs)

In humans, immunoglobulins (Igs) belong to the immunoglobulin super-family (IgSF) and consist of two polypeptide chains, a heavy chain (H) and a light chain (L) that assemble into a monomeric unit (H_2L_2). Both H and L chains contain one variable (V) domain that forms the antigen binding site (known as the paratope; VH and VL) and one or more constant (C) domains that have effector functions such as complement binding, half-life length, interactions with the fragment-crystallizable receptors (FcRs) and the isotype or subclass of the Ig (Figure 6.1). In a typical Ig, there is a hinge region linking the first ($CH1$) and second ($CH2$) C domains in the H chain (Figure 6.1)

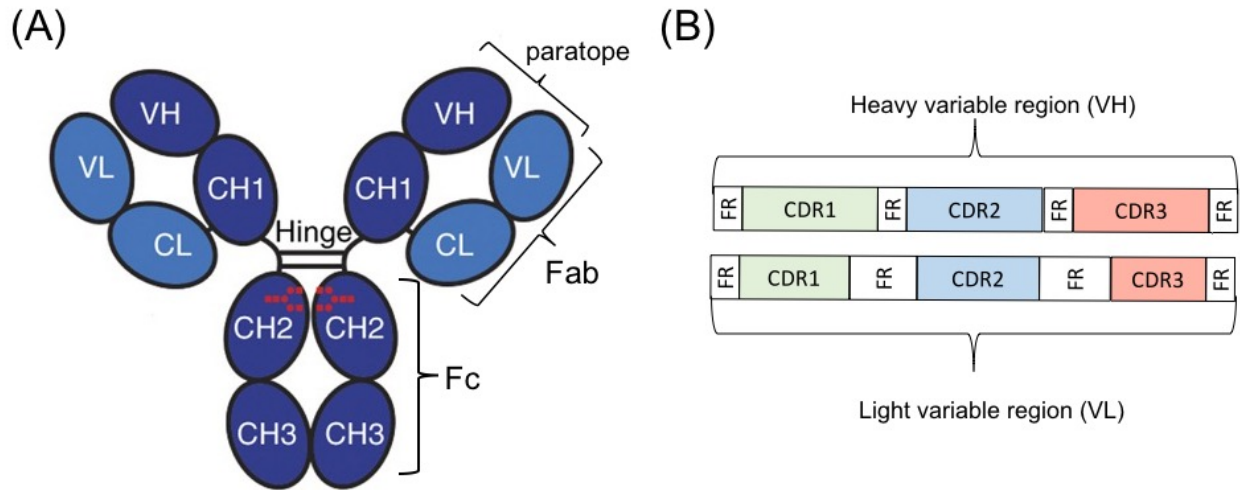


Figure 6.1 Schematic representation of the structure of a typical human immunoglobulin (based on human IgG1) and the antigen binding domain. (A) Human IgG1 consists of two heavy (H) and two light (L) chains linked by disulphide bonds. Each of the H and L chains consists of one variable (V) domain and one or more constant (C) domains. The fragment-antigen binding (Fab) region consists of CH1, VH and the L chain. The fragment crystallizable region (Fc) consists of the CH2 and CH3 domains. (B) The antigen binding domain (the paratope) of a human immunoglobulin, consists of the VL and VH domains. Each V region consists of three highly variable complementarity-determining regions (CDRs) sandwiched by four relatively conserved framework (FRs) regions. Figure A is adapted from (Rosati et al., 2014).

, the length of which differs between different Ig isotypes. The C region can consist of either a κ or a λ chain. In an intact Ig molecule, the H and L chains are linked by inter-chain disulphide bonds and covalent bonds (Figure 6.1).

6.1.2 Structure of human IgA

There are two distinct human IgA subclasses, namely IgA1 and IgA2, present in both monomeric and dimeric forms (Figure 6.2). In the serum, IgA is composed of approximately 90% IgA1 and 10% IgA2. The sub-class proportions of mucosal IgA can vary; in the nasal and male genital secretions approximately 80 to 90% is IgA1 but in the saliva, colonic and female genital secretions this is only 60% IgA1 (Woof and Russel 2011). IgA1 and IgA2 differ mainly in the length of their hinge region. IgA1 has a long and flexible hinge region (19 amino acids, rich in proline, serine and threonine, with between three and six O-linked glycosylation sites) (Tarelli et al., 2004, Woof and Kerr, 2006). IgA and their different forms are illustrated in Figure 6.2.

6.1.3 Enhancement of bioactivity of bnAb in IgA backbone

The enhancement of binding and neutralisation of recombinant IgA compared with their IgG equivalent, against influenza in *in vitro* assays, has been reported previously. Two independent research groups observed enhancement in binding and neutralising activity of humanised anti-HA antibodies in a human IgA backbone compared with their IgG equivalents (Muramatsu et al., 2014, He et al., 2015). Although the mechanism was not investigated, it was hypothesised that the enhanced bioactivity of IgA was due to the increased valency resulting from the increased flexibility of the IgA constant region (Muramatsu et al., 2014).

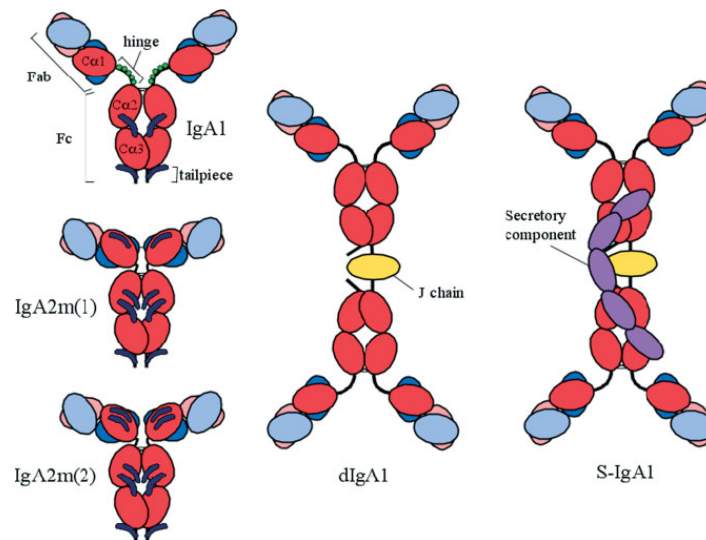


Figure 6.2 Schematic representation of the various forms of human IgA. Human IgA exists as monomeric: IgA1, IgA2m(1), and IgA2m(2), and the dimeric (dIgA) and secretory (S-IgA) forms (shown as dIgA1 and S-IgA1 in the diagram). Constant regions of the heavy chain are shown in red and the variable domains of the heavy chain in pink. Constant regions of the light chains are shown in dark blue and variable regions in light blue. The joining (J) chain connecting two monomeric IgA1 is shown in yellow and secretory component (SC) in purple. The O-linked sugars (on the IgA1 hinge) are shown as green circles, while N-linked oligosaccharides are shown in dark blue. There are two IgA2 allotypic variants, namely IgA2m(1) and IgA2m(2), with shorter hinge region (6 amino acids lacking O-linked glycosylation sites) (Kunkel et al., 1969, Endo et al., 1994). Serum IgA is predominantly monomeric while IgA in the mucosal secretions are mainly dimeric. Monomeric IgA forms dimeric IgA (dIgA) via the linking of two monomeric IgA either spontaneously via its tailpiece region (might form polymeric IgA via this mechanism) or by joining (J) chain polypeptides at the penultimate cysteine residue at the C region of the IgA H chains via covalent binding (Hendrickson et al., 1995, Brunke et al., 2013). Figure adopted from (Woof and Kerr, 2006).

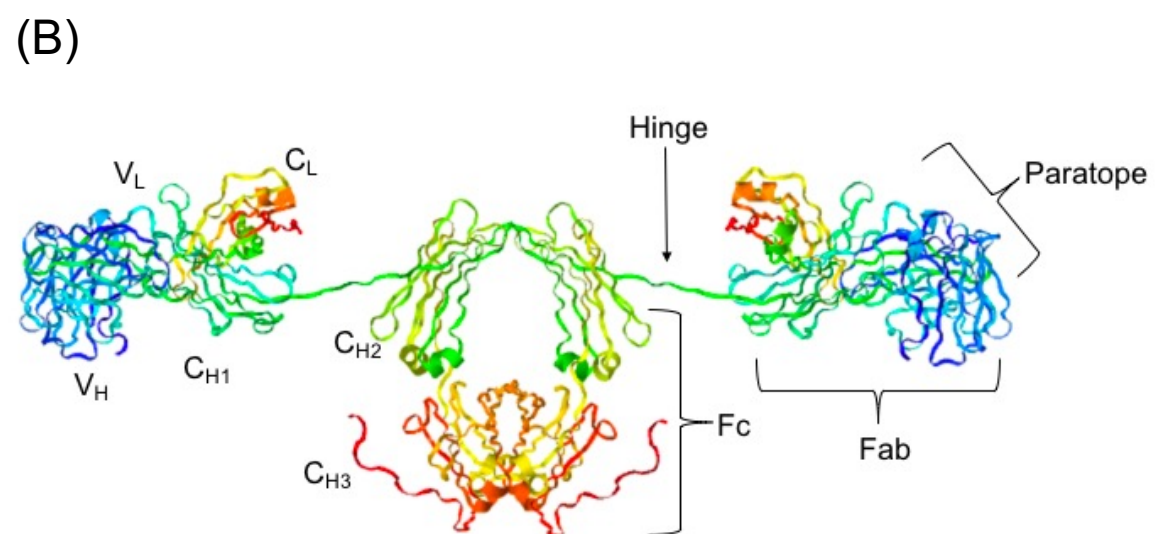
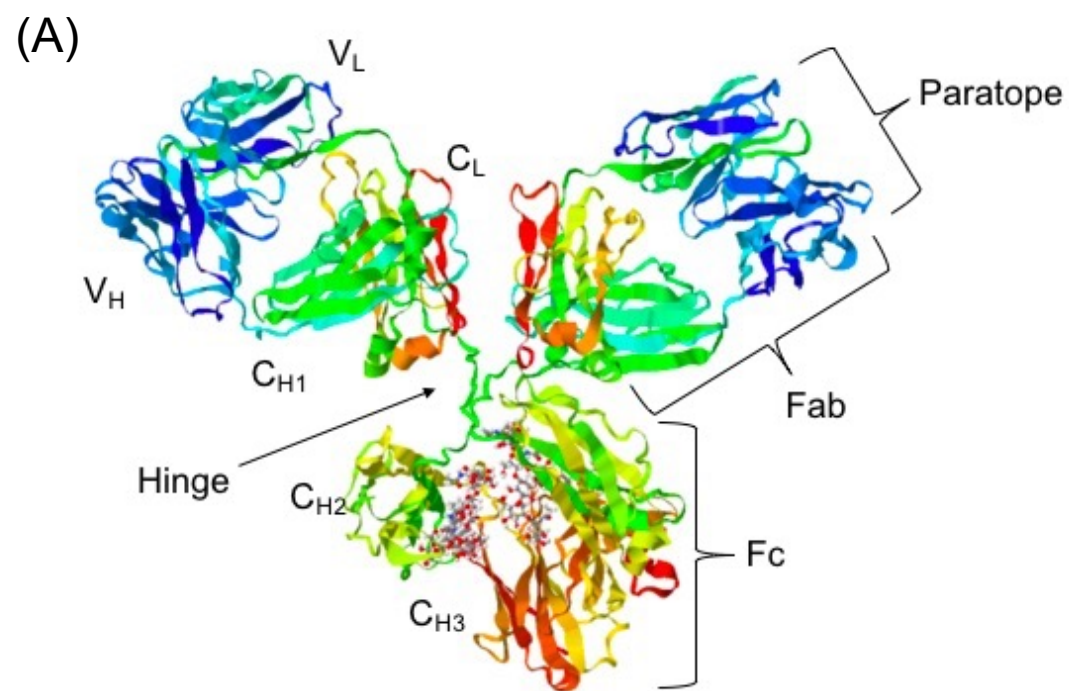


Figure 6.3 Crystallography models of intact human IgG1 and human IgA1. The ribbon diagrams show the intact structure of both monomeric human IgA1 (PDB id: 1IGA) and human IgG1 (PDB id: 1IGY). Human IgG1 and IgA1 consist of two heavy (H) and light (L) chains. Each of the H and L chains consists of a variable (V) and one or more constant (C) domains (indicated in the figure). The fragment-antigen binding (Fab) region consists of CH1, VH and the L chain. The fragment crystallizable region (Fc) consists of the CH2 and CH3 domains. Human IgA1 demonstrates a T-shape structure due to the long and more flexible hinge region. Human IgG1 demonstrates a Y-shape structure. Figures reproduced from (Harris et al., 1998, Boehm et al., 1999).

6.1.4 Aim

The studies in this chapter were designed to investigate the enhancement of bioactivity of bnAb against influenza in the IgA1 backbone.

6.2 Results

6.2.1 Generation of recombinant anti-influenza HA IgA1

In order to investigate the effect of IgA1 backbone on the neutralising activity, a panel of HA-targeting antibodies consisting of HA-head targeting specific antibodies, HA-head targeting cross-reactive and HA-stem targeting antibodies were selected (Table 6.1). With the exception of MEDI8852, where the sequence was already published (Kallewaard et al., 2016), all the IgG1 molecules studied in this chapter (Table 6.1) were isolated from recovered patients or vaccinated donors via single-B cell cloning of the H and L variable (Tiller et al., 2008, Wrammert et al., 2008). These were then sub-cloned into the human IgG1 heavy constant backbone (pIgG-AbVec) for IgG1 expression (Table 2.1). The equivalent IgA1 H expression plasmids were generated by sub-cloning the V region of the H chain into human IgA1 backbone as described in Section 2.2.3.11. Monomeric IgA1 was then expressed and affinity purified (Section 2.2.7) (Table 6.1). To confirm the production of recombinant IgA1, affinity purified IgA1 and their IgG1 equivalents were visualised using SDS-PAGE (Section 2.2.8). Results from SDS-PAGE under reducing and non-reducing conditions showed that recombinant IgA1 had been successfully produced (Figure 6.4).

6.2.2 Comparison of neutralising and binding activity of recombinant IgA1 and their IgG1 equivalents

6.2.2.1 Enhancement of neutralising activity in HA stem-targeting recombinant IgA1

The neutralisation of IgA1 and their IgG1 equivalents was compared using the MN assay against various influenza strains (Section 2.2.6.4). Neutralisation activity against tested influenza strains was reported as EC_{50} as shown in Figure 6.5 and Table 6.2. Enhancement in neutralising activity was observed in several HA-stem targeting bnAb (Figure 6.5). T1-3B IgA1 has a 11.7-fold increase in neutralising activity against H5 A/Vietnam/1203/2004 compared with T1-3B IgG1. Previous studies have observed a biased usage of the VH1-69 germline family in HA-stem targeting bnAb (Lee and Wilson, 2015). In order to investigate if the germline family plays a role in determining the enhancement observed in IgA1 backbone, T2-5A IgA1 and T1-3B IgA1 were compared, since they were both encoded by the same germline family (VH1-69) (Table 6.1). However, T2-5A IgA1, showed no enhancement in neutralisation against any of the influenza strains tested (Table 6.2), suggesting that the germline family might not predict enhancement in IgA1 backbone. MEDI8852 IgA1 showed a 10.8-fold enhancement in neutralising activity against H5 A/Vietnam/1203/2004 compared with MEDI8852 IgG1. Furthermore, MEDI8852 IgA1 also showed an enhancement in neutralising activity of three to 16-fold against all four H7 strains tested compared with MEDI8852 IgG1 (Table 6.2).

Another key observation was that switching to the IgA1 backbone enhances cross-reactivity of some antibodies against influenza. For instance, T2-5D IgG1 did not neutralise H3 A/Victoria/361/2011, but did so in the IgA1 form. The neutralisation of

T2-5D IgA1 remains similar to its IgG1 equivalent against H1 A/California/07/2009 (Table 6.2), suggesting switching to IgA1 backbone does not fundamentally change the binding epitope of the antibody. Similarly, Z3-B2 in the IgG1 form did not neutralise any of the four H7 strains tested, but neutralised H7 A/Shanghai/1/2013 in IgA1 form (Table 6.2).

6.2.2.2 Enhancement only observed in H5 and H7 strains on all recombinant IgA1 tested

In IgA1 that showed enhancement in neutralisation, it was expected that enhancement should be observed in all HA as the IgA1 constant region should enhance the general affinity of the paratope, but surprisingly, the enhancement seen with the IgA1 HA-stem targeting antibodies was only observed with H5 and H7 influenza strains and not H1 or H3 strains (Table 6.2). The exception was T2-5D which cross-reacts with H3 A/Victoria/361/2011 only in IgA1 form. This observation was best demonstrated by MEDI8852 which is known to neutralise all group 1 and group 2 influenza (Table 2.5). The results show that enhancement in neutralising activity of MEDI8852 IgA1 was only observed in H7 and H5 strains, but not H1 or H3 strains (Table 6.2). The Z3-B2 IgA1 showed cross-reactivity in one out of four H7 strains tested, but no enhancement with the three H3 strains tested in comparison with Z3-B2 IgG1 (Table 6.2). These results suggest that enhancement in neutralisation of IgA1 might be HA-dependent.

6.2.2.3 Decrease in neutralising activity of HA head-targeting recombinant IgA1

While most HA-head targeting antibodies demonstrate similar neutralising activity in the IgA1 backbone compared with their IgG1 equivalents (Table 6.2), decreased

neutralising activity was observed in several HA-head targeting antibodies: the 21-D8-5A IgA1 has a 7.9-fold and 20-fold decrease in neutralising activity against H1 A/PR/8/1934 and H5 A/Vietnam/1203/2004, respectively (Table 6.2) and the T2-6A IgA1 has a three-fold decrement in neutralising activity against A/California/07/2009 H1N1 compared with T2-6A IgG1. This suggests that the IgA1 backbone does not enhance, and might even decrease, the neutralisation of HA-head targeting antibodies, as shown in the six HA-head targeting IgA1 used in this study (Table 6.2).

6.2.2.4 No difference in binding affinity against HA was observed between IgA1 and their IgG1 equivalents

To investigate if the difference in neutralisation was associated with difference in binding affinity against HA. The binding activity of several IgG1/IgA1 pairs of bnAbs that showed differences in neutralisation (Table 6.2) was compared using HA-ELISA (Section 2.2.6.3) against various HA (Table 2.4). The ELISA results show that there was no difference in binding affinity between all of the IgG1/IgA1 pairs tested (Figure 6.6). Both Z3-B2 IgG1 and IgA1 showed similar binding affinity against H7 A/Shanghai/1/2013 (Figure 6.6 i) but only Z3-B2 IgA1 neutralised H7 A/Shanghai/1/2013 (Table 6.2), indicating that the IgA1 backbone does not change the binding epitope of Z3-B2 against H7 A/Shanghai/1/2013, but allows neutralisation to occur more potently. Similar to Z3-B2, MEDI8852 IgG1 and IgA1 showed no difference in binding activity against all four HA tested (Figure 6.6 v, vi, vii, viii) even though enhancement in neutralisation against similar influenza strains was observed (Section 6.1). 21-D8-5A IgA1 showed a 7.9 and 20.1-fold decrement in neutralising H1 A/PR/8/1934 and H5 A/Vietnam/1203/2004 respectively compared to 21-D8-5A IgG1 (Table 6.2) but there was no difference in binding activity was observed (Figure

Table 6.1 IgG1 and monomeric IgA1 produced and investigated in this study

Antibody name	VH gene usage	Antibody class	HA specificity
T2-6A	VH3-7	IgG1	HA head specific H1 (A/California/7/2009)
		IgA1	
2-12C	VH5-51	IgG1	HA head specific H1 (A/California/7/2009)
		IgA1	
Q11-C11	VH4-61	IgG1	HA head specific H3 (A/Victoria/361/2011)
		IgA1	
T2-5D	VH3-15	IgG1	HA head cross-reactive H1
		IgA1	
T3-4B	VH3-15	IgG1	HA head cross-reactive H1
		IgA1	
21-D8-5A	VH4-39	IgG1	HA head cross-reactive H1 & H5
		IgA1	
T1-3B	VH1-69	IgG1	HA stem cross-reactive H1 & H5
		IgA1	
T2-5A	VH1-69	IgG1	HA stem cross-reactive H1 & H5
		IgA1	
Z3-B2	VH3-53	IgG1	HA stem cross-reactive H1, H3 and H7
		IgA1	
MEDI8852	VH6-1	IgG1	Pan Influenza stem (all group 1 and group 2)
		IgA1	

HA: influenza haemagglutinin; VH: gene encoding the variable region of the antibody heavy chain; IgG1: human immunoglobulin G subclass 1; IgA1: human immunoglobulin A subclass

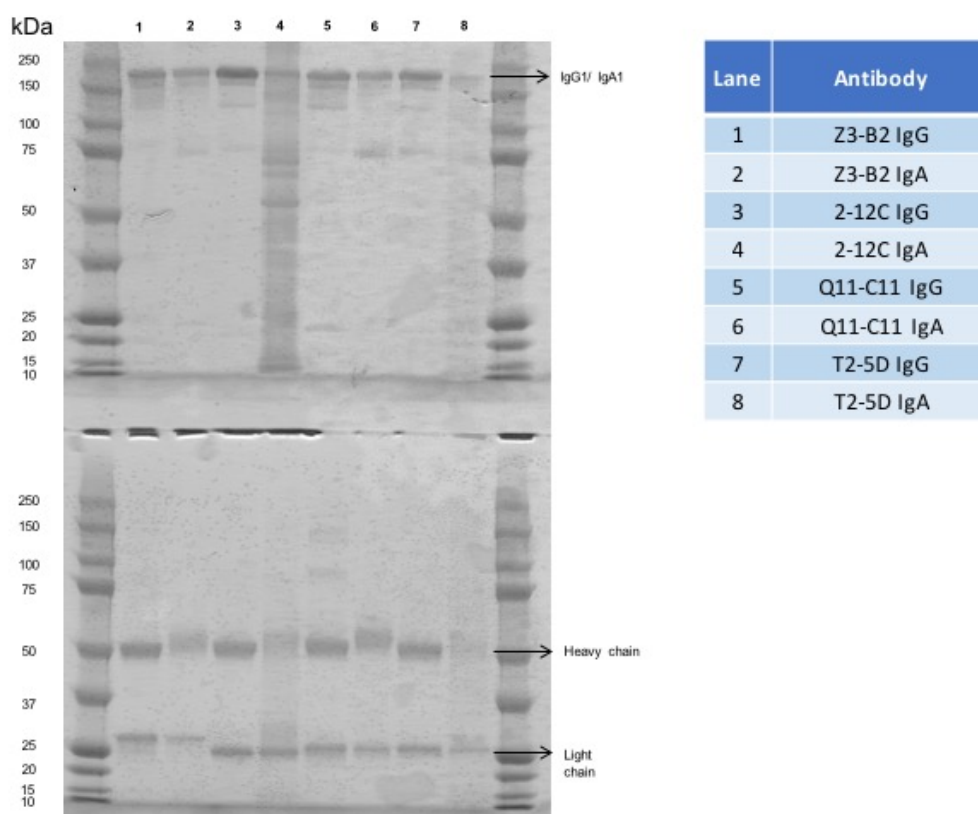


Figure 6.4 Visualisation of recombinant IgA1 using SDS-PAGE. To confirm the production of IgA1, Z3-B2 (lane 1 & 2), 2-12C (lane 3 & 4), Q11-C11 (lane 5 & 6) and T2-5D (lane 7 & 8), both IgA1 and their IgG1 equivalents, were prepared following transient transfection of HEK 293T cells (Section 2.2.4) with plasmids pCMV.Z3B2.GH, pCMV.Z3B2.AH and pCMV.Z3B2.λ; pCMV.212C.GH, pCMV.212C.AH and pCMV.212C.κ; pCMV.Q11C11.GH, pCMV.Q11C11.AH and pCMV.Q11C11.κ and pCMV.T25D.GH, pCMV.T25D.AH and pCMV.T25D.κ (Table 2.1). These were purified using a Peptide M column (Section 2.2.7.1) and were separated by SDS-PAGE (2.5 µg per lane, Section 2.2.8) under reducing (bottom gel) and non-reducing (top gel) conditions, and stained with Quick Coomassie Stain (Generon) (Section 2.2.8). The position of the bands containing the full intact IgG1, IgA1 molecule, heavy and light chains are indicated. A protein marker (Bio-Rad) ranging from 10 to 250 kDa was included.

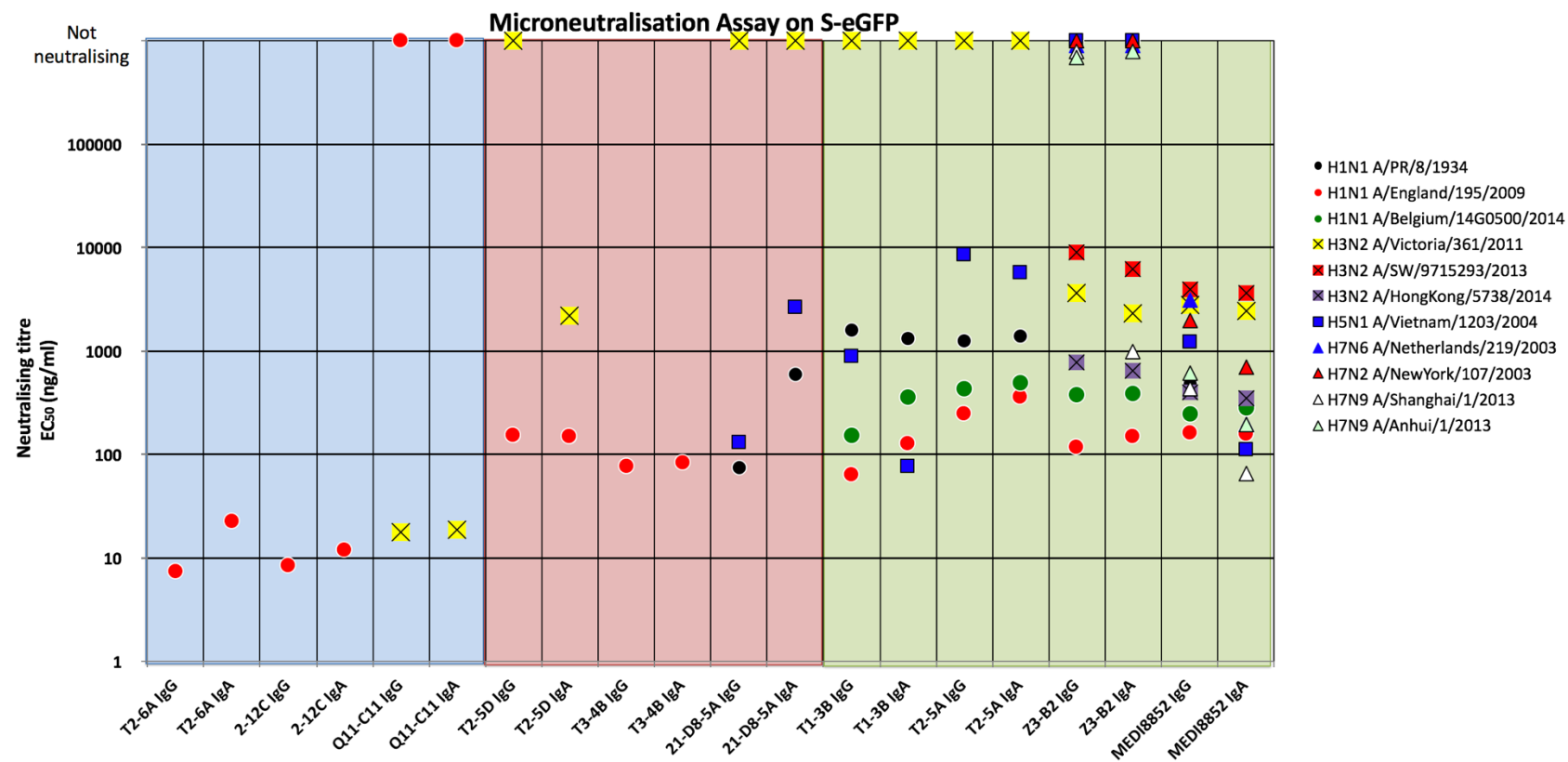


Figure 6.5 *In vitro* neutralisation titre (EC₅₀) of IgA1 and IgG1 equivalents against various influenza strains. The MEDI8852 IgG1 and all IgA1 were produced via transient transfection of HEK 293T cells with their respective expression plasmids (Table 2.1), followed by purification using a Peptide M or Sepharose A column (Section 2.2.7.1). Other IgG1s were obtained from Alain Townsend. The neutralisation titre (EC₅₀) of each IgA1 was compared to its IgG1 equivalent using the MN assay (Section 2.2.6.4) against various influenza strains (Table 2.4). The IgA1 and IgG1 tested are shown on the X-axis and the EC₅₀ neutralising titre (ng/mL) is shown on the Y-axis. The light blue background indicates HA-head targeting antibodies, the red background indicates the HA-head cross-reactive antibodies and the light green background indicates HA-stem cross-reactive antibodies. Each data point represents the mean EC₅₀ (ng/mL) obtained from at least two independent assays. The EC₅₀ was calculated as the concentration of antibody that results in half-maximal response. The fold-difference of EC₅₀ between IgA1 and their IgG1 equivalents is summarised in Table 6.2.

Table 6.2 Summary of the neutralising titre (EC₅₀) of IgA1 compared with their IgG1 equivalents

Antibody	VH usage	HA subtype	Influenza strain (S-eGFP)	EC ₅₀ (ng/mL)		IgG/ IgA	IgA/ IgG
				IgG1	IgA1		
T2-6A	VH3-7	H1	A/California/07/2009	7.5	22.5	0.3	3.0
2-12C	VH5-51	H1	A/California/07/2009	8.5	12	0.7	1.4
Q11-C11	VH4-61	H1	A/California/07/2009	-	-	-	-
		H3	A/Victoria/361/2011	18	19	0.9	1.1
T2-5D	VH3-15	H1	A/California/07/2009	156	149	1.0	1.0
		H3	A/Victoria/361/2011	-	2237.5		
T3-4B	VH3-15	H1	A/California/07/2009	78	84.5	0.9	1.1
21-D8-5A	VH4-39	H1	A/PuertoRico/8/1934	75.5	593.5	0.4	7.9
			A/California/07/2009	-	-	-	-
			A/Belgium/14G0500/2014	-	-	-	-
		H3	A/Victoria/361/2011	-	-	-	-
		H5	A/Vietnam/1203/2004	133	2674.5	0.05	20.1
T1-3B	VH1-69	H1	A/PuertoRico/8/1934	1603	1345.5	1.2	0.8
			A/California/07/2009	64	129.5	0.5	2.0
			A/Belgium/14G0500/2014	152.5	364	0.4	2.4
		H3	A/Victoria/361/2011	-	-	-	-
		H5	A/Vietnam/1203/2004	906	77.5	11.7	0.1
T2-5A	VH1-69	H1	A/PuertoRico/8/1934	1267	1402.5	0.9	1.1
			A/California/07/2009	251.5	365.5	0.7	1.5
			A/Belgium/14G0500/2014	436.5	499	0.9	1.1
		H3	A/Victoria/361/2011	-	-	-	-
		H5	A/Vietnam/1203/2004	8642	5741.5	1.5	0.7
Z3-B2	VH3-53	H1	A/PuertoRico/8/1934	-	-	-	-
			A/California/07/2009	119.5	150	0.8	1.3
			A/Belgium/14G0500/2014	378.5	393.5	1.0	1.0
		H3	A/Victoria/361/2011	3714.7	2359.7	1.6	0.6
			A/SW/9715293/2013	9041	6195	1.5	0.7
			A/HongKong/5738/2014	787.5	656.5	1.2	0.8
		H5	A/Vietnam/1203/2004	-	-	-	-
		H7	A/Netherlands/219/2003	-	-	-	-
			A/NewYork/107/2003	-	-	-	-
			A/Shanghai/1/2013	-	1008.8		
			A/Anhui/1/2013	-	-	-	-
MEDI8852	VH6-1	H1	A/PuertoRico/8/1934	482	332	1.5	0.7
			A/California/07/2009	180.3	159	1.1	0.9
			A/Belgium/14G0500/2014	246.5	282.5	0.9	1.1
		H3	A/Victoria/361/2011	2813	2454.5	1.1	0.9
			A/SW/9715293/2013	3930	3659	1.1	0.9
			A/HongKong/5738/2014	399	351.5	1.1	0.9
		H5	A/Vietnam/1203/2004	1216	112.5	10.8	0.1
		H7	A/Netherlands/219/2003	3134.7	198.3	15.8	0.1
			A/NewYork/107/2003	1975.5	707	2.8	0.4
			A/Shanghai/1/2013	433	65.7	6.6	0.2
			A/Anhui/1/2013	624.33	195	3.2	0.3

The EC₅₀ represents the average values obtained from at least two separate experiments. Fold-difference of EC₅₀ more than two-fold between IgA1 and their IgG1 equivalents are highlighted in yellow or red. Green colour indicates antibody that only neutralises in IgA1.

HA: influenza haemagglutinin; VH: gene encoding the variable region of the antibody heavy chain; S-eGFP: recombinant influenza virus expressing eGFP.

6.6 iii & iv). These results suggest that the IgA1 constant region does not affect the binding affinity of the antibodies and the difference in neutralisation observed was not due to enhanced binding affinity. 2.3 Investigation of the effect of the IgA1 hinge region on the neutralising activity of IgG1

6.2.3.1 Generation of IgG1 with the IgA1 hinge region (IgGA)

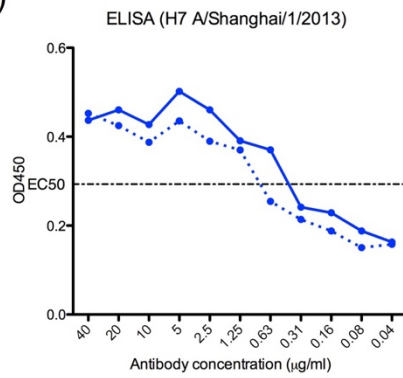
Immunoglobulins A1 and their equivalent IgG1 share the same paratope (VH and VL), and therefore the same HA specificity. It is possible that the enhanced neutralising activity and cross-reactivity, observed in Figure 6.5, might be due to an enhanced valency in IgA1, due to the wider and more flexible hinge region (Figure 6.3), as hypothesised by (Muramatsu et al., 2014). The increased flexibility of the hinge might allow the Fabs to have an extended reach towards their epitopes on the HA, especially the stem region, resulting in more potent neutralisation. To investigate this, the sequence encoding the human IgA1 hinge region was sub-cloned into the IgG1 heavy chain sequence (in between the IgG1 CH1 and the IgG1 hinge) to generate an IgG1 with an IgA1 hinge, termed IgGA (Section 2.2.3.11; Figure 6.7). In the IgGA construct, instead of replacing the IgG1 hinge region with the IgA1 hinge, an extension of the IgA1 hinge region onto the original IgG1 hinge region was designed. This is because the disulphide bonds that hold the two arms together in IgG1 are located in the IgG1 hinge region, hence the removal of the native IgG1 hinge might compromise the structure of the IgG1 (Figure 6.6). These cDNA constructs were confirmed by sequencing and used to produce Z3-B2 and MEDI8852 IgGA (Section 2.2.7). Affinity purified IgGA was visualised using SDS-PAGE to confirm the assembly of the IgGA

molecules (Section 2.2.8) and SDS-PAGE showed that IgGA was successfully produced (Figure 6.8).

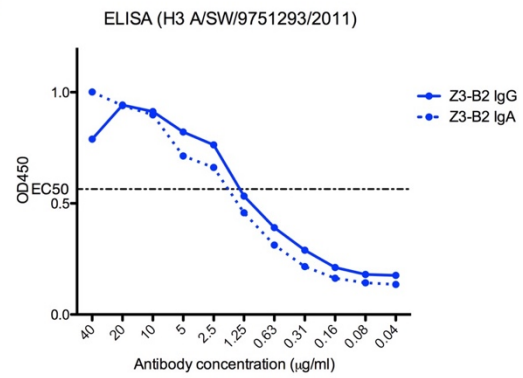
6.2.3.2 Comparison of neutralising activity of the hybrid IgGA with recombinant IgA1 and their IgG1 equivalents

The neutralisation of Z3-B2 and MEDI8852 IgGA and IgA1 and IgG1 equivalents was compared using the MN assay against various influenza strains (Section 2.2.6.4). MEDI8852 and Z3-B2 IgGA showed no enhancement in neutralisation against any of the influenza strains tested (Figure 6.9). For both MEDI8852 IgGA and Z3-B2 IgGA, the neutralisation remained similar to their IgG1 equivalents against the influenza strains tested (Figure 6.9), suggesting the inclusion of a longer and more flexible constant hinge region did not compromise the affinity of the paratope, but at the same time, did not enhance the neutralisation activity of IgGA.

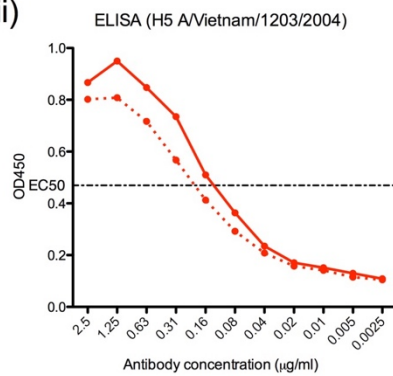
(i)



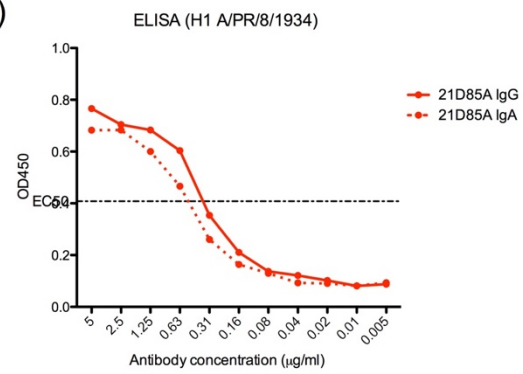
(ii)



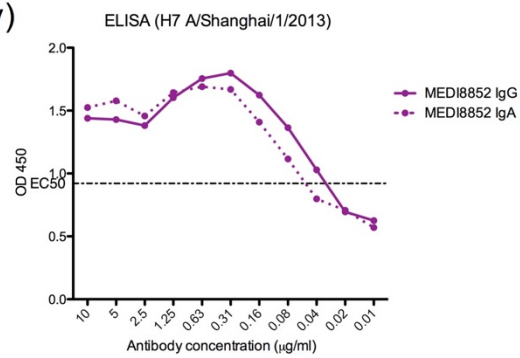
(iii)



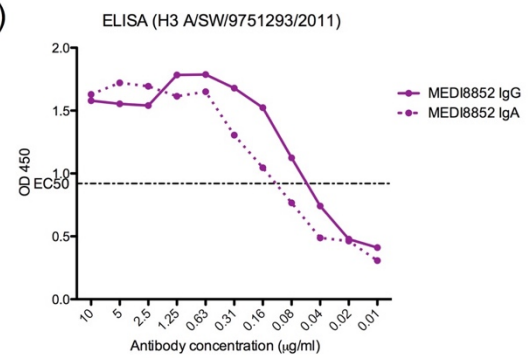
(iv)



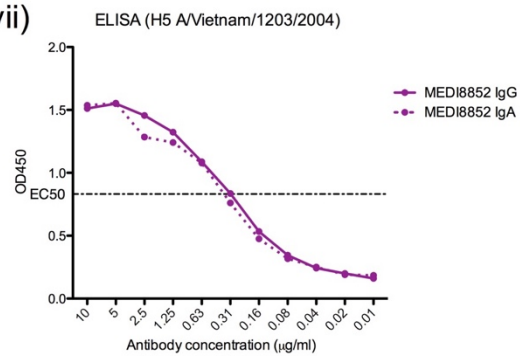
(v)



(vi)



(vii)



(viii)

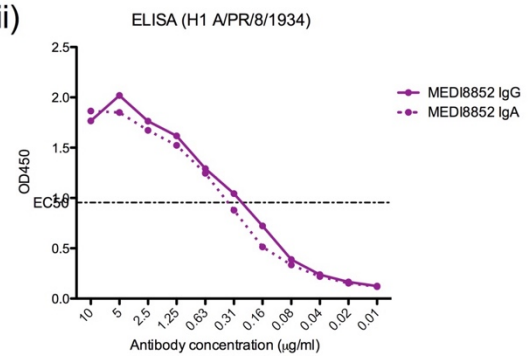


Figure 6.6 Binding activity of IgA1 compared with the respective IgG1 equivalent. Z3-B2 IgA1 (i & ii), 21-D8-5A IgA1 (iii & iv), MEDI8852 IgG1 and IgA1 (v, vi, vii, & viii) were prepared from transfection of HEK 293T cells with plasmids pCMV.Z3B2.AH and pCMV.Z3B2.λ; pCMV.21D85A.AH and pCMV.21D85A.λ; pCMV.MEDI8852.GH, pCMV.MEDI8852.AH and pCMV.MEDI8852.κ (Table 2.1), followed by purification using a Peptide M column (Section 2.2.7.1). The Z3-B2 and 21-D8-5A IgG1s were obtained from Alain Townsend. The binding activities of Z3-B2 IgA1, 21-D8-5A IgA1 and MEDI8852 IgA1 were compared with their IgG1 equivalents using HA-ELISA (Section 2.2.6.3) against various HAs (Table 2.4). Each data point represents the mean of duplicate readings. The EC₅₀ was calculated as the concentration of antibody that results in half-maximal response.

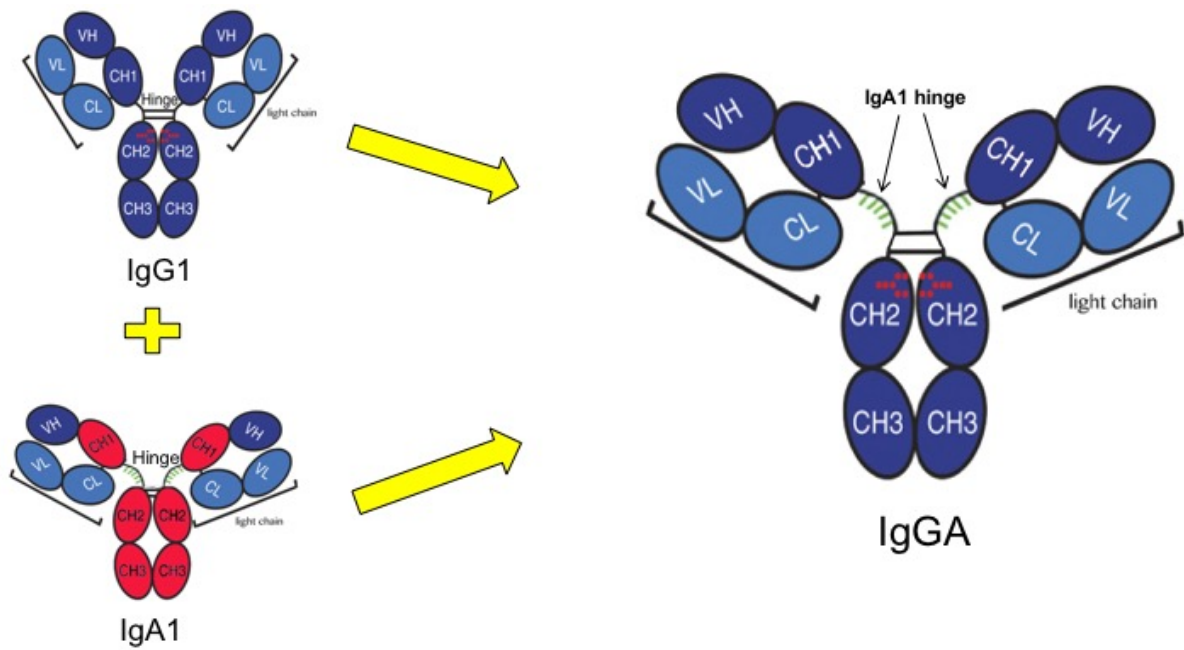
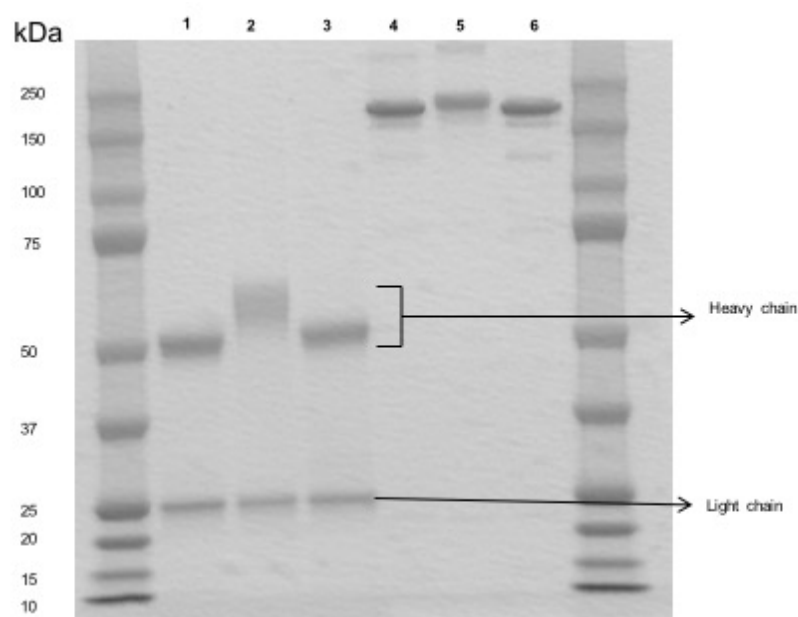


Figure 6.7 Schematic of the IgGA construct generated in this study. The hinge region of IgA1 was sub-cloned between the IgG1 C_H1 region and the IgG1 hinge region, to generate IgGA. The IgG1 hinge region was retained in the IgGA construct since it contains key disulphide bonds (black lines) that hold the two arms of IgG1 together. Variable and constant regions of the IgG1 heavy chain are shown in dark blue and the constant domains of the IgA1 heavy chain are shown in red. The light chains are shown in light blue.



Lane	Antibody	SDS-PAGE condition
1	MEDI8852 IgG	Reducing
2	MEDI8852 IgA	Reducing
3	MEDI8852 IgGA	Reducing
4	MEDI8852 IgG	Non-reducing
5	MEDI8852 IgA	Non-reducing
6	MEDI8852 IgGA	Non-reducing

Figure 6.8 Visualisation of MEDI8852 IgGA using SDS-PAGE. MEDI8852 IgGA, comprising MEDI8852 IgG1 with the IgA1 hinge region, together with the monomeric IgA1 and IgG1 equivalents, were prepared from transient transfection of HEK 293T cells with plasmids pCMV.MEDI8852.GH, pCMV.MEDI8852.AH, pCMV.MEDI8852.GAH and pCMV.MEDI8852.κ (Table 2.1), purified using Sepharose A, separated by SDS-PAGE (3.0 µg per lane; Section 2.2.7.1) under reducing and non-reducing conditions and detected with Quick Coomassie Stain (Section 2.9). The position of the bands corresponding to the heavy and light chains are indicated with arrows. Protein markers (Bio-Rad) ranging from 10 to 250 kDa were included.

6.3 Discussion

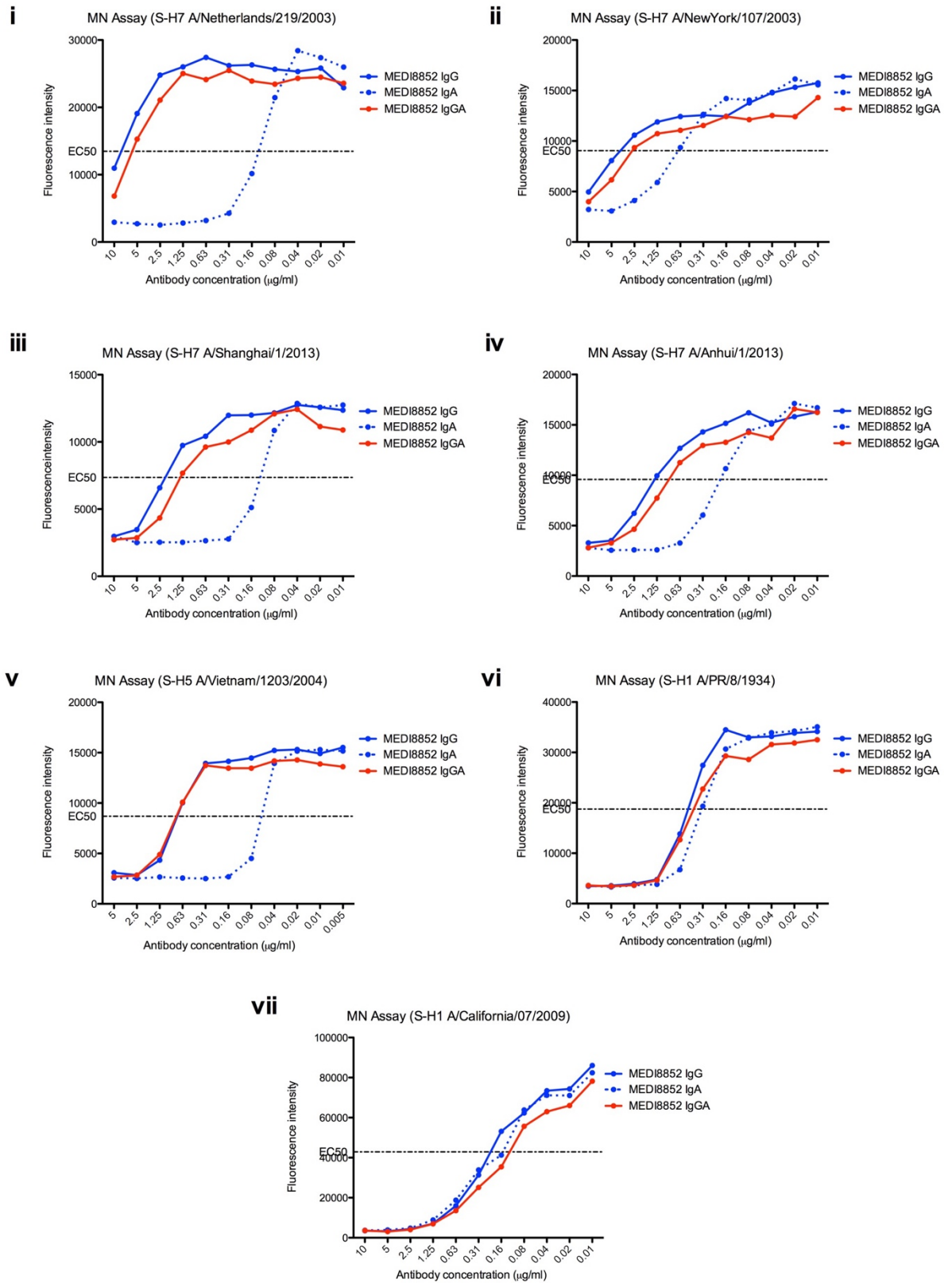
6.3.1 Enhancement of neutralising activity and cross-reactivity in HA-stem targeting IgA1

In this chapter, the enhanced bioactivity of IgA1 bnAb was explored. Two publications reported the enhancement of influenza bnAb in an IgA backbone (Section 6.1.3). Enhancement in bioactivity of HA-targeting antibodies were observed when they are switched to IgA isotype from IgG. Whereas the two published studies only investigated one antibody (Muramatsu et al., 2014), or only tested the antibodies against one influenza strain (Muramatsu et al., 2014, He et al., 2015)) this chapter describes a more extensive study, with a panel of bnAbs targeting different influenza strains and HA epitopes isolated from humans.

Several findings were observed in this study. Firstly, similar to what has been reported previously (He et al., 2015), enhancement in neutralising activity was observed in HA-stem targeting antibodies when they were switched to the IgA1 backbone, with a three- to 16-fold enhancement compared with their IgG1 equivalents, depending on influenza strains (Table 6.2). One observation that has not been previously reported, however, is the enhancement in cross-reactivity of neutralisation when influenza bnAb IgG1 are switched to IgA1. The T2-5D and Z3-B2 IgA1 were shown to neutralise influenza strains that their IgG1 equivalents did not (Table 6.2).

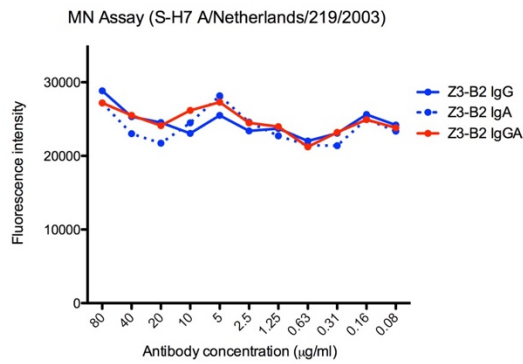
It has been previously reported that VH1-69-encoded HA-stem targeting bnAb has a distinct molecular characteristic, consisting of a triad of amino acid

A

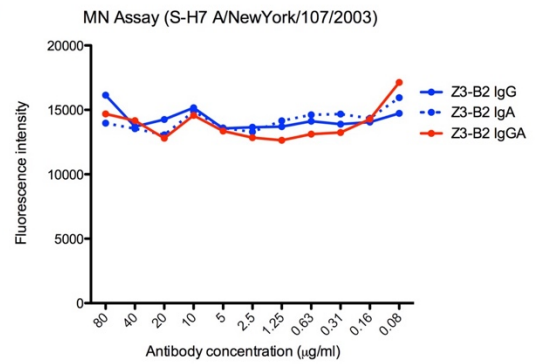


B

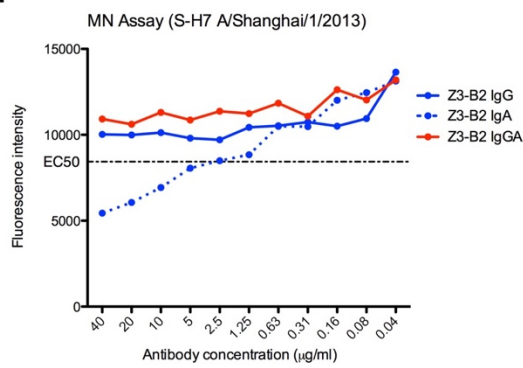
i



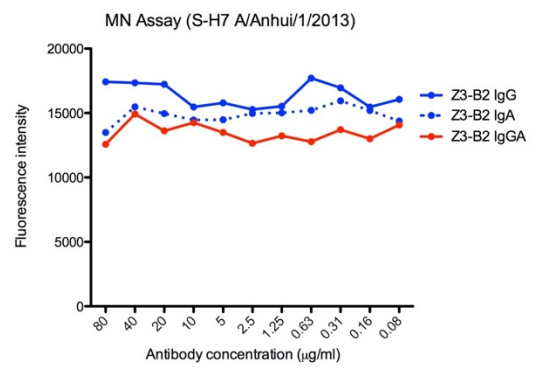
ii



iii



iv



v

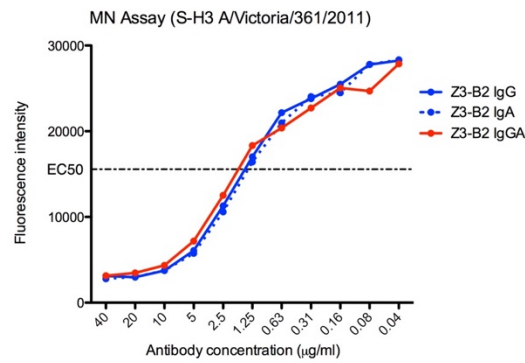


Figure 6.9 Neutralising titre of IgGA compared to the IgG1 and IgA1 equivalents.

(A) MEDI8852 IgGA, (B) Z3-B2 IgGA and their IgG1 and IgA1 equivalents were prepared from transfection of HEK 293T cells with plasmids pCMV.MEDI8852.GAH, pCMV.MEDI8852.GH pCMV.MEDI8852.AH and pCMV.MEDI8852.κ or pCMV.Z3B2.GAH, pCMV.Z3B2.GH pCMV.Z3B2.AH and pCMV.Z3B2.λ (Table 2.1) followed by purification using Sepharose A or Peptide M column (Section 2.2.7.1). Neutralising titre was determined using MN assay (Section 2.2.6.4) against various recombinant influenza strains (Table 2.4). Each data point represents the mean of duplicate readings. The EC₅₀ was calculated as the concentration of antibody that results in half-maximal response. In the MN assay, infection was expressed as fluorescence intensity when S-Flu (Table 2.4) was used.

residues (Avnir et al., 2014) and that binding of these bnAbs with HA relies on a hydrophobic amino acid, a conserved phenylalanine at position 54 in the CDR2, and a tyrosine at position 98 in the CDR3 region of the H chain (Avnir et al., 2014). This led to the speculation that switching to IgA1 backbone might enhance the neutralising activity of bnAbs that have the same molecular signatures or germline family. However, the results in Section 6.2.2.1, with at least two bnAbs encoded by VH1-69, suggest that the antibody germline family does not predict enhancement in IgA1 backbone. This would need to be confirmed by testing more bnAbs from the same germline family, although this is difficult, because apart from VH1-69, no association between another germline family and HA specificity has been reported. In the collection of bnAbs used here, there are no other antibodies from the same germline family that share the same HA specificity. Together, these data suggest that the enhancement in IgA1 backbone is antibody-dependent and enhancement cannot be predicted solely based on the antibody germline family.

To investigate if the more flexible IgA1 hinge region enhances the neutralisation of IgG1, hybrid IgGAs were constructed, produced and tested for neutralisation activity (Section 6.2.3). The results show that the inclusion of the IgA1 hinge in IgG1 did not enhance neutralisation (Figure 6.7); the hybrid IgGA retained the same neutralisation activity as the IgG equivalents. Although the structure of the hybrid IgGA was not determined, it can be speculated that with the inclusion of the long, proline-rich IgA1 hinge, IgGA should have a more flexible hinge

region and possess an overall T-shape structure like IgA1 (Figure 6.7). MEDI8852 and Z3-B2 IgGA molecules retained their neutralisation activity against all influenza strains tested compared with their IgG equivalents (Figure 6.9), a result suggesting that the flexibility of the hinge region does not influence the neutralisation activity of antibody. This finding is consistent with a study reporting that the increased length and flexibility of the hinge region did not increase the antigen binding of an antibody against *Bacillus anthracis* (Hovenden et al., 2013).

It has been a central dogma in the immunology field that antigen specificity is solely dependent on the V regions, but it was later discovered that the C regions also confer antigen binding function (Torres and Casadevall, 2008). Several groups have reported that different antibody isotypes or subclasses, all with identical V regions, possess different affinity and/or specificity in antigen binding for both polysaccharide and peptide antigen (Cooper et al., 1993, Pritsch et al., 2000, McLean et al., 2002, Torres et al., 2005, Tudor et al., 2012). These works led to the suggestion that the C region can exert an allosteric effect on the paratope and influence antigen interactions. Further studies on dissecting the mechanism of how the C regions influence the paratope showed that the C and V regions are structurally coupled and affect each other during antigen binding (Janda and Casadevall, 2010). The C region can affect the structural rigidity of the V region (Correa et al., 2013) and the energy landscape on the paratope (Janda et al., 2012). Together with the IgGA results, it is suggested that the increased length and flexibility of the hinge region alone do not enhance the neutralisation of the antibody and the enhancement observed

in IgA1 was probably due to the allosteric effect on the paratope exerted by the IgA1 C region, although this needs further confirmation.

It was surprising that there was no difference in HA binding affinity between those IgG1/IgA1 pairs which showed differences in neutralisation (Figure 6.6), as there is an expectation that an enhancement in neutralisation would be associated with enhancement in binding affinity to the antigen, as reported previously (Muramatsu et al., 2014, He et al., 2015). One explanation is that the difference in neutralisation is associated with a difference in binding kinetics (association rate (K_{on}) and dissociation rate(K_{off})) of IgG1 and IgA1 against HA that does not show up on ELISA used in this studies that measures antibody affinity at a steady equilibrium state. The binding kinetics could be measured using avidity ELISA or Surface Plasmon Resonance (SPR), which might provide more insight on the difference in the interactions between HA and IgG1 or IgA1. Changes in mAb binding kinetics have been found to affect neutralisation of respiratory syncytial virus (Bates et al., 2014).

6.3.2 Enhancement in neutralising activity in H5 and H7 strains

Another intriguing observation was that the enhancement in IgA1 HA-stem targeting was observed with H5 and H7 strains only, and not with H3 or H1 strains (Table 6.2). He et al., 2015 reported neutralisation enhancement in two recombinant HA-stem targeting IgA1, although only one influenza strain was tested in the study and it contained a chimeric HA (the globular head region of H5 A/Vietnam/1203/2004 and stem region of H1 A/PR/8/1934). It is possible that the difference in stability between HA/IgG1 and HA/IgA1 complexes within

the acidic endosome might be responsible for the enhancement neutralisation of HA-stem targeting IgA1. The HA stem region contains a fusion peptide required for viral-endosomal fusion due to gradual acidification of the endosome upon internalisation of the virus in the host cell (Wiley and Skehel, 1987). Most HA-stem targeting antibodies neutralise infection via blocking the fusion between the viral and endosomal membranes (Brandenburg et al., 2013). It is known that different HA subtypes undergo conformation change for fusion at different pH (Scholtissek, 1985). It is possible that the HA/IgA1 complex has a higher stability at its fusion pH compared with HA/IgG1. This speculation is established only if the H5 and H7 have a lower fusion pH than H3 and H1, since enhancement in neutralisation in IgA1 was observed in H5 and H7. Although the fusion pH of the HA used in this study was not determined, it has been reported that H1 and H3 subtypes have a lower fusion pH (around pH 5.2 to 5.4) compared with H5 and H7 subtypes (around pH 5.9 to 6.0) (Galloway et al., 2013, Zaraket et al., 2013, Gabbard et al., 2014). It is therefore unlikely that the enhanced neutralisation seen in H5 and H7 with IgA1 was due to enhanced stability of the HA/IgA1 complexes in the acidic endosome. Recombinant influenza virus expressing eGFP (S-eGFP) (Table 2.4) was used in the MN assays in this study. It has been previously established that S-eGFP is comparable to wild type in neutralisation (Alain Townsend, personal communication.) MN assays with wild type H5 and H7 influenza were not performed due to technical difficulties, but it could be interesting to compare the neutralisation of IgG1 and IgA1 with wild type H5 and H7 influenza to see if similar enhancement is seen in IgA1.

6.3.3 Decreased neutralising activity in HA-head targeting IgA1

Most of the HA-head targeting IgA1 tested in this study show no difference in neutralisation compared with their IgG1 equivalents. This is probably because only a tiny amount of antibody is needed to saturate the HA-head to prevent infection (via blocking attachment of virus to the host cells) compared with HA-stem (via blocking of viral membrane fusion) which requires more antibody. Approximately 10 to 100-fold less HA-head targeting IgG1 is needed to block influenza infection in cell culture compared with HA-stem-targeting IgG (Huang et al., 2015b). This could be because the HA head region is more accessible to the antibodies and can be neutralised more potently.

Decreased neutralisation was observed in HA-head targeting IgA1 (21-D8-5A and T2-6A) (Table 2.1) ranging from three- to 20-fold, a finding contrary to Muramatsu et al., 2014 who reported an enhancement of neutralising activity of a HA-head targeting cross-reactive IgA compared with its IgG equivalent. Possible differences include that the IgA used by Muramatsu *et al.*, (2014) was produced via hybridoma technology by fusing mouse antibody-secreting cells with myeloma cells (Kohler and Milstein, 1975), and the IgA subclass was unknown, whereas all IgAs used in this study were IgA1. The IgA produced was also found to be a mixture of monomeric and polymeric forms (Muramatsu et al., 2014) whereas the IgA1 produced in this study were shown to be largely monomeric (Figure 6.4). It is possible that the polymeric IgA present in the study by Muramatsu et al., (2014) might be responsible for the enhancement observed. Also, the IgA used was a chimeric mouse antibody, and such

decreased neutralisation has been reported previously for a chimeric mouse HA-stem targeting IgA (Mak et al., 2014).

HA-head targeting mAb can neutralise in one of two ways: either via inserting the CDR3 region into the receptor binding site (RBS) to mimic sialic acid binding thereby blocking sialic acid recognition when the binding site overlaps the RBS, or via steric hindrance when they bind elsewhere on the HA globular head (Knossow and Skehel, 2006). As discussed above, the C domains can affect the paratope and can influence steric hindrance of an antibody (Nason et al., 2001). The exact neutralisation mechanism of 21-D8-5A and T2-6A, whether via mimicking sialic acid binding or steric hindrance, is not known. The results suggest that switching to IgA1 might reduce the potency of HA-head targeting antibodies that act via steric hindrance. This, however, requires further investigation.

A study reported that, from the PBMC repertoire isolated from healthy human donors, a substantial portion of IgA-secreting B cells were HA-stem targeting (He et al., 2015). This indicates that, in nature at least, HA-head targeting V regions might not be favourable for class-switching into IgA, probably because of the reduced neutralisation of HA-head targeting IgA, which is consistent with the findings in this chapter.

6.3.4 Conclusion

The results presented in this chapter show that influenza HA-stem targeting bnAbs can be made more potent in neutralisation by switching them into an

IgA1 backbone; further investigation is needed to elucidate the mechanism. While this chapter focuses on the neutralisation, the effector function of IgA1, such as ADCC, could also be investigated to show if the IgA1 backbone (the Fc region) has an enhanced effector function compared with IgG1, which could result in enhanced elimination of influenza infected-cells. Furthermore, the efficacy of IgA1 could be compared with IgG1 in animal models to investigate if the enhanced neutralisation of IgA1 *in vitro* were translated into improved protection against influenza *in vivo*. Overall, this chapter demonstrates the enhanced potency of anti-influenza IgA1 in neutralising influenza infection and supports studies to express IgA1 using viral vectors. The enhanced efficacy of IgA1 could ultimately translate into lower viral vector doses, and therefore lower costs, for higher therapeutic efficacy.

Chapter 7 Discussion

7.1 Viral vectors for passive immunisation against influenza

The discovery of broadly neutralising antibodies (bnAbs) has opened up the possibility of passive immunisation by means of delivering sera from recovered patients, or pathogen-specific monoclonal antibody (mAb), as an alternative to conventional vaccines. While the parenteral administration of purified antibody is limited by high antibody production costs, and only transient protection due to the relatively short half-life of the delivered antibody in the circulation, the use of viral vectors to deliver bnAbs can perhaps overcome these limitations.

The aim of this thesis was to study the potential of rSIV.F/HN as a bnAb gene transfer agent to the lung for passive immunisation against influenza. Initially developed for cystic fibrosis (CF) gene therapy, rSIV.F/HN has shown promising results in non-clinical studies and will be tested in dose-escalating Phase I/II safety and efficacy studies in due course (Alton et al., 2017). Influenza was selected as a disease model because i) there is a need for novel, effective prophylaxis approaches for the emerging influenza pandemics; ii) of the discovery of influenza bnAbs, including some novel bnAbs isolated by collaborator Alain Townsend from recovered patients/vaccinated donors, offering broad protection against multiple strains of influenza, and iii) influenza is a respiratory disease, production of bnAb at the initial site of infection (lung) mediated by rSIV.F/HN could provide effective protection. Studies were designed to investigate expression of bnAb from rSIV.F/HN to confer protection against influenza infection in the mouse model. As rSIV.F/HN had not been previously tested in this way,

delivery of rAAV2/8 vector to the muscle was also included since this latter approach was reported to be effective against influenza challenge in mice (Balazs et al., 2013).

Studies in Chapter 3 focussed on the design of the bnAb expression construct and the production of viral vectors expressing the secreted GLux reporter protein and selected bnAbs. The latter were expressed from a single-ORF cDNA construct (Figure 3.1), to facilitate packaging of the genome into viral vectors with limited packaging capacity, and were found to be as least as potent as their dual-ORF equivalents (Section 3.2.4).

In Chapters 4 and 5, it was confirmed that rAAV2/8 delivered to the muscle expressed high levels of bnAb, and was capable of conferring 100% protection against influenza challenge in the mouse model (Sections 4.2.2 and 5.2.1), consistent with previous findings (Balazs et al., 2013). This also confirmed the efficacy of the novel T1-3B bnAb (Table 2.5) for protection against lethal influenza challenge mediated by a viral vector. Initial proof-of-principle using GLux showed that rSIV.F/HN delivered to the lung can direct expression of soluble protein both into the lung lumen and systemic circulation (Section 4.2.3). Consistent with this proof-of-principle study, the ability of rSIV.F/HN vector to express T1-3B bnAb, both locally in the lung and the circulation following lung delivery to mice, was demonstrated (Section 4.2.4). However, GLux and bnAb resulted in different expression profiles, suggesting that GLux might not be a useful proxy for bnAb expression mediated by viral vectors (Section 4.3.3). Comparing bnAb expression following rAAV2/8 IM delivery and rSIV.F/HN IN delivery, rAAV2/8 directed around 1,000-fold higher serum bnAb than rSIV.F/HN, whereas the bnAb levels in the lung lumen were comparable using both delivery strategies. This suggests that IM delivery of rAAV2/8 may be a better approach for mAb expression where high levels

of mAb are needed in the serum, whereas IN delivery of rSIV.F/HN and IM delivery of rAAV2/8 are equivalent for expression of mAb within the lung lumen.

In Chapter 5, mice receiving rSIV.F/HN expressing T1-3B were challenged with influenza to determine the extent of rSIV.F/HN mediated protection. A single dose of rSIV.F/HN conferred partial protection against lethal dose of influenza challenge (Section 5.2.2), representing, to the best of our knowledge, the first demonstration of rLV vector expressing therapeutic bnAb and conferring protection against influenza *in vivo*. As discussed above, although the IgG levels in the lung lumen for both rSIV.F/HN and rAAV2/8 treated mice prior to influenza challenge were similar, mice receiving rAAV2/8 demonstrated full protection against influenza challenge (150 LD₅₀) whereas mice receiving rSIV.F/HN only conferred partial protection against influenza challenge (15 LD₅₀). This supports the suggestion that of serum IgG helps prevent influenza infection which has been suggested previously (Ito et al., 2003, Renegar et al., 2004). One can imagine during an influenza infection, the depleted IgG in the lung lumen could be continuously replenished from the high serum IgG which was being produced in the muscle mediated by rAAV2/8 whereas the protection against influenza in mice receiving rSIV.F/HN IN probably relies solely on the IgG levels in the lung lumen. As discussed in Section 5.3.2, the mouse model serves as a convenient and relatively inexpensive animal model for influenza studies but it is not the most relevant. To better recapitulate and predict the efficacy of viral vector-mediated passive immunisation in humans, a more relevant animal model for future studies would be ferrets. Ferrets have a similar airway SA distribution to humans and are naturally susceptible to human influenza viruses. More importantly, unlike mice, ferrets can be used to study transmission of influenza via direct contact or aerosol transmission, which more

closely resembles the natural infection route of influenza in humans (Bouvier and Lowen, 2010).

In order to improve the protection against influenza mediated by rSIV.F/HN, two strategies were investigated. The first strategy was the use of daily repeated administration, which has been previously shown to increase reporter transgene expression in mice with low dose (1E6 TU) of rSIV.F/HN (Griesenbach et al., 2012c)(Section 5.2.3). Consistent with previous findings (Griesenbach et al., 2012b), bnAb expression increased significantly when lower dose (1E6 TU) of rSIV.F/HN was repeat administered daily which also led to higher survival against influenza challenge (Figure 5.6) However, this daily repeat administration strategy did not lead to increased bnAb expression, and did not improve protection, with a higher dose of rSIV.F/HN (1E7 and 1E8 TU) (Figure 5.6). As discussed in Section 5.1.1, the reason was unclear and requires further investigation. It is possible that the failure of the daily repeat administration approach to improve protection against influenza could be due to the repeated sessions of inhalation anaesthesia, the high (overall) volume of fluid delivered to the mouse lung, or a resultant cytokine response due to higher viral dose, a combination of which might increase the susceptibility of the mice to influenza infection.

The second strategy aimed to improve rSIV.F/HN-mediated protection against influenza involved antibody engineering (Chapter 6). Engineering of bnAb into human IgA was attempted, based on previous studies that reported bioactivity enhancement of influenza bnAb in IgA form (Section 6.1.3). Indeed, an enhancement in neutralisation was observed with several of our novel bnAbs, including T1-3B, against

the two potential pandemic influenza strains H5 and H7, when they were engineered into the human IgA1 isotype (Section 6.3.2.1). If expressed using viral vectors, the enhanced potency of influenza-targeting IgA1 could mean higher therapeutic efficacy, lower viral vector dose and lower overall costs. However, the enhanced potency of IgA1 compared to their IgG1 equivalents was only tested in cell culture in this study, whether the enhancement holds true *in vivo* requires further investigation.

7.2 Potential issues for the translation of rAAV2/8 and rSIV.F/HN approaches into the clinic

Recombinant AAV is a promising vector for gene therapy, becoming the first gene therapy vector licenced for gene therapy in the European Union (Yla-Herttuala, 2012). Apart from its excellent track record in animal models, rAAV is non-pathogenic in humans, has a good safety profile in the clinic (Nathwani et al., 2011, Gaudet et al., 2013) and has been shown to direct expression of various mAbs at high levels in animal models (Table 1.3). In this study, published findings were confirmed and showed that rAAV2/8 delivered IM can direct high levels of bnAb expression in the serum (Section 4.2.2), providing complete protection against influenza infection in mice (Section 5.2.1). Inconsistencies in the translation of rAAV transgene expression from animal models to humans have been reported. For example, in the Haemophilia B clinical trial using rAAV2/8 delivered via IV route, less than 1% of serum Factor IX was detected in patients (Nathwani et al., 2011) which was not proportional to levels achieved in mice (160%) and NHP (40%) (Nathwani et al., 2006), even though the vector doses per body weight used were similar or higher in humans. The discrepancy, however, was not seen when the same dose of rAAV expressing FIX was delivered IM in mice, dogs and humans (Manno et al., 2003). The published reports suggest that

the inconsistency of transgene expression following rAAV delivery between animals and humans is dependent on the delivery method and that IM delivery leads to more consistent cross-species transgene expression. Based on this, it might be reasonable to extrapolate the bnAb expression in mice obtained in this study into proportional bnAb expression in humans.

One of the biggest hurdles for the clinical realisation of rAAV gene therapy is the presence of pre-existing immunity in the human population, which can hinder rAAV transduction. It has been reported that around 30 to 60% of the human population is seropositive against various AAV serotypes, with the highest being against AAV1 and AAV2 (Calcedo et al., 2009, Boutin et al., 2010). Furthermore, humoral immunity triggered following the first rAAV administration is likely to prevent repeated administration of the same AAV serotype, as observed in non-clinical studies (Sumner-Jones et al., 2007, Wang et al., 2009). A lack of ability to repeatedly administer rAAV vectors means that a patient could only be successfully treated on one occasion; this limits the opportunity for an individual to receive multiple doses of rAAV for long-term treatment, or subsequent doses for treatment of other diseases in the future. A possible solution is that alternative AAV serotype/variants could be used in subsequent AAV administrations to avoid this issue (Riviere et al., 2006), provided the variants have similar tissue tropism and there is no cross-reactivity between them. This approach has been tested successfully in animal models (Halbert et al., 2000, Davidoff et al., 2005), but it has been reported that cross-reactive antibodies against AAV serotypes 2, 7 and 8 have developed in subjects receiving rAAV1 (Flotte et al., 2011). This problem potentially limits the wider use of rAAV for applications requiring long-term expression and/or requiring repeated administration. It is likely that gene

transfer for expression of influenza antibodies will require repeated administration of viral vectors to achieve therapeutic levels of expression and especially to keep pace with the highly evolving influenza virus.

While IM delivery of rAAV in mice is relatively simple and requires only a single injection (Section 2.2.9.4), the procedure is much more complicated in human and may require 40 or more injections at multiple muscles to maximise transduction of muscle cells (Gaudet et al., 2013). Furthermore, due to the high number of injections needed, the patient has to be put under spinal anaesthesia. This makes mass vaccination of the population during disease outbreaks with such a delivery strategy impractical.

Unlike rAAV, rSIV.F/HN offers the possibility of successful repeat administration. In mice, monthly doses of rSIV.F/HN did not result in loss of transgene expression (Griesenbach et al., 2012b), which has been observed when two or more doses of other viral vectors were delivered to the murine lung (Sumner-Jones et al., 2007). The Sendai virus (mouse parainfluenza virus) used for rSIV pseudotyping in this study shares high sequence homology with human parainfluenza virus and pre-existing neutralising mAb against parainfluenza virus in the human populations might prevent transduction of rSIV.F/HN. However, it has been shown that the lung transduction of rSIV.F/HN was not inhibited in mice pre-treated with neutralising antibodies against human parainfluenza virus isolated from the human population (Alton et al., 2017). The reason why it is possible to repeatedly administer rSIV.F/HN without loss of efficacy is currently unknown, but might relate to the speed with which the virus enters the cell via the F/HN pseudotype (Alton et al., 2017). Together, these observations

suggest that repeated administration of rSIV.F/HN in humans might also be possible, which would be beneficial for passive immunisation as well as treatment of chronic diseases.

For delivery of rSIV.F/HN, the aim is to deliver to the human lung via a non-invasive aerosol-generating nebuliser. Preliminary studies have shown that rSIV.F/HN remains stable in nasal spray bottles and clinical-grade catheters, which allow nasal and regional lung delivery (Alton et al., 2017). Although the use of a nebuliser is non-invasive, the delivery device utilised needs careful selection, as such devices can be relatively inefficient, leading to additional unwanted expense for wide-spread use in large target populations.

7.3 Potential viral vector related toxicity

The potential toxicity of viral vectors, in particular those that can integrate into the host genome, is a valid concern. Wild type AAV has been long considered a non-pathogenic virus with integration at a safe harbour in chromosome 19 (namely AAVS1 site) mediated by the Rep protein (Kotin et al., 1990). A recent study has found that wild type AAV2 can integrate within a known oncogene in around 5% of hepatocellular carcinomas, suggesting potential genotoxicity risks of AAV (Nault et al., 2015), although following this controversial report, a later publication challenged the initial findings and suggested an alternative conclusion (Berns et al., 2015). In the rAAV vector, due to the absence of *rep* gene, integration appears to occur rarely and randomly into the host genome (Miller et al., 2005, Kaepffel et al., 2013a). In the clinic, no genotoxicity has been so far reported in patients receiving rAAV1 gene therapy intramuscularly for up to 6 years (Gaudet et al., 2016), but rAAV vectors possess

genotoxicity risks in mice which were influenced by the selection of enhancers/promoter sequence, the transgene, vector dose and timing of vector delivery (Chandler et al., 2015). These findings highlight the importance of attending to rAAV vector design to reduce risk of genotoxicity.

The rSIV.F/HN vector offers several advantages as a lung gene transfer agent. The lung-targeting rSIV.F/HN efficiently transduces 15% of mouse lung epithelial cells including ciliated airway epithelial cells, goblet cells, club cells and type I and II pneumocytes at the maximum feasible dose, without the need of tight-junction openers prior to administration (Alton et al., 2017). The integration of rLV into the host genome of dividing and non-dividing cells renders rSIV.F/HN suitable for applications requiring long-term transgene expression, but possible genotoxicity has been a major concern. It has been shown in animal models and cell culture that rLV exhibits insertional mutagenesis (Bokhoven et al., 2009), although rLV is generally less mutagenic than γ -retroviral vectors (Hematti et al., 2004, Montini et al., 2009). Unlike γ -retroviral vectors, rLV has a strong preference to integrate within transcriptional units in actively transcribing genes instead of near the transcription start site (TSS), hence a reduced chance of trans-activating adjacent proto-oncogenes (Wu et al., 2003, Hematti et al., 2004, Mitchell et al., 2004, Niederer and Bangham, 2014). The integration site bias of rLV is also influenced by the activity status of the transduced cells (Brady et al., 2009), and there are many other factors that affect genotoxicity including the vector design, the transgene, the transduced cell type and host factors such as underlying diseases and age (Montini et al., 2009, David and Doherty, 2017). Integration site analysis of rSIV.F/HN in the mouse lungs revealed no preference for integration near oncogenic loci, showing the reduced likelihood of oncogenesis (Alton

et al., 2017). In terms of vector design, rSIV.F/HN is a self-inactivating (SIN) vector, lacking 3' LTR viral promoter and enhancer activity (Section 1.5.3), which greatly reduces the risk of insertional mutagenesis (Cesana et al., 2014) and the generation of splice-variant transcripts of genes located near the vicinity of integration sites compared with γ -retroviral vectors (Cesana et al., 2012, Moiani et al., 2012).

One possible adverse effect of mAb expression mediated by viral vectors is the persistent overexpression of the mAb which might lead to a pathological condition called paraproteinemia (also known as monoclonal gammopathy). Paraproteinemia is characterised by the presence of excessive (single) mAb in the circulation, often caused by immune-proliferative diseases or haematological cancers such as multiple myeloma or monoclonal gammopathy of undetermined significance (MGUS). Paraproteinemia has been linked to pathological conditions such as neuropathy, oculopathy, dermatopathy, coagulopathy, autoimmunity and nephropathy (reviewed in (Glavey and Leung, 2016)). Based on the high serum bnAb expression levels with rAAV2/8 in mice (Section 4.2.2), this delivery approach might pose a risk of paraproteinemia. Thus, careful assessment of the delivered dose may be particularly important for the successful development of rAAV2/8 vectors in this context.

These possible toxicities highlight the importance of careful vector design, extensive genotoxicity studies and fine tuning of the expression levels of bnAb by optimising the vector dose and constraining expression via careful selection of promoters/enhancers, to maximise therapeutic effect while avoiding side-effects.

7.4 Future directions

The enhanced potency of IgA1 bnAb demonstrated in Chapter 6 is supportive of studies to express IgA1 bnAb using viral vectors. If this approach translates into enhanced *in vivo* efficacy, this could ultimately result in lower viral vector doses, and therefore lower costs required to achieve immunological protection. Influenza replication is highly error-prone, and selection of 'escape mutants' is highly likely to occur if a single antibody is used (O' Donnell et al 2012). Although the HA stem region is less permissive for escape mutations, because it is structurally constrained, selection of escape mutations from HA-stem targeting bnAbs has been reported (Chai et al., 2016). Thus, the therapeutic efficacy of using of a single bnAb regimen, even with the HA-targeting bnAb, might be hampered by the emergence of escape mutants. Furthermore, a mass vaccination of the human population expressing the same single bnAb is likely to exert a greater immunological pressure on influenza, further promoting generation of escape mutations. To circumvent this problem, a cocktail of non-competing bnAbs could be used to reduce the chances of selecting escape mutants and, at the same time, confer broader protection. Delivery of a cocktail of affinity-purified bnAbs has been shown to successfully prevent formation of escape mutants in H5N1 influenza (Prabakaran et al., 2009). Furthermore, to reduce the immunological pressure on a single target on the influenza virus, antibodies targeting other influenza surface glycoproteins, such as the NA which is involved in influenza virus budding, could be included (Section 1.1.2); NA-targeting mAbs have been shown to protect against influenza challenge in the mouse model (Wan et al., 2013, Wan et al., 2015). Several NA-targeting bnAbs have been isolated (Alain Townsend, personal communication) and one in particular, Z2-B3 that cross-reacts with N1 and N9 influenza, has shown protection in mice against a lethal dose of A/PR/8 H1N1

challenge given prophylactically (data not shown). This highlights the therapeutic potential of NA-targeting bnAbs and the utility of including them in the cocktails.

One concern with the expression of a cocktail of antibodies mediated by viral vectors is that it might lead to random association of antibody heavy and light chains when two or more different antibody genes are being expressed in the same host cell, a phenomenon commonly observed in bispecific antibody production (Schaefer et al., 2016). A cocktail comprising two different bnAbs could theoretically result in 16 bnAb combinations, and this random chain association might result in non-functional or low efficacy bnAb. To overcome this problem, scFV, encoded on a single intact protein chain, instead of full-length bnAb, encoded on two chains, could be used, although, it would be important to first investigate the efficacy of scFV compared with their full length bnAb equivalents.

Influenza protection investigated in this thesis was evaluated using a standard lab influenza strain (A/PR/8 H1N1) performed in commonly available, relatively low containment Biosafety Level 2 (BSL-2) facilities. However, for future influenza pandemic preparedness, efficacy of viral vectors ought to be tested against potential future pandemic strains, such as the HPAI H5N1 and H7N9. Such strains are categorised as BSL-3 pathogens by the WHO due to their highly virulent nature and animal challenge experiments with these strains can only be carried out in BSL-3 or BSL-4 facilities. Due to the limited number of BSL-3 or 4 facilities, and their associated high experimental costs, testing of vaccines or treatments against these viruses has been restricted. To circumvent this problem, BSL-2 compatible, *firefly* luciferase expressing, replication-deficient recombinant H5 and H7 influenza (S-Lux), based on

S-Flu (Powell et al., 2012a) are being developed. The S-Lux could be used as a surrogate to study potential pandemic influenza in animal models in BSL-2 facilities, using non-invasive bioluminescent imaging, greatly simplifying progress in the field.

7.5 Conclusions

This thesis investigated the feasibility of using rSIV.F/HN to express bnAb for passive immunisation against influenza, comparing it with the published rAAV2/8 IM approach. Both vectors need further development and testing, and have challenges that need addressing. However, given the data presented in this thesis, passive immunisation clearly offers an alternative to conventional vaccines particularly in high-risk groups that often respond only poorly to conventional vaccination. One can envisage that during an influenza pandemic, viral vectors carrying bnAb matching the pandemic strain could be used immediately for ring vaccination in the affected areas. In addition, the availability of pre-tested cocktails of suitable bnAbs could facilitate a swift response to an influenza outbreak, rapidly protecting key health workers and crucial infrastructure personnel from infection. Such an approach could also be applicable for other infectious diseases; indeed, preliminary experiments show that rAAV2/8 delivered to the muscle directed high levels expression of *Zaire* Ebola neutralising antibodies at potentially therapeutic levels (data not shown). Taken together, the studies in this thesis provide evidence for use of viral vectors to mediate passive immunisation against influenza, potentially more cost-effective and long-lasting than conventional approaches.

References

- ABRAMOFF, M. D., MAGALHAES, P. J. & RAM, S. J. 2004. Image processing with ImageJ. *Biophotonics International*, 11, 36-42.
- ABRUZZESE, R. V., GODIN, D., BURCIN, M., MEHTA, V., FRENCH, M., LI, Y., O'MALLEY, B. W. & NORDSTROM, J. L. 1999. Ligand-dependent regulation of plasmid-based transgene expression in vivo. *Hum Gene Ther*, 10, 1499-507.
- AIR, G. M. 2012. Influenza neuraminidase. *Influenza Other Respir Viruses*, 6, 245-56.
- AITKEN, M. L., MOSS, R. B., WALTZ, D. A., DOVEY, M. E., TONELLI, M. R., MCNAMARA, S. C., GIBSON, R. L., RAMSEY, B. W., CARTER, B. J. & REYNOLDS, T. C. 2001. A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. *Hum Gene Ther*, 12, 1907-16.
- AIUTI, A., BIASCO, L., SCARAMUZZA, S., FERRUA, F., CICALESSE, M. P., BARICORDI, C., DIONISIO, F., CALABRIA, A., GIANNELLI, S., CASTIELLO, M. C., BOSTICARDO, M., EVANGELIO, C., ASSANELLI, A., CASIRAGHI, M., DI NUNZIO, S., CALLEGARO, L., BENATI, C., RIZZARDI, P., PELLIN, D., DI SERIO, C., SCHMIDT, M., VON KALLE, C., GARDNER, J., MEHTA, N., NEDUVA, V., DOW, D. J., GALY, A., MINIERO, R., FINOCCHI, A., METIN, A., BANERJEE, P. P., ORANGE, J. S., GALIMBERTI, S., VALSECCHI, M. G., BIFFI, A., MONTINI, E., VILLA, A., CICERI, F., RONCAROLO, M. G. & NALDINI, L. 2013. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science*, 341, 1233151.
- AKACHE, B., GRIMM, D., PANDEY, K., YANT, S. R., XU, H. & KAY, M. A. 2006. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *Journal of Virology*, 80, 9831-9836.
- ALBO, C., VALENCIA, A. & PORTELA, A. 1995. Identification of an RNA binding region within the N-terminal third of the influenza A virus nucleoprotein. *J Virol*, 69, 3799-806.
- ALFORD, R. H., KASEL, J. A., GERONE, P. J. & KNIGHT, V. 1966. Human influenza resulting from aerosol inhalation. *Proc Soc Exp Biol Med*, 122, 800-4.
- ALLOCCA, M., MUSSOLINO, C., GARCIA-HOYOS, M., SANGES, D., IODICE, C., PETRILLO, M., VANDENBERGHE, L. H., WILSON, J. M., MARIGO, V., SURACE, E. M. & AURICCHIO, A. 2007. Novel adeno-associated virus serotypes efficiently transduce murine photoreceptors. *J Virol*, 81, 11372-80.
- ALTON, E. W., ARMSTRONG, D. K., ASHBY, D., BAYFIELD, K. J., BILTON, D., BLOOMFIELD, E. V., BOYD, A. C., BRAND, J., BUCHAN, R., CALCEDO, R., CARVELLI, P., CHAN, M., CHENG, S. H., COLLIE, D. D., CUNNINGHAM, S., DAVIDSON, H. E., DAVIES, G., DAVIES, J. C., DAVIES, L. A., DEWAR, M. H., DOHERTY, A., DONOVAN, J., DWYER, N. S., ELGMATI, H. I., FEATHERSTONE, R. F., GAVINO, J., GEA-SORLI, S., GEDDES, D. M., GIBSON, J. S., GILL, D. R., GREENING, A. P., GRIESENBACH, U., HANSELL, D. M., HARMAN, K., HIGGINS, T. E., HODGES, S. L., HYDE, S. C., HYNDMAN, L., INNES, J. A., JACOB, J., JONES, N., KEOGH, B. F., LIMBERIS, M. P., LLOYD-

- EVANS, P., MACLEAN, A. W., MANVELL, M. C., MCCORMICK, D., MCGOVERN, M., MCLACHLAN, G., MENG, C., MONTERO, M. A., MILLIGAN, H., MOYCE, L. J., MURRAY, G. D., NICHOLSON, A. G., OSADOLOR, T., PARRA-LEITON, J., PORTEOUS, D. J., PRINGLE, I. A., PUNCH, E. K., PYTEL, K. M., QUITTNER, A. L., RIVELLINI, G., SAUNDERS, C. J., SCHEULE, R. K., SHEARD, S., SIMMONDS, N. J., SMITH, K., SMITH, S. N., SOUSSI, N., SOUSSI, S., SPEARING, E. J., STEVENSON, B. J., SUMNER-JONES, S. G., TURKKILA, M., URETA, R. P., WALLER, M. D., WASOWICZ, M. Y., WILSON, J. M., WOLSTENHOLME-HOGG, P. & CONSORTIUM, U. K. C. F. G. T. 2015. Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: a randomised, double-blind, placebo-controlled, phase 2b trial. *Lancet Respir Med*, 3, 684-91.
- ALTON, E. W., BEEKMAN, J. M., BOYD, A. C., BRAND, J., CARLON, M. S., CONNOLLY, M. M., CHAN, M., CONLON, S., DAVIDSON, H. E., DAVIES, J. C., DAVIES, L. A., DEKKERS, J. F., DOHERTY, A., GEA-SORLI, S., GILL, D. R., GRIESENBACH, U., HASEGAWA, M., HIGGINS, T. E., HIRONAKA, T., HYNDMAN, L., MCLACHLAN, G., INOUE, M., HYDE, S. C., INNES, J. A., MAHER, T. M., MORAN, C., MENG, C., PAUL-SMITH, M. C., PRINGLE, I. A., PYTEL, K. M., RODRIGUEZ-MARTINEZ, A., SCHMIDT, A. C., STEVENSON, B. J., SUMNER-JONES, S. G., TOSHNER, R., TSUGUMINE, S., WASOWICZ, M. W. & ZHU, J. 2017. Preparation for a first-in-man lentivirus trial in patients with cystic fibrosis. *Thorax*, 72, 137-147.
- AMBROSE, C. S. & LEVIN, M. J. 2012. The rationale for quadrivalent influenza vaccines. *Hum Vaccin Immunother*, 8, 81-8.
- ANITEI, M. & HOFLACK, B. 2011. Exit from the trans-Golgi network: from molecules to mechanisms. *Curr Opin Cell Biol*, 23, 443-51.
- AREA, E., MARTIN-BENITO, J., GASTAMINZA, P., TORREIRA, E., VALPUESTA, J. M., CARRASCOSA, J. L. & ORTIN, J. 2004. 3D structure of the influenza virus polymerase complex: localization of subunit domains. *Proc Natl Acad Sci U S A*, 101, 308-13.
- ASOKAN, A., HAMRA, J. B., GOVINDASAMY, L., AGBANDJE-MCKENNA, M. & SAMULSKI, R. J. 2006. Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. *J Virol*, 80, 8961-9.
- ATCHISON, R. W., CASTO, B. C. & HAMMON, W. M. 1965. Adenovirus-Associated Defective Virus Particles. *Science*, 149, 754-6.
- AVNIR, Y., TALLARICO, A. S., ZHU, Q., BENNETT, A. S., CONNELLY, G., SHEEHAN, J., SUI, J., FAHMY, A., HUANG, C. Y., CADWELL, G., BANKSTON, L. A., MCGUIRE, A. T., STAMATATOS, L., WAGNER, G., LIDDINGTON, R. C. & MARASCO, W. A. 2014. Molecular signatures of hemagglutinin stem-directed heterosubtypic human neutralizing antibodies against influenza A viruses. *PLoS Pathog*, 10, e1004103.
- AYUSO, E., MINGOZZI, F., MONTANE, J., LEON, X., ANGUELA, X. M., HAURIGOT, V., EDMONSON, S. A., AFRICA, L., ZHOU, S., HIGH, K. A., BOSCH, F. & WRIGHT, J. F. 2010. High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. *Gene Ther*, 17, 503-10.
- BABU, Y. S., CHAND, P., BANTIA, S., KOTIAN, P., DEHGHANI, A., EL-KATTAN, Y., LIN, T. H., HUTCHISON, T. L., ELLIOTT, A. J., PARKER, C. D., ANANTH, S. L.,

- HORN, L. L., LAVER, G. W. & MONTGOMERY, J. A. 2000. BCX-1812 (RWJ-270201): Discovery of a novel, highly potent, orally active, and selective influenza neuraminidase inhibitor through structure-based drug design. *Journal of Medicinal Chemistry*, 43, 3482-3486.
- BADR, C. E., HEWETT, J. W., BREAKEFIELD, X. O. & TANNOUS, B. A. 2007. A highly sensitive assay for monitoring the secretory pathway and ER stress. *PLoS One*, 2, e571.
- BADRANE, H., BAHLOUL, C., PERRIN, P. & TORDO, N. 2001. Evidence of two Lyssavirus phylogroups with distinct pathogenicity and immunogenicity. *J Virol*, 75, 3268-76.
- BAINBRIDGE, J. W., MEHAT, M. S., SUNDARAM, V., ROBBIE, S. J., BARKER, S. E., RIPAMONTI, C., GEORGIADIS, A., MOWAT, F. M., BEATTIE, S. G., GARDNER, P. J., FEATHERS, K. L., LUONG, V. A., YZER, S., BALAGGAN, K., VISWANATHAN, A., DE RAVEL, T. J., CASTEELS, I., HOLDER, G. E., TYLER, N., FITZKE, F. W., WELEBER, R. G., NARDINI, M., MOORE, A. T., THOMPSON, D. A., PETERSEN-JONES, S. M., MICHAELIDES, M., VAN DEN BORN, L. I., STOCKMAN, A., SMITH, A. J., RUBIN, G. & ALI, R. R. 2015. Long-term effect of gene therapy on Leber's congenital amaurosis. *N Engl J Med*, 372, 1887-97.
- BALAZS, A. B., BLOOM, J. D., HONG, C. M., RAO, D. S. & BALTIMORE, D. 2013. Broad protection against influenza infection by vectored immunoprophylaxis in mice. *Nat Biotechnol*, 31, 647-52.
- BALAZS, A. B., CHEN, J., HONG, C. M., RAO, D. S., YANG, L. & BALTIMORE, D. 2012. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature*, 481, 81-4.
- BALAZS, A. B., OUYANG, Y., HONG, C. M., CHEN, J., NGUYEN, S. M., RAO, D. S., AN, D. S. & BALTIMORE, D. 2014. Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. *Nat Med*, 20, 296-300.
- BALTIMORE, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature*, 226, 1209-11.
- BAO, R., SELVAKUMARAN, M. & HAMILTON, T. C. 2000. Use of a surrogate marker (human secreted alkaline phosphatase) to monitor in vivo tumor growth and anticancer drug efficacy in ovarian cancer xenografts? *Gynecologic Oncology*, 78, 373-379.
- BARTLETT, J. S., SAMULSKI, R. J. & MCCOWN, T. J. 1998. Selective and rapid uptake of adeno-associated virus type 2 in brain. *Hum Gene Ther*, 9, 1181-6.
- BATES, J. T., KEEFER, C. J., SLAUGHTER, J. C., KULP, D. W., SCHIEF, W. R. & CROWE, J. E. 2014. Escape from neutralization by the respiratory syncytial virus-specific neutralizing monoclonal antibody palivizumab is driven by changes in on-rate of binding to the fusion protein. *Virology*, 454, 139-144.
- BAZ, M., ABED, Y., MCDONALD, J. & BOIVIN, G. 2006. Characterization of multidrug-resistant influenza A/H3N2 viruses shed during 1 year by an immunocompromised child. *Clin Infect Dis*, 43, 1555-61.
- BEERLI, R. R., BAUER, M., SCHMITZ, N., BUSER, R. B., GWERDER, M., MUNTWILER, S., RENNER, W. A., SAUDAN, P. & BACHMANN, M. F. 2009. Prophylactic and therapeutic activity of fully human monoclonal antibodies directed against influenza A M2 protein. *J Virol* 6, 224.

- BELSHE, R. B. 2010. The need for quadrivalent vaccine against seasonal influenza. *Vaccine*, 28 Suppl 4, D45-53.
- BELSHE, R. B., EDWARDS, K. M., VESIKARI, T., BLACK, S. V., WALKER, R. E., HULTQUIST, M., KEMBLE, G., CONNOR, E. M. & GROUP, C.-T. C. E. S. 2007. Live attenuated versus inactivated influenza vaccine in infants and young children. *N Engl J Med*, 356, 685-96.
- BENJAMIN, E., WANG, W., MCAULIFFE, J. M., PALMER-HILL, F. J., KALLEWAARD, N. L., CHEN, Z., SUZICH, J. A., BLAIR, W. S., JIN, H. & ZHU, Q. 2014. A broadly neutralizing human monoclonal antibody directed against a novel conserved epitope on the influenza virus H3 hemagglutinin globular head. *J Virol*, 88, 6743-50.
- BERGER, J., HAUBER, J., HAUBER, R., GEIGER, R. & CULLEN, B. R. 1988. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene*, 66, 1-10.
- BERNS, K. I., BYRNE, B. J., FLOTTE, T. R., GAO, G., HAUSWIRTH, W. W., HERZOG, R. W., MUZYCZKA, N., VANDENDRIESCHE, T., XIAO, X., ZOLOTUKHIN, S. & SRIVASTAVA, A. 2015. Adeno-Associated Virus Type 2 and Hepatocellular Carcinoma? *Hum Gene Ther*, 26, 779-81.
- BERNS, K. I. & ROSE, J. A. 1970. Evidence for a single-stranded adenovirus-associated virus genome: isolation and separation of complementary single strands. *J Virol*, 5, 693-9.
- BEVERIDGE, W. 1977. *Influenza: The Last Great Plague, an Unfinished Story*.
- BIFFI, A., MONTINI, E., LORIOLI, L., CESANI, M., FUMAGALLI, F., PLATI, T., BALDOLI, C., MARTINO, S., CALABRIA, A., CANALE, S., BENEDICENTI, F., VALLANTI, G., BIASCO, L., LEO, S., KABBARA, N., ZANETTI, G., RIZZO, W. B., MEHTA, N. A., CICALESSE, M. P., CASIRAGHI, M., BOELEN, J. J., DEL CARRO, U., DOW, D. J., SCHMIDT, M., ASSANELLI, A., NEDUVA, V., DI SERIO, C., STUPKA, E., GARDNER, J., VON KALLE, C., BORDIGNON, C., CICERI, F., ROVELLI, A., RONCAROLO, M. G., AIUTI, A., SESSA, M. & NALDINI, L. 2013. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science*, 341, 1233158.
- BLACKBURN, S. D., STEADMAN, R. A. & JOHNSON, F. B. 2006. Attachment of adeno-associated virus type 3H to fibroblast growth factor receptor 1. *Arch Virol*, 151, 617-23.
- BLANKINSHIP, M. J., GREGOREVIC, P., ALLEN, J. M., HARPER, S. Q., HARPER, H., HALBERT, C. L., MILLER, A. D. & CHAMBERLAIN, J. S. 2004. Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Molecular Therapy*, 10, 671-678.
- BLOBEL, G. & DOBBERSTEIN, B. 1975a. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J Cell Biol*, 67, 835-51.
- BLOBEL, G. & DOBBERSTEIN, B. 1975b. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J Cell Biol*, 67, 852-62.
- BOEHM, M. K., WOOF, J. M., KERR, M. A. & PERKINS, S. J. 1999. The Fab and Fc fragments of IgA1 exhibit a different arrangement from that in IgG: A study by X-ray and neutron solution scattering and homology modelling. *Journal of Molecular Biology*, 286, 1421-1447.

- BOKHOVEN, M., STEPHEN, S. L., KNIGHT, S., GEVERS, E. F., ROBINSON, I. C., TAKEUCHI, Y. & COLLINS, M. K. 2009. Insertional gene activation by lentiviral and gammaretroviral vectors. *J Virol*, 83, 283-94.
- BONI, M. F., GOG, J. R., ANDREASEN, V. & CHRISTIANSEN, F. B. 2004. Influenza drift and epidemic size: the race between generating and escaping immunity. *Theor Popul Biol*, 65, 179-91.
- BOSTICK, B., GHOSH, A., YUE, Y., LONG, C. & DUAN, D. 2007. Systemic AAV-9 transduction in mice is influenced by animal age but not by the route of administration. *Gene Therapy*, 14, 1605-1609.
- BOTH, G. W., SLEIGH, M. J., COX, N. J. & KENDAL, A. P. 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. *J Virol*, 48, 52-60.
- BOUTIN, S., MONTEILHET, V., VERON, P., LEBORGNE, C., BENVENISTE, O., MONTUS, M. F. & MASURIER, C. 2010. Prevalence of Serum IgG and Neutralizing Factors Against Adeno-Associated Virus (AAV) Types 1, 2, 5, 6, 8, and 9 in the Healthy Population: Implications for Gene Therapy Using AAV Vectors. *Human Gene Therapy*, 21, 704-712.
- BOUVIER, N. M. & LOWEN, A. C. 2010. Animal Models for Influenza Virus Pathogenesis and Transmission. *Viruses*, 2, 1530-1563.
- BOWLES, D. E., MCPHEE, S. W. J., LI, C. W., GRAY, S. J., SAMULSKI, J. J., CAMP, A. S., LI, J., WANG, B., MONAHAN, P. E., RABINOWITZ, J. E., GRIEGER, J. C., GOVINDASAMY, L., AGBANDJE-MCKENNA, M., XIAO, X. & SAMULSKI, R. J. 2012. Phase 1 Gene Therapy for Duchenne Muscular Dystrophy Using a Translational Optimized AAV Vector. *Molecular Therapy*, 20, 443-455.
- BRADY, T., AGOSTO, L. M., MALANI, N., BERRY, C. C., O'DOHERTY, U. & BUSHMAN, F. 2009. HIV integration site distributions in resting and activated CD4+ T cells infected in culture. *AIDS*, 23, 1461-71.
- BRANDENBURG, B., KOUDSTAAL, W., GOUDSMIT, J., KLAREN, V., TANG, C., BUJNY, M. V., KORSE, H. J., KWAKS, T., OTTERSTROM, J. J., JURASZEK, J., VAN OIJEN, A. M., VOGELS, R. & FRIESEN, R. H. 2013. Mechanisms of hemagglutinin targeted influenza virus neutralization. *PLoS One*, 8, e80034.
- BRANTLY, M. L., CHULAY, J. D., WANG, L. L., MUELLER, C., HUMPHRIES, M., SPENCER, L. T., ROUHANI, F., CONLON, T. J., CALCEDO, R., BETTS, M. R., SPENCER, C., BYRNE, B. J., WILSON, J. & FLOTTE, T. R. 2009. Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy (vol 106. pg 16363, 2009). *Proceedings of the National Academy of Sciences of the United States of America*, 106, 17606-17606.
- BRANTLY, M. L., SPENCER, L. T., HUMPHRIES, M., CONLON, T. J., SPENCER, C. T., POIRIER, A., GARLINGTON, W., BAKER, D., SONG, S., BERNS, K. I., MUZYCZKA, N., SNYDER, R. O., BYRNE, B. J. & FLOTTE, T. R. 2006. Phase I trial of intramuscular injection of a recombinant adeno-associated virus serotype 2 alpha1-antitrypsin (AAT) vector in AAT-deficient adults. *Hum Gene Ther*, 17, 1177-86.
- BREKKE, O. H. & LOSET, G. A. 2003. New technologies in therapeutic antibody development. *Curr Opin Pharmacol*, 3, 544-50.

- BREMER, E. T. V. D., BEURSKENS, F. J., VOORHORST, M., ENGELBERTS, P. J., JONG, R. N. D., BOOM, B. G. V. D., COOK, E. M., LINDORFER, M. A., TAYLOR, R. P., BERKEL, P. H. V. & PARREN, P. W. 2015. Human IgG is produced in a pro-form that requires clipping of C-terminal lysines for maximal complement activation. *mAbs*, 7, 672-680.
- BRIGHT, R. A., SHAY, D. K., SHU, B., COX, N. J. & KLIMOV, A. I. 2006. Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. *JAMA*, 295, 891-4.
- BROWN, B. D., SITIA, G., ANNONI, A., HAUBEN, E., SERGI, L. S., ZINGALE, A., RONCAROLO, M. G., GUIDOTTI, L. G. & NALDINI, L. 2007. In vivo administration of lentiviral vectors triggers a type I interferon response that restricts hepatocyte gene transfer and promotes vector clearance. *Blood*, 109, 2797-805.
- BRUNKE, C., LOHSE, S., DERER, S., PEIPP, M., BOROSS, P., KELLNER, C., BEYER, T., DECHANT, M., ROYLE, L., LIEW, L. P., LEUSEN, J. H. & VALERIUS, T. 2013. Effect of a tail piece cysteine deletion on biochemical and functional properties of an epidermal growth factor receptor-directed IgA2m(1) antibody. *MAbs*, 5, 936-45.
- BUI, M., WHITTAKER, G. & HELENIUS, A. 1996. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *J Virol*, 70, 8391-401.
- BUKRINSKY, M. I., HAGGERTY, S., DEMPSEY, M. P., SHAROVA, N., ADZHUBEL, A., SPITZ, L., LEWIS, P., GOLDFARB, D., EMERMAN, M. & STEVENSON, M. 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature*, 365, 666-9.
- CALCEDO, R., VANDENBERGHE, L. H., GAO, G., LIN, J. & WILSON, J. M. 2009. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis*, 199, 381-90.
- CARTER, B. J. 1992. Adeno-associated virus vectors. *Curr Opin Biotechnol*, 3, 533-9.
- CARTER, B. J., FIFE, K. H., DE LA MAZA, L. M. & BERNIS, K. I. 1976. Genome localization of adeno-associated virus RNA. *J Virol*, 19, 1044-53.
- CARTIER, N. & AUBOURG, P. 2010. Hematopoietic stem cell transplantation and hematopoietic stem cell gene therapy in X-linked adrenoleukodystrophy. *Brain Pathol*, 20, 857-62.
- CAVAZZANA-CALVO, M., PAYEN, E., NEGRE, O., WANG, G., HEHIR, K., FUSIL, F., DOWN, J., DENARO, M., BRADY, T., WESTERMAN, K., CAVALLESCO, R., GILLET-LEGRAND, B., CACCAVELLI, L., SGARRA, R., MAOUCHE-CHRETIEN, L., BERNAUDIN, F., GIROT, R., DORAZIO, R., MULDER, G. J., POLACK, A., BANK, A., SOULIER, J., LARGHERO, J., KABBARA, N., DALLE, B., GOURMEL, B., SOCIE, G., CHRETIEN, S., CARTIER, N., AUBOURG, P., FISCHER, A., CORNETTA, K., GALACTEROS, F., BEUZARD, Y., GLUCKMAN, E., BUSHMAN, F., HACEIN-BEY-ABINA, S. & LEBOULCH, P. 2010. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature*, 467, 318-22.
- CDC 2013. Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory.
- CDC 2014. Morbidity and Mortality Weekly Report (MMWR).
- CDC 2017.

- CESANA, D., RANZANI, M., VOLPIN, M., BARTHOLOMAE, C., DUROS, C., ARTUS, A., MERELLA, S., BENEDICENTI, F., SERGI SERGI, L., SANVITO, F., BROMBIN, C., NONIS, A., SERIO, C. D., DOGLIONI, C., VON KALLE, C., SCHMIDT, M., COHEN-HAGUENAUER, O., NALDINI, L. & MONTINI, E. 2014. Uncovering and dissecting the genotoxicity of self-inactivating lentiviral vectors in vivo. *Mol Ther*, 22, 774-85.
- CESANA, D., SGUALDINO, J., RUDILOSSO, L., MERELLA, S., NALDINI, L. & MONTINI, E. 2012. Whole transcriptome characterization of aberrant splicing events induced by lentiviral vector integrations. *J Clin Invest*, 122, 1667-76.
- CHAI, N., SWEM, L. R., REICHEL, M., CHEN-HARRIS, H., LUIS, E., PARK, S., FOUTS, A., LUPARDUS, P., WU, T. D., LI, O., MCBRIDE, J., LAWRENCE, M., XU, M. & TAN, M. W. 2016. Two Escape Mechanisms of Influenza A Virus to a Broadly Neutralizing Stalk-Binding Antibody. *Plos Pathogens*, 12.
- CHAMBERS, B. S., PARKHOUSE, K., ROSS, T. M., ALBY, K. & HENSLEY, S. E. 2015. Identification of Hemagglutinin Residues Responsible for H3N2 Antigenic Drift during the 2014-2015 Influenza Season. *Cell Rep*, 12, 1-6.
- CHAMES, P., VAN REGENMORTEL, M., WEISS, E. & BATY, D. 2009. Therapeutic antibodies: successes, limitations and hopes for the future. *Br J Pharmacol*, 157, 220-33.
- CHANDLER, R. J., LAFAVE, M. C., VARSHNEY, G. K., TRIVEDI, N. S., CARRILLO-CARRASCO, N., SENAC, J. S., WU, W., HOFFMANN, V., ELKAHLOUN, A. G., BURGESS, S. M. & VENDITTI, C. P. 2015. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *J Clin Invest*, 125, 870-80.
- CHAO, H. J., LIU, Y. B., RABINOWITZ, J., LI, C. W., SAMULSKI, R. J. & WALSH, C. E. 2000. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. *Molecular Therapy*, 2, 619-623.
- CHARNEAU, P., ALIZON, M. & CLAVEL, F. 1992. A second origin of DNA plus-strand synthesis is required for optimal human immunodeficiency virus replication. *J Virol*, 66, 2814-20.
- CHEN, W. S., CALVO, P. A., MALIDE, D., GIBBS, J., SCHUBERT, U., BACIK, I., BASTA, S., O'NEILL, R., SCHICKLI, J., PALESE, P., HENKLEIN, P., BENNINK, J. R. & YEWEDELL, J. W. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nature Medicine*, 7, 1306-1312.
- CHEUNG, C. L., RAYNER, J. M., SMITH, G. J., WANG, P., NAIPOSPPOS, T. S., ZHANG, J., YUEN, K. Y., WEBSTER, R. G., PEIRIS, J. S., GUAN, Y. & CHEN, H. 2006. Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. *J Infect Dis*, 193, 1626-9.
- CHIEN, C. Y., XU, Y., XIAO, R., ARAMINI, J. M., SAHASRABUDHE, P. V., KRUG, R. M. & MONTELLIONE, G. T. 2004. Biophysical characterization of the complex between double-stranded RNA and the N-terminal domain of the NS1 protein from influenza A virus: evidence for a novel RNA-binding mode. *Biochemistry*, 43, 1950-62.
- CLAAS, E. C., OSTERHAUS, A. D., VAN BEEK, R., DE JONG, J. C., RIMMELZWAAN, G. F., SENNE, D. A., KRAUSS, S., SHORTRIDGE, K. F. & WEBSTER, R. G. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet*, 351, 472-7.

- COHEN, M., ZHANG, X. Q., SENAATI, H. P., CHEN, H. W., VARKI, N. M., SCHOOLEY, R. T. & GAGNEUX, P. 2013. Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virol J*, 10, 321.
- COLOMA, R., VALPUESTA, J. M., ARRANZ, R., CARRASCOSA, J. L., ORTIN, J. & MARTIN-BENITO, J. 2009. The structure of a biologically active influenza virus ribonucleoprotein complex. *PLoS Pathog*, 5, e1000491.
- CONE, R. A. 2009. Barrier properties of mucus. *Adv Drug Deliv Rev*, 61, 75-85.
- COOPER, L. J. N., SHIKHMAN, A. R., GLASS, D. D., KANGISSER, D., CUNNINGHAM, M. W. & GREENSPAN, N. S. 1993. Role of Heavy-Chain Constant Domains in Antibody-Antigen Interaction - Apparent Specificity Differences among Streptococcal IgG Antibodies Expressing Identical Variable Domains. *Journal of Immunology*, 150, 2231-2242.
- CORREA, A., TRAJTENBERG, F., OBAL, G., PRITSCH, O., DIGHIERO, G., OPPEZZO, P. & BUSCHIAZZO, A. 2013. Structure of a human IgA1 Fab fragment at 1.55 Å resolution: potential effect of the constant domains on antigen-affinity modulation. *Acta Crystallogr D Biol Crystallogr*, 69, 388-97.
- CORTI, D. & LANZAVECCHIA, A. 2013. Broadly neutralizing antiviral antibodies. *Annu Rev Immunol*, 31, 705-42.
- CORTI, D., VOSS, J., GAMBLIN, S. J., CODONI, G., MACAGNO, A., JARROSSAY, D., VACHIERI, S. G., PINNA, D., MINOLA, A., VANZETTA, F., SILACCI, C., FERNANDEZ-RODRIGUEZ, B. M., AGATIC, G., BIANCHI, S., GIACCHETTO-SASSELLI, I., CALDER, L., SALLUSTO, F., COLLINS, P., HAIRE, L. F., TEMPERTON, N., LANGEDIJK, J. P., SKEHEL, J. J. & LANZAVECCHIA, A. 2011. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science*, 333, 850-6.
- COX, N. J., BRAMMER, T. L. & REGNERY, H. L. 1994. Influenza: global surveillance for epidemic and pandemic variants. *Eur J Epidemiol*, 10, 467-70.
- CROYLE, M. A., CHENG, X. & WILSON, J. M. 2001. Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Therapy*, 8, 1281-1290.
- DAS, K., ARAMINI, J. M., MA, L. C., KRUG, R. M. & ARNOLD, E. 2010. Structures of influenza A proteins and insights into antiviral drug targets. *Nat Struct Mol Biol*, 17, 530-8.
- DAVID, R. M. & DOHERTY, A. T. 2017. Viral Vectors: The Road to Reducing Genotoxicity. *Toxicol Sci*, 155, 315-325.
- DAVIDOFF, A. M., GRAY, J. T., NG, C. Y., ZHANG, Y., ZHOU, J., SPENCE, Y., BAKAR, Y. & NATHWANI, A. C. 2005. Comparison of the ability of adeno-associated viral vectors pseudotyped with serotype 2, 5, and 8 capsid proteins to mediate efficient transduction of the liver in murine and nonhuman primate models. *Mol Ther*, 11, 875-88.
- DAVIDSON, B. L., STEIN, C. S., HETH, J. A., MARTINS, I., KOTIN, R. M., DERKSEN, T. A., ZABNER, J., GHODSI, A. & CHIORINI, J. A. 2000. Recombinant adeno-associated virus type 2, 4, and 5 vectors: Transduction of variant cell types and regions in the mammalian central nervous system. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 3428-3432.
- DAVIDSON, S., CROTTA, S., MCCABE, T. M. & WACK, A. 2014. Pathogenic potential of interferon alphabeta in acute influenza infection. *Nat Commun*, 5, 3864.

- DAVIES, L. A., HYDE, S. C., NUNEZ-ALONSO, G., BAZZANI, R. P., HARDING-SMITH, R., PRINGLE, I. A., LAWTON, A. E., ABDULLAH, S., ROBERTS, T. C., MCCORMICK, D., SUMNER-JONES, S. G. & GILL, D. R. 2012. The use of CpG-free plasmids to mediate persistent gene expression following repeated aerosol delivery of pDNA/PEI complexes. *Biomaterials*, 33, 5618-27.
- DAYA, S. & BERNIS, K. I. 2008. Gene therapy using adeno-associated virus vectors. *Clin Microbiol Rev*, 21, 583-93.
- DE, B. P., HACKETT, N. R., CRYSTAL, R. G. & BOYER, J. L. 2008. Rapid/sustained anti-anthrax passive immunity mediated by co-administration of Ad/AAV. *Mol Ther*, 16, 203-9.
- DE JONG, M. D., TRAN, T. T., TRUONG, H. K., VO, M. H., SMITH, G. J., NGUYEN, V. C., BACH, V. C., PHAN, T. Q., DO, Q. H., GUAN, Y., PEIRIS, J. S., TRAN, T. H. & FARRAR, J. 2005. Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N Engl J Med*, 353, 2667-72.
- DE JONG, Y. P., DORNER, M., MOMMERSTEEG, M. C., XIAO, J. W., BALAZS, A. B., ROBBINS, J. B., WINER, B. Y., GERGES, S., VEGA, K., LABITT, R. N., DONOVAN, B. M., GIANG, E., KRISHNAN, A., CHIRIBOGA, L., CHARLTON, M. R., BURTON, D. R., BALTIMORE, D., LAW, M., RICE, C. M. & PLOSS, A. 2014. Broadly neutralizing antibodies abrogate established hepatitis C virus infection. *Sci Transl Med*, 6, 254ra129.
- DE LA LUNA, S., MARTINEZ, C. & ORTIN, J. 1989. Molecular cloning and sequencing of influenza virus A/Victoria/3/75 polymerase genes: sequence evolution and prediction of possible functional domains. *Virus Res*, 13, 143-55.
- DEAL, C., BALAZS, A. B., ESPINOSA, D. A., ZAVALA, F., BALTIMORE, D. & KETNER, G. 2014. Vectored antibody gene delivery protects against *Plasmodium falciparum* sporozoite challenge in mice. *Proc Natl Acad Sci U S A*, 111, 12528-32.
- DI PASQUALE, G., DAVIDSON, B. L., STEIN, C. S., MARTINS, I. S., SCUDIERO, D., MONKS, A. & CHIORINI, J. A. 2003. Identification of PDGFR as a receptor for AAV-5 transduction. *Nature Medicine*, 9, 1306-1312.
- DICK, L. W., JR., QIU, D., MAHON, D., ADAMO, M. & CHENG, K. C. 2008. C-terminal lysine variants in fully human monoclonal antibodies: investigation of test methods and possible causes. *Biotechnol Bioeng*, 100, 1132-43.
- DICKINSON, B. L., BADIZADEGAN, K., WU, Z., AHOUSE, J. C., ZHU, X., SIMISTER, N. E., BLUMBERG, R. S. & LENCER, W. I. 1999. Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. *J Clin Invest*, 104, 903-11.
- DILILLO, D. J., TAN, G. S., PALESE, P. & RAVETCH, J. V. 2014. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. *Nat Med*, 20, 143-51.
- DOCHERTY, K. & HUTTON, J. C. 1983. Carboxypeptidase activity in the insulin secretory granule. *FEBS Lett*, 162, 137-41.
- DOHERTY, P. C. 2008. Burnet, chick embryos, viruses, clones and quantitative biology. *Immunol Cell Biol*, 86, 119-23.
- DONNELLY, M. L., HUGHES, L. E., LUKE, G., MENDOZA, H., TEN DAM, E., GANI, D. & RYAN, M. D. 2001a. The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. *J Gen Virol*, 82, 1027-41.

- DONNELLY, M. L., LUKE, G., MEHROTRA, A., LI, X., HUGHES, L. E., GANI, D. & RYAN, M. D. 2001b. Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. *J Gen Virol*, 82, 1013-25.
- DONSANTE, A., MILLER, D. G., LI, Y., VOGLER, C., BRUNT, E. M., RUSSELL, D. W. & SANDS, M. S. 2007. AAV vector integration sites in mouse hepatocellular carcinoma. *Science*, 317, 477.
- DONSANTE, A., VOGLER, C., MUZYCZKA, N., CRAWFORD, J. M., BARKER, J., FLOTTE, T., CAMPBELL-THOMPSON, M., DALY, T. & SANDS, M. S. 2001. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Therapy*, 8, 1343-1346.
- DREYFUS, C., LAURSEN, N. S., KWAKS, T., ZUIJDGEEST, D., KHAYAT, R., EKIERT, D. C., LEE, J. H., METLAGEL, Z., BUJNY, M. V., JONGENEELLEN, M., VAN DER VLUGT, R., LAMRANI, M., KORSE, H. J., GEELEN, E., SAHIN, O., SIEUWERTS, M., BRAKENHOFF, J. P., VOGELS, R., LI, O. T., POON, L. L., PEIRIS, M., KOUDSTAAL, W., WARD, A. B., WILSON, I. A., GOUDSMIT, J. & FRIESEN, R. H. 2012. Highly conserved protective epitopes on influenza B viruses. *Science*, 337, 1343-8.
- DUBRIDGE, R. B., TANG, P., HSIA, H. C., LEONG, P. M., MILLER, J. H. & CALOS, M. P. 1987. Analysis of Mutation in Human-Cells by Using an Epstein-Barr-Virus Shuttle System. *Molecular and Cellular Biology*, 7, 379-387.
- DUEZ, J. M., SIXT, N. & PECHINOT, A. 2009. Influenza virus infection: don't forget the role of the mucociliary system! *Journal of Antimicrobial Chemotherapy*, 63, 421-422.
- DULL, T., ZUFFEREY, R., KELLY, M., MANDEL, R. J., NGUYEN, M., TRONO, D. & NALDINI, L. 1998. A third-generation lentivirus vector with a conditional packaging system. *J Virol*, 72, 8463-71.
- EKIERT, D. C., BHABHA, G., ELSLIGER, M. A., FRIESEN, R. H., JONGENEELLEN, M., THROSBY, M., GOUDSMIT, J. & WILSON, I. A. 2009. Antibody recognition of a highly conserved influenza virus epitope. *Science*, 324, 246-51.
- EKIERT, D. C., FRIESEN, R. H., BHABHA, G., KWAKS, T., JONGENEELLEN, M., YU, W., OPHORST, C., COX, F., KORSE, H. J., BRANDENBURG, B., VOGELS, R., BRAKENHOFF, J. P., KOMPIER, R., KOLDIJK, M. H., CORNELISSEN, L. A., POON, L. L., PEIRIS, M., KOUDSTAAL, W., WILSON, I. A. & GOUDSMIT, J. 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. *Science*, 333, 843-50.
- EKIERT, D. C., KASHYAP, A. K., STEEL, J., RUBRUM, A., BHABHA, G., KHAYAT, R., LEE, J. H., DILLON, M. A., O'NEIL, R. E., FAYNBOYM, A. M., HOROWITZ, M., HOROWITZ, L., WARD, A. B., PALESE, P., WEBBY, R., LERNER, R. A., BHATT, R. R. & WILSON, I. A. 2012. Cross-neutralization of influenza A viruses mediated by a single antibody loop. *Nature*, 489, 526-32.
- EL-AMOURI, S. S., CAO, P., MIAO, C. & PAN, D. 2013. Secreted luciferase for in vivo evaluation of systemic protein delivery in mice. *Mol Biotechnol*, 53, 63-73.
- ENDO, T., MESTECKY, J., KULHAVY, R. & KOBATA, A. 1994. Carbohydrate heterogeneity of human myeloma proteins of the IgA1 and IgA2 subclasses. *Mol Immunol*, 31, 1415-22.
- FAHY, J. V. & DICKEY, B. F. 2010. Airway mucus function and dysfunction. *N Engl J Med*, 363, 2233-47.

- FANG, J., QIAN, J. J., YI, S., HARDING, T. C., TU, G. H., VANROEY, M. & JOOSS, K. 2005a. Stable antibody expression at therapeutic levels using the 2A peptide. *Nat Biotechnol*, 23, 584-90.
- FANG, J., YI, S., SIMMONS, A., TU, G. H., NGUYEN, M., HARDING, T. C., VANROEY, M. & JOOSS, K. 2007. An antibody delivery system for regulated expression of therapeutic levels of monoclonal antibodies in vivo. *Mol Ther*, 15, 1153-9.
- FANG, J. M., QIAN, J. J., YI, S. L., HARDING, T. C., TU, G. H., VANROEY, M. & JOOSS, K. 2005b. Stable antibody expression at therapeutic levels using the 2A peptide. *Nature Biotechnology*, 23, 584-590.
- FERRARI, F. K., SAMULSKI, T., SHENK, T. & SAMULSKI, R. J. 1996. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol*, 70, 3227-34.
- FERRARI, F. K., XIAO, X., MCCARTY, D. & SAMULSKI, R. J. 1997. New developments in the generation of Ad-free, high-titer rAAV gene therapy vectors. *Nat Med*, 3, 1295-7.
- FINKENSTADT, B. F., MORTON, A. & RAND, D. A. 2005. Modelling antigenic drift in weekly flu incidence. *Stat Med*, 24, 3447-61.
- FLOTTE, T. R., TRAPNELL, B. C., HUMPHRIES, M., CAREY, B., CALCEDO, R., ROUHANI, F., CAMPBELL-THOMPSON, M., YACHNIS, A. T., SANDHAUS, R. A., MCELVANEY, N. G., MUELLER, C., MESSINA, L. M., WILSON, J. M., BRANTLY, M., KNOP, D. R., YE, G. J. & CHULAY, J. D. 2011. Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing alpha1-antitrypsin: interim results. *Hum Gene Ther*, 22, 1239-47.
- FOECKING, M. K. & HOFSTETTER, H. 1986. Powerful and Versatile Enhancer-Promoter Unit for Mammalian Expression Vectors. *Gene*, 45, 101-105.
- FOUCHIER, R. A., MUNSTER, V., WALLENSTEN, A., BESTEBROER, T. M., HERFST, S., SMITH, D., RIMMELZWAAN, G. F., OLSEN, B. & OSTERHAUS, A. D. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol*, 79, 2814-22.
- FOUCHIER, R. A., SCHNEEBERGER, P. M., ROZENDAAL, F. W., BROEKMAN, J. M., KEMINK, S. A., MUNSTER, V., KUIKEN, T., RIMMELZWAAN, G. F., SCHUTTEN, M., VAN DOORNUM, G. J., KOCH, G., BOSMAN, A., KOOPMANS, M. & OSTERHAUS, A. D. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci U S A*, 101, 1356-61.
- FOUST, K. D., NURRE, E., MONTGOMERY, C. L., HERNANDEZ, A., CHAN, C. M. & KASPAR, B. K. 2009. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nature Biotechnology*, 27, 59-65.
- FRICKER, L. D. & HERBERT, E. 1988. Comparison of a carboxypeptidase E-like enzyme in human, bovine, mouse, *Xenopus*, shark and *Aplysia* neural tissue. *Brain Res*, 453, 281-6.
- FRICKER, L. D. & SNYDER, S. H. 1982. Enkephalin convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules. *Proc Natl Acad Sci U S A*, 79, 3886-90.
- FUCHS, S. P., MARTINEZ-NAVIO, J. M., GAO, G. P. & DESROSIERS, R. C. 2016. Recombinant AAV Vectors for Enhanced Expression of Authentic IgG. *Plos One*, 11.

- GABBARD, J. D., DLUGOLENSKI, D., VAN RIEL, D., MARSHALL, N., GALLOWAY, S. E., HOWERTH, E. W., CAMPBELL, P. J., JONES, C., JOHNSON, S., BYRD-LEOTIS, L., STEINHAEUER, D. A., KUIKEN, T., TOMPKINS, S. M., TRIPP, R., LOWEN, A. C. & STEEL, J. 2014. Novel H7N9 Influenza Virus Shows Low Infectious Dose, High Growth Rate, and Efficient Contact Transmission in the Guinea Pig Model. *Journal of Virology*, 88, 1502-1512.
- GALLOWAY, S. E., REED, M. L., RUSSELL, C. J. & STEINHAEUER, D. A. 2013. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS Pathog*, 9, e1003151.
- GAO, G. P., ALVIRA, M. R., WANG, L., CALCEDO, R., JOHNSTON, J. & WILSON, J. M. 2002a. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A*, 99, 11854-9.
- GAO, G. P., ALVIRA, M. R., WANG, L. L., CALCEDO, R., JOHNSTON, J. & WILSON, J. M. 2002b. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 11854-11859.
- GAO, G. P., VANDENBERGHE, L. H., ALVIRA, M. R., LU, Y., CALCEDO, R., ZHOU, X. Y. & WILSON, J. A. 2004. Clades of Adeno-associated viruses are widely disseminated in human tissues. *Journal of Virology*, 78, 6381-6388.
- GARDNER, M. R., KATTENHORN, L. M., KONDUR, H. R., VON SCHAEWEN, M., DORFMAN, T., CHIANG, J. J., HAWORTH, K. G., DECKER, J. M., ALPERT, M. D., BAILEY, C. C., NEALE, E. S., JR., FELLINGER, C. H., JOSHI, V. R., FUCHS, S. P., MARTINEZ-NAVIO, J. M., QUINLAN, B. D., YAO, A. Y., MOUQUET, H., GORMAN, J., ZHANG, B., POIGNARD, P., NUSSENZWEIG, M. C., BURTON, D. R., KWONG, P. D., PIATAK, M., JR., LIFSON, J. D., GAO, G., DESROSIERS, R. C., EVANS, D. T., HAHN, B. H., PLOSS, A., CANNON, P. M., SEAMAN, M. S. & FARZAN, M. 2015. AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature*, 519, 87-91.
- GAUDET, D., DE WAL, J., TREMBLAY, K., DERY, S., VAN DEVENTER, S., FREIDIG, A., BRISSON, D. & METHOT, J. 2010. Review of the clinical development of alipogene tiparvovec gene therapy for lipoprotein lipase deficiency. *Atheroscler Suppl*, 11, 55-60.
- GAUDET, D., METHOT, J., DERY, S., BRISSON, D., ESSIEMBRE, C., TREMBLAY, G., TREMBLAY, K., DE WAL, J., TWISK, J., VAN DEN BULK, N., SIER-FERREIRA, V. & VAN DEVENTER, S. 2013. Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial. *Gene Ther*, 20, 361-9.
- GAUDET, D., STROES, E. S., METHOT, J., BRISSON, D., TREMBLAY, K., BERNELOT MOENS, S. J., IOTTI, G., RASTELLETI, I., ARDIGO, D., CORZO, D., MEYER, C., ANDERSEN, M., RUSZNIEWSKI, P., DEAKIN, M. & BRUNO, M. J. 2016. Long-Term Retrospective Analysis of Gene Therapy with Alipogene Tiparvovec and Its Effect on Lipoprotein Lipase Deficiency-Induced Pancreatitis. *Hum Gene Ther*, 27, 916-925.
- GERDIL, C. 2003. The annual production cycle for influenza vaccine. *Vaccine*, 21, 1776-9.
- GIBSON, D. G., YOUNG, L., CHUANG, R. Y., VENTER, J. C., HUTCHISON, C. A., 3RD & SMITH, H. O. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*, 6, 343-5.

- GILL, D. R. & HYDE, S. C. 2014. Delivery of genes into the CF airway. *Thorax*, 69, 962-4.
- GILMORE, R., WALTER, P. & BLOBEL, G. 1982. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J Cell Biol*, 95, 470-7.
- GLAVEY, S. V. & LEUNG, N. 2016. Monoclonal gammopathy: The good, the bad and the ugly. *Blood Rev*, 30, 223-31.
- GLUSHAKOVA, L. G., LISANKIE, M. J., ERUSLANOV, E. B., OJANO-DIRAIN, C., ZOLOTUKHIN, I., LIU, C., SRIVASTAVA, A. & STACPOOLE, P. W. 2009. AAV3-mediated transfer and expression of the pyruvate dehydrogenase E1 alpha subunit gene causes metabolic remodeling and apoptosis of human liver cancer cells. *Molecular Genetics and Metabolism*, 98, 289-299.
- GORLICH, D., HARTMANN, E., PREHN, S. & RAPOPORT, T. A. 1992. A protein of the endoplasmic reticulum involved early in polypeptide translocation. *Nature*, 357, 47-52.
- GORMAN, C. M., HOWARD, B. H. & REEVES, R. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res*, 11, 7631-48.
- GORMAN, C. M., MERLINO, G. T., WILLINGHAM, M. C., PASTAN, I. & HOWARD, B. H. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc Natl Acad Sci U S A*, 79, 6777-81.
- GRAHAM, F. L., SMILEY, J., RUSSELL, W. C. & NAIRN, R. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*, 36, 59-74.
- GRANDEA, A. G., 3RD, OLSEN, O. A., COX, T. C., RENSHAW, M., HAMMOND, P. W., CHAN-HUI, P. Y., MITCHAM, J. L., CIEPLAK, W., STEWART, S. M., GRANTHAM, M. L., PEKOSZ, A., KISO, M., SHINYA, K., HATTA, M., KAWAOKA, Y. & MOYLE, M. 2010. Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza A viruses. *Proc Natl Acad Sci U S A*, 107, 12658-63.
- GREGOREVIC, P., BLANKINSHIP, M. J., ALLEN, J. M., CRAWFORD, R. W., MEUSE, L., MILLER, D. G., RUSSELL, D. W. & CHAMBERLAIN, J. S. 2004. Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nature Medicine*, 10, 828-834.
- GRIESENBACH, U., INOUE, M., MENG, C., FARLEY, R., CHAN, M., NEWMAN, N. K., BRUM, A., YOU, J., KERTON, A., SHOEMARK, A., BOYD, A. C., DAVIES, J. C., HIGGINS, T. E., GILL, D. R., HYDE, S. C., INNES, J. A., PORTEOUS, D. J., HASEGAWA, M. & ALTON, E. W. 2012a. Assessment of F/HN-pseudotyped lentivirus as a clinically relevant vector for lung gene therapy. *Am J Respir Crit Care Med*, 186, 846-56.
- GRIESENBACH, U., INOUE, M., MENG, C., FARLEY, R., CHAN, M., NEWMAN, N. K., BRUM, A., YOU, J., KERTON, A., SHOEMARK, A., BOYD, A. C., DAVIES, J. C., HIGGINS, T. E., GILL, D. R., HYDE, S. C., INNES, J. A., PORTEOUS, D. J., HASEGAWA, M. & ALTON, E. W. F. W. 2012b. Assessment of F/Hn-Pseudotyped Lentivirus as a Clinically Relevant Vector for Lung Gene Therapy. *Thorax*, 67, A105-A105.
- GRIESENBACH, U., INOUE, M., MENG, C. X., FARLEY, R., CHAN, M., NEWMAN, N. K., BRUM, A., YOU, J., KERTON, A., SHOEMARK, A., BOYD, A. C., DAVIES, J. C.,

- HIGGINS, T. E., GILL, D. R., HYDE, S. C., INNES, J. A., PORTEOUS, D. J., HASEGAWA, M. & ALTON, E. W. F. W. 2012c. Assessment of F/HN-Pseudotyped Lentivirus as a Clinically Relevant Vector for Lung Gene Therapy. *American Journal of Respiratory and Critical Care Medicine*, 186, 846-856.
- GRIESENBACH, U., VICENTE, C. C., ROBERTS, M. J., MENG, C. X., SOUSSI, S., XENARIOU, S., TENNANT, P., BAKER, A., BAKER, E., GORDON, C., VRETTOU, C., MCCORMICK, D., COLES, R., GREEN, A. M., LAWTON, A. E., SUMNER-JONES, S. G., CHENG, S. H., SCHEULE, R. K., HYDE, S. C., GILL, D. R., COLLIE, D. D., MCLACHLAN, G. & ALTON, E. W. F. W. 2011. Secreted Gaussia luciferase as a sensitive reporter gene for in vivo and ex vivo studies of airway gene transfer. *Biomaterials*, 32, 2614-2624.
- GRIMM, D., PANDEY, K. & KAY, M. A. 2005. Adeno-associated virus vectors for short hairpin RNA expression. *Methods Enzymol*, 392, 381-405.
- GRIMM, D., STAEHEL, P., HUFBAUER, M., KOERNER, I., MARTINEZ-SOBRIDO, L., SOLORZANO, A., GARCIA-SASTRE, A., HALLER, O. & KOCHS, G. 2007. Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. *Proc Natl Acad Sci U S A*, 104, 6806-11.
- GUAN, Y., SHORTRIDGE, K. F., KRAUSS, S. & WEBSTER, R. G. 1999. Molecular characterization of H9N2 influenza viruses: Were they the donors of the "internal" genes of H5N1 viruses in Hong Kong? *Proc Natl Acad Sci U S A*, 96, 9363-9367.
- GUBAREVA, L. V., TRUJILLO, A. A., OKOMO-ADHIAMBO, M., MISHIN, V. P., DEYDE, V. M., SLEEMAN, K., NGUYEN, H. T., SHEU, T. G., GARTEN, R. J., SHAW, M. W., FRY, A. M. & KLIMOV, A. I. 2010. Comprehensive assessment of 2009 pandemic influenza A (H1N1) virus drug susceptibility in vitro. *Antivir Ther*, 15, 1151-9.
- GUIBINGA, G. H., MIYANOHARA, A., ESKO, J. D. & FRIEDMANN, T. 2002. Cell surface heparan sulfate is a receptor for attachment of envelope protein-free retrovirus-like particles and VSV-G pseudotyped MLV-derived retrovirus vectors to target cells. *Mol Ther*, 5, 538-46.
- GUO, J., LI, F., HE, Q., JIN, H., LIU, M., LI, S., HU, S., XIAO, Y., BI, D. & LI, Z. 2016. Neonatal Fc Receptor-Mediated IgG Transport Across Porcine Intestinal Epithelial Cells: Potentially Provide the Mucosal Protection. *DNA Cell Biol*, 35, 301-9.
- HALBERT, C. L., ALLEN, J. M. & MILLER, A. D. 2001. Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *Journal of Virology*, 75, 6615-6624.
- HALBERT, C. L., RUTLEDGE, E. A., ALLEN, J. M., RUSSELL, D. W. & MILLER, A. D. 2000. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. *J Virol*, 74, 1524-32.
- HALE, B. G., RANDALL, R. E., ORTIN, J. & JACKSON, D. 2008. The multifunctional NS1 protein of influenza A viruses. *J Gen Virol*, 89, 2359-76.
- HARRIS, A., CARDONE, G., WINKLER, D. C., HEYMANN, J. B., BRECHER, M., WHITE, J. M. & STEVEN, A. C. 2006. Influenza virus pleiomorphy characterized by cryoelectron tomography. *Proc Natl Acad Sci U S A*, 103, 19123-7.

- HARRIS, L. J., SKALETISKY, E. & MCPHERSON, A. 1998. Crystallographic structure of an intact IgG1 monoclonal antibody. *Journal of Molecular Biology*, 275, 861-872.
- HARRIS, R. J. 1995. Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. *J Chromatogr A*, 705, 129-34.
- HATADA, E. & FUKUDA, R. 1992. Binding of influenza A virus NS1 protein to dsRNA in vitro. *J Gen Virol*, 73 (Pt 12), 3325-9.
- HATTA, M., HATTA, Y., KIM, J. H., WATANABE, S., SHINYA, K., NGUYEN, T., LIEN, P. S., LE, Q. M. & KAWAOKA, Y. 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog*, 3, 1374-9.
- HE, W., MULLARKEY, C. E., DUTY, J. A., MORAN, T. M., PALESE, P. & MILLER, M. S. 2015. Broadly neutralizing anti-influenza virus antibodies: enhancement of neutralizing potency in polyclonal mixtures and IgA backbones. *J Virol*, 89, 3610-8.
- HEGDE, R. S. & BERNSTEIN, H. D. 2006. The surprising complexity of signal sequences. *Trends Biochem Sci*, 31, 563-71.
- HEMATTI, P., HONG, B. K., FERGUSON, C., ADLER, R., HANAWA, H., SELLERS, S., HOLT, I. E., ECKFELDT, C. E., SHARMA, Y., SCHMIDT, M., VON KALLE, C., PERSONS, D. A., BILLINGS, E. M., VERFAILLIE, C. M., NIENHUIS, A. W., WOLFSBERG, T. G., DUNBAR, C. E. & CALMELS, B. 2004. Distinct genomic integration of MLV and SIV vectors in primate hematopoietic stem and progenitor cells. *PLoS Biol*, 2, e423.
- HENDRICKSON, B. A., CONNER, D. A., LADD, D. J., KENDALL, D., CASANOVA, J. E., CORTHESEY, B., MAX, E. E., NEUTRA, M. R., SEIDMAN, C. E. & SEIDMAN, J. G. 1995. Altered hepatic transport of immunoglobulin A in mice lacking the J chain. *J Exp Med*, 182, 1905-11.
- HERMAN, S. A. & COFFIN, J. M. 1987. Efficient packaging of readthrough RNA in ALV: implications for oncogene transduction. *Science*, 236, 845-8.
- HERMONAT, P. L., QUIRK, J. G., BISHOP, B. M. & HAN, L. 1997. The packaging capacity of adeno-associated virus (AAV) and the potential for wild-type-plus AAV gene therapy vectors. *FEBS Lett*, 407, 78-84.
- HETZ, C. 2012. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol*, 13, 89-102.
- HOGGAN, M. D., BLACKLOW, N. R. & ROWE, W. P. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. *Proc Natl Acad Sci U S A*, 55, 1467-74.
- HOLSINGER, L. J. & LAMB, R. A. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology*, 183, 32-43.
- HOOK, V. Y. & LOH, Y. P. 1984. Carboxypeptidase B-like converting enzyme activity in secretory granules of rat pituitary. *Proc Natl Acad Sci U S A*, 81, 2776-80.
- HORWITZ, J. A., HALPER-STROMBERG, A., MOUQUET, H., GITLIN, A. D., TRETIKOVA, A., EISENREICH, T. R., MALBEC, M., GRAVEMANN, S., BILLERBECK, E., DORNER, M., BUNING, H., SCHWARTZ, O., KNOPS, E., KAISER, R., SEAMAN, M. S., WILSON, J. M., RICE, C. M., PLOSS, A., BJORKMAN, P. J., KLEIN, F. & NUSSENZWEIG, M. C. 2013. HIV-1 suppression and durable control by combining single broadly neutralizing

- antibodies and antiretroviral drugs in humanized mice. *Proc Natl Acad Sci U S A*, 110, 16538-43.
- HOVENDEN, M., HUBBARD, M. A., AUCCOIN, D. P., THORKILDSON, P., REED, D. E., WELCH, W. H., LYONS, C. R., LOVCHIK, J. A. & KOZEL, T. R. 2013. IgG subclass and heavy chain domains contribute to binding and protection by mAbs to the poly gamma-D-glutamic acid capsular antigen of *Bacillus anthracis*. *PLoS Pathog*, 9, e1003306.
- HU, J., ZHU, Y., ZHAO, B., LI, J., LIU, L., GU, K., ZHANG, W., SU, H., TENG, Z., TANG, S., YUAN, Z., FENG, Z. & WU, F. 2014. Limited human-to-human transmission of avian influenza A(H7N9) virus, Shanghai, China, March to April 2013. *Euro Surveill*, 19.
- HU, Y. W., LU, S. H., SONG, Z. G., WANG, W., HAO, P., LI, J. H., ZHANG, X. N., YEN, H. L., SHI, B. S., LI, T., GUAN, W. C., XU, L., LIU, Y., WANG, S., ZHANG, X. L., TIAN, D., ZHU, Z. Q., HE, J., HUANG, K., CHEN, H. J., ZHENG, L. L., LI, X., PING, J., KANG, B., XI, X. H., ZHA, L. J., LI, Y. X., ZHANG, Z. Y., PEIRIS, M. & YUAN, Z. H. 2013. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. *Lancet*, 381, 2273-2279.
- HUANG, K. Y., RIJAL, P., SCHIMANSKI, L., POWELL, T. J., LIN, T. Y., MCCAULEY, J. W., DANIELS, R. S. & TOWNSEND, A. R. 2015a. Focused antibody response to influenza linked to antigenic drift. *J Clin Invest*, 125, 2631-45.
- HUANG, K. Y. A., RIJAL, P., SCHIMANSKI, L., POWELL, T. J., LIN, T. Y., MCCAULEY, J. W., DANIELS, R. S. & TOWNSEND, A. R. 2015b. Focused antibody response to influenza linked to antigenic drift. *Journal of Clinical Investigation*, 125, 2631-2645.
- HUANG, T. S., PALESE, P. & KRYSTAL, M. 1990. Determination of influenza virus proteins required for genome replication. *J Virol*, 64, 5669-73.
- HYDE, S. C., PRINGLE, I. A., ABDULLAH, S., LAWTON, A. E., DAVIES, L. A., VARATHALINGAM, A., NUNEZ-ALONSO, G., GREEN, A. M., BAZZANI, R. P., SUMNER-JONES, S. G., CHAN, M., LI, H., YEW, N. S., CHENG, S. H., BOYD, A. C., DAVIES, J. C., GRIESENBACH, U., PORTEOUS, D. J., SHEPPARD, D. N., MUNKONGE, F. M., ALTON, E. W. & GILL, D. R. 2008. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. *Nat Biotechnol*, 26, 549-51.
- IBRICEVIC, A., PEKOSZ, A., WALTER, M. J., NEWBY, C., BATTAILE, J. T., BROWN, E. G., HOLTZMAN, M. J. & BRODY, S. L. 2006. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol*, 80, 7469-80.
- INAGAKI, K., FUESS, S., STORM, T. A., GIBSON, G. A., MCTIERNAN, C. F., KAY, M. A. & NAKAI, H. 2006. Robust systemic transduction with AAV9 vectors in mice: Efficient global cardiac gene transfer superior to that of AAV8. *Molecular Therapy*, 14, 45-+.
- INGLIS, S. C., BARRETT, T., BROWN, C. M. & ALMOND, J. W. 1979. The smallest genome RNA segment of influenza virus contains two genes that may overlap. *Proc Natl Acad Sci U S A*, 76, 3790-4.
- ISON, M. G. 2010. Influenza, including the novel H1N1, in organ transplant patients. *Curr Opin Infect Dis*, 23, 365-73.

- ISON, M. G., GUBAREVA, L. V., ATMAR, R. L., TREANOR, J. & HAYDEN, F. G. 2006. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. *J Infect Dis*, 193, 760-4.
- ITO, R., OZAKI, Y. A., YOSHIKAWA, T., HASEGAWA, H., SATO, Y., SUZUKI, Y., INOUE, R., MORISHIMA, T., KONDO, N., SATA, T., KURATA, T. & TAMURA, S. 2003. Roles of anti-hemagglutinin IgA and IgG antibodies in different sites of the respiratory tract of vaccinated mice in preventing lethal influenza pneumonia. *Vaccine*, 21, 2362-71.
- ITO, T., COUCEIRO, J. N., KELM, S., BAUM, L. G., KRAUSS, S., CASTRUCCI, M. R., DONATELLI, I., KIDA, H., PAULSON, J. C., WEBSTER, R. G. & KAWAOKA, Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J Virol*, 72, 7367-73.
- ITO, T., GORMAN, O. T., KAWAOKA, Y., BEAN, W. J. & WEBSTER, R. G. 1991. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. *J Virol*, 65, 5491-8.
- JACOBSEN, K. R., KALLIOKOSKI, O., TEILMANN, A. C., HAU, J. & ABELSON, K. S. 2012. The effect of isoflurane anaesthesia and vasectomy on circulating corticosterone and ACTH in BALB/c mice. *Gen Comp Endocrinol*, 179, 406-13.
- JACOBSON, S. G., CIDECIYAN, A. V., RATNAKARAM, R., HEON, E., SCHWARTZ, S. B., ROMAN, A. J., PEDEN, M. C., ALEMAN, T. S., BOYE, S. L., SUMAROKA, A., CONLON, T. J., CALCEDO, R., PANG, J. J., ERGER, K. E., OLIVARES, M. B., MULLINS, C. L., SWIDER, M., KAUSHAL, S., FEUER, W. J., IANNACCONE, A., FISHMAN, G. A., STONE, E. M., BYRNE, B. J. & HAUSWIRTH, W. W. 2012. Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years. *Arch Ophthalmol*, 130, 9-24.
- JAGGER, B. W., WISE, H. M., KASH, J. C., WALTERS, K. A., WILLS, N. M., XIAO, Y. L., DUNFEE, R. L., SCHWARTZMAN, L. M., OZINSKY, A., BELL, G. L., DALTON, R. M., LO, A., EFSTATHIOU, S., ATKINS, J. F., FIRTH, A. E., TAUBENBERGER, J. K. & DIGARD, P. 2012. An Overlapping Protein-Coding Region in Influenza A Virus Segment 3 Modulates the Host Response. *Science*, 337, 199-204.
- JANDA, A. & CASADEVALL, A. 2010. Circular Dichroism reveals evidence of coupling between immunoglobulin constant and variable region secondary structure. *Mol Immunol*, 47, 1421-5.
- JANDA, A., ERYILMAZ, E., NAKOUZI, A., COWBURN, D. & CASADEVALL, A. 2012. Variable Region Identical Immunoglobulins Differing in Isotype Express Different Paratopes. *Journal of Biological Chemistry*, 287, 35409-35417.
- JANSON, C., MCPHEE, S., BILANIUK, L., HASELGROVE, J., TESTAIUTI, M., FREESE, A., WANG, D. J., SHERA, D., HURH, P., RUPIN, J., SASLOW, E., GOLDFARB, O., GOLDBERG, M., LARIJANI, G., SHARRAR, W., LIOUTERMAN, L., CAMP, A., KOLODNY, E., SAMULSKI, J. & LEONE, P. 2002. Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. *Hum Gene Ther*, 13, 1391-412.
- JASKI, B. E., JESSUP, M. L., MANCINI, D. M., CAPPOLA, T. P., PAULY, D. F., GREENBERG, B., BOROW, K., DITTRICH, H., ZSEBO, K. M., HAJJAR, R. J. & CALCIUM UP-REGULATION BY PERCUTANEOUS ADMINISTRATION OF

- GENE THERAPY IN CARDIAC DISEASE TRIAL, I. 2009. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. *J Card Fail*, 15, 171-81.
- JAY, F. T., LAUGHLIN, C. A. & CARTER, B. J. 1981. Eukaryotic translational control: adeno-associated virus protein synthesis is affected by a mutation in the adenovirus DNA-binding protein. *Proc Natl Acad Sci U S A*, 78, 2927-31.
- JEGASKANDA, S., JOB, E. R., KRAMSKI, M., LAURIE, K., ISITMAN, G., DE ROSE, R., WINNALL, W. R., STRATOV, I., BROOKS, A. G., READING, P. C. & KENT, S. J. 2013. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. *J Immunol*, 190, 1837-48.
- JESSUP, M., GREENBERG, B., MANCINI, D., CAPPOLA, T., PAULY, D. F., JASKI, B., YAROSHINSKY, A., ZSEBO, K. M., DITTRICH, H., HAJJAR, R. J. & CALCIUM UPREGULATION BY PERCUTANEOUS ADMINISTRATION OF GENE THERAPY IN CARDIAC DISEASE, I. 2011. Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID): a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca²⁺-ATPase in patients with advanced heart failure. *Circulation*, 124, 304-13.
- JOHNSON, P. R., SCHNEPP, B. C., ZHANG, J., CONNELL, M. J., GREENE, S. M., YUSTE, E., DESROSIERS, R. C. & CLARK, K. R. 2009. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nat Med*, 15, 901-6.
- JOSEPH, A., ZHENG, J. H., CHEN, K., DUTTA, M., CHEN, C., STIEGLER, G., KUNERT, R., FOLLENZI, A. & GOLDSTEIN, H. 2010. Inhibition of in vivo HIV infection in humanized mice by gene therapy of human hematopoietic stem cells with a lentiviral vector encoding a broadly neutralizing anti-HIV antibody. *J Virol*, 84, 6645-53.
- KAEPPEL, C., BEATTIE, S. G., FRONZA, R., VAN LOGTENSTEIN, R., SALMON, F., SCHMIDT, S., WOLF, S., NOWROUZI, A., GLIMM, H., VON KALLE, C., PETRY, H., GAUDET, D. & SCHMIDT, M. 2013a. A largely random AAV integration profile after LPLD gene therapy. *Nat Med*, 19, 889-91.
- KAEPPEL, C., BEATTIE, S. G., FRONZA, R., VAN LOGTENSTEIN, R., SALMON, F., SCHMIDT, S., WOLF, S., NOWROUZI, A., GLIMM, H., VON KALLE, C., PETRY, H., GAUDET, D. & SCHMIDT, M. 2013b. A largely random AAV integration profile after LPLD gene therapy. *Nature Medicine*, 19, 889-+.
- KALLEWAARD, N. L., CORTI, D., COLLINS, P. J., NEU, U., MCAULIFFE, J. M., BENJAMIN, E., WACHTER-ROSATI, L., PALMER-HILL, F. J., YUAN, A. Q., WALKER, P. A., VORLAENDER, M. K., BIANCHI, S., GUARINO, B., DE MARCO, A., VANZETTA, F., AGATIC, G., FOGLIERINI, M., PINNA, D., FERNANDEZ-RODRIGUEZ, B., FRUEHWIRTH, A., SILACCI, C., OGRODOWICZ, R. W., MARTIN, S. R., SALLUSTO, F., SUZICH, J. A., LANZAVECCHIA, A., ZHU, Q., GAMBLIN, S. J. & SKEHEL, J. J. 2016. Structure and Function Analysis of an Antibody Recognizing All Influenza A Subtypes. *Cell*, 166, 596-608.
- KALUDOV, N., BROWN, K. E., WALTERS, R. W., ZABNER, J. & CHIORINI, J. A. 2001. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic

- acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *Journal of Virology*, 75, 6884-6893.
- KAMLANGDEE, A., KINGSTAD-BAKKE, B., ANDERSON, T. K., GOLDBERG, T. L. & OSORIO, J. E. 2014. Broad Protection against Avian Influenza Virus by Using a Modified Vaccinia Ankara Virus Expressing a Mosaic Hemagglutinin Gene. *Journal of Virology*, 88, 13300-13309.
- KANG, Y. B., STEIN, C. S., HETH, J. A., SINN, P. L., PENISTEN, A. K., STABER, P. D., RATLIFF, K. L., SHEN, H., BARKER, C. K., MARTINS, I., SHARKEY, C. M., SANDERS, D. A., MCCRAY, P. B. & DAVIDSON, B. L. 2002. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. *Journal of Virology*, 76, 9378-9388.
- KASHIWAKURA, Y., TAMAYOSE, K., IWABUCHI, K., HIRAI, Y., SHIMADA, T., MATSUMOTO, K., NAKAMURA, T., WATANABE, M., OSHIMI, K. & DAIDA, H. 2005. Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. *Journal of Virology*, 79, 609-614.
- KEIZER, R. J., HUITEMA, A. D., SCHELLENS, J. H. & BEIJNEN, J. H. 2010. Clinical pharmacokinetics of therapeutic monoclonal antibodies. *Clin Pharmacokinet*, 49, 493-507.
- KELLER, M. A. & STIEHM, E. R. 2000. Passive immunity in prevention and treatment of infectious diseases. *Clin Microbiol Rev*, 13, 602-14.
- KILLIAN, M. L., SWENSON, S. L., VINCENT, A. L., LANDGRAF, J. G., SHU, B., LINDSTROM, S., XU, X., KLIMOV, A., ZHANG, Y. & BOWMAN, A. S. 2013. Simultaneous Infection of Pigs and People with Triple-Reassortant Swine Influenza Virus H1N1 at a U.S. County Fair. *Zoonoses and Public Health*, 60, 196-201.
- KIM, V. N., MITROPHANOUS, K., KINGSMAN, S. M. & KINGSMAN, A. J. 1998. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol*, 72, 811-6.
- KISO, M., MITAMURA, K., SAKAI-TAGAWA, Y., SHIRAISHI, K., KAWAKAMI, C., KIMURA, K., HAYDEN, F. G., SUGAYA, N. & KAWAOKA, Y. 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet*, 364, 759-65.
- KNAPPSKOG, S., RAVNEBERG, H., GJERDRUM, C., TROSSE, C., STERN, B. & PRYME, I. F. 2007. The level of synthesis and secretion of Gaussia princeps luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide. *Journal of Biotechnology*, 128, 705-715.
- KNOSSOW, M. & SKEHEL, J. J. 2006. Variation and infectivity neutralization in influenza. *Immunology*, 119, 1-7.
- KOBAYASHI, M., IIDA, A., UEDA, Y. & HASEGAWA, M. 2003a. Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *J Virol*, 77, 2607-14.
- KOBAYASHI, M., IIDA, A., UEDA, Y. & HASEGAWA, M. 2003b. Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *Journal of Virology*, 77, 2607-2614.
- KOBAYASHI, M., TOYODA, T., ADYSHEV, D. M., AZUMA, Y. & ISHIHAMA, A. 1994. Molecular dissection of influenza virus nucleoprotein: deletion mapping of the RNA binding domain. *J Virol*, 68, 8433-6.

- KOBER, L., ZEHE, C. & BODE, J. 2013. Optimized signal peptides for the development of high expressing CHO cell lines. *Biotechnol Bioeng*, 110, 1164-73.
- KOEBERL, D. D., ALEXANDER, I. E., HALBERT, C. L., RUSSELL, D. W. & MILLER, A. D. 1997. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. *Proc Natl Acad Sci U S A*, 94, 1426-31.
- KOELLE, K., COBEY, S., GRENFELL, B. & PASCUAL, M. 2006. Epochal evolution shapes the phylodynamics of interpandemic influenza A (H3N2) in humans. *Science*, 314, 1898-903.
- KOHLER, G. & MILSTEIN, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256, 495-7.
- KOSZALKA, P., TILMANIS, D. & HURT, A. C. 2017. Influenza antivirals currently in late-phase clinical trial. *Influenza Other Respir Viruses*, 11, 240-246.
- KOTIN, R. M., SINISCALCO, M., SAMULSKI, R. J., ZHU, X. D., HUNTER, L., LAUGHLIN, C. A., MCLAUGHLIN, S., MUZYCZKA, N., ROCCHI, M. & BERNIS, K. I. 1990. Site-specific integration by adeno-associated virus. *Proc Natl Acad Sci U S A*, 87, 2211-5.
- KOYAMA, K., TAKAHASHI, M., NAKAI, N., TAKAKUSA, H., MURAI, T., HOSHI, M., YAMAMURA, N., KOBAYASHI, N. & OKAZAKI, O. 2010. Pharmacokinetics and disposition of CS-8958, a long-acting prodrug of the novel neuraminidase inhibitor laninamivir in rats. *Xenobiotica*, 40, 207-16.
- KRAMMER, F. & PALESE, P. 2015. Advances in the development of influenza virus vaccines. *Nat Rev Drug Discov*, 14, 167-82.
- KUNKEL, H. G., SMITH, W. K., JOSLIN, F. G., NATVIG, J. B. & LITWIN, S. D. 1969. Genetic Marker of the γ A2 Subgroup of γ A Immunoglobulins. *Nature*, 223, 1247-1248.
- KURZEDER, C., KOPPOLD, B., SAUER, G., PABST, S., KREIENBERG, R. & DEISSLER, H. 2007. CD9 promotes adeno-associated virus type 2 infection of mammary carcinoma cells with low cell surface expression of heparan sulphate proteoglycans. *International Journal of Molecular Medicine*, 19, 325-333.
- LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-5.
- LAMB, R. A. & CHOPPIN, P. W. 1979. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. *Proc Natl Acad Sci U S A*, 76, 4908-12.
- LAVER, W. G., COLMAN, P. M., WEBSTER, R. G., HINSHAW, V. S. & AIR, G. M. 1984. Influenza virus neuraminidase with hemagglutinin activity. *Virology*, 137, 314-23.
- LE, Q. M., KISO, M., SOMEYA, K., SAKAI, Y. T., NGUYEN, T. H., NGUYEN, K. H., PHAM, N. D., NGYEN, H. H., YAMADA, S., MURAMOTO, Y., HORIMOTO, T., TAKADA, A., GOTO, H., SUZUKI, T., SUZUKI, Y. & KAWAOKA, Y. 2005. Avian flu: isolation of drug-resistant H5N1 virus. *Nature*, 437, 1108.
- LEBHERZ, C., MAGUIRE, A., TANG, W., BENNETT, J. & WILSON, J. M. 2008. Novel AAV serotypes for improved ocular gene transfer. *J Gene Med*, 10, 375-82.
- LEE, P. S. & WILSON, I. A. 2015. Structural characterization of viral epitopes recognized by broadly cross-reactive antibodies. *Curr Top Microbiol Immunol*, 386, 323-41.

- LEONE, P., SHERA, D., MCPHEE, S. W., FRANCIS, J. S., KOLODNY, E. H., BILANIUK, L. T., WANG, D. J., ASSADI, M., GOLDFARB, O., GOLDMAN, H. W., FREESE, A., YOUNG, D., DURING, M. J., SAMULSKI, R. J. & JANSON, C. G. 2012. Long-term follow-up after gene therapy for canavan disease. *Sci Transl Med*, 4, 165ra163.
- LEVER, A., GOTTLINGER, H., HASELTINE, W. & SODROSKI, J. 1989. Identification of a sequence required for efficient packaging of human immunodeficiency virus type 1 RNA into virions. *J Virol*, 63, 4085-7.
- LEWIS, A. D., CHEN, R., MONTEFIORI, D. C., JOHNSON, P. R. & CLARK, K. R. 2002. Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. *J Virol*, 76, 8769-75.
- LIMBERIS, M. P., ADAM, V. S., WONG, G., GREN, J., KOBASA, D., ROSS, T. M., KOBINGER, G. P., TRETIAKOVA, A. & WILSON, J. M. 2013. Intranasal antibody gene transfer in mice and ferrets elicits broad protection against pandemic influenza. *Sci Transl Med*, 5, 187ra72.
- LIN, Y. C., BOONE, M., MEURIS, L., LEMMENS, I., VAN ROY, N., SOETE, A., REUMERS, J., MOISSE, M., PLAISANCE, S., DRMANAC, R., CHEN, J., SPELEMAN, F., LAMBRECHTS, D., VAN DE PEER, Y., TAVERNIER, J. & CALLEWAERT, N. 2014. Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat Commun*, 5, 4767.
- LING, C., LU, Y. A., KALSI, J. K., JAYANDHARAN, G. R., LI, B. Z., MA, W. Q., CHENG, B. B., GEE, S. W. Y., MCGOOGAN, K. E., GOVINDASAMY, L., ZHONG, L., AGBANDJE-MCKENNA, M. & SRIVASTAVA, A. 2010. Human Hepatocyte Growth Factor Receptor Is a Cellular Coreceptor for Adeno-Associated Virus Serotype. *Human Gene Therapy*, 21, 1741-1747.
- LOILER, S. A., TANG, Q., CLARKE, T., CAMPBELL-THOMPSON, M. L., CHIODO, V., HAUSWIRTH, W., CRUZ, P., PERRET-GENTIL, M., ATKINSON, M. A., RAMIYA, V. K. & FLOTTE, T. R. 2005. Localized gene expression following administration of adeno-associated viral vectors via pancreatic ducts. *Mol Ther*, 12, 519-27.
- LOWEN, A. C., MUBAREKA, S., TUMPEY, T. M., GARCIA-SASTRE, A. & PALESE, P. 2006. The guinea pig as a transmission model for human influenza viruses. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 9988-9992.
- LU, Y., WAMBACH, M., KATZE, M. G. & KRUG, R. M. 1995. Binding of the Influenza-Virus Ns1 Protein to Double-Stranded-Rna Inhibits the Activation of the Protein-Kinase That Phosphorylates the Elf-2 Translation Initiation-Factor. *Virology*, 214, 222-228.
- LUO, X. M., MAARSCHALK, E., O'CONNELL, R. M., WANG, P., YANG, L. & BALTIMORE, D. 2009. Engineering human hematopoietic stem/progenitor cells to produce a broadly neutralizing anti-HIV antibody after in vitro maturation to human B lymphocytes. *Blood*, 113, 1422-31.
- MACLAREN, R. E., GROPE, M., BARNARD, A. R., COTTRIAL, C. L., TOLMACHOVA, T., SEYMOUR, L., CLARK, K. R., DURING, M. J., CREMERS, F. P., BLACK, G. C., LOTERY, A. J., DOWNES, S. M., WEBSTER, A. R. & SEABRA, M. C. 2014. Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial. *Lancet*, 383, 1129-37.

- MACPHERSON, A. J., MCCOY, K. D., JOHANSEN, F. E. & BRANDTZAEG, P. 2008. The immune geography of IgA induction and function. *Mucosal Immunology*, 1, 11-22.
- MAGUIRE, A. M., HIGH, K. A., AURICCHIO, A., WRIGHT, J. F., PIERCE, E. A., TESTA, F., MINGOZZI, F., BENNICELLI, J. L., YING, G. S., ROSSI, S., FULTON, A., MARSHALL, K. A., BANFI, S., CHUNG, D. C., MORGAN, J. I., HAUCK, B., ZELENIAIA, O., ZHU, X., RAFFINI, L., COPPIETERS, F., DE BAERE, E., SHINDLER, K. S., VOLPE, N. J., SURACE, E. M., ACERRA, C., LYUBARSKY, A., REDMOND, T. M., STONE, E., SUN, J., MCDONNELL, J. W., LEROY, B. P., SIMONELLI, F. & BENNETT, J. 2009a. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. *Lancet*, 374, 1597-605.
- MAGUIRE, A. M., SIMONELLI, F., PIERCE, E. A., PUGH, E. N., JR., MINGOZZI, F., BENNICELLI, J., BANFI, S., MARSHALL, K. A., TESTA, F., SURACE, E. M., ROSSI, S., LYUBARSKY, A., ARRUDA, V. R., KONKLE, B., STONE, E., SUN, J., JACOBS, J., DELL'OSSO, L., HERTLE, R., MA, J. X., REDMOND, T. M., ZHU, X., HAUCK, B., ZELENIAIA, O., SHINDLER, K. S., MAGUIRE, M. G., WRIGHT, J. F., VOLPE, N. J., MCDONNELL, J. W., AURICCHIO, A., HIGH, K. A. & BENNETT, J. 2008. Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med*, 358, 2240-8.
- MAGUIRE, C. A., DELIOLANIS, N. C., PIKE, L., NIER, J. M., TJON-KON-FAT, L. A., SENA-ESTEVEZ, M. & TANNOUS, B. A. 2009b. Gaussia luciferase variant for high-throughput functional screening applications. *Anal Chem*, 81, 7102-6.
- MAK, T. M., HANSON, B. J. & TAN, Y. J. 2014. Chimerization and characterization of a monoclonal antibody with potent neutralizing activity across multiple influenza A H5N1 clades. *Antiviral Res*, 107, 76-83.
- MANNO, C. S., CHEW, A. J., HUTCHISON, S., LARSON, P. J., HERZOG, R. W., ARRUDA, V. R., TAI, S. J., RAGNI, M. V., THOMPSON, A., OZELO, M., COUTO, L. B., LEONARD, D. G., JOHNSON, F. A., MCCLELLAND, A., SCALLAN, C., SKARSGARD, E., FLAKE, A. W., KAY, M. A., HIGH, K. A. & GLADER, B. 2003. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood*, 101, 2963-72.
- MARTIN, K. & HELENIUS, A. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell*, 67, 117-30.
- MATROSOVICH, M., MATROSOVICH, T., CARR, J., ROBERTS, N. A. & KLENK, H. D. 2003. Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J Virol*, 77, 8418-25.
- MATROSOVICH, M. N., GAMBARYAN, A. S., TENEBERG, S., PISKAREV, V. E., YAMNIKOVA, S. S., LVOV, D. K., ROBERTSON, J. S. & KARLSSON, K. A. 1997. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. *Virology*, 233, 224-34.
- MCCARTY, D. M. 2008. Self-complementary AAV vectors; advances and applications. *Mol Ther*, 16, 1648-56.

- MCKAY, T., PATEL, M., PICKLES, R. J., JOHNSON, L. G. & OLSEN, J. C. 2006. Influenza M2 envelope protein augments avian influenza hemagglutinin pseudotyping of lentiviral vectors. *Gene Therapy*, 13, 715-724.
- MCLEAN, G. R., TORRES, M., ELGUEZABAL, N., NAKOUZI, A. & CASADEVALL, A. 2002. Isotype can affect the fine specificity of an antibody for a polysaccharide antigen. *Journal of Immunology*, 169, 1379-1386.
- MCPHEE, S. W., JANSON, C. G., LI, C., SAMULSKI, R. J., CAMP, A. S., FRANCIS, J., SHERA, D., LIOUTERMANN, L., FEELY, M., FREESE, A. & LEONE, P. 2006. Immune responses to AAV in a phase I study for Canavan disease. *J Gene Med*, 8, 577-88.
- MEASE, P. J., HOBBS, K., CHALMERS, A., EL-GABALAWY, H., BOOKMAN, A., KEYSTONE, E., FURST, D. E., ANKLESARIA, P. & HEALD, A. E. 2009. Local delivery of a recombinant adenoassociated vector containing a tumour necrosis factor alpha antagonist gene in inflammatory arthritis: a phase 1 dose-escalation safety and tolerability study. *Ann Rheum Dis*, 68, 1247-54.
- MEASE, P. J., WEI, N., FUDMAN, E. J., KIVITZ, A. J., SCHECHTMAN, J., TRAPP, R. G., HOBBS, K. F., GREENWALD, M., HOU, A., BOOKBINDER, S. A., GRAHAM, G. E., WIESENHUTTER, C. W., WILLIS, L., RUDERMAN, E. M., FORSTOT, J. Z., MARICIC, M. J., DAO, K. H., PRITCHARD, C. H., FISKE, D. N., BURCH, F. X., PRUPAS, H. M., ANKLESARIA, P. & HEALD, A. E. 2010. Safety, tolerability, and clinical outcomes after intraarticular injection of a recombinant adeno-associated vector containing a tumor necrosis factor antagonist gene: results of a phase 1/2 Study. *J Rheumatol*, 37, 692-703.
- MEDINA, M. F., KOBINGER, G. P., RUX, J., GASMI, M., LOONEY, D. J., BATES, P. & WILSON, J. M. 2003. Lentiviral vectors pseudotyped with minimal filovirus envelopes increased gene transfer in murine lung. *Mol Ther*, 8, 777-89.
- MENA, I., JAMBRINA, E., ALBO, C., PERALES, B., ORTIN, J., ARRESE, M., VALLEJO, D. & PORTELA, A. 1999. Mutational analysis of influenza A virus nucleoprotein: identification of mutations that affect RNA replication. *J Virol*, 73, 1186-94.
- MENDELL, J. R., RODINO-KLAPAC, L. R., ROSALES, X. Q., COLEY, B. D., GALLOWAY, G., LEWIS, S., MALIK, V., SHILLING, C., BYRNE, B. J., CONLON, T., CAMPBELL, K. J., BREMER, W. G., TAYLOR, L. E., FLANIGAN, K. M., GASTIER-FOSTER, J. M., ASTBURY, C., KOTA, J., SAHENK, Z., WALKER, C. M. & CLARK, K. R. 2010. Sustained alpha-sarcoglycan gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D. *Ann Neurol*, 68, 629-38.
- MENDELL, J. R., RODINO-KLAPAC, L. R., ROSALES-QUINTERO, X., KOTA, J., COLEY, B. D., GALLOWAY, G., CRAENEN, J. M., LEWIS, S., MALIK, V., SHILLING, C., BYRNE, B. J., CONLON, T., CAMPBELL, K. J., BREMER, W. G., VIOLLET, L., WALKER, C. M., SAHENK, Z. & CLARK, K. R. 2009. Limb-girdle muscular dystrophy type 2D gene therapy restores alpha-sarcoglycan and associated proteins. *Ann Neurol*, 66, 290-7.
- MENDELL, J. R., SAHENK, Z., MALIK, V., GOMEZ, A. M., FLANIGAN, K. M., LOWES, L. P., ALFANO, L. N., BERRY, K., MEADOWS, E., LEWIS, S., BRAUN, L., SHONTZ, K., ROUHANA, M., CLARK, K. R., ROSALES, X. Q., AL-ZAIDY, S., GOVONI, A., RODINO-KLAPAC, L. R., HOGAN, M. J. & KASPAR, B. K. 2015. A

- phase 1/2a follistatin gene therapy trial for becker muscular dystrophy. *Mol Ther*, 23, 192-201.
- MILLER, D. G., TROBRIDGE, G. D., PETEK, L. M., JACOBS, M. A., KAUL, R. & RUSSELL, D. W. 2005. Large-scale analysis of adeno-associated virus vector integration sites in normal human cells. *J Virol*, 79, 11434-42.
- MITCHELL, R. S., BEITZEL, B. F., SCHRODER, A. R., SHINN, P., CHEN, H., BERRY, C. C., ECKER, J. R. & BUSHMAN, F. D. 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol*, 2, E234.
- MITOMO, K., GRIESENBACH, U., INOUE, M., SOMERTON, L., MENG, C., AKIBA, E., TABATA, T., UEDA, Y., FRANKEL, G. M., FARLEY, R., SINGH, C., CHAN, M., MUNKONGE, F., BRUM, A., XENARIOU, S., ESCUDERO-GARCIA, S., HASEGAWA, M. & ALTON, E. W. 2010a. Toward gene therapy for cystic fibrosis using a lentivirus pseudotyped with Sendai virus envelopes. *Mol Ther*, 18, 1173-82.
- MITOMO, K., GRIESENBACH, U., INOUE, M., SOMERTON, L., MENG, C. X., AKIBA, E., TABATA, T., UEDA, Y., FRANKEL, G. M., FARLEY, R., SINGH, C., CHAN, M., MUNKONGE, F., BRUM, A., XENARIOU, S., ESCUDERO-GARCIA, S., HASEGAWA, M. & ALTON, E. W. F. W. 2010b. Toward Gene Therapy for Cystic Fibrosis Using a Lentivirus Pseudotyped With Sendai Virus Envelopes. *Molecular Therapy*, 18, 1173-1182.
- MIYAZAKI, J., TAKAKI, S., ARAKI, K., TASHIRO, F., TOMINAGA, A., TAKATSU, K. & YAMAMURA, K. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene*, 79, 269-77.
- MIYOSHI, H., BLOMER, U., TAKAHASHI, M., GAGE, F. H. & VERMA, I. M. 1998. Development of a self-inactivating lentivirus vector. *J Virol*, 72, 8150-7.
- MODA, F., VIMERCATI, C., CAMPAGNANI, I., RUGGERONE, M., GIACCONE, G., MORBIN, M., ZENTILIN, L., GIACCA, M., ZUCCA, I., LEGNAME, G. & TAGLIAVINI, F. 2012. Brain delivery of AAV9 expressing an anti-PrP monovalent antibody delays prion disease in mice. *Prion*, 6, 383-90.
- MOIANI, A., PALEARI, Y., SARTORI, D., MEZZADRA, R., MICCIO, A., CATTOGLIO, C., COCCHIARELLA, F., LIDONNICI, M. R., FERRARI, G. & MAVILIO, F. 2012. Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts. *J Clin Invest*, 122, 1653-66.
- MONTINI, E., CESANA, D., SCHMIDT, M., SANVITO, F., BARTHOLOMAE, C. C., RANZANI, M., BENEDICENTI, F., SERGI, L. S., AMBROSI, A., PONZONI, M., DOGLIONI, C., DI SERIO, C., VON KALLE, C. & NALDINI, L. 2009. The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *Journal of Clinical Investigation*, 119, 964-975.
- MONTO, A. S. 2008. Antivirals and influenza: frequency of resistance. *Pediatr Infect Dis J*, 27, S110-2.
- MORI, S., WANG, L., TAKEUCHI, T. & KANDA, T. 2004. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. *Virology*, 330, 375-83.
- MOSCONA, A. 2005. Neuraminidase inhibitors for influenza. *N Engl J Med*, 353, 1363-73.
- MOSS, R. B., MILLA, C., COLOMBO, J., ACCURSO, F., ZEITLIN, P. L., CLANCY, J. P., SPENCER, L. T., PILEWSKI, J., WALTZ, D. A., DORKIN, H. L., FERKOL, T.,

- PIAN, M., RAMSEY, B., CARTER, B. J., MARTIN, D. B. & HEALD, A. E. 2007. Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. *Hum Gene Ther*, 18, 726-32.
- MOSS, R. B., RODMAN, D., SPENCER, L. T., AITKEN, M. L., ZEITLIN, P. L., WALTZ, D., MILLA, C., BRODY, A. S., CLANCY, J. P., RAMSEY, B., HAMBLETT, N. & HEALD, A. E. 2004. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest*, 125, 509-21.
- MUKAIGAWA, J. & NAYAK, D. P. 1991. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. *J Virol*, 65, 245-53.
- MURAMATSU, M., YOSHIDA, R., YOKOYAMA, A., MIYAMOTO, H., KAJIHARA, M., MARUYAMA, J., NAO, N., MANZOOR, R. & TAKADA, A. 2014. Comparison of antiviral activity between IgA and IgG specific to influenza virus hemagglutinin: increased potential of IgA for heterosubtypic immunity. *PLoS One*, 9, e85582.
- MURAMOTO, Y., NODA, T., KAWAKAMI, E., AKKINA, R. & KAWAOKA, Y. 2013. Identification of Novel Influenza A Virus Proteins Translated from PA mRNA. *Journal of Virology*, 87, 2455-2462.
- MUTHUMANI, K., FLINGAI, S., WISE, M., TINGEY, C., UGEN, K. E. & WEINER, D. B. 2013. Optimized and enhanced DNA plasmid vector based in vivo construction of a neutralizing anti-HIV-1 envelope glycoprotein Fab. *Hum Vaccin Immunother*, 9, 2253-62.
- MUZYCZKA, N. 1992. Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr Top Microbiol Immunol*, 158, 97-129.
- NACHBAGAUER, R. & KRAMMER, F. 2017. Universal influenza virus vaccines and therapeutic antibodies. *Clin Microbiol Infect*, 23, 222-228.
- NAKAJIMA, T., NAKAMARU, K., IDO, E., TERAOKA, K., HAYAMI, M. & HASEGAWA, M. 2000. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther*, 11, 1863-74.
- NAKAYAMA, K. 1997. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J*, 327 (Pt 3), 625-35.
- NALDINI, L., BLOMER, U., GALLAY, P., ORY, D., MULLIGAN, R., GAGE, F. H., VERMA, I. M. & TRONO, D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*, 272, 263-7.
- NASON, E. L., WETZEL, J. D., MUKHERJEE, S. K., BARTON, E. S., PRASAD, B. V. & DERMODY, T. S. 2001. A monoclonal antibody specific for reovirus outer-capsid protein sigma3 inhibits sigma1-mediated hemagglutination by steric hindrance. *J Virol*, 75, 6625-34.
- NATHWANI, A. C., GRAY, J. T., NG, C. Y. C., ZHOU, J. F., SPENCE, Y., WADDINGTON, S. N., TUDDENHAM, E. G. D., KEMBALL-COOK, G., MCINTOSH, J., BOON-SPIJKER, M., MERTENS, K. & DAVIDOFF, A. M. 2006. Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver. *Blood*, 107, 2653-2661.

- NATHWANI, A. C., TUDDENHAM, E. G., RANGARAJAN, S., ROSALES, C., MCINTOSH, J., LINCH, D. C., CHOWDARY, P., RIDDELL, A., PIE, A. J., HARRINGTON, C., O'BEIRNE, J., SMITH, K., PASI, J., GLADER, B., RUSTAGI, P., NG, C. Y., KAY, M. A., ZHOU, J., SPENCE, Y., MORTON, C. L., ALLAY, J., COLEMAN, J., SLEEP, S., CUNNINGHAM, J. M., SRIVASTAVA, D., BASNER-TSCHAKARJAN, E., MINGOZZI, F., HIGH, K. A., GRAY, J. T., REISS, U. M., NIENHUIS, A. W. & DAVIDOFF, A. M. 2011. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med*, 365, 2357-65.
- NAULT, J. C., DATTA, S., IMBEAUD, S., FRANCONI, A., MALLET, M., COUCHY, G., LETOUZE, E., PILATI, C., VERRET, B., BLANC, J. F., BALABAUD, C., CALDERARO, J., LAURENT, A., LETEXIER, M., BIOULAC-SAGE, P., CALVO, F. & ZUCMAN-ROSSI, J. 2015. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet*, 47, 1187-93.
- NEGRE, D., MANGEOT, P. E., DUISIT, G., BLANCHARD, S., VIDALAIN, P. O., LEISSNER, P., WINTER, A. J., RABOURDIN-COMBE, C., MEHTALI, M., MOULLIER, P., DARLIX, J. L. & COSSET, F. L. 2000. Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells. *Gene Therapy*, 7, 1613-1623.
- NEMEROFF, M. E., QIAN, X. Y. & KRUG, R. M. 1995. The influenza virus NS1 protein forms multimers in vitro and in vivo. *Virology*, 212, 422-8.
- NG, R., GOVINDASAMY, L., GURDA, B. L., MCKENNA, R., KOZYREVA, O. G., SAMULSKI, R. J., PARENT, K. N., BAKER, T. S. & AGBANDJE-MCKENNA, M. 2010. Structural characterization of the dual glycan binding adeno-associated virus serotype 6. *J Virol*, 84, 12945-57.
- NIEDERER, H. A. & BANGHAM, C. R. M. 2014. Integration Site and Clonal Expansion in Human Chronic Retroviral Infection and Gene Therapy. *Viruses-Basel*, 6, 4140-4164.
- NIEMEYER, G. P., HERZOG, R. W., MOUNT, J., ARRUDA, V. R., TILLSON, D. M., HATHCOCK, J., VAN GINKEL, F. W., HIGH, K. A. & LOTHROP, C. D. 2009. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood*, 113, 797-806.
- O'NEILL, R. E., TALON, J. & PALESE, P. 1998. The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO J*, 17, 288-96.
- OBER, R. J., RADU, C. G., GHETIE, V. & WARD, E. S. 2001. Differences in promiscuity for antibody-FcRn interactions across species: implications for therapeutic antibodies. *International Immunology*, 13, 1551-1559.
- OKUNO, Y., ISEGAWA, Y., SASAO, F. & UEDA, S. 1993. A Common Neutralizing Epitope Conserved between the Hemagglutinins of Influenza-a Virus H1 and H2 Strains. *Journal of Virology*, 67, 2552-2558.
- OLSEN, C. W., KARASIN, A. I., CARMAN, S., LI, Y., BASTIEN, N., OJKIC, D., ALVES, D., CHARBONNEAU, G., HENNING, B. M., LOW, D. E., BURTON, L. & BROUKHANSKI, G. 2006. Triple reassortant H3N2 influenza A viruses, Canada, 2005. *Emerg Infect Dis*, 12, 1132-5.
- OLSEN, J. C. 1998. Gene transfer vectors derived from equine infectious anemia virus. *Gene Ther*, 5, 1481-7.

- OSTERHAUS, A., FOUCHIER, R. & RIMMELZWAAN, G. 2011. Towards universal influenza vaccines? *Philos Trans R Soc Lond B Biol Sci*, 366, 2766-73.
- PADGETT, D. A. & GLASER, R. 2003. How stress influences the immune response. *Trends in Immunology*, 24, 444-448.
- PALFI, S., GURRUCHAGA, J. M., RALPH, G. S., LEPETIT, H., LAVISSE, S., BUTTERY, P. C., WATTS, C., MISKIN, J., KELLEHER, M., DEELEY, S., IWAMURO, H., LEFAUCHEUR, J. P., THIRIEZ, C., FENELON, G., LUCAS, C., BRUGIERES, P., GABRIEL, I., ABHAY, K., DROUOT, X., TANI, N., KAS, A., GHALEH, B., LE CORVOISIER, P., DOLPHIN, P., BREEN, D. P., MASON, S., GUZMAN, N. V., MAZARAKIS, N. D., RADCLIFFE, P. A., HARROP, R., KINGSMAN, S. M., RASCOL, O., NAYLOR, S., BARKER, R. A., HANTRAYE, P., REMY, P., CESARO, P. & MITROPHANOUS, K. A. 2014. Long-term safety and tolerability of ProSavin, a lentiviral vector-based gene therapy for Parkinson's disease: a dose escalation, open-label, phase 1/2 trial. *Lancet*, 383, 1138-46.
- PATEL, P., KRIZ, J., GRAVEL, M., SOUCY, G., BAREIL, C., GRAVEL, C. & JULIEN, J. P. 2014. Adeno-associated virus-mediated delivery of a recombinant single-chain antibody against misfolded superoxide dismutase for treatment of amyotrophic lateral sclerosis. *Mol Ther*, 22, 498-510.
- PERALES, B., DE LA LUNA, S., PALACIOS, I. & ORTIN, J. 1996. Mutational analysis identifies functional domains in the influenza A virus PB2 polymerase subunit. *J Virol*, 70, 1678-86.
- PERALES, B. & ORTIN, J. 1997. The influenza A virus PB2 polymerase subunit is required for the replication of viral RNA. *J Virol*, 71, 1381-5.
- PESCOVITZ, M. D. 2006. Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. *Am J Transplant*, 6, 859-66.
- PINTO, L. H., HOLSINGER, L. J. & LAMB, R. A. 1992. Influenza virus M2 protein has ion channel activity. *Cell*, 69, 517-28.
- PLOTKIN, J. B., DUSHOFF, J. & LEVIN, S. A. 2002. Hemagglutinin sequence clusters and the antigenic evolution of influenza A virus. *Proc Natl Acad Sci U S A*, 99, 6263-8.
- PLOTKIN, S. A. 2010. Correlates of protection induced by vaccination. *Clin Vaccine Immunol*, 17, 1055-65.
- POESCHLA, E., GILBERT, J., LI, X., HUANG, S., HO, A. & WONG-STAAAL, F. 1998a. Identification of a human immunodeficiency virus type 2 (HIV-2) encapsidation determinant and transduction of nondividing human cells by HIV-2-based lentivirus vectors. *J Virol*, 72, 6527-36.
- POESCHLA, E. M., WONG-STAAAL, F. & LOONEY, D. J. 1998b. Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat Med*, 4, 354-7.
- PORTER, D. L., LEVINE, B. L., KALOS, M., BAGG, A. & JUNE, C. H. 2011. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med*, 365, 725-33.
- POTTER, C. W. 2001. A history of influenza. *J Appl Microbiol*, 91, 572-9.
- POWELL, T. J., SILK, J. D., SHARPS, J., FODOR, E. & TOWNSEND, A. R. 2012a. Pseudotyped influenza A virus as a vaccine for the induction of heterotypic immunity. *J Virol*, 86, 13397-406.
- POWELL, T. J., SILK, J. D., SHARPS, J., FODOR, E. & TOWNSEND, A. R. M. 2012b. Pseudotyped Influenza A Virus as a Vaccine for the Induction of Heterotypic Immunity. *Journal of Virology*, 86, 13397-13406.

- PRABAKARAN, M., PRABHU, N., HE, F., HONGLIANG, Q., HO, H. T., QIANG, J., MENG, T., GOUTAMA, M. & KWANG, J. 2009. Combination Therapy Using Chimeric Monoclonal Antibodies Protects Mice from Lethal H5N1 Infection and Prevents Formation of Escape Mutants. *Plos One*, 4.
- PRITSCH, O., MAGNAC, C., DUMAS, G., BOUVET, J. P., ALZARI, P. & DIGHIRO, G. 2000. Can isotype switch modulate antigen-binding affinity and influence clonal selection? *European Journal of Immunology*, 30, 3387-3395.
- QIAN, X. Y., CHIEN, C. Y., LU, Y., MONTELIONE, G. T. & KRUG, R. M. 1995. An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure. *RNA*, 1, 948-56.
- QING, K., MAH, C., HANSEN, J., ZHOU, S. Z., DWARKI, V. & SRIVASTAVA, A. 1999. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nature Medicine*, 5, 71-77.
- QUAN, F. S., STEINHAUER, D., HUANG, C. Z., ROSS, T. M., COMPANS, R. W. & KANG, S. M. 2008. A bivalent influenza VLP vaccine confers complete inhibition of virus replication in lungs. *Vaccine*, 26, 3352-3361.
- QUAN, F. S., VUNNAVA, A., COMPANS, R. W. & KANG, S. M. 2010. Virus-like particle vaccine protects against 2009 H1N1 pandemic influenza virus in mice. *PLoS One*, 5, e9161.
- RABINOWITZ, J. E., ROLLING, F., LI, C. W., CONRATH, H., XIAO, W. D., XIAO, X. & SAMULSKI, R. J. 2002. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *Journal of Virology*, 76, 791-801.
- RAGHAVAN, M., BONAGURA, V. R., MORRISON, S. L. & BJORKMAN, P. J. 1995. Analysis of the Ph-Dependence of the Neonatal Fc Receptor Immunoglobulin-G Interaction Using Antibody and Receptor Variant. *Biochemistry*, 34, 14649-14657.
- RENEGAR, K. B., SMALL, P. A., JR., BOYKINS, L. G. & WRIGHT, P. F. 2004. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. *J Immunol*, 173, 1978-86.
- RICHTER, M., IWATA, A., NYHUIS, J., NITTA, Y., MILLER, A. D., HALBERT, C. L. & ALLEN, M. D. 2000. Adeno-associated virus vector transduction of vascular smooth muscle cells in vivo. *Physiological Genomics*, 2, 117-127.
- RIO, D., CLARK, S. & TJIAN, R. 1985. A mammalian host-vector system that regulates expression and amplification of transfected genes by temperature induction. *Science*, 227, 23-28.
- RIVIERE, C., DANOS, O. & DOUAR, A. M. 2006. Long-term expression and repeated administration of AAV type 1, 2 and 5 vectors in skeletal muscle of immunocompetent adult mice. *Gene Ther*, 13, 1300-8.
- ROBB, N. C., CHASE, G., BIER, K., VREEDE, F. T., SHAW, P. C., NAFFAKH, N., SCHWEMMLE, M. & FODOR, E. 2011. The influenza A virus NS1 protein interacts with the nucleoprotein of viral ribonucleoprotein complexes. *J Virol*, 85, 5228-31.
- ROBINSON, J. L., LEE, B. E., PATEL, J., BASTIEN, N., GRIMSRUD, K., SEAL, R. F., KING, R., MARSHALL, F. & LI, Y. 2007. Swine influenza (H3N2) infection in a child and possible community transmission, Canada. *Emerg Infect Dis*, 13, 1865-70.

- ROGERS, G. N. & D'SOUZA, B. L. 1989. Receptor binding properties of human and animal H1 influenza virus isolates. *Virology*, 173, 317-22.
- ROGERS, G. N. & PAULSON, J. C. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology*, 127, 361-73.
- ROSATI, S., YANG, Y., BARENDREGT, A. & HECK, A. J. R. 2014. Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. *Nature Protocols*, 9, 967-976.
- RUIGROK, R. W., CALDER, L. J. & WHARTON, S. A. 1989. Electron microscopy of the influenza virus submembranal structure. *Virology*, 173, 311-6.
- RUSSELL, R. J., HAIRE, L. F., STEVENS, D. J., COLLINS, P. J., LIN, Y. P., BLACKBURN, G. M., HAY, A. J., GAMBLIN, S. J. & SKEHEL, J. J. 2006. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature*, 443, 45-9.
- SAITO, R., SUZUKI, Y., LI, D., ZARAKET, H., SATO, I., MASAKI, H., KAWASHIMA, T., HIBI, S., SANO, Y., SHOBUGAWA, Y., OGUMA, T. & SUZUKI, H. 2008. Increased incidence of adamantane-resistant influenza A(H1N1) and A(H3N2) viruses during the 2006-2007 influenza season in Japan. *J Infect Dis*, 197, 630-2; author reply 632-3.
- SALGANIK, M., HIRSCH, M. L. & SAMULSKI, R. J. 2015. Adeno-associated Virus as a Mammalian DNA Vector. *Microbiology Spectrum*, 3.
- SAMULSKI, R. J., BERNS, K. I., TAN, M. & MUZYCZKA, N. 1982. Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci U S A*, 79, 2077-81.
- SAMULSKI, R. J. & SHENK, T. 1988. Adenovirus-E1b 55-Mr Polypeptide Facilitates Timely Cytoplasmic Accumulation of Adeno-Associated Virus Messenger-Rnas. *Journal of Virology*, 62, 206-210.
- SCHAEFER, W., VOLGER, H. R., LORENZ, S., IMHOF-JUNG, S., REGULA, J. T., KLEIN, C. & MOLHOJ, M. 2016. Heavy and light chain pairing of bivalent quadroma and knobs-into-holes antibodies analyzed by UHR-ESI-QTOF mass spectrometry. *MAbs*, 8, 49-55.
- SCHINDELIN, J., RUEDEN, C. T., HINER, M. C. & ELICEIRI, K. W. 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. *Mol Reprod Dev*, 82, 518-29.
- SCHMIDT, M., VOUTETAKIS, A., AFIONE, S., ZHENG, C., MANDIKIAN, D. & CHIORINI, J. A. 2008. Adeno-associated virus type 12 (AAV12): a novel AAV serotype with sialic acid- and heparan sulfate proteoglycan-independent transduction activity. *J Virol*, 82, 1399-406.
- SCHOLTISSEK, C. 1985. Stability of Infectious Influenza-a Viruses at Low Ph and at Elevated-Temperature. *Vaccine*, 3, 215-218.
- SCHOLTISSEK, C. 1990. Pigs as the 'mixing vessel' for the creation of new pandemic influenza A viruses. . *Med Princip Prac*, 2, 65-71.
- SCHOLTISSEK, C. 1995. Molecular evolution of influenza viruses. *Virus Genes*, 11, 209-15.
- SCHOLTISSEK, C. 1996. Molecular evolution of influenza viruses. . *Virus Genes*, 11, 209-215.
- SCHRODER, M. & KAUFMAN, R. J. 2005. The mammalian unfolded protein response. *Annu Rev Biochem*, 74, 739-89.

- SEILER, M. P., MILLER, A. D., ZABNER, J. & HALBERT, C. L. 2006. Adeno-associated virus types 5 and 6 use distinct receptors for cell entry. *Human Gene Therapy*, 17, 10-19.
- SHEPARD, H. M., PHILLIPS, G. L., C, D. T. & FELDMANN, M. 2017. Developments in therapy with monoclonal antibodies and related proteins. *Clin Med (Lond)*, 17, 220-232.
- SIDWELL, R. W. & SMEE, D. F. 2000. In vitro and in vivo assay systems for study of influenza virus inhibitors. *Antiviral Res*, 48, 1-16.
- SILVA, S. R. D., MCCLEMENTS, M. E., HANKINS, M. W. & MACLAREN, R. E. 2015. Adeno-Associated Viral Gene Therapy for Retinal Disorders. *Gene Delivery and Therapy for Neurological Disorders*, 98, 203-228.
- SILVERSTEIN, A. M. 1989. *A History of Immunology*, Academic Press.
- SIMONSEN, L. 1999. The global impact of influenza on morbidity and mortality. *Vaccine*, 17 Suppl 1, S3-10.
- SINN, P. L., ARIAS, A. C., BROGDEN, K. A. & MCCRAY, P. B. 2008a. Lentivirus Vector Can Be Readministered to Nasal Epithelia without Blocking Immune Responses. *Journal of Virology*, 82, 10684-10692.
- SINN, P. L., ARIAS, A. C., BROGDEN, K. A. & MCCRAY, P. B., JR. 2008b. Lentivirus vector can be readministered to nasal epithelia without blocking immune responses. *J Virol*, 82, 10684-92.
- SKARICIC, D., TRAUBE, C., DE, B., JOH, J., BOYER, J., CRYSTAL, R. G. & WORGALL, S. 2008. Genetic delivery of an anti-RSV antibody to protect against pulmonary infection with RSV. *Virology*, 378, 79-85.
- SMITH, D. J., LAPEDES, A. S., DE JONG, J. C., BESTEBROER, T. M., RIMMELZWAAN, G. F., OSTERHAUS, A. D. & FOUCHIER, R. A. 2004. Mapping the antigenic and genetic evolution of influenza virus. *Science*, 305, 371-6.
- SMITH, W., ANDREWES, C. H. & LAIDLAW, P. P. 1995. A virus obtained from influenza patients (Reprinted from Lancet, July 8, pg 66-68, 1933). *Reviews in Medical Virology*, 5, 187-191.
- SMORODINTSEFF AA, T. M., DROBYSHEVSKAYA AI, KOROVIN AA, OSETROFF AI 1937. Investigation on volunteers infected with the influenza virus. *Am J Med Sciences*, 194, 159-170.
- STROBEL, B., MILLER, F. D., RIST, W. & LAMLA, T. 2015. Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. *Hum Gene Ther Methods*, 26, 147-57.
- STROES, E. S., NIERMAN, M. C., MEULENBERG, J. J., FRANSSSEN, R., TWISK, J., HENNY, C. P., MAAS, M. M., ZWINDERMAN, A. H., ROSS, C., ARONICA, E., HIGH, K. A., LEVI, M. M., HAYDEN, M. R., KASTELEIN, J. J. & KUIVENHOVEN, J. A. 2008. Intramuscular Administration of AAV1-Lipoprotein Lipase(S447X) Lowers Triglycerides in Lipoprotein Lipase-Deficient Patients. *Arteriosclerosis Thrombosis and Vascular Biology*, 28, 2303-U264.
- SUI, J., HWANG, W. C., PEREZ, S., WEI, G., AIRD, D., CHEN, L. M., SANTELLI, E., STEC, B., CADWELL, G., ALI, M., WAN, H., MURAKAMI, A., YAMMANURU, A., HAN, T., COX, N. J., BANKSTON, L. A., DONIS, R. O., LIDDINGTON, R. C. & MARASCO, W. A. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat Struct Mol Biol*, 16, 265-73.

- SUMMERFORD, C. & SAMULSKI, R. J. 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *Journal of Virology*, 72, 1438-1445.
- SUMNER-JONES, S. G., GILL, D. R. & HYDE, S. C. 2007. Lack of repeat transduction by recombinant adeno-associated virus type 5/5 vectors in the mouse airway. *J Virol*, 81, 12360-7.
- SUZUKI, T., TAKAHASHI, T., HIDARI, K. I. P. J., MIYAMOTO, D. & SUZUKI, Y. 2004. A molecular mechanism for the low pH stability of sialidase activity of influenza A virus N2 neuraminidases. *Options for the Control of Influenza V*, 1263, 781-786.
- SUZUKI, Y. 1994. Gangliosides as Influenza-Virus Receptors - Variation of Influenza-Viruses and Their Recognition of the Receptor Sialo-Sugar Chains. *Progress in Lipid Research*, 33, 429-457.
- SUZUKI, Y. & NEI, M. 2002. Origin and evolution of influenza virus hemagglutinin genes. *Mol Biol Evol*, 19, 501-9.
- TAKEDA, S., TAKAHASHI, M., MIZUKAMI, H., KOBAYASHI, E., TAKEUCHI, K., HAKAMATA, Y., KANEKO, T., YAMAMOTO, H., ITO, C., OZAWA, K., ISHIBASHI, K., MATSUZAKI, T., TAKATA, K., ASANO, Y. & KUSANO, E. 2004. Successful gene transfer using adeno-associated virus vectors into the kidney: Comparison among adeno-associated virus serotype 1-5 vectors in vitro and in vivo. *Nephron Experimental Nephrology*, 96, E119-E126.
- TAKEUCHI, K. & LAMB, R. A. 1994. Influenza virus M2 protein ion channel activity stabilizes the native form of fowl plague virus hemagglutinin during intracellular transport. *J Virol*, 68, 911-9.
- TAN, G. S., LEE, P. S., HOFFMAN, R. M. B., MAZEL-SANCHEZ, B., KRAMMER, F., LEON, P. E., WARD, A. B., WILSON, I. A. & PALESE, P. 2014. Characterization of a Broadly Neutralizing Monoclonal Antibody That Targets the Fusion Domain of Group 2 Influenza A Virus Hemagglutinin. *Journal of Virology*, 88, 13580-13592.
- TANNOUS, B. A. 2009. Gaussia luciferase reporter assay for monitoring biological processes in culture and in vivo. *Nat Protoc*, 4, 582-91.
- TANNOUS, B. A., KIM, D. E., FERNANDEZ, J. L., WEISSLEDER, R. & BREAKFIELD, X. O. 2005. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. *Mol Ther*, 11, 435-43.
- TARELLI, E., SMITH, A. C., HENDRY, B. M., CHALLACOMBE, S. J. & POURIA, S. 2004. Human serum IgA1 is substituted with up to six O-glycans as shown by matrix assisted laser desorption ionisation time-of-flight mass spectrometry. *Carbohydr Res*, 339, 2329-35.
- TAUBENBERGER, J. K. & KASH, J. C. 2010. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe*, 7, 440-51.
- TAYMANS, J. M., VANDENBERGHE, L. H., VAN DEN HAUTE, C., THIRY, I., DEROOSE, C. M., MORTELMANS, L., WILSON, J. M., DEBYSER, Z. & BAEKELANDT, V. 2007. Comparative analysis of adeno-associated viral vector serotypes 1, 2, 5, 7, and 8 in mouse brain. *Human Gene Therapy*, 18, 195-206.
- TEMIN, H. M. & MIZUTANI, S. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature*, 226, 1211-3.
- TERAJIMA, M., CRUZ, J., CO, M. D., LEE, J. H., KAUR, K., WRAMMERT, J., WILSON, P. C. & ENNIS, F. A. 2011. Complement-dependent lysis of influenza a virus-

- infected cells by broadly cross-reactive human monoclonal antibodies. *J Virol*, 85, 13463-7.
- TESAR, D. B., TIANGCO, N. E. & BJORKMAN, P. J. 2006. Ligand valency affects transcytosis, recycling and intracellular trafficking mediated by the neonatal Fc receptor. *Traffic*, 7, 1127-1142.
- THOMPSON, W. W., SHAY, D. K., WEINTRAUB, E., BRAMMER, L., COX, N., ANDERSON, L. J. & FUKUDA, K. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA*, 289, 179-86.
- THORLUND, K., AWAD, T., BOIVIN, G. & THABANE, L. 2011. Systematic review of influenza resistance to the neuraminidase inhibitors. *BMC Infect Dis*, 11, 134.
- THROSBY, M., VAN DEN BRINK, E., JONGENELEN, M., POON, L. L., ALARD, P., CORNELISSEN, L., BAKKER, A., COX, F., VAN DEVENTER, E., GUAN, Y., CINATL, J., TER MEULEN, J., LASTERS, I., CARSETTI, R., PEIRIS, M., DE KRUIF, J. & GOUDSMIT, J. 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. *PLoS One*, 3, e3942.
- TILLER, T., MEFFRE, E., YURASOV, S., TSUIJI, M., NUSSENZWEIG, M. C. & WARDEMAN, H. 2008. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods*, 329, 112-24.
- TJELLE, T. E., CORTHAY, A., LUNDE, E., SANDLIE, I., MICHAELSEN, T. E., MATHIESEN, I. & BOGEN, B. 2004. Monoclonal antibodies produced by muscle after plasmid injection and electroporation. *Mol Ther*, 9, 328-36.
- TORRES, M. & CASADEVALL, A. 2008. The immunoglobulin constant region contributes to affinity and specificity. *Trends Immunol*, 29, 91-7.
- TORRES, M., MAY, R., SCHARFF, M. D. & CASADEVALL, A. 2005. Variable-region-identical antibodies differing in isotype demonstrate differences in fine specificity and idiotype. *J Immunol*, 174, 2132-42.
- TUDOR, D., YU, H., MAUPETIT, J., DRILLET, A. S., BOUCEBA, T., SCHWARTZ-CORNIL, I., LOPALCO, L., TUFFERY, P. & BOMSEL, M. 2012. Isotype modulates epitope specificity, affinity, and antiviral activities of anti-HIV-1 human broadly neutralizing 2F5 antibody. *Proc Natl Acad Sci U S A*, 109, 12680-5.
- TUTYKHINA, I. L., SEDOVA, E. S., GRIBOVA, I. Y., IVANOVA, T. I., VASILEV, L. A., RUTOVSKAYA, M. V., LYSENKO, A. A., SHMAROV, M. M., LOGUNOV, D. Y., NARODITSKY, B. S., TILLIB, S. V. & GINTSBURG, A. L. 2013. Passive immunization with a recombinant adenovirus expressing an HA (H5)-specific single-domain antibody protects mice from lethal influenza infection. *Antiviral Res*, 97, 318-28.
- VAN RIEL, D., MUNSTER, V. J., DE WIT, E., RIMMELZWAAN, G. F., FOUCHIER, R. A., OSTERHAUS, A. D. & KUIKEN, T. 2007. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *Am J Pathol*, 171, 1215-23.
- VANDENDRIESSCHE, T., THORREZ, L., ACOSTA-SANCHEZ, A., PETRUS, I., WANG, L., MA, L., DE WAELE, L., IWASAKI, Y., GILLIJNS, V., WILSON, J. M., COLLEN, D. & CHUAH, M. K. L. 2007. Efficacy and safety of adeno-associated viral vectors based on serotype 8 and 9 vs. lentiviral vectors for hemophilia B gene therapy. *Journal of Thrombosis and Haemostasis*, 5, 16-24.

- WALTER, P., IBRAHIMI, I. & BLOBEL, G. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J Cell Biol*, 91, 545-50.
- WAN, H., YANG, H., SHORE, D. A., GARTEN, R. J., COUZENS, L., GAO, J., JIANG, L., CARNEY, P. J., VILLANUEVA, J., STEVENS, J. & EICHELBERGER, M. C. 2015. Structural characterization of a protective epitope spanning A(H1N1)pdm09 influenza virus neuraminidase monomers. *Nat Commun*, 6, 6114.
- WAN, H. Q., GAO, J., XU, K. M., CHEN, H. J., COUZENS, L. K., RIVERS, K. H., EASTERBROOK, J. D., YANG, K. V., ZHONG, L., RAJABI, M., YE, J. Q., SULTANA, I., WAN, X. F., LIU, X. F., PEREZ, D. R., TAUBENBERGER, J. K. & EICHELBERGER, M. C. 2013. Molecular Basis for Broad Neuraminidase Immunity: Conserved Epitopes in Seasonal and Pandemic H1N1 as Well as H5N1 Influenza Viruses. *Journal of Virology*, 87, 9290-9300.
- WANG, C., WANG, C. M., CLARK, K. R. & SFERRA, T. J. 2003. Recombinant AAV serotype 1 transduction efficiency and tropism in the murine brain. *Gene Ther*, 10, 1528-34.
- WANG, D., ZHONG, L., NAHID, M. A. & GAO, G. 2014. The potential of adeno-associated viral vectors for gene delivery to muscle tissue. *Expert Opin Drug Deliv*, 11, 345-64.
- WANG, J., XIE, J., LU, H., CHEN, L., HAUCK, B., SAMULSKI, R. J. & XIAO, W. 2007. Existence of transient functional double-stranded DNA intermediates during recombinant AAV transduction. *Proc Natl Acad Sci U S A*, 104, 13104-9.
- WANG, T. T., TAN, G. S., HAI, R., PICA, N., PETERSEN, E., MORAN, T. M. & PALESE, P. 2010. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog*, 6, e1000796.
- WANG, Y. J., POLLARD, A., ZHONG, J. H., DONG, X. Y., WU, X. B., ZHOU, H. D. & ZHOU, X. F. 2009. Intramuscular delivery of a single chain antibody gene reduces brain Abeta burden in a mouse model of Alzheimer's disease. *Neurobiol Aging*, 30, 364-76.
- WANG, Z., ZHU, T., QIAO, C. P., ZHOU, L. Q., WANG, B., ZHANG, J., CHEN, C. L., LI, J. & XIAO, X. 2005. Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. *Nature Biotechnology*, 23, 321-328.
- WARD, P., DEAN, F. B., O'DONNELL, M. E. & BERNIS, K. I. 1998. Role of the adenovirus DNA-binding protein in in vitro adeno-associated virus DNA replication. *J Virol*, 72, 420-7.
- WATANABE, K., HANDA, H., MIZUMOTO, K. & NAGATA, K. 1996. Mechanism for inhibition of influenza virus RNA polymerase activity by matrix protein. *J Virol*, 70, 241-7.
- WEBBY, R. J. & WEBSTER, R. G. 2001. Emergence of influenza A viruses. *Philos Trans R Soc Lond B Biol Sci*, 356, 1817-28.
- WEBER, M., RABINOWITZ, J., PROVOST, N., CONRATH, H., FOLLIO, S., BRIOT, D., CHEREL, Y., CHENUAUD, P., SAMULSKI, J., MOULLIER, P. & ROLLING, F. 2003. Recombinant adeno-associated virus serotype 4 mediates unique and exclusive long-term transduction of retinal pigmented epithelium in

- rat, dog, and nonhuman primate after subretinal delivery. *Molecular Therapy*, 7, 774-781.
- WEBSTER, R. G., BEAN, W. J., GORMAN, O. T., CHAMBERS, T. M. & KAWAOKA, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev*, 56, 152-79.
- WEINBERG, J. B., MATTHEWS, T. J., CULLEN, B. R. & MALIM, M. H. 1991. Productive human immunodeficiency virus type 1 (HIV-1) infection of nonproliferating human monocytes. *J Exp Med*, 174, 1477-82.
- WEIS, W., BROWN, J. H., CUSACK, S., PAULSON, J. C., SKEHEL, J. J. & WILEY, D. C. 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature*, 333, 426-31.
- WELLER, M. L., AMORNPHIMOLTHAM, P., SCHMIDT, M., WILSON, P. A., GUTKIND, J. S. & CHIORINI, J. A. 2010. Epidermal growth factor receptor is a co-receptor for adeno-associated virus serotype 6. *Nature Medicine*, 16, 662-664.
- WHITTLE, J. R., ZHANG, R., KHURANA, S., KING, L. R., MANISCHEWITZ, J., GOLDING, H., DORMITZER, P. R., HAYNES, B. F., WALTER, E. B., MOODY, M. A., KEPLER, T. B., LIAO, H. X. & HARRISON, S. C. 2011. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc Natl Acad Sci U S A*, 108, 14216-21.
- WHO. 2016. *Influenza (Seasonal)* [Online]. Available: <http://www.who.int/mediacentre/factsheets/fs211/en/>
- WHO. 2017a. Available: <http://www.who.int/csr/don/19-july-2017-ah7n9-china/en/>
- WHO 2017b. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003-2017.
- WILEY, D. C. & SKEHEL, J. J. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem*, 56, 365-94.
- WILSON, I. A., SKEHEL, J. J. & WILEY, D. C. 1981. Structure of the Hemagglutinin Membrane Glycoprotein of Influenza-Virus at 3-Å Resolution. *Nature*, 289, 366-373.
- WISE, H. M., FOEGLEIN, A., SUN, J. C., DALTON, R. M., PATEL, S., HOWARD, W., ANDERSON, E. C., BARCLAY, W. S. & DIGARD, P. 2009. A Complicated Message: Identification of a Novel PB1-Related Protein Translated from Influenza A Virus Segment 2 mRNA. *Journal of Virology*, 83, 8021-8031.
- WONG, S. S. & WEBBY, R. J. 2013. Traditional and new influenza vaccines. *Clin Microbiol Rev*, 26, 476-92.
- WOOF, J. M. & KERR, M. A. 2006. The function of immunoglobulin A in immunity. *J Pathol*, 208, 270-82.
- WRAMMERT, J., SMITH, K., MILLER, J., LANGLEY, W. A., KOKKO, K., LARSEN, C., ZHENG, N. Y., MAYS, I., GARMAN, L., HELMS, C., JAMES, J., AIR, G. M., CAPRA, J. D., AHMED, R. & WILSON, P. C. 2008. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature*, 453, 667-71.
- WRIGHT, P. N., G.; KAWAOKA, Y. 2007. *Orthomyxoviruses*, Philadelphia: Lippincott Williams & Wilkins.
- WU, X., LI, Y., CRISE, B. & BURGESS, S. M. 2003. Transcription start regions in the human genome are favored targets for MLV integration. *Science*, 300, 1749-51.

- WU, Z., ASOKAN, A. & SAMULSKI, R. J. 2006a. Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther*, 14, 316-27.
- WU, Z., MILLER, E., AGBANDJE-MCKENNA, M. & SAMULSKI, R. J. 2006b. Alpha2,3 and alpha2,6 N-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. *J Virol*, 80, 9093-103.
- WUERTZER, C. A., SULLIVAN, M. A., QIU, X. & FEDEROFF, H. J. 2008. CNS delivery of vectored prion-specific single-chain antibodies delays disease onset. *Mol Ther*, 16, 481-6.
- WURDINGER, T., BADR, C., PIKE, L., DE KLEINE, R., WEISSLEDER, R., BREAKEFIELD, X. O. & TANNOUS, B. A. 2008. A secreted luciferase for ex vivo monitoring of in vivo processes. *Nat Methods*, 5, 171-3.
- XIAO, X., XIAO, W., LI, J. & SAMULSKI, R. J. 1997. A novel 165-base-pair terminal repeat sequence is the sole cis requirement for the adeno-associated virus life cycle. *J Virol*, 71, 941-8.
- XU, Q., WANG, W., CHENG, X., ZENGEL, J. & JIN, H. 2010. Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. *J Virol*, 84, 4936-45.
- YANG, S., COHEN, C. J., PENG, P. D., ZHAO, Y., CASSARD, L., YU, Z., ZHENG, Z., JONES, S., RESTIFO, N. P., ROSENBERG, S. A. & MORGAN, R. A. 2008. Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. *Gene Therapy*, 15, 1411-1423.
- YANG, Y. T. & EVANS, C. A. 1961. Hypothermia in Mice Due to Influenza Virus Infection. *Proceedings of the Society for Experimental Biology and Medicine*, 108, 776-&.
- YLA-HERTTUALA, S. 2012. Endgame: Glybera Finally Recommended for Approval as the First Gene Therapy Drug in the European Union. *Molecular Therapy*, 20, 1831-1832.
- YOSHIDA, R., IGARASHI, M., OZAKI, H., KISHIDA, N., TOMABECHI, D., KIDA, H., ITO, K. & TAKADA, A. 2009. Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog*, 5, e1000350.
- YUE, Y. P., SHIN, J. H. & DUAN, D. S. 2011. Whole Body Skeletal Muscle Transduction in Neonatal Dogs with AAV-9. *Muscle Gene Therapy: Methods and Protocols*, 709, 313-329.
- ZARAKET, H., BRIDGES, O. A. & RUSSELL, C. J. 2013. The pH of Activation of the Hemagglutinin Protein Regulates H5N1 Influenza Virus Replication and Pathogenesis in Mice. *Journal of Virology*, 87, 4826-4834.
- ZHANG, K., WANG, Z., LIU, X., YIN, C., BASIT, Z., XIA, B. & LIU, W. 2012. Dissection of influenza A virus M1 protein: pH-dependent oligomerization of N-terminal domain and dimerization of C-terminal domain. *PLoS One*, 7, e37786.
- ZHANG, L., LENG, Q. & MIXSON, A. J. 2005. Alteration in the IL-2 signal peptide affects secretion of proteins in vitro and in vivo. *J Gene Med*, 7, 354-65.
- ZINCARELLI, C., SOLTYS, S., RENGO, G., KOCH, W. J. & RABINOWITZ, J. E. 2010. Comparative Cardiac Gene Delivery of Adeno-Associated Virus Serotypes

- 1-9 reveals that AAV6 Mediates the Most Efficient Transduction in Mouse Heart. *Cts-Clinical and Translational Science*, 3, 81-89.
- ZINKERNAGEL, R. M. 2001. Maternal antibodies, childhood infections, and autoimmune diseases. *N Engl J Med*, 345, 1331-5.
- ZOLOTUKHIN, S., BYRNE, B. J., MASON, E., ZOLOTUKHIN, I., POTTER, M., CHESNUT, K., SUMMERFORD, C., SAMULSKI, R. J. & MUZYCZKA, N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy*, 6, 973-985.
- ZUFFEREY, R., DONELLO, J. E., TRONO, D. & HOPE, T. J. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol*, 73, 2886-92.
- ZUFFEREY, R., DULL, T., MANDEL, R. J., BUKOVSKY, A., QUIROZ, D., NALDINI, L. & TRONO, D. 1998. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol*, 72, 9873-80.
- ZUFFEREY, R., NAGY, D., MANDEL, R. J., NALDINI, L. & TRONO, D. 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol*, 15, 871-5.

Appendix I Amino acid sequence of the variable regions of the antibodies used in this study

Antibody	Heavy Chain		Light chain	
	V-gene and allele	Variable region amino acid sequence	Locus	Variable region amino acid sequence
T1-3B	1-69*01	TGVHSEVQLVQSGAEVRKPGSSVKVSCPKPSGGIFNK YAVTWVRQAPGQGLEWMGGIIAVFRTTNYAHKFQG RVTISADESMNTVYLELRSLRSDDTAVYYCARGPNYY NSYMDVWVGKGTTVTVSSA	kappa	EIVLTQSPGTLSSLSPGERATLSCRVSQSLTNR DLAWYQQQPGQAPRLIHGASTRATGIPDRFS GSGSGTEFTLTISRLEPEDFAVYYCQQYGTSP QTFGQGTKVEIK
21-D8-5A	4-39*03	QLQLQESGPGLVKSSETLSLTCTVSGDSVSNKIFYW GWVRQPPGKGLEWIGSLYSGGNTYYNPSLQSRVTM SVDTSKNQFSLKLTSVTAAADTAMYFCARHFPYDDV LTGYSSHFDYWGGGILVTVSSASTK	lambda	TGSWAQSALTQPPSASGTPGQRVTVSCSGS RSNIGNNAVNWYQQLPGTAPKLIYTNDQRPS GVPDRFSGSKSGTSASLAITGLQSEDEADYYC ATWDDSVNGVFGTGTRVTVLGQPK
T2-5D	3-15*01	EVQLVESGGDLVKPGGSLRLSCAASGFTFSYASMTW VRQAPGKGLEWVGRIKSLEWGGTTDYAAPVRGRFTI SRDDSKNTLYLQMNSLNTEDTAVYFCVTAATHDYD TYGYSNYWGRGTLTVSS	kappa	DIVMTQSPLSLPVTPGEPASISCRSSRSVQRS NGYNYLEWYLQKPGQSPQLLIYVASNRASGV PDRFSGSGSGTDFTLKISRVEAEDVGVYYCM QTLETPQTFGQGTKVEIK
MED18852	6-1*01	HSQVQLQQSGPGLVKPSQTLSTCAISGDSVSSYNA VWNWIRQSPSRGLEWLGRTYRSGWYNDYAESVKS RITINPDTSKNQFSLQLNSVTPEDTAVYYCARSGHITV FGVNVDAFDMWGGQTMVTVSS	kappa	DIQMTQSPSSLSASVGDRVTITCRTSQSLSSY THWYQQKPGKAPKLLLYAASSRSGSVPSRFS GSGSGTDFTLTISLQPEDFATYYCQQSRTFG QGTKVEIK
T2-5A	1-69*01	EVQLVESGAIEVKKPGSSVKVSCRTSGGIFNSYGITW VRQAPGQGLEWMGGILAFRTTKYAQRFGGRVTISA DESMNTVYMELSLRSDDTAVYYCARGPTYYNYSYM DVWVGKGTTVTVSS	kappa	EIVLTQSPGTLSSLSPGERATLSCRVSQSLSSN NLAWYQKKPGQAPRLIHGAFTTRATGIPDRFS GSGSGTEFTLSISRLEAEDFAVYYCQQYGSSP QTFGQGTKVEIK
T3-4B	3-15*01	EVQLLESGGDLIKPGGSLRLSCVASGFTFSYASMSW VRQAPGKGLEWVGRIKSLEWGGTTDYSAAVNGRFTI	kappa	DIVMTQSPLSLPVTPGEPASISCRSSQSVQRD NGYYYLDWYVQKPGQSPQLLIYVASNRAPGV

		SRDDSKNTVYLHMTSLKIEDTAVYYCVTAATNDYYDT YGYS DYWGQGLTVTVSS		PDRFSGRSGSGTDFTLKISRVEAEDVGVYYCM QTLQTPQTFGQGTKVEIK
T2-6A	3-7*01	TGVHSEVQLVESGGGLVQPGGSLRLTCATSGFSFNS YWMTWVRQAPGKGLEWVANINQVGSEIYYVDSVKG RFTISRDNANKSLYLQMNSLRADDTAVYYCARQGSF ADTYFYYYAMDVWGQGT TVTVSS	lambda	TGSWAQS VLTQPPSVSGAPGQRVTISCTGSS SNIGAGYDVHWYQLLPGTAPKLLIYAFSDRPS GVPDRFSGSKSGTSASLAITGLQAEDEGDYYC QSYDTGLSGLEVVF GGGTKLTVLGQPK
Z3-B2	3-53*01	TGVHSQVQLVESGGGLIQPGGSLRLSCAASGFTVSN NYMTWVRQAPGRGLEWVSVIFSGGTTYADSVKDR FTISRDNANKNTLYLQMNSLRVEETAVYYCARFLVLR FDWPNYAMDVWGRGTTTVTVSSASTK	lambda	TGSWAQSALTQPPSASGSPGQSVTISCTGTS SDVGSYNVSWYQQHPGKAPKLMIYEVSKRP SGVPDRFSGSKSGNTASLTVSGLQTEDEADY YCSTHAGSNNLVLF GGGTRL SVLGQPK
2-12C	5-55*01	TGVHSEVQLVQSGAEVKKPGESLKISCKGSGNGFTT YWIGWVRQVPGKGLEWMGIYPDDSDTRYSPSFQG QVSISADKSISTAFLQWSSLKASDTAMYFCARLGDVE TAMVGQDAFHIWGQGTMTVTVSS	kappa	TGVHSEIVLTQSPGTLSPGDRATLSCRASQ SVSSSFLAWYQQKPGQAPRLLMYGASRRATG IPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQ QYDSSPFTFGGGTKVEIKRTV
Q11-C11	4-61*02	TGVHSQLQLQESGPGLVKPSQTLSTCTVSGGSISS GAYYWNWIRQPAGKGLEWIGHVYFSGTTNYSPSLKS RVTISVDTSKNQFSLNLSVTAADTAVYYCARSPCGG NCYIGEGEGWDFYYSYMDVWGRGTTTVTVSS	lambda	TGSWAQS VLTQPPSVSAAPGQTVTISCSGSS PNIGINYVSWYLHLPGTAPKLLIYDNDKRPSGI PDRFSGSKSGTSATLVITGLQSGDEADYYCGT WDSSLSAVVF GGGTKLTVLGQPK