

Evaluation of a single cycle influenza virus as a candidate vaccine

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Declaration of authorship

I declare that the work described in this thesis is my own, except where indicated below. Contributions by others are also acknowledged in the appropriate text and figure legends.

- Alain Townsend conceived the CLEARFLU concept and selected candidate mutations. He helped to develop the research strategy of this project and provided supervision and training.
- Figure 4.8 investigating co-expression of hemagglutinin and Ebola glycoprotein was the result of a collaboration with Dr Julie Xiao and her student Declan Pang. The data and graph were generated by Julie Xiao using a plasmid I produced. Figure 4.7 describes work entirely performed by Julie Xiao and is included for explanatory purposes.
- Primers used for sequencing the PR8 viral genome were provided by Dr Jack Tan.
- All *in vivo* work in mice was conducted under the supervision of Dr Pramila Rijal and Dr Jack Tan. Both also provided extensive support in processing animal samples and designing the MHC tetramer staining antibody panel. Dr Pramila Rijal assisted with microneutralisation and ELLA in the first mouse study.
- The pig study was designed and conducted by Elma Tchilian and Veronica Martini. Data analysis was conducted by Veronica Martini. Barbara Holzer and Sophie Morgan assisted with tissue harvest. The presentation and interpretation of data are my own.

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Abstract

Evaluation of a single cycle influenza virus as a candidate vaccine

Influenza A continues to impose a considerable burden on humans and animals, including farmed poultry and pigs. Current vaccination approaches successfully protect certain populations against specific strains, but none are effective at consistently inducing broad and long-lasting protection to the diversity of circulating strains.

Single cycle viruses may offer a promising solution as they are able to safely present a wide array of viral antigens in the respiratory tract by undergoing just one round of cell infection in their host. This is thought to be advantageous for stimulating broadly protective resident memory T-cell responses in the lung as well as mucosal antibody responses.

This thesis describes the design and evaluation of a candidate vaccine platform called CLEARFLU, building on the previously described single cycle virus S-Flu designed by Alain Townsend. CLEARFLU viruses are unable to replicate *in vivo* due to the introduction of several independently inactivating mutations into the hemagglutinin protein, blocking the cell entry step of infection. They can however be propagated in cell culture by providing complementing hemagglutinin in trans.

We demonstrate that CLEARFLU viruses do not cause disease in mice or pigs, even when administered to the respiratory tract. They induce a strong T-cell response in the lung, including to the highly conserved viral component nucleoprotein which is known to be associated with broad protection. CLEARFLU viruses also stimulate both systemic and local strain-specific antibody to neuraminidase and neutralising antibody to hemagglutinin in mice. This neutralising response to hemagglutinin is a notable advantage over other hemagglutinin-inactivated single cycle viruses like S-Flu which do not induce such responses and can thus limit but not prevent future infection.

Together with investigations into their genomic stability, we demonstrate that CLEARFLU viruses show promise as vaccine candidates able to generate both non-neutralising protection against a broad range of strains and neutralising protection against strain-matched challenge.

Glossary and commonly used abbreviations

AF	Alexa Fluor
BAL	bronchoalveolar lavage
bp	base pairs
CID	cell infectious dose, see methods
eGFP	enhanced green fluorescent protein
ELLA	enzyme-linked lectin assay
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FSC	forward scatter
GAH	goat anti-human
GAM	goat anti-mouse
GISAID	data repository for sharing of influenza virus sequences and clinical information, available at www.gisaid.org
GP	glycoprotein
H1N1pdm09	standardised name for the H1N1 virus which resulted in the 2009 influenza pandemic
HA/H	hemagglutinin
HA0	precursor hemagglutinin which is cleaved at amino acid 329 to yield HA1 at the N-terminus and HA2 at the C-terminus
HA1	hemagglutination inhibition assay
heterologous protection	immunity which protects against a virus from a different strain to the virus used for immunisation
heterosubtypic protection	immunity which protects against a virus from a different subtype to the virus used for immunisation
HK125	A/Hong Kong/125/2017
LAIV	live attenuated influenza virus
M1/M2	matrix 1/matrix 2 proteins
MN	microneutralisation assay
MOI	multiplicity of infection. e.g. infection of 1 million cells with 1 million TCID ₅₀ of virus equates to a MOI of 1 by TCID ₅₀ .

MUNANA	neuraminidase inhibition assay which measures the cleavage of 20-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid by neuraminidase to release a fluorescent product
NA/N	neuraminidase
neutralising response	immune response which prevents cell entry of viruses
NP	nucleoprotein
PA	polymerase acidic protein
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDB	protein database
PR8	A/Puerto Rico/8/1934
RBC	red blood cell
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT-PCR	reverse transcription polymerase chain reaction
SSC	side scatter
sterile immunity	immunity which does not permit future infection
TBLN	tracheobronchial lymph nodes
TCID	tissue culture infectious dose, see methods
UTR	untranslated region
VGM	virus growth medium
vRNA	viral ribonucleic acid
X31-PM	passage mutant hemagglutinin described by Hooper and Bloom, 2013

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Chapter 1

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1.1 Influenza biology & challenges for control

1.1.1 Influenza burden in humans and animals

Despite the existence of several vaccines, the influenza virus continues to burden human and animal populations. Influenza A viruses are a significant cause of mortality and morbidity in swine and birds and are therefore the focus of this thesis. In chickens and other farmed birds, viruses can cause up to 100% mortality and spread quickly through flocks (Causey & Edwards, 2008). In affected areas, animals may need to be culled to prevent the spread of disease. In swine, most infections cause respiratory issues and fever but the mortality rate is lower (Ma, 2020). The principal economic losses are through stunted growth and reduction in birth rate. Influenza infections are therefore a considerable threat to the health and welfare of animals and can have severe economic repercussions for the agricultural sector.

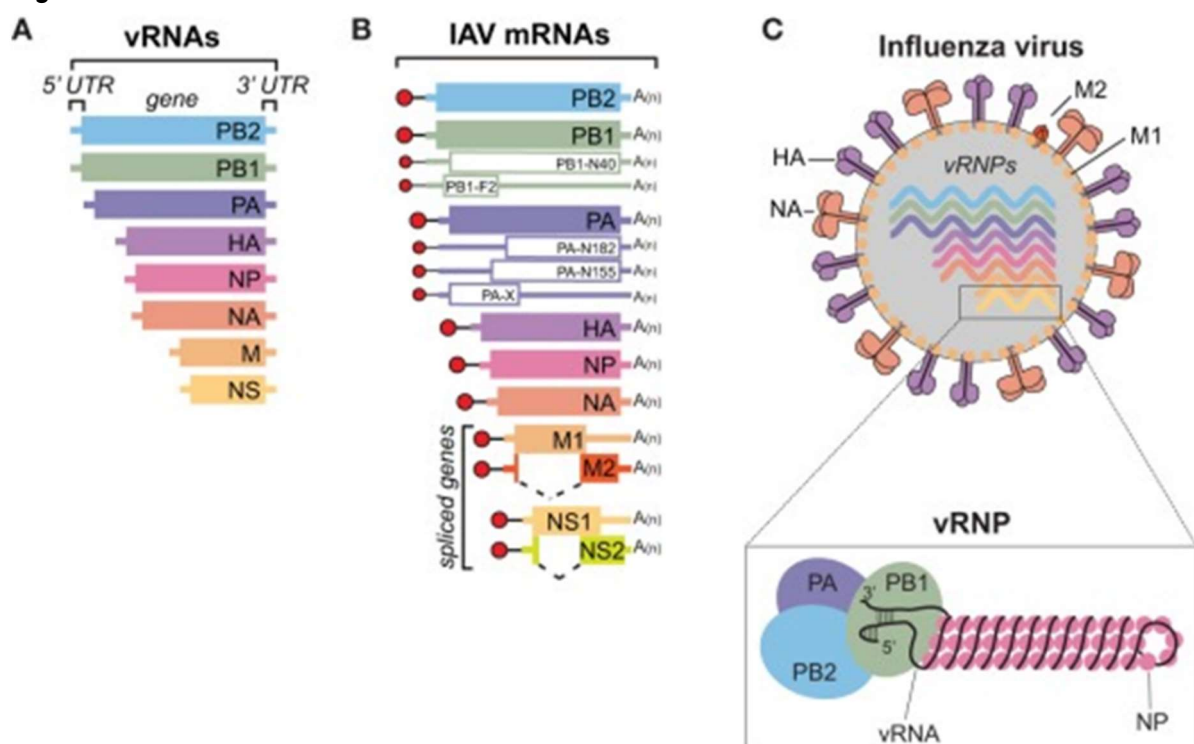
Influenza also poses a threat to human health. Influenzas A and B cocirculate endemically in some areas and seasonally in others, leading to approximately 290 000–650 000 deaths and 3–5 million severe illnesses annually (Iuliano et al., 2018). Additionally, influenza A viruses are known to occasionally undergo antigenic shift and cause pandemics, such as in 1918, 1957, 1968 and 2009. The 1918 pandemic is thought to have killed at least 50 million people globally while the most recent, the swine flu pandemic of 2009, spread to over 200 countries but caused relatively minor illness and an estimated 200 000 deaths (Simonsen et al., 2013). The current SARS-CoV-2 pandemic illustrates the immense health as well as economic impacts of pandemics and the importance of preparedness and surveillance for respiratory viruses.

Influenzas C and D are less prevalent and cause mild symptoms. They therefore only contribute marginally to the burden of disease in humans and animals.

1.1.2 Viral genomics, infection cycle and evolution

Influenza viruses are enveloped viruses with a segmented negative-sense single-stranded RNA genome. Each segment encodes one or more protein products and is packaged inside virions as a ribonucleoprotein complex (RNP). Influenza types vary slightly in the genes they encode on each segment. Figure 1.1 shows the genome structure of influenza A virus.

Figure 1.1



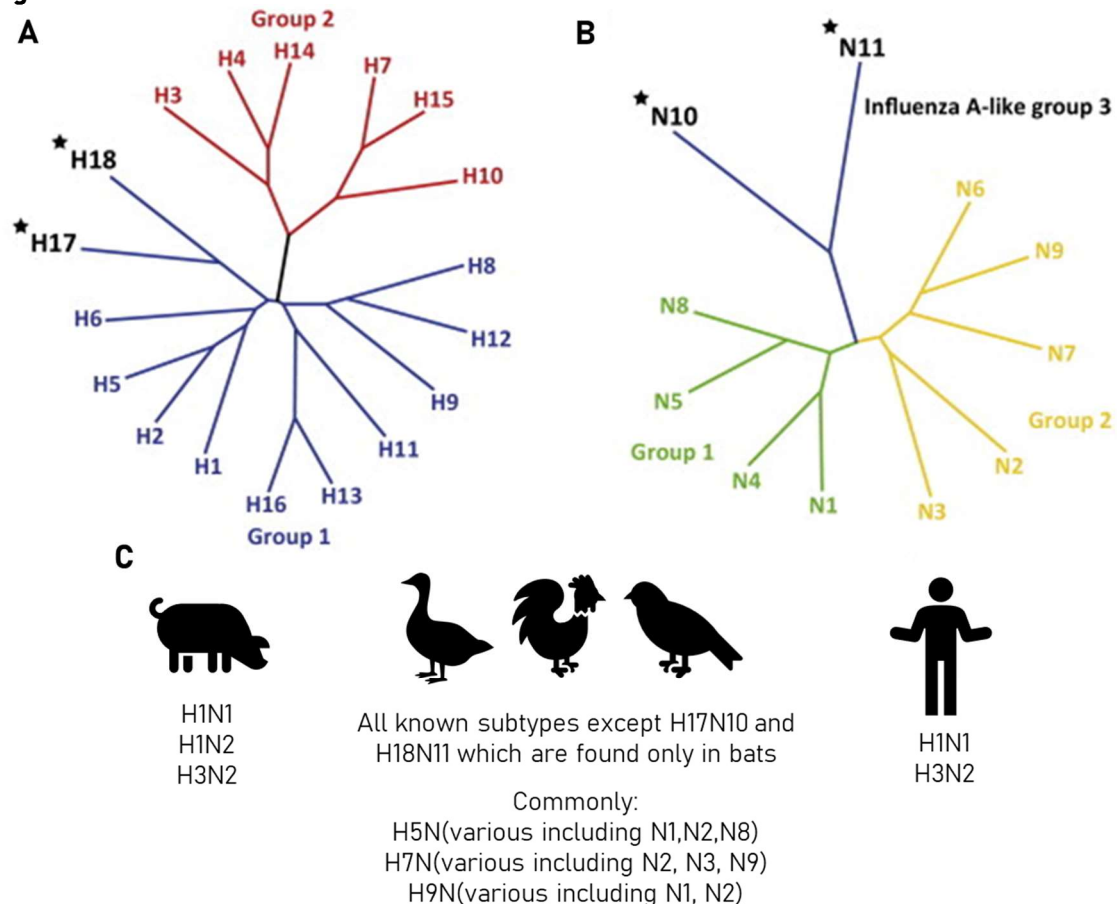
Structure of the influenza A virus genome, adapted from Dou et al., 2018*

A. Viral RNA (vRNA) segments constituting the influenza A genome. **B.** Viral messenger RNAs (mRNA) encoded by each viral RNA segment, with transcripts encoding common non-essential accessory proteins shown in empty boxes. **C.** Surface proteins hemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2) are displayed on the viral membrane, which encapsulates eight viral ribonucleoproteins (vRNPs) each containing nucleoprotein (NP), a polymerase unit (PA, PB1 & PB2), and an RNA segment.

*Reproduced with permission from Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement, Dou et al, 2018, Frontiers in Immunology

Segments 4 and 6 encode hemagglutinin (HA or H) and neuraminidase (NA or N), the major surface proteins and main antigenic determinants which give influenza A virus subtypes their name (see Figure 1.2). Hemagglutinins and neuraminidases are each classed into groups 1 and 2 according to their antigenic similarities. Both of these proteins decorate the viral coat, which is derived from host lipid membrane and encapsulates the viral genome.

Figure 1.2



Influenza A virus subtypes and current circulation in key host groups

A&B. Phylogeny of hemagglutinin and neuraminidase genes in Influenza A viruses, Figure 1 from Wu et al, 2014*. Hemagglutinins are divided into group 1 and group 2. H17 & H18 discovered recently in bats belong to group 1. Neuraminidases are divided into group 1 and 2, with the exception of bat-derived N10 and N11 which do not belong to either group. *Reprinted from Trends in Microbiology, Volume 22, Ying Wu, Yan Wu, Boris Tefsen, Yi Shi & George F. Gao, Bat-derived influenza-like viruses H17N10 and H18N11, 183-191, 2014, with permission from Elsevier

C. Key subtypes currently circulating in swine, birds and humans. H1N1 and H3N2 are currently endemic in humans, but other subtypes including H1N2 and avian H7 and H5 viruses have been known to cause sporadic infections or small outbreaks. H2N2 has previously caused a pandemic in humans but is no longer in circulation.

The hemagglutinin mediates cell entry by using sialylated glycans present on the cell surface as a receptor to initiate internalisation into endosomes (Weis et al., 1988). As conditions acidify along the endocytic pathway, hemagglutinin then mediates fusion of the viral and endosomal membranes to release the contents of viral particles into the cytosol. Hemagglutinins from different strains have different binding specificities, which are categorised by the linkage between the terminal sialic acid and penultimate galactose on their sialylated receptors. α 2-6 linked sialic acids dominate the human and swine upper respiratory tract, while α 2-3 linkages are found on cells in the lungs and bronchi (Kumlin et al., 2008; Trebbien et al., 2011). In birds, α 2-3 linkages are dominant. Human and swine viruses therefore typically bind well to α 2-6 sialic acids, whereas avian viruses show greater affinity towards α 2-3 and infect humans infrequently. As a critical determinant of host range, mutations in hemagglutinin can be linked to zoonoses.

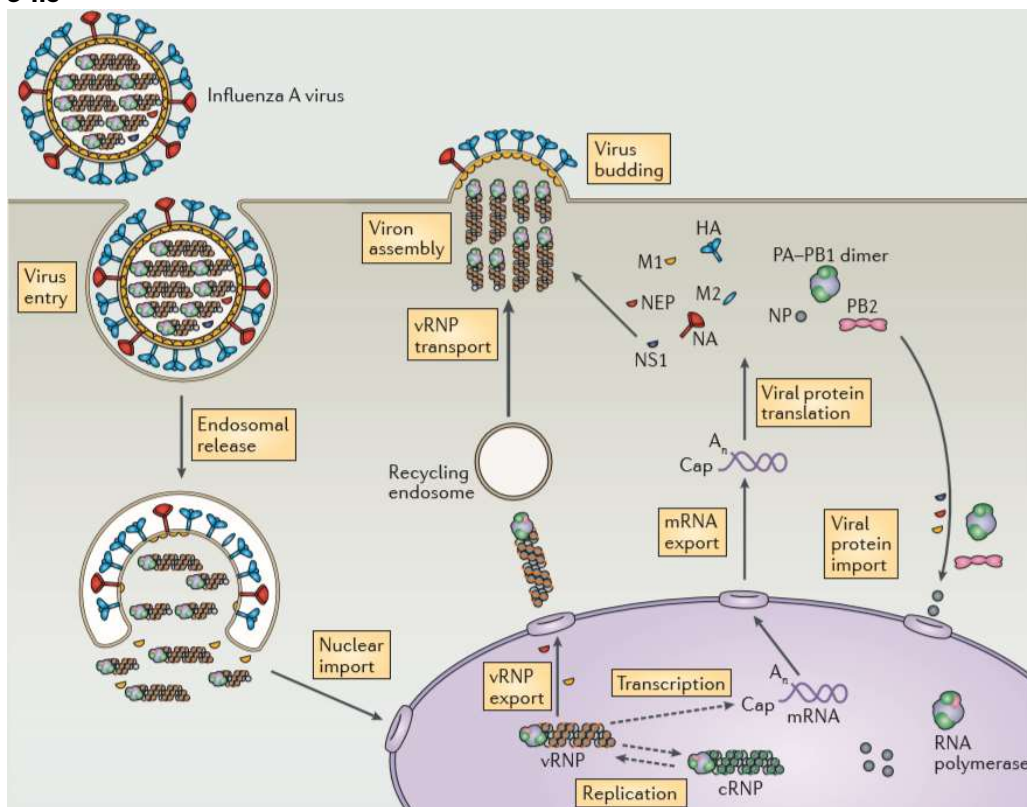
Conversely, neuraminidase acts as a sialidase, cleaving sialic acids to allow hemagglutinin to be released from its receptor. This is critical for the release of viral particles following budding, for preventing agglutination of viral particles to each other and for mediating release from decoy sialic acids such as mucins (McAuley et al., 2019). Sialic acid binding by hemagglutinin and cleavage by neuraminidase must be balanced to ensure viral entry and release is efficient. As hemagglutinins and neuraminidases vary in their strength and specificity, not all combinations work well together. Although neuraminidase contributes to viral proliferation rather than cell entry, premature cleavage of sialic acids can prevent hemagglutinin binding and therefore reduce viral entry.

Segment 7 encodes the minor surface protein matrix 2 (M2), a proton channel which by acidifying the contents of virions in the endosome triggers dissociation of viral RNPs from matrix 1 (M1), allowing RNPs to be released into the cytosol after fusion of the viral and endosomal membranes. M2 also has a role in promoting budding from the cell surface. M1, encoded in the same segment, oligomerises at the cell membrane and facilitates

budding by mediating interactions between ribonucleoprotein complexes and the viral envelope. It has additional roles, including in nuclear export of viral RNAs.

Non-structural protein 1 (NS1) on segment 8 has multiple functions which affect viral replication and pathogenicity (reviewed by Hale et al., 2008). These include regulating RNA replication, selectively translating viral mRNAs over cellular transcripts, antagonising the host anti-viral response and modulating apoptosis. Segment 8 also encodes NS2 (also called nuclear export protein (NEP)) which mediates nuclear export of newly synthesised RNPs and regulates transcription and replication of the viral genome.

Figure 1.3



Influenza virus lifecycle, from Fodor & Te Velthuis, 2016*

The virus hemagglutinin binds sialic acid residues on cell surface glycoproteins to initiate cell entry through the endosomal pathway. Fusion of the viral and endosomal membrane triggers release of vRNPs into the cytosol. The viral genome is replicated and transcribed to produce viral proteins. Viral surface proteins hemagglutinin, neuraminidase and matrix 2 are expressed on the cell surface. Nascent vRNPs are transported to the cell membrane where they are packaged into virions by budding of the cell membrane.

*Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, NATURE REVIEWS MICROBIOLOGY, INFLUENZA VIRUS RNA POLYMERASE: INSIGHTS INTO THE MECHANISMS OF VIRAL RNA SYNTHESIS, AARTJAN J. W. TE VELTHUIS & ERVIN FODOR, 2016

Nucleoprotein on segment 5 oligomerises and associates with the vRNA segments to form ribonucleoprotein complexes (RNPs) so that they can be packaged into virions. Each RNP also includes a polymerase unit, as illustrated in Figure 1.1c and Figure 1.3. RNPs are selectively packaged into budding virions with the aid of segment-specific RNA packaging signals at the ends of each segment (reviewed by Goto et al., 2013; Hutchinson et al., 2010). These signals are key to efficiently producing virions with one of each segment. Mutating the segment ends disrupts these signals and reduces the incorporation efficiency of RNPs. It is not uncommon for virions missing one or more segments to be produced (Nakatsu et al., 2016). These virions are only replication competent if they co-infect a cell alongside a virus which can complement for their deficiency.

The viral RNA polymerase is encoded by three vital genes called PB2, PB1 and PA on segments 1, 2 and 3 respectively. These segments also encode common non-essential accessory proteins through alternative start codons, ribosomal frameshifting and mRNA splicing (Pinto et al., 2020). For example, PB1-F2 and PA-X are able to modulate the immune response to influenza, with consequences on virulence. The lack of proof-reading activity in the RNA polymerase enables the virus to accumulate mutations rapidly and therefore evade host immunity through small but constant changes, a process called antigenic drift.

Further to this, the division of the genome into eight parts allows segments to reassort when multiple viruses coinfect a cell. As either variant of each segment may be packaged into a budding virion, new strains with novel combinations of segment variants can arise. This form of virus evolution is termed antigenic shift, reflecting the vast leaps it can cause in virus characteristics (reviewed by Kim et al., 2018). Not all genetic combinations lead to viable viruses – indeed some segments tend to remain associated as they have become co-adapted to working efficiently together (White & Lowen, 2018). These leaps can be advantageous by enabling viruses to target new host species and escape host immunity.

Pandemics, in which infectious agents cause widespread outbreaks, are therefore often linked to reassortment events. Pandemics are also sometimes associated with higher morbidity and mortality than endemic infections due to the lack of pre-existing immunity and can disproportionately cause disease in younger people relative to endemic influenza (reviewed by Taubenberger & Morens, 2010).

Both antigenic drift and shift pose a challenge to the control of influenza A virus. As influenza drifts at a fast rate, there are many circulating strains at any time, not all of which can be represented in a vaccine. Crucially, these strains are sufficiently antigenically different from each other that hosts immune to one strain are not protected against all others, even from the same subtype. Additionally, dominant strains change from year to year depending on the susceptibility of the population to infection by these different strains. This turnover is a problem as it continuously exposes populations to new strains and requires vaccines to be reformulated, usually on an annual basis. Antigenic shift complicates control by introducing novel viruses with new and unknown characteristics for which we have not developed vaccines or therapies.

1.1.3 Immune responses to viral components

Antigenic drift and shift in the surface proteins hemagglutinin and neuraminidase are particularly relevant to the control of influenza. This is because they are major targets of adaptive immune responses, are important determinants of viral characteristics and are the most variable components. The challenge with controlling influenza virus is therefore that the most readily targeted protective epitopes are not well conserved and stimulate strain-specific immunity to secondary infection.

On an average virion, there are approximately 300–400 hemagglutinin spikes and 40–50 neuraminidase spikes (Harris et al., 2006). Hemagglutinin is a highly immunogenic viral

component, stimulating a particularly strong antibody response as well as innate and T-cell responses (Chen et al., 2018; Kreijtz et al., 2011). Antibody responses can be highly protective as blocking sialic acid binding by the head region can neutralise cell entry and therefore provide sterile immunity against secondary infection. This sterile immunity is highly valuable as it prevents disease and transmission to other hosts. However, being subject to immense immune pressure, the hemagglutinin head region evolves particularly rapidly and is highly variable, making protective responses targeted to this region highly strain-specific. Some broadly neutralising epitopes have been identified in less variable regions in the stem, which mediates fusion (reviewed by Air, 2015; de Jong et al., 2020; Lee & Wilson, 2015; Mallajosyula et al., 2014). Additionally, non-neutralising antibodies to the stem can be protective through antibody Fc effector mechanisms including antibody dependent cytotoxicity (ADCC), antibody dependent complement mediated lysis (ADCL) and antibody dependent phagocytosis (ADP) (reviewed by Jegaskanda, 2018). Broadly protective antibodies to these epitopes tend to be rare in infected or immunised individuals.

Neuraminidase is less immunogenic than hemagglutinin, perhaps because less is expressed on the viral surface, or because it does not protrude as far from the cell membrane (Broecker et al., 2019). Nevertheless, antibody responses to neuraminidase are commonly seen in human serological surveys and reduce the severity of infections, suggesting that they could play an important part in protecting populations (reviewed by Wohlbold & Krammer, 2014). These protective responses are strain or subtype-specific as, while the catalytic site is highly constrained, neighbouring residues which form part of antibody binding footprints are more variable. The variability of the neuraminidase protein also affects our ability to treat active infections using neuraminidase inhibitors such as oseltamivir (sold as Tamiflu).

Internal viral components are generally more highly conserved than those presented on the cell surface, both due to functional constraints and because they are not pressured to evade host antibody responses (Li et al., 2019). These conserved features have the potential to generate broad protection through peptide presentation to T-cells.

CD8+ cytotoxic T-cell responses clear infected cells which present endogenous viral peptides on their surface through major histocompatibility complex (MHC) class I proteins. They also recognise exogenous peptides cross-presented by antigen presenting cells (APCs). CD8+ T-cells therefore do not provide sterile immunity, but can reduce the severity of infection. They are associated with reduced viral titres, viral shedding and symptoms and can protect against widely divergent strains (reviewed by Schotsaert et al., 2012). All eight viral segments are targeted by the CD8+ T-cell response, with nucleoprotein often observed to be dominant in humans (Grant et al., 2013; Hayward et al., 2015).

CD4+ helper T-cells respond to exogenous peptides presented through MHC class II by APCs. They are important in supporting antibody production, B-cell and CD8+ memory T-cell persistence, immune cell recruitment through cytokine secretion and can themselves be cytotoxic (Wilkinson et al., 2012). They therefore have roles in both strain-specific antibody-mediated immunity and broader cross-protective responses. CD4+ T-cell responses target all viral genes, with no one segment consistently dominating the response (Babon et al., 2009; Chen et al., 2014).

T-cell responses wane and their protective effects do not last more than a few years unless they are maintained by regular exposure through vaccination or infection (McMichael et al., 1983). Non-neutralising vaccines which present T-cell epitopes and therefore limit but do not entirely block subsequent infection may therefore provide long-term protection against severe disease from endemic strains as well as novel zoonotically acquired viruses with pandemic potential, but this has yet to be shown in humans.

1.2 Influenza vaccine approaches and mechanisms of protection

Ideal swine or avian influenza vaccines should generate lasting protection against a broad spectrum of strains across influenza A groups 1 and 2. Ideal human vaccines should also protect against the two major lineages of influenza B: Yamagata and Victoria. Broad protection might be generated by stimulating responses to viral features which are shared between many strains, or by stimulating responses against many epitopes, each of which are shared between some strains.

Despite this, the most commonly administered vaccines accord strain-specific protection primarily or entirely through antibody responses to hemagglutinin. The hemagglutinin content of vaccines and the hemagglutination inhibition titre generated in the serum of vaccinees remain key measures of immunogenicity for regulators. Hemagglutination inhibition (HAI) titres measure the antibody response to the hemagglutinin receptor binding site by detecting blocking of virus binding to sialic acid on red blood cells. Quick and easy to perform, it is a convenient assay for evaluating potential vaccine candidates, but only accounts for one facet of the potential immune response to influenza viruses.

In recent years there has been a drive to establish more diverse correlates of protection for vaccines, especially as focus shifts towards novel vaccine candidates which do not rely on receptor binding site-associated mechanisms of protection (Trombetta & Montomoli, 2016). Alternative mechanisms include generating antibodies to the less immunogenic surface proteins neuraminidase and matrix 2 and the hemagglutinin stem, as well as using more complete formulations which present several or all viral components as immune targets (reviewed by Corder et al., 2020; Harding & Heaton, 2018; Treanor, 2015).

Currently licensed influenza vaccines can be broadly categorised into replicative viruses and non-replicative platforms, each with their own advantages and drawbacks. An

emerging class, referred to as single cycle vaccines, able to undergo a single round of cell infection are being developed. They may draw on the advantages of both replicative and non-replicative vaccines and are the focus of this thesis.

1.2.1 Currently licensed non-replicative vaccines

The majority of vaccines approved for use in humans and animals are produced from inactivated virus and use hemagglutinin as their main or sole immunogen. Viruses must first be cultured in embryonated eggs or cell culture and then inactivated, for instance using formaldehyde (Gerdil, 2003). In split virion vaccines, detergents are then used to disrupt viral particles. Hemagglutinin and neuraminidase are sometimes purified to produce a subunit vaccine. Both split virion and subunit vaccines mainly accord protection through antibody responses to hemagglutinin, although using certain adjuvants can stimulate cross-priming and generate some CD8+ T-cell responses. As these responses are directed to a highly variable protein, vaccines contain hemagglutinin from three (trivalent) or four (quadrivalent) representative strains which must be reselected twice annually. The neuraminidase content of vaccines is not routinely measured or standardised like it is for hemagglutinin. In the United Kingdom, both split and subunit vaccines are available for eligible adults (see Table 1.1). In pigs, intramuscular injection with whole inactivated virus (WIV) is conventional, although across Europe only 10–20% of sows are vaccinated (reviewed by Mancera Gracia et al., 2020). Vaccination is particularly targeted at pregnant sows to pass maternal antibodies to offspring *in utero*. Using whole virus with adjuvants can generate T-cell responses to conserved internal proteins, though, similarly to other inactivated vaccines, these are weak as WIVs do not infect host cells.

Table 1.1

Influenza vaccines marketed in the UK for the 2020–2021 season			
Adapted from The National Flu Immunisation Programme 2020/21, published 14th May 2020 by Department of Health and Social Care & Public Health England			
Supplier	Product Name	Vaccine Type	Age indications
Astra Zeneca	Fluenz Tetra	Quadrivalent live attenuated vaccine supplied as nasal spray suspension	From 24 months to 18 years
GSK	Fluarix Tetra	Quadrivalent egg-grown vaccine, split virion, inactivated	From 6 months
MASTA	Quadrivalent Influenza Vaccine	Quadrivalent egg-grown vaccine, split virion, inactivated	From 6 months
Sanofi Pasteur	Quadrivalent Influenza Vaccine	Quadrivalent egg-grown vaccine, split virion, inactivated	From 6 months
Mylan	Quadrivalent Influvac subunit Tetra	Quadrivalent egg-grown vaccine, subunit, inactivated	From 3 years
Seqirus UK Ltd	Flucelvax Tetra	Quadrivalent cell-grown vaccine, subunit, inactivated	From 9 years
Seqirus UK Ltd	Adjuvanted Trivalent Influenza Vaccine	Trivalent egg-grown vaccine, subunit, inactivated, adjuvanted with MF59C.1	From 65 years
Sanofi Pasteur	Trivalent Influenza Vaccine, High-Dose	High dose, trivalent egg-grown vaccine, split virion, inactivated	From 65 years

An alternative to conventionally inactivated vaccines is producing recombinant hemagglutinin in tissue culture, removing the need to grow viruses. The vaccine Flublok contains hemagglutinin produced by baculovirus expression in insect cells (Cox et al., 2015). Flublok in trivalent or quadrivalent form has been used in the United States of America since 2016 and was approved for temporary usage in the United Kingdom for the 2020/2021 season (MHRA, 2020). Like inactivated viruses, strain-specific immunity is generated to hemagglutinins, so vaccine efficacy is poor when there is a mismatch between the strains used in the vaccine and circulating viruses. High quality virus surveillance data is therefore needed for optimal vaccine strain selection and very little protection to pandemic viruses is expected.

1.2.2 Currently licensed replicative virus vaccines

Viruses can be attenuated through various mechanisms to allow them to infect host cells without causing disease. This infection is beneficial for stimulating strong CD8⁺ T-cell responses to intracellular and often highly conserved viral components. Live attenuated influenza vaccines (LAIV) are usually delivered into the respiratory tract to mimic natural infection, which generates strong local immunity (Holzer et al., 2019; Szabo et al., 2019; Tchilian & Holzer, 2017).

The swine vaccine Ingelvac Provenza is produced by truncation of the NS1 protein, which prevents the virus from evading antiviral responses and therefore limits viral replication (reviewed by Mancera Gracia et al., 2020). The bivalent vaccine includes two H3N2 strains and reduces viral shedding from the nose after challenge with H3N2 or H1N1 virus, demonstrating that it can generate broad protection. It has been commercially available in the United States since 2017. Temperature-sensitive cold-adapted viruses which grow well in the nose but poorly in the warmer lower respiratory tract due to mutations in the polymerase genes have also shown promise in pigs. Temperature-sensitive viruses with the desired immunogenicity are produced by pairing the internal genes from a temperature-sensitive virus with the hemagglutinin and neuraminidase of circulating strains. These vaccines lead to reduced viral shedding, reduced transmission to other pigs and faster clearance on subsequent infection.

In the UK, the quadrivalent cold-adapted live-attenuated vaccine Fluenz Tetra is used in children aged 2-18 as it appears more effective than subunit vaccines. Fluenz vaccine viruses (called Flumist in the US) are produced by plasmid rescue in Vero cells, a process in which the desired viral genes are expressed together in cell culture to make viruses *de novo*. Viruses are then expanded in eggs, purified and administered by nasal spray. Fluenz Tetra stimulates antibodies to hemagglutinin, particularly mucosal IgA, as well as T-cell

responses in children, leading to decreased prevalence of symptomatic infection, shorter duration of illness and reduced shedding of wildtype viruses following natural infection (Carter & Curran, 2011). In adults, T-cell responses are weaker than they are in children (Carter & Curran, 2011) and antibody responses to hemagglutinin and neuraminidase are weaker than for inactivated viruses (Couch et al., 2012). The lower efficacy of Fluenz/Flumist in adults relative to children may be because pre-existing immunity can prevent attenuated vaccines from replicating sufficiently to generate strong immune responses. Conversely, LAIV is not recommended for immunocompromised people, in which the virus may replicate too effectively and cause pathology. This is problematic as the immunocompromised are highly vulnerable to severe disease and stand to gain the most from enhanced T-cell mediated immunity.

One of the other major problems with live attenuated vaccines is the risk of reversion to pathogenicity. As viruses replicate *in vivo*, there is opportunity for them to acquire mutations which may compensate for attenuating mutations and cause disease in healthy individuals. Attenuated vaccines may also reassort with circulating wildtype viruses to produce pathogenic viruses. Reassortment between vaccine Ingelvac Provenza and endemic viruses has been observed in swine in the United States (reviewed by Mancera Gracia et al., 2020). Inactivated vaccines are usually therefore the preferred vaccination approach for pigs.

1.2.3 The future of influenza vaccines

Several novel vaccination approaches are in development. Some constitute novel platforms for presenting antigens, while others focus on engineering antigens themselves to strengthen and redirect responses. Some of the most promising are briefly discussed here.

The recent Medicines and Healthcare products Regulatory Agency (MHRA) approval for use of the first (MHRA News Centre, 2020) and second (MHRA News Centre, 2021) messenger RNA (mRNA) vaccines in response to the COVID-19 pandemic may accelerate the development and licensing of mRNA vaccines for influenza and other respiratory diseases. mRNA-based vaccines are composed of a messenger RNA expressing a viral gene with or without stabilising adjuvants, usually encapsulated by a carrier molecule (reviewed by Scorza & Pardi, 2018). By not expressing viral antigens on the surface of the carrier, pre-existing immunity is circumvented for efficient uptake into cells. mRNA vaccines can be self-amplifying, increasing their immunogenicity, and can be made multivalent by expressing several viral genes or the same gene- typically hemagglutinin- from various strains. Several mRNA vaccine candidates have shown protective efficacy in animal models and have entered clinical development. They have the potential to present both folded protein and short peptides to the immune system and therefore stimulate both antibody and T-cell responses to the encoded protein.

Viral vectors function in a similar way in that they induce host cells to express the desired viral protein. Several viral vectored vaccines, mostly driving surface expression of a hemagglutinin protein, have progressed to clinical trials (Sebastian & Lambe, 2018). Viral vectors can drive high-level expression, with the vector itself acting as an adjuvant. Depending on the application, a vector that is replication-competent, attenuated or

replication-deficient can be selected. However, stimulating immunity to the vector can be problematic, especially as multiple doses are often required for effective responses.

Viral-like particles (VLPs) have been developed to enhance antibody responses to influenza virus surface proteins (reviewed by Kang et al., 2012). VLPs provide a scaffold which mimics the structure of viral particles, onto which proteins can be presented to increase their valency. They do not contain any virus genomic material and so cannot reassort with other viruses or become integrated into the host genome. Antibody and some T-cell responses are generated to the selected protein, as VLPs can stimulate cross-presentation by dendritic cells. VLPs are particularly useful for presenting surface antigens which are immunologically subdominant and highly conserved as they can focus the response to a single protein. For example, VLPs expressing the ectodomain of matrix 2 (M2e) or neuraminidase are known to stimulate antibodies which provide heterosubtypic protection by reducing the spread of viruses (Schotsaert et al., 2016).

Gamma irradiated viruses (γ -flu) have been proposed as potential broadly protective vaccines (Müllbacher et al., 1988). Gamma radiation inactivates viruses by generating strand breaks in the viral genome. Unlike other inactivation methods, this occurs without disrupting the structure and antigenicity of viral proteins. Irradiated hemagglutinins still agglutinate red blood cells and may mediate uptake of γ -flu into cells *in vivo* (Müllbacher et al., 2006). This may be what enables intranasally administered γ -flu to generate strong broadly reactive cytotoxic T-cells in mice, which accord heterosubtypic protection (Alsharifi et al., 2009). As well as generating stronger CD8⁺ T-cell responses than formalin-inactivated virus, higher serum IgG, mucosal IgA and inhibiting antibody to hemagglutinin and neuraminidase may contribute to enhanced protection against challenge in mice (Chen et al., 2020).

Alongside advances in antigen presentation platforms, there has been substantial innovation in immunogen design (reviewed by Mathew & Angeletti, 2020; Sautto et al., 2018). Epitopes can be engineered or contextualised to improve the strength, breadth or focus of immune responses. For instance, immunising with strain-matched neuraminidase but mismatched hemagglutinin with respect to the challenge virus can lead to stronger secondary antibody responses to neuraminidase. Similarly, the immunogenicity of the relatively conserved hemagglutinin stem can be enhanced by sequentially immunising with two chimeric hemagglutinins each with the same stem but different heads. Broad responses to the stem can also be improved by shielding head epitopes through glycosylation, engineering headless hemagglutinins or mini-HAs or by selectively chemically modifying the head region with polyethylene glycol chains. Alternatively, by studying consensus sequences, computationally optimized broadly reactive antigens (COBRA) can be designed to boost and broaden immunogenicity to proteins like hemagglutinin or neuraminidase. Each of these approaches could be paired with replicative or non-replicative, conventional or novel vaccine platforms to optimise responses.

1.2.4 The potential of single cycle viruses

Viruses which undergo a single replication cycle offer a promising platform for generating broadly protective immune responses (Dudek & Knipe, 2006; Nogales et al., 2016). They are designed to lack a functional copy of one of the genes required for influenza function and are grown in an environment which compensates for this loss by supplying the related gene product in trans. While single cycle viruses are able to grow well in this permissive environment, they are unable to replicate in the tissues of their natural hosts. Instead, thanks to the externally provided gene product, they are able to effectuate only a single cycle of cell infection.

Multiplasmid transfection is commonly used to produce viruses *de novo* in cell culture (Fodor et al., 1999). To generate single cycle viruses, one or more plasmids can be mutated or omitted. Single cycle influenza virus (SCIV) vaccine candidates with deficiencies in various genes have been developed, including in matrix 2, neuraminidase, the polymerase genes and, most commonly, the hemagglutinin. Depending on the nature of the deficiency, it can be compensated for by adding a soluble protein or expressing the gene in a non-viral expression vector in cell culture.

SCIVs offer an advantage over inactivated vaccines in that they infect host cells. Single cycles with a functioning polymerase undergo genome replication and expression in their host, leading to amplification of viral epitopes inside infected cells and cell surface expression of viral coat proteins. As SCIV typically contain all but one intact viral segment, responses can be targeted to a wide array of protein, peptide and RNA epitopes. SCIV are therefore capable of generating strong antibody and T-cell responses, including broadly protective cytotoxic CD8⁺ T-cell responses to peptides from highly conserved internal proteins. Although other emerging technologies including mRNA and viral vectored vaccines also induce T-cells, they do not do so to the full complement of viral antigens.

Their advantages over conventional live-attenuated vaccines stem from their safety, as they infect very few cells and so are not at risk of causing pathology or mutating or reassorting into virulent forms. This approach could therefore be suitable for both healthy and vulnerable people, as well as swine where co-infection risks can be significant. Additionally, SCIV can be safely administered by aerosol to immunise cells in the lung, which is associated with local immunity through tissue-resident T-cells (Mueller & Mackay, 2016) and mucosal IgA (van Riet et al., 2012). Their safety could allow them to be used as pre-pandemic vaccines, as they can be given before an outbreak without risk of introducing new viruses into populations. Such a safe platform allows single-cycle versions of highly pathogenic influenza strains to be developed as laboratory tools and vaccines with close antigenic similarity to high risk strains.

Practical advantages of SCIV include avoiding reliance on biosecure eggs for virus isolation and expansion, the ability to individually tailor the sequence of each viral segment, and the fast and reliable method of production in cell culture.

Most single cycle vaccine candidates are produced by inactivating hemagglutinin. This is advantageous as it prevents the hemagglutinin from acting as a donor sequence for reassortment with circulating strains. Hemagglutinin activity is easy to compensate for *in trans* by growing viruses in cells which express functional hemagglutinin on the surface. As viruses bud from cells, they coat themselves in host-derived membrane containing this functional hemagglutinin. Altering the coating proteins, called pseudotyping, can allow single cycle viruses with the same core genes but different coating hemagglutinins to be produced quickly from a single seed virus.

One notable single cycle vaccine candidate is M2SR (M2-deficient Single Replication) developed by Flugen. It is produced by deletion of the M2 transmembrane domain and insertion of upstream stop codons, and is pseudotyped in functional M2 (Sarawar et al., 2016). Intranasal M2SR vaccines are able to protect mice and ferrets against highly

pathogenic avian influenza (Hatta et al., 2017; Sarawar et al., 2016). M2SR induced sterile immunity against strain-matched challenge, and non-sterile immunity when vaccine and challenge strain were from different subtypes. This was associated with high levels of serum and mucosal antibody to hemagglutinin, antibody to neuraminidase and influenza-specific CD4+ and CD8+ T-cell responses in bronchoalveolar lavage. M2SR has completed trials evaluating safety and immunogenicity in adults and in children. In a recent challenge trial, an intranasal M2SR vaccine containing hemagglutinin and neuraminidase from an H3N2 virus was shown to accord protection against challenge with a highly divergent H3N2 virus. An 18% reduction in viral load was observed in the vaccinated group after challenge, increasing to 34% in the 54% of recipients who developed neutralising antibody responses in sera. Reduced rates of infection and symptomatic illness were also observed (Eiden et al., 2019).

1.3 Thesis premise and aims

The Townsend laboratory have previously described the single cycle virus S-Flu, which is produced by inactivation of the hemagglutinin signal sequence, generating a viral RNA called S-HA (signal minus hemagglutinin) (Powell et al., 2012). Like for most other SCIVs lacking hemagglutinin activity, no hemagglutinin epitopes are presented on the surface of cells infected with S-Flu. In a second design, the majority of the hemagglutinin coding sequence is replaced by the gene for eGFP (enhanced green fluorescent protein), causing the cytosol of infected cells to fluoresce. S-Flu viruses have been helpful laboratory tools for evaluating the susceptibility of different coat proteins to chemical inhibitors and antibodies. Recently, S-Flu viruses coated in avian H7 hemagglutinins (Powell et al., 2019) and the glycoprotein from Ebola viruses (Xiao et al., 2017) have been evaluated as safe and accurate pseudotypes for assaying inhibition of cell entry. This enables the characteristics of coat proteins from highly virulent viruses to be investigated in biosafety containment 1 or 2 rather than in high containment facilities.

S-Flu viruses have shown promise as broadly protective vaccines in mice, ferrets and pigs. They can be administered into the lung intranasally (mice and ferrets) or by aerosol (pigs) without causing pathology. Heterologous protection, even against strains in different subtypes, is induced in the absence of a neutralising antibody response to hemagglutinin. This lack of response is unsurprising given that hemagglutinin is not expressed on the cell surface. Protection is instead associated with strong T-cell responses, including CD8⁺ T-cell responses in the lung, and a strain-specific antibody response to neuraminidase.

Mice immunised with S-Flu are protected from severe illness and weight loss and have lower virus titres in their lungs after challenge than unvaccinated animals. This protection lasts at least four months, even when vaccine strain (group 1 H1N1 PR8 S-Flu) and

challenge strain (group 2 H3N2 X31 virus) are from different groups (Powell et al., 2012). In ferrets, S-Flu gives good protection against challenge with a highly pathogenic strain, reduces challenge virus titres and can prevent transmission to unvaccinated neighbours (Baz et al., 2015; Holzer et al., 2018). In pigs, S-Flu reduces pathology in the lung following heterosubtypic challenge with live virus and may (Morgan et al., 2016) or may not (Holzer et al., 2018) reduce viral titres in the nose and lungs.

The lack of neutralising antibody response incurs that no sterile immunity is evoked in any model species following local administration of S-Flu. This means that while S-Flu viruses reduce disease severity, they are not able to neutralise viruses to prevent infection, even if they are perfectly antigenically matched to the challenge strain. In contrast, intraperitoneal injection of S-Flu stimulates neutralising antibody to the pseudotyping hemagglutinin coat in mice. In pigs, intramuscular immunisation of S-Flu leads to strong anti-hemagglutinin and anti-neuraminidase antibody but weak T-cell responses relative to aerosol administration of the same dose (unpublished manuscript, Martini 2020). This echoes findings from a mouse study showing that lung-resident memory T-cells are associated with LAIV administration to the lung, whereas strong serum antibody responses are associated with peripheral immunisation with inactivated viruses (Zens et al., 2019).

Neutralising antibody responses are desirable as they limit or abolish virus shedding and onward transmission, reduce the risk of pathology in individuals with weak T-cell responses, restrict the opportunity for wildtype virus evolution and, as a relatively established correlate of protection, may facilitate vaccine licensing.

The aim of this thesis is to determine whether neutralising antibody responses can be generated by a locally administered, hemagglutinin-inactivated single cycle vaccine. Previous studies have illustrated that locally administered S-Flu generates antibodies to its virally expressed neuraminidase but not its coating hemagglutinin, which is not

displayed on the surface of infected cells. M2SR virally expresses neuraminidase and hemagglutinin proteins and generates antibodies to both. Similarly, natural infection and LAIV induce cell surface expression and local antibody responses to both surface proteins. This may suggest that virally-driven cell surface expression is required for locally delivered attenuated or single cycle viruses to generate strong antibody responses to hemagglutinin epitopes. We therefore wished to explore whether expressing hemagglutinin epitopes from a non-functional hemagglutinin molecule instead of S-HA or eGFP in the S-Flu expression cassette could drive their cell surface expression and stimulate neutralising antibody responses without impacting on the existing immunogenicity and single cycle nature of S-Flu viruses.

A multitude of single site mutations which significantly ablate or abolish hemagglutinin function have been described. As single nucleotide changes, they likely impact minimally on the antigenicity of the overall protein and can be selected to avoid key antigenic sites. While alone these mutations generate single cycle viruses with a high rate of reversion to infectivity, using them in combination could allow for the generation of a full length, thoughtfully designed, multi-mutated hemagglutinin molecule which is irreversibly non-functional but antigenically preserved.

Expressing such a protein in the place of a functional hemagglutinin gene could generate a single cycle virus with considerable advantages over alternative vaccination approaches. As well as the general benefits of single cycle vaccines, the presence of multiple independent mutations scattered throughout the hemagglutinin renders negligible the probability of reversion to pathogenicity, and reduces the already low risk of homologous recombination with wildtype hemagglutinins to yield functional new variants (Boni et al., 2008). As hemagglutinin is a key contributor to antigenic shift, it is particularly beneficial to prevent it from acting as a donor sequence for wildtype viruses. Moreover, as the compensating pseudotyping hemagglutinin is the determinant of cell

entry but no longer the antigen driving neutralising antibody responses, pseudotyping cell lines can be selected to avoid pre-existing immunity while maximising sialic acid binding. Meanwhile, the multi-mutated hemagglutinin can be chosen entirely for its immunogenicity. This could be a key advantage over other approaches such as LAIV where pre-existing immunity reduces vaccination efficiency. Finally, unlike many single cycle viruses which lack all or a large part of a viral gene, the full complement of viral antigens should be expressed in RNA, protein and peptide form in the appropriate cellular location, maximising immunogenicity.

In this thesis, we describe the iterative design of a non-functional multi-mutated hemagglutinin immunogen named CLEARFLU. We go on to evaluate the potential of single cycle viruses expressing CLEARFLU instead of functional hemagglutinin as laboratory tools and broadly protective influenza vaccines. The following chapters investigate the antigenicity of CLEARFLU hemagglutinins as well as the genomic stability, growth dynamics and immunogenicity in mice and pigs of single cycle viruses expressing them.

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Chapter 2

METHODS

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2.1 Lists of reagents and materials used in this thesis

2.1.1 General reagents

Table 2.1

General reagents used in this thesis	
Product	Supplier
Molecular cloning	
BspQI	New England Biolabs R0712
SapI	New England Biolabs R0596
AgeI-High Fidelity	New England Biolabs R3552
NotI-High fidelity	New England Biolabs R3189
EcoRI-High fidelity	New England Biolabs R3101
Cutsmart Buffer	New England Biolabs B7204
NEBuffer 3.1	B7203S
T4 DNA Ligase	Invitrogen 15224017
Ligation Buffer	Invitrogen B69
Ethidium Bromide	Sigma E1510
Agarose	Sigma A6013
50x TAE Buffer	Distilled water + 242g/L Tris base + 57.1g/L glacial acetic acid + 0.05M EDTA
OrangeG	O3756
QIAquick Gel Extraction Kit	Qiagen 28704
Platinum Superfi DNA Polymerase	Invitrogen 12351010
SuperScript III Reverse Transcriptase	Life Technologies 18080044
RNasin	Promega N261B
dNTPs	GE Healthcare Life Sciences 28406560
MgSO ₄	Invitrogen P/N 52044
Phusion High-Fidelity DNA Polymerase	Thermo Scientific F530
QuikChange Lightning Site-Directed Mutagenesis Kit	Agilent 210519
QIAprep Spin Miniprep Kit	Qiagen 27104
DH5α High Efficiency competent <i>E.coli</i>	New England Biolabs C2987
Ampicillin	Sigma A5354
LB broth (Lennox)	Sigma L3022 (10g LB powder in 500ml distilled water)
LB + ampicillin	0.1ml/ml ampicillin + 10g LB powder in 500ml distilled water
Bacto-Agar	BD 214010
Agar plates	7.5g Bacto-Agar + 10g LB powder + 0.1mg/ml ampicillin in 500ml distilled water

10X Hogness solution	4.9g/l K_2PO_4 + 1.77g/l KH_2PO_4 + 2.5g/l $MgSO_4 \cdot 7H_2O$ + 5.88g/l $Na_3cit \cdot 2H_2O$ + 44% glycerol in distilled water
Cell culture	
DMEM - high glucose	Sigma-Aldrich D5796
D10	DMEM + 10% Foetal calf serum + 2mM L-glutamine + 100 units/ml Penicillin & 100µg/ml Streptomycin
OPTIMEM	Gibco 31985062
Trypsin/EDTA	Sigma T3924
293T cells	William Dunn School of Pathology
MDCK-SIAT cells	ECACC 05071502 (Matrosovich et al., 2003)
Polybrene	H9268-5G
N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin	Sigma T1426
Foetal calf serum (FCS), heat inactivated	Sigma F9665
PBS (phosphate buffered saline)	Sigma P4417
Lipofectamine 2000 Transfection Reagent	Invitrogen 11668019
Blocker Bovine Serum Albumin	Life Technologies 37525
Bovine Serum Albumin solution	Sigma A0336
EDTA	Sigma E6758
Viral growth medium (VGM)	DMEM + 2mM L-glutamine + 100 units/ml Penicillin & 100µg/ml Streptomycin + 0.01M HEPES + 3.2ml/l bovine serum albumin solution
1M HEPES Buffer	Gibco 15630049
Penicillin + Streptomycin	Sigma P0781 (10 000 U penicillin & 10 mg streptomycin/mL)
Freezing medium	FCS + 10% DMSO Sigma D8418
L-glutamine	Sigma G7513
0.22 micron syringe filter	Millipore SLGP033RS
Immunofluorescence	
Sodium azide	Sigma-Aldrich 438456
FACS fixative	1% FCS + 1% Formalin in PBS
FACS wash	1% FCS + 0.01% Azide in PBS
10% Formalin	Sigma 252549
Permeabilisation buffer	PBS + 20mm Glycine + 0.5% Triton X100
Viral assays	
Red Blood Cells	Human volunteers
Alsever's solution	Sigma A3351

Fetuin solution	25 microgram/ml fetuin (Sigma F3385) + 0,02% azide in PBS
PNA-HRP (peanut agglutinin conjugated to horseradish peroxidase)	Sigma L7759 made up to 1 microgram per ml in PBS with 0.1% BSA
Sigmafast OPD	o-Phenylenediamine dihydrochloride solution, Sigma P9187
RNA extraction kit	QIAamp viral RNA mini kit 52904

Animal work

RPMI-1640	Sigma R8758
R10	10% FCS in RPMI-1640 + 2mM L-glutamine + 100 units/ml Penicillin & 100µg/ml Streptomycin
Blood tubes	BD microtainer with SST and polymer gel
RBC lysis buffer	Qiagen 158904
Cell strainer 70um	VWR 732-2758
Percoll	GE Healthcare L17-0891-0
Collagenase, type IV	Sigma-Aldrich C5138
DNase I	Sigma-Aldrich D5025
29G needle + 0.5ml insulin syringe	BD 324892
23G needle	BD 305143

2.1.2 Plasmids

Table 2.2

Plasmids used in this thesis		
Insert	Vector	Origin
Transient transfection of 293T cells		
H1 A/England/195/2009 CFv1	pcDNA3.1-	Generated in this thesis
H3 A/Hong Kong/5738/2014 CFv1	pcDNA3.1-	Generated in this thesis
H1 A/England/195/2009 wildtype	pcDNA3.1-	Generated in this thesis
H3 A/Hong Kong/5738/2014 wildtype	pcDNA3.1-	Generated in this thesis
Templates and products of mutagenesis		
H7 A/Hong Kong/125/2017 wildtype	pcDNA3.1-	Generated in this thesis for use as mutagenesis template
H7 HK125 single mutants x 9 (Y98F, R329Q, G1Q, L2G, I6G, G8A, 212/216C, 30/47C, 63/70P)	pcDNA 3.1-	Generated in this thesis from H7 HK125 wildtype by mutagenesis
N1 A/Puerto Rico/8/1934 wildtype	pcDNA3.1-	Generated in this thesis for use as mutagenesis template
N1 A/Puerto Rico/8/1934 T148A	pcDNA3.1-	Generated in this thesis from N1 wildtype by mutagenesis
Lentivirus infection for stable transduction		
VSV-G	pCMV-VSV-G	Demaison et al., 2002
Gag/Pol	pCMVR8.91	Demaison et al., 2002
X31 wildtype	pHR-SIN	In-house
X13-PM (passage mutant)	pHR-SIN	Generated in this thesis using sequence from Hooper & Bloom, 2013
H1 A/England/195/2009 wildtype	pHR-SIN	Generated in this thesis
H3 A/Hong Kong/5738/2014 wildtype	pHR-SIN	Generated in this thesis
H1 A/England/195/2009 CFv1	pHR-SIN	Generated in this thesis
H3 A/Hong Kong/5738/2014 CFv1	pHR-SIN	Generated in this thesis
H1 A/England/195/2009 CFv2	pHR-SIN	Generated in this thesis
H3 A/Hong Kong/5738/2014 CFv2	pHR-SIN	Generated in this thesis
H7 A/Hong Kong/125/2017 wildtype	pHR-SIN	Generated in this thesis
H7 A/Hong Kong/125/2017 CFv2	pHR-SIN	Generated in this thesis
H7 A/Hong Kong/125/2017 CFv3	pHR-SIN	Generated in this thesis
H7 HK125 single mutants x 9 (Y98F, R329Q, G1Q, L2G, I6G, G8A, 212/216C, 30/47C, 63/70P)	pHR-SIN	Generated in this thesis
H7 A/Guangdong/17SF003/2016 CFv2	pHR-SIN	Generated in this thesis

Multi-plasmid mix for S-Flu production		
Core initiators (all H1 A/Puerto Rico/8/1934)		
PB1, PB2, PA, NP	pcDNA 3.1-	Ervin Fodor, Fodor et al., 1999
Viral expression of internal genes (all H1 A/Puerto Rico/8/1934)		
PB1, PB2, PA, NP, M, NS	pPol	Ervin Fodor, Fodor et al., 1999
Source of viral neuraminidase (all H1 A/Puerto Rico/8/1934)		
NA wildtype	pPol	Generated in this thesis
NA T148A	pPol	Generated in this thesis
Source of pseudotyping hemagglutinin		
H1	pcDNA 3.1-	In-house
Insert replacing viral expression of hemagglutinin		
eGFP	pPol S-Flu expression cassette (Powell et al., 2012)	In-house
H7 A/Hong Kong/125/2017 CFv3	pPol S-Flu expression cassette (Powell et al., 2012)	Generated in this thesis
H1 A/England/195/2009 CFv2	pPol S-Flu expression cassette (Powell et al., 2012)	Generated in this thesis
H3 A/Hong Kong/5738/2014 CFv2	pPol S-Flu expression cassette (Powell et al., 2012)	Generated in this thesis

2.1.3 Cell lines

Table 2.3

Cell lines used in this thesis	
Cell line	Origin
293T transient transfections	
293T	William Dunn School of Pathology
H3 WT-293T (A/Hong Kong/5738/2014)	Generated in this thesis
H1 WT-293T (A/England/195/2009)	Generated in this thesis
H3 CFv1-293T (A/Hong Kong/5738/2014)	Generated in this thesis
H1 CFv1-293T (A/England/195/2009)	Generated in this thesis
MDCK-SIAT stable transductions	
MDCK-SIAT	ECACC 05071502 (Matrosovich et al., 2003)
E-SIAT (Zaire)	In-house
E-SIAT (Sudan)	In-house
H1 WT-SIAT (A/Puerto Rico/8/1934)	In-house
X31 WT-SIAT	In-house
H7 WT-SIAT (A/Netherlands/219/2003)	In-house
X31 PM-SIAT	Generated in this thesis, sequence from Hooper & Bloom, 2013
H1 WT-SIAT (A/England/195/2009)	Generated in this thesis
H3 WT-SIAT (A/Hong Kong/5738/2014)	Generated in this thesis
H7 WT-SIAT (A/Hong Kong/125/2017)	Generated in this thesis
H1 CFv1-SIAT (A/England/195/2009)	Generated in this thesis
H3 CFv1-SIAT (A/Hong Kong/5738/2014)	Generated in this thesis
H1 CFv2-SIAT (A/England/195/2009)	Generated in this thesis
H3 CFv2-SIAT (A/Hong Kong/5738/2014)	Generated in this thesis
H7 CFv2-SIAT (A/Hong Kong/125/2017)	Generated in this thesis
H7 CFv3-SIAT (A/Hong Kong/125/2017)	Generated in this thesis
H7 single mutants x 9 (Y98F, R329Q, G1Q, L2G, I6G, G8A, 212/216C, 30/47C, 63/70P) - SIAT	Generated in this thesis
H7 GDCFv2-SIAT (A/Guangdong/17SF003/2016)	Generated in this thesis
HeLa	
HeLa wildtype	Julie Xiao
HeLa NPC1 exon 2 knockout	Julie Xiao
HeLa NPC1 exon 4 knockout	Julie Xiao

2.1.4 Primers

Table 2.4

PCR primers used in this thesis	
Sequence	Name
Amplifying and sequencing H7 HK125 hemagglutinins with internal primers	
TGTAAAACGACGGCCAGTCCACCATGAACACCCAGATC	HK125-frag1F-M13F
TGTAAAACGACGGCCAGTCATGAACACCCAGATCCTG	HK125-frag1F-M13F(2)
CAGGAAACAGCTATGACCGCTTCTCTGTTTCACGTATCTG	HK125-frag1R-M13R
TGTAAAACGACGGCCAGTACAGAGCCAGCTTTCTGAGAG	HK125-frag2F-M13F
CAGGAAACAGCTATGACCCATGTTGCCGTTCTTCACG	HK125-frag2R-M13R
GTTTCACGTATCTGGGGCAC	HK125seq_1005_R
CGGCACCATCATCAGCAACC	H7HKseq_R329Q
CGACAACGCCGCTTTCCAC	H7HKseq_HA1-212
TCCTGGTGTTTGCCCTGATCG	H7HKseq_HA1-30
Amplifying and sequencing all viral RNAs from the segment ends	
AGCAAAAGCAGG	Uni12
AGCAAAAGCAGGGGAA	Uni13
Amplifying and sequencing H1 PR8 from the segment ends	
AGTAGAAACAAGG	Frensing12
AGTAGAAACAAGGGTGTTTT	Frensing13
Specific amplification of each segment of the PR8 genome with internal primers for sequencing with M13 primers (provided by Jack Tan)	
TGTAAAACGACGGCCAGTAGCGAAAGCAGGCAAACCAT	A-PB1-I M13F
CAGGAAACAGCTATGACCGTTCAAGTTTCTCACATAT	A-PB1-I-M13R
TGTAAAACGACGGCCAGTCRATGACCAAAGATGCWGA	A-PB1-II-M13F
CAGGAAACAGCTATGACCAAGATCATTGTTTATCATRTTG	A-PB1-II-M13R
TGTAAAACGACGGCCAGTGTTGCTAATTTAGCATGGAG	A-PB1-III-M13F
CAGGAAACAGCTATGACCAAGTAGGAACAAGGCATT	A-PB1-III-M13F
TGTAAAACGACGGCCAGTAGCGAAAGCAGGTCA	A-PB2-I-M13F
CAGGAAACAGCTATGACCGATGCTARTGGRTCTGCTG	A-PB2-I-M13R
TGTAAAACGACGGCCAGTCAGTGTGTACATTGAAGT	A-PB2-II-M13F
CAGGAAACAGCTATGACCYTGYGTTTCACTGAC	A-PB2-II-M13R
TGTAAAACGACGGCCAGTGCAAAATGGGTGTAGATG	A-PB2-III-M13F
CAGGAAACAGCTATGACCTAGAAACAAGGTCGTT	A-PB2-III-M13R
TGTAAAACGACGGCCAGTAGCGAAAGCAGGTACTGAT	A-PA-I-M13F
CAGGAAACAGCTATGACCGGTTCTTCCATCCAAAG	A-PA-I-M13R
TGTAAAACGACGGCCAGTCAAATTCCTGCTGATG	A-PA-II-M13F
CAGGAAACAGCTATGACCTCAAGTCTTGGGTCAGTGAG	A-PA-II-M13R
TGTAAAACGACGGCCAGTGCATCTTGTGCAGCAATGGA	A-PA-III-M13F

CAGGAAACAGCTATGACCAGTAGAAACAAGGTACTTTT	A-PA-III-M13R
TGTAACACGACGGCCAGTCAGGGTAGATAATCACTCACTG	A-NP-I-M13F
CAGGAAACAGCTATGACCTGACTCTTGTGTGCTGG	A-NP-I-M13R
TGTAACACGACGGCCAGTATGAAAGAATGTGCAAC	A-NP-II-M13F
CAGGAAACAGCTATGACCAGTAGAAACAAGGGTATTTTC	A-NP-II-M13R
TGTAACACGACGGCCAGTAGCAAAAGCAGGTAG	A-M-M13F
CAGGAAACAGCTATGACCAGTAGAAACAAGGTAGT	A-M-M13R
TGTAACACGACGGCCAGTAGCAAAAGCAGGGTGACAAAGACA	A-NS-M13F
CAGGAAACAGCTATGACCAGTAGAAACAAGGGTGTTTTTAT	A-NS-M13R
TGTAACACGACGGCCAGTAGCGAAAGCAGGGGT	A-N1-I-M13F
CAGGAAACAGCTATGACCGTACGAGGCCAGCCCA	A-N1-I-M13R
TGTAACACGACGGCCAGTGGAATTCYGGTCCAG	A-N1-II-M13F
CAGGAAACAGCTATGACCAGTAGAAACAAGGAG	A-N1-II-IM13R

Table 2.5

Sequencing primers used in this thesis	
Sequence	Name
Sequencing of N1 PR8 plasmid pre- and post-mutagenesis	
CATCCGTGGGTGGGCTATATA	NA_T148Aseq
CGATTGTTAGCCAGCCCATG	N1_PR8_seq1R
GTGTGCAGAGACAATTGGCA	N1_PR8_seq2F
Sequencing X31PM plasmid to confirm as described by Hooper and Bloom, 2013	
GCGGTACCATAAGAATGCG	X31PMID_seq1F
CTGGAAGGGCTTGTCATTG	X31PMID_seq2R
AAGCTGTACATCTGGGGCAT	X31PMID_seq3F
CGAGCTCCTAGGCCTTGAAT	X31PMID_seq4R
Sequencing inserts in pHR-SIN plasmids before transfection (provided by Townsend group)	
ATCCAGAGGTTGATTATCGATAAG	pHRSIN-3p-SP
Sequencing H7 HK125 hemagglutinin in plasmids or PCR products	
GTTTCACGTATCTGGGGCAC	HK125seq_1005_R
CGGCACCATCATCAGCAACC	H7HKSeq_R329Q
CGACAACGCCGCCTTTCCAC	H7HKSeq_HA1-212
TCCTGGTGTGTCCTGATCG	H7HKSeq_HA1-30
Generic primers supplied by sequencing facility	
GTAAACGACGGCCAG	M13F
CAGGAAACAGCTATGAC	M13R

Table 2.6

Mutagenesis primers used in this thesis	
Sequence	Name
Introduction of T148A into wild-type PR8 neuraminidase	
TGTTTCGTAAGTTCACCCCGACAATTCCTGTCTTCG	NA_N1_T148A_F
ACAAGCATTCAAGTGGGGCTGTTAAGGACAGAAGC	NA_N1_T148A_R
Generation of single mutants in H7 A/Hong Kong/125/2017 hemagglutinin	
TTGCCGGGGAAGCACACATCGGAGCCT	H7HK_Y98F_1
AGGCTCCGATGTGTGCTTCCCCGGCAA	H7HK_Y98F_2
AATGGCGCCAAACAGGCCCTGGCCTTTAGGGATCTCAGG	H7HK_R329Q_1
CCTGAGATCCCTAAAGGCCAGGGCCTGTTTGGCGCCATT	H7HK_R329Q_2
GGCAATGGCGCCAAACAGCTGTCTGCCTTTAGGGATCTC	H7HK_G1Q_1
GAGATCCCTAAAGGCAGACAGCTGTTTGGCGCCATTGCC	H7HK_G1Q_2
GCAATGGCGCCAAACCCGCCTCTGCCTTTAGG	H7HK_L2G_1
CCTAAAGGCAGAGGCGGGTTTGGCGCCATTGC	H7HK_L2G_2
TCGATAAAGCCGGCACCCGGCGCCAAACAGGC	H7HK_I6G_1
GGCCTGTTTGGCGCCGGTGCCGGCTTTATCG	H7HK_I6G_2
CCATTCTCGATAAAGGCGGCAATGGCGCCAA	H7HK_G8A_1
TTGGCGCCATTGCCGCCTTTATCGAGAATGG	H7HK_G8A_2
CCACGCCGCGCTCGCACAGTGTGTTCACTT	H7HK_HA1-30_1
AAGTGAACACACTGTGCGAGCGCGGCGTGG	H7HK_HA1-30_2
TTCAGCTTGGCCGGTGATGCAGTCGATGGCGCTCTGTG	H7HK_HA1-47_1
CACAGAGCGCCATCGACTGCATCACCGGCCAAGCTGAA	H7HK_HA1-47_2
CAGCAATTACCAGCAGTGCTTTGTGCCAAGTCC	H7HK_212C_1
GGA CT TGGCACA AAGCACTGCTGGTAATTGCTG	H7HK_212C_2
CTGGCGCCTGGACATGGCACAAAGCTC	H7HK_216C_1
GAGCTTTGTGCCATGTCCAGGCGCCAG	H7HK_216C_2
CCAAGACCAACCAGCAGCCCGAGCTGATCGACAACG	H7HK_F63P_1
CGTTGTCGATCAGCTCGGGCTGCTGGTTGGTCTTGG	H7HK_F63P_2
GAGCTGATCGACAACGAGCCCAACGAGGTGGAAAAGCA	H7HK_F70P_1
TGCTTTTCCACCTCGTTGGGCTCGTTGTCGATCAGCTC	H7HK_F70P_2

2.1.5 Viruses

Table 2.7

Viruses produced in this thesis		
	Core virus	Pseudotypes
H3N1 CLEARFLUv2	[H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)]	H1(A/Puerto Rico/8/1934) H5(A/Vietnam/1203/2004) H7(A/Netherlands/219/2003)
H1N1 CLEARFLUv2	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)]	H1(A/Puerto Rico/8/1934) H5(A/Vietnam/1203/2004) H7(A/Netherlands/219/2003)
H7N1 CLEARFLUv3	[H7CFv3(A/Hong Kong/125/2017)/N1(A/Puerto Rico/8/1934)]	H1(A/Puerto Rico/8/1934) H3(A/X31) wild-type H3(A/X31) Passage Mutant Ebola GP (Zaire) Ebola GP (Sudan)
N1 (WT) S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)]	H1(A/Puerto Rico/8/1934) Ebola GP (Zaire)
N1 (T148A) S-Flu	[S-eGFP/N1_T148A(A/Puerto Rico/8/1934)]	H1(A/Puerto Rico/8/1934) Ebola GP (Zaire)

Table 2.8

Other viruses used in this thesis		
	Core virus	Pseudotypes
For general assays		
N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)]	H7(A/Hong Kong/125/2017) H7(A/Netherlands/219/2003)
N2 S-Flu	[S-eGFP/N2(A/Victoria/361/2011)]	H3(A/Switzerland/9715293/2013)
E-S-Flu (N1)	[S-eGFP/N1(A/Puerto Rico/8/1934)]	Ebola GP (Zaire)
For immunisation		
N1 S-Flu	[S-eGFP/N1(A/England/195/2009)]	H1(A/England/195/2009)
N2 S-Flu	[S-eGFP/N2(X217)]	H3(A/Hong Kong/5738/2014)
N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)]	H7(A/Hong Kong/125/2017) H7(A/Netherlands/219/2003)
For immunological assays		
N1 S-Flu	[S-eGFP/N1(A/England/195/2009)]	H1(A/England/195/2009) H7(A/Netherlands/219/2003)
N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)]	H5(A/Vietnam/1203/2004) H7(A/New York/107/2003) H7(A/Netherlands/219/2003) H7(A/Hong Kong/125/2017) H7(A/Taiwan/1/2017) H1(A/Puerto Rico/8/1934)
N2 S-Flu	[S-eGFP/N2(X217)]	H3(A/X31) H3(A/Hong Kong/5738/2014) H3(A/Switzerland/9715293/2013)
H1N1 wildtype virus	A/sw/H1N1/1535/2009 virus	

2.1.6 Antibodies

Table 2.9

Antibodies used in this thesis				
Staining for cell sorting				
Antibody	Target	Species	Concentration	Reference
T3-3C	H1	Human	20µg/ml	In-house
AM3C	H1	Human	20µg/ml	In-house
MEDI8852	HA	Human	20µg/ml	Kallewaard et al, 2016
Staining for flow cytometry				
Antibody	Target	Species	Concentration	Reference
Q11C9	H3	Human	20µg/ml	In-house
AR10D	H3	Human	20µg/ml	In-house
AM3C	H3	Human	20µg/ml	In-house
AQ6A	H3	Human	20µg/ml	In-house
Z3B2	H1/H3	Human	20µg/ml	In-house
AR1D	H3	Human	20µg/ml	In-house
4B18	H7	Human	20µg/ml	In-house
4A14	H7	Human	20µg/ml	In-house
9B122	H7	Human	20µg/ml	In-house
W3A1	H7	Human	20µg/ml	In-house
2-12C	H1	Human	20µg/ml	In-house
T2-7D	H1	Human	20µg/ml	In-house
T3-3C	H1	Human	20µg/ml	In-house
M6-B11	H1	Human	20µg/ml	In-house
Q11A6	H1	Human	20µg/ml	In-house
T1-3B	H1	Human	20µg/ml	In-house
MEDI8852	HA	Human	20µg/ml	Kallewaard et al., 2016
T2-5C9	H1	Human	20µg/ml	In-house
T3-5D	H1	Human	20µg/ml	In-house
6541	GP	Human	20µg/ml	Rijal et al., 2019
6662	GP	Human	20µg/ml	Rijal et al., 2019
040	GP	Human	20µg/ml	Rijal et al., 2019
Staining for plate assays				
Antibody	Target	Species	Concentration	Reference
4A14	H7	Human	5µg/ml	In-house
2-8C-biotinylated	NP	Human	1µg/ml	In-house (available from Absolute Antibody)
AA5H	NP	Mouse	1µg/ml	Abcam ab20343
AG7C	N1	Human	5µg/ml	Rijal et al., 2020
3CSY (KZ52)	GP	Human	5µg/ml	Maruyama et al., 1999

Neutralisation assays				
Antibody	Target	Species	Concentration	Reference
MEDI8852	HA	Human	20µg/ml	Kallewaard et al., 2016
AG7C	N1	Human	20µg/ml	Rijal et al., 2020
AF9C	N1	Human	20µg/ml	Rijal et al., 2020
Z2B3	NA	Human	20µg/ml	Rijal et al., 2020
4A14	H7	Human	20µg/ml	In-house
21-D8-5A	H1	Human	20µg/ml	Arthur Huang
3CSY (KZ52)	GP (Zaire)	Human	20µg/ml	Maruyama et al., 1999
P6	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
040	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
125	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
105	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
141	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
66-3-9C	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
66-3-2C	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
6662	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
114.24	GP (Zaire)	Human	20µg/ml	Misasi et al., 2016
6541	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
66-4C-12	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
6663	GP (Zaire)	Human	20µg/ml	Rijal et al., 2019
CA45	GP (Zaire +Sudan)	Human	20µg/ml	Janus et al., 2018
6D6	GP (Sudan)	Human	20µg/ml	Furuyama et al., 2016
Hemagglutination assays				
Antibody	Target	Species	Concentration	Reference
BS-2D	H3	Human	25µg/ml	In-house
21-D8-5A	H1	Human	25µg/ml	Arthur Huang
4A14	H7	Human	25µg/ml	In-house
2-12C	H1	Human	25µg/ml	In-house
Secondary layers for staining assays				
Antibody	Target	Concentration	Reference	
GAH AF647	Human mAbs: various	5µg/ml	Invitrogen 21445	
GAM 647	Mouse mAbs: AA5H	5µg/ml	Invitrogen A32728	
Strep 647	2-8C-bio	5µg/ml	Invitrogen S32357	
GAH FITC	Human mAbs: various	1:160	Invitrogen H10301	
GAH 488	Human mAbs: various	5µg/ml	Invitrogen A11013	
GAM 488	Mouse mAbs: AA5H	5µg/ml	Invitrogen A11001	

2.2 Experimental models

2.2.1 Cell lines

Madin-Darby Canine Kidney-sialyltransferase (MDCK-SIAT1/MDCK-SIAT) cells generated by transducing MDCK cells with human 2,6-sialtransferase (SIAT) to express higher levels of sialyl- α 2,6-galactose moieties (Matrosovich et al., 2003) were obtained from the European Collection of Authenticated Cell Cultures (ECACC).

Human Embryonic Kidney (HEK) 293T cells (HEK cells transduced to express SV40 Large T antigen) were obtained from the William Dunn School of Pathology, Oxford.

HeLa wild type, NPC1 exon 2 and NPC1 exon 4 knockout cells were provided by Julie Xiao.

Culture and growth conditions. Cells were grown in D10 at 37°C with 5% CO₂, usually in T175 flasks. Cells were passaged when confluent. MDCK-SIAT and HeLa cells were harvested by incubating with enough Trypsin-EDTA to cover the cells for 10 minutes and tapping the flask or plate to detach cells. Cells were spun down in 50ml of D10. 293T cells were harvested using 2mM EDTA in phosphate buffered saline (PBS) and spun in 50ml PBS. Pellets were resuspended in D10 for passaging, VGM for viral assays or at about 10 million cells/ml in freezing medium for storage.

2.2.2 Animal models

C57BL/6 and BALB/c female mice were obtained from Envigo RMS Inc, Bicester and maintained at the Biomedical Services facility at the John Radcliffe hospital. They were 6–8 weeks old at the start of experiments. Experiments were conducted under Project License number PBA43A2E4 in accordance with the 3Rs. Mice were housed in small groups in individually ventilated cages.

Experiments with **Babraham large white inbred pigs** were conducted at the Pirbright Institute. Pigs were aged 4–6 weeks at the start of experiments.

2.3 Method details

Materials availability statement. All products generated in this thesis can be made available to other laboratories for research use.

Quantification and statistical analyses. Statistical analyses were performed in Microsoft Excel and GraphPad Prism 8.3.1. Details of the statistical tests performed are available in figure legends.

2.3.1 Molecular biology

Design of CLEARFLU hemagglutinin sequences. CLEARFLU hemagglutinins were designed by incorporating inactivating mutations already described in the literature into wild-type hemagglutinin sequences from A/England/195/2009 (H1), A/Hong Kong/5738/2014 (H3) and A/Hong Kong/125/2017 (H7). DNAs were human codon-optimised. Restriction enzyme cloning sites for NotI and EcoRI were added to either end of the sequence to facilitate cloning into vectors pcDNA3.1-/pHR-SIN or the S-Flu cassette for mammalian or viral expression respectively. All sequences were ordered from GeneArt and resuspended in 50µl 10mM Tris/0.1mM EDTA. Sequences are available in the appendix.

Design of neuraminidase sequences for virus production. The sequence of the wild-type neuraminidase from A/Puerto Rico/8/1934, flanked by SapI/BspQI restriction sites to facilitate cloning into the pPol vector, was ordered from GeneArt.

Subcloning. Hemagglutinin sequences were subcloned into vectors pcDNA3.1- and pHR-SIN to generate transient and stable cell lines respectively using restriction enzymes EcoRI and NotI. EcoRI/NotI digestion was also used for cloning CLEARFLUs into the S-Flu expression cassette. 2µg of GeneArt plasmid was digested using 1µl EcoRI and 2µl NotI in NEBuffer 3.1 for 1 hour at 37°C in 100µl total volume. Neuraminidase sequence was subcloned into the pPol vector by digesting 2µg of plasmid with 1µl BspQI at 50°C for 15 minutes in the same buffer and volume. Digestion products were run on gels containing 0.7% Agarose and 0.5µg/ml Ethidium Bromide in TAE buffer. Samples were loaded using Orange G gel loading dye. Bands were imaged and extracted under UV light, purified using a QIAquick Gel Extraction Kit from Qiagen and eluted in 50µl of provided Elution Buffer. Inserts were ligated into new vectors in a 30µl reaction containing 1.5µl T4 DNA ligase, 3µl T4 DNA ligase buffer, 3µl pre-digested and phosphatase treated vector and 15µl of insert. The reactions were incubated at room temperature for at least 1 hour.

Transformation. All plasmids were grown up in DH5α *E.coli*. 2µl of plasmid was added to 5µl of DH5α ultracompetent cells in Eppendorf tubes on ice. After a 40-minute incubation, a heat shock was administered for 30 seconds at 42°C. 1ml of LB was added before incubating at 37°C for 1 hour and then spreading the broth on LB-Agar plates containing ampicillin. After overnight incubation at 37°C, colonies were picked into 13ml LB containing ampicillin for a further overnight incubation at 37°C on a 200rpm shaking platform. 0.9ml of the broth was stored at -80°C in Hogness solution for future use. The remaining cells were then pelleted by centrifugation at 3000rpm for 45 minutes and the supernatant removed. Plasmids were extracted from the pellets using a QIAprep Spin Miniprep Kit.

Diagnostic digests. To check pcDNA3.1- and pHR-SIN plasmids for correct insert length, 20µl digests with 2µl plasmid DNA and 0.5µl each of NotI and EcoRI were set up. As the BspQI site is lost after digestion, it could not be used to check insert length.

Sanger sequencing services were provided by the WIMM internal sequencing facility and Source Bioscience.

Polymerase chain reaction (PCR). Phusion PCR (Thermo Scientific) was performed as described in Table 2.10. Unless stated otherwise, PCR products were run on 0.7% agarose gels containing ethidium bromide as described for digestion products. All PCR primers are listed in 2.1.4.

Table 2.10 – Phusion PCR

Phusion PCR reaction		Volume (μl)
dH ₂ O		12.3
5x High Fidelity Buffer		4
25mM dNTPs		0.4
DMSO		0.6
Forward Primer (10μM)		1
Reverse Primer (10μM)		1
Template Plasmid		0.5 (20ng total)
Phusion Polymerase		0.2
		20μl total
Phusion PCR cycling conditions		
Step	Temperature	Duration
1	98°C	1 min
2	98°C	10s
3	63°C	30s
4	72°C	45s
5	72°C	7mins
6	4°C	hold

**Steps
2-4 X35
cycles**

RNA extraction from virus supernatant and One-step reverse-transcription polymerase chain reaction (RT-PCR). RNA was extracted from 100µl of tissue culture supernatant from infected MDCK-SIAT cells using a Qiagen QIAamp Viral RNA Mini Kit. One-Step RT-PCR reactions were set up as described in Table 2.11.

Table 2.11 – One-step RT-PCR

RT-PCR reaction			Volume (µl)
RNase free H2O			31.1
5 x buffer			10
50mM MgSO ₄			1
25mM dNTPs			0.9
RNasin Ribonuclease inhibitor			0.5
SuperScript III Reverse Transcriptase			1
Platinum Superfi			0.5
Forward Primer (10µM)			1.5
Reverse Primer (10µM)			1.5
Template RNA (80-90ng/µl)			2
			50µl total
One-Step RT-PCR cycling conditions			
Step	Temperature	Duration	Steps 3-5 X40 cycles
1	50°C	30 min	
2	94°C	10 min	
3	98°C	30 sec	
4	55°C	30 sec	
5	72°C	1 min	
6	72°C	5 min	
7	4°C	hold	

Site-directed mutagenesis. Agilent QuickChange Lightning Site-Directed Mutagenesis kits were used to introduce specific mutations by PCR into the hemagglutinin sequence of A/Hong Kong/125/2017 to generate versions with a single inactivating mutation. The same protocol was used to introduce T148A into PR8 neuraminidase. The bacteria provided were grown up in the same conditions as described previously for DH5α *E.coli*. The primers used are described in 2.1.4.

2.3.2 Tissue culture

Transient transfection of HEK 293T cells. 2×10^6 293T cells in Opti-MEM were added to 1 well of a six well plate. 16 μ l of Lipofectamine was diluted in 200 μ l Opti-MEM and incubated at room temperature for 5 minutes before adding 4 μ g of miniprep DNA in plasmid pcDNA3.1- in 200 μ l Opti-MEM. The mixture was incubated at room temperature for a further 20 minutes before being added to the cells. After at least four hours at 37°C, the supernatant was replaced with 2.5ml D10. When confluent, the cells were harvested and expanded in T175 flasks in D10.

Transduction of MDCK-SIAT cells for stable expression. 293T cells were transfected as above, but with 1.33 μ g each of plasmids containing VSV-G, Gag/Pol and the gene of interest to produce lentivirus (Demaision et al., 2002). Rather than harvesting the cells, the supernatant was harvested after 48 hours and 1ml was added to 10^5 MDCK-SIAT cells in 1ml D10 containing 8 μ g/ml polybrene in a six well plate. The culture medium was replaced with fresh D10 the next morning and, 24 hours after the first infection, cells were re-infected with a further 1ml of lentivirus with polybrene. When confluent, the cells were harvested and expanded in T175 flasks in D10. The medium was replaced with fresh D10 the following morning. Leftover lentivirus was stored at -80°C.

Multi-plasmid transfection of HEK 293T cells for generating recombinant viruses (Fodor et al., 1999). 10^6 293T cells in D10 were plated in a single well of a 6-well plate and left overnight. Mixes containing 1 μ g of DNA of each of 13 plasmids were prepared in Opti-MEM to a final volume of 250 μ l according to Table 2.12. 13 μ l of Lipofectamine was added to 237 μ l of Opti-MEM and incubated for 5 minutes before adding the DNA mixture dropwise and incubating for a further 20 minutes at room temperature. The cells were then washed in Opti-MEM and 1.5ml of fresh Opti-MEM was added, along with the DNA-Lipofectamine mix.

After 4 hours at 37°C, the media was replaced with 3ml of VGM containing 0.75µg/ml TPCK Trypsin.

After 48 hours, the supernatant was harvested and 1.5ml was used to infect 5×10^5 MDCK cells transduced to express PR8 hemagglutinin. After 2 hours at 37°C, the cells were washed in PBS and 3ml of VGM containing 0.75µg/ml TPCK Trypsin was added. After 48 hours, the supernatant was harvested, diluted in VGM and used to infect confluent T175 flasks of MDCK-SIAT cells transduced to express a chosen hemagglutinin. After 1 hour, flasks were topped up with 30ml of VGM containing 1µg/ml TPCK Trypsin. Viruses were harvested from these flasks after 48-72 hours and viral clones were isolated by limiting dilution before being expanded to produce virus stocks (**see isolation of viral clones by limiting dilution and quantification by TCID₅₀**).

Table 2.12

Multi-plasmid mixes for S-Flu and CLEARFLU virus production				
	H1 PR8 S-Flu	H1 CLEARFLU version 2	H3 CLEARFLU version 2	H7 CLEARFLU version 3
4 pcDNA3.1- Core Initiators				
pcDNA PB1-PR8	x	x	x	x
pcDNA PB2-PR8	x	x	x	x
pcDNA PA-PR8	x	x	x	x
pcDNA NP-PR8	x	x	x	x
6 pPol Internal genes				
pPol PB1-PR8	x	x	x	x
pPol PB2-PR8	x	x	x	x
pPol PA-PR8	x	x	x	x
pPol NP-PR8	x	x	x	x
pPol M-PR8	x	x	x	x
pPol NS-PR8	x	x	x	x
Source of expressed functional NA				
pPol N1-NA-PR8	x	x	x	x
Source of pseudotyping HA				
pcDNA 3.1- H1 HA-PR8	x	x	x	x
Source of insert replacing HA in S-Flu cassette				
pPol/US/eGFP	x			
pPol/US/H1(Eng195)CFv2		x		
pPol/US/H3(HK5738)CFv2			x	
pPol/US/H7(HK125)CFv3				x

Plasmid mixes include four core initiating pcDNA3.1- plasmids containing the genes PB1, PB2, PA and NP and seven viral expression pPol plasmids containing these genes as well as M, NA and NS genes. Functional pseudotyping HA was provided in a pcDNA3.1- plasmid so that it was expressed by the cell but not the virus. Fluorescent S-Flu was made by adding a pPol plasmid with an eGFP insert described by Powell et al, 2012. CLEARFLU viruses were made by replacing the eGFP sequence with a CLEARFLU hemagglutinin gene by subcloning using EcoRI/NotI restriction sites.

Virus naming convention. All S-Flu and CLEARFLU viruses are named according to the sequence expressed in the hemagglutinin cassette, the neuraminidase and pseudotyping coat protein as follows:

[gene in hemagglutinin expression cassette/neuraminidase group(neuraminidase strain)] coated in coat protein group(coat protein strain)

e.g. [S-eGFP/N1(A/England/195/2009)] coated in H1(A/England/195/2009)

or [H1CFv3(A/Hong Kong/125/2017)/N1(A/Puerto Rico/8/1934)] coated in GP(Zaire)

Staining cells for fluorescence-activated cell sorting (FACS). Confluent T175 flasks of MDCK-SIAT cells were harvested and spun in D10 at 1400rpm. The cell pellet was resuspended in 1ml of primary antibody in D10 with 10mM HEPES and incubated on ice for 1 hour. Cells were then washed with 50ml of D10 and resuspended in 1ml of secondary antibody and incubated on ice for 1 hour. Cells were again washed with D10, spun and resuspended in 7 ml of D10 before being passed through a 0.22µm syringe filter and sorted using a BD FACS Aria IIIu at the WIMM FACS facility. Sorted cells were grown up and passaged in T175 flasks to produce cell stocks for experiments.

Staining cells for analysis by flow cytometry. 293T or MDCK-SIAT cells infected with virus or transfected/transduced to express a chosen plasmid were harvested and aliquoted into FACS tubes at approximately 1 million cells per tube. The tubes were spun at 1400rpm and the cells were resuspended in 50µl of primary antibody diluted in cold FACS wash. After 1 hour at 4°C, 2ml of FACS wash was added and the cells were spun and resuspended in 50µl of secondary antibody. After a further hour at 4°C, the cells were washed in FACS wash and resuspended in 300µl of FACS fixative. Cells were analysed using a CyAn ADP flow cytometer by Beckman or an Attune NxT flow cytometer by Invitrogen. Data was

analysed using FlowJo v10.6.1. Forward and side scatter were used to gate for single cells only.

Staining cells for plated viral assays. Confluent MDCK-SIAT cells in 96 well plates were stained with 40µl of primary antibody per well for 1 hour at 4°C, washed thoroughly with PBS and then stained with 40µl of secondary antibody in the same conditions. For staining nucleoprotein, cells were first fixed by incubation with 100µl of 10% formalin for 30 minutes at 4°C and then incubated with 100µl of permeabilization buffer at room temperature for 20 minutes. For stains not involving nucleoprotein, cells were fixed after staining and were not permeabilised. Double stains were performed by incubating with both primary antibodies- one mouse and one human- simultaneously and then both secondary antibodies, as they do not cross-react significantly. Antibodies were diluted in FACS wash. Fixed cells were stored in PBS. The same protocol was used to stain cells in 6-well plates, using 500µl of each antibody, 1ml of FACS FIX and storing in 1ml of PBS.

Fluorescence microscopy and quantification. Images were taken with a Zeiss fluorescence microscope using 10x magnification. Images were managed using Fiji/Image J. Fluorescence was quantified using a CLARIOstar microplate reader.

2.3.3 Viral assays

Isolation of viral clones by limiting dilution and quantification by TCID₅₀. 96 well flat-bottomed plates were seeded with 3×10^4 MDCK-SIAT cells expressing a pseudotyping coat protein (influenza hemagglutinin or Ebola glycoprotein) per well. The following day, confluent monolayers were washed with PBS. In a separate plate, half-log dilutions of virus were set up in VGM and 50 μ l from each well was transferred to the respective well on the assay plate. After 1–2 hours, 150 μ l of VGM was added (containing 0.75 μ g/ml TPCK-Trypsin if hemagglutinin was used as the coat protein). After 48 hours, infected wells were identified by staining cells for nucleoprotein expression (see **staining cells for plated viral assays**). For cloning by limiting dilution, the supernatant from wells containing a single growing viral clone was harvested and expanded in T175 flasks. For quantifying virus, the dilution at which 50% of wells were infected (TCID₅₀) was calculated using eight replicate rows and the Reed Muench method (Reed & Muench, 1938). Wells with fluorescence more than four standard deviations above background (based on eight replicates) were scored as positive. Viruses were typically diluted between 100 and 30000-fold in VGM before titration.

Virus quantification by CID₅₀. Serial two-fold dilutions of virus were set up in a 96 well flat-bottomed plate in a 100 μ l volume of VGM per well. 3×10^4 MDCK-SIAT cells in 100 μ l VGM were added to each well and incubated overnight. The dilution of virus at which 50% of cells were infected was determined by linear interpolation and used to calculate virus titre. In experiments where fluorescence dipped at the highest virus concentrations due to cell detachment, the highest recorded value was used for the 100% infection point. Cell infection was measured either using GFP fluorescence or nucleoprotein staining. This procedure was also used to titrate viruses for microneutralisation by finding the minimum amount of virus required for maximum signal. Viruses were usually diluted 1 in 2 in the

first column, but were used neat when expected titres were low. Occasionally, high titre viruses were pre-diluted before titration.

Microneutralisation assays to investigate viral entry. Serial two-fold dilutions of antibody in 50µl of PBS were set up in duplicate in a 96-well flat-bottomed plate. Antibodies used in this assay are listed in 2.1.6. Column 11 was kept as a no antibody control and column 12 as a cell only control. 50µl of virus was added to each well, except the cell only control wells, and incubated for 2 hours at 37°C. 3×10^4 MDCK-SIAT cells in 100µl VGM were added to each well and the plates were incubated overnight at 37°C. The plates were fixed and stained for nucleoprotein (see **staining cells for plated viral assays**) or measured for GFP fluorescence.

Enzyme-linked lectin assay (ELLA) for neuraminidase activity. 96 well ELISA plates were coated in fetuin by incubating in 50µl of fetuin solution overnight at 4°C. Antibody doubling dilutions were set up in 60µl per well in a separate plate. 60µl of virus was added to each well in the antibody dilution plate except the VGM only controls, and the plate was incubated at 37°C for 2 hours. Fetuin plates were washed four times with PBS before transferring 100µl of the antibody and virus mix to the respective well. The plates were incubated for 18 hours at 37°C, after which the mix was removed and the plates were washed with PBS four times. 50µl of PNA-HRP (peanut agglutinin conjugated to horseradish peroxidase) solution was added to each well. After 1.5 hours on a shaking platform at room temperature, the solution was removed and the plates washed four times with PBS. 50µl of peroxidase substrate solution (OPD) was added to each well. The plates were developed for approximately 15 minutes at room temperature before stopping the reaction using 50µl of 1M H_2SO_4 . Absorbance at 492nm was read immediately using a CLARIOstar microplate reader.

Hemagglutination inhibition assay. Human red blood cells were pelleted by centrifugation and washed by adding PBS. Centrifugation was at 14000rpm for 10 minutes using a slow deceleration to avoid disturbing the pellet. The supernatant was discarded and the cells were washed with PBS two further times. The pellet was resuspended in an equal volume of Alsever's solution to prepare 50% RBCs. The cells were stored at 4°C for up to two weeks. The hemagglutination assay was performed using a fresh solution of 1% RBCs obtained by resuspending 1ml of 50% RBC solution in approximately 50ml of PBS. The exact volume of PBS was adjusted to obtain an optical density reading of 1.8 at 600nm. Assays were performed in 96 well V-bottomed plates. Doubling dilutions of viruses in PBS were set up in duplicate rows. The final column was kept as a no virus control. 50µl of 1% RBC solution was added to each well. The plate was incubated for 1 hour at room temperature before being tipped vertically to allow teardrops to form. Wells with no red cell pellet or teardrop were recorded as being positive for agglutination. Wells with a pinkish hue in which a small pellet with no teardrop formed were recorded as partial positives. To test for blocking of agglutination, viruses were incubated with antibody or oseltamivir for 1 hour before adding RBCs.

Niemann-Pick Type C1 (NPC1) knockout experiment. HeLa NPC1 exon 2 and exon 4 knockout cells were generated previously by Julie Xiao. HeLa cells were seeded in a 96-well plate at 3×10^4 per well in VGM. The next day, the cells were washed with PBS and infected with 50µl of virus. Viruses were diluted in PBS in serial two-fold dilutions in quadruplicate as VGM has previously been observed by Julie Xiao to inhibit infection of HeLa cells. Few dilutions could be performed as cell survival overnight in PBS is poor. After 24 hours, the cells were fixed with 10% formalin, permeabilised and stained for nucleoprotein expression. Fluorescence microscopy images were taken at 10x magnification.

2.3.4 *In vivo* experiments in mice

Viruses were filtered prior to administration using a 0.22µm syringe filter.

Intranasal immunisation. Anaesthesia was induced using 4.5% isoflurane (Abbott) and maintained using 2.5% isoflurane in air. Anaesthetised mice were held on their back with their mouth held shut and drops of virus were pipetted onto the nose to be inhaled one by one, to a total volume of 50µl. Mice were observed until they recovered from the anaesthetic.

Intraperitoneal immunisation. 500µl of virus was injected into the peritoneal space as per standard practice using a 0.5ml insulin syringe and a 29G needle.

Harvesting mouse BAL, sera and tissues. Mice were humanely killed using schedule 1 methods. Mice were exposed to a slowly rising concentration of CO₂ and death was confirmed by cervical dislocation. Bronchoalveolar lavage (BAL) was obtained by nicking the trachea and using a syringe to wash 1ml of sterile PBS through the lungs 3 times. BAL fluid was stored in Eppendorf tubes at -20°C. Blood was harvested by cardiac puncture using a 23-25G needle and a 1 or 2ml syringe. Blood was stored in a BD microcontainer with SST gel for 30 minutes to allow clotting before spinning at 10000rpm for 5 minutes. Serum was collected from above the interface gel and heat-inactivated at 56°C for 30 minutes. Lungs and spleens were removed and placed in R10 on ice.

Microneutralisation and ELLA for investigating serum antibody. The assays were performed as described previously in this thesis, using mouse sera or bronchoalveolar lavage fluid instead of purified monoclonal antibody. Sera were diluted 1 in 20 before use, so that the first dilution represents a 1 in 40 dilution. BAL samples were not diluted. For some experiments, sera from multiple animals were pooled. S-Flu viruses with known neutralisation and ELLA profiles were used as sources of hemagglutinin and

neuraminidase respectively. All S-Flus were titrated before use to ensure maximum signal on microneutralisation and ELLA.

Lymphocyte preparation & MHC class I tetramer staining.

Spleens were cut into small pieces and then pushed through a 70µm cell strainer mesh using a 2ml syringe plunger. Cells were washed through with R10.

Lungs were minced with scissors. 2mg/ml Collagenase IV and 200 units/ml of DNase I were diluted one part each in three parts RPMI-1640 to make up an enzyme solution. 4ml of this was added to each sample of minced lungs and incubated for 30 minutes at 37° with occasional shaking. Lung pieces in enzyme mix were poured onto a 70µm cell strainer mesh and a 2ml syringe plunger was used to push the tissue through. The cell suspensions were then spun at 1200rpm for 5 minutes and the pellets were treated with 2ml of RBC lysis solution at room temperature for 10 minutes. 8ml of R10 was then used to wash the cells. The cell pellets were resuspended in 5ml of 40% Percoll in a 15ml tube. A 5ml layer of 80% Percoll was carefully laid down at the bottom of the tubes using a Pasteur pipette. The cells were spun at 2000rpm at room temperature for 25 minutes using the slowest deceleration setting and an intermediate acceleration rate. Leukocytes were collected from the interface between the two Percoll concentrations using a Pasteur pipette and were washed in 10ml of R10 twice. The cells were resuspended in R10 to achieve a single cell suspension.

Staining. Approximately one million cells were pipetted for each spleen or lung sample into one well of a round-bottomed 96 well plate. The cells were washed with R10, spinning at 1400rpm for 5 minutes and discarding the supernatant. MHC Class I tetramers were diluted 1 in 200 in R10 and 20µl was added to each well. After 30 minutes at 37°C, the cells were washed with PBS. 25µl of Zombie Violet was used at 1:1000 in PBS to stain dead cells. The cells were incubated in the dark at room temperature for

10 minutes. An antibody mix was then used to simultaneously stain for surface markers CD8, CD44, CD11b and B220 (see Table 2.13). The mix was made up by diluting each antibody to 1 in 200 in a final volume of 25µl FACS buffer per well. This was added without removing the Zombie Violet, and was incubated for 30 minutes on ice in the dark. The cells were then washed with PBS and fixed using 100µl of cold 5% formalin for 10 minutes at 4°C. As APC-Cy7 may degrade in the fixative, cells were washed in PBS and resuspended in PBS after fixation. Results were analysed using an Attune NxT flow cytometer by Invitrogen and FlowJo v10.6.1 software. Ultracompensation beads were used to account for overlapping emission spectra.

Table 2.13

Spleen and lung lymphocyte staining			
Target	Label	Further	Origin
MHC Tetramer	APC (Allophycocyanin)	H-2D(b) Influenza A NP 366-374 ASNENMETM	NIH (National Institutes of Health)
CD8	APC-Cy7	Rat IgG2a kappa, clone 53-6.7	Biolegend 100713
CD44	Alexa Fluor 488	Rat IgG2a kappa, clone IM7	Biolegend 103016
CD11b	Brilliant Violet 421 (Dump channel)	Rat IgG2a kappa, clone M1/70	Biolegend 101235
B220 (CD45R)	Brilliant Violet 421 (Dump channel)	Rat IgG2a kappa, clone RA3-6B2	Biolegend 103239
Live/Dead stain	Zombie Violet		Biolegend 423113
Ultracompensation beads			eBioscience 01-2222-41

2.3.5 *In vivo* experiments in pigs

All experiments were performed by Elma Tchilian and Veronica Martini at the Pirbright Institute. Data were analysed by Veronica Martini. Barbara Holzer and Sophie Morgan assisted with bleeds and tissue harvest.

Immunisation. Babraham pigs were sedated using 1mg/kg stesnil and 5mg/kg ketamine prior to immunisation. 2ml of S-Flu or CLEARFLU version 2 virus was administered using an Aerogen Solo nebuliser (Aerogen Ltd, Ireland) as described by Holzer et al., 2018. A second identical dose was administered after 21 days.

Harvest and analysis. Sera were harvested on days 7, 14 and 21 post-boost for use in ELLA and microneutralisation assays and to isolate peripheral blood mononuclear cells (PBMCs) for ELISPOT. Animals were culled 21 days post-boost by overdose of anaesthesia, and lungs, spleen, BAL, tracheobronchial lymph nodes (TBLN) and PBMCs were harvested for microneutralisation, ELLA, ELISPOT and MHC class I tetramer staining. ELLA and microneutralisation were conducted as described previously in this thesis and ELISPOT and tetramer staining were performed as described by Tungatt et al., 2018. Additional details are given in figure legends.

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Chapter 3

DESIGN, EXPRESSION AND ANTIGENICITY OF CLEARFLU HEMAGGLUTININS

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3.1 Introduction

This chapter describes the rationale behind the CLEARFLU design and the process through which it has been refined to improve antigenicity and the stability of inactivation. It begins by discussing ways in which hemagglutinin can be inactivated, and then goes on to consider the importance of maintaining antigenicity in the design of an immunogen. Results presented in this chapter show that hemagglutinins can be designed to be stably inactivated without losing structural features, and that they can be expressed in mammalian cell lines and in recombinant viruses.

3.1.1 Disrupting hemagglutinin function

Several mutations capable of inactivating hemagglutinin have previously been described. Some are fully inhibiting while others partially attenuate viral replication. They alter hemagglutinin activity in a multitude of ways, including during receptor binding, endosomal membrane attachment and fusion-associated conformational changes. The potential for some of these mutations to be used to generate single-cycle virus vaccines has been recognised. However, many inactivating mutations are unstable and easily acquire compensatory mutations or revert to a functional form. This is particularly concerning for the design of vaccines based on highly pathogenic strains. As such, the concept for CLEARFLU vaccines is centred on being able to combine multiple independently inactivating mutations to engineer a single hemagglutinin which is highly unlikely to revert to a functional form. The following paragraphs describe how different classes of mutation disrupt hemagglutinin function. These are identified according to the H3 numbering by Burke & Smith (Burke & Smith, 2014).

RECEPTOR BINDING

The first role of the hemagglutinin protein is to bind target cells through sialic acid residues on cell surface glycoproteins and glycolipids. The receptor binding site is situated in a shallow pocket at the top of the head domain delineated by the 130-, 150- and 220-loops and the 190- α helix (Weis et al., 1988)

Several mutations in the site are known to affect receptor binding by disrupting hydrogen bonds or van der Waals contacts with sialic acid (Martin et al., 1998). A notable residue is the tyrosine at position 98, which forms a hydrogen bond with the 8-hydroxyl group on sialic acid. This is believed to be important for binding as the mutation Y98F disrupts this bond and greatly reduces binding to erythrocytes (reviewed by Skehel & Wiley, 2000). Similarly, the mutation H183F which prevents the formation of a hydrogen bond between positions 183 and 98 reduces binding to erythrocytes, again supporting the importance of position tyrosine 98 in sialic acid binding. Meisner et al report that reduced binding activity in Y98F hemagglutinin allows the growth of mutant viruses in cell culture and in eggs but leads to an attenuated phenotype in infected mice (Meisner et al., 2008). They suggest that a 20-fold decrease in sialic acid binding leads to a 1000-fold decrease in viral titre in the lung, sufficient to prevent weight loss and other signs of illness. This decrease was not observed for other mutations around the receptor binding site. Immunisation with Y98F virus also protected against future challenge with wild-type virus. However, several of the viruses isolated from infected mice had reverted to wildtype at position 98 *in vivo*. Although the mice did not develop pathology, likely because a strong innate immune response was established before the reversion, this emphasises the need for multiple inactivating mechanisms in vaccines.

THE CLEAVAGE SITE

With the exception of high pathogenicity H5 or H7 strains which can be cleaved by furin due to the presence of a polybasic cleavage site, hemagglutinins must be cleaved by trypsin-like enzymes to achieve their fusion-competent conformation. For cleavage to occur, an arginine (or rarely lysine) residue must be present at the cleavage site at position 329. Viruses with mutations which alter the protease sensitivity of hemagglutinin, making it resistant to trypsin but sensitive to other enzymes like chymotrypsin, elastase or thermolysin, can be grown in cell culture (Orlich et al., 1995). Due to the absence of the required protease *in vivo*, these cleavage mutants do not cause disease in the chicken respiratory tract (Orlich et al., 1995). They are however able to protect chickens against challenge with wildtype virus. Similarly, an elastase-sensitive mutant is apathogenic in mice and able to protect against wildtype challenge (Stech et al., 2005). However, when grown in the presence of trypsin, mutant viruses were able to revert to a trypsin-susceptible form at a frequency of 1 in 10^7 pfu (plaque forming units) (Stech et al., 2005). As such, these mutants would be unsuitable as vaccine candidates without additional mutations to prevent reactivation.

The identification of residues essential for cleavage has paved the way for the design of protease resistant hemagglutinins. In our design, we have therefore used a mutation-R329Q - that removes rather than alters the sensitivity of the hemagglutinin to proteolytic cleavage (Chen et al., 1998). By pseudotyping viruses with trypsin-sensitive functional hemagglutinin instead of relying on alternative protease activity for growth in cell culture, the risk that alternative protease sensitivity allows infection of unexpected cell types can be circumvented.

THE FUSION PEPTIDE

Cleavage of the HA0 precursor removes residue R329 to give HA1 at the N-terminus and HA2 at the C-terminus. The first 20-25 amino acids of the HA2 fragment constitute the fusion peptide; a hydrophobic portion which, after cleavage, becomes buried inside the molecule. At the pH of activation, the molecule undergoes extensive conformational changes including the dilation of membrane distal regions and the formation of an extended central trimeric coiled coil which results in the fusion peptide being thrust into the endosomal membrane (Benton et al., 2020; reviewed by Sriwilaijaroen & Suzuki, 2012). The fusion peptide is very well conserved, particularly at the N-terminal end. It contains a series of interspersed glycine residues separated by long chain hydrophobic amino-acids. The first amino acid in the fusion peptide, glycine 1, is very highly conserved across strains. Versions of HA2 generated by alternative protease digestion lack this first residue and are not fusion competent (Garten et al., 1981). Certain substitutions such as G1Q are also sufficient to abolish fusion (Gething et al., 1986; Qiao et al., 1999).

Cross et al established that L2G and I6G in H3 (X31) hemagglutinin lead to changes in the pH required for conformational changes (Cross et al., 2001). The mutations also decreased or abolished fusion as measured by fluorescent dye transfer, erythrocyte haemolysis and polykaryon formation. Their attempts to produce H1N1 (WSN) virus with either L2G or I6G failed, consistent with the suggestion that the mutated hemagglutinins are non-functional. Similarly, Steinhauer et al showed that G8A decreases the stability of the pre-fusion hemagglutinin and inhibits fusion as measured by the formation of polykaryons (Steinhauer et al., 1995).

The tryptophan residues at positions 14 and 21 are also highly conserved and not very tolerant to substitution. Mutation of either residue to alanine prevents fusion and does not yield infectious virus (Cross et al., 2009; Lai et al., 2006).

Combinations of these fusion loop mutations are included together in the design of CLEARFLU hemagglutinins to lower the probability of back-mutation to a competent fusion peptide.

THE B LOOP

The B loop (HA2 55 to HA2 75) undergoes a critical conformational change during hemagglutinin activation. The B loop transforms into an alpha-helix which connects together existing helices on either end to form a single extended helical structure in each monomer. Each monomer contributes one helix to form a central coiled coil which projects out from the viral membrane, ending in the fusion peptide. This is called the spring-loaded conformational change. This change is essential for relocation of the fusion peptide to the endosomal membrane.

Mutations in the B loop disrupt the formation of the coiled coil structure. Double proline mutant F63P/F70P prevents the coiled coil from achieving its full length due to a kink in the helices which means that they are splayed rather than bunched together at the N-terminal end (Gruenke et al., 2002). Although this mutant undergoes conformational changes at low pH and is able to bind the target membrane, it is not fusion competent, suggesting that a step after membrane attachment is blocked. This may be because the fusion peptides of the three monomers are no longer in proximity or because the kinked coil only projects half as far as wild-type hemagglutinin towards the target membrane.

STABILISATION BY DISULPHIDE BRIDGES

Although the HA1 portions of the hemagglutinin trimer are not directly involved in the presentation of the fusion peptide at the target membrane, they must separate to make way for the formation of the central coiled coil (reviewed by Stegmann, 2000). As such, mutations which restrict the movement of HA1 may block fusion.

Following cleavage, a disulphide bridge between HA1 14 and HA2 137 holds HA1 and HA2 fragments together. Three other intra-monomer disulphide bonds are present within HA1 and one within HA2. Godley et al describe how the introduction of further disulphide bonds in the HA1 domain of X31 hemagglutinin can prevent conformational changes required for fusion (Godley et al., 1992). They substituted residues 212 and 216 for cysteine, so that the 212 cysteine from one monomer is positioned appropriately to form a disulphide bridge with the 216 cysteine of its neighbour. This allows the formation of three disulphide bonds per trimer which lock hemagglutinin in its pre-fusion conformation (Kemble et al., 1992). These changes do not noticeably disrupt the native structure of hemagglutinin, are permissive of receptor binding and cleavage but prevent the dissociation of HA1 monomers required for fusion.

An alternative design for a disulphide-stabilised trimer has been proposed by Lee et al (Lee et al., 2015). They engineered mutations which crosslink the stem regions of the trimer by introducing disulphide bridges between HA1 30 on one monomer and HA2 47 on another. In hemagglutinin from A/Hong Kong/1/1968, this inter-monomer linkage enhanced thermostability by reducing dissociation of hemagglutinin trimers, while preserving the structure of antigenic sites. The enhanced stability of trimeric hemagglutinin may serve to enhance its antigenicity, a valued characteristic for a vaccine immunogen. Although not previously tested, HA1 30/HA2 47 hemagglutinins may have a reduced ability to mediate fusion and therefore may attenuate or abolish viral replication.

3.1.2 Maintaining and optimising antigenicity

Selecting antigenically neutral mutations

Maintaining the antigenicity of the hemagglutinin is critical to ensure that immunisation generates a strong and protective anti-hemagglutinin response. Mutations must therefore be carefully selected to minimise their effect on hemagglutinin structure, particularly in common antibody binding regions.

Most neutralising antibody epitopes are located in the head region in the Sa, Sb, Ca1, Ca2 and Cb sites in H1 or A, B, C, D and E sites in H3 hemagglutinin (Caton et al., 1982; Gerhard et al., 1981; Skehel et al., 1984; Webster & Laver, 1980). Antibodies targeted to the head region tend to be more potent neutralisers than those to the stem, and so stimulating these *in vivo* may be particularly protective. Responses to the sialic acid binding site are especially significant as these neutralise the virus by preventing cell attachment. Hemagglutination inhibition (HAI) assays, which are used as a correlate of protection and as a key measure in assessing vaccine candidates, rely on detecting antibodies which block this activity by binding to the sialic acid binding region. As such, maintaining epitopes around the sialic acid binding site is critical not only for generating a neutralising antibody response *in vivo*, but also for conforming to established methods of virus and antigen quantification.

Other neutralising antibodies, sometimes with broad reactivity, are known to target the stem (reviewed by Air, 2015; Lee & Wilson, 2015). Neutralising stem antibodies usually function by blocking cleavage or fusion and so are targeted to the HA1-HA2 boundary. Stem antibodies which are non-neutralising may also be protective through Fc-mediated mechanisms (reviewed by Jegaskanda, 2018).

Antibodies which show broad strain reactivity tend to achieve breadth by targeting highly conserved areas. Such areas include a small region in the head domain around the

receptor binding site and the fusion peptide in the stem. The need for residues in these two sites to make direct contacts with sialic acid and the endosomal membrane, respectively, explains both their high degree of conservation and the effectiveness of antibodies which target them. The critical role of these sites in effectuating hemagglutinin function also make them prime candidates for inactivating mutations. As such, many of the mutations identified in the previous section are located in or near important antibody binding epitopes. For instance, residues towards the C-terminal end of the fusion peptide make contacts with the antibody MEDI8852 which potently neutralises a vast range of hemagglutinins across groups 1 and 2 (Kallewaard et al., 2016). Testing the effect of candidate mutations on antibody binding is critical, and mutations which disrupt the most common protective targets should be avoided.

Avoiding egg-adaptations

Egg adaptation mutations are known to arise in mammalian viruses grown in embryonated eggs (Raymond et al., 2016). This is largely due to differences in sialic acid expression, with avian cells typically expressing α -2,3 rather than α -2,6 linked terminal sialic acid. Egg-grown vaccines may therefore acquire adaptations which improve their growth in eggs but alter their ability to bind mammalian sialic acids.

As the receptor binding region is highly immunogenic, mutations which alter the structure of the site have significant effects on antibody responses to hemagglutinin. This is especially problematic in some recent human H3N2 viruses which grow particularly poorly in eggs and therefore are under greater pressure to acquire mutations during growth (Parker et al., 2016). One such mutation is T160K, which removes an N-glycosylation motif present in many H3 hemagglutinins circulating after 2014 (Zost et al., 2017). Antibodies generated by immunisation with egg-adapted vaccines containing T160K tend to bind well to other non-glycosylated hemagglutinins but react poorly with circulating viruses which are glycosylated. This is thought to have contributed to poor vaccine efficacy in some seasons after 2014. Other mutations around the receptor binding region associated with passage in eggs such as at positions 190 and 156 also alter antigenicity, although not necessarily alongside large effects on sialic acid binding. Conversely, egg-adaptations at other nearby sites - for example at position 186 - affect binding without affecting antigenicity (Parker et al., 2016).

Strain choice

With the design of CLEARFLU, we set out to propose a complement of mutations which can be used to inactivate any type A hemagglutinin. Paired with the ability to generate single-cycle recombinant viruses in cell culture, this could offer a universal platform for generating vaccines against all strains. In this thesis, CLEARFLU hemagglutinins are designed both in human/swine seasonal subtypes (H1 and H3) and in a non-seasonal subtype with pandemic potential (H7) as proof-of-concept.

The hemagglutinin from A/Hong Kong/5738/2014 was selected as the exemplar H3 strain. It sits in the 3C.2a clade which is ancestral to currently circulating seasonal H3N2 strains and is known to generate broad reactivity both within and across clades, including current strains (ECDC Influenza Virus Characterisation Reports, Summary Europe, March 2019).

The H1 representative hemagglutinin is from A/England/195/2009, a strain isolated during the 2009 pandemic which originated from a reassortment event in pigs and went on to transmit well between humans. It usually caused mild disease, and its descendants now circulate in humans as a seasonal infection.

We were also interested in whether we could use CLEARFLU hemagglutinins to produce vaccines against high-threat viruses. Human H7N9 infections from poultry have been recurring in waves in China since 2013, with the fifth wave identified in 2017. This thesis uses the strain A/Hong Kong/125/2017 as a representative of the fifth wave Yangtze Delta viruses, which are the highest rated in the CDC's influenza risk assessment tool (IRAT) at the time of writing. The IRAT is used to assess the potential for novel viruses to develop human-to-human transmission and the impact this could have on public health. The A/Hong Kong/125/2017 virus itself is a lower pathogenicity strain relative to some other avian viruses, with a case fatality rate of 40%.

3.1.3 Summary and CLEARFLU candidate mutations

We call our candidate vaccine platform CLEARFLU, referring to the eight central characteristics of its design:

Cleavage resistance

Locked by inter-monomer disulphide bonds

Egg adaptations avoided

Antigenicity maintained

Receptor binding abolished

Fusion peptide inhibited

Loop inactivation by proline residues

Universal platform for all strains

The candidate mutations selected for testing as part of the CLEARFLU approach are listed in Table 3.1 and their locations in the hemagglutinin molecule and sequence are shown in Figures 3.1 and 3.2 respectively. The CLEARFLU design was refined over three iterations to optimise its properties. This chapter describes that process.

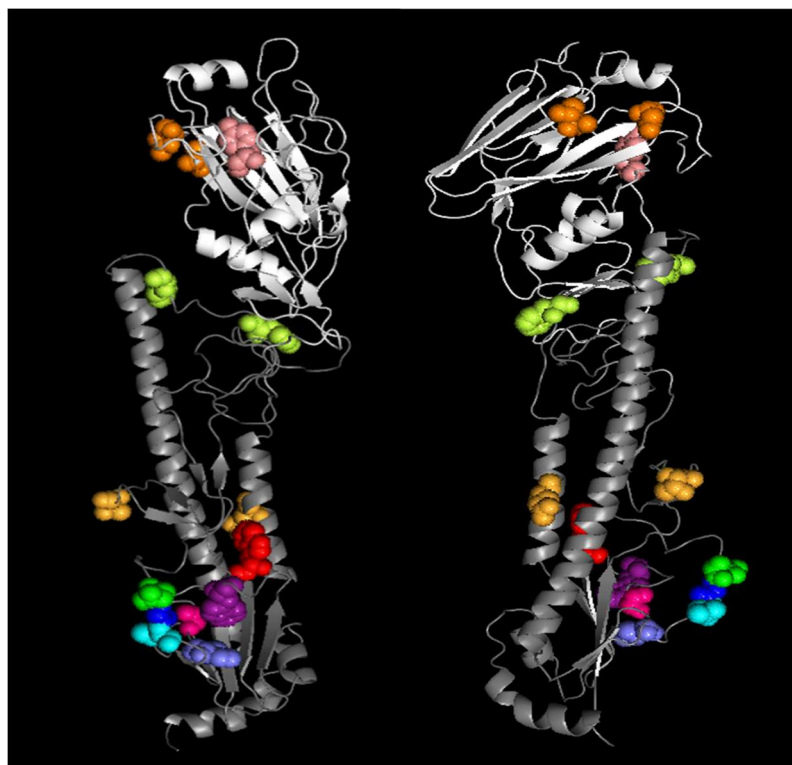


Figure 3.1 - CLEARFLU mutation sites in an H3 hemagglutinin

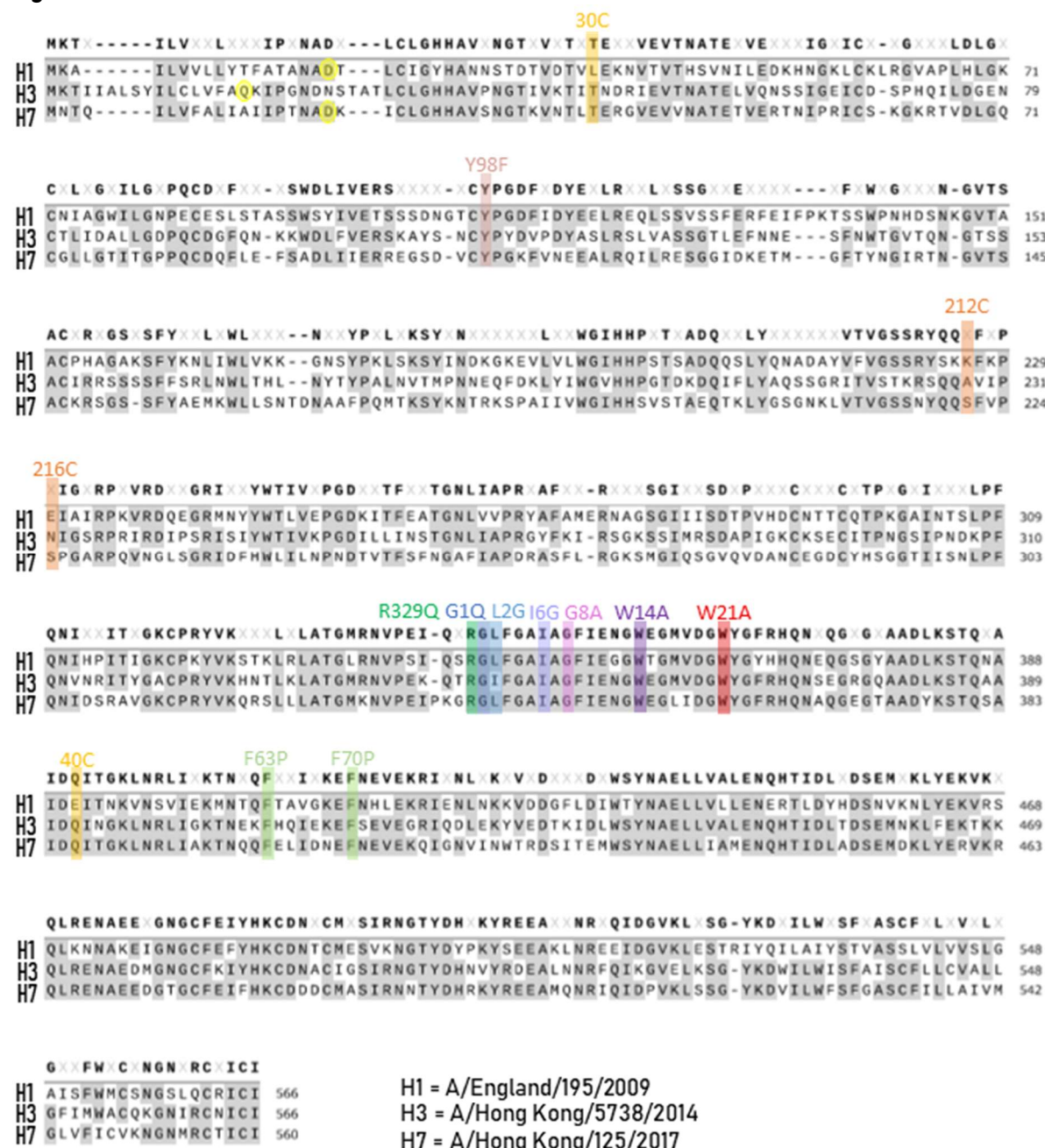
H3N2 (A/Aichi/2/1968) from Chen et al., 1998 (PDB: 1HA0).

Mutated residues are shown as coloured spheres according to the scheme in Table 1 on a ribbon diagram background. The globular head domain is shown in light grey and the stalk in dark grey.

Table 3.1 – Mutations introduced to inactivate wild-type hemagglutinin genes

Mutation type	Mutation position	Rationale	CLEARFLU version		
	(H3 numbering system- see Burke & Smith, 2014)		1	2	3
Cleavage resistance	HA1- R329Q	Cleavage of the precursor HA0 into HA1 and HA2 by trypsin is necessary for conformational changes required for cell entry. Viruses with mutations at the cleavage site cannot replicate due to changes in protease sensitivity (Chen et al, 1998; Orlich et al, 1995).	X	X	X
Locked by inter-monomer disulphide bonds	Head: HA1- 212C & HA1- 216C	The introduction of inter-chain disulphide bridges between head regions prevents membrane fusion for cell entry (Godley et al, 1992). Disulphide bridges between stem regions increase the stability of the hemagglutinin trimer and may prevent fusion (Lee et al, 2015).	X	X	X
	Stem: HA1- 30C & HA2- 47C		X	X	X
Receptor binding abolished	HA1- Y98F	The change from tyrosine to phenylalanine prevents the formation of a hydrogen bond with sialic acid residues necessary for binding (Skehel & Wiley, 2000) and attenuates influenza in mice, but has a high rate of back-mutation (Meisner et al, 2008).	X	X	
Fusion peptide inhibited	HA2- G1Q , HA2- L2G	Mutations in the fusion peptide abolish fusion. The residues which are most highly conserved are most likely to block function when mutated (Cross et al, 2009).	X	X	X
	HA2- W14A , HA2- W21A		X		
	HA2- I6G , HA2- G8A			X	X
B loop inactivation by proline residues	HA2- F63P & HA2- F70P	Introducing two proline residues in the B loop prevents conformational changes needed for fusion (Gruenke et al, 2002).			X

Figure 3.2



MUSCLE alignment of H1, H3 and H7 wild-type hemagglutinin protein sequences with candidate CLEARFLU mutations highlighted

The consensus sequence is shown above the alignment where >50% consensus is met. Yellow circles indicate the first residue in the mature protein after cleavage of the signal peptide, as determined using the SignalP server (version 5.0) (Petersen et al., 2011) and described in Burke and Smith, 2014.

3.2 Results

3.2.1 CLEARFLU version 1

CLEARFLU version 1 sequences in H1 (A/England/195/2009) and H3 (A/Hong Kong/5738/2014) genetic backgrounds were subcloned into vectors pcDNA3.1- and pHR-SIN. The plasmids were sequenced before being used to generate transiently transfected 293T or stably transduced MDCK-SIAT cells, respectively. Cell lines expressing wild-type versions of the same hemagglutinins were also produced.

The proper folding of CLEARFLU proteins expressed in cell lines was assayed using monoclonal antibody binding panels. These antibodies are described in Table 3.2. Figures 3.3 and 3.4 show staining profiles for transfected 293T cells. As the transfection is transient and cells were not sorted, not all cells express hemagglutinin and a range of fluorescence values are observed. Although this experiment is not powered to make quantitative comparisons of the quality of binding to wild-type and CLEARFLU hemagglutinins, it is evident from median fluorescence values that antibodies targeted to the head of hemagglutinin are able to bind to CLEARFLU. The exception to this is AR1D, which binds site B on the H3 head. This suggests that the structure and antibody binding epitopes of the head are largely conserved despite the introduction of Y98F, 212C and 216C in the head region.

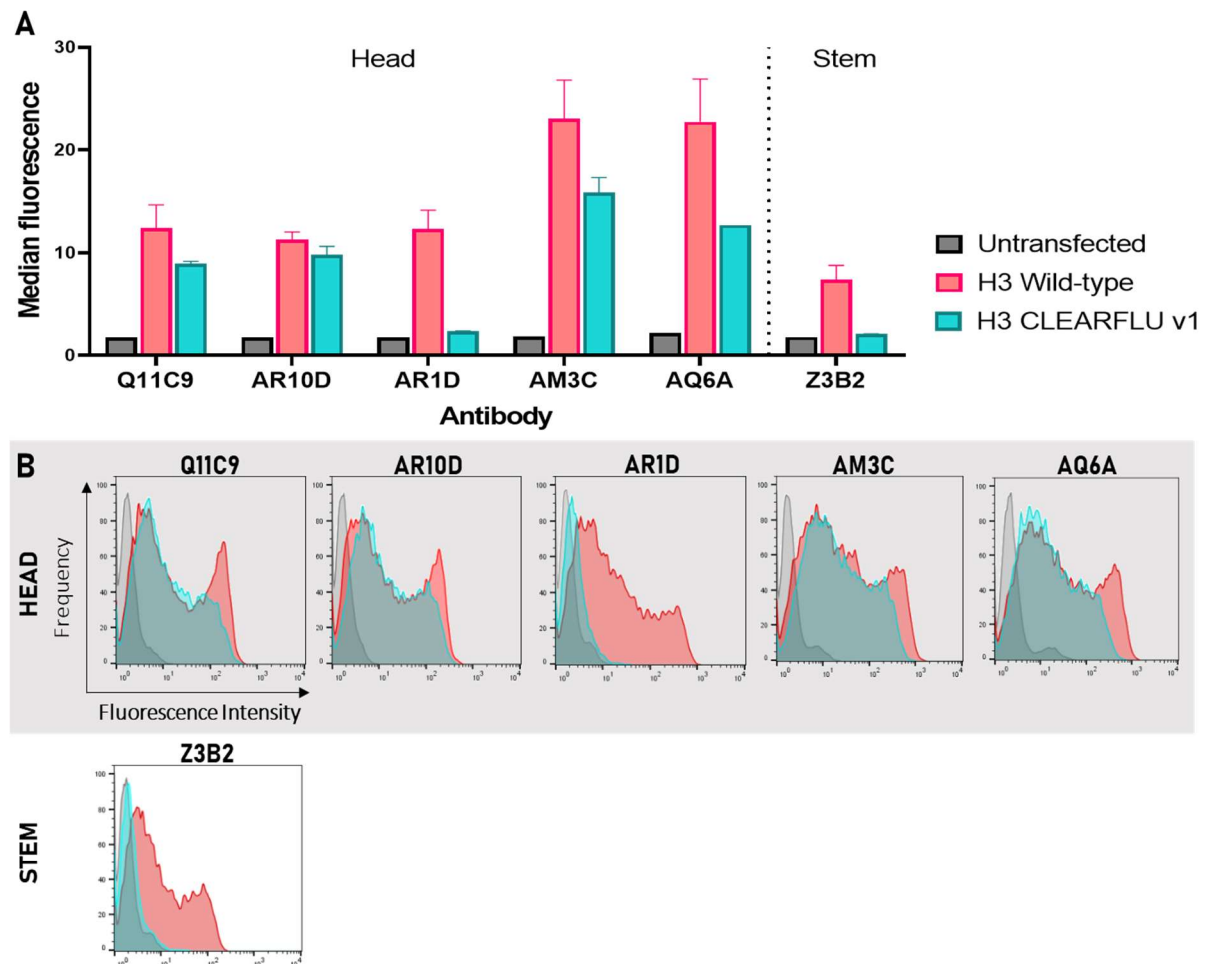
In contrast, binding of monoclonal antibodies to the stem region was greatly reduced for CLEARFLU relative to wild-type hemagglutinin. This was observed both for H1 and H3 hemagglutinins. The only indication that there was any specific binding by a stem antibody was for MEDI8852 on H1 CLEARFLU, where there appears to be a small population of cells with some low-level binding.

Table 3.2 – Key antibodies used in this chapter

Antibody	Specificity	Site	Comments	Reference
Q11C9	H3 head	R142 (Site A)	Selected R142G escape with virus X-217 (A/Victoria/361/2011)	In-house
AR10D	H3 head	no information		In-house
AM3C	H3 head	F193 (Site B)	Selected F193Y escape with virus X-263A (A/Hong Kong/4801/2014)	In-house
AQ6A	H3 head	Site B	Selected F159S escape with virus X-217 (A/Victoria/361/2011)	In-house
BS-2D	H3 head	Site B	Selected F193S escape with virus X-263A (A/Hong Kong/4801/2014)	In-house
AR1D	H3 head	Site B	Selected F193V/L escape with virus X-263A (A/Hong Kong/4801/2014)	In-house
2-12C	H1 head (H1N1pdm09 only)	K130 (Ca site)	Selected K130E escape with virus X-179A (A/California/07/2009)	Huang et al., 2015
T2-7D	H1 head (H1N1pdm09 only)	K163 (Sa site)	Does not bind to K163E/Q virus (A/California/07/2009)	Huang et al., 2015
T3-3C	H1 head (H1N1pdm09 and pre-2009 seasonal H1N1)	K163 (Sa site)	Does not bind K163E/Q virus (A/California/07/2009)	Huang et al., 2015
M6-B11	H1 head	Ca site	Does not bind to K130E virus (X-179A)	In-house
Q11A6	H1 head	Ca site	Does not bind to K130E virus (X-179A)	Huang et al., 2015
4A14 (L4A-14)	H7 head	Site B	Neutralised 2013 and 2016–2017 avian H7 but not 2003 avian H7 or seasonal H3	Huang et al., 2019
9B26 (L9B-26)	H7 head	no information	Neutralised 2013 and 2016–2017 avian H7 but not 2003 avian H7 or seasonal H3	Huang et al., 2019
11B46 (L11B-46)	H7 head	no information	Neutralised 2013 and 2016–2017 avian H7 but not 2003 avian H7 or seasonal H3	Huang et al., 2019
12B36 (L12B-36)	H7 head	no information	Neutralised 2013 avian H7 but not 2016–2017 or 2003 avian H7 or seasonal H3	Huang et al., 2019
W3A1	H7/H3 head	no information	Neutralised 2003, 2013, 2016–2017 avian H7s and seasonal H3	Huang et al., 2019
Z1B10	H7/H3 head	no information	Neutralised 2003, 2013, 2016–2017 avian H7s and seasonal H3	Huang et al., 2019
T1-3B	Group 1	Stem	Neutralised H1 and H5 viruses	Huang et al., 2015
MEDI8852	Group 1 and group 2	Stem	Binds to all H1–H16 subtype viruses	Kallewaard et al., 2016
T2-5C	Group 1	Stem	Neutralised H1 and H5 viruses	Huang et al., 2015

T3-5D	Group 1	Stem	Neutralised H1 and H5 viruses	Huang et al., 2015
Z3B2	Group 1 and group 2	Stem	Neutralised H1, H5 and H7 viruses	Huang et al., 2019

Figure 3.3



Antibody binding to H3 CLEARFLU and wild-type hemagglutinins expressed in transiently transfected 293T cells

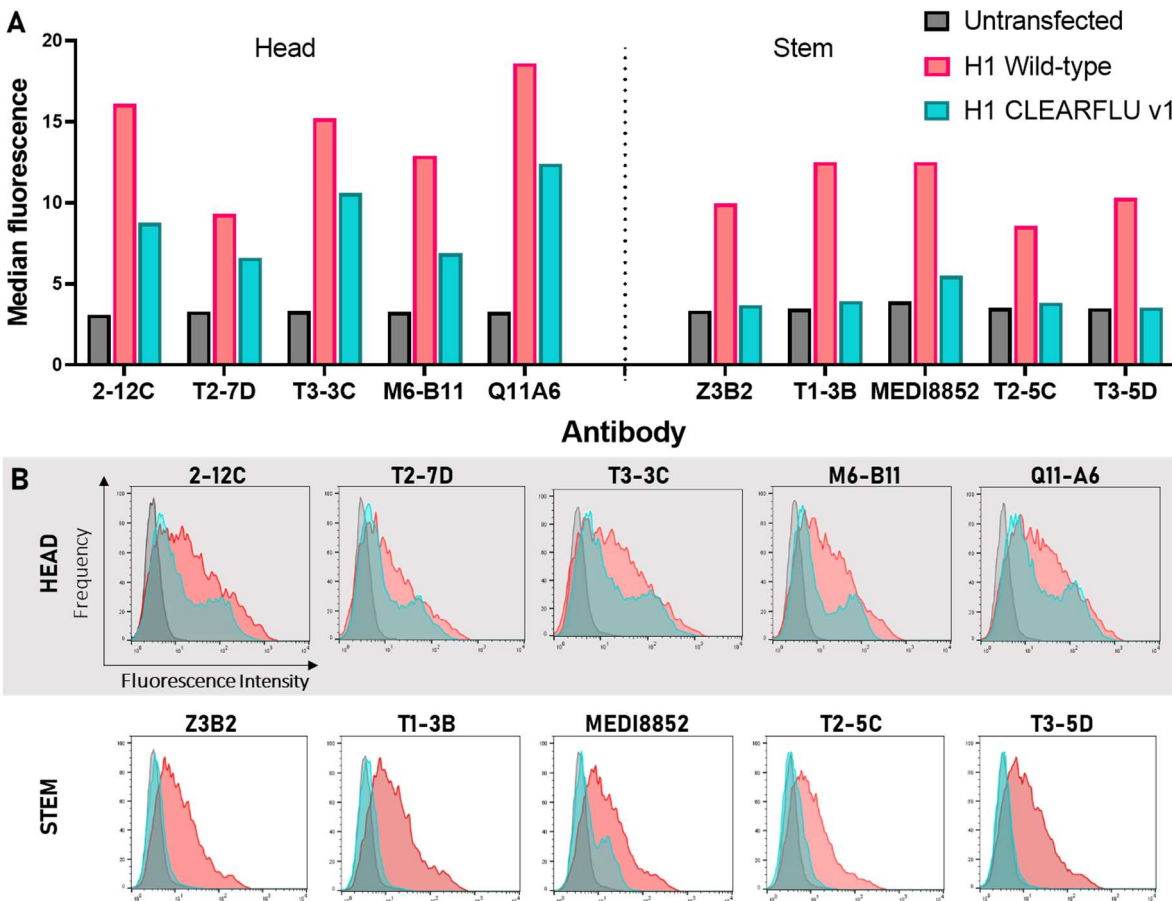
Hemagglutinins were bound with a range of head or stem specific human primary antibodies and binding was detected with a FIT-C labelled secondary antibody. This experiment was performed on unsorted cells.

A. Bar graph comparing median fluorescence intensity of cells bound by different primary antibodies. Error bars represent standard deviation based on two repeat populations within a single experiment. No error bars are shown for untransfected cells as only one measurement was made for each antibody.

B. Histogram plots showing the relative frequency of cells detected with a given fluorescence intensity. For cells expressing wildtype or CLEARFLU hemagglutinin, data from one of two repeats is shown.

The antibody binding epitope for MEDI8852 is known to include latter parts of the fusion peptide, certain residues of helix A in HA2 and a nearby hydrophobic groove (Kallewaard et al., 2016). Notably, contacts are made with the tryptophan at position 21. Z3B2 isolated by our lab is thought to have an overlapping binding site with MEDI8852 as it competes for binding (Huang et al., 2015). The loss of stem antibody binding to CLEARFLU is therefore perhaps caused by mutations we have introduced towards the end of the fusion peptide, namely W21A and W14A.

Figure 3.4



Antibody binding to H1 CLEARFLU and wild-type hemagglutinins expressed in transiently transfected 293T cells

Hemagglutinins were bound with a range of head or stem specific human primary antibodies and binding was detected with a FIT-C labelled secondary antibody. This experiment was performed on unsorted cells. Data represent a single experiment, with the results supporting previous work on H3 CLEARFLU in 293T cells and later experiments in sorted MDCK-SIAT cells.

A. Bar graph comparing median fluorescence intensity of cells bound by different primary antibodies.

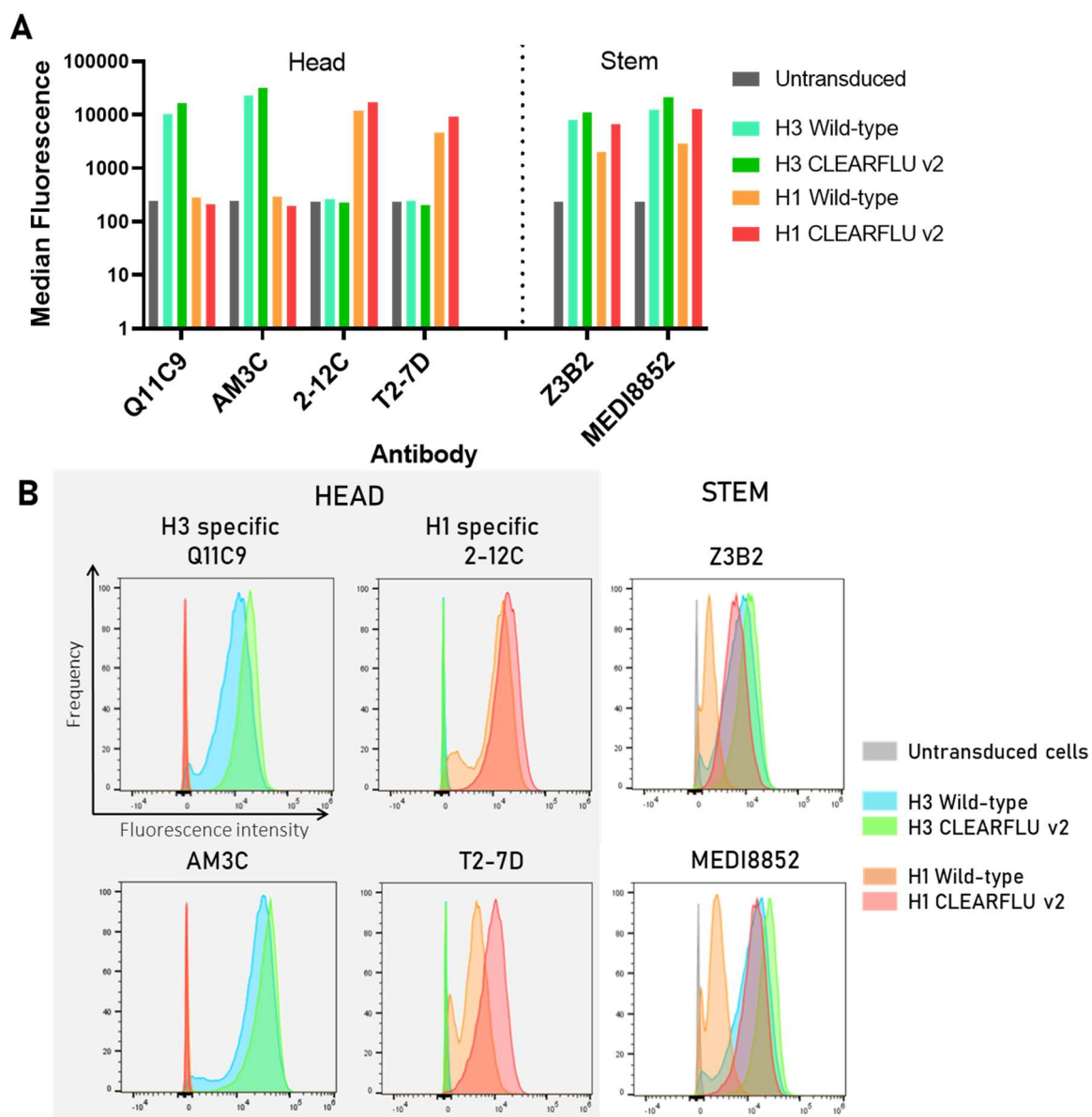
B. Histogram plots showing the relative frequency of cells detected with a given fluorescence.

3.2.2 CLEARFLU version 2

The hypothesis that the loss of stem epitopes was due to mutations at positions 21 and 14 of the fusion peptide was tested by designing an alternative CLEARFLU with a change in the mutations used to inactivate the fusion peptide. W14A and W21A were replaced with I6G and G8A to attempt to restore stem antibody binding while maintaining the same number of inactivating mutations. This time, MDCK-SIAT cells stably expressing hemagglutinin were used to assay antibody binding (see Figure 3.5). After being transduced to express hemagglutinin, the cells were sorted by fluorescence-activated cell sorting with a head binding antibody (AM3C for H3 or T3-3C for H1) to ensure high level expression of the desired protein. These sorted cells were cultured to produce cell stocks for use in experiments.

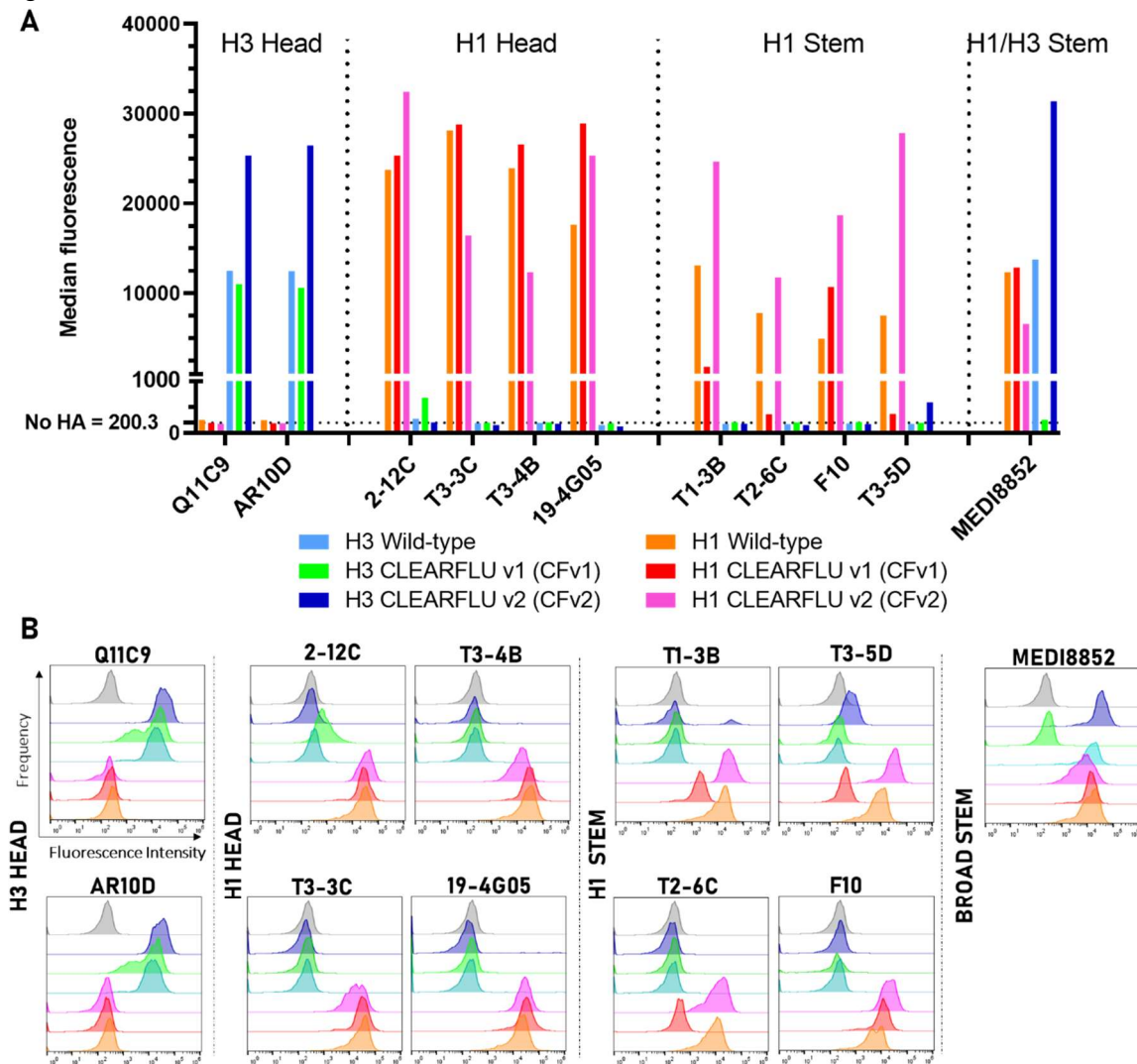
Due to possible variation in protein expression level, caution must be exercised when trying to directly compare fluorescence between cell lines. The median fluorescence for wild-type H1 is brought down by a small population of cells with low fluorescence. Even accounting for this, binding by T2-7D, MEDI8852 and Z3B2 appears to be stronger for H1 CLEARFLU version 2 than H1 wild-type as the main peak is shifted to the right (Figure 3.5.B). It is possible that this is due to increased epitope stability caused by the introduction of disulphide bridges. It is unlikely to be fully explained by differences in protein expression level as this difference is not observed for antibody 2-12C. For H3 hemagglutinins, binding appears to be similar for wild-type and CLEARFLU for all antibodies tested. These results suggest that the change in fusion peptide mutations has been sufficient to restore antibody binding epitopes in the stem of CLEARFLU in both H1 and H3 backgrounds.

Figure 3.5



A further experiment was conducted to confirm that stem epitopes were lost in CLEARFLU version 1 (CFv1) and restored in CLEARFLU version 2 (CFv2). Both versions were expressed in MDCK-SIAT cells and compared to each other and to wildtype hemagglutinin (see Figure 3.6).

Figure 3.6



Antibody binding to CLEARFLU versions 1 & 2 and wild-type hemagglutinins expressed in sorted stably transduced MDCK-SIAT cells

Hemagglutinins were bound with head or stem-targeted human primary antibodies and binding was detected with a FIT-C labelled goat anti-human secondary antibody. The data represent the results of a single experiment conducted to confirm results from earlier studies.

A.. Bar graphs comparing median fluorescence of each cell line when bound by different primary antibodies. The average median fluorescence of three populations of unstained cells which were not transduced to express hemagglutinin is shown as a no HA control.

B. Histogram plots showing the relative frequency of cells detected with a given fluorescence intensity. The plots are staggered for easier visualisation of the peaks. For baseline comparison, grey plots show a population of unstained cells which were not transduced to express hemagglutinin.

In support of previous data, antibodies targeted to the hemagglutinin head were able to bind both versions of the CLEARFLU design in both strains. Looking at the H1 stem antibodies, F10 was able to bind CFv1 and CFv2, while T2-6C, T3-5D and T1-3B showed reduced binding against CFv1. This data confirms results for T1-3B and T3-5D from Figure 3.4 showing binding is lost for CFv1, and demonstrates for the first time that, like for MEDI8852 and Z3B2 in Figure 3.5, binding is restored for CFv2. For four of the five stem antibodies tested here, median fluorescence for H1 CFv2 was stronger than for the wildtype hemagglutinin. This was not seen for MEDI8852 as had been observed in Figure 3.5.

Interestingly, the broadly reactive stem antibody MEDI8852 seems to bind to all the hemagglutinins except for H3 CFv1. This confirms the result from Figure 3.5 that MEDI8852 is able to bind well to H1 and H3 CFv2, and appears to suggest that MEDI8852 binds differently to H1 and H3 CFv1. Such an effect could be caused by differences in the angle of antibody binding to H1 and H3 hemagglutinins or differences in the effect of mutations on the structure of the antibody binding footprint. This observation is not investigated further as this thesis is concerned more with the general loss of stem epitopes than the binding characteristics of individual antibodies.

In summary, the fluorescent antibody binding assays presented in Figures 3.3–3.6 show that of seven stem antibodies tested, six showed reduced binding to CFv1. Five of these were tested against CFv2 and all were able to bind, showing that binding can be recovered by altering residues in the fusion peptide.

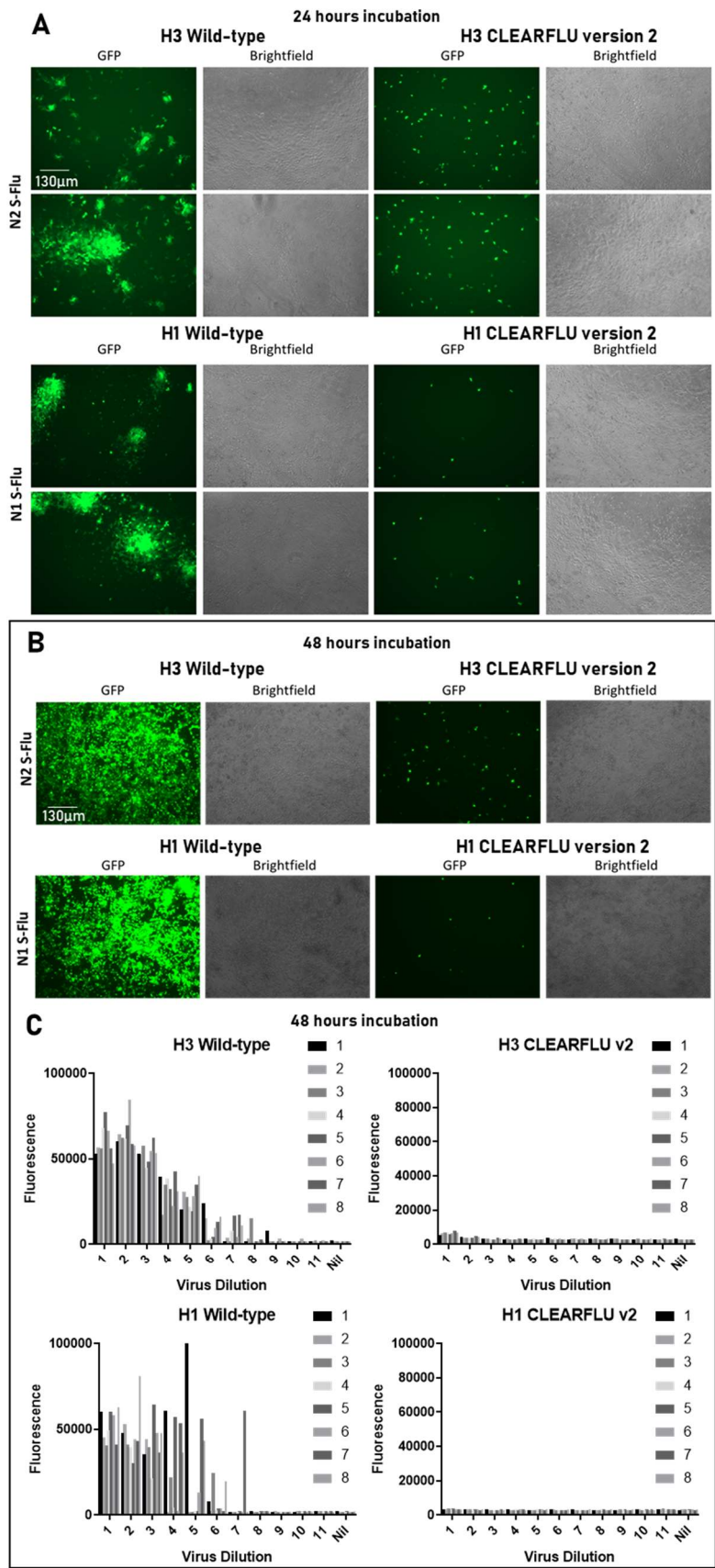
While broadly cross-reactive stem antibody Z2B3 showed the same pattern of epitope loss and recovery for both H1 and H3 CLEARFLU, MEDI8852 only lost binding to H3 CFv1. F10, an antibody which binds H1 but not H3 hemagglutinins and has a binding footprint that includes W21 (Sui et al., 2009) still bound well to H1 CFv1. This demonstrates variation in how stem antibody binding is affected by stem mutations.

3.2.3 Testing hemagglutinin function

Having determined that the CLEARFLU version 2 mutations do not cause major disruptions to protein folding, it was important to confirm that they do indeed abolish hemagglutinin function as expected.

Hemagglutinin function was assayed by infecting cell lines with fluorescent S-Flu virus. S-Flu expressing eGFP is able to replicate only in cells expressing functional hemagglutinin, producing green comma-shaped plaques as it spreads between adjacent cells. Figure 3.7 shows that S-Flus do not replicate in cells expressing H1 or H3 CLEARFLU version 2 hemagglutinins, suggesting that viruses with CLEARFLU version 2 but no wildtype hemagglutinin on their surface are not infection competent. Two different S-Flus with N1 and N2 neuraminidases were used in this experiment to ensure that compatible combinations of neuraminidase and hemagglutinin genes were used for efficient growth. This experiment was performed simultaneously alongside the immunofluorescence assay described in Figure 3.5 to show that the lack of replication in CLEARFLU expressing cells was not due to low expression.

Figure 3.7



Replication of S-Flu viruses after 24 or 48 hours in MDCK-SIAT cells transduced with wild-type and CLEARFLU version 2 hemagglutinins

GFP-expressing S-Flu viruses were used to infect cell lines stably expressing wild-type or CLEARFLU v2 hemagglutinins. Cells expressing H1 hemagglutinins were infected with N1 S-Flu and those expressing H3 were infected with N2 S-Flu. Viruses were added to cells in 96-well plates in half-log dilutions as per the quantification by TCID50 protocol, starting at approximately 1000 TCID50 per well. No virus was added to the final well. 8 replicates were used at each dilution.

Viruses:
[S-eGFP/N2(A/Victoria/361/2011)]
pseudotyped with
H3(A/Switzerland/9715293/2013)
& [S-eGFP/N1(A/England/195/2009)]
pseudotyped with
H7(A/Netherlands/219/2003)

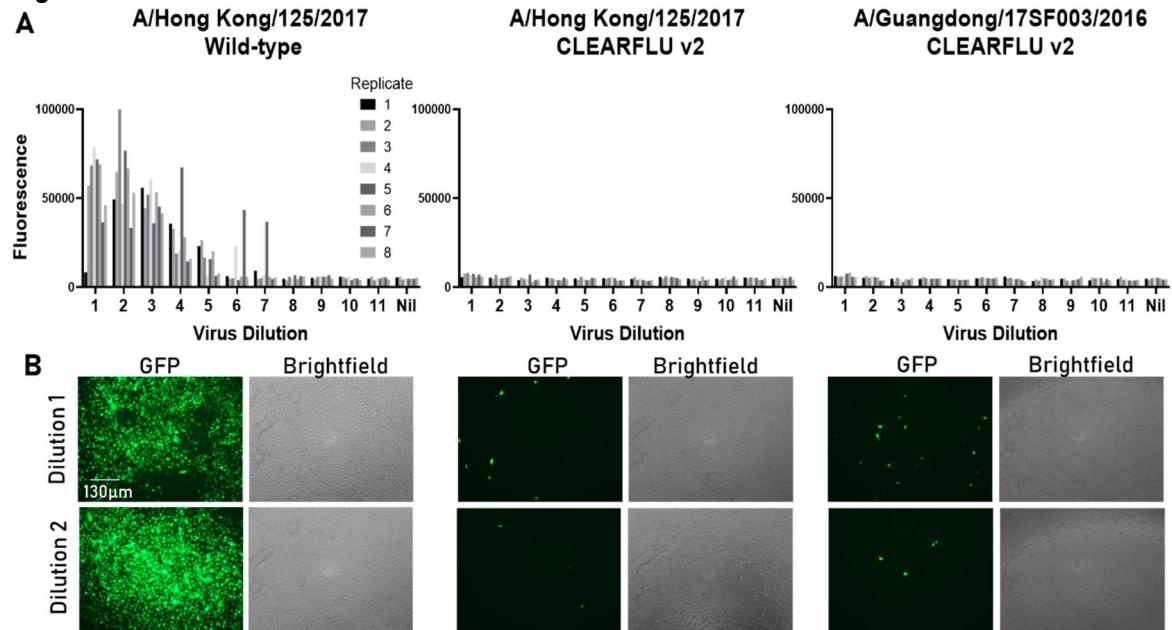
A. Brightfield and fluorescence images show single cell infections in a confluent cell monolayer in cell lines transduced with CLEARFLU v2 hemagglutinins, but clusters of infection in cells expressing functional hemagglutinin. Images were taken at dilution 1, equivalent to approximately 1000 TCID50 per well, after 24 hours.

B. Images show that after 48 hours in the presence of wild-type hemagglutinin the virus has spread to infect almost all cells. Images were taken at dilution 1.

C. Bar graphs showing the intensity of GFP fluorescence in each well after 48 hours. Each plot represents a single 96-well plate, with 8 replicates at each half-log dilution. At later dilutions, expansions of single viral clones can be seen as erratic peaks.

S-Flu was also unable to grow in cells expressing CLEARFLU version 2 hemagglutinins from H7 A/Hong Kong/125/2017 and from H7 A/Guangdong/17SF003/2016 (see Figure 3.8, cell surface expression confirmed in Figure 3.11).

Figure 3.8



Replication of S-Flu viruses after 24 hours in sorted MDCK-SIAT cells transduced with H7 wild-type and CLEARFLU version 2 hemagglutinins

H7N1 S-Flu ([S-eGFP/N1(Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)) was used to infect cell lines stably expressing wild-type or CLEARFLU version 2 hemagglutinins. Viruses were added to cells in 96-well plates in half-log dilutions as per the quantification by TCID50 protocol, starting at approximately 1000 TCID50 per well. No virus was added to the final well. 8 replicates were used at each dilution.

A. Bar graphs showing the intensity of GFP fluorescence in each well after 48 hours. Each plot represents a single 96-well plate, with 8 replicates at each half-log dilution.

B. Brightfield and fluorescence images show single cell infections or expanding plaques. Images were taken at dilutions 1 and 2.

From these results, it is clear that the combination of mutations in the CLEARFLU version 2 design is sufficient to abolish hemagglutinin function. It is important to understand how the individual mutations contribute to inactivation to estimate the rate at which we expect CLEARFLU to back-mutate to a functional form. This would also help to refine the design by removing mutations which do not contribute to inactivation but may disturb peptide or conformational epitopes.

Figure 3.9 describes an experiment in which nine cell lines, each expressing hemagglutinin with a single inactivating mutation, are assayed for their ability to permit replication of S-Flu. Mutations were introduced by site-directed mutagenesis of hemagglutinin in a pcDNA3.1- plasmid, then the mutated hemagglutinins were subcloned into pHR-SIN plasmids to generate lentiviruses for stable transduction of MDCK-SIAT cells. The cell lines were sorted for high expression with antibody 4A14. The single mutants include all eight mutations in the CLEARFLU version 2 design, as well as one additional mutation over two sites (F63P/F70P) in the B loop. The mutations were introduced into the sequence from the H7 A/Hong Kong/125/2017 hemagglutinin rather than the human seasonal strains used for the CFv1 and CFv2 work. As the mutations were originally described in seasonal strains, this serves to test the generalisability of their action. Furthermore, given the pandemic potential of H7 hemagglutinins, it was important to verify that the mutations function in this background specifically before using them in a candidate vaccine.

Seven of the nine mutations were able to independently block S-Flu replication, suggesting they fully abolish hemagglutinin function. There was no evidence to suggest that there was any reduction in growth in the Y98F sialic acid binding site mutant. A hemagglutination inhibition assay conducted on CLEARFLU version 2 viruses which contain this mutation did not detect any CLEARFLU-mediated agglutination of red blood cells to indicate sialic acid binding activity (see Figure 3.10). All agglutination activity instead appeared to be attributable to the pseudotyping hemagglutinin which has an intact sialic acid binding site. These results support observations by others that Y98F leads to very poor binding and agglutination of erythrocytes but that this only leads to reduced infection in cells with low sialic acid expression (Martin et al., 1998; Meisner et al., 2008). This effect is therefore not seen in MDCK-SIAT cells, which over-express α -2,6 sialic acids (Matrosovich et al., 2003).

In contrast, the G8A mutation appeared to significantly reduce the size of viral plaques as even at the highest concentration of virus in dilution 1, only small clusters of infected cells were observed. Looking at the plots in Figure 3.9.B, unlike for wild-type or Y98F hemagglutinin, there were no erratic peaks in fluorescence in the middle dilutions to suggest the efficient expansion of single clones. This supports the conclusion that the intermediate fluorescence level for G8A is due to many viruses replicating poorly rather than a few viruses replicating efficiently. This suggests that G8A has reduced but not abolished fusion activity.

Figure 3.9

A

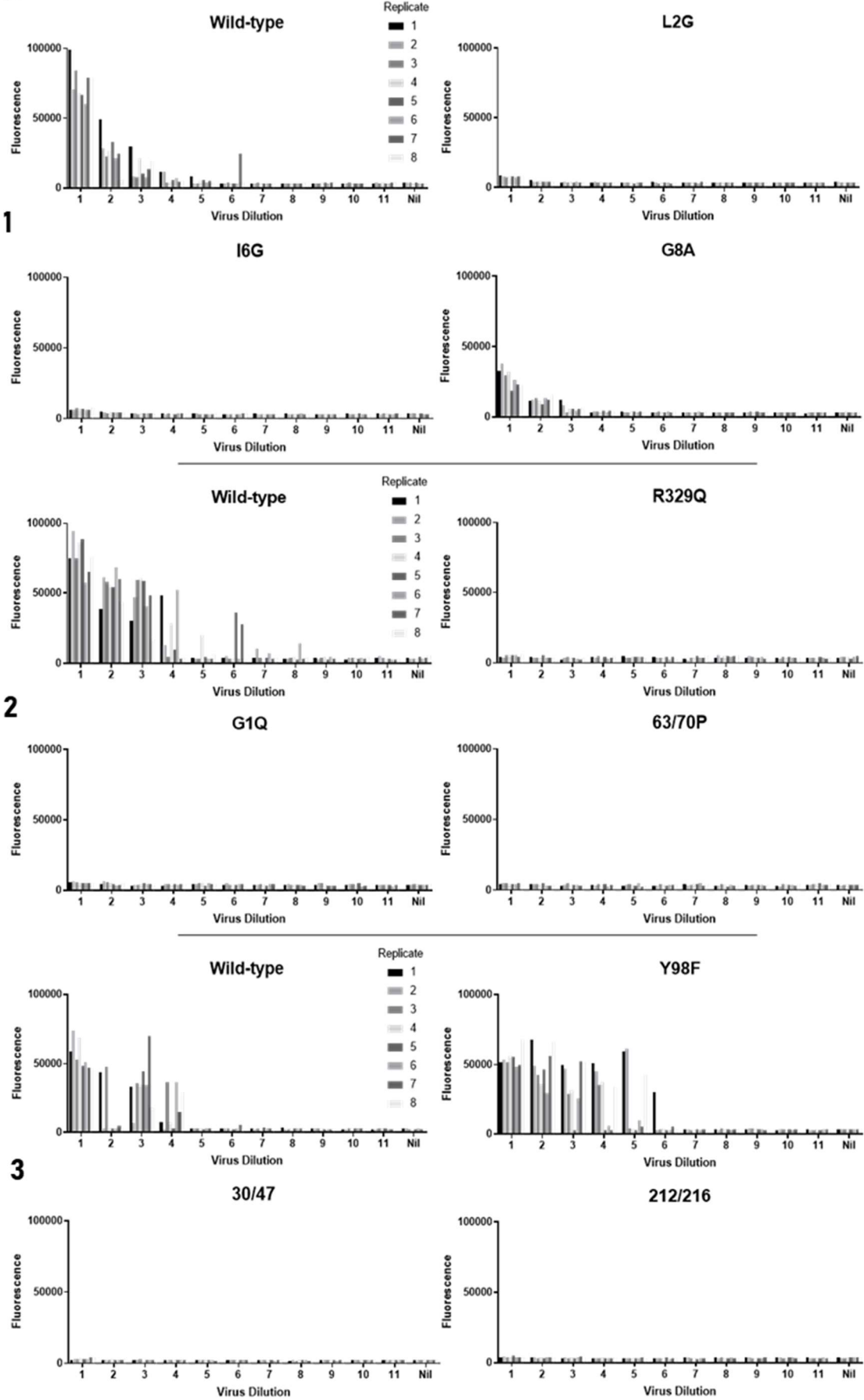
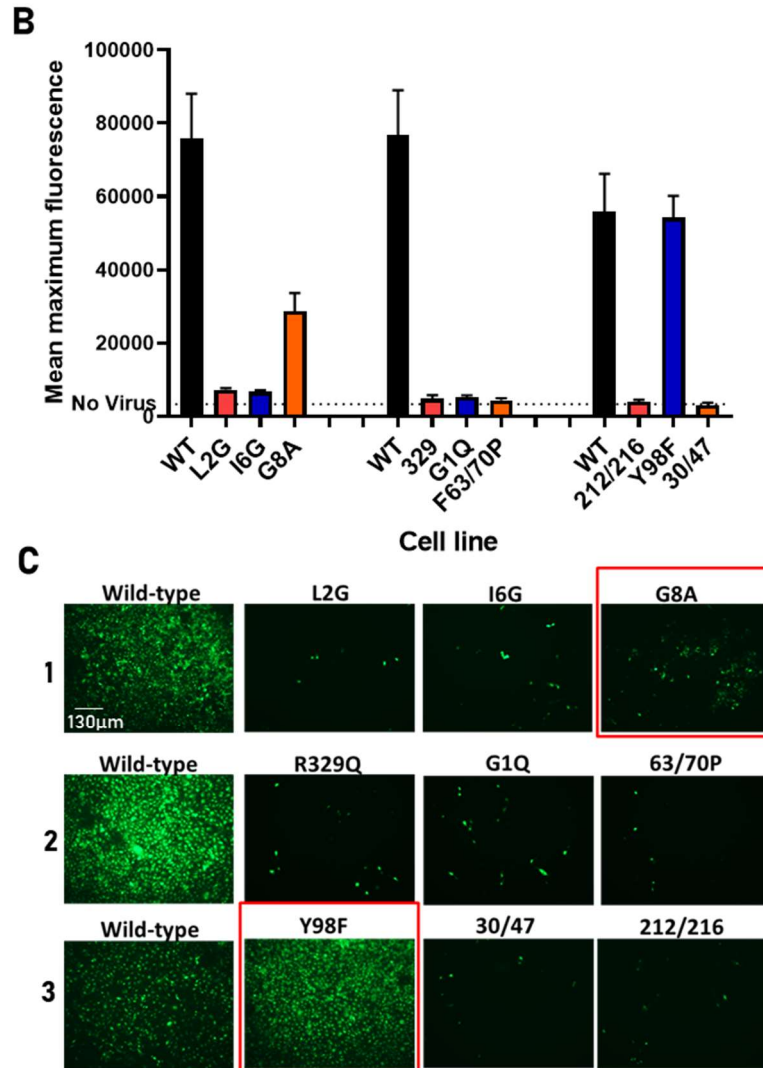


Figure 3.9 continued



Replication of S-Flu viruses in sorted MDCK-SIAT cells transduced with hemagglutinin each containing a single CLEARFLU candidate mutation

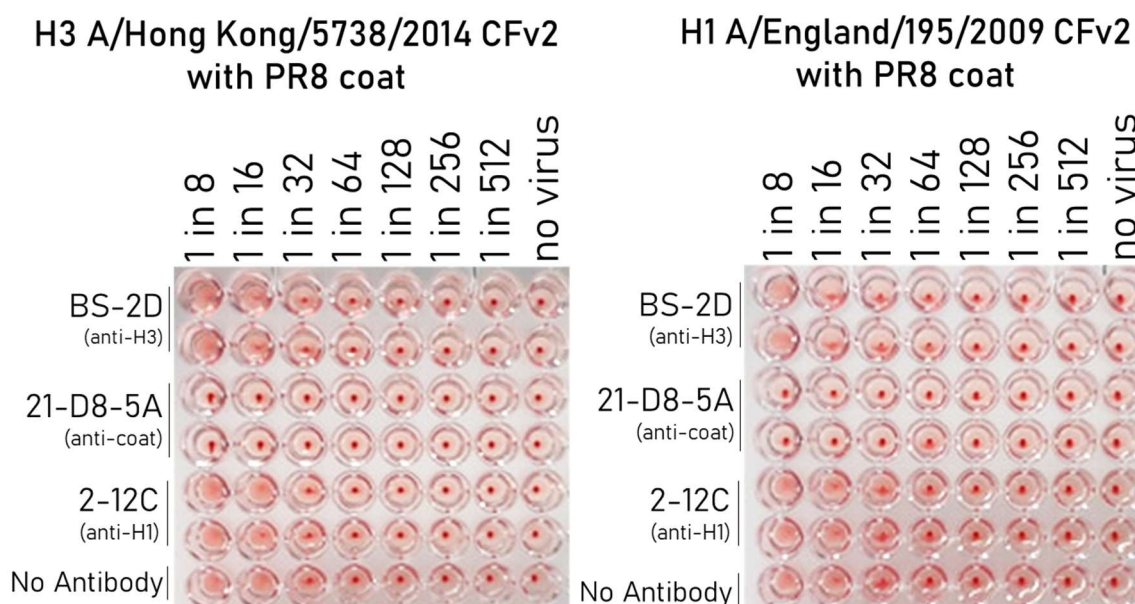
H7N1 S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)) was used to test whether individual mutations in the CLEARFLU design were sufficient to abolish hemagglutinin function. S-Flu was added to cells expressing mutated hemagglutinins in 96-well plates in half-log dilutions as per TCID50 protocol, starting at around 1000 TCID50 of virus per well. No virus was added to row 12. 8 replicates were used at each dilution. This experiment was split into 3 sets (1-3), each with a wild-type control. The data shown is from one experiment, which was repeated with the same results.

A. Bar graphs showing GFP fluorescence in each well. Each plot represents a 96 well plate with eight replicates at each viral dilution. Lone peaks around dilutions 5 and 6 show the expansion of single clones.

B. Mean fluorescence at maximum S-Flu concentration (dilution 1) in each cell line, with standard deviation calculated on eight replicate wells. The no virus line shows the mean of the no virus control wells across all plates.

C. Fluorescence microscopy images of cells expressing GFP after infection with fluorescent S-Flu. Images were taken at dilution 1, equivalent to approximately 1000 TCID50 per well. Clusters of infected cells indicate virus replication.

Figure 3.10

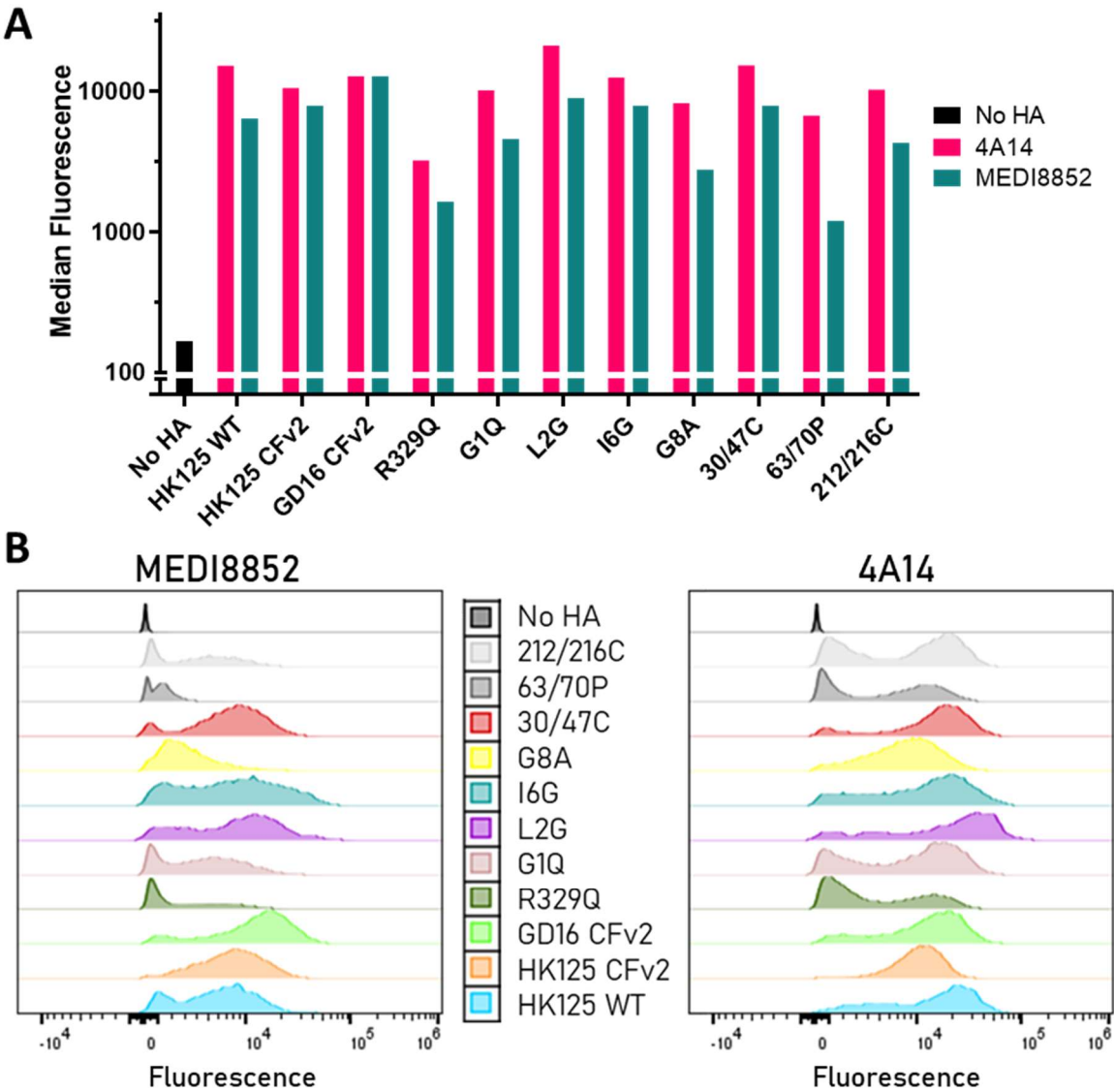


Agglutination of red blood cells by H1 and H3 CLEARFLU version 2 viruses

CLEARFLU version 2 viruses were titrated in doubling dilutions and incubated with 2µl of antibody at 25µg/ml for 1 hour before adding red blood cells to determine whether antibodies were able to prevent viruses from binding sialic acid on red blood cells and therefore prevent agglutination. BS-2D and 2-12C inhibit sialic acid binding by H3 A/Hong Kong/5738/2014 and H1 A/England/195/2009 respectively. 21-D8-5A inhibits binding by H1 A/Puerto Rico/8/1934, which was used to pseudotype both viruses. Agglutination was determined by the lack of red blood cell pellet at the bottom of a well. Rows 1,2,5 and 6 on each plate demonstrate that antibody blocking of the H3 or H1 CLEARFLU sialic acid binding site does not affect agglutination. Rows 3 and 4 show that inhibiting sialic acid binding by the pseudotyping PR8 coat abolishes agglutination, suggesting that it is the only protein mediating detectable sialic acid binding. Both assays were repeated with the same results.

Figure 3.11 shows the results of a FACS experiment performed on the same cell stocks described in Figure 3.9 to show that each cell line expressed the desired hemagglutinin on its surface.

Figure 3.11



Antibody binding to H7 A/Hong Kong/125/2017 hemagglutinins each containing a single CLEARFLU candidate mutation

MDCK-SIAT cells were stably transduced to express hemagglutinins and were sorted with antibody 4A14 before use. Hemagglutinins were bound with MEDI8852 or 4A14 and binding was detected with a FIT-C labelled secondary antibody. Unstained and untransduced cells are shown as a negative control (no HA). Parts A and B show the results of a single experiment.

A. Bar graphs comparing median fluorescence of different cell lines when bound by MEDI8852 or 4A14. Data are plotted on a log₁₀ scale as the data span a large range.

B. Histogram plots showing the relative frequency of cells detected with a given fluorescence intensity after staining with MEDI8852 or 4A14.

The inclusion of two prolines in the B-loop (63/70P) appeared to reduce binding by MEDI8852 to the stem relative to the other hemagglutinins (3.11.B; dark grey). Binding by 4A14 to the head of 63/70P appeared to be stronger than for MEDI8852, suggesting that the mutation may specifically disrupt epitopes in the stem. 212/216C and R329Q also appeared to show lower binding by MEDI8852 than 4A14 (3.11.B; light grey & dark green). However, binding to both A/Hong Kong/125/2017 CLEARFLU version 2 and A/Guangdong/17SF003/2016 CLEARFLU version 2 - which include all mutations except for 63/70P - was maintained for both antibodies, suggesting that this effect is not seen when mutations are used in combination.

These results together indicate that Y98F does not contribute to inactivation. How the attenuated growth in cell culture of the G8A mutant translates to virus characteristics *in vivo* is not as clear. We might therefore disregard G8A and conservatively estimate the back-mutation rate for CLEARFLU version 2 to a functional protein based on 6 independent mutations. Using an estimate of 10^{-5} to 10^{-4} for the per nucleotide per cell-infection mutation rate (Sanjuán et al., 2010), we might expect 1 in 10^{30} to 1 in 10^{24} cell infections to result in infectious virus if we assume that any change at a mutated site is restorative. As the virus is single-cycle, it does not have the opportunity to accrue mutations *in vivo* and the number of cell infections is directly proportional to the immunisation dose. With a world population of just under 8×10^9 , this means that we would need 10^{14} cell infections in every person to expect to generate a single replicative virus by back-mutation. This is approximately equivalent to immunising every cell in the human body (Sender et al., 2016). Further work investigating the stability of the viral genome during cell culture expansion is described in Chapters 4 and 5.

Although a useful illustration, mutations may have different effects in different strains, and so the actual rate of reversion to functionality may vary. For instance, the presence

of a poly-basic cleavage site in some H7 hemagglutinins may require mutations to prevent furin-based cleavage in addition to R329Q at the trypsin cleavage site.

In Figure 3.9, the presence of two proline residues in the B loop (F63/70P) was shown to abolish hemagglutinin function. Adding this mutation to the CLEARFLU design would therefore further decrease the likelihood of hemagglutinin reactivation to between 10^{-35} and 10^{-28} . As the Y98F mutation does not abolish function but has the potential to disrupt untested epitopes, we wished to investigate a further refinement to the CLEARFLU design in which the Y98F mutation is replaced with F63/70P.

In Figure 3.11 we had observed a possible reduction in the binding of MEDI8852 to hemagglutinin containing the F63/70P mutation. A similar effect was observed for 212/216C and R329Q, but these same mutations did not appear to disrupt binding to CLEARFLU version 2, which includes them alongside six other mutations. Therefore, we were interested in confirming the effect of F63/70P on stem binding in the context of other mutations before dismissing it as a candidate.

3.2.4 CLEARFLU version 3

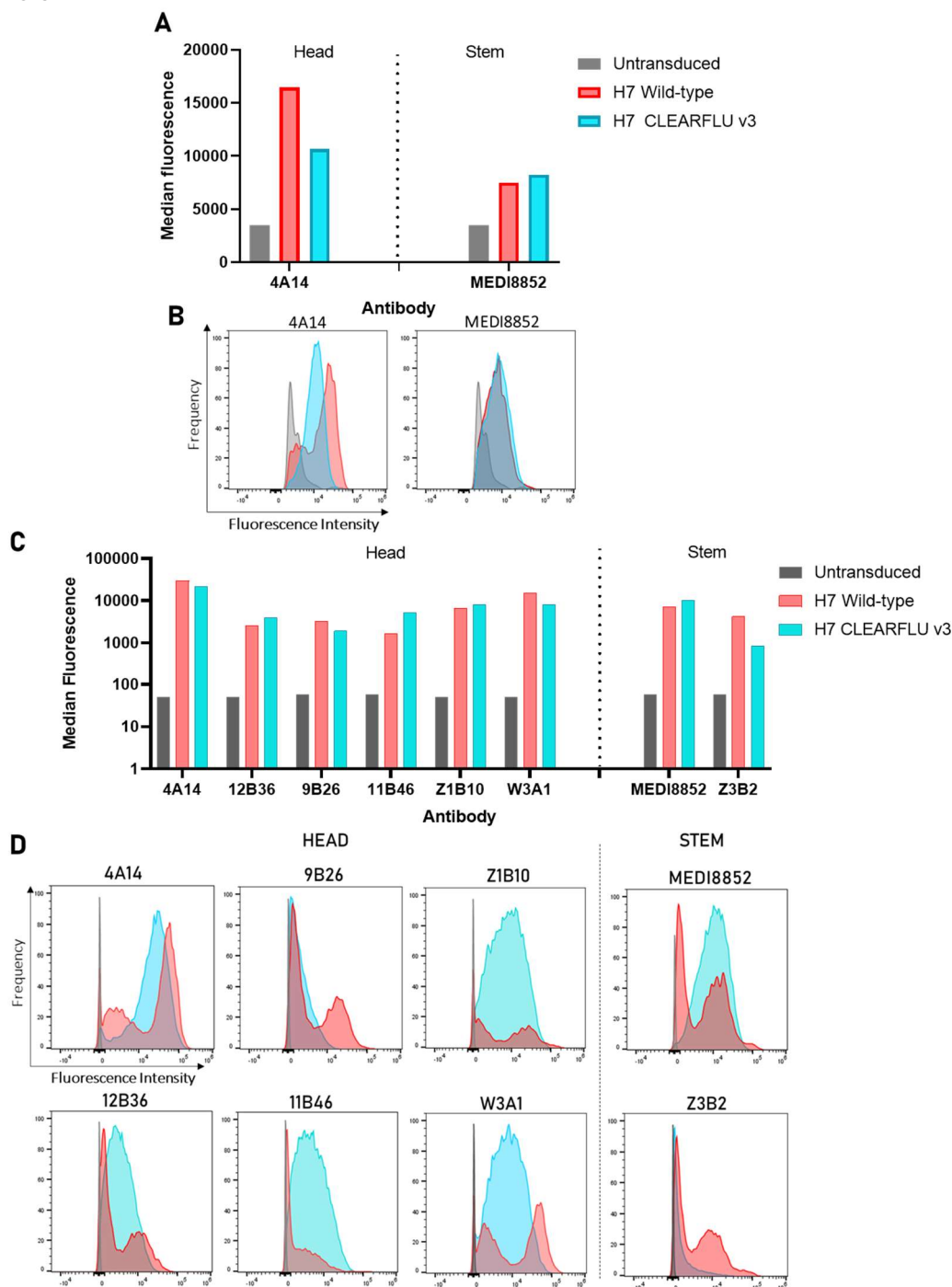
A CLEARFLU version 3 hemagglutinin was designed in an H7 A/Hong Kong/125 genetic background. Unlike previous designs, CLEARFLU version 3 lacks the Y98F mutation and therefore contains an intact sialic acid binding site. It includes the partially attenuating G8A and the 7 independently inactivating mutations described in Figure 3.9, including F63/70P.

As previously, CLEARFLU hemagglutinin was expressed in pHR-SIN vectors to generate lentivirus for the stable transduction of MDCK-SIAT cells. The head antibody 4A14 was used to sort cells for high hemagglutinin expression.

As CLEARFLU version 3 includes a novel combination of mutations, it was important to again check for the loss of antibody epitopes. Figure 3.12 shows the results of two independent flow cytometry experiments. Antibody binding was similar to CLEARFLU version 3 and to wild-type hemagglutinin from the same subtype. Histogram plots showed bimodal fluorescence distributions for the wild-type cell line with all antibodies, suggesting that median fluorescence values are brought down by some cells with low protein expression.

Although the first experiment appeared to show decreased binding to CLEARFLU version 3 by 4A14, this difference was less marked in the second experiment. Antibodies 9B26 and Z2B3 had the weakest fluorescence intensity profiles for both CLEARFLU and wild-type hemagglutinin, with a significant part of the fluorescence distribution overlapping with the negative control (part D). In both cases, the right-hand tail of the distribution shows that some binding does occur. Overall, these experiments showed that antibody binding to the head and stem of the CLEARFLU version 3 molecule was not dissimilar to wild-type hemagglutinin. This indicated that the inclusion of F63/70P in the CLEARFLU version 3 design did not disrupt its antigenicity.

Figure 3.12



Antibody binding to H7 A/Hong Kong/125/2017 CLEARFLU version 3 and wild-type hemagglutinin expressed in sorted stably transduced MDCK-SIAT cells

Hemagglutinins were bound with human primary antibodies and binding was detected with an Alexa Fluor 647 labelled secondary antibody. Parts A and B show the results of a single experiment which was repeated and extended in parts C and D.

A. Bar graphs comparing median fluorescence of different cell lines when bound by a head or stem primary antibody.

B. Histogram plots showing the relative frequency of cells detected with a given fluorescence intensity.

C. Bar graphs comparing median fluorescence of different cell lines when bound by various primary antibodies. Data are plotted on a log₁₀ scale as the data span a large range.

D. Histogram plots showing the relative frequency of cells detected with a given fluorescence intensity.

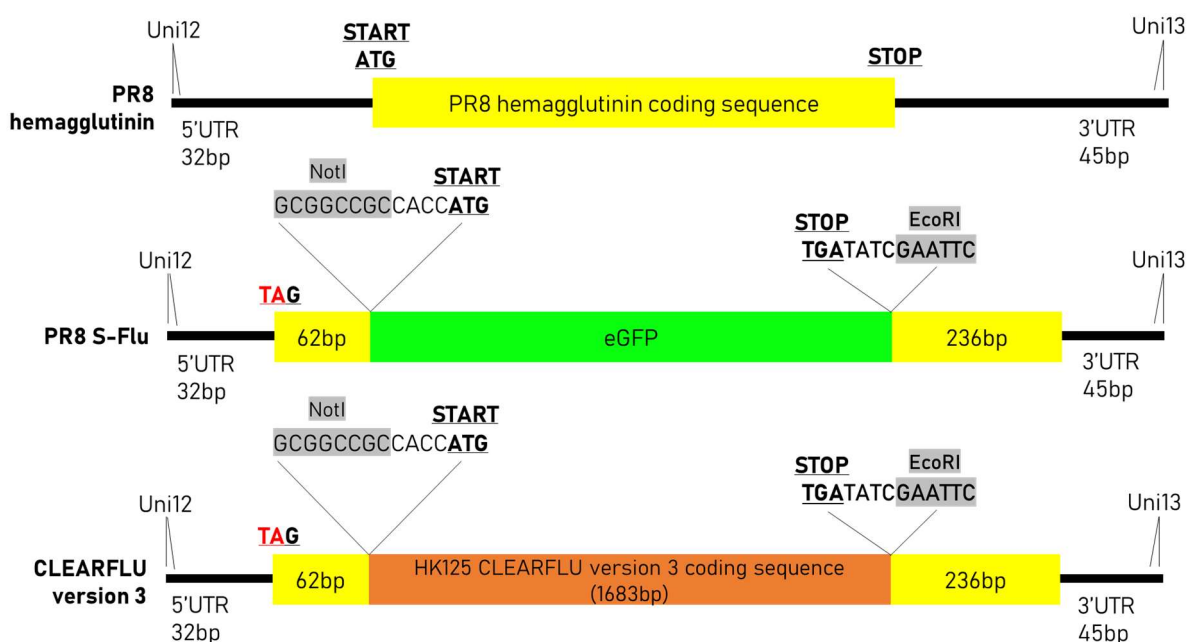
3.2.5 Viral expression of CLEARFLU hemagglutinins

We have shown that CLEARFLU hemagglutinins expressed in mammalian cell lines are displayed on the cell membrane and fold correctly. However, viral expression poses an additional challenge as it requires an extended RNA segment to be packaged into RNPs, for it to be replicated and for the protein to be expressed correctly on viral particles and the cell membrane.

Viruses expressing CLEARFLU version 3 were generated by multiplasmid transfection of 293T cells. 13 plasmids were used, including one mammalian expression plasmid (pcDNA3.1-) expressing a functional PR8 hemagglutinin to permit replication. Core polymerase genes were also expressed in pcDNA3.1- plasmids to initiate viral replication. Each viral segment except hemagglutinin was encoded in full, including packaging sequences, in a separate viral expression plasmid (pPol) so that expression and replication were driven by the viral polymerase and segments were incorporated into viral ribonucleoparticles. All gene sequences were from PR8 virus unless stated otherwise.

Instead of a functional hemagglutinin pPol plasmid, A/Hong Kong/125/2017 CLEARFLU version 3 was expressed in the S-Flu cassette described by Powell et al, 2012 (see Figure 3.13). The cassette contains packaging signals from the ends of the PR8 hemagglutinin to ensure that CLEARFLU is efficiently packaged into viral particles with the other PR8 genes (Marsh et al., 2007). The PR8 signal sequence is inactivated by the mutation of the start codon to TAG, but the CLEARFLU coding sequence has its own start codon and signal sequence, allowing CLEARFLU to be exported via the secretory pathway to the cell surface.

Figure 3.13



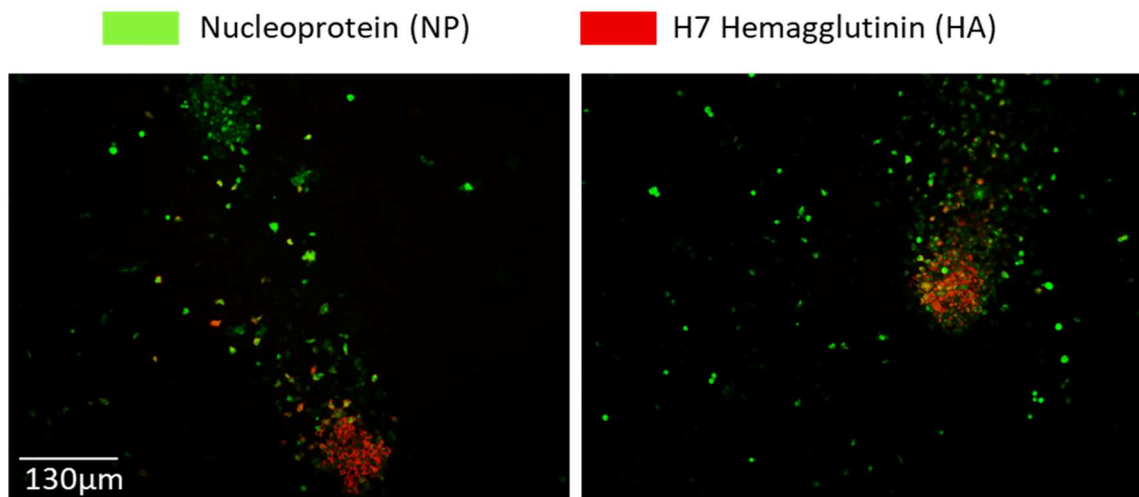
S-Flu and CLEARFLU expression cassettes

The S-Flu expression cassette includes the untranslated regions and the first and last bases of coding sequence from wild-type hemagglutinin to provide packaging signals. The original start codon is mutated to TAG and a stop codon is present in the eGFP/CLEARFLU insert so that the partial wild-type PR8 hemagglutinin sequences at either end are not expressed. The NotI/EcoRI sites allows easy substitution of any sequence, such as GFP or CLEARFLU into the cassette. The original S-Flu design is described in Powell et al, 2012.

Yellow = PR8 coding sequence, Black = PR8 untranslated regions, Grey = restriction enzyme cloning sites, Green = eGFP sequence, Orange = CLEARFLU hemagglutinin sequence

Following transfection, CLEARFLU virus was harvested and expanded in MDCK-SIAT cells expressing a PR8 pseudotyping hemagglutinin, first in a well of a six-well plate, and then in large flasks (see Figure 3.14). From this initial expansion, two viral clones (B7 and E4) were isolated by limiting dilution in MDCK-SIAT-PR8 cells (see TCID₅₀ protocol) and grown up to make virus seed stocks. The seed stocks were then titrated by CID₅₀ (See Figure 3.15).

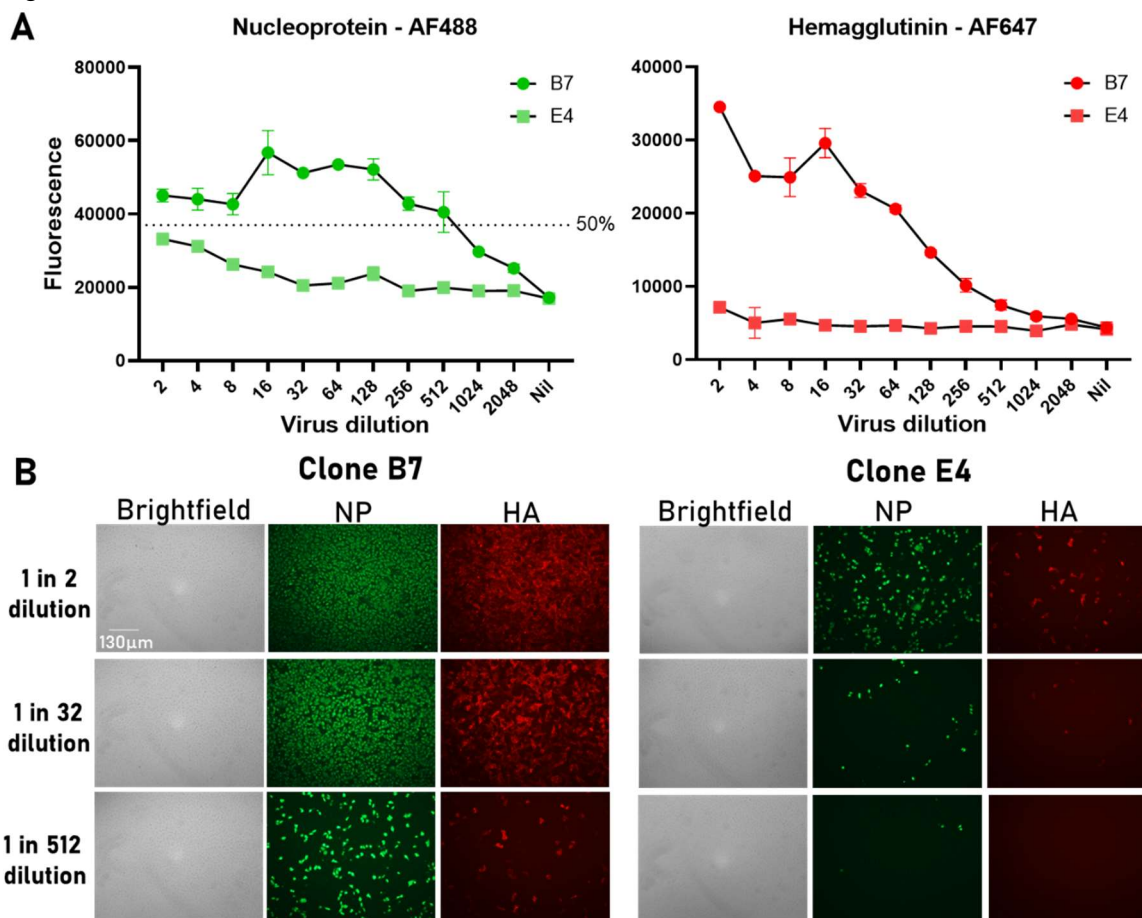
Figure 3.14



CLEARFLU version 3 viruses growing in MDCK-SIAT cells expressing PR8 hemagglutinin

Cells in one well of a 6-well plate were used to expand CFv3 viruses produced by multi-plasmid transfection. After 24 hours, the supernatant was used to expand the virus at larger scale in flasks and the cells in the plate were stained to determine whether transfection had yielded any virus. The cells are stained for nucleoprotein and H7 hemagglutinin using primary antibodies AA5H and 4A14 and secondary layers goat anti-mouse Alexa Fluor 488 (green) and goat anti-human Alexa Fluor 647 (red) respectively. Clusters of double-stained cells indicate replicating viruses express CLEARFLU hemagglutinin as part of the viral proteome.

Figure 3.15



Titration of two CLEARFLU version 3 virus stocks by cell infectious dose (CID)

Viruses were added to cells in doubling dilutions and incubated overnight. The titration was performed in duplicate. Cells were stained with the human hemagglutinin antibody 4A14 and the mouse nucleoprotein antibody AA5H. Goat anti-human Alexa Fluor 647 (red) and goat anti-mouse Alexa Fluor 488 (green) were used as secondary layers. Nucleoprotein staining is used as a measure of virus titre and hemagglutinin staining is used to assay for the expression of CLEARFLU.

A. Titration curves according to nucleoprotein and hemagglutinin staining. Error bars represent standard deviation based on two replicates. The 50% point is calculated based on the maximum value for B7 at dilution 1 in 16 due to cell loss in the first dilutions. B7 has a high titre by NP as fluorescence is saturated even at 128-fold dilution and the point where 50% of cells are infected is beyond the 1 in 512 dilution. Clone E4 has a much lower titre as even at the first dilution, 50% fluorescence is not reached. No 50% point is calculated for hemagglutinin staining due to the absence of a clear plateau. The titre by HA is therefore lower than for NP, reflecting that not all infected cells express CLEARFLU.

B. Images show single cell infections at three different virus dilutions for both B7 and E4. Fewer fluorescent cells are observed for clone E4, supporting that reduced fluorescence overall is due to a lower rate of infection rather than decreased fluorescence intensity in infected cells.

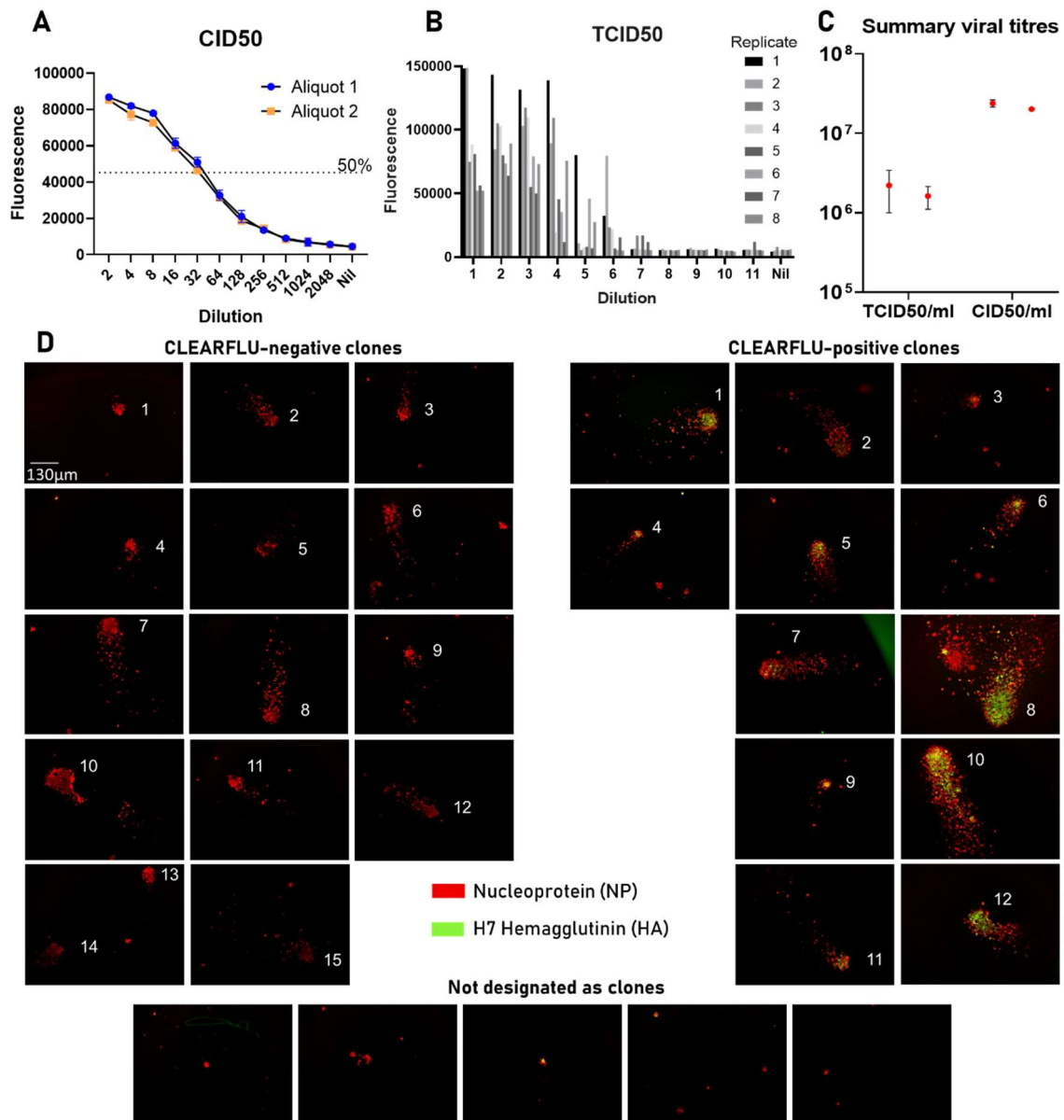
Due to its higher titre, the B7 clone seed was selected for further expansion to generate 400ml of reference virus stock which was used for all further experiments. This reference stock was titrated by TCID₅₀ (50% tissue culture infectious dose) and CID₅₀ (50% cell infectious dose) (see Figure 3.16). As CID₅₀ is performed on cells in the absence of pseudotyping hemagglutinin or trypsin, it measures single infections and so quantifies the number of virus units capable of cell entry. TCID₅₀ is performed with pseudotyping hemagglutinin and trypsin and therefore is a measure of growth and the number of replicative units. This is an important distinction as many infection events do not yield replicative virus. This is usually associated with the generation of defective interfering particles which do not package a functional copy of every segment.

Replicative assays highlighted that a proportion of viral plaques did not stain with the hemagglutinin head antibody 4A14. This indicated instability in the CLEARFLU gene, which has led to loss of expression, large-scale structural changes or localised disruption of the 4A14 epitope. A level of instability would be anticipated given that expression of CLEARFLU is not expected to confer a fitness advantage and that the CLEARFLU expression cassette is larger than wildtype hemagglutinin and so may be particularly demanding to replicate.

The true proportion of viruses expressing the 4A14 epitope is difficult to determine because it is not always easy to distinguish between neighbouring clones, because large plaques may hide smaller plaques (therefore leading to different results at varying virus densities), and because the distinction between smaller plaques and single infection events is at times unclear. Instability in the CLEARFLU RNA and the rest of the viral genome is the focus of the next chapter.

H1 A/England/195/2009 CLEARFLU version 2 and H3 A/Hong Kong/5738/2014 CLEARFLU version 2 viruses were also produced in this way and are referred to in later chapters. A PR8 genetic background was used for all genes with some exceptions (see methods).

Figure 3.16



Titration of CLEARFLU version 3 virus reference stock by CID50 and TCID50

A. CID50 titration curve on nucleoprotein using antibody 2-8C and fluorescence label AF647. Two separate aliquots of virus were titrated in 2-fold dilution in duplicate and incubated overnight. The mean fluorescence and standard deviation of duplicates is plotted at each dilution. The curves were used to calculate the dilution at which 50% of cells are infected, which is plotted as CID50/ml in part C. Viruses were diluted 2-fold before use.

B. TCID50 titration plate measuring nucleoprotein using 2-8C and fluorescence label AF647. Bars show the intensity of fluorescence in each well after 48 hours. Viruses were added to MDCK-SIAT cells expressing PR8 hemagglutinin in 96-well plates in half-log dilutions as per the quantification by TCID50 protocol. Viruses were diluted 100-fold before use. 8 replicates were used at each dilution. Erratic peaks of high fluorescence indicate the expansion of viral clones. This plot is representative of four titration plates used to estimate the dilution at which 50% of wells are infected, the results of which are summarised in part C.

C. Summary viral titres by CID50/ml and TCID50/ml, both by NP (2-8C) staining. Error bars show the standard deviation of two duplicate measurements on the same aliquot of virus. Two different aliquots were tested for each measure.

D. Fluorescence microscopy images show the expansion of CLEARFLU version 3 virus clones in MDCK-SIAT-PR8 cells. Cells were infected as per TCID50 protocol, but were stained after 24 hours to ensure individual clones could be distinguished. The virus was diluted 1 in 10000 before use and all photos were taken at the first dilution, equivalent to dilution 5 in part B. Nucleoprotein was stained with 2-8C and a secondary layer labelled with AF647; hemagglutinin was stained using 4A14 and AF488. Clones were defined as clusters of nucleoprotein stained cells, often with comet-like trails.

3.3 Discussion

The results presented in this chapter show that highly mutated hemagglutinins can be presented at the cell surface through both mammalian and viral expression mechanisms.

Firstly, this has allowed us to test the effect of mutations on antigenicity by testing antibody binding to cells expressing mutated hemagglutinins. Cellular expression has also been a helpful tool for assessing the contribution of single mutations to hemagglutinin inactivation.

Secondly, by investigating viral expression we have shown that viruses expressing multi-mutated hemagglutinins are viable when grown in a cell line which compensates for the lack of hemagglutinin activity. This allows the controlled propagation in cell culture of viruses which should not pose a pathological threat to human or animal hosts due to their single-cycle nature.

In this chapter, the design for CLEARFLU hemagglutinin was refined through 3 iterations. While some antibody epitopes were lost in the stem region of CLEARFLU version 1, the second and third iterations overcame this problem and therefore show promise as B-cell antigens. This was shown using a wide range of human antibodies and several viral strains to establish that patterns of binding are both generalisable and relevant to the human immune response.

In CLEARFLU version 3, the Y98F mutation in the sialic acid binding site was removed as it was shown not to inactivate function. Although this mutation did not appear to disrupt antibody binding to the sialic acid binding site in CLEARFLU versions 1 or 2, the site is known to be a target of broadly neutralising antibodies and so removing it may improve the immunogenicity of the CLEARFLU version 3 virus. Whilst the Y98F mutation did not appear to restrict virus growth *in vitro*, no CLEARFLU-mediated sialic acid binding was

observed. It is therefore possible that an intact binding site may enhance cell attachment and thus improve viral titres in some conditions or viral strains.

Together with data showing that mutation of these hemagglutinins to a functional form is highly improbable, viruses expressing CLEARFLU version 2 or 3 show initial promise as single-cycle vaccine candidates. CLEARFLU version 3, with its 63/70P mutation in the B loop, offers a particularly low probability of reversion to functionality.

However, it remains to be seen whether these viruses stimulate a strong antibody response to CLEARFLU *in vivo* and offer a true improvement over S-Flu viruses which do not express hemagglutinin epitopes on the cell surface. We observed a loss of binding for head antibody 4A14 to CLEARFLU version 3 viruses after expansion. For CLEARFLU viruses to offer an advantage as a vaccine over S-Flu viruses, hemagglutinin epitopes must be stably expressed even after many rounds of virus replication. Therefore, further investigations will involve determining the rate at which epitopes are lost and whether sufficient expression can be maintained to stimulate strong immune responses *in vivo*.

The following chapters aim to further characterise CLEARFLU viruses to assess their potential as vaccines. In Chapter 4, the potential for CLEARFLU version 3 viruses to be propagated at large scale is investigated, testing their genomic stability and trialling mechanisms to select for expression of the CLEARFLU antigen. In Chapter 5, we explore the relationship between surface proteins on pseudotyped viruses and how the expression of CLEARFLU version 3 may affect the role of wild-type hemagglutinin and neuraminidase in cell entry and release. In Chapter 6, observations on antigenicity in this chapter are extended to test immune responses to CLEARFLU version 2 and version 3 viruses, particularly to the CLEARFLU hemagglutinin itself, *in vivo* in mice and pigs.

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Chapter 4

GENOMIC STABILITY OF CLEARFLU VIRUSES

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4.1 Introduction

This chapter explores whether CLEARFLU viruses could be produced at a large scale for vaccine manufacture. It builds on the observation that CLEARFLU viruses appear to lose expression of an antibody binding epitope in the head region during virus expansion by investigating the extent of this loss and ways to ensure expression is maintained.

Two approaches for selecting for CLEARFLU expression are evaluated, and CLEARFLU viruses are characterised after 6 passages at low multiplicity of infection (MOI) under the most promising approach. All experiments in this chapter are conducted using A/H7/Hong Kong/125 CLEARFLU version 3 virus unless otherwise stated.

4.1.1 Interpreting loss of expression

Previously, we observed that CLEARFLU version 3 viruses grew to good titres in cell culture but that the antibody binding epitope for 4A14 was lost in some clones. The loss of binding by this antibody is particularly interesting as 4A14 binds to the sialic acid binding region and neutralises entry of H7 viruses. This suggests that the changes that have caused loss of 4A14 binding will likely disrupt sialic acid binding activity by CLEARFLU. This loss is not expected to be disadvantageous to replication as binding activity is provided by the pseudotyping hemagglutinin. However, a mechanism which selects for the retention of sialic acid binding by CLEARFLU may select for continued expression of this and - depending on the mechanism underlying loss of 4A14 binding- other valuable epitopes.

Influenza viruses are known to package their eight RNA segments into budding viral particles imperfectly, generating semi-infectious particles when one or more is missing (Hutchinson et al., 2010; Nakatsu et al., 2016). These particles are only able to replicate if they are coinfecting alongside another virus which can compensate for the missing segment. The CLEARFLU segment is larger than the hemagglutinin in wild-type viruses (~2.1kb vs 1.7kb) due to the inclusion of short PR8 hemagglutinin sequence at both ends to provide packaging signals (Marsh et al., 2007). This increased size may decrease the packaging efficiency of this segment. Unlike the other segments, failure to package CLEARFLU should not prevent the virus from replicating. We might therefore expect that some viruses should naturally fail to package CLEARFLU, and that these viruses should remain competitive with CLEARFLU-expressing viruses. Indeed, avoiding the need to invest in replicating or expressing the CLEARFLU gene may be advantageous.

Alternatively, CLEARFLU epitope loss may be explained by internal deletion of parts of the segment. This is known to occur in wild-type viruses, leading to the generation of a truncated non-functional segment which still contains intact packaging signals at either end and can therefore still be incorporated into budding virions. These virions are called defective interfering particles because they are only able to replicate in the presence of complementing virus and they reduce the amount of replicative virus budding from the cell as the defective segment competes for packaging (von Magnus, 1954; Huang & Baltimore, 1970; reviewed by Frensing, 2015; Alnaji & Brooke, 2020). Deletions in the CLEARFLU segment would not disrupt the virus' ability to replicate, and so we might expect that deletions could arise which trim down the segment to an optimal size for efficient replication and packaging (Jennings et al., 1983).

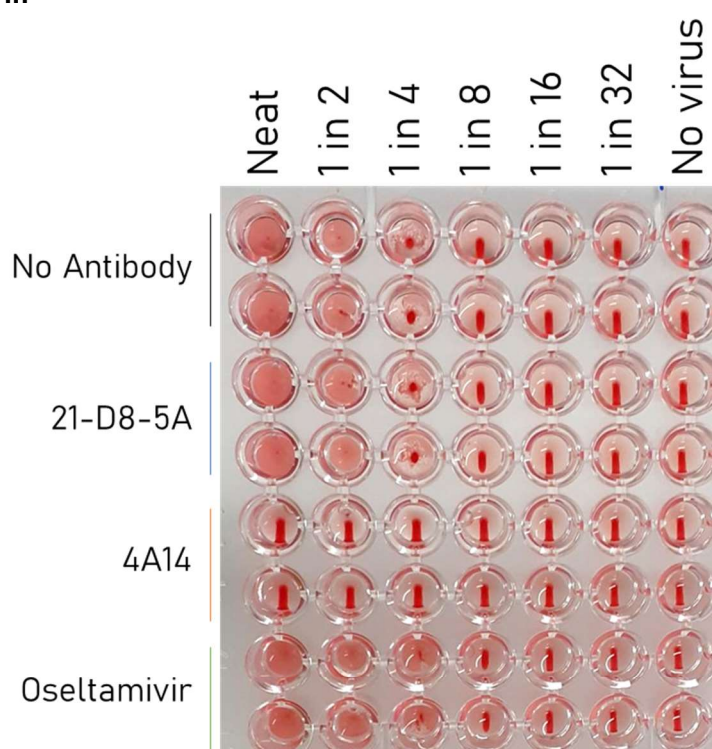
Thirdly, the loss of the 4A14 epitope may be caused by more specific, local changes. There may be mutations in a regulatory region or in the signal sequence which prevent the CLEARFLU gene from being expressed or exported to the cell surface, respectively. Alternatively, specific mutations may have arisen in the coding sequence. Although the sialic acid binding site is usually well conserved, we would not expect this to be so constrained in the presence of a pseudotyping hemagglutinin. The loss of the 4A14 epitope may therefore not be indicative of widespread loss of other epitopes.

Depending on which mechanism underlies epitope loss, a full or partial CLEARFLU protein may or may not be expressed at the cell surface.

4.1.2 Using alternative coat proteins to select for CLEARFLU expression

Two approaches for taking advantage of the intact sialic acid binding site in CLEARFLU version 3 to select for its expression are presented in this chapter. Figure 4.1 confirms that S-Flu viruses pseudotyped in CLEARFLU version 3 agglutinate red blood cells and therefore bind sialic acids. This binding is inhibited by 4A14, indicating that it is mediated by H7 CLEARFLU rather than neuraminidase or contaminating PR8 hemagglutinin.

Figure 4.1



Agglutination of red blood cells by CLEARFLU version 3 hemagglutinin

MDCK-SIAT cells stably expressing A/Hong Kong/125/2017 CLEARFLU version 3 hemagglutinin were infected with S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)) at MOI=1 by TCID50. After 2 hours, the cells were washed three times with PBS to remove non-internalised virus. After 48 hours, the supernatant was harvested and titrated in doubling dilutions in duplicate in the plate shown above. Viruses were incubated for 1 hour with 2µl of antibody at 25µg/ml or 5µl Oseltamivir at 200nM before adding 1% red blood cells as per HAI protocol. Agglutination is demonstrated by the lack of a red cell pellet, which forms a teardrop on tilting the plate. The first two rows show agglutination of red blood cells by S-FLU pseudotyped in HK125 CLEARFLU version 3. The antibody 21-D8-5A to H1 A/Puerto Rico/8/1934 hemagglutinin does not block agglutination. Neuraminidase inhibitor oseltamivir also fails to inhibit agglutination. 4A-14, a monoclonal antibody to the head of the H7 CLEARFLU hemagglutinin, completely inhibits agglutination, suggesting H7 CLEARFLU is the sole significant contributor to sialic acid binding activity.

The first approach uses a mutant hemagglutinin (X31-PM) which is fusion competent but lacks sialic acid binding and therefore cell binding activity. The second uses the coating glycoprotein (GP) from Ebola viruses which mediates cell attachment and fusion but is known to generate only very low titre pseudotyped viruses. When pseudotyped with these proteins instead of wild-type hemagglutinin, sialic acid binding activity from the CLEARFLU hemagglutinin should give CLEARFLU-expressing viruses a replicative advantage through enhanced cell attachment. This system should be particularly effective with X31-PM hemagglutinin as viruses without CLEARFLU expression should not be able to replicate at all due to a total lack of cell receptor binding.

These two examples serve to illustrate how any coat protein which mediates fusion and has weak or no cell attachment capacity might be used to select for CLEARFLU hemagglutinin expression and thus enhance the presentation of CLEARFLU epitopes. Other enveloped viruses which have separate proteins for each of these activities, such as Sendai virus which has a fusion protein and a hemagglutinin-neuraminidase protein (Faísca & Desmecht, 2007), might offer alternatives.

Previous studies on S-Flu have shown that the pseudotyping hemagglutinin protein does not stimulate a strong neutralising antibody response when administered intranasally to mice or ferrets or by aerosol in pigs (reviewed in Chapters 1 & 6). However, a strong response is seen after intraperitoneal or intramuscular immunisation. Depending on the chosen route of administration, the coating protein may therefore have immunological consequences.

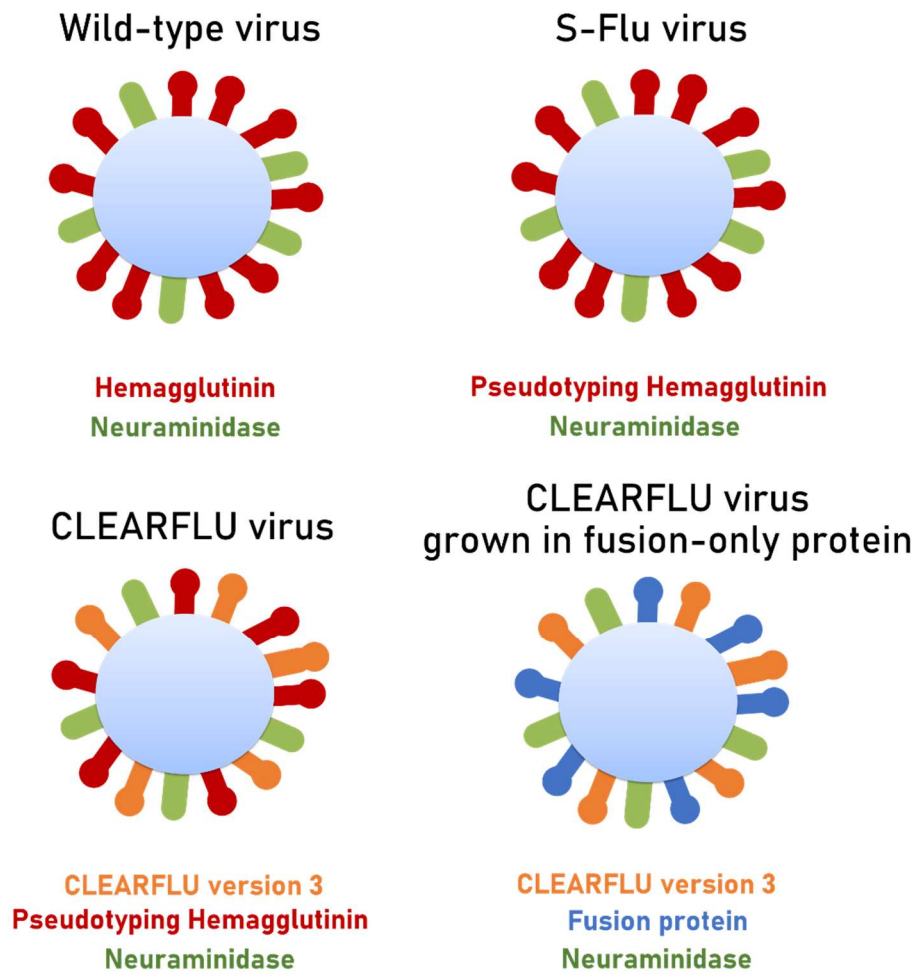
Equally, the use of a non-influenza protein may affect virus homing *in vivo*, as Ebola GP for instance does not use the same cell receptors for entry or home to the same tissues as influenza. Although GP is co-expressed with CLEARFLU which should also contribute to cell-targeting through sialic acid binding, we might expect reduced local responses in the lung after aerosol or intra-nasal administration due to inefficient infection of the lung

by Ebola GP. To maximise the local expression of influenza epitopes and avoid anti-GP responses, viruses grown in GP cells could be passaged once in hemagglutinin-expressing cells before administration. Viruses grown in X31-PM hemagglutinin could be passaged to switch the hemagglutinin or used directly for immunisation.

4.1.3 Balancing CLEARFLU expression and viral fitness

While wildtype and S-Flu viruses have just two major surface proteins, CLEARFLU viruses have CLEARFLU in addition to neuraminidase and a coating hemagglutinin. One might envisage that high expression of CLEARFLU on the viral surface could reduce fusion capacity by competing with the fusion-competent pseudotyping hemagglutinin for space. Equally, CLEARFLU expression might reduce the level of neuraminidase on the viral surface and affect the balance between cell attachment and release. In the previous chapter, both the CLEARFLU and pseudotyping hemagglutinin coat were able to mediate cell attachment through an intact sialic acid binding site. In this chapter, we discuss the use of pseudotyping proteins which lack or have little cell binding activity. As such, not only is the fusion capacity limited to only one of three major surface proteins, but the cell binding function is also offered exclusively by one of three proteins (see Figure 4.2). CLEARFLU expression may therefore come at the cost of lower infectivity and virus titre. This may act as a driving force for reducing CLEARFLU expression.

Figure 4.2 – Surface proteins of influenza pseudotypes



	Wild-type virus	S-Flu virus	CLEARFLU version 3 virus	CLEARFLU version 3 virus grown in fusion-only protein
Total surface proteins*	2	2	3	3
Mediating cell attachment	1	1	2	1
Mediating fusion	1	1	1	1
Mediating release	1	1	1	1

*Not including minor surface protein M2

4.2 Growth in cells expressing X31 Passage Mutant hemagglutinin (X31-PM)

This approach takes advantage of a mutant hemagglutinin which is fusion competent but does not bind sialic acid to select for viruses which bind sialic acid through CLEARFLU version 3.

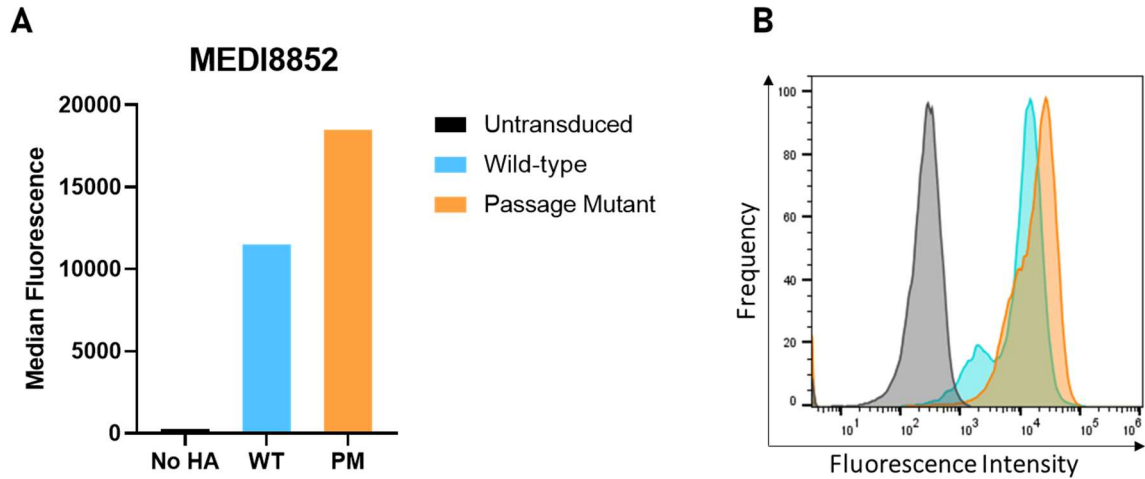
Hooper & Bloom engineered a binding mutant X31 hemagglutinin (X31-BM) with a disrupted receptor binding pocket due to several mutations (Hooper & Bloom, 2013). These include the Y98F mutation previously discussed as well as H183F, L194A and the deletion of residues 221 to 228 (all according to H3 numbering). They showed that viruses expressing this hemagglutinin were not able to grow unless they had a compensatory mutation in the neuraminidase gene which allowed it to take over sialic acid binding. After serial passage of these mutant viruses, the hemagglutinin acquired K62E in HA2 which improved virus titres. This version was named passage mutant hemagglutinin (X31-PM). Hooper and Bloom showed that the passage mutant hemagglutinin, similarly to the binding mutant, requires cell attachment to be provided from the mutant neuraminidase for viral growth.

MDCK-SIAT cell lines stably expressing either passage mutant or wildtype X31 (X31-WT) hemagglutinin were produced and sorted for high expression with MEDI8852 as described previously (see Figure 4.3). These cells were used to pseudotype CLEARFLU viruses to test whether those grown in X31-PM cells maintained higher levels of CLEARFLU expression.

Figure 4.4 describes an experiment in which the proportion of viral clones expressing the CLEARFLU 4A14 binding epitope was estimated before and after a round of expansion in X31-WT or X31-PM cells. Even after growth in a cell line which should only permit viruses expressing CLEARFLU to replicate, clones without expression could be identified. Although only a small number of clones were counted, there was no increase in the

proportion of nucleoprotein and CLEARFLU double-positive clones in passage mutant relative to wild-type cells. This therefore provided no evidence that CLEARFLU expression was selectively maintained in the passage mutant cell line.

Figure 4.3



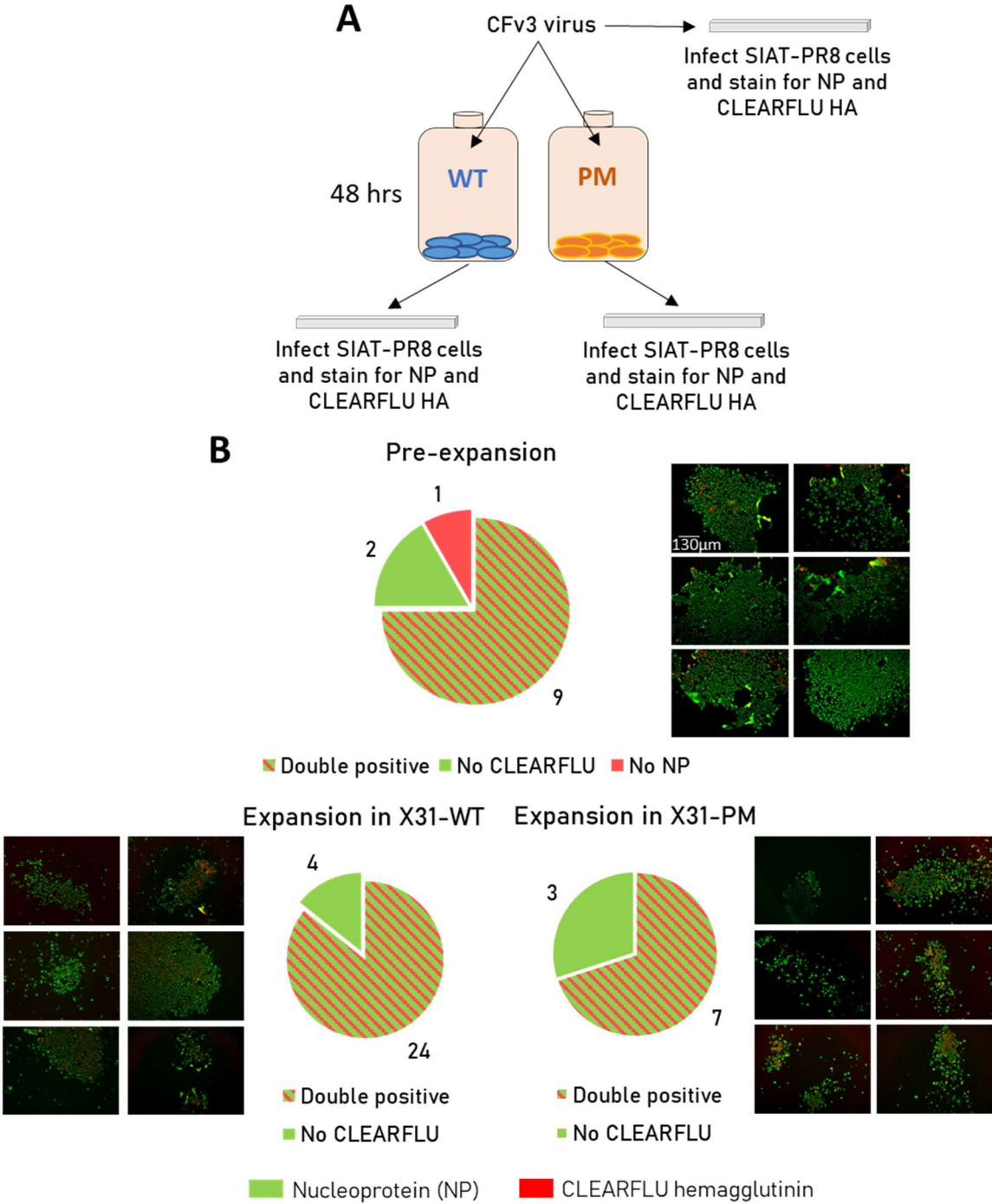
Antibody binding to X31 Passage Mutant and wild-type hemagglutinin expressed in sorted stably transduced MDCK-SIAT cells

Cells were bound with MEDI8852 and binding was detected with an Alexa Fluor 647 labelled secondary antibody.

A. Bar graph comparing median fluorescence of cells.

B. Histogram plots showing the frequency of cells detected with a given fluorescence intensity.

Figure 4.4



Testing for the selection of CLEARFLU expression in sorted MDCK-SIAT cells stably expressing X31-PM and X31-WT

H7 A/Hong Kong/125/2017 CLEARFLU version 3 virus was added to cells as per TCID50 protocol. Cells were fixed after 48 hours and double stained for NP and CLEARFLU using antibody AA5H with an AF488 secondary layer and 4A14 with a AF647 secondary layer respectively.

A. Experimental scheme

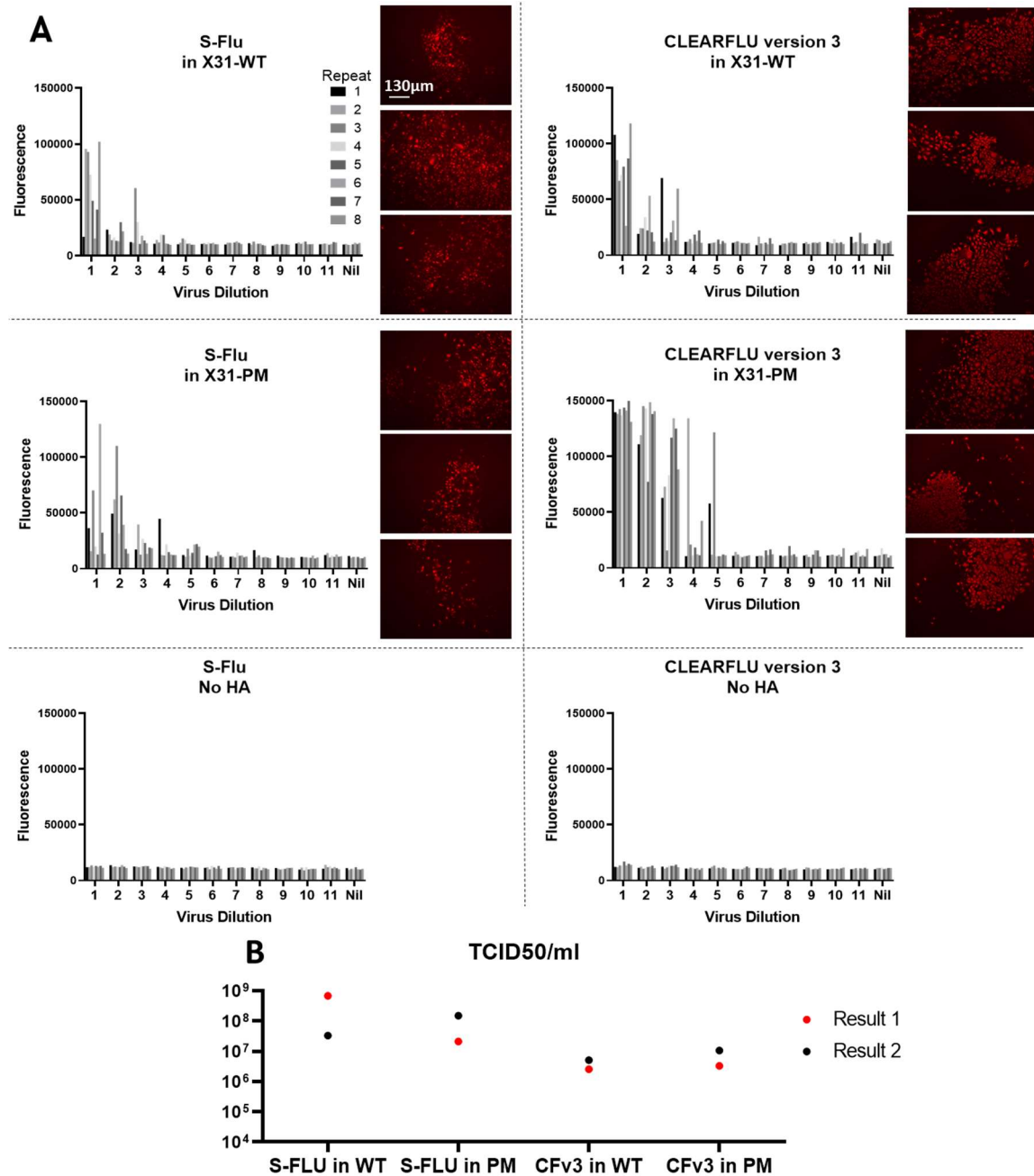
B. Pie graphs showing the number of viral clones double or single positive for NP and CLEARFLU pre- and post-expansion in X31-WT or X31-PM cells, and exemplar images.

To confirm this, the replication of CLEARFLU virus was tested directly using our TCID₅₀ protocol, using an H7 S-Flu as a negative control. Figure 4.5 shows that the CLEARFLU virus was able to grow in both X31-PM and X31-WT cells as expected, forming plaques of nucleoprotein-expressing cells. However, S-Flu was also able to grow in both cell lines with no obvious difference in titre after growth in X31-PM or X31-WT cells. This is surprising as S-Flu should be unable to replicate in X31-PM cells as there should be no protein competent for cell attachment.

The plasmid used to make the passage mutant cell line was sequenced to confirm that it contained the expected mutations. This plasmid was then used to produce a second X31-PM cell line, which was again sorted with MEDI8852. The ability of S-Flu to replicate in these cells was verified by the appearance of plaques of eGFP expressing cells (see Figure 4.6).

These results confirmed that the expression of CLEARFLU hemagglutinin was not essential for virus replication. This implies that sialic acid binding is somehow mediated by another protein. As the PR8 N1 neuraminidase used in these assays is not known to bind sialic acid and has not been seen to do so in other experiments, this suggests that, in our hands, the passage mutant hemagglutinin is still able to perform this function. As S-Flu appears to replicate well in passage mutant cells, there is no evidence to suggest that growing viruses in this environment would select for CLEARFLU expression.

Figure 4.5



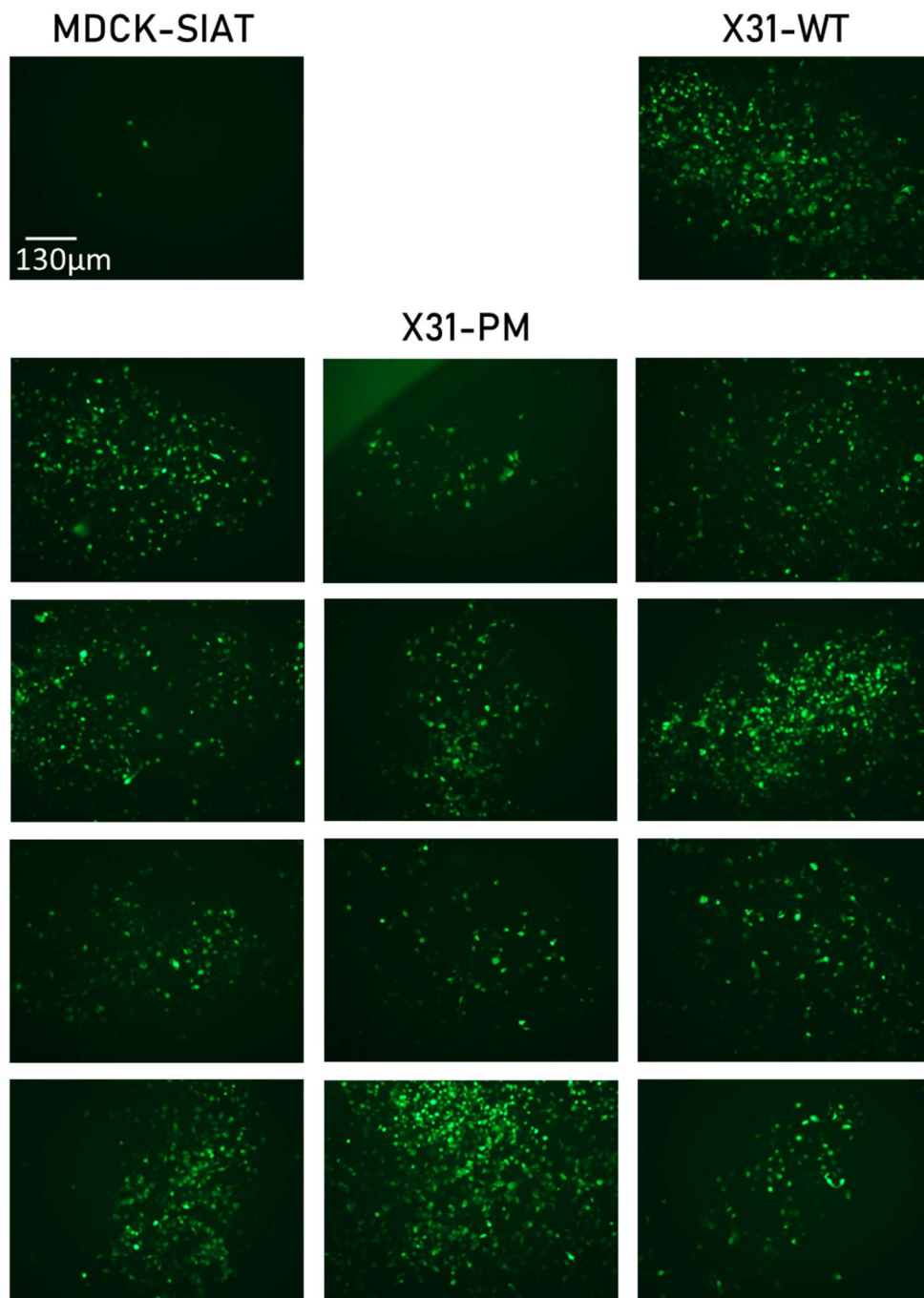
Replication of S-Flu and CLEARFLU viruses in sorted MDCK-SIAT cells transduced with wildtype or passage mutant X31 hemagglutinin

H7N1 S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)) and H7 A/Hong Kong/125/2017 CLEARFLU version 3 virus were added to cells in a 96 well plate in half-log dilutions as per TCID50 protocol. The viruses were diluted 1 in 35000 and 1 in 7000 respectively. The data in Part A are from a single experiment which was repeated exactly with similar results. The TCID50 values for both repeats are shown in part B. Cells were stained with nucleoprotein antibody AA5H and a goat anti-mouse secondary layer labelled with AF647.

A. Fluorescence by nucleoprotein staining for eight replicate wells at each dilution. Bars in the first four plots show high fluorescence, indicating that both S-Flu and CLEARFLU were able to replicate well in X31-WT and X31-PM mutant cells.

B. Viral titres by TCID50/ml. Each point represents one assay plate. Two estimates are presented from independent identical experiments.

Figure 4.6



Verification of S-Flu replication in a second MDCK-SIAT cell line stably expressing X31-PM

H7N1 S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)) was diluted 1 in 35000 and added to cells in a 96 well plate in half-log dilutions as per TCID₅₀ protocol. Cells were fixed and infected cells were imaged using eGFP fluorescence.

4.3 Growth in cells expressing Ebola glycoprotein (GP)

4.3.1 Preliminary observations

Previous work by Dr Julie Xiao in the Townsend group has shown that S-Flu viruses can be pseudotyped with the Ebola glycoprotein (GP) to make E-S-Flu (Xiao et al., 2017). This has proven to be a useful laboratory tool for screening drugs and antibodies which inhibit GP-mediated entry, and can be used in biosafety level 1/2 containment.

However, GP-coated S-Flu viruses grow to lower titres in MDCK-SIAT cells than those coated in hemagglutinin. With permission, Figure 2 from Xiao et al is reproduced below and shows how viruses coated in GP from the Ebola strain Zaire form diffuse rather than dense plaques and grow to a lower maximum titre (see Figure 4.7). It was hypothesised that this difference is due to weaker initial cell attachment as GP-coated viruses appear to travel further on average before entering new cells.

This theory was tested in collaboration with Julie Xiao and her student Declan Pang by testing the replication of S-Flu in cells co-expressing GP and CLEARFLU version 3 to see whether the presence of a sialic acid binding protein improved titres. The cell line was produced by transducing MDCK-SIAT cells first with GP and then with H7 A/Hong Kong/125/2017 CLEARFLU version 3, and sorting the cells for high expression of both proteins using antibodies 6D6 and 4A14. Figure 4.8 shows that S-Flu grew to a higher titre in cells expressing GP and CLEARFLU together than in cells expressing only GP. This provides promising evidence that sialic acid binding activity provided by CLEARFLU hemagglutinin can enhance viral replication in GP cells.

In the following experiments, we investigate whether this effect extends to virally- as opposed to cellularly- expressed CLEARFLU. We explore whether this observation translates to selection for continued viral expression of CLEARFLU.

Figure 4.7

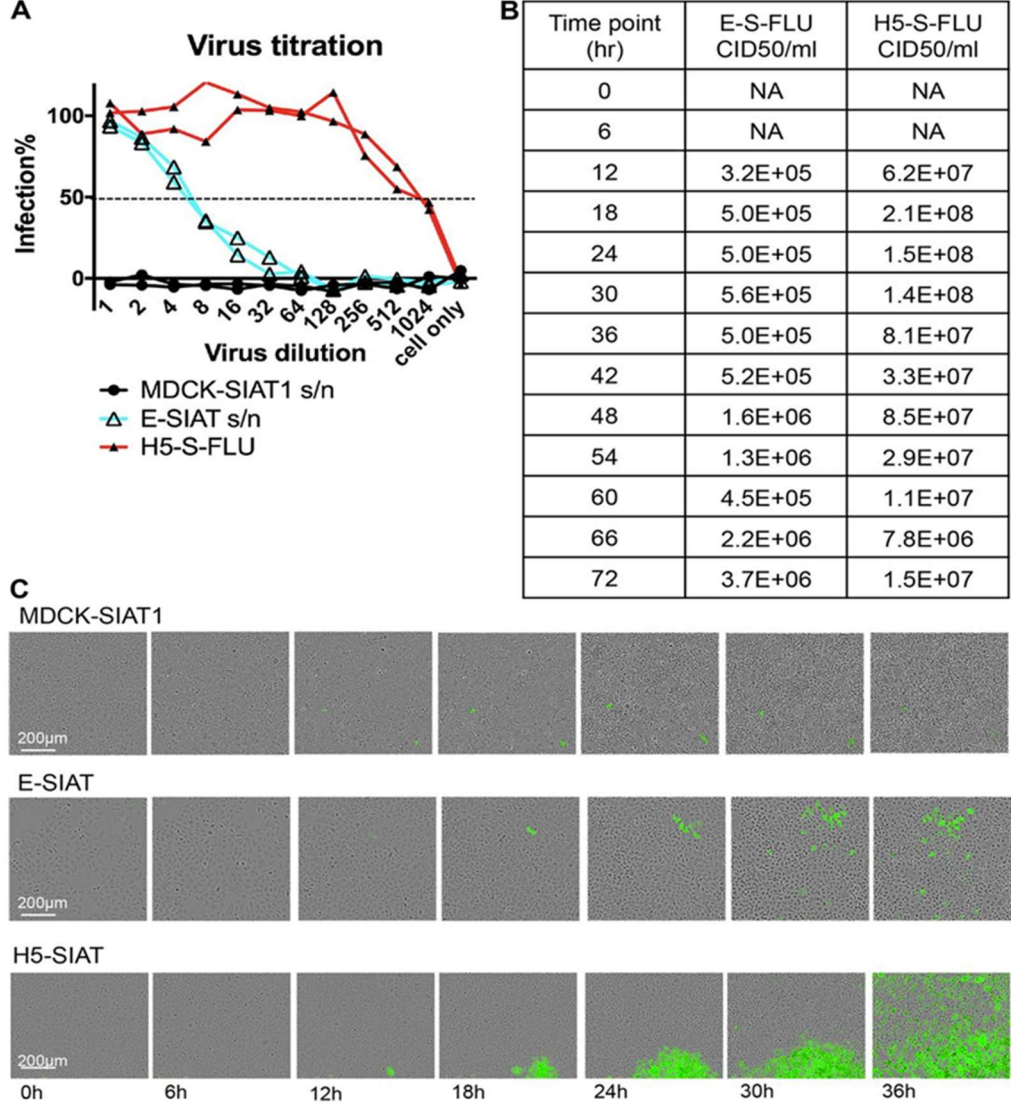


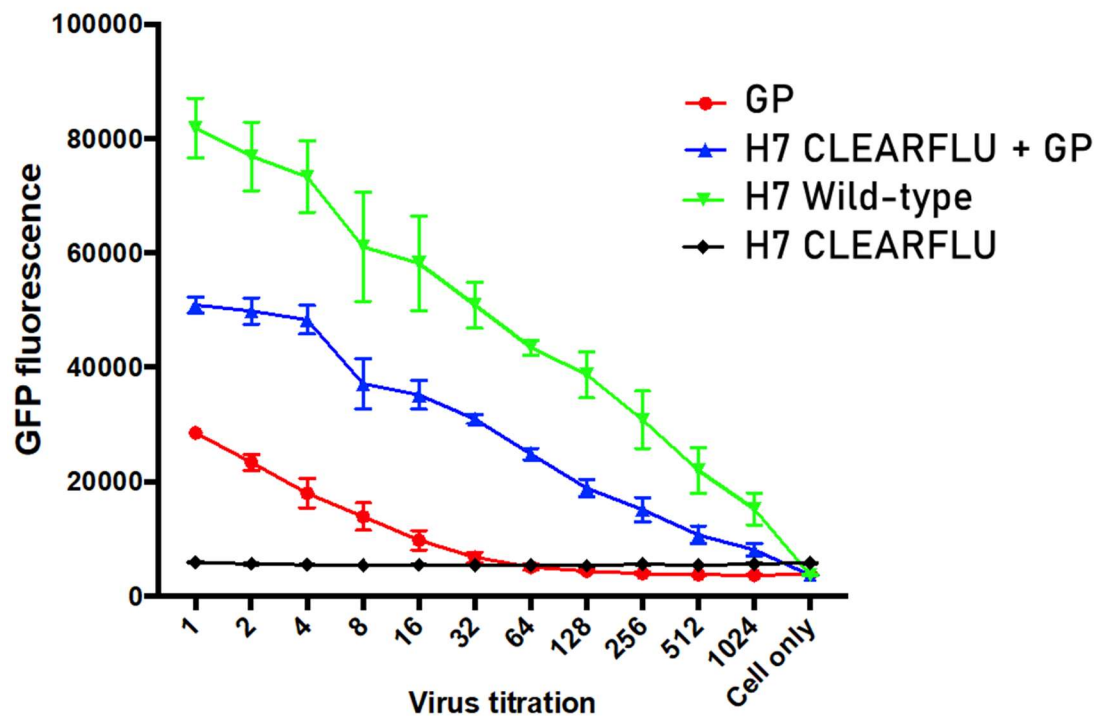
Figure 2 from Xiao et al, 2017 comparing the growth of S-Flu in sorted MDCK-SIAT cells stably expressing H5 hemagglutinin or Ebola GP.

A. E-SIAT or H5-SIAT cells were seeded with 1 TCID₅₀ PR8 S-FLU virus per cell to generate EBOV-GP (cyan) and H5 (red) pseudotyped S-FLU viruses. Untransduced MDCK-SIAT1 cells were also included as a control. Culture supernatants harvested after 48 h were titrated in 2-fold dilutions on MDCK-SIAT1 indicator cells for 24 h of infection. Cell infection was detected using eGFP fluorescence. The undiluted E-S-FLU and H5-S-FLU viruses gave saturated infection, and the maximum fluorescence readout was used to calculate the percent infection at lower dilutions.

B. E-SIAT and H5-SIAT cells were seeded with 1 TCID₅₀ of seed PR8 S-FLU virus per cell. Culture supernatant harvested at different time points were titrated on MDCK-SIAT1 cells. CID₅₀/ml was calculated using eGFP fluorescence.

C. A total of 100 TCID₅₀ of seed PR8 S-FLU virus was added to 3×10^4 MDCK-SIAT1, E-SIAT, or H5-SIAT cells. Live images of representative plaques were taken using IncuCyte every 3 hours. E-SIAT cells support the formation of small diffuse plaques, unlike the dense plaques formed on H5-SIAT cells.

Figure 4.8



Titration of S-Flu after growth in MDCK-SIAT cells expressing GP, wild-type H7, CLEARFLU H7 or co-expressing GP and CLEARFLU H7.

Cells were seeded with 1 TCID₅₀/cell of PR8 S-FLU to produce pseudotyped viruses. Culture supernatants were harvested after 48 hours and titrated by CID₅₀ using eGFP fluorescence. Viruses were used neat in the first dilution. Error bars show standard deviation based on two duplicate readings. This graph is reproduced from Figure 3.17 from Julie Xiao's DPhil thesis, 2018.

4.3.2 An introduction to Ebola glycoprotein

The surface glycoprotein of Ebola viruses is responsible for both the cell attachment and fusion steps of cell entry (reviewed in Lee & Saphire, 2009). The glycoprotein precursor is cleaved into disulphide-linked subunits called GP1 and GP2 by furin, which assemble into a trimer of heterodimers at the viral surface. Cell attachment is mediated by the GP1 subunit which is formed of the base, head and glycan cap (see Figure 4.9). The head mediates cell attachment through its receptor binding region but the ligands it binds are not fully elucidated.

Figure 4.9 – Structure of Ebola glycoprotein

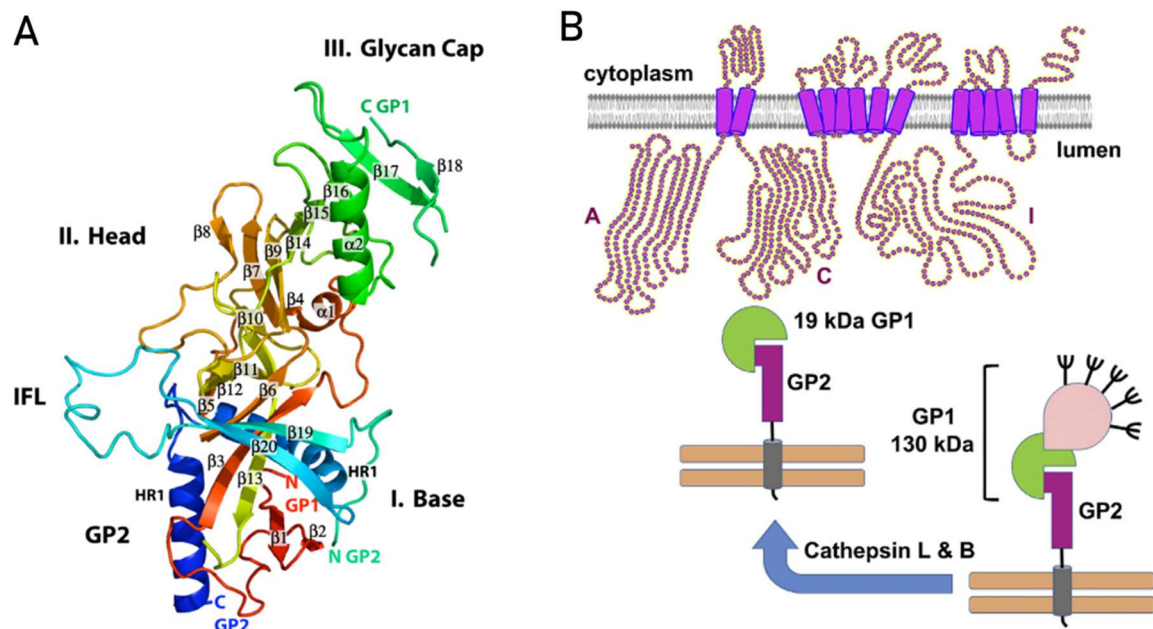


Figure S2 from Lee et al, 2008 supplementary data showing the structure of Ebola virus GP.

The chain is coloured by residue number in a rainbow gradient from red (N terminus of GP1) to blue (C terminus of GP2). β strands are shown as arrows and are numbered starting from the N terminus of GP1. Coils indicate α helices. GP2 contains the internal fusion loop (IFL) and heptad repeats (HR).

Figure 1 from Wang et al, 2016 showing binding of Ebola GP to the endosomal receptor NPC1.

GP is primed by cathepsins L and B in the late endosome, leaving only 19kDa of the 130kDa GP1 with GP2. The cleaved form binds to domain C of NPC1.

The virus is then internalised through macropinocytosis and transported to the endosome where removal of much of GP1 (including the glycan cap and mucin-like domain) by cathepsins B & L primes GP for fusion. Fusion is mediated by the GP2 subunit and requires interaction with NPC1 (Niemann-Pick Type C1) on the endosomal membrane (Wang et al., 2016).

Unlike in hemagglutinin-expressing cells where trypsin must be added, S-Flu viruses can be grown in GP-expressing cells without adding a cleavage enzyme as furin and cathepsins B & L are ubiquitously expressed.

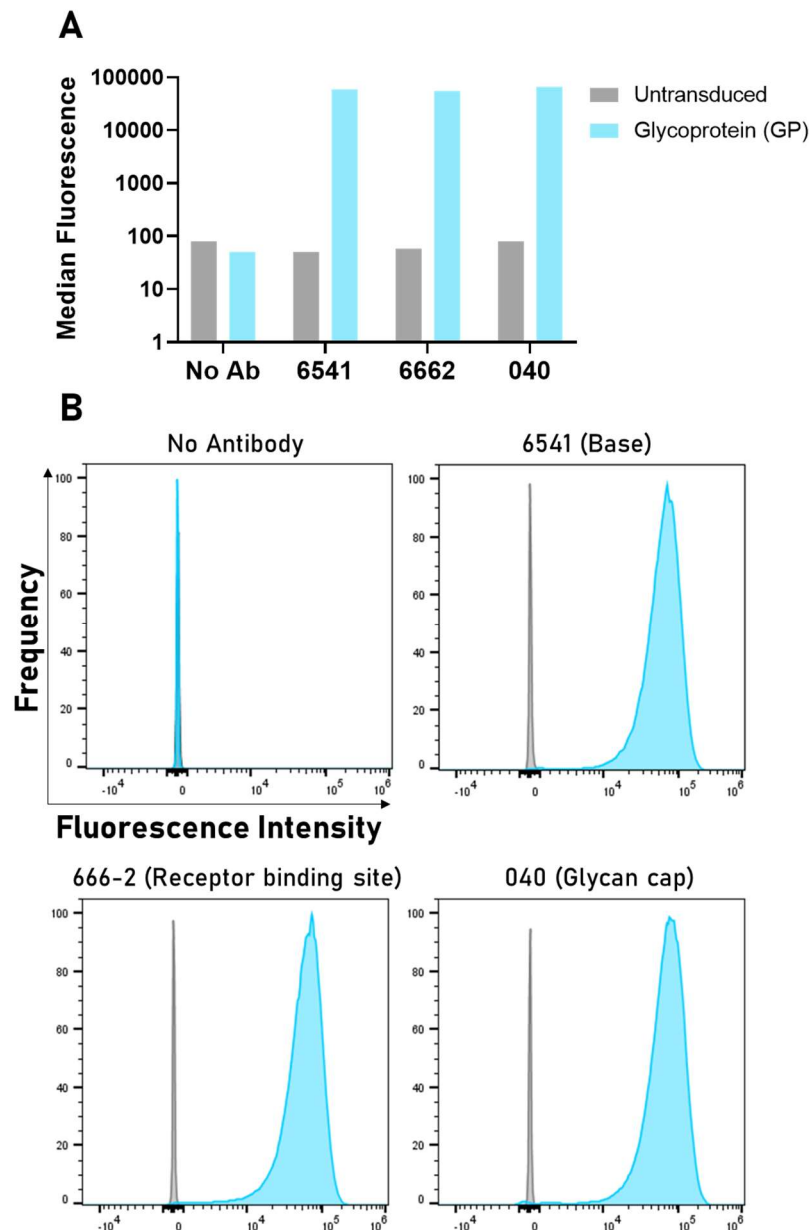
Glycoprotein from the Zaire species is the standard ebolavirus protein used in our lab. The Zaire species has the highest pathogenicity, was responsible for recent human outbreaks in the Democratic Republic of the Congo (most recently 2018–2020) and in West Africa (2014–2016). Other species include Bundibugyo, Sudan, Taï Forest, Reston and Bombali, of which Bundibugyo, Sudan and Taï Forest are known to cause disease in people.

4.3.3 Replication of CLEARFLU viruses in Zaire-GP cells

MDCK-SIAT cells transduced to express Zaire-GP were tested using a monoclonal antibody binding panel to ensure that any differences in viral growth dynamics observed in subsequent experiments could not be attributed to low glycoprotein expression at the cell surface. Figure 4.10 shows that all three of the Zaire-GP antibodies tested bound well to the cell line.

Figure 4.11 shows the morphology of viral plaques formed by S-Flu and CLEARFLU version 3 viruses in GP-expressing cells. Plaques formed by S-Flu viruses are visibly less dense than those formed by CLEARFLU. Weaker plaques, reminiscent of those formed by S-Flu, are observed for CLEARFLU viruses which do not stain for CLEARFLU with monoclonal antibody 4A14. This suggests that CLEARFLU expression is correlated with increased replication potential, compatible with our hypothesis that sialic acid binding is advantageous for growth.

Figure 4.10



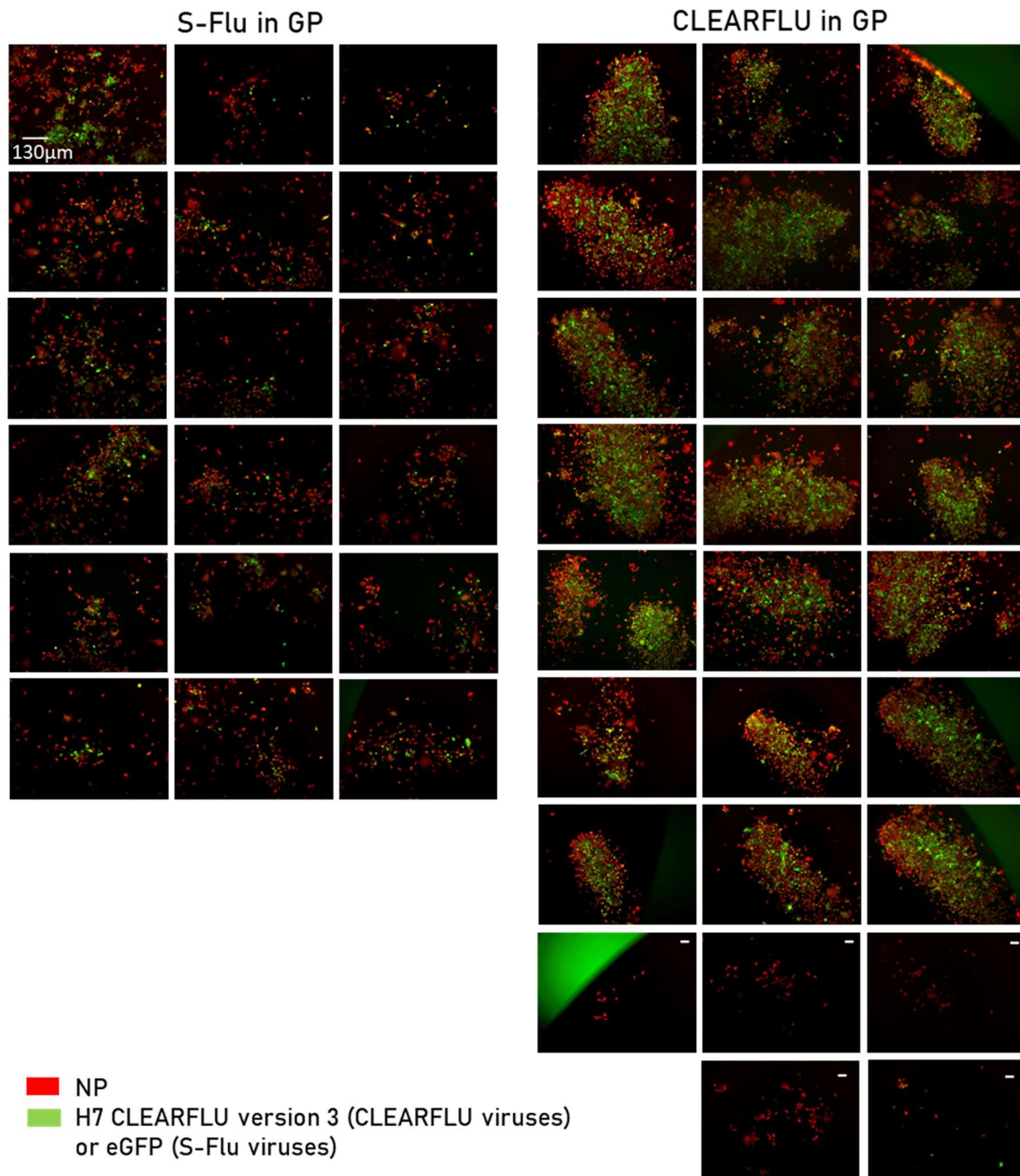
Antibody binding to Ebola Glycoprotein (Zaire strain) expressed in sorted stably transduced MDCK-SIAT cells

Cells were bound with human primary antibodies to the base, receptor binding site or glycan cap and binding was detected with an Alexa Fluor 647 labelled secondary antibody. Data are from a single experiment.

A. Bar graph comparing median fluorescence of cells bound by three antibodies to the glycoprotein.

B. Histogram plots showing the relative frequency of cells detected with a given fluorescence intensity.

Figure 4.11



Plaque morphology of S-Flu and CLEARFLU version 3 viruses in MDCK-SIAT cells stably expressing Ebola glycoprotein

Cells were infected as per TCID₅₀ protocol and stained after 48 hours with nucleoprotein antibody AA5H (AF647 secondary layer). Cells infected with CLEARFLU virus were also stained with hemagglutinin antibody 4A14 (AF488 secondary layer). Fluorescence microscopy images were taken of wells with plaques indicative of clonal expansion. CLEARFLU virus plaques which do not express the binding epitope for 4A14 are shown with a white dash.

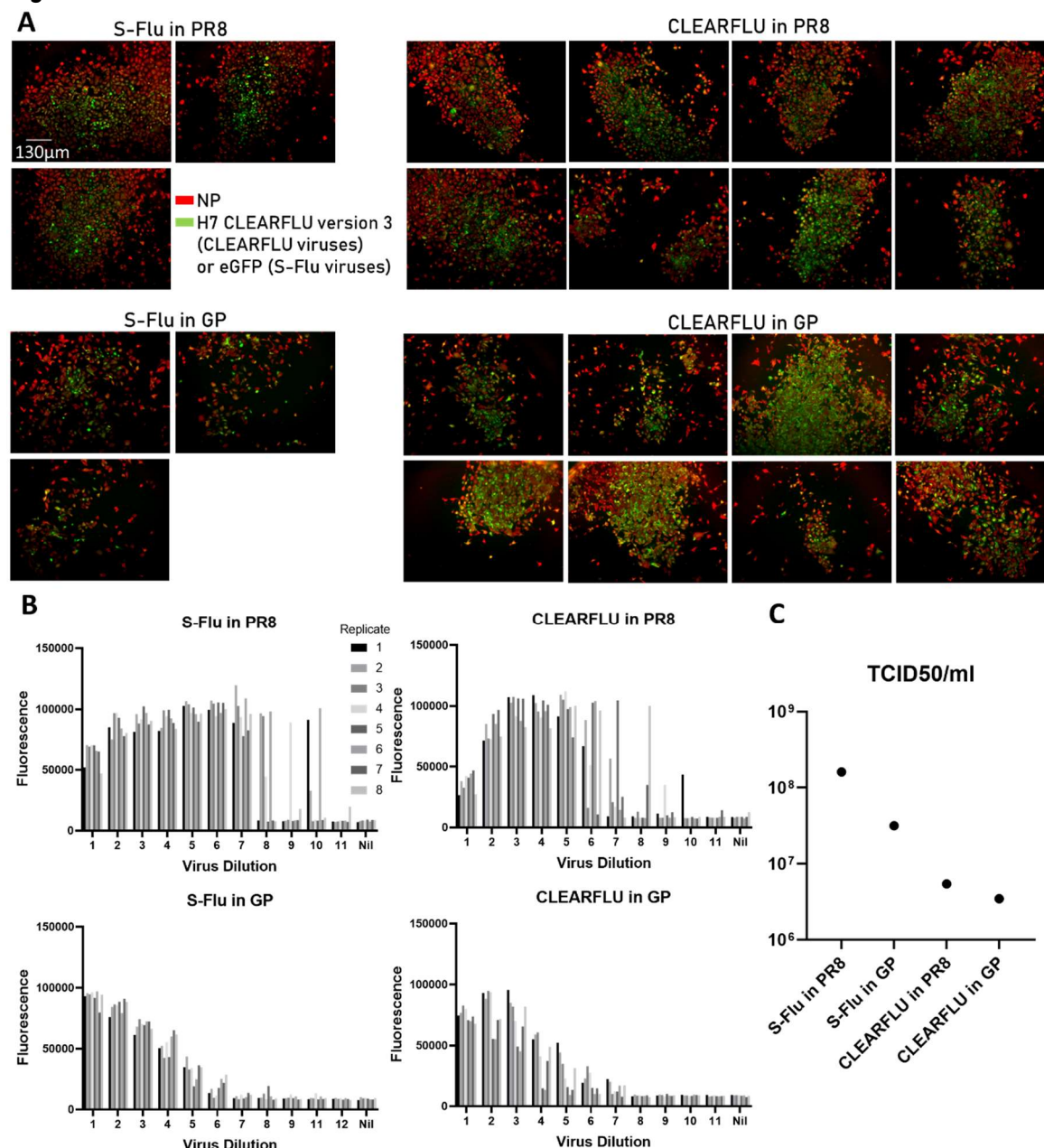
Viruses: H7N1 S-Flu ([S-eGFP/N1(Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2014)) & H7 A/Hong Kong/125 CLEARFLU version 3 reference stock.

Figure 4.12 shows the results of a similar experiment to Figure 4.11 in which the initial virus inoculum was increased to ensure that virus TCID₅₀/ml could be calculated. CLEARFLU viruses formed strong plaques in PR8 hemagglutinin or glycoprotein-expressing cells. Plaques formed by S-Flu are visibly less dense and smaller in cells expressing GP compared to PR8 hemagglutinin. These weaker plaques lead to a lower virus titre by TCID₅₀. Although the CLEARFLU virus may have grown to slightly higher titre in PR8 cells than in GP, the difference is minimal compared to that seen for S-Flu. This might be explained by the poor replication of a fraction of CLEARFLU viruses which have lost expression of the CLEARFLU protein and/or by the reduction in sialic acid binding activity of all viral particles as binding is now mediated largely by CLEARFLU with little support from the pseudotyping glycoprotein.

Finally, we wished to measure the output titre of virus in the supernatant after growth in the different cell lines. Viruses were prediluted to achieve similar titres at infection and facilitate comparisons. S-Flu and CLEARFLU viruses were grown as described in Figures 4.11 and 4.12 and virus titre in the supernatant after 48 hours was measured by CID₅₀. A larger difference is seen in absolute titre and in titration curves between S-Flu groups than between CLEARFLU groups (see Figure 4.13), indicating that growth in GP is less disadvantageous to CLEARFLU viruses than it is to S-Flu viruses. These results are consistent with Julie Xiao's observation from her equivalent experiment in Figure 4.7 that the S-Flu virus does not just grow more slowly, but also peaks at a lower titre in GP cells, around $2-4 \times 10^6$ CID₅₀/ml.

We have therefore shown by plaque morphology and quantitatively by TCID₅₀ on growing plaques and CID₅₀ on viral supernatants that viruses expressing CLEARFLU hemagglutinin have a replicative advantage over those which do not.

Figure 4.12



S-Flu and CLEARFLU growth in MDCK-SIAT cells stably expressing GP or PR8 hemagglutinin

CLEARFLU virus was diluted 1 in 50 and S-Flu 1 in 500 and were added to cells in half-log dilutions as per TCID50 protocol. After 48 hours, cells were stained with AA5H with an AF647 secondary antibody and 4A14 with a AF488 secondary antibody.

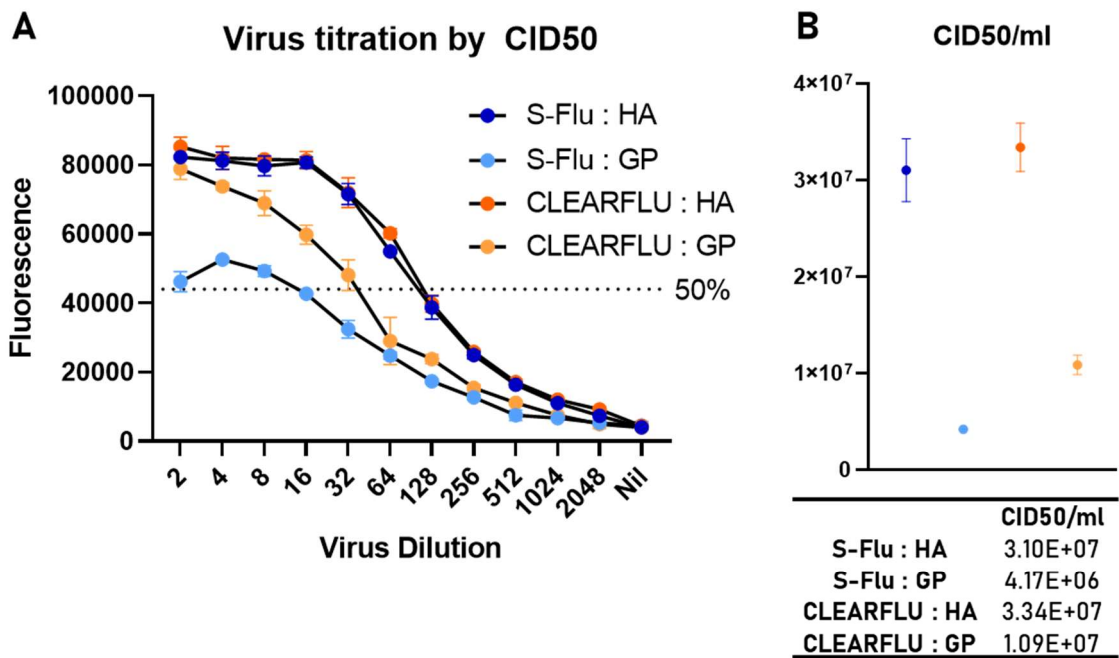
Viruses: H7 A/Hong Kong/125/2017 CLEARFLU version 3 reference stock & H7N1 S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2014))

A. Images show that S-Flu forms weaker plaques in cells transduced with GP than in cells transduced with PR8 hemagglutinin. CLEARFLU virus forms dense plaques in both cell lines.

B. Bars show AF647 fluorescence intensity in each well, measuring NP staining. For both viruses, peaks are seen at a higher dilution factor in hemagglutinin-expressing cells compared to glycoprotein-expressing cells, indicating higher titre.

C. Estimates of TCID50/ml calculated from the readings shown in part B.

Figure 4.13



Titration of S-Flu and CLEARFLU viruses by CID50 after growth in MDCK-SIAT cells expressing GP or PR8 hemagglutinin

S-Flu and CLEARFLU viruses were diluted 1 in 1000 and 1 in 100 respectively to achieve a similar titre and used to infect MDCK-SIAT cells expressing GP or HA according to TCID50 protocol. After 48 hours, the supernatants in the first column were pooled and titrated on untransduced MDCK-SIAT cells as per CID50 protocol. Cells were stained with nucleoprotein antibody AA5H and a secondary layer labelled with AF647.

A. Titration curves by CID50. Error bars show standard deviation based on two duplicates.

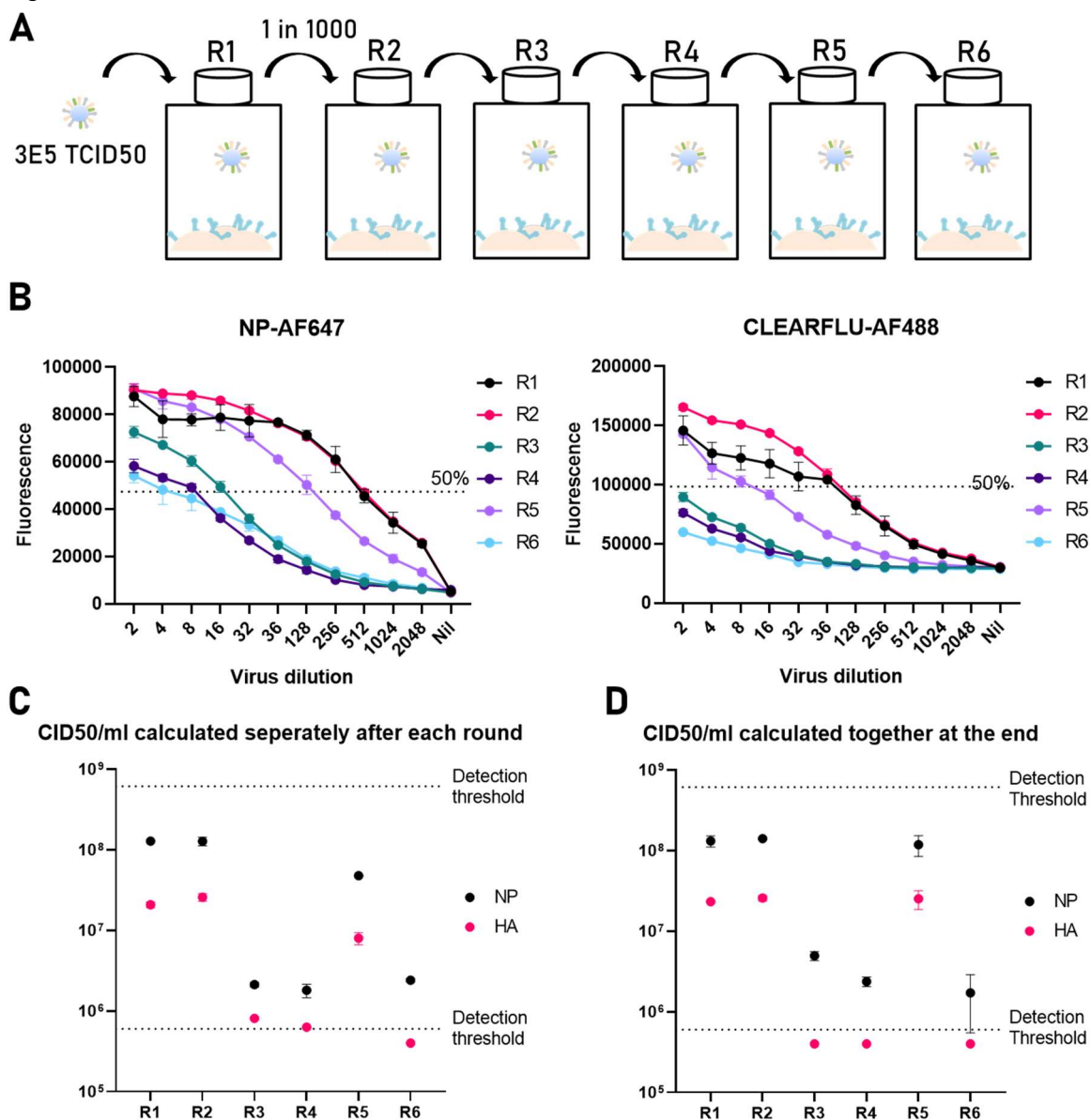
B. Estimated CID50/ml values. Error bars show standard deviation based on two duplicates - for S-Flu : GP they are too narrow to be shown.

4.3.4 Selection for CLEARFLU expression over serial passage at low MOI

To determine whether there is sufficient selective pressure to maintain CLEARFLU expression during large scale expansion, the CLEARFLU version 3 reference virus was passaged six times at low multiplicity of infection in cells expressing GP. At the end of six rounds, the proportion of viral clones which stained with 4A14 was estimated and clones were isolated for characterisation.

Figure 4.14 shows how the CLEARFLU reference virus was expanded for six rounds, each time undergoing a dilution factor of 1 in 1000. In addition to this, the reference virus was diluted 4000-fold to inoculate the first flask. The virus in round 6 (R6) has therefore undergone a 4×10^{18} -fold expansion relative to the cloned reference virus (R0). The nucleoprotein (NP) and CLEARFLU (HA) titre at each round was measured twice- once alongside the experiment in real time to see whether an appropriate dilution factor had been selected (4.14.C) and once at the end of the experiment so that aliquots from all six rounds could be tested simultaneously as part of a single assay (4.14.B & 4.14.D). The results across the two assays were highly similar. The virus titre, as measured by NP, was around 2×10^6 CID50/ml in round 6 virus. This is relatively low, and within the range achieved by S-Flu viruses grown in GP cells. This may therefore be the maximum titre round 6 viruses reach due to a loss of CLEARFLU expression, or alternatively could be limited by the dilution factor and time point used for harvest. CLEARFLU expression was detectable but did not reach the 50% infection point necessary for calculation of CID50/ml.

Figure 4.14



Virus titration by NP and HA during 6 rounds of expansion at low MOI in MDCK-SIAT cells stably expressing GP

A. Experimental set-up. The first T175 flask was seeded with CLEARFLU version 3 reference virus at an MOI of approximately 0.005-0.01 by TCID50. After 48 hours, the virus was harvested and 4 μ l was used to infect the next confluent flask in a total volume of 40ml.

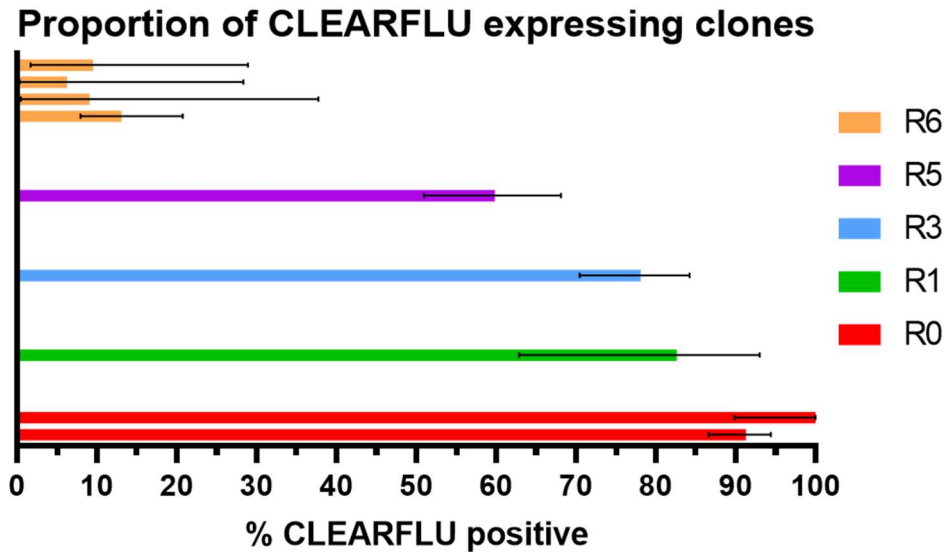
B. Titration curves based on NP or CLEARFLU staining. Viruses from all six rounds were titrated together at the end of the experiment as per CID50 protocol. Cells were stained for NP (primary: AA5H, secondary: AF647) and for CLEARFLU (primary: 4A14, secondary: AF488). The mean fluorescence and standard deviation of duplicates is plotted at each dilution. The dotted line indicates 50% maximum fluorescence.

C. Estimated CID50/ml values for NP and HA calculated in separate assays along the course of the experiment. All points show the mean of two values calculated from duplicate curves. Error bars show the standard deviation for duplicates and are too narrow to be seen in some cases. Where the 50% point was reached either before the first or after the last dilution, points are shown just beyond the respective detection threshold.

D. As above, except fresh aliquots of stored viruses were thawed and titrated together as shown in part B.

To better estimate the extent of CLEARFLU loss, the proportion of viruses positive for 4A14 binding was determined. This was done by double-staining cells infected with expanding clones at limiting dilution and counting the number of plaques with and without 4A14 binding. Figure 4.15 shows the results of 9 independent counts which show that the proportion of clones which expressed the 4A14 epitope decreased over the course of expansion. This confirms that low binding by 4A14 at the population level is due to total loss of binding in some clones, rather than weaker binding in all clones. The lack of overlap in 95% confidence intervals indicates that the decrease between round 3 and round 5 and between round 5 and round 6 are significant at the $p=0.05$ level. The drop in expression from around 60% positivity in round 5 to around 10% in round 6 was particularly large.

Figure 4.15



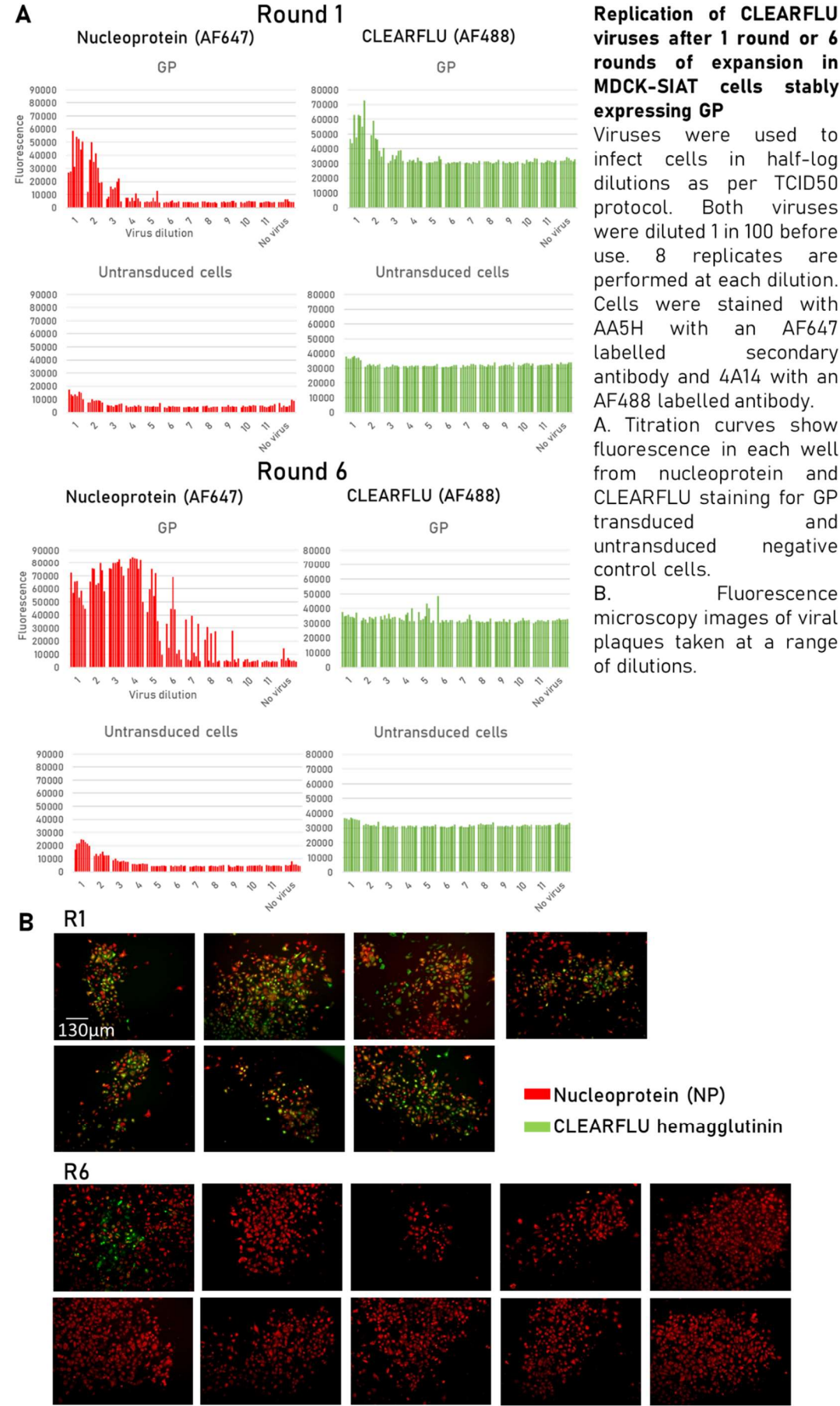
	R0	R0	R1	R3	R5	R6	R6	R6	R6
Positive	188	34	19	107	73	14	1	1	2
Negative	18	0	4	30	49	93	10	15	19
% positive	91.3	100	82.6	78.1	59.8	13.1	9.09	6.25	9.52

Proportion of clones expressing CLEARFLU hemagglutinin after passage at low MOI in MDCK-SIAT cells expressing GP

Cells in 96-well plates were stained 48 hours after infection. Clones were identified by NP staining with AA5H and scored as positive if they also stained with 4A14. Each bar shows an independent count. Error bars shows show 95% confidence interval calculated using the Wilson/Brown method for proportions.

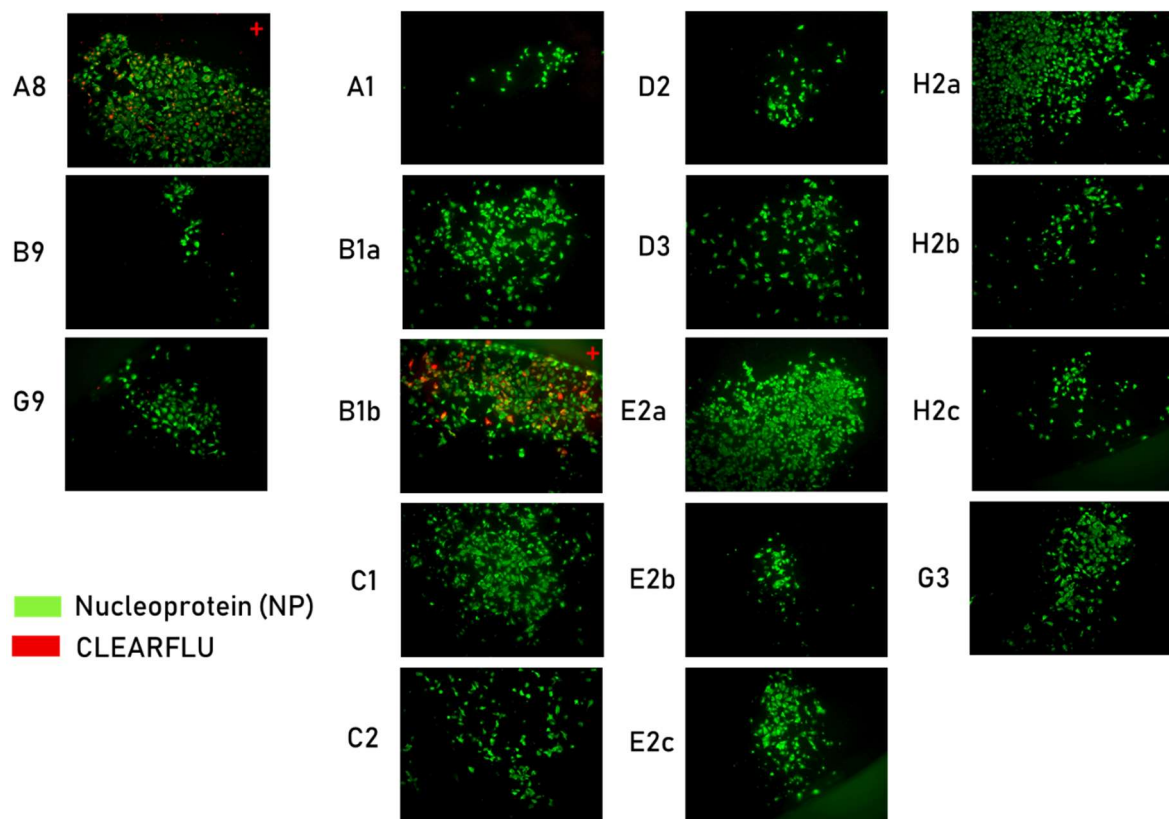
Given that expression of CLEARFLU should impart a replicative advantage to viruses using GP for entry, an *in vitro* replication assay was used to check that the virus had not adapted to circumvent the requirement for GP expression and to study the characteristics of expanding clones. Figure 4.16 confirms that GP expression was still required for cell entry, but that clones were able to form dense plaques in the absence of the 4A14 binding epitope. This suggests either that the 4A14 binding epitope specifically has been lost but that CLEARFLU is still able to mediate cell attachment, or that CLEARFLU no longer mediates binding and the virus has adapted to compensate for this. For example, another protein may have adapted to bind a cell surface receptor, or a change in neuraminidase activity may have altered the optimum level of sialic acid binding. As the 4A14 epitope is around the sialic acid binding site, it seems unlikely that CLEARFLU would still be able to mediate cell attachment even if only local changes have occurred.

Figure 4.16



The possibility that round 6 CLEARFLU hemagglutinin may be truncated or mutated in the head region led us to consider whether the loss of 4A14 binding was a head-specific effect. This was investigated by double-staining infected cells with the stem-binding antibody MEDI8852 alongside nucleoprotein staining. The existence of MEDI8852-positive and MEDI8852-negative plaques indicated that this stem epitope (which we know from the previous chapter is expressed well by CLEARFLU) is also lost in some clones by round 6, and therefore that epitope loss is not a head-specific effect (see Figure 4.17). Viral clones A8, B9 and G9 were identified and isolated in an initial experiment which was repeated to obtain the others. Three types of plaque were identified. A8 and B1b both show the expansion of clones expressing CLEARFLU version 3 and, as expected, grow well. C1, E2a and H2a show the efficient expansion of viral clones despite a lack of CLEARFLU epitope expression. The others show either small or low-density plaques formed by viruses which do not stain for CLEARFLU. Determining what allows clones like C1, E2a and H2a to grow well in the absence of detectable CLEARFLU expression may be key to understanding the loss of CLEARFLU expression over time.

Figure 4.17



Round 6 clones isolated by limiting dilution in MDCK-SIAT cells expressing GP

Round 6 virus was added to cells stably expressing GP in a 96-well plate as per TCID₅₀ protocol. After 48 hours, the supernatant from each well was collected. The cells were bound with the hemagglutinin stem antibody MEDI8852, which was detected using an AF647 labelled secondary antibody, and the NP antibody AA5H which was detected with an AF488 labelled secondary layer. Some wells contained multiple independent clones (B1, E2, H2). Viruses from these wells were cloned out by limiting dilution a second time before being used for further analysis. Red crosses indicate plaques which are positive for MEDI8852 binding.

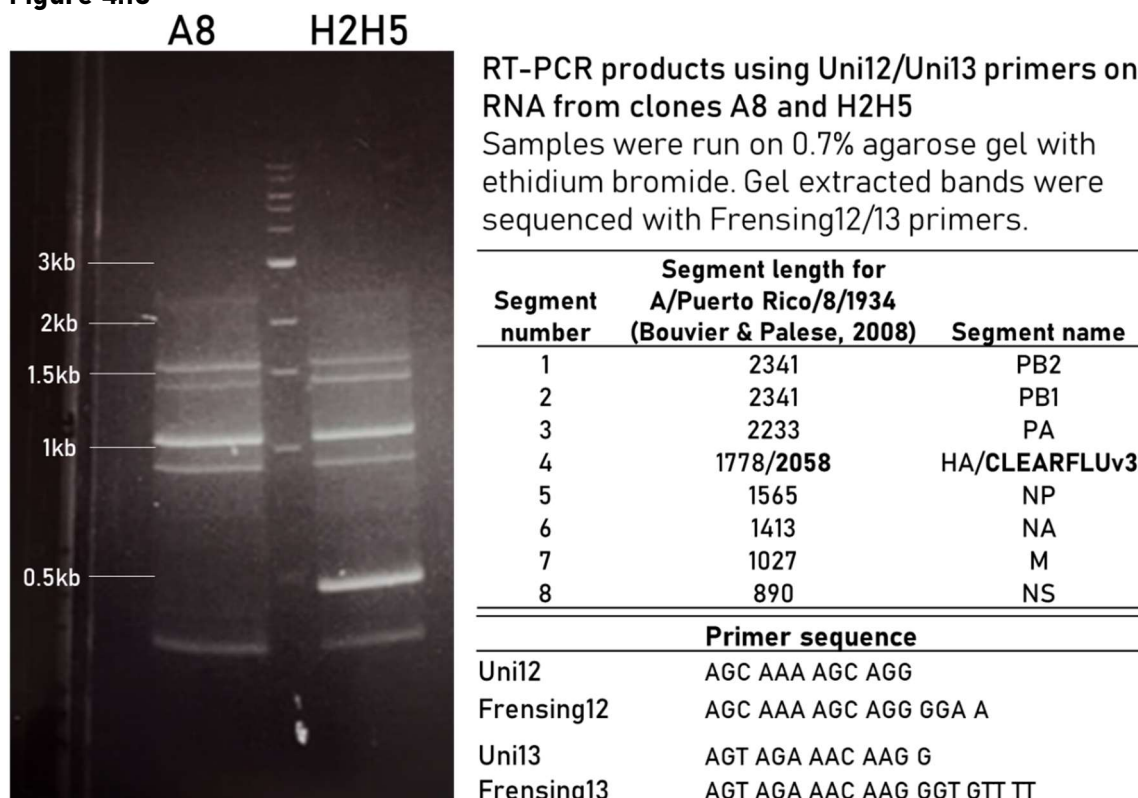
4.3.5 Sequencing of the round 6 genome

To determine how the 4A14 and MEDI8852 epitopes were lost and how this might be compensated for in other genes, the viral genome at round 1 and round 6 was sequenced by reverse transcription polymerase chain reaction (RT-PCR) using primers provided by Dr Jack Tan (see 2.1.4). The coding sequence of each of the viral genes was obtained. This revealed no mutations in the polymerase or other core genes. However, a single nucleotide mutation arose in the neuraminidase gene. This leads to a substitution from threonine to alanine at position 132 in the N1 amino acid sequence, equivalent to 148 in the N2 sequence.

There were difficulties in sequencing the full CLEARFLU hemagglutinin from round 1 and round 6 viruses, likely because the samples contained a mixture of sequences. Therefore, a selection of R6 viral clones isolated in Figure 4.17 were sequenced independently. A8, C1, G3 and G9 were selected for analysis, as well as clones H2H5 and E2B4 which were isolated by re-cloning viruses in wells H2 and E2 respectively.

Attempts to sequence the CLEARFLU segment using a range of primers targeted to the coding region were successful only for clone A8. To understand this issue, we took advantage of the 12 universal bases at the 5' end (Uni12) and 13 universal bases at the 3' end (Uni13) common to all eight segments of influenza viruses (Hoffmann et al., 2001). Using primers which contained the Uni12 or Uni13 sequence, RNA from clones A8 and H2H5 was reverse-transcribed by RT-PCR and size separated by gel electrophoresis to visualise the length of each segment. Figure 4.18 shows that H2H5 has an unexpected short segment just under 0.5kb in length.

Figure 4.18

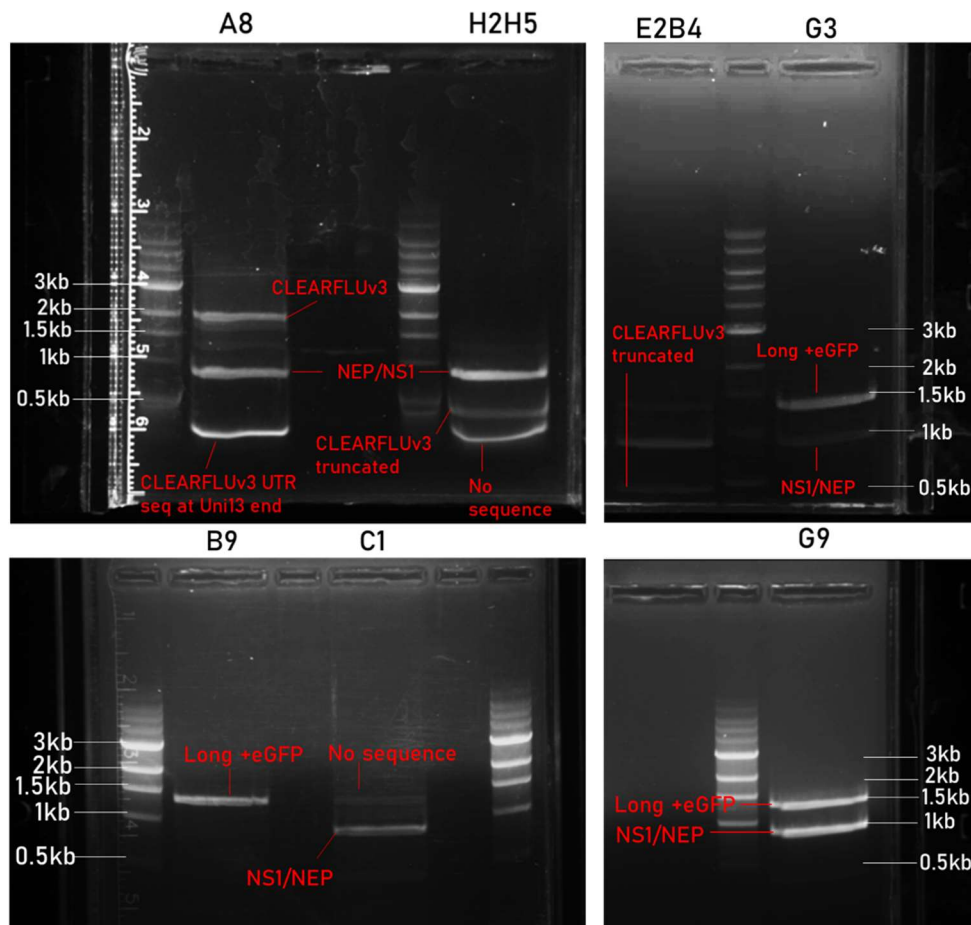


Attempts to sequence this band using Uni12/13 primers were unsuccessful. However, Frensing et al have previously described similar primers which contain the Uni12/13 sequence with the addition of the first few bases of untranslated region (UTR) sequence to allow segment-specific amplification starting from the segment ends (Frensing et al., 2014). Sequencing the 0.5kb band using these primers showed that the H2H5 sequence contained the full CLEARFLU expression cassette and 63 nucleotides of the CLEARFLU coding region, indicating a 1620 nucleotide internal deletion.

A full length CLEARFLU segment was not clearly visible after multi-segment PCR, even for clone A8 which expresses the 4A14 and MEDI8852 epitopes. Therefore, we used the Frensing primers to specifically amplify and sequence the CLEARFLU segment from a range of round 6 clones (see Figure 4.19). This revealed that E2B4 encoded an identical truncated CLEARFLU segment to H2H5, measuring 438 nucleotides long (see Figure 4.20, full sequence in Appendix). A8 encoded a full length, unmutated segment. No CLEARFLU sequence was obtained for the other clones. It is possible that they encode segments of

varying size and sequence, which make them challenging to detect and sequence. Alternatively, they may entirely lack a CLEARFLU segment. Faint bands of approximately 438 nucleotides in size were seen for clones C1 and G9, although these were too weak to be isolated on two separate attempts. Four clones appeared to be contaminated with GFP sequence, possibly through a reagent used in transfection for virus production or RT-PCR. No GFP expression is detectable when these clones are grown in cell culture.

Figure 4.19



RT-PCR amplification products using Frensing12/13 primers

Samples were run on 0.7% Agarose gel with ethidium bromide. Gel extracted bands were sequenced with Frensing12/13 primers.

Sequence from the NS1/NEP segment was detected for several clones, indicating off-target amplification. Clones G3, G9 and B9 all yielded a band containing eGFP sequence and a longer version of the expression cassette, indicating contamination with an eGFP expression plasmid. Clone A8 generated a full length CLEARFLU sequence, as well as a short band of 200bp from the Uni13 end of the CLEARFLU segment, which may be indicative of off-target amplification.

Figure 4.20

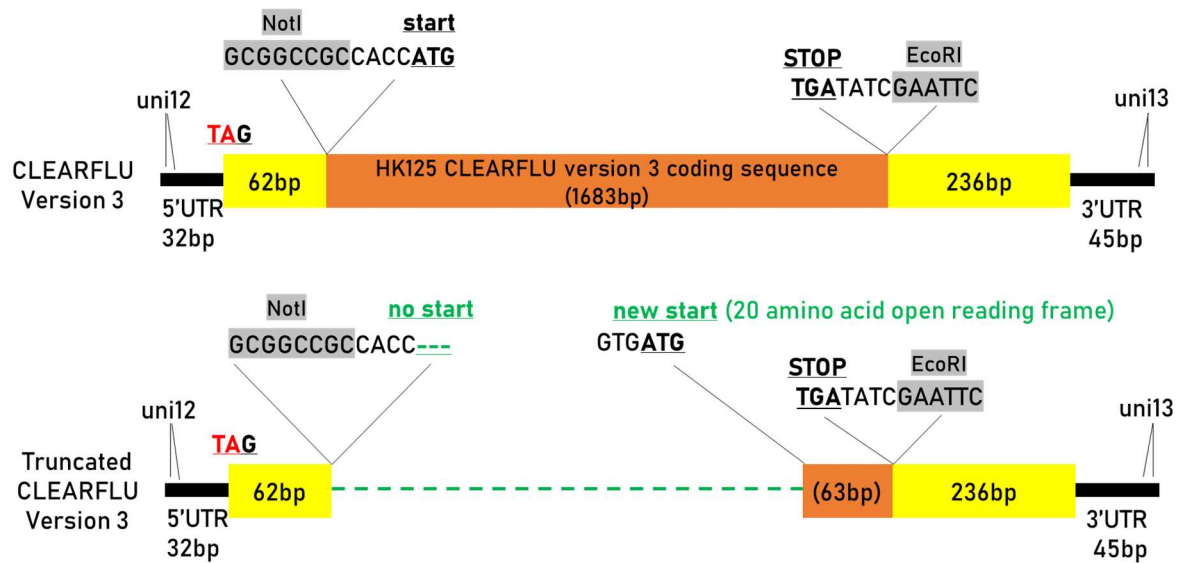


Diagram illustrating the structure of the truncated CLEARFLU version 3 segment observed in clones E2B4 and H2H5

Differences between the expected sequence observed in clone A8 and the truncated sequences observed in clones E2B4 and H2H5 are shown in green. The 1620 nucleotide deletion generates a truncated segment with a 20 amino acid open reading frame in frame with the original construct.

Yellow = PR8 coding sequence, Black = PR8 untranslated regions, Grey = restriction enzyme cloning sites, Orange = CLEARFLU hemagglutinin sequence

4.3.6 Mediators of cell entry

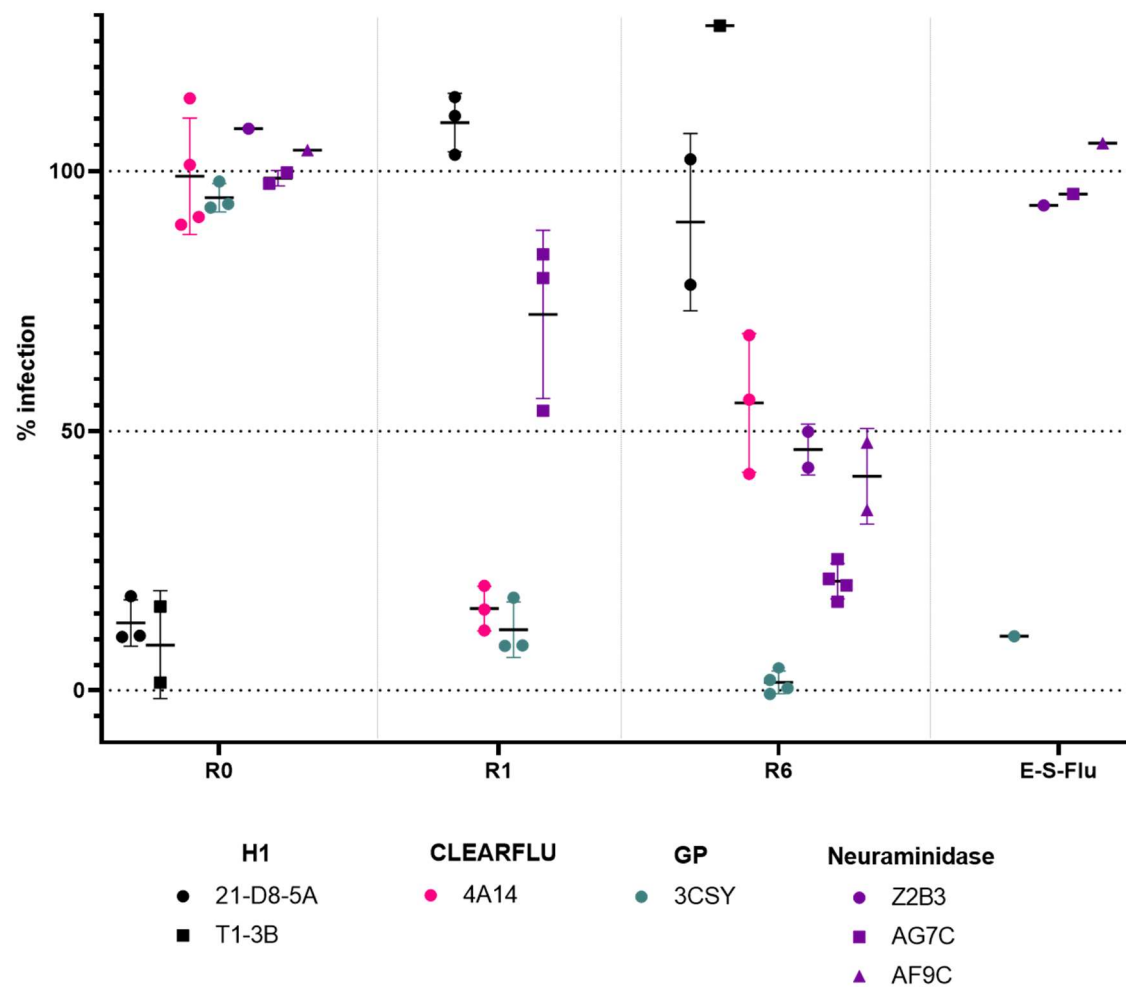
Given that the requirement for viruses to express CLEARFLU to thrive in GP cells appeared to wane between round 1 and round 6, we investigated the role of the different viral surface proteins in mediating cell entry using a neutralisation assay (see Figure 4.21).

R0 reference virus - which is coated in H1 PR8 hemagglutinin - was neutralised by antibodies 21-D8-5A and T1-3B which prevent PR8 hemagglutinin-mediated entry (black), as expected. Similarly, E-S-Flu was strongly neutralised by anti-GP antibody 3CSY (teal). In R1 virus, anti-CLEARFLU (pink) and anti-GP (teal) antibody both dramatically reduced cell infection by 85-90%, which is compatible with complete or near-complete neutralisation. By round 6, anti-CLEARFLU antibody only reduced infection by around 40%, while anti-GP antibody maintained its capacity to inhibit infection. This suggests that sialic acid binding by CLEARFLU has become less important for entry. Antibodies against neuraminidase (purple), which do not ordinarily affect entry (see R0 and E-S-Flu), reduced infection by R1 viruses by 30%. By round 6, this had increased to up to 80% inhibition. Entry therefore switches from being mediated predominantly by CLEARFLU and GP in round 1 to CLEARFLU, GP and neuraminidase in round 6. This suggests that neuraminidase has acquired and then consolidated a role in cell entry.

The observation that PR8 neuraminidase does not play a role in cell entry of E-S-Flu virus shows that the role of neuraminidase is not simply hidden by effective binding through HA and subsequently revealed in viruses with weaker cell binding. While sequencing of the neuraminidase gene in R1 viruses did not detect any deviations from the wild-type sequence, it is possible that the T132/148A mutation detected in R6 was already present at low frequency and would have been detected with a deep sequencing approach.

The next chapter is dedicated to understanding the role of neuraminidase in cell entry for GP-pseudotyped CLEARFLU viruses and whether this is linked to the T132/148A mutation.

Figure 4.21



Surface proteins involved in viral entry as determined by microneutralisation

Viruses were incubated with antibody before being added to cells according to microneutralisation protocol. After overnight infection, infected cells were stained for nucleoprotein with AA5H and an AF647 secondary layer. All antibodies were titrated in two fold dilutions, starting at 20µg/ml in the first dilution to ensure a plateau was reached. Percentage infection was calculated using the fluorescence observed in the absence of antibody and the fluorescence observed at saturating antibody concentration. Error bars show the standard deviation around the mean based on 1-4 independent experiments, each of which is represented by a symbol.

4.4 Discussion

4.4.1 Summary

In the previous chapter, we experimented with introducing the Y98F mutation into the CLEARFLU design to inhibit sialic acid binding activity. In our latest iteration - CLEARFLU version 3 - we opted not to include this mutation as we reasoned that it might disrupt neutralising antibody binding epitopes around the sialic acid binding site or reduce viral titres in some culture conditions. This decision proved fortuitous as it has permitted us to investigate whether sialic acid binding can be used to select for continued viral expression of CLEARFLU, and therefore hemagglutinin epitopes, over the course of large-scale expansion.

In this chapter, we investigated two possible methods for maintaining high levels of CLEARFLU expression. In our hands, the passage mutant hemagglutinin described by Hooper and Bloom did not select for CLEARFLU expression despite supposedly entirely lacking cell attachment capacity. There was no evidence that S-Flu was able to grow less well in this cell line than in cells expressing wildtype hemagglutinin. Contrastingly, CLEARFLU-expressing viruses did appear to have a growth advantage in cells expressing Zaire glycoprotein as they formed denser plaques and grew to higher titres. Even after six rounds of expansion at low MOI, equivalent to a dilution factor of over 10^{18} from reference stock, approximately 10% of viral clones still expressed CLEARFLU.

Functionally, CLEARFLU still plays an important role in facilitating cell entry of round 6 virus as inhibiting CLEARFLU-mediated sialic acid binding using 4A14 reduces cell entry by 40%. Despite the dramatic 90% loss in CLEARFLU expression, CLEARFLU must therefore still be beneficial for cell entry. This might indicate that the resource costs of replicating and expressing CLEARFLU outweigh the benefits of enhanced cell entry. Particularly, this could be influenced by the properties of other viral proteins, notably the

neuraminidase which acquired the T132/148A mutation and a role in cell entry over the course of expansion. For instance, a decrease in neuraminidase enzymatic activity might reduce the optimal level of sialic acid binding and therefore reduce selection for CLEARFLU. Likewise, if the T148A mutation has permitted neuraminidase to acquire sialic acid binding activity, contributions by CLEARFLU may become redundant. The acquired role for neuraminidase in cell entry and the effect of the T132/148A mutation is further explored in the next chapter.

One purpose of this chapter was to investigate whether CLEARFLU expression could be maintained sufficiently well to produce highly antigenic vaccine candidates at a large scale. Ultimately, *in vivo* immunogenicity trials must be performed to determine whether expression levels are sufficient for use in vaccination and therefore whether a selection mechanism has been successful. After 5 rounds in Zaire-GP cells, CLEARFLU version 3 virus grew to high titre ($\sim 10^8$ CID50/ml) and showed high CLEARFLU expression at the population ($\sim 10^7$ CID50/ml) and individual clone level (60% CLEARFLU positivity). Limiting expansion to five rounds at this MOI – equivalent to a dilution factor of 10^{15} – or recloning virus after 5 rounds may therefore be a viable approach for generating large volumes of virus for vaccination. How CLEARFLU expression translates to immunogenicity is explored in Chapter 6, where CLEARFLU version 3 reference virus (R0) which has approximately 90-100% CLEARFLU positivity is tested for immunogenicity in mice.

No stabilising, silent or reversion mutations were identified in clones which expressed full length CLEARFLU. This was perhaps surprising given the length of the segment and the known infidelity of the RNA polymerase. This is encouraging as in viruses which express full length CLEARFLU, antigenic sites are maintained and there is no evidence that any inactivating mutations are lost. The lack of stabilising mutations provides no evidence that altering the CLEARFLU sequence could improve its genetic stability. We also note that the viral plaques which do stain with CLEARFLU antibodies stain strongly, indicating that high

levels of CLEARFLU are expressed on the surface of infected cells. At face value, this implies that the challenge is in maintaining the proportion of clones encoding full length segments rather than preventing the accumulation of mutations in the coding or regulatory regions which may decrease antigenicity, expression or the number of changes needed for reversion to a functional form. Fortunately, viral populations can be monitored for segment truncations by PCR and antibody staining and enriched for full length segments by cloning out viruses which stain for CLEARFLU, as described in this chapter. However, we cannot rule out that other mechanisms including base substitutions or low efficiency in packaging the CLEARFLU segment into virions do not also play a role in decreasing CLEARFLU expression and antigenicity. A deep sequencing approach may provide more insights into the true diversity of CLEARFLU segment variants, in terms of sequence, segment size or absence.

We were able to identify two round 6 clones (H2H5 and E2B4) with an identical heavily truncated CLEARFLU segment reminiscent of defective interfering RNAs. The 438nt size of the truncated fragment draws a clear parallel with observations made by Jennings et al (Jennings et al., 1983). Following passage of PR8 virus at high MOI, they identified 35 unique sub-genomic RNAs (sgRNAs) from all but one viral segment. These sgRNAs contain conserved 5' and 3' ends from their parent segment (and thus contain packaging signals) and arise from deletions or rearrangements of viral RNA sequence. Notably, 84% of the sgRNAs observed measured between 300 and 500 nucleotides long, with extremes of 178nt and 857nt. Jennings et al describe a high proportion (36/41) of identified sgRNAs as being generated by a simple large internal deletion. These were mostly comprised of similarly sized contributions from either end of the original segment. Although the fragment we observed does not contain equal contributions from the 5' and 3' ends, it does contain more than the minimal sequences described for each end. Other groups have also observed subgenomic RNAs of a similar size, including Winter et al (Winter et al., 1981)

who proposed that such deletions occur as the viral polymerase jumps between parts of its template. They proposed that the size of these fragments is determined by the tertiary structure of viral RNAs in ribonucleoparticles during replication, which brings the beginning and end of segments into close proximity.

It is unsurprising that we have observed a similar truncation. Replicating and expressing a full length CLEARFLU segment is likely costly and may be of little benefit. Encoding a small segment may be better for segment packaging efficiency than lacking the CLEARFLU segment entirely due to the network of packaging signal interactions involved in virion formation (Goto et al., 2013; Hutchinson et al., 2010). CLEARFLU expression could reduce viral fitness by competing for space on the viral surface and driving down the expression of neuraminidase and the pseudotyping coat, therefore altering the balance between sialic acid binding, cleavage and fusion activity. The deletion we have observed is so significant that we would expect no protein to be expressed on the cell surface, thus enabling higher expression of neuraminidase and the fusion protein and a return to having only two major surface proteins.

In this chapter we have shown that CLEARFLU viruses can be expanded quickly to high titre. Viral stocks were expanded 4×10^{15} fold between R0 and R5 in ten days, resulting in a titre at R5 that was slightly higher than the titre at R0 ($\sim 8.32 \times 10^7$ and 3.13×10^7 CID₅₀/ml). Just one microlitre of reference virus (R0) could therefore yield sufficient virus in ten days to immunise over 10^{13} mice intraperitoneally at the titre used for CLEARFLU version 3 in Chapter 6.

We observed that viral titres fluctuated from round to round. Cycling in virus titre over serial (Kirkwood & Bangham, 1994) or continuous passage (Frensing, 2014) is a known characteristic of populations containing defective interfering particles (DIPs). This occurs as virions containing DIPs replicate poorly at low MOI but rapidly accumulate in high titre environments where they are likely to coinfect cells alongside the standard virus which

they rely on to replicate. As the proportion of fully competent viruses decreases, so does the infectivity of the population, which leads to a decrease in titre. As the CLEARFLU segment is not essential for replication in GP cells, viruses with a truncated CLEARFLU segment should not behave as true defective interfering particles. However, as our microneutralisation assay showed that CLEARFLU enhances cell entry even of round 6 virus, a full-length segment might be sufficiently advantageous to drive cyclical dynamics. This could however be complicated by the growing role for neuraminidase in cell entry. To establish whether these are true cyclical behaviours, further work could determine whether the number of truncated CLEARFLU segments also cycles and whether this pattern continues with further passage. The role of CLEARFLU in facilitating cell entry may also fluctuate. We did not observe fluctuation in the proportion of clones which stain for CLEARFLU at rounds 1, 3, 5 and 6 suggestive of cyclical behaviour. We instead observed a stable decline over time. The possibility that cyclical dynamics are at play is worthy of investigation as higher titres and higher CLEARFLU expression might be achieved with one or more additional passages. Alternatively, passaging viruses after shorter intervals or using less seed virus may lead to better maintenance of CLEARFLU expression by keeping the multiplicity of infection low.

The second purpose of this chapter was to investigate the stability of other parts of the viral genome. With the exception of the single mutation observed in the neuraminidase, no genomic changes were detected outside of the CLEARFLU hemagglutinin. This is promising for a vaccine candidate as it suggests that there is a low probability that CLEARFLU viruses will develop altered characteristics including pathogenicity and transmissibility. As CLEARFLU viruses should undergo only a single cycle of infection after immunisation, we expect that very few changes, including CLEARFLU reversion mutations, should arise in the viral genome *in vivo*.

4.4.2 Implications of sialic acid binding by CLEARFLU version 3

CLEARFLU version 3 viruses differ from S-Flu and CLEARFLU version 2 viruses in that they express a viral protein with an intact sialic acid binding site. Although CLEARFLU version 3 viruses cannot replicate in untransduced cells or *in vivo*, they are able to undergo a single cycle of infection thanks to their pseudotyping hemagglutinin or glycoprotein coat. As the viral segments involved in replication, gene expression, viral assembly and release are all as wildtype, we might expect that virus-like particles with CLEARFLU hemagglutinin but no functional pseudotyping coat on their surface bud from infected cells and are released into the supernatant. In Figure 4.1, we showed that S-Flu grown in cells expressing CLEARFLU version 3 agglutinates erythrocytes. This confirms that S-Flu viruses coated in CLEARFLU version 3 are able to bud from MDCK-SIAT cells and bind sialic acid on erythrocytes. This implies that infection of untransduced cells with CLEARFLU version 3 could yield viral-like particles which are able to bind sialic acid on a second generation of host cells.

As the CLEARFLU hemagglutinin cannot mediate fusion, this binding could not trigger infection. However, we might expect these virus-like particles to behave like viruses which have been neutralised by antibodies which block hemagglutinin function in the same ways as the mutations we have introduced, namely through blocking cleavage or fusion. Brandenburg et al have shown that viruses bound by neutralising stem-binding antibodies which block either cleavage or fusion are internalised and delivered to the late endosome (Brandenburg et al., 2013). A lack of nucleoprotein expression confirms that this occurs in the absence of infection. Viruses bound by head antibodies are not internalised, providing no evidence that the presence of antibody influences virus localisation. We propose that CLEARFLU virus-like particles may also reach the late endosome and consequently may present protein and RNA antigens to these secondary cells. We therefore postulate that

viral expression of hemagglutinin epitopes in the form of CLEARFLU version 3 may be a more efficient antigen delivery system than using a non-sialic acid binding protein.

In Chapter 3 we discussed how although viruses expressing hemagglutinin with the Y98F mutation in the sialic acid binding site do not agglutinate red blood cells, they do not show reduced replication in cell culture unless cells with particularly low sialic acid expression are used (Meisner et al., 2008; Martin et al., 1998). They do show reduced replication *in vivo*, likely because sialic acid availability is lower. This suggests that Y98F lowers but does not abolish sialic acid binding, and that relatively weak binding is sufficient for strong viral growth in MDCK-SIAT cells. This indicates that there is unlikely to be powerful selective pressure for viruses to maintain strong sialic acid binding when grown in MDCK-SIAT cells, which overexpress sialic acid (Matrosovich et al., 2003). Only low levels of CLEARFLU expression may therefore be required for viruses pseudotyped in GP to achieve strong viral growth. This raises the question of whether using a cell line with lower sialic acid expression might select for a higher proportion of CLEARFLU expressing clones. Counter-intuitively, this may also suggest that a weaker sialic acid binding protein such as CLEARFLU version 2 might be more strongly maintained in MDCK-SIAT cells. The benefits of this may be outweighed by the advantages of expressing a stronger sialic acid binding protein once *in vivo*, where stronger binding increases efficiency of cell infection and therefore may improve immunogenicity.

We therefore propose that using alternative cell lines, lower seeding MOI, shorter passages and experimenting with sialic acid affinity may optimise this system for maintaining expression of CLEARFLU epitopes over long term expansion.

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Chapter 5

EXPLORING THE EFFECT OF THE T132/148A MUTATION ON NEURAMINIDASE FUNCTION

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5.1 Introduction

The neuraminidase protein is important for the release of viral particles from the cell surface after budding. It mediates release by cleaving terminal sialic acid residues from cell surface glycoproteins so that hemagglutinin on the viral membrane no longer binds to them. It also prevents viruses from forming clumps through binding between hemagglutinin and sialic acids on the surface of neighbouring virions.

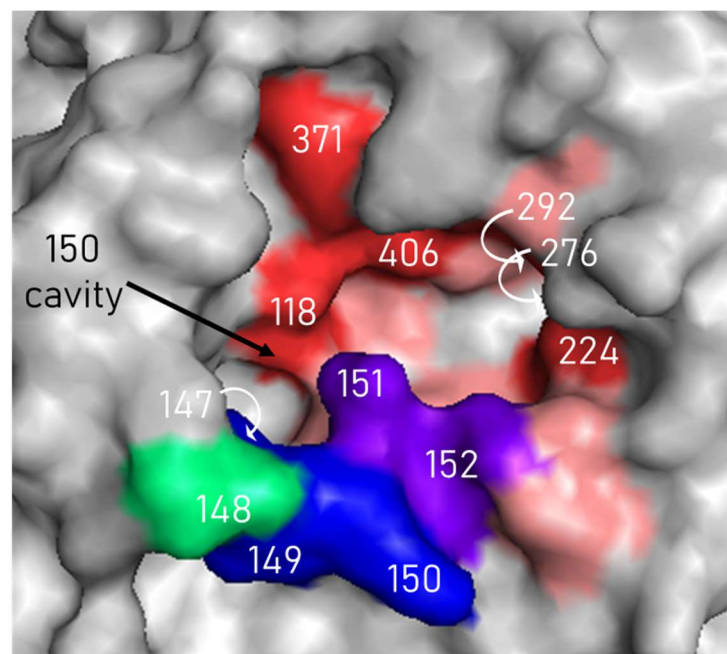
In the previous chapter, a mutation in the neuraminidase gene was observed over the course of large-scale expansion of CLEARFLU version 3 virus in cells expressing Zaire-GP. This appeared to be correlated with an acquired role for neuraminidase in cell entry and decreased expression of the sialic acid binding protein CLEARFLU. This evidence hinted that the neuraminidase gene had perhaps taken-over cell binding activity, rendering CLEARFLU hemagglutinin expression redundant.

This chapter explores whether the T132/148A mutation is associated with the changes observed in neuraminidase function and explores the implications of this on selection for CLEARFLU expression.

5.1.1 The 150-loop

The neuraminidase tetramer does not have a canonical role in cell entry. While neuraminidase inhibitors restrict viral growth by inhibiting viral release and preventing detachment from decoy receptors such as mucins, they therefore do not prevent cell entry. The neuraminidase enzymatic site is made up of eight highly conserved residues which contact sialic acid and a further ten framework residues with important structural roles (see Figure 5.1, reviewed in McAuley et al., 2019).

Figure 5.1



The N1 neuraminidase active site

Residues which directly contact sialic acid (Arg118, Arg224, Glu276, Arg292, Arg371, Tyr406) are shown in red and framework residues in pink (reviewed in McAuley et al, 2019). The 150-loop is shown in blue except for residues 151 and 152 (purple) which also contact sialic acid and residue 148 (green). Sites are numbered except for framework residues. PDB 2HTY showing N1 A/Vietnam/1203/2004 was obtained from Russell et al, 2006

Examples of variants at each site in the 150-loop are discussed below. Several studies have identified mutations in the 150-loop which lead to neuraminidase-mediated sialic acid binding, usually measured by red blood cell (RBC) agglutination. This phenomenon is particularly common in recent H3N2 isolates, and may compensate for the decreased sialic acid binding activity of some H3 hemagglutinins. In some cases, these neuraminidase mutations increase sensitivity to neuraminidase inhibitors such as oseltamivir and zanamivir, most notably by rendering them neutralising. This suggests that neuraminidase has taken on a vital role in cell entry.

Other mutations in the 150-loop decrease neuraminidase activity. This is perhaps an alternative adaptation to poor hemagglutinin-mediated sialic acid binding as the balance between sialic acid binding and cleavage is maintained when both activities are low. Some mutations both decrease enzymatic activity and enable neuraminidase-mediated sialic acid binding.

These examples illustrate that single changes in the 150-loop are able to dramatically alter neuraminidase characteristics and suggest that T148A could conceivably facilitate a role in cell entry through sialic acid binding. This may or may not be accompanied by changes in enzymatic cleavage activity.

152

One of the most conserved residues in the 150-loop is the arginine at 152. Lentz et al mutated 11 conserved sites around the substrate binding pocket and determined the effect on binding and catalysis in an N2 neuraminidase (Lentz et al., 1987). Mutations at many but not all of these sites led to complete ablation of enzymatic activity, including R152K and R152I. Introducing R152K into an H1N1 2009 pandemic virus can decrease susceptibility to oseltamivir (Baek et al., 2015).

Mutations at site 151 occur naturally in H3N2 viruses and tend to be most often seen in cell cultured viruses (reviewed by Xue et al., 2016). Common mutations include D151G and D151N, amongst others.

Lin et al found that D151G does not affect cleavage activity but does lead to neuraminidase-mediated agglutination of RBCs (Lin et al., 2010). This agglutination was resistant to enzymatic cleavage by neuraminidase, indicating that the sialic acids bound by neuraminidase are different to those bound by hemagglutinin, which are sensitive to cleavage. This suggests that neuraminidase and hemagglutinin therefore exhibit different sialic acid binding specificities and that neuraminidase can bind different sialic acids than it cleaves.

Zhu et al also studied H3N2 viruses with naturally arising D151G (Zhu et al., 2012). In agreement with Lin et al, they observed that D151G leads to α -2,3 and α -2,6 sialic acid binding by neuraminidase, with a preference for α -2,3. Crystal structures show that this binding occurs in the enzyme active site. However, they observed drastically reduced sialidase activity relative to wildtype for both linkage types. While Lin et al tested activity of virus on MUNANA and ELLA, Zhu et al used recombinant neuraminidase protein and tested activity on MUNANA and a glycan-based enzyme assay. The glycan array showed that neuraminidase D151G exhibited similar binding and cleavage specificities, contrasting with Lin et al's suggestion that the mutant neuraminidase binds different acids than it cleaves. Zhu et al suggest that the differences in their observations, particularly in relation to reduced neuraminidase cleavage activity, are likely due to their use of purified neuraminidase protein, as a small population of virus with wildtype neuraminidase could have affected Lin et al's results. Zhu et al extended their observations to show that D151G also imparted sialic acid binding and decreased cleavage activity in another N2 and three further N1 neuraminidases.

Also studying H3N2 viruses, Xue et al have shown that mixed 151D/G populations grow better than clonal populations, as one genotype contributes stronger attachment and the other provides stronger cleavage (Xue et al., 2016). This effect is also seen for D151N mutants, which grow poorly on their own but cooperate with D151. 151D/G cooperation is not observed when hemagglutinins with strong sialic acid binding are used, presumably because the contribution by 151G to sialic acid binding becomes redundant. When a hemagglutinin with no sialic acid binding is used, both D and G variants are necessary as they provide sialidase and cell attachment activity respectively.

150

The H150R mutation is thought to have first arisen in 2006 in human H3N2 isolates and is associated with loss of sialic acid binding by hemagglutinin (Mögling et al., 2017). Viruses with this mutation agglutinate red blood cells through their neuraminidase.

149

Group 1 neuraminidases typically have a valine residue at 149, with the exception of 2009 N1 pandemic viruses which, similarly to group 2 neuraminidases, encode isoleucine. Position 149, along with the presence of a salt-bridge between residues 147 and 150, appears to be linked to the conformation of the 150-loop and therefore the presence of the 150-cavity (Amaro et al., 2011). The conformation of the 150-loop affects sensitivity to neuraminidase inhibitors, as the open 150-cavity in most N1 neuraminidases favours binding by zanamivir while the absence of a cavity in group 2 neuraminidases favours binding by oseltamivir (Sriwilaijaroen et al., 2016). Similarly, mutation at 149 has been linked to modulation of sensitivity to neuraminidase inhibitors, with I149V reducing sensitivity of 2009 pandemic N1 to oseltamivir and V149I increasing sensitivity of avian N1 to oseltamivir (Yongkiettrakul et al., 2013).

The G147R mutation arises naturally in chicken H5N1 and human H1N1 isolates. When Hooper and Bloom attempted to grow N1 viruses with an H3 hemagglutinin defective for sialic acid binding, they observed that the only viruses they rescued contained the G147R neuraminidase mutation (Hooper & Bloom, 2013). Viruses containing this mutation agglutinate RBCs in a manner which is sensitive to neuraminidase inhibitor oseltamivir, indicating that neuraminidase is able to take over sialic acid binding. This mutation did not appear to affect neuraminidase activity.

Gentles et al showed that this virus is totally neutralised by anti-neuraminidase antibodies, suggesting that neuraminidase-mediated sialic acid binding plays an essential role in cell binding (Gentles et al., 2020). The ability of antibodies to block entry correlated well with their ability to block activity on ELLA, suggesting that sialic acid binding and enzymatic activities are co-localised within the neuraminidase.

In H3N2 viruses, aspartic acid (D) is usually found at 147, contributing to the formation of a salt bridge between positions 147 and 150 which stabilises the closed formation of the 150-loop.

Tamura et al searched GISAID and found variants I, K, P, S and M alongside the standard T148 in H3N2 human isolates (Tamura et al., 2013). A/Saitama/103/2014 and A/Hongkong/4801/2014 are examples of known T148I and T148K variants in human H3N2 clade 3C2a (Nextflu/GISAID). N1 neuraminidases also vary at this site, for instance A/Taiwan/29/2010 and A/Auckland/528/2013 both contain T148S.

T148I has been detected as a cell culture adaptation which has a small effect increasing zanamivir resistance but no effect on resistance to other neuraminidase inhibitors, including oseltamivir (Tamura et al, 2013). It also leads to 50% reduction in neuraminidase activity on MUNANA and may contribute to receptor binding.

Mok et al describe 148 as one of the highly conserved sites at the oligomerisation interface (Mok et al., 2013). They showed that unlike many neighbouring sites, mutation at T148 to alanine is not deleterious to survival of an H1N1 virus. While this mutation does not trigger changes in structure at the oligomerisation interface or drastically alter the stability of the tetramer, it may affect the conformation of other regions. T148A has been identified in human pdm09 isolates and may be associated with weak zanamivir resistance (Takashita et al., 2020).

Determining how the T148A mutation affects viral characteristics may therefore be relevant to influenza evolution and control. This chapter begins by exploring whether T148A facilitates cell entry by permitting sialic acid binding and how this could affect the sensitivity of viruses to cell entry inhibitors, before turning to investigate sialidase activity and expression. Finally, the wider implications of neuraminidase mutations on viral evolution and the protectiveness of immune responses and chemical inhibitors are discussed.

5.2 Results

5.2.1 Properties of round 6 clones

In the previous chapter, an increase in the prevalence of the T148A mutation appeared to be correlated with an increasing role for neuraminidase in cell entry in a CLEARFLU virus which had undergone 6 rounds of replication in cells expressing Ebola GP. In this section, individual viral clones are studied to determine whether those with T148A exhibit different characteristics to those with a wildtype neuraminidase.

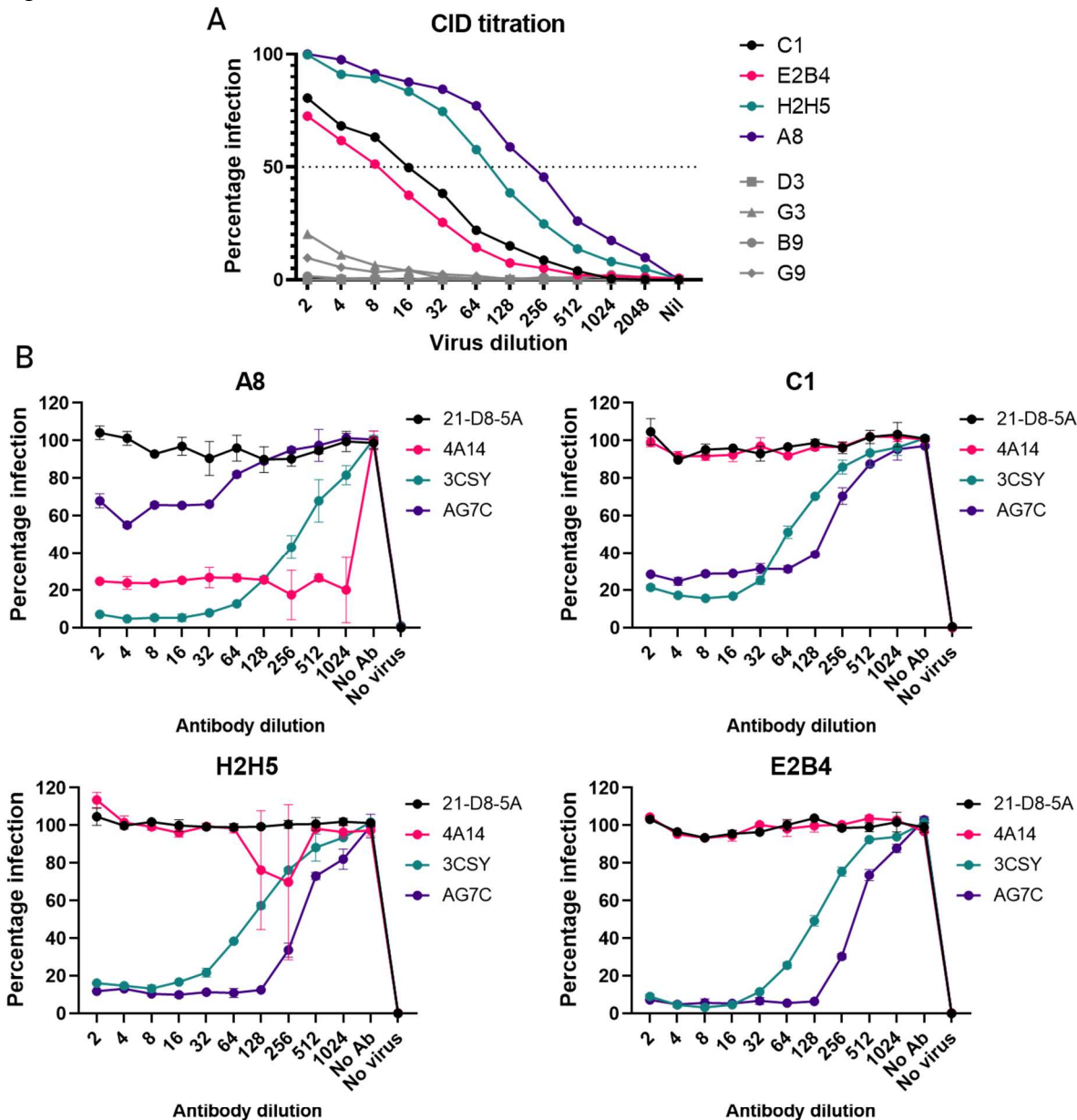
Viral clones A8, G9, B9, C1, D3, G3, E2B4 and H2H5 isolated in Chapter 4 from round 6 virus were expanded and then titrated by CID50. Clones B9, G9, D3 and G3 had not grown to a sufficient titre to be tested in a microneutralisation assay. This was not surprising given that they formed weak plaques in Figure 4.17 in the previous chapter. The effect of antibodies targeted to hemagglutinin, neuraminidase and glycoprotein on cell entry was determined for the other clones (see Figure 5.3).

All four clones were neutralised by anti-GP antibody 3CSY, as expected. A8 – the only clone which previously stained for H7 CLEARFLU with MEDI8852 and for which a full-length CLEARFLU segment could be detected – was partially neutralised by H7 antibody 4A14. This confirms that CLEARFLU has a role in promoting cell entry of clone A8, but is not essential.

AG7C, an antibody which blocks neuraminidase activity and reduces viral spread but does not ordinarily affect cell entry was also tested. Entry of A8 was reduced a little in the presence of AG7C, while H2H5, E2B4 and C1 were fully or almost fully neutralised. This indicates that neuraminidase has a more important role in cell entry for these clones than for A8. This neuraminidase involvement in promoting cell entry might explain why these clones were able to grow to high titre and form dense plaques in the absence of CLEARFLU expression. For H2H5 and E2B4 in particular, anti-neuraminidase antibodies appeared to

have a similar effect on cell entry to anti-GP antibodies. This is surprising as GP alone should be necessary and sufficient for cell entry. It may be that AG7C is not fully neutralising but reduces viral entry to such an extent that entry is undetectable in this assay.

Figure 5.3



Surface proteins involved in cell entry of CLEARFLU round 6 clones by microneutralisation

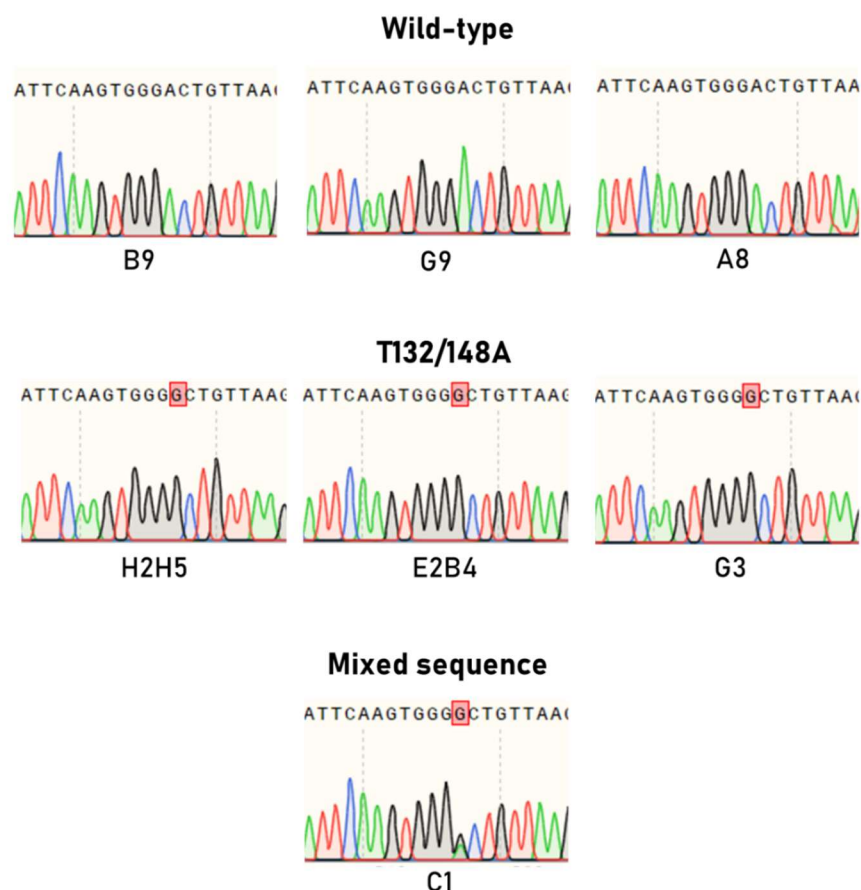
A. CID titration of eight CLEARFLU round 6 clones. A8, H2H5, E2B4 and C1 were used at 1 in 20, 1 in 8, 1 in 2 and 1 in 2 respectively for microneutralisation (B). D3, G3, B9 and G9 (grey) yielded titres too low for use in microneutralisation.

B. Neutralisation of A8, C1, H2H5 and E2B4 by H1 head antibody 21-D8-5A, H7 head antibody 4A14, GP antibody 3CSY and neuraminidase antibody AG7C. 21-D8-5A is used as a negative control. Error bars show standard deviation based on two duplicate titrations. Results are from a single experiment which was repeated with the same result.

Sequencing of the neuraminidase gene of these four clones identified the T148A mutation in E2B4, H2H5 and C1 but not in A8 (Figure 5.4). T148A was therefore associated with strong neutralisation by AG7C and efficient growth in the absence of CLEARFLU expression. Notably, C1 generated mixed sequence, indicating that it was not a true clone and explaining the intermediate level of AG7C neutralisation observed.

Of the clones which were at too low titre to assess microneutralisation, G3 (with the highest titre of the four) contained the T148A mutation and B9 and G9 did not. G3 was expanded successfully on a second attempt and used in later experiments (see 5.2.2). RT-PCR for sequencing D3 failed twice and was not pursued further given that virus was not detectable by CID.

Figure 5.4

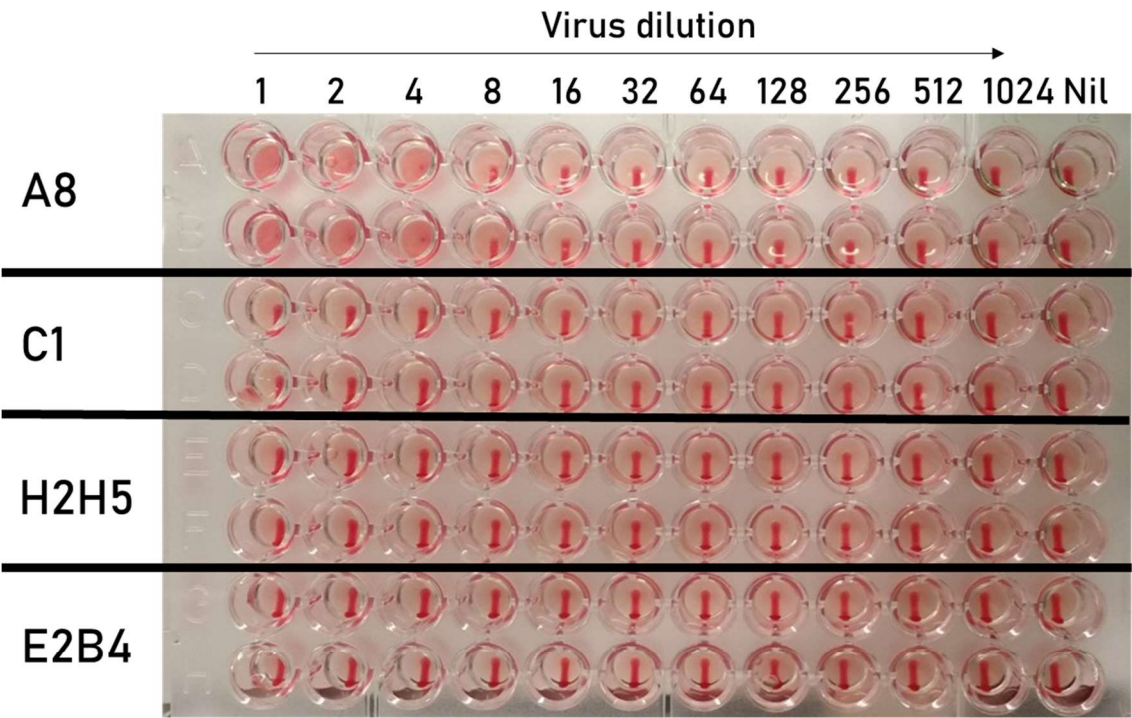


Sequencing of the neuraminidase gene

One non-synonymous adenine to guanine mutation was identified in several clones, causing a change from threonine to alanine at position 132(N1)/148(N2) in the amino acid sequence. Red boxes show the position of the mutation.

A hemagglutination assay did not provide any evidence to suggest that this mutation allowed the neuraminidase in clones C1, H2H5 or E2B4 to bind sialic acid and agglutinate red blood cells (Figure 5.5). The result is compatible with the hypothesis that the lack of CLEARFLU expression in these clones leads to poor sialic acid binding activity. However, it is possible that agglutination would have been observed at higher viral titres, especially as C1, H2H5 and E2B4 all have lower titres than A8. This is explored further in sections 5.2.2 and 5.2.3 where higher titre T148A viruses are produced.

Figure 5.5

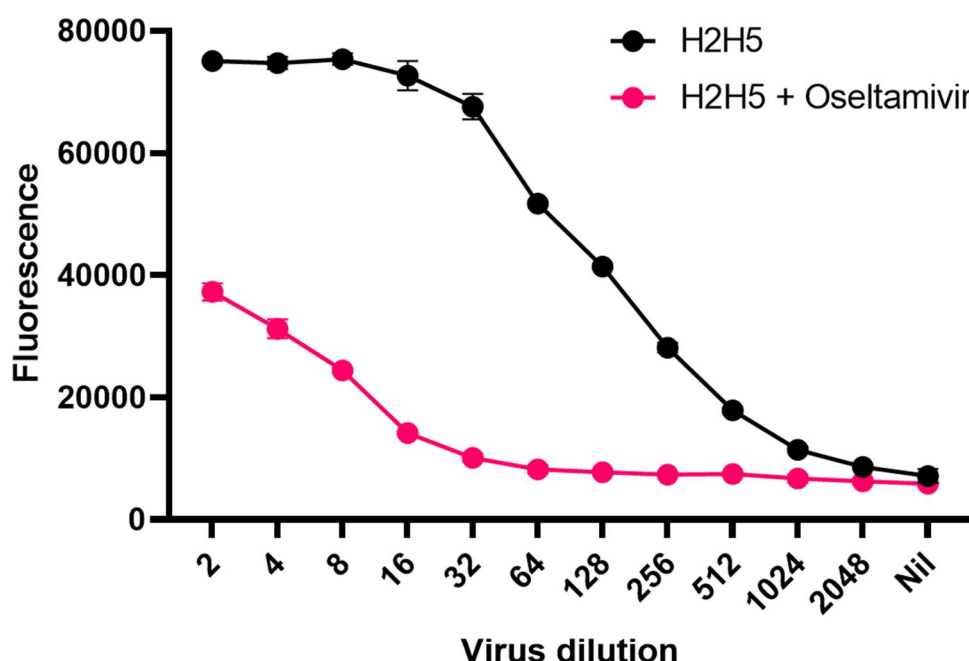


Agglutination of red blood cells by CLEARFLU round 6 clones

Viruses were added to RBCs in doubling dilutions as per hemagglutination assay protocol, starting from neat virus in the first column. Agglutination was observed for clone A8 in the first three dilutions but not for any other clones.

H2H5 was selected as the standard T148A virus for further analysis on account of its high titre. To verify that neutralisation by AG7C was directly caused by effects on the neuraminidase rather than steric inhibition of GP activity, the neuraminidase-inhibiting drug oseltamivir was tested for its effect on viral entry. Figure 5.6 shows that oseltamivir inhibits cell entry of clone H2H5 and therefore that this is not due to steric hindrance.

Figure 5.6



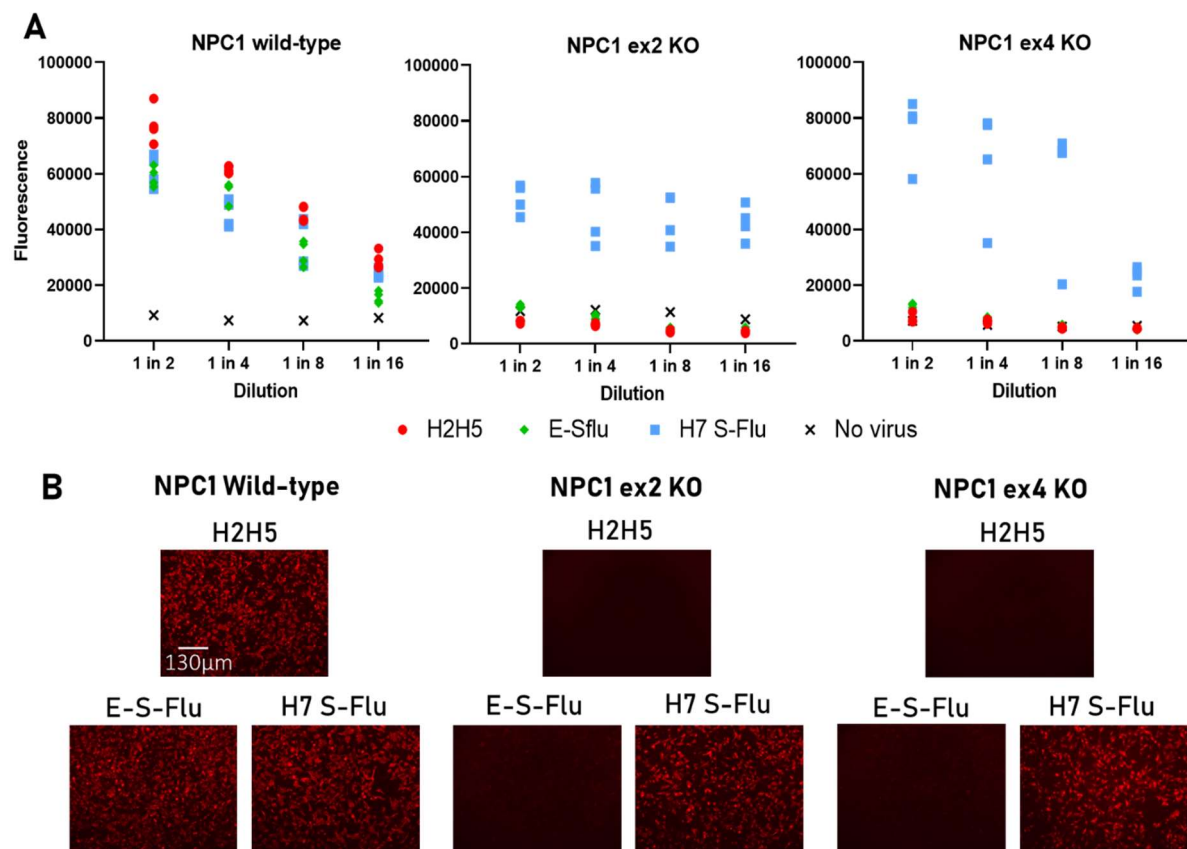
Titration by CID of clone H2H5 with and without oseltamivir

Doubling dilutions of H2H5 were set up in quadruplicate in 96 well plates. Two sets were incubated for 1 hour with 25µl of 50nM Oseltamivir and the other two with 25µl of PBS before adding cells as per CID50 protocol. Fluorescence was measured by staining for nucleoprotein (NP) (primary: AA5H, secondary: AF647). Error bars show the standard deviation of duplicates.

As GP alone should be sufficient for both cell attachment and fusion, it was important to determine how neuraminidase contributes to cell entry. An experiment was designed to test whether the fusion step of cell entry was still dependent on GP and therefore its endosomal receptor NPC1 (see Figure 5.7). HeLa cell lines were obtained from Julie Xiao. These included two NPC1 knock-out cell lines; one lacking the second exon (NPC ex2 KO) and one lacking the fourth (NPC ex4 KO). These cells were infected with H7 S-Flu, E-S-

Flu (both with standard PR8 neuraminidase) or clone H2H5 which has the T148A neuraminidase mutation. Hemagglutinin provided fusion activity for H7 S-Flu, allowing this virus to infect NPC1 knock-out cells. H2H5 behaved like standard E-S-Flu, indicating that the T148A mutation did not circumvent the requirement for NPC1 and therefore that GP mediates fusion. The implication of this is that T148A neuraminidase likely contributes to attachment while GP alone mediates fusion.

Figure 5.7



Dependence on NPC1 for viral entry

Viruses H2H5, E-S-Flu and H7 S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2017)) were added to cells in doubling dilutions and incubated overnight. The experiment was performed in quadruplicate. Infection was detected by NP fluorescence by permeabilising cells and staining with biotinylated antibody 2-8C and a streptavidin-AF647 secondary layer.

A. Plots show the fluorescence of individual wells infected with H2H5, E-S-Flu or H7 S-Flu at four dilutions. Four values are shown for each virus at each dilution. Crosses show background fluorescence in uninfected cells.

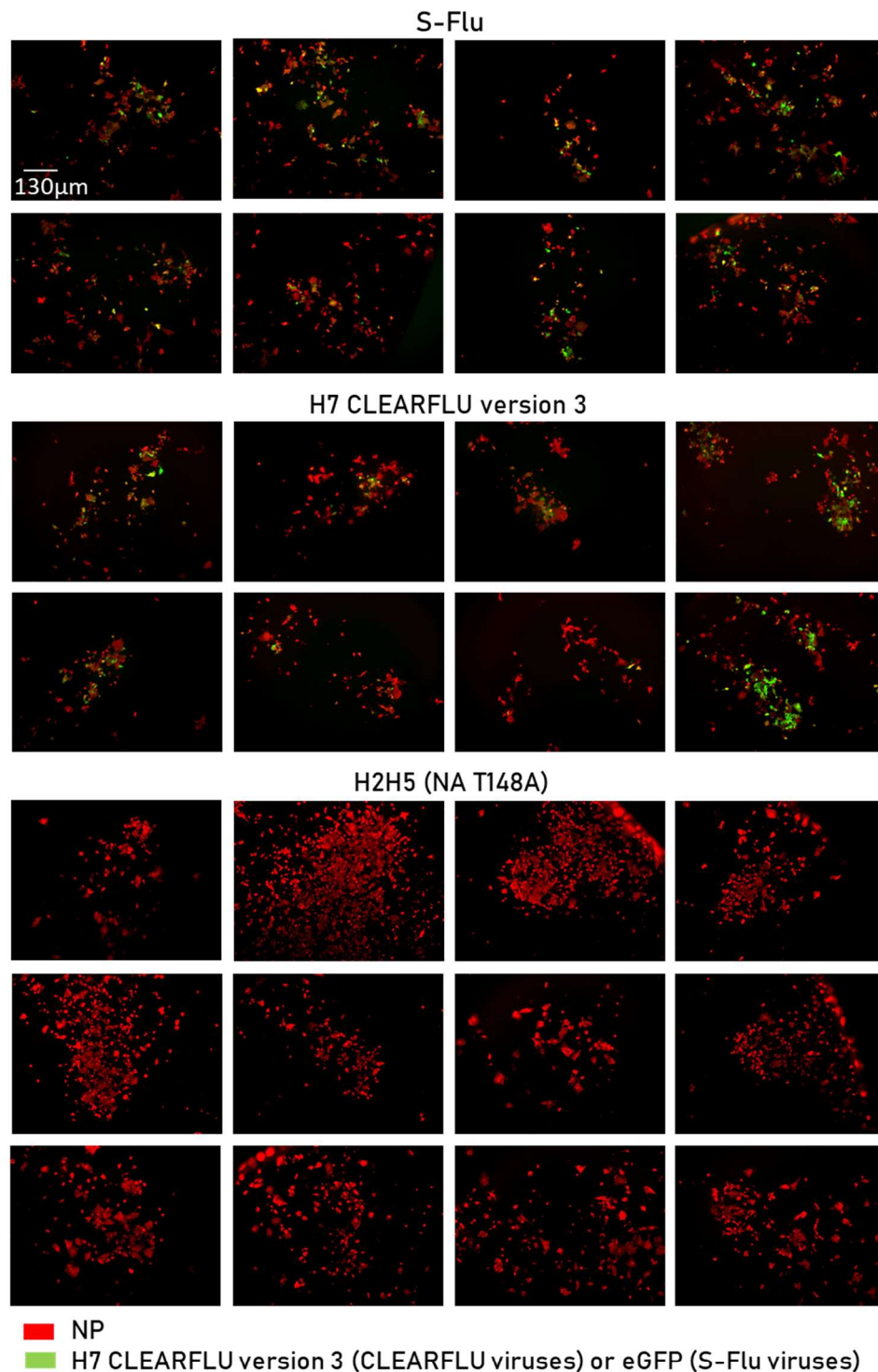
B. Fluorescence microscopy images show NP staining of infected cells in the first dilution. No infected cells were observed for H2H5 or E-S-Flu in either NPC1 knock-out cell line.

5.2.2 Properties of Sudan-GP-pseudotyped viruses

We were interested to explore whether viruses pseudotyped with a glycoprotein from another strain of ebolavirus would similarly show T148A-associated enhanced cell entry. The glycoprotein from the Sudan strain was selected as Sudan pseudotypes of E-S-Flu grow particularly poorly, even relative to other Ebola glycoproteins (Julie Xiao, personal communication). The expression of T148A neuraminidase or CLEARFLU might therefore be even more advantageous in Sudan pseudotypes.

Figure 5.8 shows the morphology of plaques formed by S-Flu and CLEARFLU viruses in Sudan-GP cells. Unlike in Zaire-GP cells, the expression of CLEARFLU did not appear to improve the density of plaques in Sudan-GP cells. However, denser plaques were observed for the H2H5 clone. This suggests that Sudan-GP is not complemented by CLEARFLU expression in the same way as Zaire-GP, and that in Sudan-GP cells the neuraminidase mutation provides better complementation than CLEARFLU.

Figure 5.8

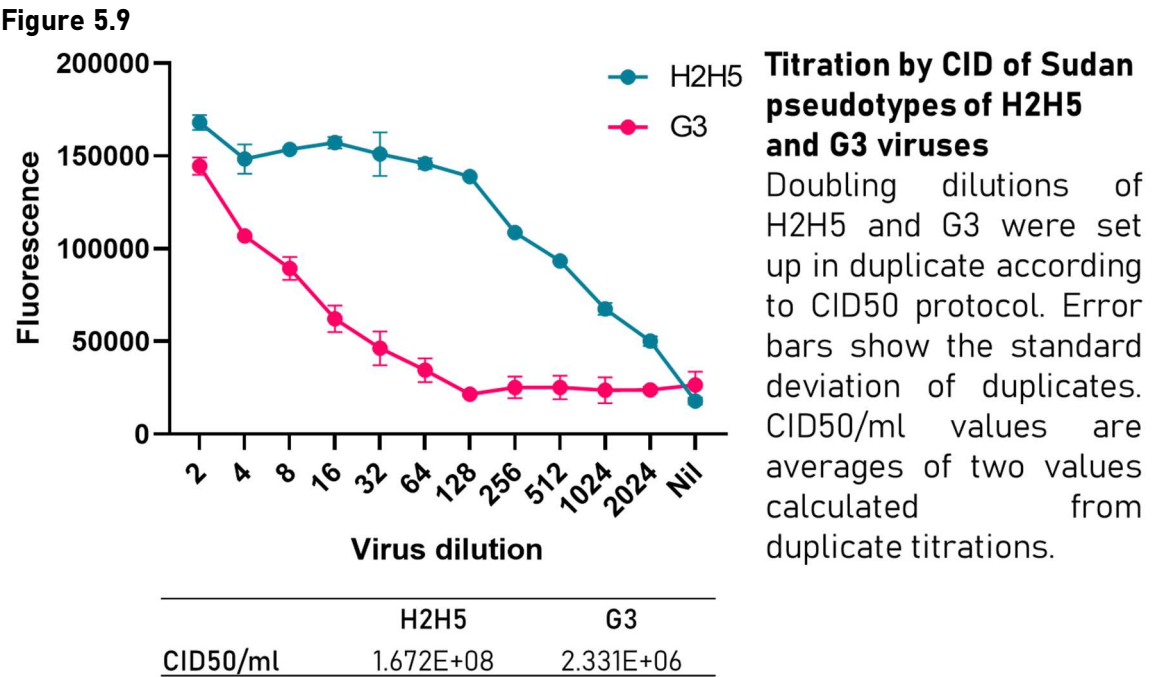


Plaque morphology of S-Flu, CLEARFLU version 3 and H2H5 viruses in MDCK-SIAT cells stably expressing Sudan glycoprotein

Cells were infected as per TCID₅₀ protocol and stained after 48 hours with AA5H and an AF647 secondary antibody. Cells infected with CLEARFLU or H2H5 were also stained with 4A14 and an AF488 secondary antibody to detect CLEARFLU hemagglutinin. Fluorescence microscopy images were taken of wells with distinguishable clones. This experiment was performed twice with concordant results.

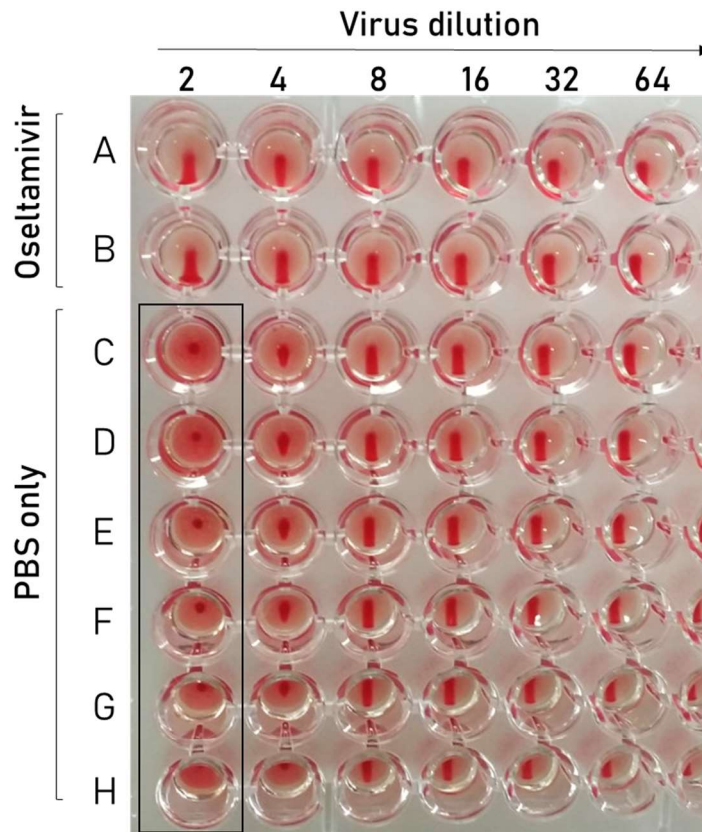
Viruses: H7N1 S-Flu ([S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2017)), H7 A/Hong Kong/125/2017 CLEARFLU version 3 reference stock and clone H2H5.

To ascertain whether enhanced growth in Sudan-GP cells was related to cell entry, Sudan pseudotypes of two round 6 clones carrying the T148A mutation were produced (see Figure 5.9). The titre generated by H2H5 was extraordinarily high for a Sudan pseudotype, as titres are usually difficult to quantify as fluorescence does not reach saturation. This high titre allowed for sialic acid binding to be tested by agglutination.



Despite not expressing full-length CLEARFLU, the H2H5 Sudan pseudotype agglutinated red blood cells (Figure 5.10). This activity could be blocked by oseltamivir, confirming that neuraminidase was mediating sialic acid binding. Previously, we had not detected agglutination activity by H2H5 when coated in Zaire-GP (see Figure 5.5), likely because the virus was at much lower titre. No agglutination was detected for the G3 Sudan pseudotype, presumably also due to low titre.

Figure 5.10



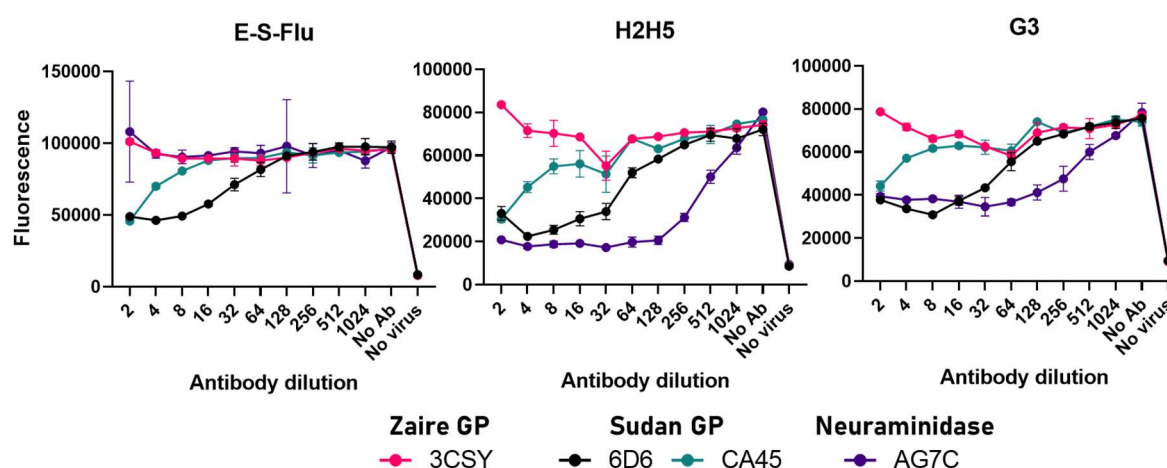
Agglutination of red blood cells by H2H5 coated in Sudan-GP

Doubling dilutions of virus were set up in a 96 well plate and pre-incubated for 1 hour with 25 μ l of 50nM Oseltamivir or PBS only before being added to RBCs as per hemagglutination assay protocol. H2H5 agglutinated RBCs in the first dilution (C-H), but not in the presence of Oseltamivir (A&B). This experiment was repeated with concordant results.

A microneutralisation assay was used to determine whether the T148A neuraminidase adopts a role in cell entry in Sudan-GP coated viruses. Figure 5.11 shows that Sudan-GP antibodies CA45 and 6D6 neutralise Sudan pseudotypes of H2H5 and G3. Neuraminidase antibody AG7C does not neutralise standard Sudan E-S-Flu but strongly neutralises H2H5 and G3.

Together, these data support that neuraminidase has been able to co-opt a role in cell entry in both Zaire and Sudan GP-pseudotyped viruses and that this is linked to acquiring the T148A mutation and sialic acid binding activity (see Table 5.1).

Figure 5.11



Surface proteins involved in cell entry of Sudan-GP coated viruses by microneutralisation
Neutralisation of Sudan-GP coated E-S-Flu, H2H5 and G3 viruses by Sudan GP antibodies 6D6 and CA45 and neuraminidase antibody AG7C. Zaire specific GP antibody 3CSY is used as a negative control. Error bars show the standard deviation of duplicates. Low titre viruses which must be used almost neat for microneutralisation (here E-S-Flu and G3) pose a technical challenge due to high background fluorescence even when viruses are fully neutralised. CA45 and 6D6 are known to be neutralising for Sudan E-S-Flu. Results are from a single experiment which was repeated with concordant results.

Table 5.1 – Summary of the properties of round 6 clones pseudotyped in Zaire or Sudan glycoprotein. Pseudotyping proteins are shown in brackets.

CLONE	PLAQUE DENSITY IN ZAIRE-GP CELLS	MEDI8852 STAIN	CLEARFLU	NA	STRONG NEUTRALISATION BY ANTI-NA ANTIBODY OR OSELTAMIVIR	RBC AGGLUTINATION
A8	HIGH	YES	FULL	WT	NO (Zaire)	YES (Zaire)
B9	LOW	NO		WT		
C1	HIGH	NO		WT/T148A	PARTIAL (Zaire)	
E2B4	HIGH	NO	TRUNCATED	T148A	YES (Zaire)	
G9	MEDIUM	NO		WT		
H2H5	HIGH	NO	TRUNCATED	T148A	YES (Zaire & Sudan)	YES (Sudan, NA-mediated)
G3	MEDIUM	NO		T148A	YES (Sudan)	

5.2.3 T148A in S-Flu pseudotypes

To confirm that the T148A mutation was the cause of changes in cell entry behaviour, S-Flus which differ only at the T148A site were produced by site-directed mutagenesis of the neuraminidase plasmid. All other plasmids used for virus production were the same and the mutagenized plasmid was sequenced to confirm identical sequence except at the target site.

Wild-type and T148A mutant S-Flus were pseudotyped in H7 (HK125) hemagglutinin and then Zaire glycoprotein and tested for hemagglutination and microneutralisation. The viral neuraminidase was sequenced after growth in both cell lines to confirm that no mutations had arisen over the course of expansion.

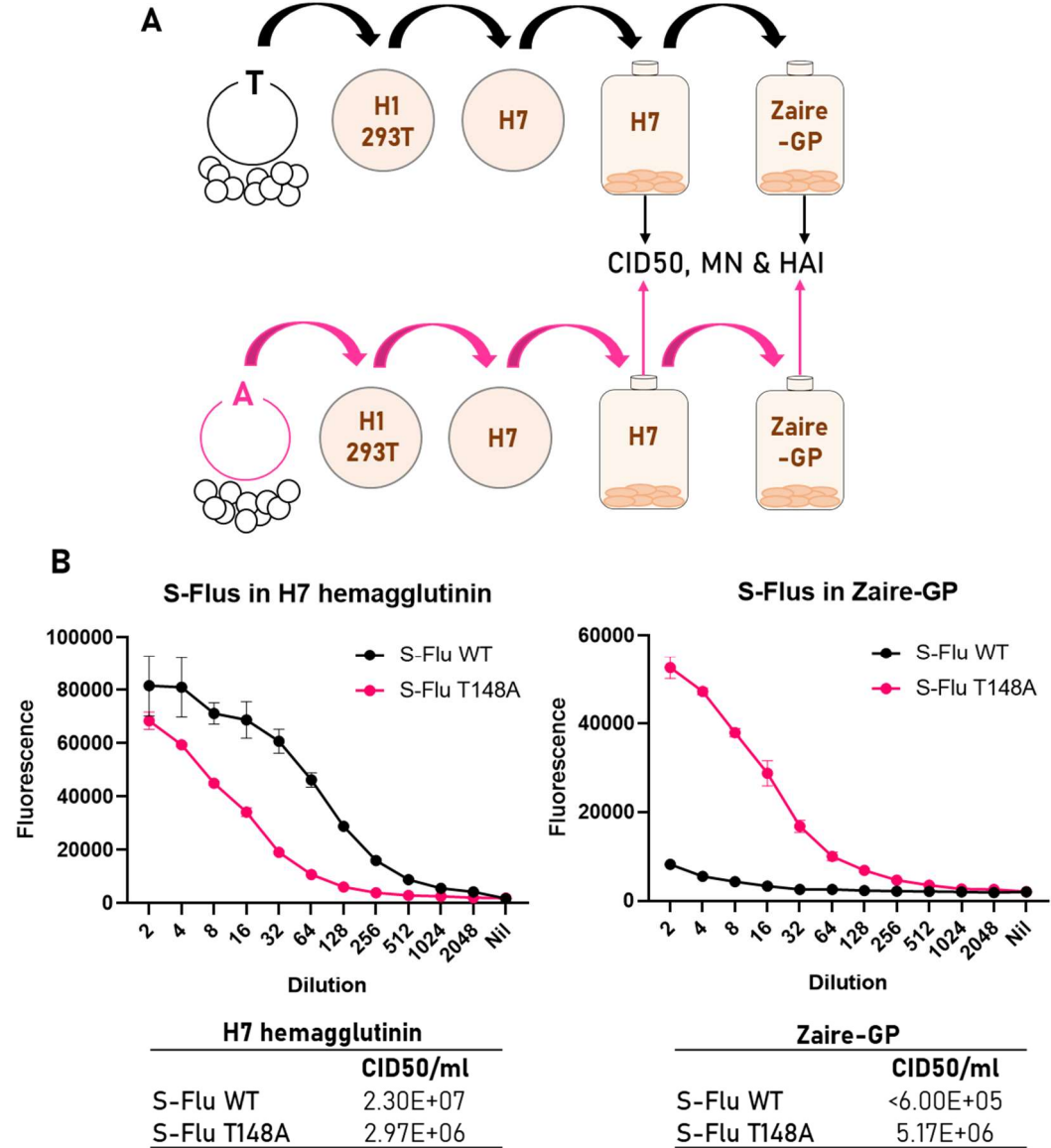
Figure 5.12 shows the CID titration of wild-type and T148A S-Flu after growth in each cell line. The titre of wildtype S-Flu decreased dramatically after passage in Zaire-GP cells, yielding a titre too low to calculate according to our CID50 protocol. T148A S-Flu grew much better than the wildtype virus, supporting our earlier observation that T148A confers a growth advantage in Zaire-GP cells.

To improve the titre of wildtype S-Flu in GP cells for further experiments, another set of Zaire-GP cells was inoculated with 5 times more virus. Although the titre remained too low to calculate CID50/ml, it was raised sufficiently for use in a microneutralisation assay.

Figure 5.13 shows that entry of H7-pseudotyped viruses was neutralised by H7 antibody 4A14 but was not reduced by neuraminidase antibody AG7C or inhibitor oseltamivir, irrespective of the residue at 148. This indicates that neuraminidase does not play an important role in mediating entry. While GP-coated wildtype S-Flu was only neutralised by GP antibody 3CSY, the T148A mutant also appeared to be neutralised by oseltamivir and AG7C. Overall, this suggests that H7 provides strong enough cell attachment for additional binding through T148A neuraminidase to be redundant. However, in viruses which are

pseudotyped with a protein like GP which mediates weaker cell attachment, the mutant neuraminidase becomes an important contributor to cell entry.

Figure 5.12



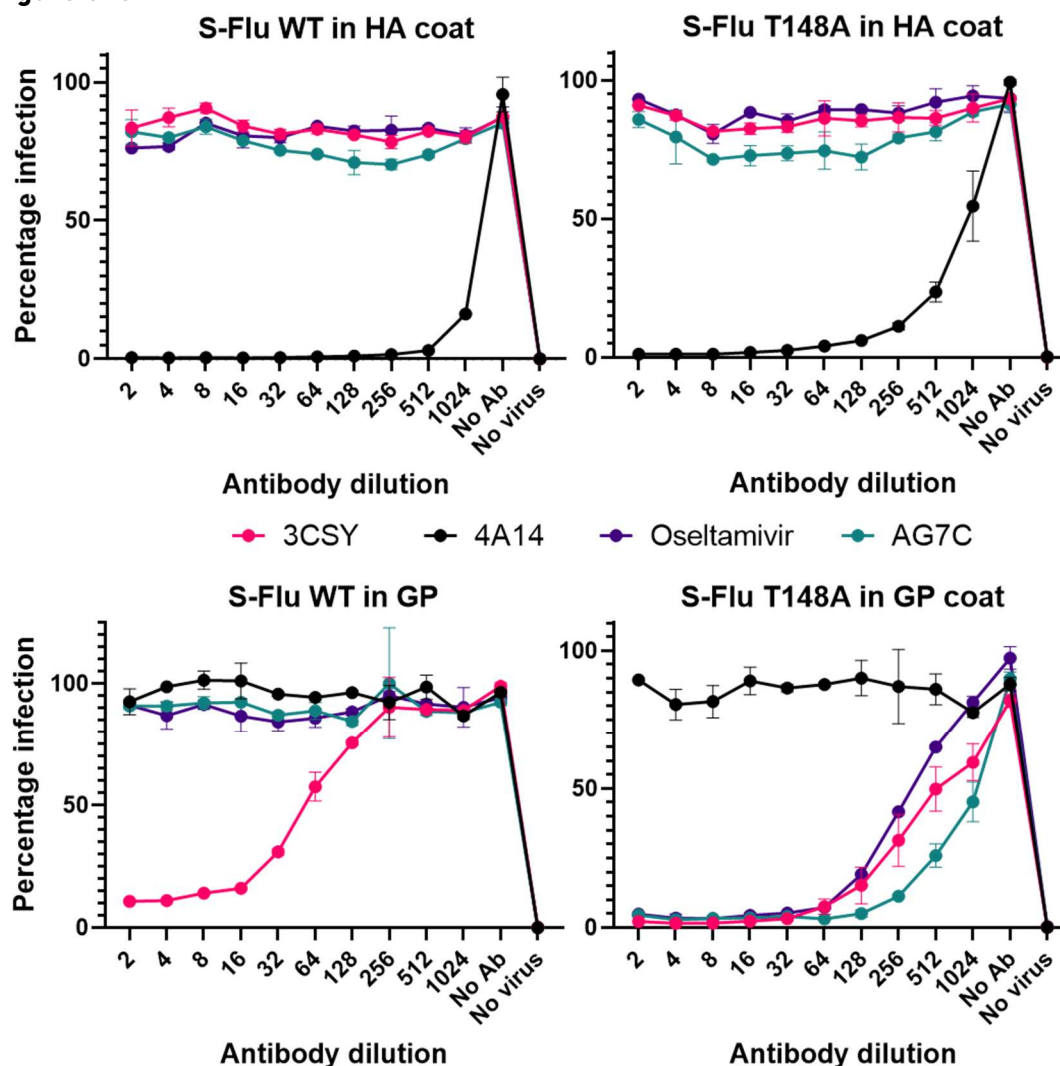
Titration of wildtype and T148A S-Flu viruses by CID after growth in MDCK-SIAT cells expressing H7 HK125 hemagglutinin and Zaire-GP

Wildtype and T148A S-Flu produced by multiplasmid transfection in 293T cells were used to infect MDCK-SIAT cells stably transduced with H7 hemagglutinin. After 48 hours, virus was harvested and used to infect a flask of MDCK-SIAT-H7 cells. After a further 48 hours, the virus was harvested and titrated by CID50. 200µl of the 40ml viral supernatant was used to infect MDCK-SIAT cells transduced with Zaire-GP. 48 hours later, virus was again harvested and titrated by CID50. Cell infection was determined by eGFP expression.

A. Experimental scheme showing expansion of wildtype (T, black) and T148A (A, pink) S-Flu viruses.

B. CID titration curves and CID50/ml estimates. Error bars show standard deviation based on two duplicates.

Figure 5.13



Neutralisation of wildtype and T148A S-Flu viruses by neuraminidase inhibiting antibody AG7C and chemical inhibitor oseltamivir

Antibodies were diluted to 20 μ g/ml and oseltamivir to 200nM before being added to virus in doubling dilutions. 4A14 and 3CSY were used as control antibodies as they neutralise wildtype S-Flu viruses coated in H7 hemagglutinin and Zaire-GP respectively. Cell entry was measured by eGFP fluorescence. Points and error bars indicate the mean and standard deviation of two duplicate measures. Data are shown from a single experiment which was repeated with the same result.

To better understand how entry can be dependent on both GP and neuraminidase, a panel of neutralising anti-GP monoclonal antibodies with diverse binding properties was assembled. We hypothesised that antibodies which neutralise cell entry by blocking fusion should neutralise both wildtype and T148A viruses, while antibodies which block only cell attachment may be compensated for by the mutant neuraminidase in T148A viruses. T148A viruses may therefore help us to determine the mechanism of action of antibodies targeted to different sites on the glycoprotein.

In Figure 5.14.1, the neutralisation curves of wildtype and T148A viruses are plotted together for each antibody. Antibodies 66-4C-12 and 66-6-3 directed to the base region of Ebola GP appeared to neutralise T148A more effectively than wildtype virus. These antibodies are known to compete with antibody 6D6 which binds the fusion loop (Rijal et al., 2019) and therefore likely neutralise by blocking fusion activity. Increased sensitivity to fusion inhibitors could occur because there is higher cell binding in T148A virus and therefore fusion becomes the limiting step for entry. We did not observe any differences in the neutralisation curves of wildtype and T148A S-Flu for antibodies targeted to the receptor binding site or glycan cap. Antibodies 66-3-2C (glycan cap), CA45 (base), 66-6-3 (base) and 6541 (base) fully neutralised T148A virus, showing that the activity they block cannot be compensated for by the ability of T148A neuraminidase to bind sialic acid. This therefore suggests that all of these antibodies block fusion despite binding different parts of the GP molecule. For some, this may be indirect through blocking removal of the glycan cap.

Neutralisation by anti-neuraminidase antibodies was also measured as part of the same experiment (Figure 5.14.2). Comparison with anti-GP antibodies revealed that neuraminidase inhibition may not be absolute, as approximately 3% infection was still observed at saturating inhibitor concentration, while infection could be reduced to less than 0.5% by certain anti-GP antibodies, notably 66-6-3, 66-3-2C, CA45 and 6541.

Figure 5.14.1

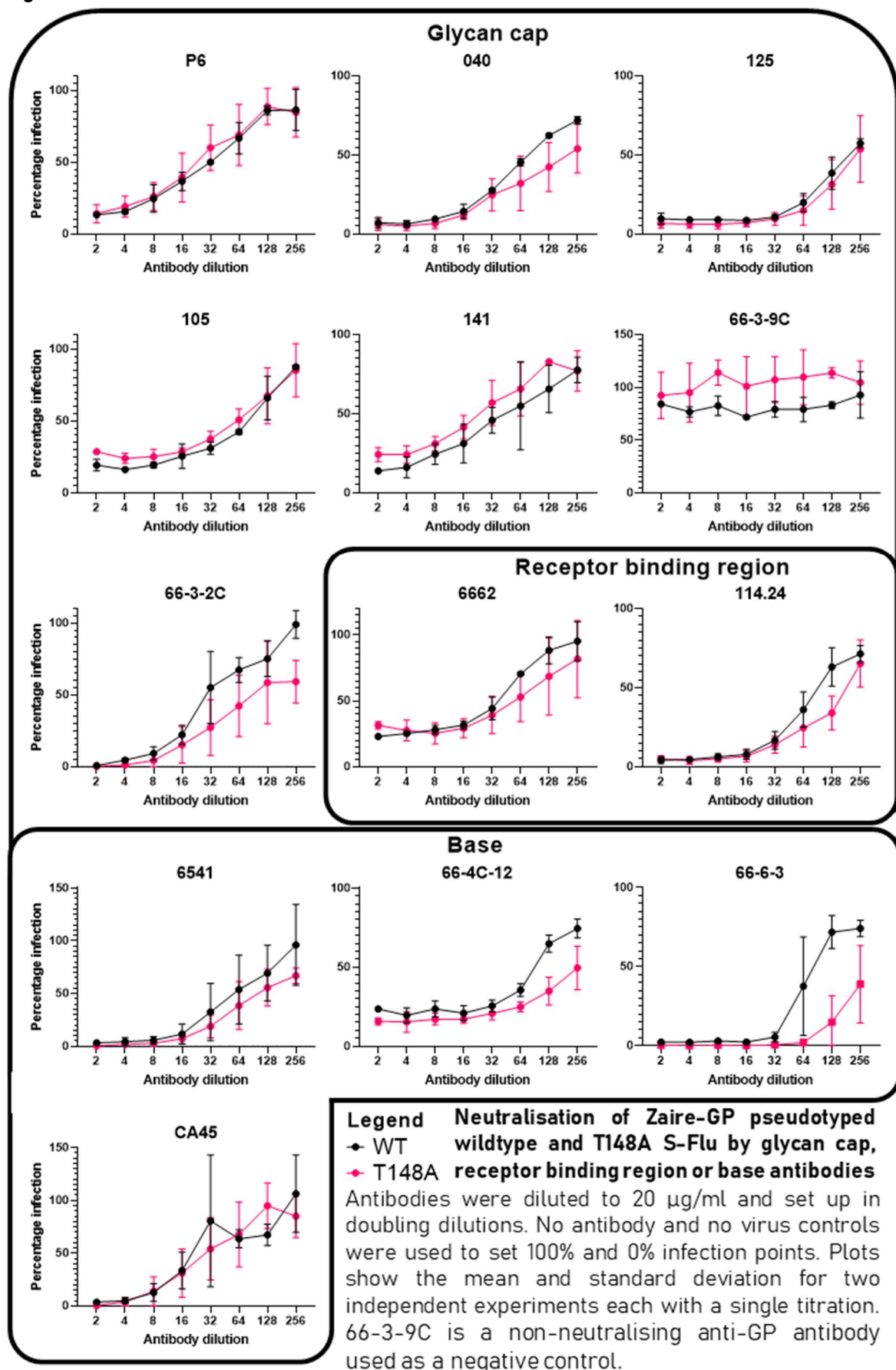
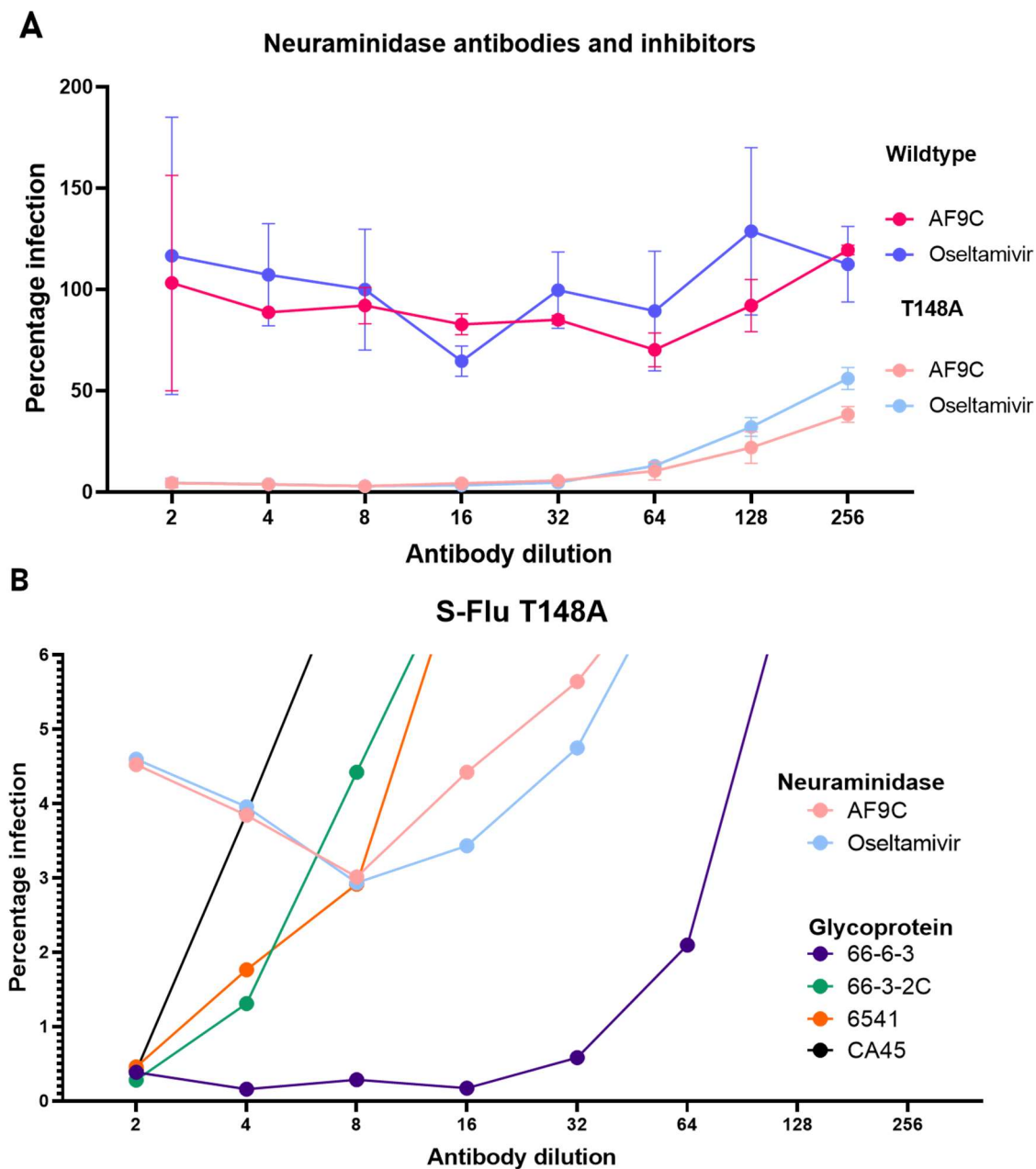


Figure 5.14.2



Neutralisation of Zaire GP pseudotyped wildtype and T148A S-Flu by neuraminidase antibody AF9C and oseltamivir

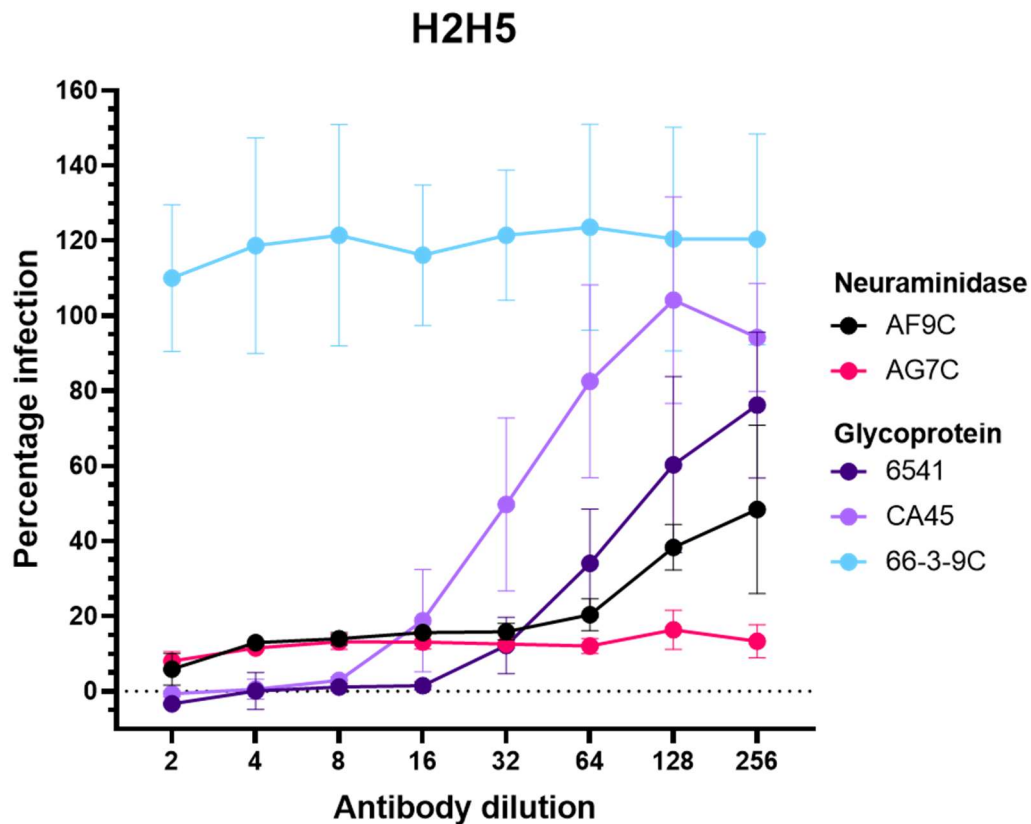
Antibodies were diluted to 20 $\mu\text{g/ml}$ and set up in doubling dilutions. No antibody and no virus controls were used to set 100% and 0% infection points.

A. Plots show the mean and standard deviation for two independent experiments each with a single titration.

B. The four most strongly neutralising anti-GP antibodies from Figure 14.1 are compared to neuraminidase antibody AF9C and chemical inhibitor oseltamivir from part A for neutralisation of T148A S-Flu. Points show the mean of two values from independent experiments. AF9C and oseltamivir are approximately 97% neutralising when used at a saturating concentration while the selected GP-targeted antibodies appear to be fully neutralising.

The observation that T148A viruses coated in Zaire-GP are very strongly but not fully neutralised by neuraminidase inhibition is confirmed in Figure 5.15 using round 6 clone H2H5. The maximum reduction in cell infection for an anti-neuraminidase antibody was 97.1%, while some GP antibodies reduced infection to below the no virus control, indicating 100% neutralisation.

Figure 5.15



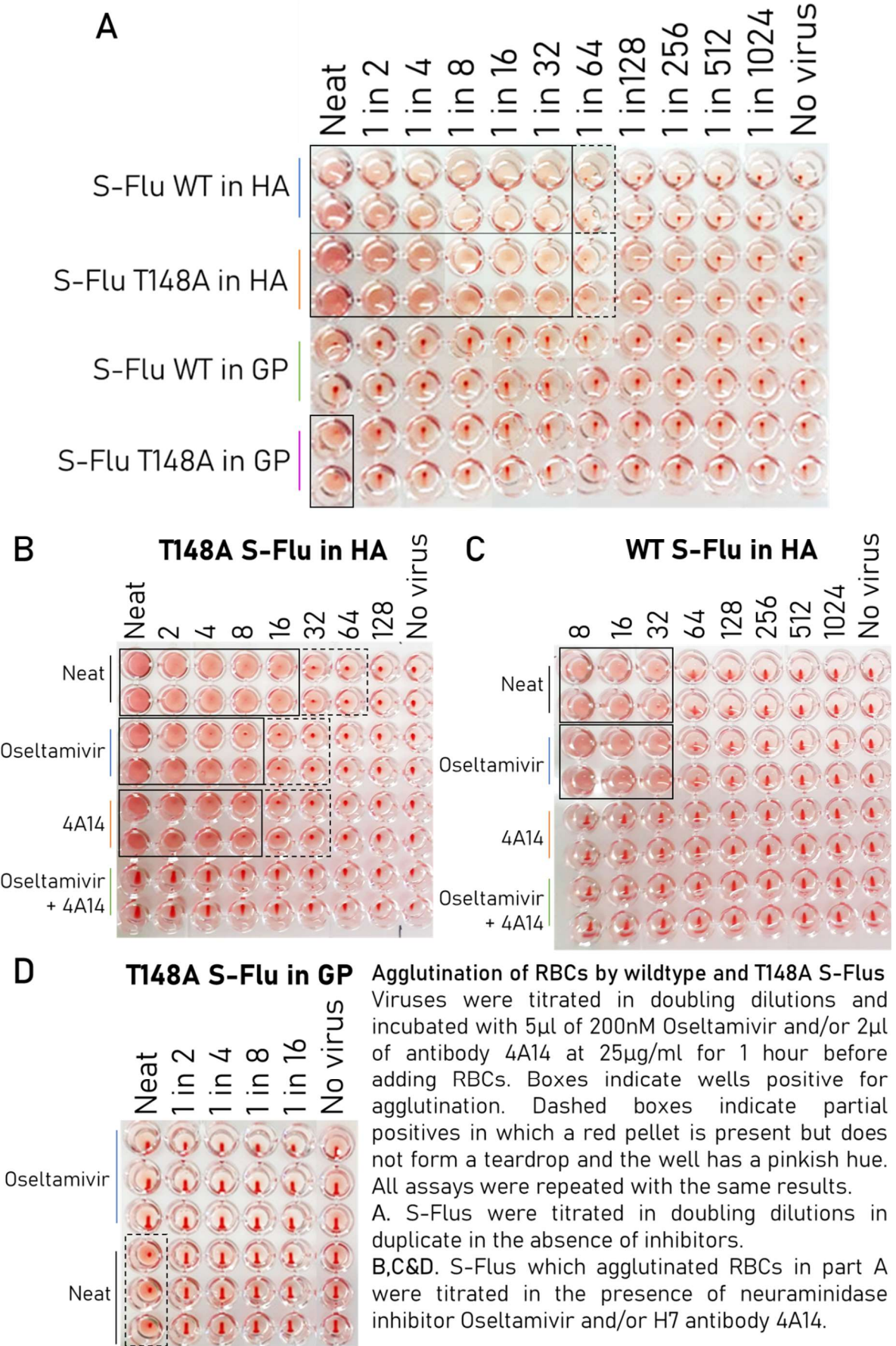
Neutralisation of Zaire-GP pseudotyped round 6 clone H2H5

Antibodies were diluted to 20 $\mu\text{g}/\text{ml}$ and set up in doubling dilutions. No antibody and no virus controls were used to set 100% and 0% infection points. Plots show the mean and standard deviation for two independent experiments each with a single titration. 66-3-9C is a non-neutralising anti-GP antibody used as a negative control.

To confirm that neutralisation behaviours and the T148A mutation are associated with neuraminidase-mediated sialic acid binding, the wildtype and mutant S-Flus were tested for RBC agglutination. Previously, Sudan-pseudotyped round 6 clone H2H5 was observed to agglutinate RBCs unless blocked by oseltamivir. This was not detected in Zaire-coated viruses, presumably due to their lower titre. Figure 5.16 shows that the T148A mutation is sufficient to allow GP-pseudotyped S-Flu to acquire oseltamivir-sensitive agglutinating activity. S-Flus coated in H7 agglutinate RBCs, and complementing this activity with a T148A neuraminidase generates a virus which is able to agglutinate in the presence of a hemagglutinin or neuraminidase inhibitor, but not both. This suggests that these viruses are able to bind sialic acid through both their H7 coat and the T148A neuraminidase. This redundancy could explain why CLEARFLU expression is no longer selectively maintained in populations which have acquired the T148A change.

In conclusion, this section confirms that T148A is the true cause of changes to viral characteristics described in the last two chapters.

Figure 5.16

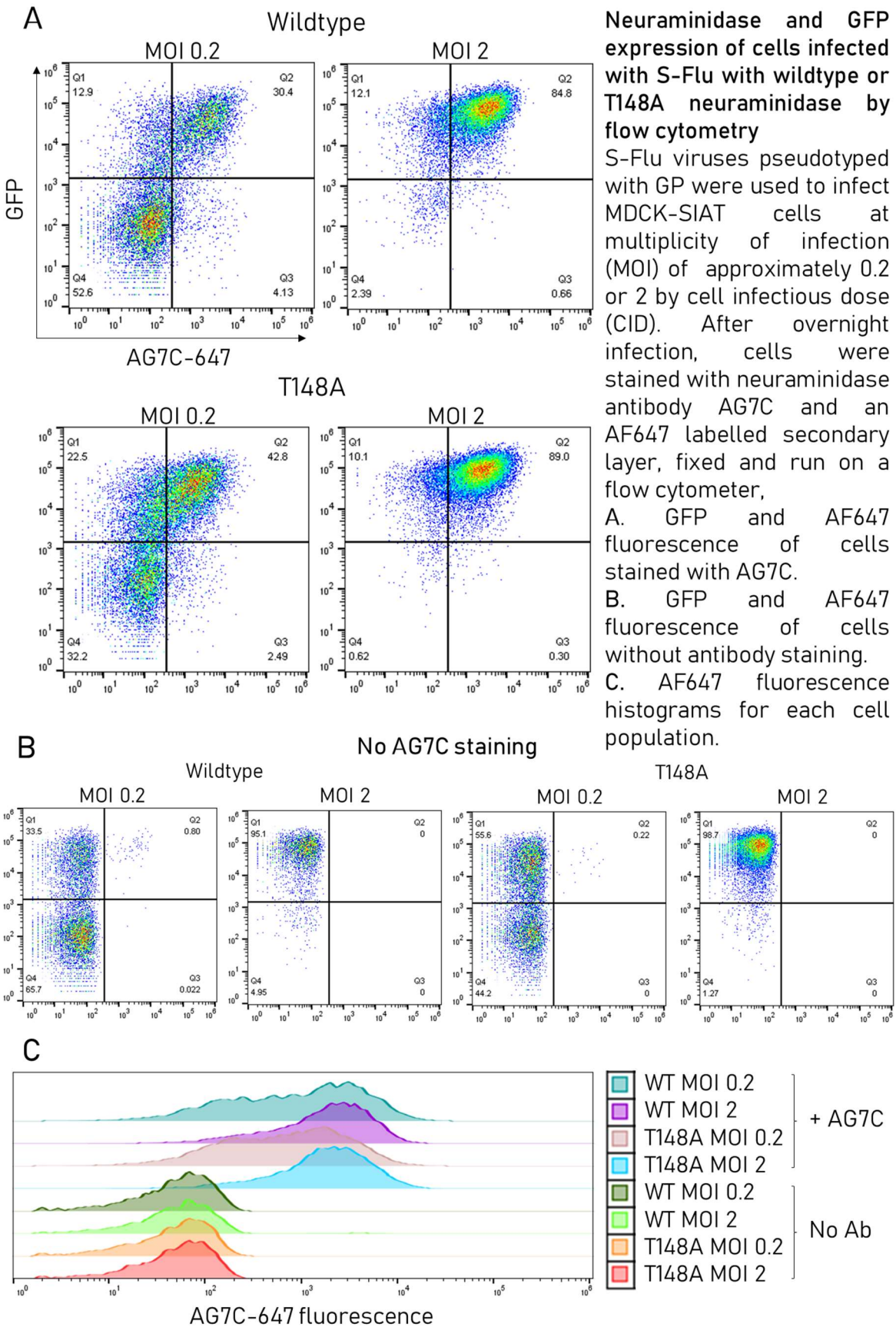


5.2.4 Neuraminidase expression and sialidase activity

Previous reports have suggested that mutations in the 150-loop can drastically affect neuraminidase activity. To determine whether this is the case for T148A, the neuraminidase expression levels and sialidase activities of wildtype and T148A S-Flu viruses were compared.

The neuraminidase expression level in mutant viruses was examined by flow cytometry (see Figure 5.17). Cells infected with wildtype or T148A S-Flu virus were measured for GFP fluorescence and neuraminidase expression was detected by antibody AG7C. Neuraminidase-associated fluorescence was equally strong for cells infected with wildtype or T148A S-Flu, with similar fluorescence distributions. This indicates that the mutant neuraminidase is highly expressed and experiences no major issues with expression or folding.

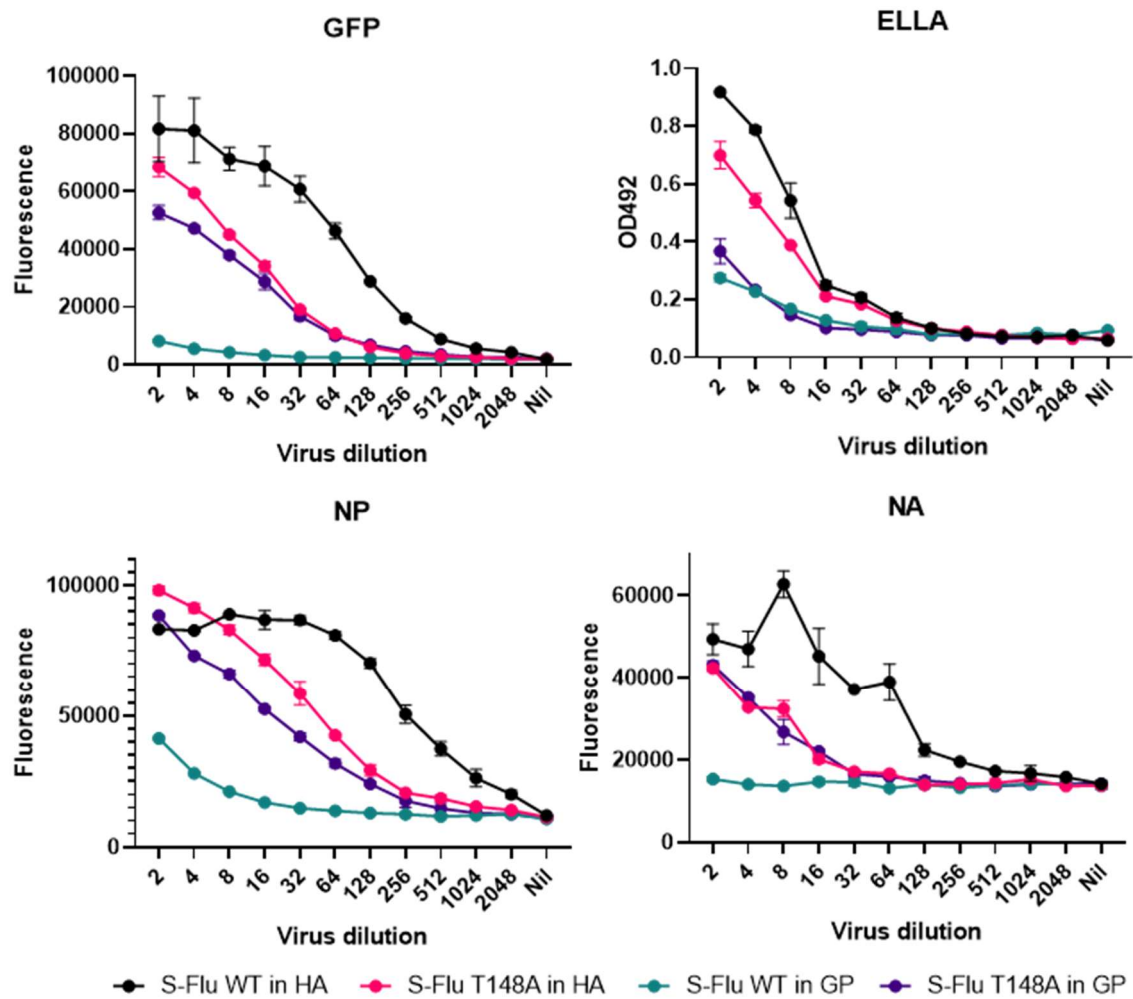
Figure 5.17



In Figure 5.18, S-Flu viruses are titrated by GFP, nucleoprotein and neuraminidase expression and tested for neuraminidase activity by ELLA. Titration curves for GFP and nucleoprotein correlate well as expected. Neuraminidase titration curves also appear to correlate well with these measures of viral titre, again providing no evidence that the T148A mutation drastically alters viral neuraminidase expression.

On ELLA, S-Flu T148A pseudotyped in GP (purple) had a similar titration curve to S-Flu WT in GP (teal) despite having a higher titre by NP, GFP and NA. This could suggest that sialidase activity is slightly lower than expected for this T148A virus. Whether S-Flu T148A in HA (pink) showed reduced activity could not be determined due to the low activity observed for all viruses overall, as none of the viruses reached a plateau indicative of maximum activity allowing for calculation of 50% endpoint titres. Using purified neuraminidase proteins may facilitate the detection of differences in sialidase activity (Zhu et al, 2012). Although we cannot determine with this assay whether there are small differences, it is clear that neuraminidase activity has not been drastically ablated. This is supported by the observation that T148A viruses grow to high titre in H7 cells, suggesting that viral egress is not inhibited by poor neuraminidase activity.

Figure 5.18



Neuraminidase expression and sialidase activity of S-Flu viruses

Neuraminidase expression was measured by titrating S-Flu viruses in doubling dilutions in duplicate and staining for neuraminidase (primary: AG7C, secondary: AF647). Nucleoprotein staining (primary: AA5H, secondary: AF647) and GFP expression are also shown as measures of viral titre for comparison. S-Flu viruses were similarly titrated by ELLA (enzyme-linked lectin assay) to measure neuraminidase activity. Error bars show standard deviation based on duplicate readings.

5.3 Discussion

5.3.1 Summary

Using a variety of viral clones and S-Flu of known sequence to ensure results were not affected by undetected genetic changes at other sites, we showed that T148A enhances growth in Ebola glycoprotein-expressing cells, allowing viruses to form denser plaques and reach higher titres. T148A was associated with agglutination of erythrocytes, indicating that the mutation enabled sialic acid binding. Together with microneutralisation assays showing that cell entry could be almost fully neutralised by neuraminidase inhibitors or antibodies, this suggested that T148A neuraminidases enhance cell entry by facilitating cell attachment. Inhibition of T148A neuraminidase led to a 97% decrease in cell entry, suggesting that T148A enhanced cell entry by a factor of around 30-fold. T148A did not alter reliance on interaction with the NPC1 receptor for fusion with the endosomal membrane or reduce sensitivity to antibodies against GP, providing no evidence that the mutant neuraminidase is involved in the fusion step of cell entry. The neuraminidase activity of T148A S-Flu did not appear to be significantly ablated as has been shown for mutations at site 152. The effects we have observed are likely due to changes in the conformation of the 150-loop, at the edge of the neuraminidase catalytic site.

5.3.2 Further characterisation of the T148A neuraminidase catalytic site

Unlike T148I which leads to a 50% reduction in catalytic activity and mutations at 152 which fully ablate function, we did not observe significant changes in neuraminidase catalytic activity for the T148A mutant. Producing purified proteins may be necessary to identify subtle differences in the catalytic properties of wildtype and T148A neuraminidases. It may be possible to observe indirect signs that the mutation causes a change in enzymatic activity by passaging a mixed population of wildtype and T148A viruses in cells with a strong sialic acid binding pseudotyping protein, as this might select for expression of the neuraminidase with the highest activity.

Antibodies AG7C and AF9C and chemical inhibitor oseltamivir block neuraminidase catalytic activity. They also block sialic acid binding by T148A neuraminidase, providing evidence that sialic acid binding occurs at or very close to the enzymatic site. As T148A does not prevent binding by these inhibitors, the structure of the catalytic site must not be majorly disrupted. Further study with other inhibitors may provide more detailed insights as some mutations in the 150-loop alter sensitivity to certain inhibitors and not others. For instance, 2009 pandemic H1N1 mutant R151K shows an 18-fold reduction in inhibition by oseltamivir, but normal inhibition by zanamivir and peramivir (Baek et al., 2015).

It would be interesting to determine how T148A alters the characteristics of neuraminidases which do not have a 150-cavity, namely pandemic 2009 H1N1 or group 2 neuraminidases. Interestingly, D151G had the same effect in several N1 and N2 neuraminidases despite differences in the conformation of their 150-loop (Zhu et al., 2012).

5.3.3 Selection for neuraminidase takeover of sialic acid binding

Neuraminidase-mediated sialic acid binding can be acquired through single mutations at several different residues close to the catalytic site, and is therefore likely to arise after serial passage in an environment where enhanced sialic acid binding is beneficial. Indeed, T148A may be a superior solution to CLEARFLU for enhancing the growth of viruses with weak cell attachment if CLEARFLU is expensive to replicate and express, or if expression of a third major surface protein drives down expression of the other two.

Is it inevitable then that mechanisms which select for CLEARFLU through sialic acid binding activity will also select for neuraminidase mutations? While we have observed that T148A is able to reduce reliance on CLEARFLU expression for cell entry, we note that even after 6 rounds of expansion at low MOI in Zaire-GP cells, CLEARFLU expression is still beneficial as it enhances cell entry. Not all clones isolated at the end of this expansion process have acquired the T148A mutation, including some which have lost CLEARFLU expression. Moreover, even after a single round of expansion, there was evidence that neuraminidase inhibition could reduce cell entry. All of these observations suggest that takeover of sialic acid binding by T148A was a slow process, which remains incomplete after 6 rounds of expansion. This implies that expression of T148A neuraminidase has only marginally increased viral fitness compared to maintenance of CLEARFLU expression.

Supporting this idea, H7 pseudotyped S-Flu viruses with T148A are not at all neutralised by neuraminidase inhibitors, showing that binding through H7 is sufficiently strong that contributions by T148A are negligible. This suggests either that H7 (and therefore likely also our H7 CLEARFLU of the same subtype) is a much stronger binder than T148A or that the limiting factor for cell entry is fusion activity.

However, in microneutralisation curves for viruses with either T148A or CLEARFLU, blocking neuraminidase-mediated cell entry appears to lead to a greater decrease in infection than blocking CLEARFLU-mediated entry (97% vs 75%) (Figures 5.3, 5.14.2 & 5.15). This is compatible with a hypothesis that although CLEARFLU binds more strongly than T148A, this is offset by decreased fusion activity as GP competes with CLEARFLU for expression on the viral surface. We therefore propose that the relative benefits of T148A and CLEARFLU expression may vary according to the balance in selective pressures for increased binding and fusion activity. In MDCK-SIAT cells expressing Zaire glycoprotein, T148A appears slightly superior for enhancing cell entry.

In Sudan GP cells, expression of CLEARFLU did not visibly enhance growth. This is confirmed by Dr Julie Xiao who observed only a two-fold increase in maximum fluorescence for S-Flu in cells co-expressing Sudan-GP and CLEARFLU version 3 compared to Sudan-GP alone (personal communication). Both viruses had titres too low to quantify by CID50. The titre achieved by clone H2H5 which contains the T148A mutation (Figure 5.9) therefore reached more than 100-fold higher titre. Given that CLEARFLU improves titres in Zaire-GP cells and strongly agglutinates RBCs, it is unlikely that CLEARFLU's inability to improve titres in Sudan cells is because it binds sialic acids less strongly than T148A neuraminidase. It is possible that CLEARFLU is not incorporated effectively into the coat of budding virions expressing Sudan glycoprotein. This could be tested by sandwich ELISA. Alternatively, the Sudan glycoprotein may fuse less efficiently than Zaire glycoprotein so that the benefit of binding sialic acid through CLEARFLU is almost negated by reduced fusion activity due to a decrease in expression of GP on the viral surface. This could be investigated by determining whether a virus which expresses both CLEARFLU and T148A neuraminidase grows less well in Sudan cells than one which expresses only T148A.

5.3.4 Avoiding neuraminidase mutations

Reducing selection for mutation

Adjusting growth conditions could help to slow the accrual of mutations like T148A and maintain CLEARFLU expression. This might include using a pseudotyping coat with strong fusion activity or which expresses well on the viral surface in the presence of CLEARFLU (perhaps due to a smaller size or a more compatible stalk length) to ensure that CLEARFLU does not drive down fusion activity. Alternatively, using cells with fewer sialic acids may increase selection for the stronger binding protein.

In this chapter, we have not compared the sialic acid binding specificities of T148A neuraminidase and H7 CLEARFLU version 3. This is important to fully understand the selective pressures driving the rise in prevalence of T148A. Previous reports have given mixed results as to whether neuraminidase mutants bind sialic acids with the same or different specificity as their cleavage activity. We saw no evidence that T148A neuraminidase binds sialic acids it cannot cleave itself from, as viruses were able to grow well in both GP and H7 cells. Some reports suggest neuraminidases tend to bind preferentially to α -2,3 sialic acids, whereas hemagglutinins from human viruses typically favour α -2,6. Our T148A viruses agglutinated human RBCs, but we might have observed different results with RBCs from other species, for instance with horse RBCs which contain predominantly α -2,3 or turkey and guinea pig RBCs which contain more α -2,6 linked sialic acids (Ovsyannikova et al., 2014). If neuraminidase is indeed binding sialic acids differently to H7 CLEARFLU, then our agglutination assay could have over or underestimated the strength of sialic acid binding by neuraminidase relative to CLEARFLU.

Sialic acid specificity could be investigated using a glycan binding array as Zhu et al have described or by comparing the growth of viruses in cell lines with differing expression of α -2,3 and α -2,6 sialic acids. MDCK-SIAT cells overexpress an α -2,6-sialyltransferase and therefore express high levels of α -2,6 relative to α -2,3 sialic acids. If T148A neuraminidase

preferentially binds α -2,3 sialic acids, growing viruses in MDCK-SIAT or other cells with few α -2,3 sialic acids may favour binding by an α -2,6 specific CLEARFLU hemagglutinin over neuraminidase and prevent neuraminidase from acquiring a role in cell entry. Simultaneously designing CLEARFLUs based on hemagglutinins which bind particularly well to α -2,6 may help to maintain their expression. Supporting this, Tamura et al have shown that growing viruses in MDCK-SIAT cells compared to standard MDCK cells reduces the prevalence of site 148 neuraminidase mutants (Tamura et al., 2013). They proposed that this was due to reduced selection for sialic acid binding. We consider that more specifically this could be due to decreased selection for α -2,3 binding rather than for sialic acid binding in general.

Removing potential for mutation

A more drastic approach to avoid neuraminidase mutation could be to remove the neuraminidase coding sequence and replace it with another sequence of choice, taking care to preserve packaging signals required for segment incorporation into virions. The loss of sialidase activity could be rescued by growing the virus in a cell line expressing neuraminidase or by adding a soluble version during virus culturing.

Neuraminidase deficient viruses have been produced previously by replacing the neuraminidase sequence with green fluorescent protein (Fujii et al., 2003; Rimmelzwaan et al., 2007). While incorporating such a segment into a CLEARFLU vaccine candidate may help to boost hemagglutinin antigenicity, the lack of neuraminidase epitopes may be detrimental to protection. Expressing a soluble neuraminidase within the CLEARFLU virus may be the preferred solution. An expression cassette similar to that used for CLEARFLU could be designed, incorporating wildtype neuraminidase sequence at the segment ends to allow packaging, but ensuring that they are not expressed by removing the start codon. Between these signals, a neuraminidase sequence with the transmembrane domain and

stalk removed and replaced with a tetramerization domain could be encoded to allow a stable neuraminidase tetramer to be secreted from infected cells (Job et al., 2018). As it would not be displayed on the viral membrane, a soluble neuraminidase could not mediate cell attachment and circumvent the requirement for CLEARFLU-mediated binding. A soluble neuraminidase would however be able to cleave sialic acids to facilitate virion release and may stimulate anti-neuraminidase T-cell and antibody responses. These responses are increasingly understood to be highly valuable (Krammer et al., 2018) and would be even more so against viruses which use neuraminidase for cell entry.

The alternative approach of heavily disrupting the sialic acid binding site by substitution or deletion to prevent the acquisition of sialic acid binding would likely also disrupt enzymatic cleavage as well as protective antibody epitopes around the catalytic site. Using such a design and providing external sialidase activity during culturing may however enable some neuraminidase T-cell epitopes to be expressed and may therefore be better than expressing no neuraminidase virally at all.

5.3.5 Implications of findings

Research tools

In this chapter, we have shown that T148A enables Ebola pseudotypes of S-Flu to grow to very high titre. This increases their value as research tools by making titration and microneutralisation curves easier to interpret and by reducing the resources and time needed to produce virus, ultimately making screening of Ebola cell entry inhibitors more efficient. Importantly, as T148A is not essential for cell entry, T148A Ebola pseudotypes show similar neutralisation profiles to viruses with wildtype neuraminidase.

The T148A mutation also diversifies the possible S-Flu pseudotypes that can be produced to encompass fusion-only proteins and proteins which bind cell surface receptors not expressed in MDCK-SIAT cells.

In viruses pseudotyped with a protein engineered to only mediate fusion (for example by mutating the hemagglutinin receptor binding site), T148A neuraminidase may become the sole mediator of cell attachment. T148A viruses could therefore be used to specifically screen for fusion inhibitors, helping to elucidate the mode of action of neutralising antibodies to hemagglutinin.

Finally, T148A has allowed the production of an S-Flu pseudotype that is strongly neutralised by inhibitors of neuraminidase catalytic activity due to the colocalization of the catalytic and sialic acid binding sites. This could allow microneutralisation assays to be used to screen for antibodies and drugs likely to block neuraminidase activity. This could be a fast, sensitive, scalable and quantitative approach compared to current methods which measure neuraminidase activity by ELLA or MUNANA or measure inhibited egress by reduced plaque size. Similarly, T148A could facilitate the process of selecting for neuraminidase escape mutants in the presence of antibodies, immune sera or chemical inhibitors as neutralisation exerts a stronger selective pressure than inhibition of viral release.

T148A in vaccines and circulating viruses

Improved titre reduces the need for concentrating viruses after production, which can also concentrate contaminants and cause reactions *in vivo*. Higher titre allows animal models and target populations to be immunised with higher doses because of limitations on administration volume. Using T148A viruses might therefore improve our ability to generate immune responses to vaccine candidates such as E-S-Flu safely, increasing the power of *in vivo* trials.

If T148A viruses exhibit altered sialic acid binding specificities, this may affect their behaviour *in vivo*. An increase in α -2,3 binding could lead to enhanced binding to mucins in the human respiratory tract and a decrease in infectivity. This could translate to reduced vaccine efficacy for single-cycle viruses. As cells in the human upper respiratory tract tend to express α -2,6 sialic acids and cells in the lower tract express more α -2,3, T148A viruses might be able to establish stronger infections in the lung if they are able to reach cells in the lower respiratory tract, as is observed for some avian influenza strains. While this would be concerning for infectious virus, it may enable single cycle viruses to generate strong local immune responses in the lung if they can be delivered there.

The ease with which neuraminidase is able to acquire sialic acid binding activity highlights the importance of using a hemagglutinin with reduced fusion activity in single-cycle or attenuated vaccines. Using a sialic acid binding mutant hemagglutinin such as Y98F or X31-PM as a form of attenuation would be dangerous as compensation by the neuraminidase could lead to pathogenicity *in vivo*. This is especially true for attenuated viruses such as Y98F which have additional opportunities for mutations to arise, but is ill-advised even for single-cycle viruses as neuraminidase mutations may already exist at a low level in the vaccine virus.

The fact that T148A and similar neuraminidase mutations are already present in human viruses should be of concern to vaccine regulators. If cell attachment becomes dependent

solely on neuraminidase, antibodies to the hemagglutinin head which function by blocking sialic acid binding may cease to be neutralising. Current subunit vaccines rely heavily on generating neutralising antibody responses to the hemagglutinin head due to the relative rarity of neutralising stem antibodies. Including neuraminidase epitopes in vaccines may therefore be critical in the future not only to reduce the severity of infections, but also to provide sterile immunity.

Reassortment between viruses generates virions with new genetic combinations and properties. Reassortment of hemagglutinin segments is the greatest cause of concern due to the role hemagglutinin has in cell entry and its antigenic dominance, which make it a key determinant of host susceptibility or immunity to infection. Mutations like T148A highlight that neuraminidase can also alter the characteristics of viruses, affecting susceptibility to neuraminidase and even hemagglutinin antibodies and inhibitors. Although the risk of neuraminidase reassortment is low in single-cycle viruses as coinfection is unlikely with few cell infection events, reassortment between T148A and wildtype viruses could introduce T148A into new strains and reduce the efficacy of vaccines which block hemagglutinin-mediated cell attachment. This risk should be considered before using viruses with T148A neuraminidase as vaccines.

5.3.6 Conclusion

Although we have identified challenges with using sialic acid binding to select for CLEARFLU expression, we suggest that it remains the most promising mechanism for this. Several refinements have been proposed here to optimise selection for CLEARFLU. The best approach for maintaining CLEARFLU expression and balancing binding, fusion and cleavage activities may vary between virus strains due to differences in the properties of hemagglutinins and neuraminidases.

T148A is just one of several mutations now known to enable neuraminidase to take on sialic acid binding. Knowledge of a range of mutations which have differing effects on catalytic activity and varying sensitivities to chemical inhibitors and antibodies allows the most suitable to be chosen for any given purpose. It would be interesting to compare the strength and specificity of sialic acid binding in different mutants, and to determine their compatibility with each other. It may be that using one sialic acid binding mutation can prevent another with undesirable characteristics, such as effects on enzyme activity or antiviral resistance, from arising. T148A may be particularly helpful as unlike some other mutations, it does not appear to drastically reduce enzymatic activity. Contrary to G147R which arose as a rescue mutation in non-permissive conditions, T148A became established in a partially selective environment. This suggests that it can thrive even under relatively low selection pressure and therefore does not drastically compromise viral fitness through other mechanisms.

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Chapter 6

IN VIVO RESPONSES TO CLEARFLU VIRUSES

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6.1 Introduction

This chapter explores the immune response in mice and pigs following immunisation with CLEARFLU viruses. We expect CLEARFLU viruses to induce a response very similar to S-Flu viruses, with the addition of a neutralising antibody response to the CLEARFLU hemagglutinin.

As discussed in Chapter 1, S-Flu viruses have previously been shown to generate cross-protective T-cell responses in mice and pigs. Subtype-specific neuraminidase responses are also observed. These responses decrease shedding and reduce the severity of disease after heterologous or homologous challenge but do not provide sterile immunity. While systemic administration by injection (intraperitoneal in mice, intramuscular in pigs) stimulates a neutralising strain-specific antibody response to the pseudotyping hemagglutinin, this is not usually observed during local administration to the lung (intranasal in mice, aerosol in pigs).

With S-Flu according heterologous protection through cellular immune mechanisms, local immunisation may be particularly advantageous as infection of cells in the lung should lead to locally concentrated anti-viral immunity (Mueller & Mackay, 2016; Tchilian & Holzer, 2017). It is hoped that the expression of CLEARFLU within the hemagglutinin expression cassette may generate a neutralising antibody response to hemagglutinin after local immunisation, much like is seen after natural infection. Particularly, it may generate neutralising antibodies in the lung and respiratory tract as well as in the serum (van Riet et al., 2012).

The experiments described in this chapter are therefore designed primarily to measure neutralising antibody responses to the CLEARFLU hemagglutinin. Two experiments in mice and pigs also confirm that CLEARFLU viruses generate T-cell responses and anti-neuraminidase antibody responses similar to S-Flu.

6.1.1 Animal models

Mice are a convenient and common model for studying immune responses to candidate vaccines. Two mouse strains are used in this thesis. BALB/c were used for preliminary experiments as they are docile and easy to work with. C57BL/6 were used later as they express the MHC haplotype for which tetramers for CD8⁺ T-cell analyses were available (H-2Db). All mouse work described in this thesis was conducted under the supervision of Dr Pramila Rijal and Dr Jack Tan.

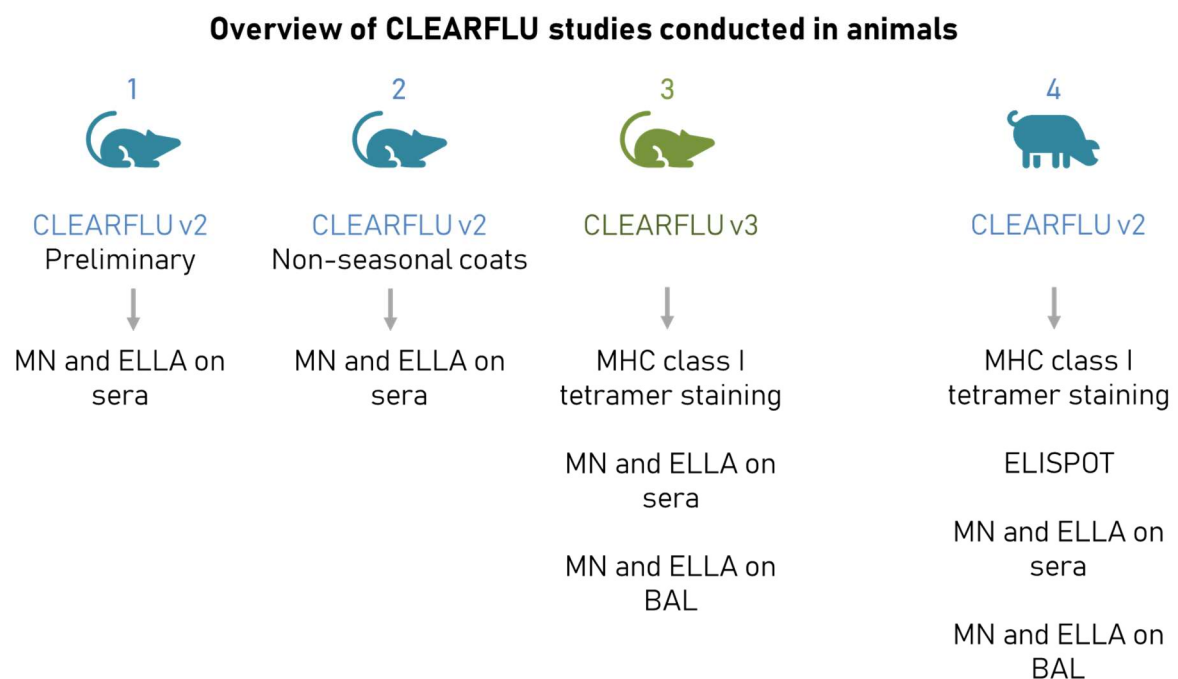
Pigs offer an opportunity to test vaccine candidates in a natural host relevant to influenza control and one of the intended target populations for CLEARFLU and S-Flu vaccines. As large mammals with an immune system and lung physiology relatively similar to humans they are also a sophisticated model for human responses (reviewed by Rajao & Vincent, 2015). Notably, they have a similar distribution of sialic acid receptors in the respiratory tract and are susceptible to human viruses (Trebbien et al., 2011).

Recently, tools for evaluating responses in the inbred Babraham pig have been developed, including MHC tetramers for studying the antigen specificity of T-cell responses (Tungatt et al., 2018). Babraham pigs are derived from Large White commercial pigs and are functionally homozygous at MHC loci, making them a reproducible model relevant to commercial agriculture (reviewed by Holzer et al., 2019). Previous work on S-Flu has been conducted in Babraham pigs (Tungatt et al., 2018) and outbred landrace pigs (Holzer et al., 2018; Morgan et al., 2016) and is discussed in section 6.2.4.

6.1.2 Experimental design

Three experiments were performed in mice, and one in pigs. The first two studies in mice and the study in pigs were conducted using CLEARFLU version 2 viruses, prior to the development of the CLEARFLU version 3 design.

Figure 6.1



The first mouse experiment tests whether any neutralising antibody response to CLEARFLU hemagglutinins can be detected in sera. The second investigates responses to the same viruses, but this time pseudotyped with hemagglutinins from subtypes which do not circulate seasonally in humans or pigs. The third mouse study confirms that similar antibody responses are seen following immunisation with CLEARFLU version 3 virus, and shows that cellular responses are comparable to S-Flu. A final experiment conducted at the Pirbright Institute compares antibody and T-cell responses generated by S-Flu and CLEARFLU version 2 viruses in pigs.

6.1.3 Regimen, route & dosage

A prime-boost regimen stimulates greater numbers of NP-reactive CD8+ T-cells in the lung in mice and generates stronger protection against homologous and heterologous challenge in both mice and ferrets than a single dose (Baz et al., 2015). Animals which have received two doses experience reduced weight loss and lower virus titres in nasal turbinates and lungs post-challenge. All three S-Flu studies previously conducted in pigs have therefore followed a prime-boost regimen.

Previous S-Flu studies have shown that the route of administration is a critical determinant of responses, most notably for generating neutralising antibody (Powell et al., 2012). Efficient delivery to the lung can be achieved by intranasal immunisation in mice and by aerosol in pigs. Aerosol is more efficient than intratracheal immunisation in pigs for stimulating responses in bronchoalveolar lavage (BAL) and protecting against challenge (Morgan et al., 2016). In this chapter, mouse studies compare local immunisation of the lung and peripheral administration to the intraperitoneal space. To minimize animal use, only aerosol administration is investigated in pigs.

Dosages are calculated by CID (cell infectious dose) or TCID (tissue culture infectious dose). CID quantifies viral particles able to infect cells in an overnight incubation, while TCID quantifies particles capable of clonal expansion over 48 hours. The relationship between CID and TCID varies between viruses as they generate different proportions of virions which are able to infect cells but not replicate due to a failure to express at least one essential protein (Brooke et al., 2013). For non-replicating vaccine viruses where the number of initial cell infections is proportional to the antigen dose, CID may be the better measure to standardise doses. TCID may be more appropriate for a virus that is replicating, where the rate of secondary and further infection is also important in

determining the antigen dose. TCID is the standard preferred by reviewers and most often seen in the literature, and is therefore used in our later experiments.

6.1.4 Measuring immune responses

Antibody to hemagglutinin

Several studies have described the antibody and T-cell response to S-Flu (Powell et al., 2012; Powell et al., 2019; Baz et al., 2015; Morgan et al., 2016; Holzer et al., 2018). As we expect all other responses to be similar for CLEARFLU, measuring the antibody titre to CLEARFLU hemagglutinin is the primary aim of this chapter.

Hemagglutination inhibition assays (HAI) and ELISA are common methods for measuring responses to hemagglutinin (reviewed by Trombetta & Montomoli, 2016). Hemagglutination inhibition assays detect the presence of antibodies which block sialic acid binding. They therefore do not account for protective stem-binding antibodies which block fusion. Technical issues also arise when using hemagglutinins which are poorly agglutinating, such as in some recent H3N2 viruses (Lin et al., 2012). Additionally, Chapter 5 illustrates how HAI results can be misleading due to neuraminidase-mediated agglutination. While ELISA detects the presence of antibodies directed to any part of the hemagglutinin, it cannot discriminate between protective and non-protective antibodies.

In our experiments we wished to measure antibodies which are likely to be protective. More specifically, given that S-Flu is known to be protective in the absence of sterile immunity, we were interested in neutralising responses which may prevent infection. Such a response would be advantageous both to the individual and to populations as transmission requires infection. Therefore, we wished to directly and specifically measure neutralising antibody titres, even though this does not measure responses which may be beneficial through Fc-mediated mechanisms.

Microneutralisation (MN) titres indicate a capacity to block infection by a test virus. In this thesis, S-Flu viruses are used as test viruses as they are safe to work with and cause infected cells to express eGFP. Unlike for HAI where a serum titre of 1 in 40 is thought to be associated with 50% protection from infection, there is no agreed value for a correlate of protection for neutralisation assays. Suggested values vary greatly, partly due to differences in how the experiment is conducted in different laboratories, but also according to the type of immunogen (natural infection versus live-attenuated virus versus inactivated virus) and host characteristics.

Antibody to neuraminidase

Antibody responses capable of inhibiting neuraminidase activity were assayed using an enzyme-linked lectin assay (ELLA). This detects the action of neuraminidase on fetuin, removing sialic acids to reveal β -D-Galactose-N-acetyl-galactosamine, which can be bound by Peanut Agglutinin-Horse Radish Peroxidase (PNA-HRP) and detected using o-Phenylenediamine dihydrochloride (OPD).

S-Flu viruses were used as the source of neuraminidase. Viruses were selected to ensure a mismatch between the S-Flu pseudotyping hemagglutinin and the hemagglutinins used for immunisation so that anti-hemagglutinin antibodies could not sterically inhibit neuraminidase activity (Kosik & Yewdell, 2017).

Although significant evidence exists to suggest that neuraminidase responses are protective (reviewed by Krammer et al., 2018), there is no agreed correlate of protection for ELLA. Memoli et al have shown that people with serum neuraminidase inhibition titres above 1 in 40 by ELLA before challenge score better on measures of disease severity than those with lower titres after intranasal challenge with H1N1pdm09 virus (Memoli et al., 2016). These measures include the duration of viral shedding and the number, duration and severity of symptoms. They found neuraminidase inhibition titres to be more prevalent and

a better correlate of protection than HAI titre, suggesting that neuraminidase-mediated responses are perhaps of greater relevance to population immunity.

T-cell responses

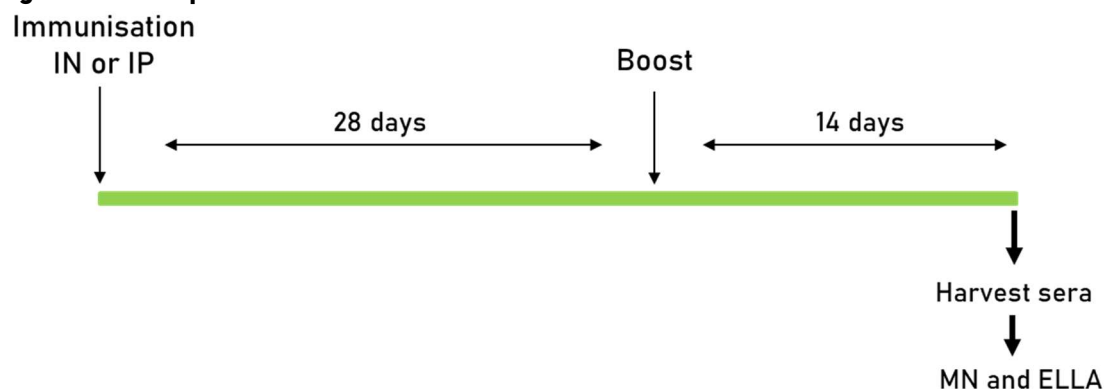
T-cell responses in mice were measured using MHC class I tetramer staining to determine the proportion of CD8⁺ T-cells in the lungs and spleen specific for a selected nucleoprotein (NP) peptide. Hayward et al have shown that cross-reactive T-cell responses to nucleoprotein are common in human populations (Hayward et al., 2015). They are associated with decreased viral shedding in patients with influenza independently of other measures. Nucleoprotein was the most common target for T-cell responses in their study and has also been shown by others to be a frequent target for both CD8⁺ and CD4⁺ T-cells (Chen et al., 2014; Grant et al., 2013). Here, we chose to focus on CD8⁺ T-cells as these make up the majority of NP-responsive T-cells in humans (Hayward et al., 2015), using the peptide NP₃₆₆₋₃₇₄ (ASNENMETM) which is known to be a dominant CD8⁺ T-cell epitope in mice (Townsend et al., 1984, 1985, 1986) and was previously used in S-Flu studies (Powell et al., 2012, 2019). Like all other internal components, the nucleoprotein sequence is identical between S-Flu and CLEARFLU viruses, allowing for comparison. T-cells in both the lung and spleen were measured to assess local as well as systemic responses.

In pigs, tetramer staining was performed using three nucleoprotein CD8⁺ T-cell epitopes recently defined by Tungatt et al (Tungatt et al., 2018). ELISPOT was also used to detect IFN γ -secreting CD4⁺ and CD8⁺ T-cells in peripheral blood mononuclear cells (PBMCs), the spleen and bronchoalveolar lavage (BAL) as described in Morgan et al (Morgan et al., 2016). This allowed us to capture a broader picture of T-cell activity by CD4⁺ and CD8⁺ T-cells, irrespective of target epitope.

6.2 Results

6.2.1 H1 and H3 CLEARFLU version 2 with H1 PR8 coats in mice

Figure 6.2 – Experimental scheme 1



Group	n.	Virus	Full virus designation	Route	Dose
1	5	H1 CLEARFLU	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IN	< 3E4 CID50
2	5	H1 S-Flu	[S-eGFP/N1(A/England/195/2009)] coated in H1 (A/England/195/2009)	IN	
3	5	H3 CLEARFLU	[H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IN	6.90E+05 CID50
4	5	H3 S-Flu	[S-eGFP/N2(NYMC X-217)] coated H3(A/Hong Kong/5738/2014)	IN	1.19E+06 CID50
5	4	VGM		IN	
6	5	H1 CLEARFLU	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IP	< 3E5 CID50
7	5	H1 S-Flu	[S-eGFP/N1(A/England/195/2009)] coated in H1 (A/England/195/2009)	IP	
8	5	H3 CLEARFLU	[H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IP	6.90E+06 CID50
9	5	H3 S-Flu	[S-eGFP/N2(NYMC X-217)] coated H3(A/Hong Kong/5738/2014)	IP	1.19E+07 CID50
10	4	VGM		IP	
	S-Flu viruses used in assays				
	MN		ELLA		
H1N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)				
H1N1 S-Flu	[S-eGFP/N1(A/England/195/2009)] coated in H1(England/195/2009)				
H3N2 S-Flu	[S-eGFP/N2(NYMC X-217)] coated in H3(A/Hong Kong/5738/2014)				
H3N2 S-Flu	[S-eGFP/N2(NYMC X-217)] coated in H3(X-31)				
H3N2 S-Flu			[S-eGFP/N2(NYMC X-217)] coated in H3(A/Switzerland/9715293/2013)		
H7N1 S-Flu			[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)		
H7N1 S-Flu			[S-eGFP/N1(A/England/195/2009)] coated in H7(A/Netherlands/219/2003)		

This preliminary experiment constituted the first trial of CLEARFLU viruses *in vivo*. The primary aim of this experiment was to determine whether serum antibody responses were generated against the CLEARFLU hemagglutinin following intranasal (IN) or intra-peritoneal (IP) immunisation.

Viruses expressing the H1 (A/England/195/2009) and H3 (A/Hong Kong/5738/2014) CLEARFLU version 2 hemagglutinins described in Chapter 3 were used. They were pseudotyped with functional H1 hemagglutinin from the A/Puerto Rico/8/1934 (PR8) strain to match their PR8 neuraminidase. S-Flu viruses pseudotyped with hemagglutinin from the same strains as the CLEARFLU hemagglutinins were used for comparison.

Viruses were used neat to offer the best chance of eliciting a response, and sera from mice in the same experimental group were pooled to test for a neutralising antibody response to the expressed CLEARFLU hemagglutinin. Serum antibody to neuraminidase was also assayed using ELLA. Where possible, doses were calculated retrospectively using leftover virus.

Microneutralisation

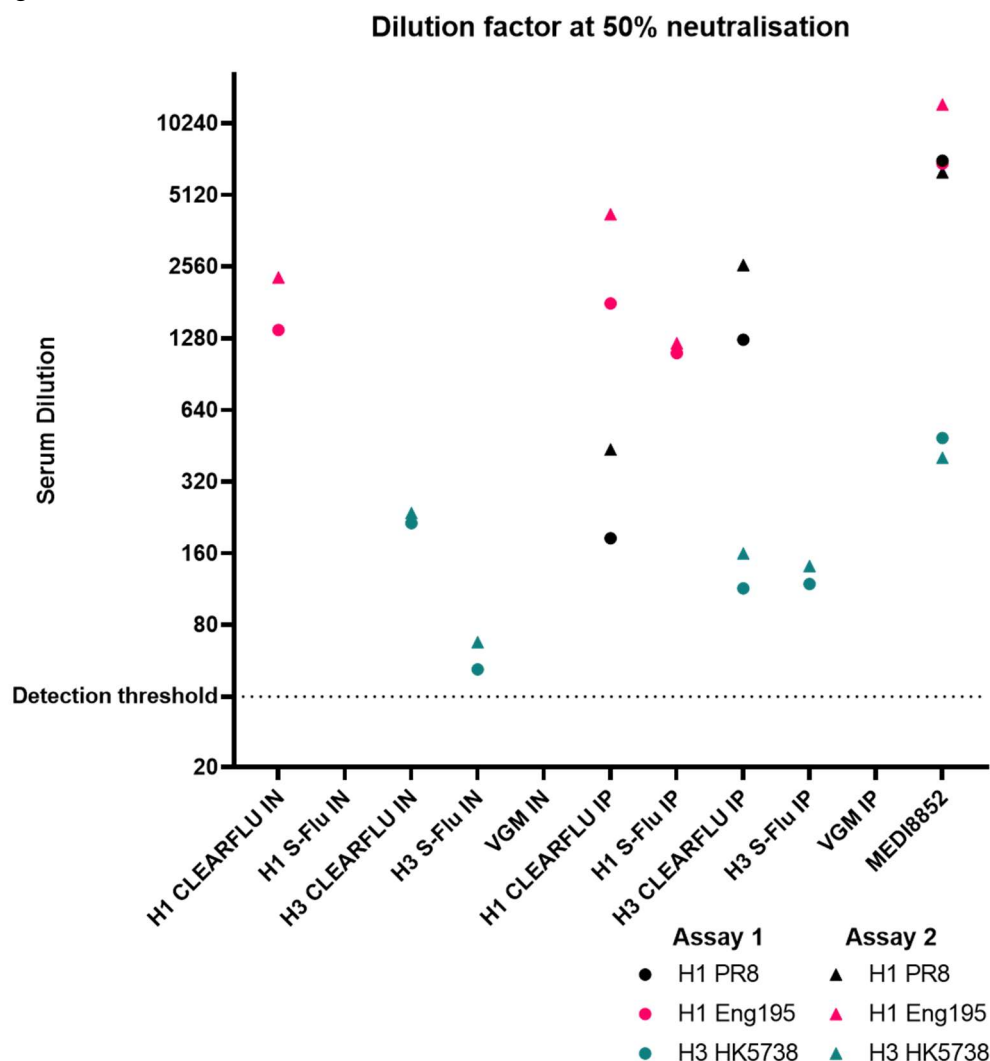
Figure 6.3 shows the results of microneutralisation assays conducted on the pooled sera. Sera were diluted 1 in 40 in the first dilution and set up in doubling dilutions after that. The serum dilution which reduces viral entry by 50% in a microneutralisation assay is plotted. The first five groups were immunised intranasally. Responses to the S-Flu pseudotyping hemagglutinin were not detected (H1) or were close to the detection threshold (H3). Similarly, the pseudotyping PR8 hemagglutinin in CLEARFLU viruses did not stimulate a detectable response. The CLEARFLU hemagglutinin however stimulated strong responses, particularly for the H1 virus which had a 50% neutralisation titre of around 1 in 2000. This is especially high considering that the H1 CLEARFLU virus has a titre too low to be quantified by our CID50 assay.

The next five groups were immunised by intra-peritoneal injection. This stimulated a neutralising antibody response to the coating hemagglutinin of all viruses as well as to the CLEARFLU hemagglutinin. CLEARFLU viruses administered in this way therefore stimulated an antibody response capable of neutralising viruses from two different subtypes. For both S-Flu and CLEARFLU viruses, the neutralising antibody response is strain specific. Once again, the response to H1 CLEARFLU appeared to be strong relative both to the response to the PR8 pseudotyping hemagglutinin and to the H3 CLEARFLU.

These results align with previous observations that immunising intranasally typically leads to low or no neutralising antibody. Powell et al found no neutralising response (detection threshold 1 in 20) following intranasal immunisation of BALB/C mice with PR8 S-Flu (both eGFP and S-HA version), while intraperitoneal immunisation led to strong neutralisation (Powell et al., 2012). Intranasal immunisation of C57BL/6 mice generated a weak neutralising response at 1 in 20 (Powell et al., 2012 supplementary information). In ferrets, Baz et al describe low microneutralisation titres (1 in 80-113) after intranasal immunisation with two doses of H1 PR8 S-Flu (Baz et al., 2015 supplementary information). In pigs, Morgan et al., show that protection against challenge is generated in the absence of any neutralising antibody response after aerosol administration of H1 (A/England/195/2009) S-Flu or intratracheal administration of H1 or H5 (A/Vietnam/1203/2004) S-Flu (Morgan et al., 2016).

The serum response to the pseudotyping coat therefore varies with animal model and antigen strain, but is consistently lower following local administration to the lung compared to peripheral administration. Here, we have shown that viral expression of a hemagglutinin antigen in the form of CLEARFLU allows for a strong neutralising response in sera following local administration.

Figure 6.3



Neutralisation of H1 or H3 S-Flu by sera from immunised mice

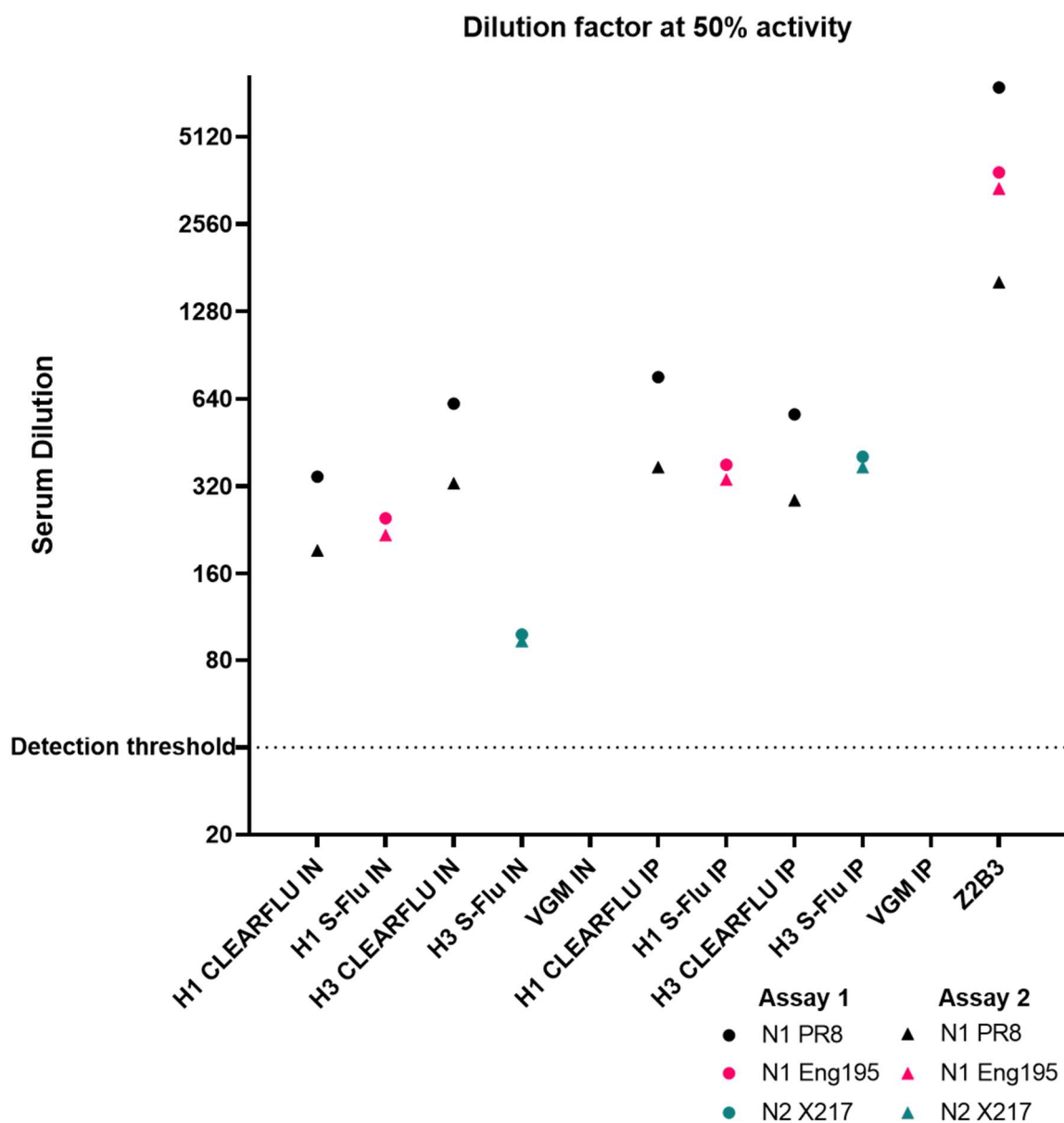
Inhibition of cell entry by S-Flu viruses after incubation with immune sera or control antibody MEDI8852 was determined as per microneutralisation protocol. The sera from mice in the same group were pooled and diluted 1 in 20 before being added to virus in doubling dilutions, giving a dilution factor of 1 in 40 in the first dilution. The dilution at which sera reduced infection by 50% is plotted for two identical experiments. The neutralisation of four viruses was tested, including X31 (H3) which is not plotted as it was not neutralised by any sera.

ELLA

Figure 6.4 shows the results of an ELLA assay conducted on pooled sera to detect antibody responses capable of blocking neuraminidase activity. Each S-Flu or CLEARFLU virus stimulated a strain-specific response which blocked the activity of matched neuraminidase, but not neuraminidase from another strain. Responses to both CLEARFLU viruses were strong, providing no indication that expressing an additional protein on the infected cell surface has a detrimental effect on neuraminidase-mediated immunity.

This result concurs with Powell et al who showed that intraperitoneal or intranasal administration of S-Flus expressing PR8 neuraminidase leads to strong serum responses by ELLA against PR8 N1 (1 in 1000-10000) but not X31 N2 or A/England/195/09 N1 (Powell et al., 2019). In ferrets, Baz et al describe relatively low serum titres (around 1 in 50 by ELLA) following intranasal administration of H1 S-Flu expressing N1 from A/England/195/09, which are also strain-specific (Baz et al., 2015).

Figure 6.4

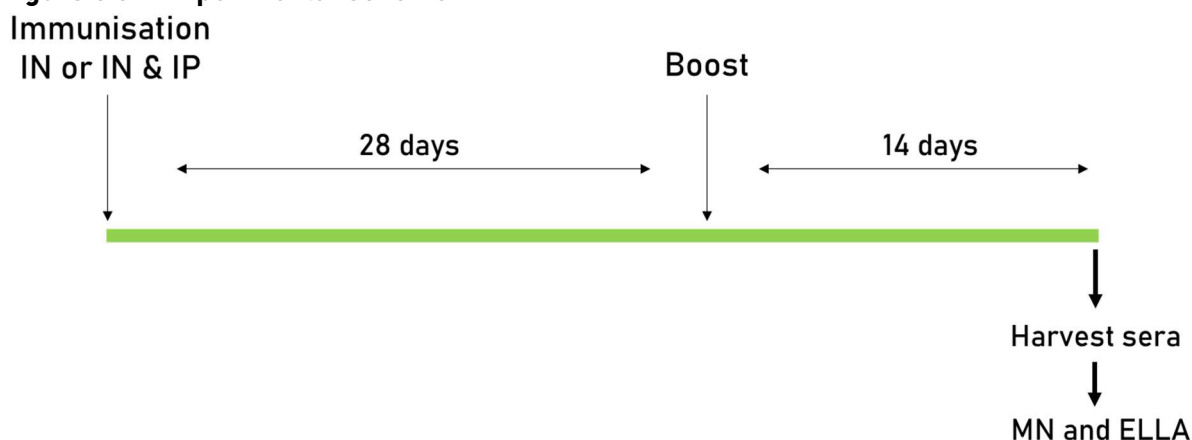


Inhibition of N1 and N2 neuraminidase activity by sera from immunised mice

The neuraminidase activity of N1 (Eng195), N1 (PR8) and N2 (X31) S-Flu after incubation with immune sera or control antibody Z2B3 was determined as per ELLA protocol. The sera from mice in the same group were pooled and diluted 1 in 20 before being added to virus in doubling dilutions, giving a dilution factor of 1 in 40 in the first dilution. The dilution at which the neuraminidase activity of S-Flu was reduced by 50% was calculated. All experimental groups showed a positive titre by ELLA against S-Flu with a neuraminidase matched to the one used for immunisation. No sera reduced the activity of neuraminidase from a different strain. The results of two independent experiments are shown.

6.2.2 H1 and H3 CLEARFLU version 2 with non-seasonal coats in mice

Figure 6.5 – Experimental scheme 2



Group	n.	Virus	Full virus designation	Route	Dose
1	5	H1 CLEARFLU (H5 coat)	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)	IN	2E6 CID50
2	5	H1 CLEARFLU (H7 coat)	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)	IN	2E6 CID50
3	5	H3 CLEARFLU (H5 coat)	[H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)	IN	2E6 CID50
4	5	H3 CLEARFLU (H7 coat)	[H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)	IN	2E6 CID50
5	5	H1 CLEARFLU (H7 coat) & H3 CLEARFLU (H5 coat)	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003) & [H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)	IN	1E6 CID50 each
6	5	H1 CLEARFLU (H7 coat) & H3 CLEARFLU (H5 coat)	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003) & [H3CFv2(A/Hong Kong/5738/2014)/N1(A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)	IN & IP	1E6 CID50 each IN & 1E7 CID50 each IP
7	4	VGM		IN & IP	

	S-Flu viruses used in assays	
	MN	ELLA
H1N1 S-Flu	[S-eGFP/N1(A/England/195/2009)] coated in H1(A/England/195/2009)	
H5N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)	
H3N2 S-Flu	[S-eGFP/N2(NYMC X-217)] coated in H3(A/Hong Kong/5738/2014)	
H7N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)	
H7N1 S-Flu		[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/New York/107/2003)
H3N2 S-Flu		[S-eGFP/N2(NYMC X-217)] coated in H3(X-31)

This experiment was designed to further characterise antibody responses to CLEARFLU viruses following intranasal administration. A set titre of virus was used to allow for clearer comparisons between groups. The core viruses were the same as for the previous experiment, but they were pseudotyped with H5 or H7 hemagglutinins. Using pseudotyping coats from non-circulating strains in vaccines may be advantageous for avoiding existing immunity and ensuring that a single cycle of infection in the lung can take place efficiently. We also investigated whether administration of viruses IP in addition to IN resulted in increased antibody titres.

In the previous experiment, we observed that a single CLEARFLU virus administered IP could generate a neutralising response against two different subtypes. Here, two experimental groups received a mix of two CLEARFLU viruses together to determine whether a neutralising response to H1, H3, H5 and H7 viruses –the key subtypes in human, avian and swine infection– can be generated simultaneously.

As previously, sera from animals in the same group were pooled and used to assay for neutralisation and inhibition of neuraminidase activity. Microneutralization and ELLA were both conducted in technical duplicates within assays and then independently repeated.

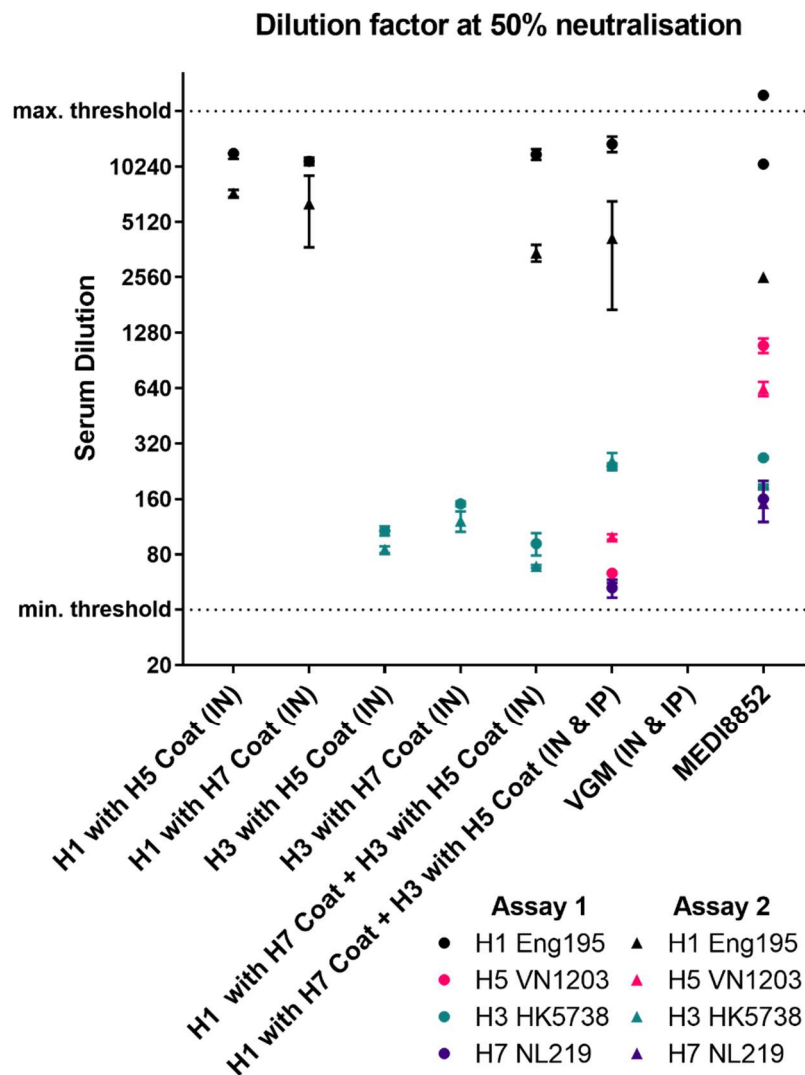
Microneutralisation

Figure 6.6.A shows the serum dilution at which 50% neutralisation is observed for four test viruses of different subtypes. Figure 6.6.B shows the neutralisation curves for each test virus for each experimental group from one of two identical repeats used to generate Figure 6.6.A.

As seen previously, neutralisation was specific to the immunising hemagglutinin and no neutralising response to the pseudotyping coat was observed for any group which received only intranasal virus. A neutralising response to the CLEARFLU hemagglutinin was observed in all cases, and was especially strong towards the H1 CLEARFLU, as was observed in the previous experiment. This is particularly evident for the group which received both H1 and H3 viruses, as this difference cannot be due to poor immunisation technique. In chapter 3 we had observed particularly strong antibody binding to H1 CLEARFLU. Together these data may indicate that H1 CLEARFLU has a higher immunogenicity than H3 CLEARFLU in mice, which is perhaps enhanced by stabilising disulphide bonds.

There is no evidence that the H7 or H5 coat offers an advantage over the other in facilitating cell entry and generating neutralising antibody responses. Immunising with a mixture of H1 and H3 CLEARFLU viruses with H7 and H5 coats respectively leads to a neutralising response to H1 and H3 when administered intranasally and all four subtypes when an intraperitoneal injection is also given. This shows that although neutralising responses are strain-specific, mixed preparations can be given to elicit a response with multiple specificities. Giving an intraperitoneal injection in addition to intranasal immunisation did not seem to increase the response to H1 CLEARFLU, but perhaps increased titres to H3 CLEARFLU (6.6.b, right hand plots, comparing purple and blue lines).

Figure 6.6

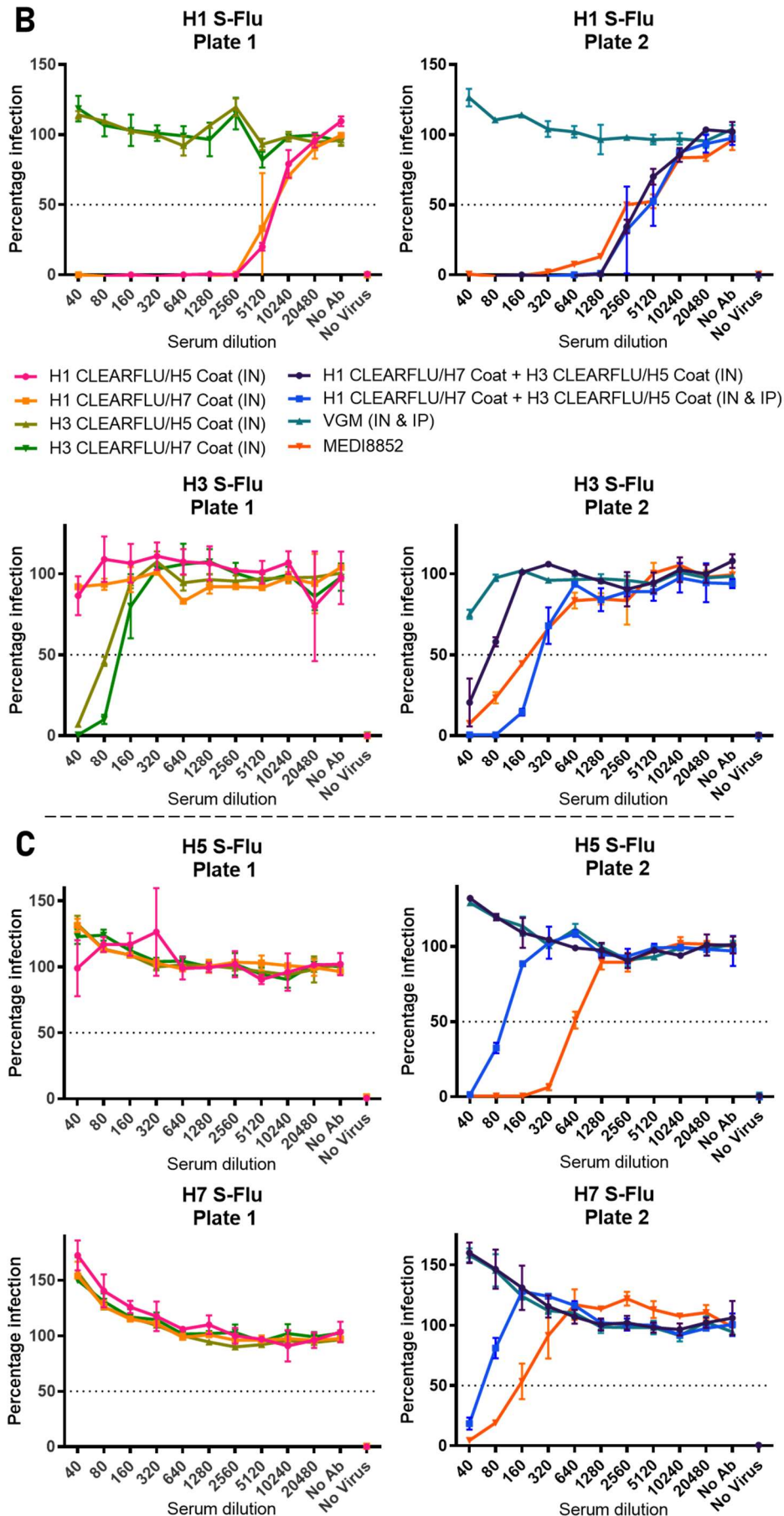


Neutralisation of H1, H3, H5 and H7 S-Flu by sera from immunised mice

Inhibition of cell entry of S-Flu viruses after incubation with immune sera or control antibody MEDI8852 was determined as per microneutralisation protocol. Sera from mice in the same group were pooled and diluted 1 in 20 before being added to virus in doubling dilutions, giving a minimum detection threshold of 1 in 40 and a maximum threshold of 20480. The experiment was performed twice with concordant results.

A. The dilution at which sera reduced infection by 50% is plotted for two identical experiments. Error bars show the standard deviation of duplicates within a single experiment. No error bar is generated for MEDI8852 for H1N1 in Assay 1 as one duplicate reading fell above the maximum detection threshold. **B&C.** Plots show the inhibition of infection in the presence of sera containing neutralising antibodies. Error bars show the mean and standard deviation of duplicates within an experiment, which was repeated to generate part A. Sera neutralised S-Flu viruses coated in hemagglutinin from the same strain as the CLEARFLU used for immunisation, indicating a strain-specific neutralising antibody response (**B**). Immunising with H1 and H3 CLEARFLU together generated a neutralising antibody response to both H1 and H3 (**B**, right panel). No neutralising response was seen to the coating hemagglutinin (H5 or H7) when immunisation was performed intranasally, but intraperitoneal administration did generate a response (**C**). MEDI8852 neutralises all 4 viruses (**B&C**, right panels).

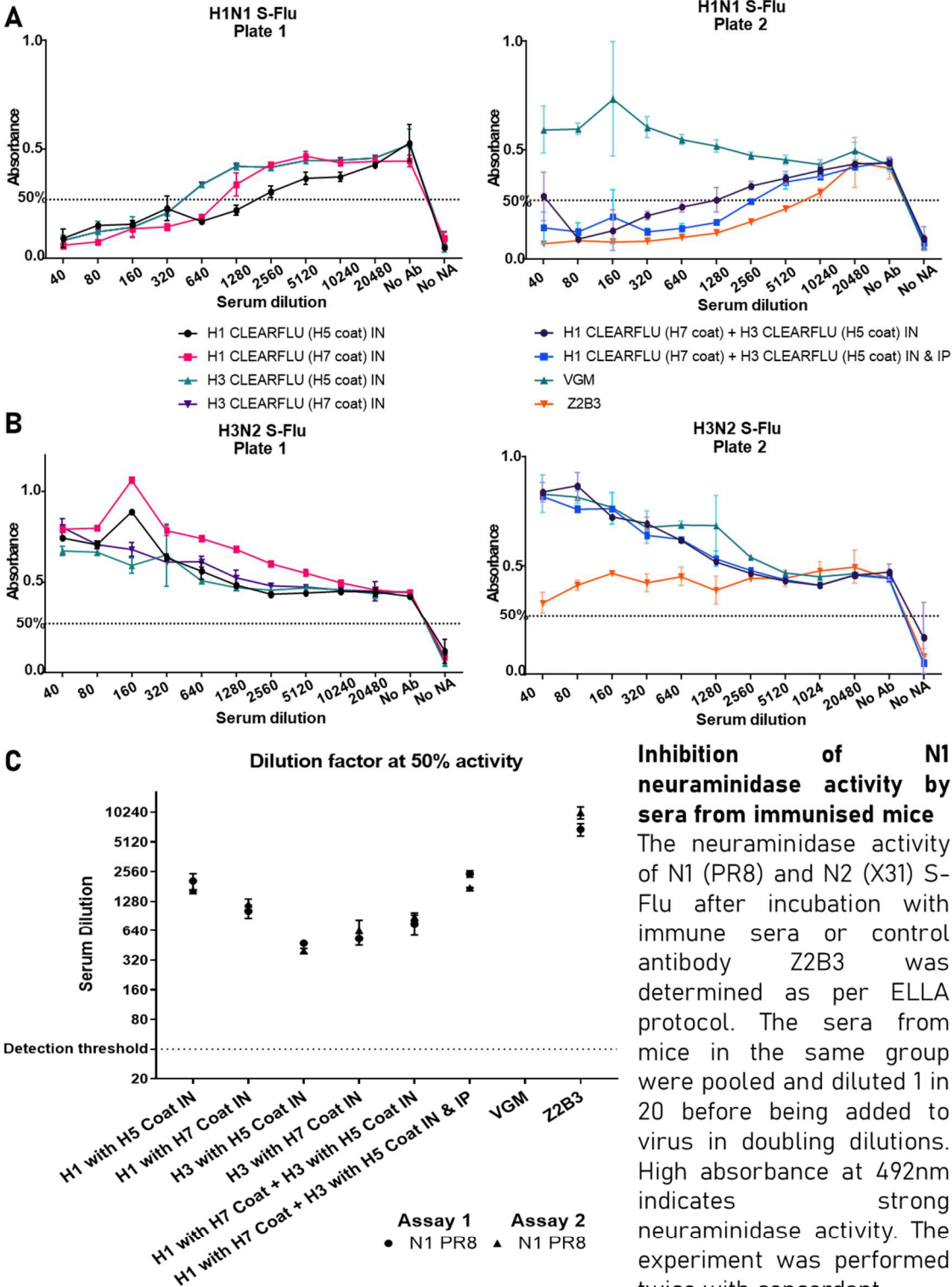
Figure 6.6 continued



ELLA

All vaccine viruses in this experiment express PR8 neuraminidase. Figure 6.7 shows that sera from all groups inhibited PR8 neuraminidase activity. The activity of X31 N2 neuraminidase was not inhibited by sera from any experimental group, as expected for a strain-specific response. There was no evidence to suggest the H7 or H5 coating hemagglutinin was superior to the other for stimulating responses to neuraminidase.

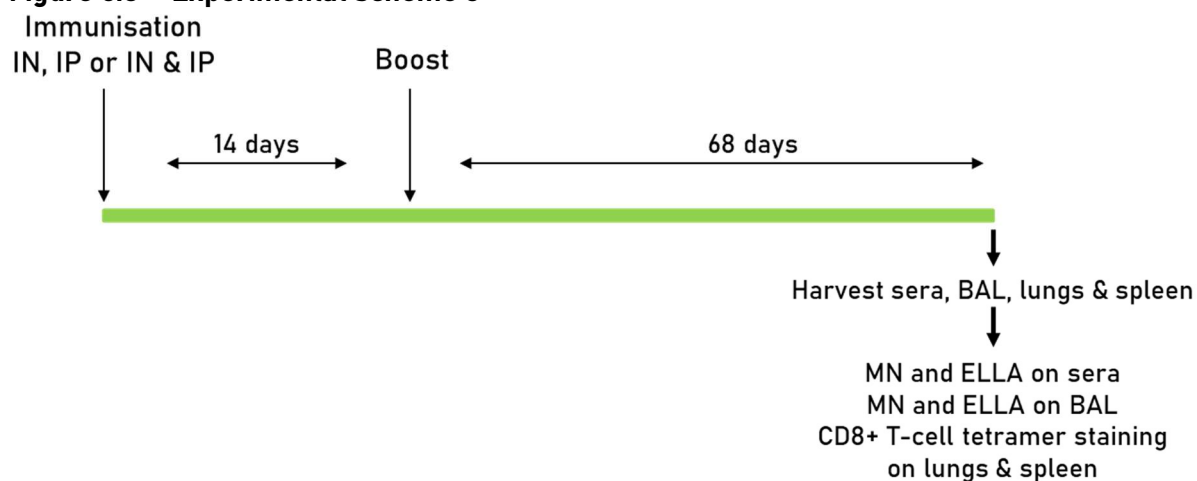
Figure 6.7



results (see C). A&B. Neuraminidase activity of N1 (A) or N2 (B) S-Flu in the presence of mouse sera, VGM or antibody Z2B3. Error bars represent the standard deviation of duplicates within a single experiment. C. The dilution at which neuraminidase activity of N1 (PR8) S-Flu was reduced by 50% was calculated using the data above and an identical repeat experiment. Results for N2 (X31) are not plotted as no sera inhibited activity. Circles show the mean of duplicate readings from the experiment shown in parts A and B, and triangles show the same for an identical repeat experiment. Error bars represent the standard deviation of duplicates within a single experiment.

6.2.3 H7 CLEARFLU version 3 in mice

Figure 6.8 – Experimental scheme 3



Group	n.	Virus	Full virus designation	Route	Dose
1	4	H7 CLEARFLU v3	[H7CFv3(A/Hong Kong/125/2017)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IN	1E5 TCID50 (equivalent to 1.24E6 CID50)
2	4	H7 CLEARFLU v3	[H7CFv3(Hong Kong/125/2017)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IP	1E6 TCID50 (equivalent to 1.24E7 CID50)
3	4	H7 CLEARFLU v3	[H7CFv3(Hong Kong/125/2017)/N1(A/Puerto Rico/8/1934)] coated in H1(A/Puerto Rico/8/1934)	IN & IP	1E5 TCID50 IN & 1E6 TCID50 IP
4	4	H7 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2017)	IN	1E5 TCID50 (equivalent to 1.11E6 CID50)
5	4	H7 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2017)	IP	1E6 TCID50 (equivalent to 1.11E7 CID50)
6	4	H7 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2017)	IN & IP	1E5 TCID50 IN & 1E6 TCID50 IP
7	4	VGM		IN & IP	
S-Flu viruses used in assays					
	MN		ELLA		
H7N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Hong Kong/125/2017)				
H7N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)				
H7N1 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Taiwan/1/2017)				
H5N1 S-Flu			[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)		

This experiment explores antibody and T-cell responses to an H7 CLEARFLU version 3 virus. It therefore varies from previous experiments in that it

- A) extends previous observations on antibody responses to also encompass T-cell mediated immune mechanisms
- B) explores responses to a CLEARFLU version 3 design which should more strongly bind sialic acid than CLEARFLU version 2 due to the replacement of the Y98F mutation with double prolines F63/70P in the B-loop
- C) uses a CLEARFLU hemagglutinin based on an avian influenza strain with pandemic potential.

As previously, sera were collected for microneutralisation and ELLA. In addition, bronchoalveolar lavage fluid (BAL) was harvested from the lungs for microneutralisation and ELLA and CD8⁺ T-cells in the lungs and spleen were stained for reactivity to nucleoprotein (NP). As we had access to a H-2Db MHC tetramer for nucleoprotein, C57BL/6 mice were used.

Microneutralisation

Previous experiments have shown strong neutralising antibody responses in pooled sera following immunisation with CLEARFLU viruses. In Figure 6.9, sera from individual mice rather than group pools were assayed for neutralisation of a strain-matched S-Flu test virus. As expected, all animals except those in the S-Flu IN or VGM (viral growth medium) only groups showed a response.

Neutralising responses in the BAL were much weaker than in the sera. Even using undiluted BAL fluid, observed responses were close to the detection threshold (see Figure 6.10). At least one individual in each group - except for the S-Flu IN group and VGM group which did not generate responses in BAL or sera - generated a detectable response in

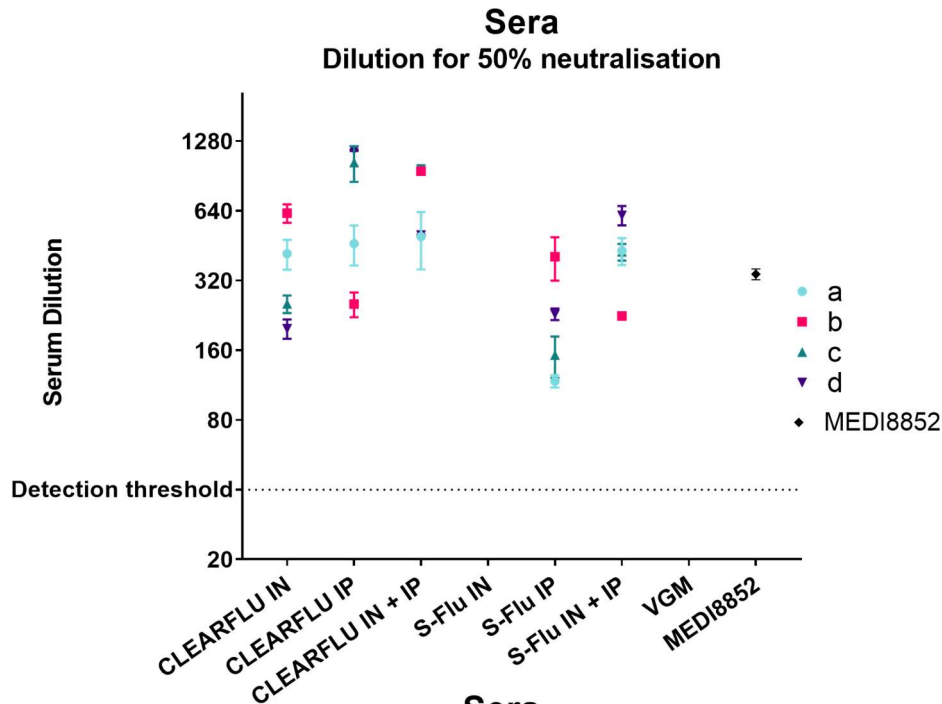
BAL, indicating that all of these regimens are capable of stimulating a local neutralising response.

To determine whether there was any within-subtype breadth to the neutralising response to H7 CLEARFLU, sera were tested for neutralisation of two other H7 strains: A/Taiwan/1/2017 and A/Netherlands/219/2003. Both are classed as highly pathogenic avian influenza (HPAI) viruses. Like A/Hong Kong/125/2017, A/Taiwan/1/2017 is a member of the fifth wave Yangtze Delta H7N9 viruses. Its hemagglutinin has close sequence identity (97%) to A/Hong Kong/125/2017 (Yang et al., 2018). S-Flu viruses with these hemagglutinins have previously shown similar but distinct sensitivities to head-specific neutralising monoclonal antibodies (Huang et al., 2019). The hemagglutinin from A/Netherlands/219/2003 is less closely related with 91% sequence identity, having been isolated from a cluster of human infections following an outbreak of HPAI H7N7 in birds. It is not neutralised by head-specific monoclonal antibodies such as 4A14 and W3A1 which neutralise the Yangtze Delta hemagglutinins (Huang et al., 2019).

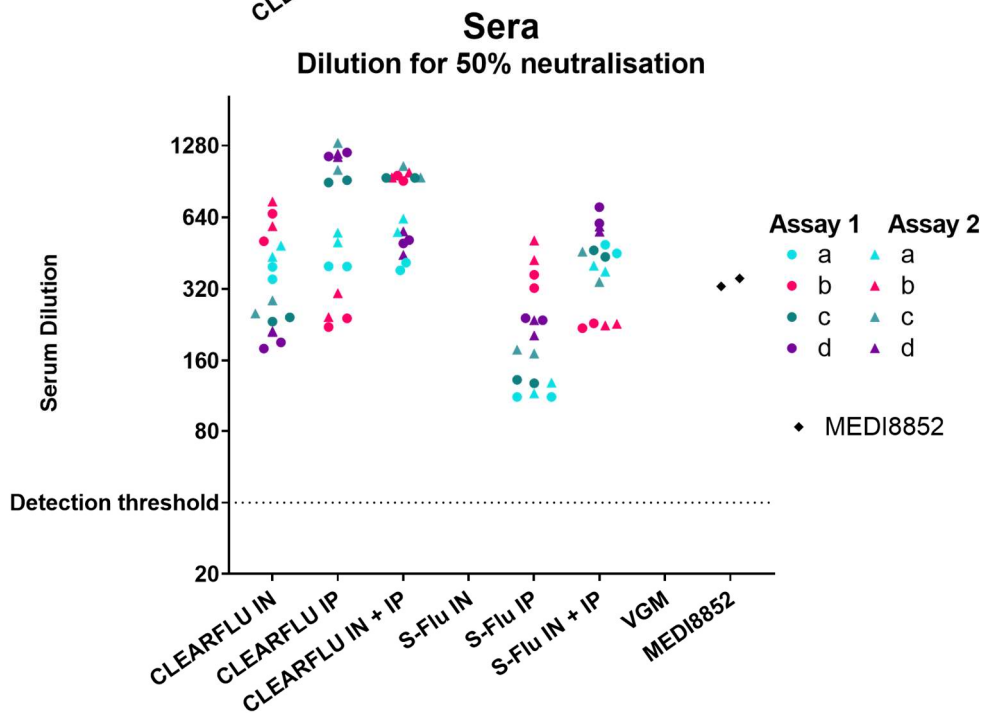
Although there was variability in the titres observed across repeats, cross-reactivity to both strains was observed following CLEARFLU immunisation (see Figure 6.11). Neutralisation titres were lower than for strain-matched hemagglutinin and were close to the detection threshold. A cross-reactive neutralising response was seen in the S-Flu IN & IP group but not to S-FLU administered IN or IP only. This is possibly as titres sit close to the detection threshold and the IN & IP group received a larger dose. In any case, cross-neutralising responses to the pseudotyping coat of S-Flu administered IN or IP were weaker than those generated to CLEARFLU administered by the same route. Unlike for S-Flu, the response to CLEARFLU did not appear to be improved by administering both IN and IP.

Figure 6.9

A



B



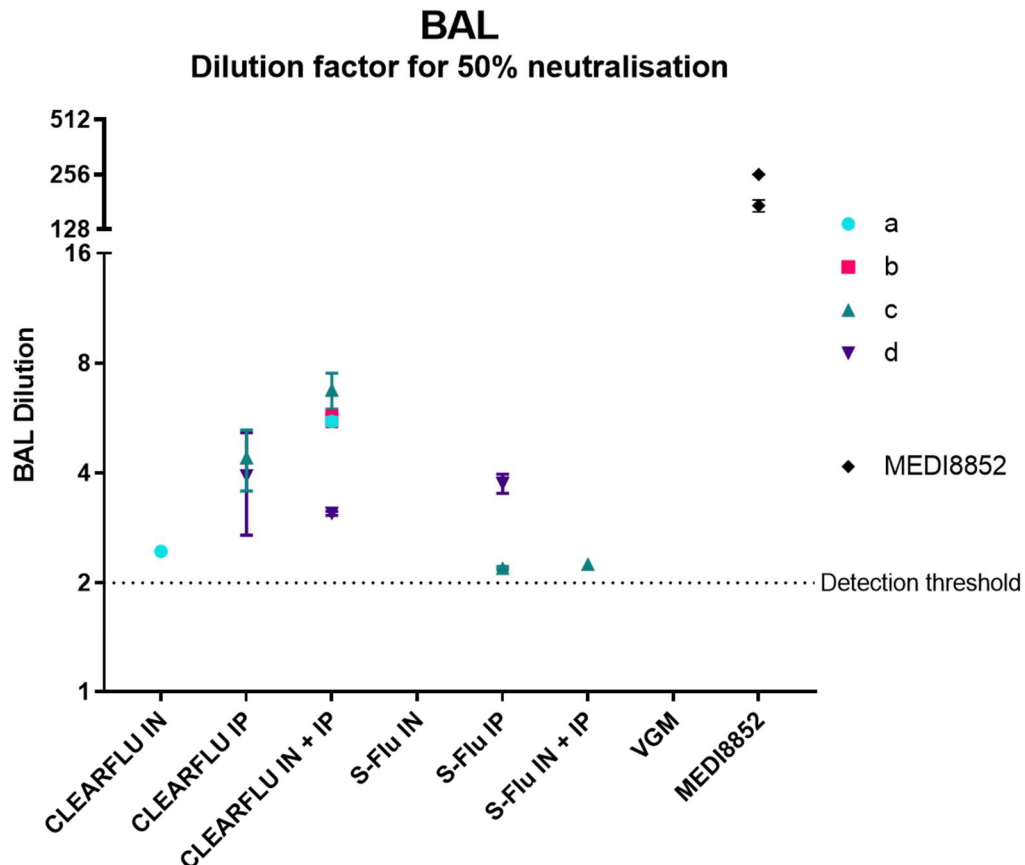
Neutralisation of H7 A/Hong Kong/125/2017 S-Flu by sera from immunised mice

Inhibition of cell entry by S-Flu after incubation with immune sera or control antibody MEDI8852 was determined as per microneutralisation protocol. The response of individual mice (a,b,c,d) in each experimental group was assayed. The sera were diluted 1 in 20 before being added to virus in doubling dilutions. The dilution at which sera reduced infection by 50% is plotted for individual mice. Two identical assays were performed, each with duplicate measurements.

A. Overall mean serum dilution at 50% neutralisation for each mouse. Error bars show the standard deviation of two mean values generated from independent experiments.

B. Individual measurements of serum dilution at 50% neutralisation for each mouse. Two values are shown for each of the two assays. Only individuals in the S-Flu IN or VGM groups failed to generate a detectable response. Points cluster by individual (colour) rather than by assay (shape), indicating that the variation in response between individuals is greater than within an individual between assays.

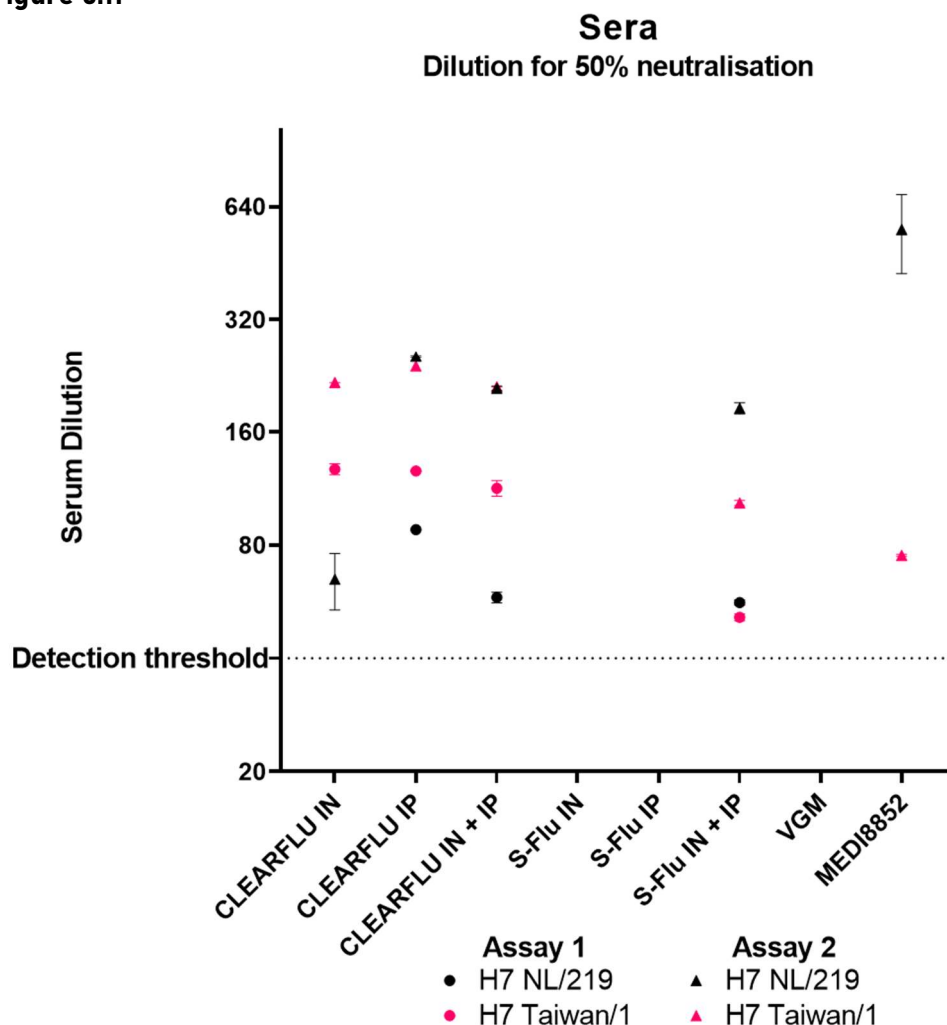
Figure 6.10



Neutralisation of H7 A/Hong Kong/125/2017 S-Flu by bronchoalveolar lavage fluid (BAL) from immunised mice

Inhibition of cell entry by S-Flu after incubation with BAL or control antibody MEDI8852 was determined as per microneutralisation protocol. The response of individual mice (a,b,c,d) in each experimental group was assayed. BAL samples were not pre-diluted before being added to virus in doubling dilutions. The dilution at which BAL reduced infection by 50% is plotted. Error bars show the standard deviation of duplicates within a single experiment, except for individual c in the S-Flu IN +IP group as one duplicate reading fell below the detection threshold. No sample was obtained from individual a in the S-Flu IN +IP group or individual a in the VGM control group.

Figure 6.11



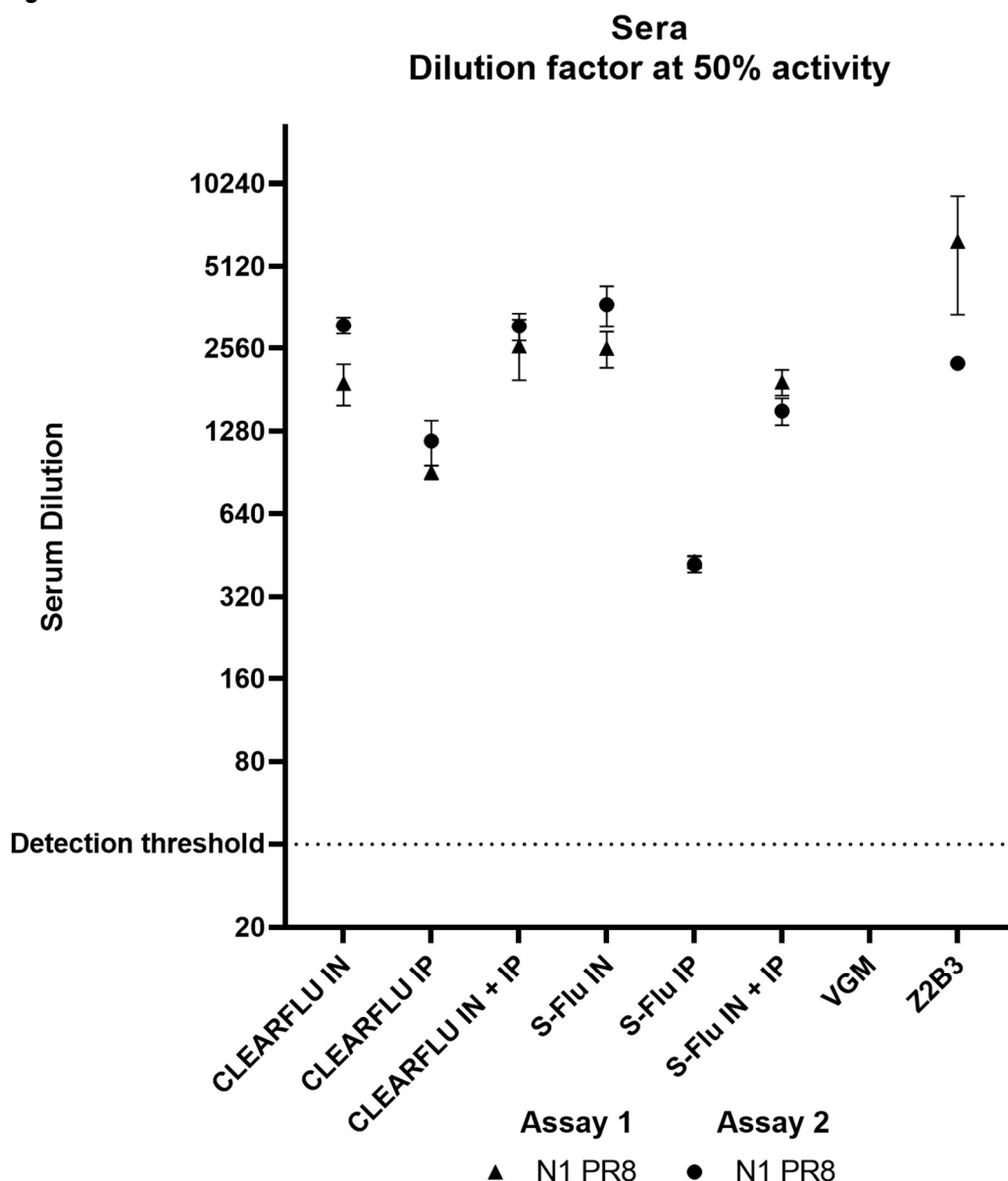
Neutralisation of unmatched H7 A/Netherlands/219/2003 and A/Taiwan/1/2017 S-Flu viruses by sera from immunised mice

Inhibition of cell entry by S-Flu viruses after incubation with immune sera or control antibody MEDI8852 was determined as per microneutralisation protocol. The sera from mice in the same group were pooled and diluted 1 in 20 before being added to virus in doubling dilutions. The dilution at which sera reduced infection by 50% is plotted for two identical experiments. Error bars show the standard deviation of duplicates within a single experiment. Titres for CLEARFLU IN (NL/219, Assay 1), S-Flu IN (all), S-Flu IP (all) and VGM (all) were not detected.

ELLA

All immunised groups generated a neuraminidase-inhibiting antibody response in the sera (Figure 6.12) and BAL (Figure 6.13), including the S-Flu IN group which did not generate a neutralising response in either sera or BAL. As with neutralisation, neuraminidase inhibition titres in the BAL were weaker than in sera but could be detected for at least one individual in every group.

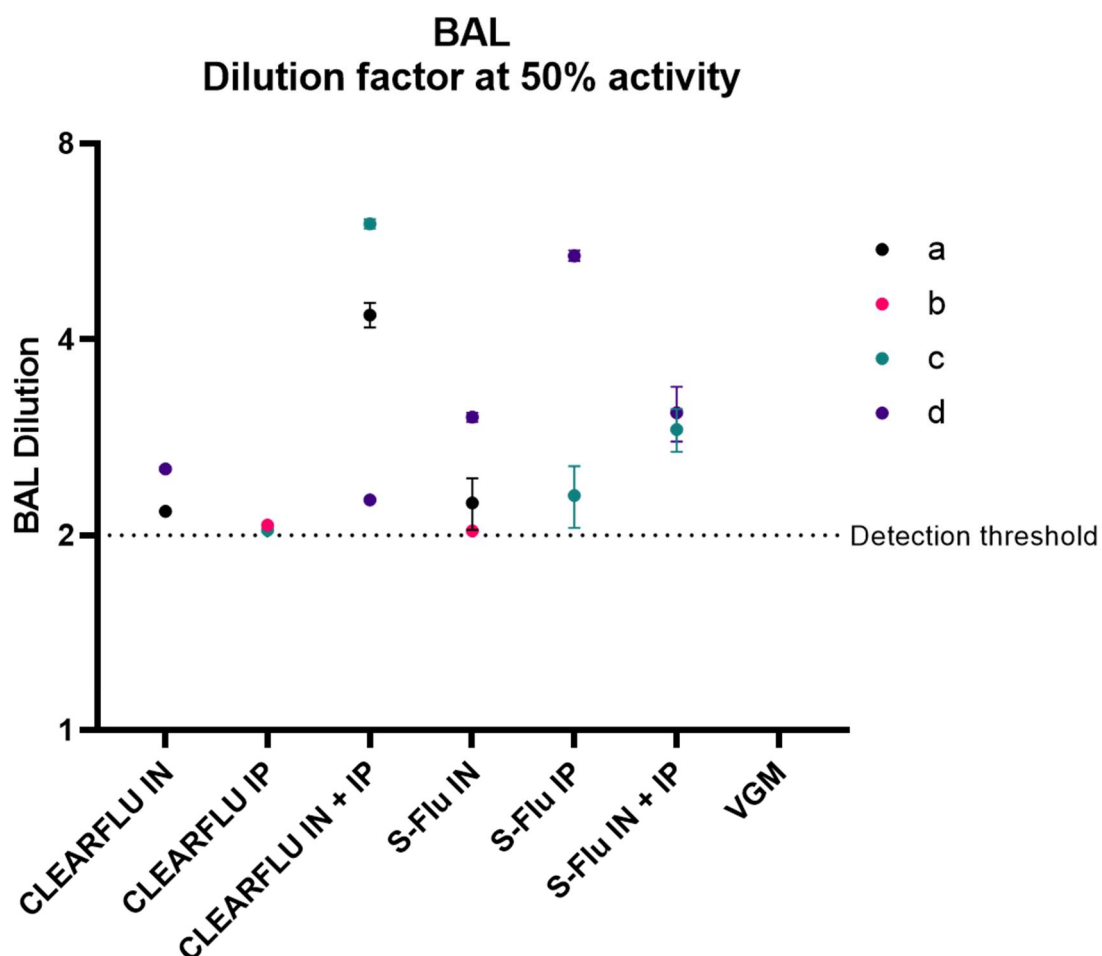
Figure 6.12



Inhibition of neuraminidase activity by sera from immunised mice

The neuraminidase activity of N1 (PR8) after incubation with immune sera or control antibody Z2B3 was determined as per ELLA protocol. The sera from mice in the same group were pooled and diluted 1 in 20 before being added to virus in doubling dilutions. The serum dilution at which neuraminidase activity of N1 (PR8) S-Flu was reduced by 50% is shown for two independent experiments, each performed in duplicate. All experimental groups showed a strong titre by ELLA in both assays except the negative control group (VGM only). Error bars represent the standard deviation of duplicates within a single experiment.

Figure 6.13



Inhibition of N1 neuraminidase activity by BAL from immunised mice

The neuraminidase activity of N1 (PR8) after incubation with BAL was determined by ELLA. Data shown are from a single experiment conducted in duplicate which could not be repeated due to limited sample volume. The mean and standard deviation are shown for two duplicate measurements, except for the following individuals where one measurement fell just below the detection threshold – CLEARFLU IN a, CLEARFLU IP b, CLEARFLU IN + IP d, S-Flu IN b. No sample was available for CLEARFLU IN + IP b, S-Flu IN +IP a, VGM a.

NP reactive T-cells

Protection from heterologous challenge following immunisation with S-Flu is associated with the presence of T-cells in the lung and spleen (Powell et al., 2012; Powell et al., 2019). In this experiment, we hoped to show similar T-cell responses to CLEARFLU viruses, indicating likely protection against a broad range of challenge strains.

Powell et al., 2019 showed that 6% of CD8⁺ T-cells in the lung and 1.5% in the spleen are specific for a single nucleoprotein (NP) peptide after intranasal immunisation of C57BL/6 mice with H7N1 (A/Anhui/1/2013) S-Flu. Mice immunised in this way were protected from disease after challenge with PR8 (H1N1) or X31 (H3N2) virus despite generating no cross-reactive neutralising antibody response, nor, in the case of X31 which has an N2 neuraminidase, a neuraminidase-inhibiting response. Here, we use the same NP peptide and a similar immunisation regimen (Powell et al.: 7.35×10^5 TCID₅₀ intranasally twice two weeks apart and harvest 40 days after second dose) to compare CD8⁺ T-cell responses following immunisation with H7 S-Flu and H7 CLEARFLU version 3.

Lung and spleen tissues were processed to produce single cell suspensions which could be stained for cell surface markers and analysed by flow cytometry. Figure 6.14 shows the staining and gating strategy used for analysis. Forward and side scatter were used to isolate single lymphocytes, after which B cells and dead cells were excluded. CD8 and CD44 were used to identify CD8⁺ T-cells, and the proportion of these which stained for MHC tetramer to NP peptide (H-2D(b) NP₃₆₆₋₃₇₄ ASNENMETM) was measured. This strategy was designed with Dr Pramila Rijal and Dr Jack Tan.

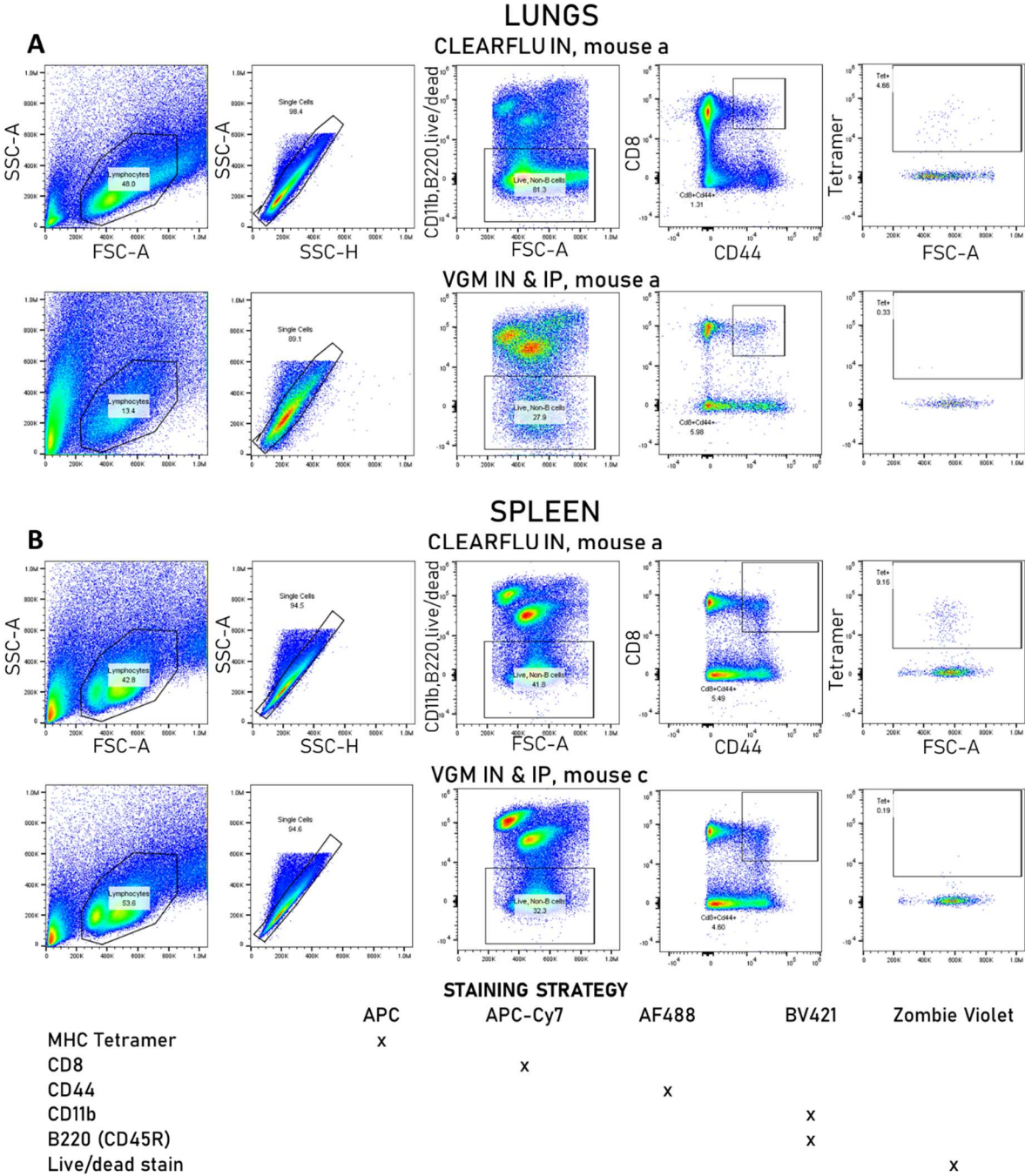
Figure 6.15 shows the population of CD8⁺ T-cells isolated from the lungs and spleen of each mouse plotted according to forward scatter and MHC tetramer binding. For some spleens, sample quality was too poor for analysis due to cell death and clumping. The percentages of tetramer positive CD8⁺ T-cells in the lungs and spleen are summarised in Figure 6.16.

For some groups, there was significant variation in tetramer binding between individuals, leading to large confidence intervals. Notably, 84% positivity in the lung was seen for one mouse in the S-Flu IP group, and 0.7% in another. The groups which varied the most were not the same for the lungs and spleens and included all three types of administration route, providing no evidence to suggest that this was caused by poor delivery of the vaccine. This may instead be due to different epitope dominance in some mice. Responses were generally more variable in the lung than spleen.

Multiple t tests were used to determine whether CLEARFLU or S-Flu stimulated a stronger T-cell response than the other for each administration regimen. No significant differences were detected except for between the IP groups in the spleen (two-tailed, $p=0.0078$). This was due to a low response in the CLEARFLU IP group relative to all other immunised groups. The response in this group was however still significantly stronger than the VGM control group (one-tailed t test with Welch's correction, $p=0.0003$).

Overall these results show that CLEARFLU, like S-Flu, generates a detectable NP-specific CD8+ T-cell response in the lungs and spleen two months after the last immunisation. While one CLEARFLU group showed reduced responses in one tissue, there is no evidence to suggest that CLEARFLU consistently stimulates weaker T-cell responses and would therefore generate inferior protection relative to S-Flu in a heterologous challenge model. In particular, there was no evidence that T-cell responses in the lung were lower for any CLEARFLU group.

Figure 6.14

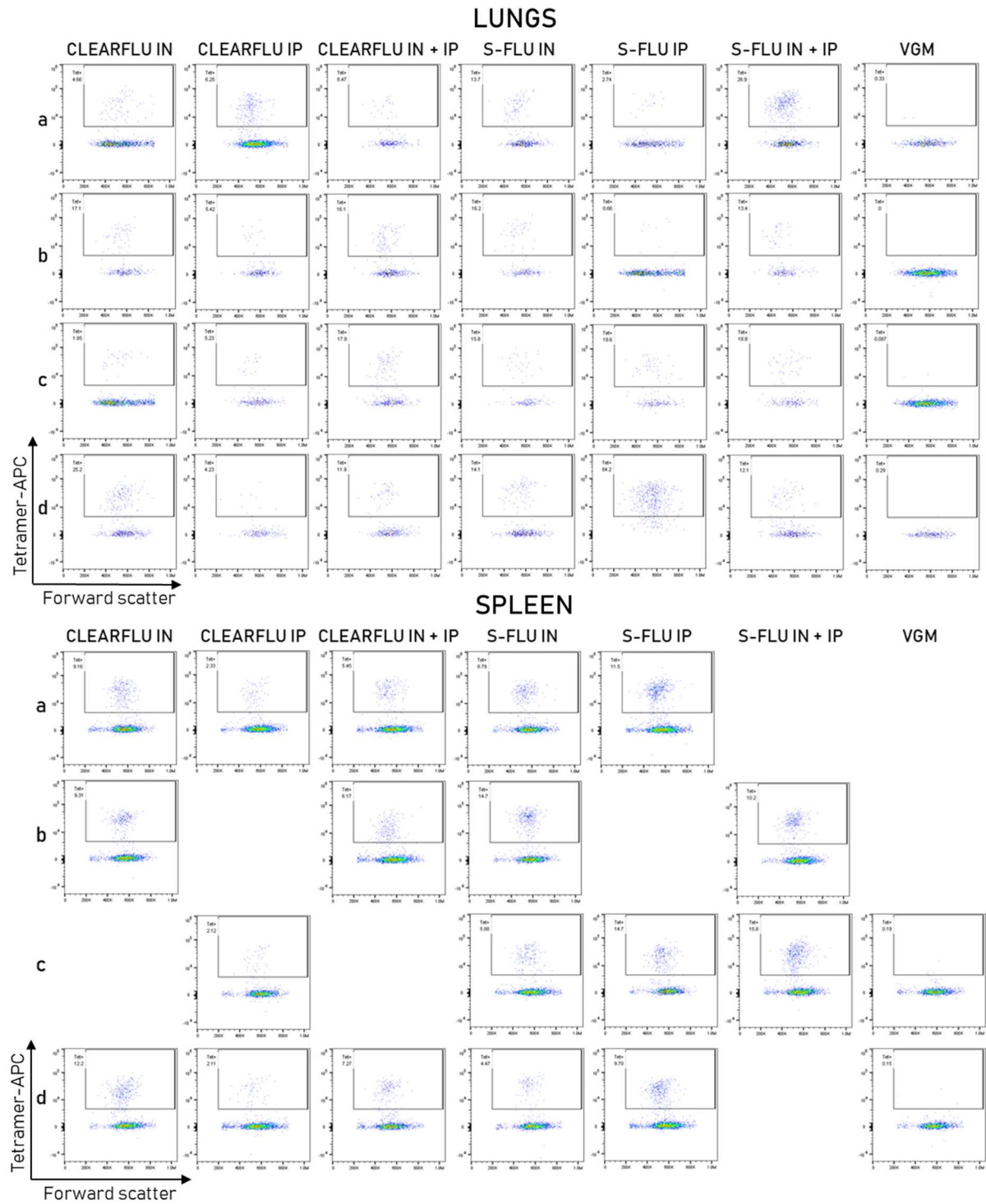


Gating strategy for studying NP peptide specificity of CD8+ T cells in the lungs and spleen

Forward (FSC) and side scatter (SSC) were used to identify single lymphocytes. A live/dead marker, B-cell marker (B220) and myeloid/NK cell marker (CD11b) with the same emission wavelength were used to exclude unwanted cell types and dead cells. CD44 and CD8 were used to identify CD8+ T cells. Of these, the proportion of cells bound by the MHC tetramer was determined.

- A. Gates used for cells obtained from the lungs
B. Gates used for cells obtained from the spleen

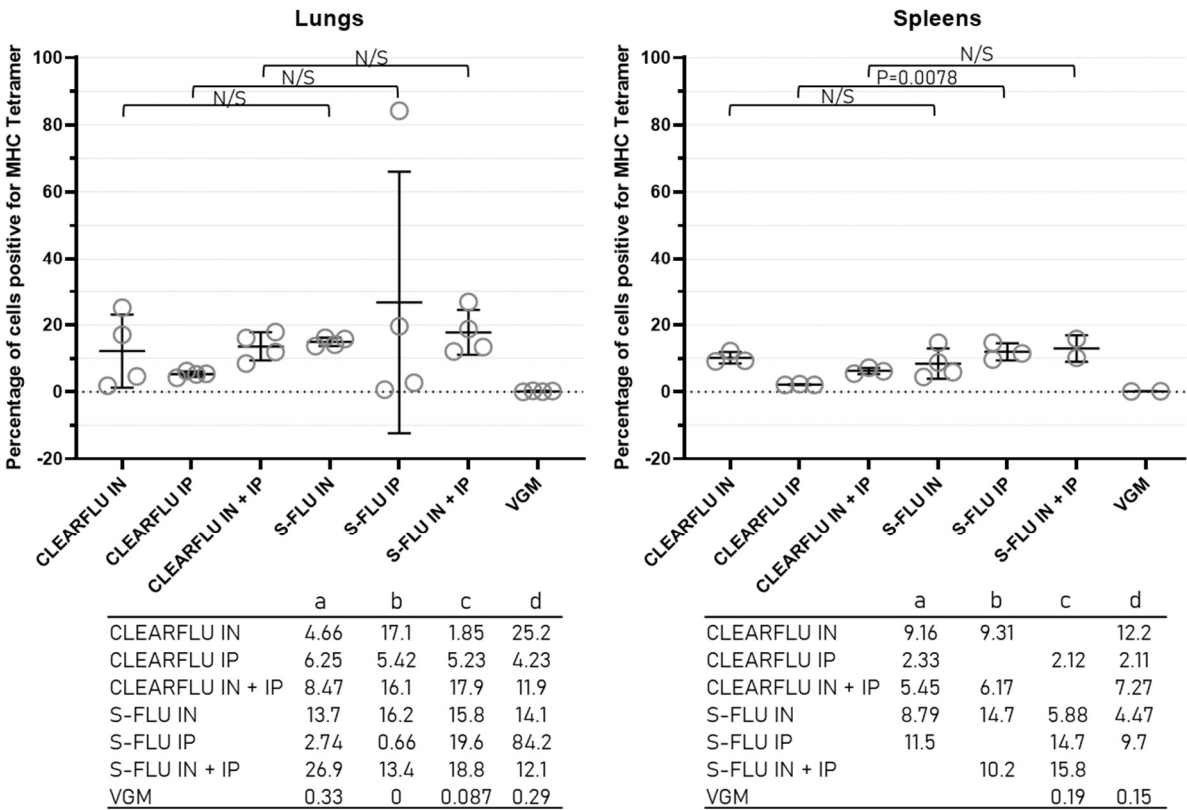
Figure 6.15



MHC tetramer binding to CD8+ T cells from the lungs and spleen of immunised mice

Cells were gated as described previously to analyse only CD8+ T cells. Fluorescence from APC-labelled MHC tetramer is plotted against forward scatter. The population of CD8+ T-cells in the top right-hand box are scored as positive for tetramer staining. Plots from up to four mice (a-d) are shown per group for each tissue.

Figure 6.16



Percentage of CD8+ T cells positive for MHC tetramer

Circles indicate the percentage of positive cells in each mouse. Bars show the mean and standard deviation for each group. For spleens, n=3 for groups 1,2,3 and 5, n=4 for group 4 and n=2 for groups 6 and 7. Multiple t tests were used to compare CLEARFLU and S-Flu groups for each administration method. The Holm-Sidak method was used to correct for multiple comparisons. All differences were non-significant at the p=0.05 level except for IP groups in the spleen where p=0.0078.

6.2.4 H1 CLEARFLU version 2 in pigs

Three key studies of S-Flu in pigs have been published. They use nebulisers (Aerogen Solo or Philips InnoSpire Deluxe) to deliver aerosolised virus evenly to the lungs and upper respiratory tract, as opposed to using a syringe with atomiser which delivers virus less uniformly (Martini et al., 2020).

Morgan et al., 2016

Morgan et al compare immunisation of Landrace pigs with H1 S-Flu intratracheally or by aerosol. Aerosol was shown to be a more efficient route to deliver vaccine to the lung, generating a strong CD4+ and CD8+ T-cell response by ELISPOT in the BAL which correlated with a greater decrease in viral titre in the lung and nose following challenge with a closely matched H1N1 virus. Immunisation by either route protected from severe lung pathology. No hemagglutinating or neutralising antibody response was detected in BAL or serum and IgA and IgG titres by ELISA in the BAL were low for both administration routes.

In the same study, Morgan et al immunised intratracheally with an H5 S-Flu and tested protection against heterologous challenge with H1N1 virus. This showed reduction in viral load in the nose after challenge, demonstrating that immunisation with S-Flu can generate broad protection.

Holzer et al., 2018

Holzer et al compared responses in landrace pigs and ferrets to an H3N2 S-Flu administered by aerosol, and investigated protection against challenge with H1N1 virus. In ferrets, S-Flu reduced viral load and transmission of challenge virus to naïve ferrets in neighbouring cages.

In pigs, S-Flu induced resident memory T-cells and reduced gross and microscopic lung pathology after heterologous challenge but did not reduce viral titres in BAL or nasal swabs. No neutralising antibody to H1N1 was detected in sera, due either to a lack of antibody response to the H3N2 S-Flu coating hemagglutinin or because the response is not cross-reactive to H1N1. In any case, protection against challenge arises in the absence of neutralising antibody to the challenge virus and is thus mediated by other mechanisms.

S-Flu stimulated weak responses by IFN γ ELISPOT in peripheral blood mononuclear cells (PBMCs) after immunisation but, after challenge, ELISPOT titres were much higher than in challenged naïve animals, indicating that immunisation primed for a strong T-cell response. A good titre was elicited by both H1N1 and H3N2 stimuli. S-Flu also generated many IFN γ and TNF α producing T-cells in the BAL, lung and tracheobronchial lymph nodes (TBLN), which could be stimulated by exposure to H3N2 or H1N1 epitopes. S-Flu therefore generated a strong and broadly-reactive local T-cell response in the lung and respiratory tract. Cytokine secretion was particularly strong in resident memory cells relative to intravascular T-cells, as determined by intravenous injection with anti-CD3 antibody prior to culling.

These results show that partial protection against heterologous challenge does not require a neutralising antibody response and is independent of reduction in challenge virus titre. Protection is instead associated with strong and broad T-cell responses in the respiratory tract. The observation that viral titres are not significantly reduced in the lungs

and nose contrasts with conclusions from Morgan et al, who did observe a decrease in viral titre in the lungs and nose. This could be due to differences in the strains used for immunisation and challenge or because Holzer et al used ten times more challenge virus.

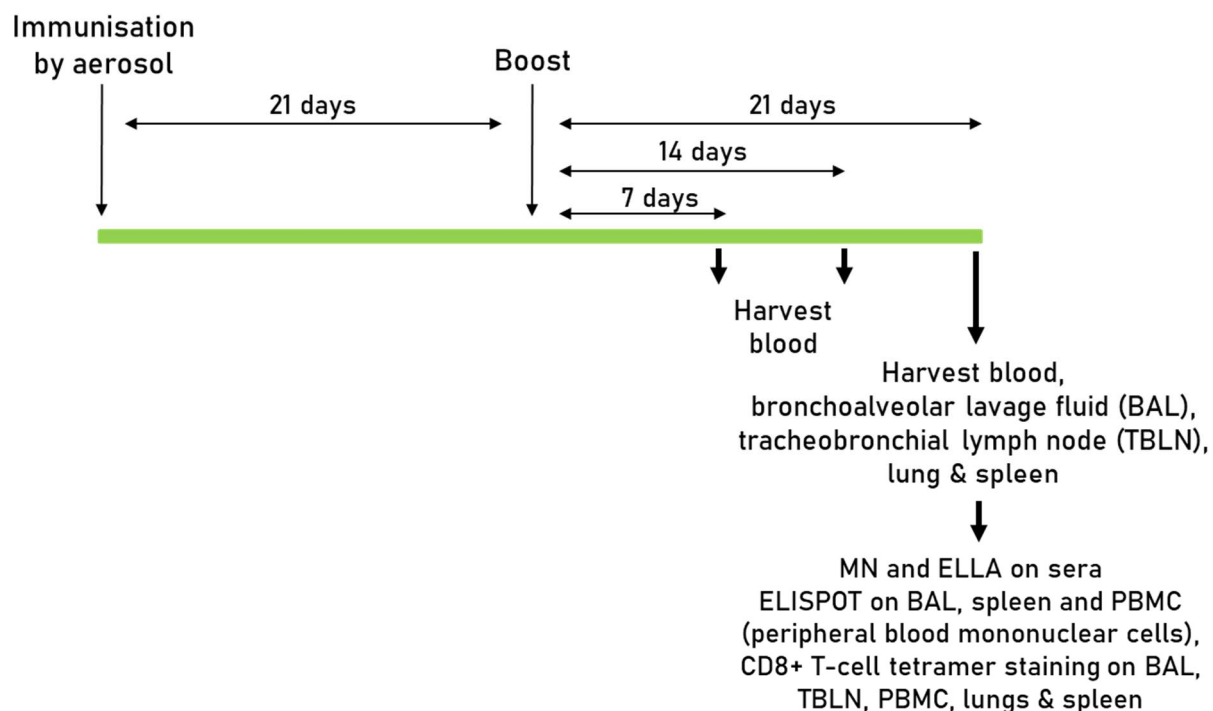
Tungatt et al., 2018

Tungatt et al developed MHC tetramers for staining T-cells from Babraham pigs and used these to study the nucleoprotein specificity of CD8+ T-cells in the BAL of pigs immunised with H1N1 S-Flu by aerosol. In immunised pigs, a very high proportion of CD8+ T-cells in the BAL responded to one of three nucleoprotein epitopes (around 22%, 28% and 40% for each of three pigs). Responses to two of these epitopes were measured following intranasal infection with an H1N1 swine virus, with similar results. These data demonstrate that immunisation with S-Flu by aerosol, similarly to infection, stimulates a strong local CD8+ T-cell response to conserved nucleoprotein epitopes in the lung.

Together these three studies show that aerosol administration of S-Flu protects pigs against homologous and heterologous challenge through local T-cells. Although S-Flu protects from lung pathology, it does not prevent infection, and may not reduce challenge virus titre in the respiratory tract. While titre is reduced in mice and ferrets locally immunised with S-Flu, the lack of a strong neutralising antibody response is common to all animal models studied. This contrasts with peripheral immunisation which does stimulate a convincing neutralising response to the coating hemagglutinin in all models, including pigs (Martini et al, unpublished manuscript). CLEARFLU viruses may make more effective aerosol-administered swine vaccines than S-Flu if they can generate a neutralising antibody response, as this may lower viral titres in the respiratory tract after challenge, reducing virus shedding and onward transmission.

Testing responses to CLEARFLU in pigs

Figure 6.17 – Experimental scheme 4



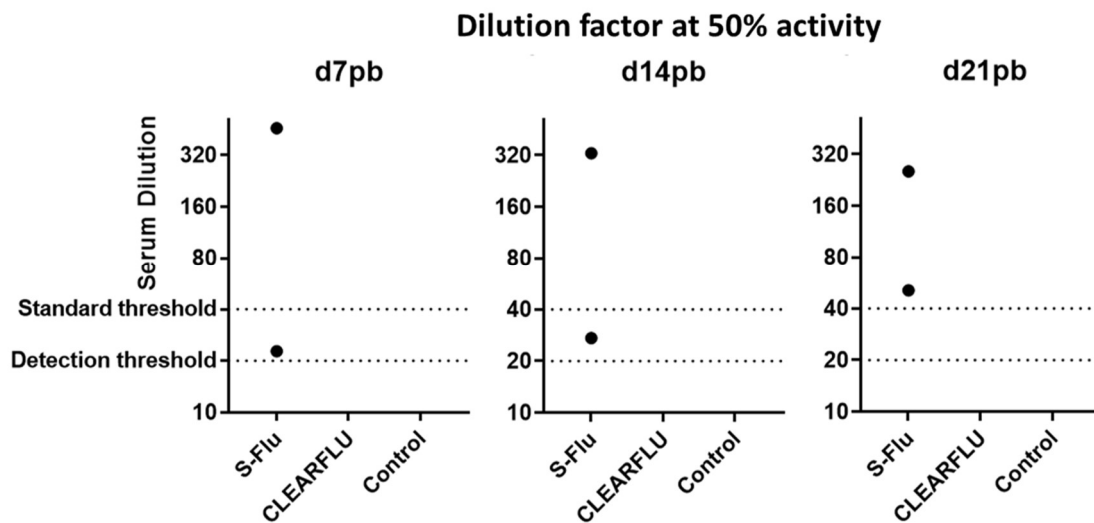
Group	n.	Virus	Full virus designation	Route	Titre
1	3	H7 S-Flu	[S-eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)	Aerosol	2.4E8 TCID50
2	3	H1 CLEARFLU with H7 coat	[H1CFv2(A/England/195/2009)/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)	Aerosol	1.3E8 TCID50
3	2		Not immunised		
		Viruses used in assays			
		MN		ELLA	
H1N1 virus		A/swine/England/1353/2009			
H7N1 S-Flu		[eGFP/N1(A/Puerto Rico/8/1934)] coated in H7(A/Netherlands/219/2003)			
H5N1 S-Flu				[eGFP/N1 (A/Puerto Rico/8/1934)] coated in H5(A/Vietnam/1203/2004)	

Dr Elma Tchilian and her student Veronica Martini conducted an experiment to compare antibody and T-cell responses to CLEARFLU version 2 virus and S-Flu in Babraham pigs. Animals were immunised by aerosol with H7 S-Flu or CLEARFLU virus expressing an H1 CLEARFLU hemagglutinin pseudotyped in an H7 coat. All other viral components were identical in the two viruses. All animal work and assays were conducted by Dr Tchilian and Ms Martini at the Pirbright Institute. I produced the CLEARFLU virus and observed immunisations.

No serum neutralising response to H1 A/swine/England/1353/2009 (a close match to A/England/195/2009 with 99.29% sequence identity, Veronica Martini by personal communication) or H7 A/Netherlands/219/2003 hemagglutinin was detected in any animal in the S-Flu or CLEARFLU immunised groups (detection threshold 1 in 40), suggesting that neither coating nor expressed CLEARFLU hemagglutinins stimulated a response. To determine whether the H1 CLEARFLU had stimulated a local neutralising response, BAL was tested for a neutralising response to H1 A/swine/England/1353/2009. No response was detected (detection threshold 1 in 2).

No neuraminidase inhibiting activity was detected by ELLA in BAL. In sera, two of three animals in the S-Flu group showed a response against matched PR8 neuraminidase, one of which was very weak and would not have been detected using our standard detection threshold for two of the three timepoints tested (see Figure 6.18). No response was detected in the CLEARFLU or naïve groups. Given the low animal numbers and the variability in response seen in the S-Flu group, it is unclear whether the response to CLEARFLU was truly weaker than that to S-Flu. Responses might have been observed with lower assay detection thresholds, altered vaccination regimens or with larger experimental groups.

Figure 6.18

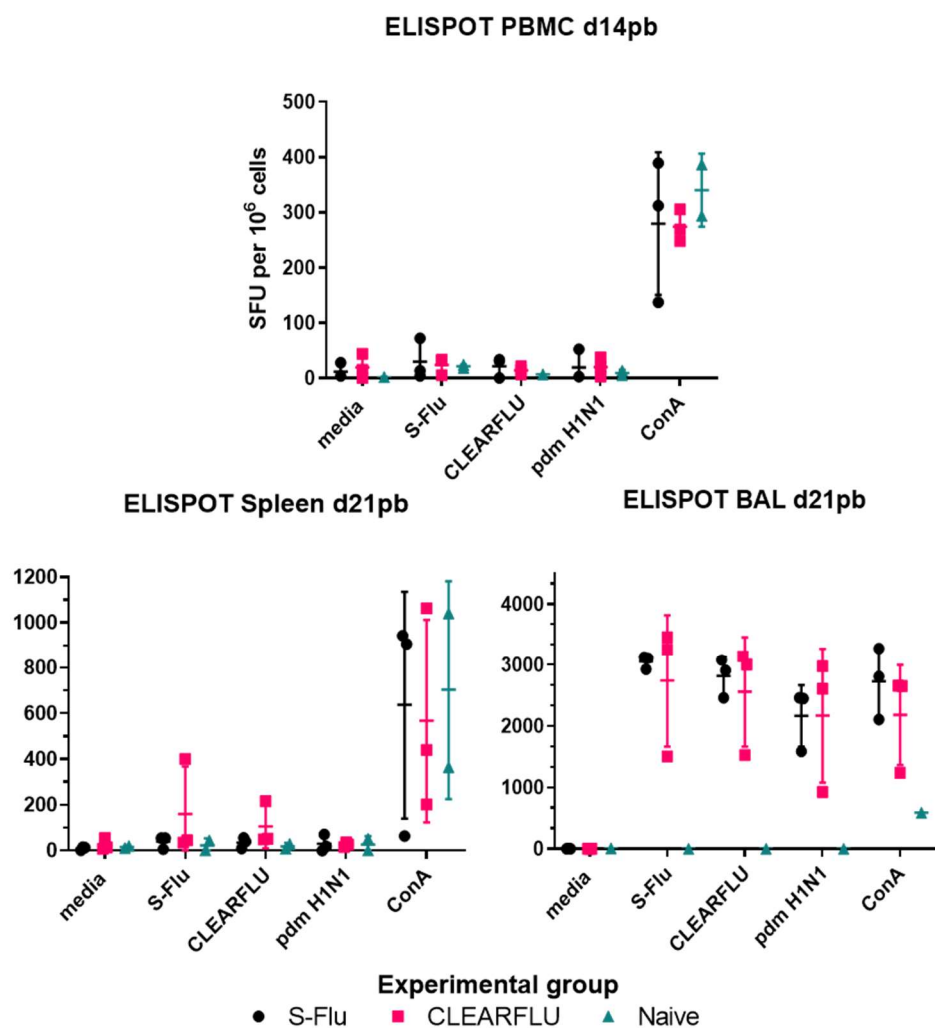


Inhibition of neuraminidase activity by sera from pigs immunised with S-Flu or CLEARFLU version 2

The neuraminidase activity of N1 (PR8) S-Flu after incubation with immune sera was measured by ELLA as described previously. The standard procedure was amended to lower the detection threshold from 1 in 40 to 1 in 20. Each point represents the response of one animal measured in a single assay. Sera from animals immunised with CLEARFLU did not yield a 50% inhibition titre.

No T-cell response was detected by IFN γ ELISPOT in peripheral blood mononuclear cells (PBMCs) or the spleen, but both S-Flu and CLEARFLU groups did generate a response in the BAL (see Figure 6.19). Stimulation of these cells with H7 S-Flu, CLEARFLU or pandemic H1N1 virus activated IFN γ producing T-cells in the BAL, showing that responses could be stimulated by a range of stimuli.

Figure 6.19

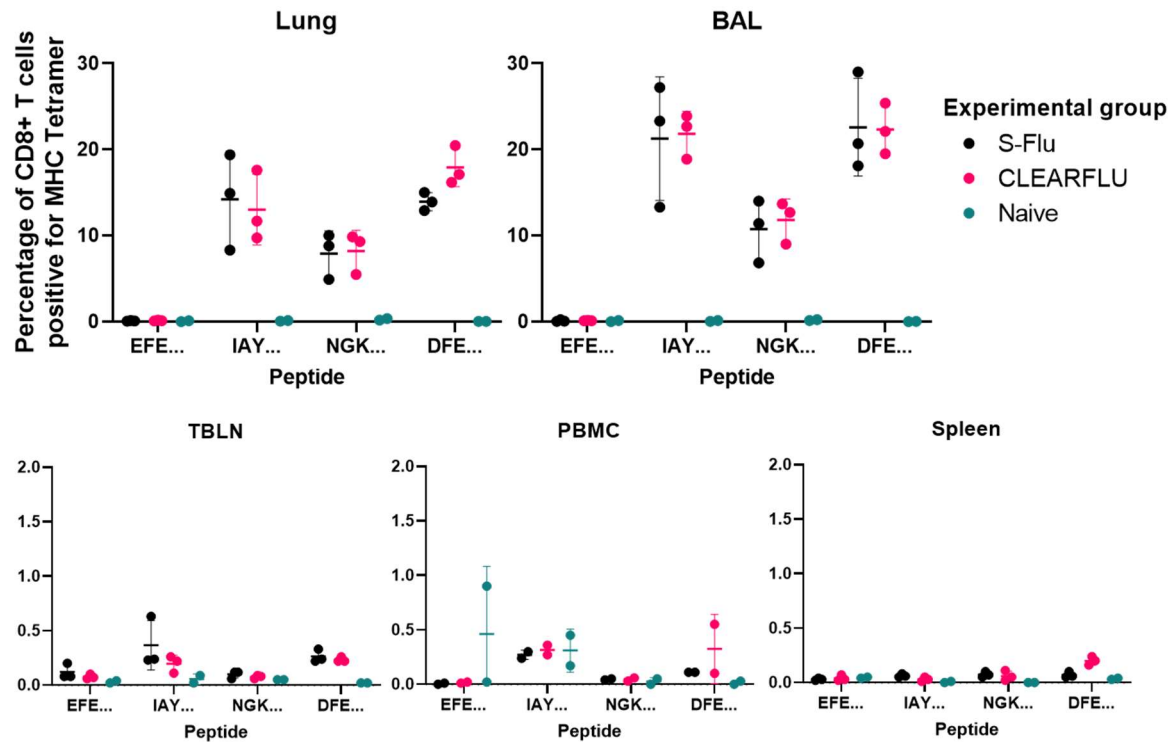


IFN γ ELISPOT on PBMCs, spleens and BAL

IFN γ ELISPOT was conducted on PBMCs harvested 14 days post-boost and on spleens and BAL 21 days post-boost. Each point represents a single pig, shown with the group mean and standard deviation. The assay was conducted as per Tungatt et al, 2018. Briefly, anti-pig IFN γ antibody was used to coat plates. BAL, PBMC and spleen cells were cultured in R5 medium with Babraham kidney epithelial cells to act as antigen presenting cells. Cells were stimulated with H7 S-Flu, H1 CLEARFLU, wild-type pandemic H1N1 virus (A/swine/England/1353/2009), media alone or concanavalin A (positive control). The S-Flu and CLEARFLU viruses were the same as for immunisation. Viruses were added at MOI=1 by TCID50 and concanavalin A at 10 μ g/ml for overnight incubation. Plates were washed and incubated with 1 μ g/ml biotin-linked mouse anti-pig IFN γ antibody for 2 hours. Plates were then incubated with streptavidin-alkaline phosphatase and developed. Spot forming units (SPU) were counted in each well and scaled to SPU per 10⁶ cells.

Finally, MHC tetramers described by Tungatt et al. were used to determine the specificity of CD8⁺ T-cells for nucleoprotein (Tungatt et al., 2018). Tetramer staining was conducted on BAL, PBMCs, tracheobronchial lymph node (TBLN), spleen and lung cells (see Figure 6.20). In the BAL, CLEARFLU and S-Flu generated very similar results. Approximately 20% of CD8⁺ T-cells were specific for each of peptide IAYERMCNI and peptide DFEREGYSL, and a further 10% were specific for peptide NKGWMRELI, indicating that at least 50% of CD8⁺ T cells in the BAL were specific for nucleoprotein. These results are consistent with those described for S-Flu by Tungatt et al., who showed that 40% of CD8⁺ T-cells in the BAL were specific for one of the three peptides and up to 25% were specific for a single peptide (observed for peptide IAYERMCNI). Both S-Flu and CLEARFLU generated strong responses in the lung, where around 40% of CD8⁺ T-cells were NP-specific. Responses in the tracheobronchial lymph node (TBLN), the spleen and peripheral blood mononuclear cells (PBMCs) were negligible.

Figure 6.20



Staining of CD8+ T-cells with MHC tetramers specific for four different nucleoprotein peptides

Forward and side scatter were used to isolate single lymphocytes, which were stained with a live/dead marker and anti-CD3 antibody to distinguish live T-cells. CD8 β + T-cells were identified and the proportion of these positive for staining with each MHC tetramer are plotted. All tissues were harvested 21 days post-boost. Each point represents one individual, with error bars indicating standard deviation within experimental groups. S-Flu and CLEARFLU groups contain three measurements for each tissue except for PBMCs where only two samples could be obtained. Each naïve group contains two measurements. Nucleoprotein target peptides: EFEDLTFLA, IAYERMCN, NGKWMREL, DFEREGYS (Tungatt et al, 2018).

In summary, these results indicate that aerosol administration of CLEARFLU generates strong T-cell responses in the lung and BAL similar to S-Flu. The lack of ELISPOT response or tetramer staining in the spleen and PBMCs suggests that the T-cell response is targeted to the site of immunisation. While an absence of neutralising antibody following S-Flu immunisation was expected from previous studies, it was disappointing to observe no improvement with CLEARFLU given the strong responses observed in mice. This experiment ought to be repeated to confirm this new observation. The lack of anti-neuraminidase antibodies to the CLEARFLU virus was also disappointing. Our results are in line with other studies indicating weak or absent serum antibody responses overall following aerosol administration of S-Flu, including to neuraminidase (Morgan et al., 2016; Veronica Martini, unpublished manuscript). However, we might have expected to see a response in the BAL, where aerosol has since been shown to stimulate stronger responses by MN and ELLA than intramuscular administration (Veronica Martini, unpublished manuscript).

Our results suggest that the T-cell response to CLEARFLU is equivalent to that generated by S-Flu, and that both S-Flu and CLEARFLU show potential as non-neutralising T-cell based vaccines in pigs. Such vaccines could be valuable for maintaining broad immunity against a wide variety of strains, preventing severe disease and supporting animal welfare.

6.3 Discussion

In this chapter, immune responses to CLEARFLU vaccine candidates in mice and in pigs were evaluated. We were particularly interested in investigating delivery of vaccines to the lung, as this is known to stimulate strong T-cell responses in pigs and humans. The main aim of this work was to determine whether adding hemagglutinin epitopes in the form of CLEARFLU to S-Flu viruses could elicit a neutralising antibody response following local administration without compromising T-cell or anti-neuraminidase antibody responses.

Inducing a neutralising response is desirable as S-Flu accords heterologous protection against severe disease but does not induce sterile immunity even against strain-matched challenge. Neutralising antibody responses may therefore enhance protection and decrease transmission by reducing viral titres and shedding. Eliciting neutralising antibody responses with a locally administered vaccine is particularly desirable as it may generate mucosal antibodies which are associated with protection (reviewed by Kim & Jang, 2017; Krammer, 2019; van Riet et al., 2012). Moreover, vaccines administered locally have not been associated with vaccine-associated enhanced respiratory disease (VAERD) in pigs, unlike some peripherally administered vaccines (Rajão et al., 2016).

6.3.1 Antibody responses

In mice immunised intranasally, strong serum antibody responses were observed to CLEARFLU and neuraminidase but not the coating hemagglutinin. These viral proteins are likely rendered more immunogenic than the coating protein by their amplification and expression on the surface of infected cells.

In pigs, anti-neuraminidase and neutralising antibody responses were weak or undetected for both S-Flu and CLEARFLU, highlighting a key difference in how the two animal models responded to immunisation. Given the strong T-cell responses elicited, this is unlikely to be due to low vaccine dosage or cell infectivity. It is perhaps more likely caused by poor antigen synthesis, weak cell surface expression or low immunogenicity in pigs. Trialling CLEARFLUs and neuraminidases from a range of strains may improve responses. For instance, trialling neuraminidases with different stalk lengths may improve antibody access and hence immunogenicity (Broecker et al., 2019). Interestingly, in a study from Powell et al., intranasal S-Flu did not generate neutralising antibody in mice but was associated with higher neutralisation titres after challenge (Powell et al., 2012). It may be that CLEARFLU viruses are similarly able to prime for enhanced neutralising responses after challenge in pigs.

Much like in previous studies, when mice were immunised intraperitoneally, strong serum neutralising antibody responses were generated to the coating hemagglutinin. Exactly why this response is stronger than following intranasal immunisation is unknown, but could be because intraperitoneal immunisation targets the spleen, a site critical for the stimulation of B-cells. This is also likely influenced by the 10 times higher dosage given intraperitoneally. Notably, intraperitoneal administration does not appear to similarly improve antibody responses to the virally expressed surface proteins neuraminidase and CLEARFLU.

In our swine study, no pigs were immunised peripherally to allow a comparison between administration routes. However, it has recently been shown that intramuscular administration of S-Flu generates strong serum antibody responses by microneutralisation, ELISA and ELLA but very weak local T-cell responses relative to aerosol administration of the same dose (Veronica Martini, unpublished manuscript). Simultaneous intramuscular and aerosol administration generated strong T-cell and antibody responses. While it is disappointing that CLEARFLU was not able to overcome the requirement for peripheral immunisation to generate strong antibody responses, simultaneous immunisation offers an alternative method for achieving the desired result.

Anti-hemagglutinin and neuraminidase responses in the BAL were barely detectable in mice. It is therefore perhaps unsurprising that they were not detected in pigs, especially as titres depend on how much fluid volume is used for lavage. In mice, antibody responses in the BAL did not appear to differ according to immunisation route, except to the coating hemagglutinin which only generated a response when administered intraperitoneally, echoing results in the sera.

6.3.2 T-cell responses

Strong T-cell responses were observed to locally administered CLEARFLU viruses in both mice and pigs. They were comparable to those stimulated by S-Flu. This suggests that CLEARFLU likely has a similar ability to generate T-cell mediated heterologous protection. It would be interesting in future studies to confirm this directly, and to determine whether CLEARFLU peptide epitopes enhance T-cell mediated protection, as hemagglutinin is known to be rich in T-cell epitopes (Babon et al., 2009).

In pigs, T-cell responses were highly localised to the site of immunisation; the lung and BAL. Responses in the spleen and PBMCs were not above those seen in naïve pigs. In mice, NP-specific CD8⁺ T-cells could be found in both the lungs and spleen, suggesting less specific targeting of the respiratory tract. Interestingly, the proportion of NP-specific cells in the lungs and spleen in mice did not appear to be associated with immunisation route, providing no evidence that intranasal administration enhanced immunisation of the lung or that intraperitoneal administration more effectively targeted the spleen. In contrast, aerosol administration of S-Flu leads to stronger ELISPOT responses in the BAL of pigs than intratracheal administration (Morgan et al., 2016), indicating that the site of immunisation does affect the localisation of T-cell responses. Similarly, intramuscular immunisation generates a negligible NP-specific T-cell response in the lung and BAL (Veronica Martini, unpublished manuscript). These disparities between mice and pigs in the site-specificity of immune responses might have arisen because the vaccine viruses reach a broader range of tissues in mice, perhaps suggesting that these local immunisation routes are non-equivalent or that virus homing was affected by the choice of pseudotyping and CLEARFLU hemagglutinin. S-Flu viruses expressing luciferase (developed by Dr Jack Tan, Townsend group) could be used to visualise the homing of bioluminescent viruses with different surface proteins *in vivo*. Alternatively, the pattern

we observed may be explained by a higher number of circulating relative to tissue-resident T-cells being stimulated in mice than in pigs. This could be investigated by staining with resident memory T-cell markers or by comparing the accessibility of T-cells *in vivo* to intravenous anti-CD3 antibody as has been done previously (Holzer et al., 2018).

6.3.3 Prospects as a swine vaccine

The aim of vaccinating sows is to prevent mortality in naïve piglets, minimise illness which leads to stunted growth and reduced fertility, and to decrease transmission to other pigs and handlers. We have shown that CLEARFLU viruses induce T-cell responses in pigs similar to those associated with protection against heterologous challenge following natural infection or immunisation with S-Flu. Aerosol immunisation with CLEARFLU would therefore be expected to reduce the severity of symptoms and perhaps reduce challenge virus titres and shedding but would not provide sterile immunity.

There are advantages to T-cell only vaccines. For instance, a vaccine that doesn't stimulate a neutralising antibody response to its coating proteins can be given many times as a T-cell boost, rendering vaccine production cost-efficient as only a single universal coating cell line needs to be licensed. Secondly, certain antibodies can be detrimental to swine health and welfare. Vaccines which induce non-neutralising antibodies to the hemagglutinin stem have been shown to sometimes enhance viral fusion and the severity of future infections in pigs (Rajão et al., 2016). Vaccine-associated enhanced respiratory disease (VAERD) is associated with poor hemagglutinin matching between inactivated vaccines and infecting strains. When achieving a good match is unlikely, for instance due to poor virus surveillance or high variation in circulating strains, it may therefore be better to avoid generating anti-hemagglutinin antibodies.

Conversely, a potential downside for a non-neutralising vaccine could be that outbreaks become more difficult to detect and trace in animals which display fewer symptoms, complicating surveillance. If the vaccine does not significantly reduce transmission, this could lead to higher numbers of infections overall. As well as posing a threat to swine welfare, the expansion of this virus reservoir increases pandemic risk to humans.

Data on protection against challenge and transmission are needed to determine whether S-Flu and CLEARFLU could be cost-effective in pigs as T-cell only vaccines. With some amendments, including optimisation of strain selection for immunogenicity and simultaneous intramuscular and aerosol administration, S-Flu and CLEARFLU vaccines could be investigated as antibody-generating vaccines for swine.

6.3.4 Prospects as a human vaccine

In humans, locally administered LAIV are associated with protective T-cell and mucosal antibody responses, particularly in children. Zens et al have shown that both the infectious nature of the vaccine and the site of administration are critical to its mode of function, with neither locally administered inactivated virus or peripherally administered LAIV generating strong lung-resident memory T-cell responses in mice (Zens et al., 2019). This reiterates the importance of achieving efficient local infection by administering LAIV directly to the lung and respiratory tract and overcoming pre-existing immunity. This contrasts with our finding that intraperitoneal immunisation with CLEARFLU and S-Flu viruses stimulates T-cells in the lung in mice. The tissue homing of LAIV and single cycle viruses administered intranasally, intraperitoneally and through other peripheral routes such as intramuscularly and their subsequent ability to generate T-cell responses in the lung requires further investigation.

CLEARFLU viruses resemble LAIV in that they can infect the respiratory tract and express the full complement of viral antigens. The ability to use a non-seasonal coating hemagglutinin conveys a clear advantage over LAIV in enabling CLEARFLU viruses to be used in populations with existing neutralising responses to seasonal hemagglutinins. This could be critical in delivering a T-cell based vaccine which is highly effective in immunocompetent adults.

A T-cell only vaccine could work well by reducing severe disease, strain on health care systems and deaths. By still allowing mild infections to occur, it could enable T-cells to be boosted regularly and safely by natural infection as well as by vaccination. As T-cell vaccines are more resilient to antigenic drift, they likely need updating less often than antibody-based vaccines. However, neutralising antibodies may be deemed necessary for protecting those with weaker immune systems such as the elderly and immunocompromised, in whom innate immunity and T-cells may not be sufficient to control the virus.

The lack of antibody response in pigs, one of the closest models for human influenza infection, does not necessarily suggest that responses would also be weak in humans. The suitability of CLEARFLU and S-FLU as human vaccines can only be determined by directly measuring responses in clinical trials. If antibodies are weaker than desired, simultaneous local and peripheral immunisation might be used to boost these, as has been done in pigs (Veronica Martini, unpublished manuscript).

6.3.5 Remaining questions and future studies

In future studies, it would be valuable to investigate the effect of different coat proteins on the efficiency of cell infection, particularly in field evaluations using natural hosts with a history of exposure and immunity. In our studies, we only compared different coating hemagglutinins in mice raised in sterile conditions, enabling us to consider their affinities for host cells independently of immune effects. While we detected no difference in the efficacy of infection using an H5 or H7 coat, the choice of coating protein could play an important role in vaccine virus homing in species like humans and pigs which are known to have different sialic acid receptors in the upper and lower respiratory tract (reviewed by Kumlin et al., 2008). Indeed, Morgan et al's observations on immunisation of the upper (intratracheal) and lower (aerosol) respiratory tract reiterate that immunising specific cells is critical to generating protective responses (Morgan et al., 2016). Furthermore, single cycle viruses with preference for α -2,3 sialic acids could be developed as vaccines able to safely immunise cells deep in the lung susceptible to avian influenzas, which often cause exceedingly high mortality. Single cycle vaccines are uniquely positioned for this as they can be safely aerosolised to reach the lung and trigger tightly regulated infection of local cells.

Optimal design of vaccine viruses requires that the activities of viral proteins be well-matched. The need for balance between neuraminidase and hemagglutinin activity for efficient infection is well documented, but may be further complicated by the presence of CLEARFLU if it contributes significantly to cell entry through its sialic acid binding site. Similarly, mutations such as T148A which enable the neuraminidase to contribute to sialic acid binding may enhance the efficiency of immunisation and allow highly antigenic hemagglutinins with low sialic acid binding activity to be formulated into effective vaccines. Different combinations of coating, neuraminidase and CLEARFLU proteins

should be trialled to find combinations which a) balance cell attachment and detachment to allow high titre vaccine production and efficient *in vivo* immunisation, b) express optimal levels of each protein on the viral and cell surface, and c) contain highly immunogenic, protective and preferably well conserved B and T-cell epitopes.

We observed that H1 CLEARFLU elicited stronger antibody responses in mice than H3 CLEARFLU, illustrating that strain choice can be an important factor in the strength of antibody responses. Trialling immunogens from different strains may therefore help to improve responses to CLEARFLU and neuraminidase in pigs. Determining whether CLEARFLU and neuraminidase are expressed at high levels on the surface of CLEARFLU infected swine cells, as they are in other cell types, might be helpful for assessing potential immunogens, as inefficient protein synthesis, folding or transport to the cell membrane could explain why antibody responses to these proteins were weak or absent in pigs. Better understanding why CLEARFLU and neuraminidase fail to be immunogenic may allow us to design more efficient swine immunogens. This might be achieved by pairing CLEARFLU mutations with immunogen design approaches such as COBRA.

Finally, future work with CLEARFLU viruses and other vaccine candidates may seek to better understand the strain-specificity of antibody responses, particularly to hemagglutinin. The antibody responses to neuraminidase and CLEARFLU hemagglutinins observed in this thesis were subtype specific. This is expected as natural infection generates antibodies with limited breadth, and responses to vaccination are usually even narrower. Some cross-neutralising activity between H7 strains was observed in mice (Figure 6.11), but these responses were weak when compared to neutralisation of the vaccine strain. This is probably because the majority of neutralising activity is attributable to antibodies binding the highly variable head region. Although multiple CLEARFLU viruses can be administered together to generate antibody responses capable of neutralising divergent viruses from distinct antigenic groups (e.g. Figure 6.6), it is unclear to what

extent this represents multi-specificity as opposed to true breadth in the antibody response. Having measured only neutralisation and not the cross-reactivity of stem antibodies acting through Fc-mediated mechanisms, we have likely underestimated the breadth of protective antibody responses to CLEARFLU. Several studies have explored the value of presenting divergent hemagglutinins simultaneously or sequentially to drive broadly protective antibody responses to conserved regions (Levine et al., 2019; Wang et al., 2010; reviewed by De Jong et al., 2020). For instance, a recent study suggests that expressing a mixture of four hemagglutinins on nanoparticles can improve the breadth of antibody responses by enhancing the immunogenicity of the stem, particularly when multiple hemagglutinins are expressed together on a single particle (Boyoglu-Barnum et al., 2021). Conversely, a similar study showed that nanoparticles expressing eight different hemagglutinins generated an antibody response no broader than the sum of their specificities (Cohen et al., 2021). While it remains unclear whether hemagglutinins can be expressed together to stimulate broader antibody responses, CLEARFLU hemagglutinins may be particularly efficient inducers of antibodies to the pre-fusion structure of hemagglutinin due to the disulphide bridges which stabilise their conformation. As such, the breadth of neutralising and non-neutralising protective antibody responses to CLEARFLU viruses deserves further investigation.

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Chapter 7

DISCUSSION

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7.1 Summary

This thesis describes the design, production and characterisation of single cycle influenza viruses which express hemagglutinin proteins rendered non-functional by the presence of multiple inactivating mutations. These hemagglutinins, which have been named CLEARFLUs, fold into tertiary structures with similar antigenicity to wildtype hemagglutinins and are expressed on the surface of infected cells and budding virions, much like is observed during natural infection.

We have shown that viruses expressing CLEARFLU hemagglutinin can be grown to high titre in cell culture, but undergo only a single round of cell infection when hemagglutinin activity is not externally provided. This property ensures the non-infectiousness of CLEARFLU viruses *in vivo*, and accords such vaccine candidates hybrid characteristics of both inactivated and live-attenuated vaccines.

In vivo, CLEARFLU viruses generate strong NP-specific T-cell responses without causing disease, whether administered peripherally or locally to the lung and respiratory tract. In mice, this is also accompanied by an antibody response to neuraminidase and a neutralising antibody response to CLEARFLU hemagglutinin which shows at least some within-subtype cross-reactivity. This antibody response to CLEARFLU hemagglutinin accords an advantage over the related vaccine candidate S-Flu which does not express a hemagglutinin protein and generates only non-neutralising responses.

As generating neutralising responses relies on continued expression of CLEARFLU epitopes, two approaches were trialled to help maintain CLEARFLU expression over the course of large-scale virus expansion. Both take advantage of the ability of the CLEARFLU hemagglutinin to facilitate cell attachment by binding sialic acid. In our hands, pseudotyping CLEARFLU viruses in the passage mutant hemagglutinin (X31-PM) designed to be competent for fusion but not cell attachment (Hooper & Bloom, 2013) did not selectively enhance the growth of viral clones expressing CLEARFLU. However, in cells expressing the Zaire Ebola glycoprotein, viruses expressing CLEARFLU had a competitive advantage which allowed them to form denser plaques and grow to higher titres.

After over 10^{18} -fold expansion in cells expressing Zaire glycoprotein, CLEARFLU viruses showed remarkable genomic stability, with only a single mutation arising outside of the CLEARFLU vRNA segment. Approximately 10% of clones still strongly expressed CLEARFLU antibody epitopes. Some viruses encoded a truncated CLEARFLU segment with only a twenty amino acid open reading frame reminiscent of defective interfering RNAs.

The gradual decrease in CLEARFLU expression over the course of expansion was associated with the increasing prevalence of a single nucleotide mutation in the 150-loop of neuraminidase. We identified that this substitution enables neuraminidase to adopt sialic acid binding activity in or near its catalytic site. This is sufficient to enhance cell entry and improve viral growth in cells expressing Ebola glycoprotein, reducing selection for CLEARFLU hemagglutinin expression.

Table 7.1 - Desirable characteristics for influenza vaccine candidates exhibited by CLEARFLU viruses

Characteristic	Implication	Evidence in CLEARFLU viruses
Long lasting protection	May need to vaccinate less frequently, especially if there is little antigenic evolution.	<u>T-cells:</u> In pigs, T-cell responses to CLEARFLU are detected in the lung and BAL and are equivalent to responses to S-Flu. S-Flu has previously been shown to generate resident memory T-cells in pigs (Holzer et al., 2018). In mice, strong T-cell responses are detected in the lung and spleen over two months after immunisation with CLEARFLU and are similar to those to S-Flu. Heterosubtypic protection accorded by S-Flu immunisation lasts at least four months in mice (Powell et al., 2012). Resident memory T-cells in the lung are known to last months in mice and years in humans (Szabo et al., 2019), perhaps suggesting that in humans equivalent responses could be protective for several years. <u>Antibody:</u> Antibody responses to hemagglutinin and neuraminidase are detected in mice over two months after immunisation with CLEARFLU.
Broad protection against many strains	Vaccines may need to be reformulated less often than current strain-specific vaccines as vaccine-induced protection against seasonal strains may last longer if immunity can protect against related variants. Seasonal vaccination may offer some protection against zoonotic viruses.	T-cells generated by CLEARFLU and S-Flu show broad reactivity by ELISPOT to viruses from a different subtype than the vaccine virus. Both CLEARFLU and S-Flu generate T-cell responses to nucleoprotein, which is highly conserved. For S-Flu, these responses are associated with protection against heterologous challenge in mice and pigs.
Reduced transmission	Lowers the prevalence of infection and prevents surges in infection which can overwhelm health and outbreak management systems.	Antibody responses to CLEARFLU are neutralising in mice, which is associated with sterile immunity to future exposure. Even in the absence of a neutralising antibody response, S-Flu reduces challenge virus titres in mice, which may help to reduce transmission (Powell et al, 2012). S-Flu reduces transmission in ferrets (Baz et al., 2015; Holzer et al., 2018).

Responses to many viral components	Some components are highly conserved. Diverse immune responses are more resilient to antigenic changes in highly variable targets and more likely to accord protection in diverse populations with different immune biases and deficiencies. Stimulating immune responses to RNA as well as peptides and protein may offer an adjuvant effect.	All viral RNAs are present and express protein, as wildtype virus does. Conformational epitopes in CLEARFLU are maintained, as well as T-cell epitopes in non-mutated regions.
Adaptable platform for different species or viral strains	Maximises usefulness of the platform and minimises long-term development costs. Can be used for strains which do not grow well in eggs.	Equivalent mutations can be introduced into any genetic background. New constructs and viruses can be made easily and quickly by subcloning into the S-Flu hemagglutinin expression cassette.
Safety: low risk of vaccine-induced disease	Vaccine can be administered to the lung to mimic infection, generating local responses including mucosal IgA and resident memory T-cells without causing illness. Local administration is dose-efficient in mice. Can be given to vulnerable populations.	Single cycle viruses initiate few cell infection events and the presence of multiple independent inactivating mutations ensures irreversible inactivation of CLEARFLU. Limited infection of few cells is unlikely to cause pathology. No adverse effects to CLEARFLU have been observed in mice or pigs.
Safety: low risk of contributing donor sequence to generate new variants	Allows for highly pathogenic and non-circulating hemagglutinins to be used as CLEARFLU antigens without risk of causing outbreaks.	Reassortment of the CLEARFLU hemagglutinin into wildtype viruses would not yield competent virus. For other segments, few cell infection events reduce the probability of reassortment. Multiple inactivation mutations are spread throughout the CLEARFLU hemagglutinin sequence so that reversion mutations or homologous recombination to generate a functional donor sequence for wildtype viruses is highly improbable. The CLEARFLU coding region is human codon optimised so has reduced homology to wildtype viral sequences.
Ease and speed of production	Accelerates mass production of vaccines, allowing faster responses to outbreaks and allowing antigen selection to be based upon most recent surveillance information.	Viruses can be expanded quickly and to high titre with relatively few resources and no reliance on biosecure eggs.

7.2 Contributions of this thesis to its field

We have described a methodology for designing inactivated, stabilised immunogens which can be safely expressed as part of single cycle viruses. In doing so, we have developed a promising influenza vaccine platform which may stimulate a combination of broad T-cell responses and neutralising antibodies in animals and humans (see Table 7.1). As this platform can be used to express antigens from human and animal viruses, it could provide a unified vaccine platform for human and animal use. CLEARFLU viruses are particularly suited to this purpose as they induce multiple immune effectors and may therefore function through different mechanisms in species with dissimilar immune systems.

This is of immense economical and societal value as the control of influenza infection in any one species relies also on control within its reservoirs. The need for a One Health approach (Mackenzie & Jeggo, 2019) to long term influenza control must be recognised, as driving down prevalence in just one species through strain-specific vaccine-derived immunity could leave populations more susceptible to highly divergent zoonotic infections in the future. Vaccine platforms like CLEARFLU which generate broad immunity have important roles to play in reducing disease while maintaining population resilience to zoonoses. By reducing the demand for antivirals and even antibiotics for secondary bacterial infections in healthcare and agriculture, they may also help to slow the emergence of pathogen resistance to current therapies.

The CLEARFLU approach could be adapted for diverse other purposes. In this thesis, we show that antigens expressed in the S-Flu hemagglutinin cassette can be displayed on the cell surface and stimulate antibody responses without altering the single cycle nature of the virus. This platform could be used to express antigens from other viruses or indeed any protein to which immune responses may be desired, such as cancer antigens. Depending on the characteristics of the antigen, it may or may not need to be inactivated

by mutation to maintain virus non-viability. Although these would function as single antigen vaccines, innate, T-cell and antibody responses would likely be generated due to the presence and amplification of RNA, peptide and protein epitopes.

Likewise, the CLEARFLU approach could be used to develop single cycle influenza viruses based on the inactivation of a protein other than hemagglutinin, so long as it can be complemented by externally provided activity through soluble protein or a pseudotyping cell line and that suitable inactivating mutations can be identified. This might be most effective for proteins which do not share structural and functional similarities with other constituents of the viral proteome, which otherwise might evolve to compensate for deficiencies. The neuraminidase is an obvious target, and blocking catalysis while including mutations like T148A to enable sialic acid binding may offer a mechanism for selecting for high expression. However, the risks of inadvertent complementation must be considered, as if co-infection were to occur alongside a virus expressing a functional sialidase, its trans activity might support replication of the vaccine virus. The strategy of inactivating essential viral proteins by multiple mutation may also be applied to other viruses which can be made in cell culture by multiplasmid transfection, including influenza B.

Of course, there are alternative ways to inactivate hemagglutinin to generate single cycle viruses. Hemagglutinins with large domain deletions like stem-only or mini hemagglutinins may be thought of as safer than CLEARFLU, as excluding entire domains more definitively removes the risk of reversion to functionality. Potential advantages of excluding the head include focussing responses purely to the immunologically subdominant but highly conserved stem, the absence of CLEARFLU mutations in the stem which could disrupt epitopes, and a shortened construct length which might reduce selection for segment truncation or improve segment incorporation. Moreover, the shorter surface protrusion of the hemagglutinin could improve the immunogenicity of

neuraminidase. However, we have shown that the risk of reversion for CLEARFLU is remote and that sialic acid binding mediated by the head region can be used to select for continued expression. Additionally, head epitopes are likely a major driver of neutralising antibody titres after immunisation and, without a head region to prevent it, stem-only constructs can sometimes spontaneously adopt the post-fusion conformation, leading to loss of pre-fusion epitopes (Chen et al., 1995). Both domain-deleted and CLEARFLU hemagglutinins therefore offer their own advantages as inactivated immunogens.

The properties of CLEARFLU hemagglutinins may make them useful reagents and immunogens in their own right. Due to the presence of additional conformation-locking disulphide bonds, CLEARFLU hemagglutinins may show enhanced pH or temperature stability. In particular, introduction of the three stem intermonomer disulphide bonds (mutation 30/47C) has previously been shown to improve thermostability of the hemagglutinin trimer and prevent its dissociation into monomers (Lee et al., 2015). By pairing stability with an ability to enhance cell entry through sialic acid binding, CLEARFLU hemagglutinins may be useful laboratory tools for identifying antibodies to the prefusion conformation, both as part of single cycle viruses for assaying neutralisation through sialic acid binding inhibition and as a purified protein for measuring antibody binding. This stability could also be advantageous for developing protein or viral-like particle conjugated protein vaccines, as stability might improve immunogenicity and reduce reliance on a temperature-controlled supply chain. A collaborative project involving our group has recently shown that the SARS-CoV-2 spike protein receptor binding domain can be conjugated onto the mi3 particle to make a vaccine candidate for COVID-19 (Tan et al., 2021). The vaccine can be stored at room temperature for two weeks with no change in antigenicity and can be lyophilised or repeatedly frozen without altering its immunogenicity. It generates strong neutralising antibody in mice and pigs and is dose-efficient relative to using the protein alone, likely because the structure of VLPs enhances

B-cell cross-linking and increases uptake into antigen presenting cells. This platform might be used to display CLEARFLU hemagglutinins, perhaps from several different strains simultaneously or alongside neuraminidase to increase the breadth of responses (Boyoglu-Barnum et al., 2021; Cohen et al., 2021) and produce a highly practical antibody-based influenza vaccine.

Finally, this study has advanced understanding of how the roles of hemagglutinin and neuraminidase in the viral lifecycle can be affected by mutations around the neuraminidase catalytic site. Characterising a mutation that is present in natural isolates, we have shown how neuraminidase takeover of sialic acid binding can enhance the efficacy of neuraminidase inhibitors and antibodies. This may have implications on the evolution of antiviral resistance and the protection accorded by vaccines which function through blocking sialic acid binding by hemagglutinin. We have also shown that the T148A mutation can improve the yield of influenza virus pseudotypes, expanding the suitability of the S-Flu pseudotyping approach to the study of viral proteins which bind MDCK-SIAT cells poorly. Viruses expressing mutant neuraminidase may make useful tools for selecting neuraminidase inhibitor escape mutants as well as for identifying antibodies and chemical inhibitors to neuraminidase. In contributing to the understanding of how reciprocal selection between neuraminidase and hemagglutinin drives virus evolution in cell culture, this work has raised important questions on how cellular conditions might affect viral genome stability during culturing for vaccine production. This is critical to manufacturing standardised, licensable vaccine products.

7.3 Further investigations leading on from this thesis

7.3.1 Characterisation of CLEARFLU hemagglutinins

Further characterisation of CLEARFLU hemagglutinins and viral particles may help to interpret some of the data presented in this thesis. Determining how much CLEARFLU is expressed on viral particles and how this affects the expression of the pseudotyping coat protein or neuraminidase could help to elucidate whether the limiting factors for growth in different conditions are virus binding, fusion, release or factors independent of cell surface proteins. This could improve understanding of the functional relationships between influenza surface proteins as well as potential trade-offs in immunogenicity between proteins which compete for space on the viral and cell surface. Equally, whether during coinfection mixed trimers (Sklyanskaya et al., 1988) containing monomers from CLEARFLU and wildtype circulating viruses arise, and what the functional and immunogenic properties of such mixed trimers might be, ought to be investigated.

Another unanswered question is how viruses coated with CLEARFLU version 3 hemagglutinin and neuraminidase but no functional pseudotyping coat interact with sialic acid expressing cells. While we know that these particles are non-infectious, they can bind sialic acid and may reach the endosome like viruses which are bound by fusion-blocking antibodies (Brandenburg et al., 2013). This might enable CLEARFLU coated particles budding from infected cells to present antigens to a second cell, influencing the immunogenicity of CLEARFLU vaccines *in vivo*. By comparing these CLEARFLU non-infectious viral-like particles to similar hemagglutinin-pseudotyped CLEARFLU single cycle viruses, studies may help to evaluate the importance of cell infection in driving different immune responses to live attenuated and single cycle viruses.

7.3.2 Characterisation of the T148A neuraminidase

Future investigations might further characterise the properties of T148A and other neuraminidase mutants. While we have identified that this mutation imparts sialic acid binding activity, we have not convincingly determined whether the mutation also affects sialidase activity. This might easily be done by producing and assaying recombinant proteins. Alternatively, fluorescent S-Flu viruses might be used to determine whether viruses with wildtype or T148A neuraminidase are able to influence the growth dynamics of the other through their sialidase activity, which unlike sialic acid binding activity, can act in trans.

Having observed that neuraminidase can affect cell entry, the result that the X31-PM mutations did not appear to abolish sialic acid binding warrants revisiting. We confirmed our observations with two independently produced viruses, but have not sequenced the neuraminidase to determine whether T148A or another neuraminidase mutation arose in both viruses independently to explain this result. However, we note that Hooper & Bloom used the neuraminidase and internal genes from A/WSN/1933, while we used those from a different H1N1 virus- A/Puerto Rico/8/1934 (PR8). Weaker sialidase activity in PR8 neuraminidase could lower the sialic acid binding activity needed for efficient viral replication, and thus permit the replication of viruses expressing X31-PM hemagglutinin as their only sialic acid binding protein. In fact, a recent preprint suggests that differences in activity in the neuraminidases themselves may not be required, as the WSN internal genes may promote higher expression of neuraminidase on the viral surface than those from PR8, and hence increase overall sialidase activity (Gao et al., 2020). Investigation of this effect may demonstrate that hemagglutinins which appear inactivated in some conditions in fact retain replicative abilities in others with different sialic acid availabilities. This is reminiscent of the effect we previously discussed for Y98F viruses which replicate well only in sialic acid rich environments (Martin et al., 1998; Meisner et al., 2008). As sialic

acid availability can be influenced both by host cell type and local sialidase activity, this reinforces the importance of considering neuraminidase properties when designing inactive hemagglutinins.

7.3.3 Evaluation of CLEARFLU viruses as animal and human vaccines

The development of CLEARFLU or S-Flu viruses as human vaccines will require investigations into how they can be produced in conditions compliant with good manufacturing practice (GMP) standards. This will mean growing viruses in approved cell lines and ensuring that all reagents used during production meet GMP requirements. This may necessitate some alterations to virus production protocols, particularly as cell line choice will have consequences on viral growth dynamics and on the selective pressures driving *in vitro* evolution and genome instability. Licensing a single universal pseudotyping cell line expressing a non-circulating hemagglutinin to avoid pre-existing immunity would be more cost-effective than producing multiple cell lines expressing different hemagglutinins. CLEARFLU viruses may therefore be more cost-effective to develop than S-Flu, as just one cell line is needed to produce viruses expressing a range of CLEARFLU hemagglutinins capable of generating neutralising antibody responses with different specificities.

Our studies in mice indicate that CLEARFLU has potential as a candidate vaccine which provides both broad-spectrum non-sterile immunity and enhanced strain-specific neutralising protection against matched seasonal viruses for at least several months. Our work in pigs indicates that the ability of CLEARFLU viruses to generate strong neutralising antibody responses may vary across species or with different immunogen strains. A critical point of evaluation is how these responses translate to protection against challenge, both in controlled trials and in field evaluations where challenge comes from

natural exposure to circulating viruses. Protection against challenge studies must be designed to ensure that there is power to detect an improvement relative to S-Flu, which is already partially protective against heterologous challenge. While we may not expect to see drastic differences in outcomes such as survival or severe disease, decreases in mild and asymptomatic infections, challenge virus titres and transmission rates might be helpful measures for evaluating the protection accorded by the induction of sterile immunity.

For human vaccines, challenge with seasonal and low pathogenicity strains can be conducted in quarantine units to investigate homologous or heterologous protection. Results from this type of study would likely be easier to interpret than from immunogenicity only studies, due to the lack of established correlates of protection and the invasiveness of procedures for analysing local, particularly T-cell, responses in humans. For pandemic vaccines, protection against homologous challenge will be difficult to evaluate directly outside of a pandemic setting. Protection against heterologous challenge can however be ascertained, and immunogenicity studies could investigate indicators of likely enhanced protection to matched challenge, particularly neutralising responses to the vaccine strain. Once an outbreak has begun, observational study methods can be used to more directly investigate protection against homologous challenge.

Key measures which will determine whether CLEARFLU is superior to S-Flu include the longevity of responses and how antigenically close to circulating strains the vaccine strains need to be to provide sterile protection. This will affect the breadth of sterile immunity as well as its longevity in populations exposed to drifting variants, and therefore determine how often populations need to be immunised to maintain it. Designing CLEARFLUs based on antigenically advanced future strains rather than current or near-

future strains might help to improve this breadth (Fonville et al., 2014), as might pairing CLEARFLU with immunogen design techniques such as COBRA.

Importantly, the nature and longevity of responses will vary by species as well as within populations as a result of differences in immune armouries. Particularly for their development as human vaccines, it will be important to understand whether CLEARFLU or S-Flu offer better or worse protection than alternative vaccines in certain subpopulations, such as those with weakened T-cell responses. Indeed, CLEARFLU and S-Flu vaccines may be most effectively used as part of a combinatorial approach, for instance as a T-cell boost every few years alongside annual strain-specific vaccines for at-risk groups.

Understanding the effect of vaccination on transmission and the overall burden of disease is critical to evaluating new vaccine candidates, especially those that in reducing the severity of disease may complicate outbreak surveillance and lead to greater incidence. This is a major concern in agricultural species, and is one of the reasons – alongside the impracticalities of vaccination and the risks accompanying increased contact between handlers and animals – why vaccination is not recommended for domesticated birds in the UK (Defra, 2019). Virus transmission by direct contact can be measured in pig studies both under controlled exposure and field conditions (e.g. Canini et al., 2020). Ferrets are also useful models as they are naturally infected by the respiratory route, demonstrate similar sialic acid distributions to both swine and humans and can be housed to test transmission through non-contact exposure between neighbouring cages. Human transmission studies have been conducted previously, but have encountered difficulties due to low rates of transmission under controlled conditions (Nguyen-Van-Tam et al., 2020). If attack rates are sufficiently high, community studies with enhanced surveillance may therefore be more useful for investigating the effect of vaccination on transmission.

It will be important to track the incidence of adverse events and the broader implications of vaccination in the short and long term. Some adverse reactions to aerosol administration might be expected (reviewed by Laube, 2005), such as lung irritation in individuals with a history of respiratory inflammation, or neurological issues such as Bell's palsy caused by exposure of the nervous system to vaccine virus through the nasal mucosa (Mutsch et al., 2004). Similarly, while vaccine-associated enhanced respiratory disease (VAERD) has not been observed in pigs in any S-Flu studies, this should be monitored during large scale trials, particularly in field trials where exposure to different natural isolates may trigger VAERD. Given that VAERD is associated with the induction of antibodies to hemagglutinin which cross-react weakly with circulating strains, this might be a higher risk for CLEARFLU viruses than for S-Flu.

Additionally, several studies have observed lower vaccine efficacy in people who have received repeated vaccinations over several years (reviewed by Belongia et al., 2017). The effect of previous vaccination on vaccine efficacy is highly variable and appears to be affected by the antigenic distance between vaccine and infecting strains. The infection block hypothesis suggests that immune responses induced by vaccines prevent circulating viruses from establishing infections, and therefore limit the opportunity for new epitopes to be presented and stimulate their own immune responses. This effect should be particularly pronounced for vaccines which generate neutralising responses. Antigen trapping, where the binding of pre-existing vaccine-induced antibodies shields certain epitopes and specifically prevents them from generating their own responses, may also be at play. Antigenic imprinting, also called original antigenic sin, describes a phenomenon in which exposure to a drifted strain favours a memory recall response to previously encountered epitopes – which may only be weakly cross-reactive – over a primary response to new epitopes (first described by Francis, 1960 and recently reviewed by Zhang et al., 2019). This can lead to hierarchical immunity in which responses are

strongest towards the strains encountered earliest in life, particularly the strain of first exposure, which then have a dominant influence on future responses. The implication of this is that vaccination can have wide ranging effects, possibly over the entire lifetime of the host, by biasing immune repertoires and determining when and which antigens from future exposures are effectively presented to the immune system. Vaccinating young people, especially as a first exposure, can be particularly influential and combining this with knowledge of immunogen design offers an opportunity to take advantage of imprinting to generate “better than nature” responses.

Which types of immune responses elicit imprinting is still not fully understood. It is therefore unclear how immunisation with CLEARFLU viruses or other vaccines might shape long-term immune biases, or how vaccines with certain characteristics might be selected to exploit these effects. As our understanding of influenza immunity and our access to diverse vaccination technologies continues to improve, it will be important to expand evaluation of influenza control strategies to consider these effects, to not only offer better solutions to annual influenza, but also develop longer term strategies for managing population susceptibility and pandemic risk.

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Hemagglutinin sequences

A/England/195/2009 wildtype hemagglutinin (human codon-optimised)

ATAAGAATGCGGCCGCCACCATGAAGGCCATCCTGGTGGTGCTGCTGTACACCTTCGCCACCGCC
AACGCCGACACCCTGTGCATCGGCTACCACGCCAACAAACAGCACCGACACCGTGGATACCGTGC
TGGAAAAGAACGTGACCGTGACCCACAGCGTGAACATCCTGGAAGATAAGCACAACGGCAAGCT
GTGCAAGCTGAGAGGCGTGGCCCCCTGCACCTGGGCAAGTGCAATATCGCCGGCTGGATTCTGG
GCAACCCCGAGTGCGAGAGCCTGAGCACCGCCAGCAGCTGGTCCTACATCGTGGAGACAAGCAG
CAGCGACAACGGCACCTGTTACCCCGGCGACTTCATCGACTACGAGGAACTGCGGGAGCAGCTG
AGCAGCGTGTCCAGCTTCGAGCGGTTTCGAGATCTTCCCCAAGACCTCCTCCTGGCCCAACCACGA
CAGCAACAAGGGCGTGACAGCCGCCTGTCCTCACGCTGGCGCCAAGAGCTTCTACAAGAACCTG
ATCTGGCTGGTGAAGAAGGGCAACAGCTACCCCAAGCTGAGCAAGAGCTACATCAACGACAAGG
GCAAGGAGGTGCTCGTCCTCTGGGGCATCCACCACCCTAGCACCAAGCGCCGACCAGCAGAGCCT
GTACCAGAACGCCGACGCCTACGTGTTCTGTTGGGCGAGCAGCCGGTACAGCAAGAAGTTCAAGCCC
GAGATCGCCATCCGGCCCCAAAGTGCGGGACCAAGAGGGGCCGGATGAACTACTACTGGACCCTGG
TGGAGCCCGGCGACAAGATCACCTTCGAGGGCCACCGGCAATCTGGTGGTGCCAGATACGCCCTTC
GCCATGGAACGGAACGCCGGCAGCGGCATCATCATCAGCGACACCCCCGTGCACGACTGCAACA
CCACCTGTCAGACCCCCAAGGGCGCCATCAACACCAGCCTGCCCTTCCAGAACATCCACCCCATC
ACCATCGGCAAGTGCCCTAAGTACGTGAAGTCCACCAAGCTGCGGCTGGCCACCGGCCTGAGAA
ACGTGCCCAGCATCCAGAGCAGAGGCCTGTTTCGGCGCCATTGCCGGCTTTATCGAGGGCGGATGG
ACCGGCATGGTGGACGGGTGGTACGGCTACCACCACCAGAATGAGCAGGGCAGCGGCTACGCCG
CCGACCTGAAGTCCACACAGAACGCCATCGACGAGATACCAACAAAGTGAAGTCCGTGATCGA
GAAGATGAACACCCAGTTACCGCCGTGGGCAAAGAGTTCAACCACCTGGAAAAGCGGATCGAG
AACCTGAACAAGAAGGTGGACGACGGCTTCCTGGACATCTGGACCTACAACGCCGAGCTGCTGGT
GCTGCTGGAAAACGAGCGGACCCTGGACTACCACGACTCCAACGTGAAGAACCTGTACGAGAAA
GTGCGGAGCCAGCTGAAGAACAAACGCCAAAGAGATCGGCAACGGCTGCTTCGAGTTCTACCACA
AGTGCGACAACACCTGTATGGAAGCGTGAAGAACGGCACCTACGACTACCCCAAGTACAGCGA
GGAAGCCAAGCTGAACCGGGAAGAGATCGACGGCGTGAAGCTGGAAAGCACCCGGATCTACCA
GATCCTGGCCATCTACAGCACCGTGGCCTCCAGCCTGGTGGTGGTGTCCCTGGGCGCCATCTC
CTTTGGATGTGCTCCAACGGCAGCCTGCAGTGCCGGATCTGCATCTGATGATATCGAATTC AAGG
CCTAGAGCTCCTGGGCCTCATGGGCCTTCCTTCACTGCC

NotI/EcoRI recognition sites

Start/stop codons

A/England/195/2009 CLEARFLU version 1 (human codon-optimised)

ATAAGAATGCGGCGCCACCATGAAGACCATCATTGCCCTGAGCTACATCCTGTGCCTGGTGTTCG
CCCAGAAGATCCCCGGCAACGATAATAGCACCGCCACACTGTGTCTGGGACACCACGCTGTGCCT
AACGGCACCATCGTGAAAACCATCTGCAACGACCGGATCGAAGTGACCAATGCCACCGAGCTGG
TGCAGAATAGCAGCATCGGCGAGATCTGCGACAGCCCTCATCAGATCCTGGACGGCGAGAACTGT
ACCCTGATTGATGCCCTGCTGGGCGACCCTCAGTGTGACGGCTTTCAGAACAAGAAAGTGGGACCT
GTTCGTCGAGCGGAGCAAGGCCTACAGCAACTGCTTCCCCTACGATGTGCCTGACTACGCCAGCC
TGAGAAGCCTGGTGGCCTCTTCTGGCACCCCTCGAGTTCAACAACGAGAGCTTCAACTGGACCGGC
GTGACCCAGAATGGCACAAGCAGCGCCTGTATCAGACGGTCCAGCAGCAGCTTCTTCAGCAGACT
GAATTGGCTGACCCACCTGAACTACACATACCCCGCTCTGAACGTGACCATGCCTAACAACGAGC
AGTTCGACAAGCTGTACATCTGGGGCGTGACCATCCTGGCACCGACAAGGATCAGATCTTCCTGT
ATGCCCAGAGCAGCGGCGAGAATCACCGTGTCCACCAAGAGAAGCCAGCAGTGCGTGATCCCTTG
CATCGGCAGCAGACCCAGGATCAGAGACATCCCCAGCCGGATCAGCATCTACTGGACAATCGTG
AAGCCCGGCGACATCCTGCTGATCAACAGCACCGGAAACCTGATCGCCCCCTCGGGGCTACTTTAA
GATCAGAAGCGGCAAGAGCAGCATCATGCGGAGCGACGCCCTATCGGCAAGTGCAAGTCTGAG
TGCATCACCCCAAACGGCAGCATCCCCAACGACAAGCCCTTCCAGAACGTGAACAGGATCACCT
ACGGCGCCTGTCTAGATACGTGAAGCACAAACACCCTGAAGCTGGCCACCGGCATGAGAAATGT
GCCCCGAGAAGCAGACACAGCAAGGCTTTGGAGCCATTGCCGGCTTCATCGAAAATGGCGCCGAA
GGCATGGTGGATGGCGCCTATGGCTTCAGACACCAGAATAGCGAAGGCAGAGGACAGGCCGCTG
ACCTGAAATCTACACAGGCCGCCATCGACTGCATCAACGGCAAGCTGAACAGACTGATCGGCAA
GACCAACGAGAAGTTCCACCAGATCGAGAAAGAGTTCAGCGAGGTCGAGGGCAGAATCCAGGAC
CTCGAGAAATACGTGGAAGATACCAAGATCGACCTGTGGTCCTACAACGCCGAAGTGTGGTGGC
CCTGGA AAAACAGCACACCATCGATCTGACCGACAGCGAGATGAACAAGCTGTTGAAAAGACC
AAGAAGCAGCTGCGCGAGAACGCCGAGGATATGGGCAACGGCTGCTTCAAGATCTACCACAAGT
GCGACAACGCCTGCATCGGCTCCATCAGAAACGGCACCTACGACCACAACGTGTACAGAGATGA
GGCCCTGAACAACAGGTTCCAGATCAAAGGCGTGGAAGTGAAGTCCGGCTACAAGGATTGGATTG
TGTGGATCAGCTTCGCCATCAGCTGCTTTCTGCTGTGTGTGGCTCTGCTGGGCTTCATCATGTGGGCC
TGCCAGAAAGGCAACATCCGGTGCAACATCTGCATCGATGATGATATCGAATTC AAGGCCTAG

NotI/EcoRI recognition sites

Start/stop codons

A/England/195/2009 CLEARFLU version 2 (human codon-optimised)

ATAAGAAT**BCGGCCGC**CACC**ATG**AAGGCTATCCTGGTGGTGCTGCTGTACACCTTCGCCACCGCC
AATGCCGATACACTGTGTATTGGCTACCACGCCAACACAGCACCGACACCGTGGATACCGTGTG
CGAGAAGAACGTGACCGTGACACACAGCGTGAACATCCTGGAAGATAAGCACAAACGGCAAGCTG
TGCAAGCTGAGAGGCGTTGCACCTCTGCACCTGGGCAAGTGAATATCGCCGGCTGGATCCTGGG
CAACCCTGAGTGTGAAAGCCTGAGCACAGCCAGCAGCTGGTCCTACATCGTGGAAACCAGCAGC
AGCGACAACGGCACCTGTTTTCCCGGCGACTTCATCGACTACGAGGAACTGAGAGAGCAGCTGAG
CAGCGTCAGCAGCTTCGAGAGATTGAGATCTTCCCCAAGACCTCCAGCTGGCCCAACCACGATT
CTAACAAGGGCGTGACAGCCGCCTGTCTCATGCCGGCGCTAAGAGCTTCTACAAGAACCTGATC
TGGCTGGTCAAGAAGGGCAACAGCTACCCCAAGCTGAGCAAGAGCTACATCAACGACAAGGGCA
AAGAGGTGCTGGTCTCTGGGGCATCCACCATCCTTCTACATCTGCCGACCAGCAGAGCCTGTACC
AGAATGCCGATGCCTACGTGTTCTGGTGGCAGCAGCAGATACAGCAAGTGCTTCAAGCCCTGTATC
GCCATCAGACCCAAAGTGCGGGACCAAGAGGGCAGAATGAACTACTACTGGACCCTGGTGGAAAC
CCGGCGACAAGATCACATTTGAGGCCACCGGCAACCTGGTGGTCCCTAGATATGCCTTCGCCATG
GAAAGAAACGCCGGCAGCGGCATCATCATCAGCGATACACCCGTGCACGACTGCAACACCACCT
GTCAGACACCCAAAGGGCGCCATCAATACAGCCTGCCTTTCCAGAACATTCACCCCATCACCATC
GGCAAGTGCCCCAAATACGTGAAGTCCACAAAGCTGAGACTGGCCACAGGCCTGAGAAACGTGC
CATCCATTAGAGCCAGCAAGGCTTTGGAGCCGGCGCTGCTTTTATCGAAGGCGGATGGACAGGC
ATGGTGGACGGATGGTACGGCTACCACCATCAGAATGAGCAAGGCAGCGGATACGCCGCCGATC
TGAAGTCTACACAGAACGCCATCGATTGCATCACCAACAAAGTGAACAGCGTGATCGAGAAGATG
AACACCCAGTTCACCGCCGTGGGAAAAGAGTTCAACCACCTGGAAAAGCGCATCGAGAACCTGA
ACAAGAAGGTGGACGACGGCTTCCTGGACATCTGGACCTATAATGCCGAGCTGCTGGTGCTCCTG
GAAAACGAGAGAACCCTGGACTACCACGACAGCAACGTGAAGAACCTGTACGAGAAAGTGCGG
AGCCAGCTGAAGAACACGCCAAAGAGATCGGCAACGGCTGCTTCGAGTTCTACCACAAGTGCG
ACAATACCTGCATGGAAAGCGTGAAGAATGGCACCTACGACTACCTAAGTACAGCGAGGAAGC
CAAGCTGAACCGGGAAGAGATTGACGGCGTGAAGCTGGAAAGCACCCGGATCTATCAGATCCTG
GCCATCTACAGCACAGTGGCCTCTAGCCTGGTGGTGGTGTCTCTGGGAGCCATCAGCTTTTGG
ATGTGCAGCAATGGCAGCCTGCAGTGCCGGATCTGCATC**TGAT**GATATC**GAATTC**AAGGCCTAG

NotI/EcoRI recognition sites

Start/stop codons

A/Hong Kong/5738/2014 wildtype hemagglutinin (human codon-optimised)

ATAAGAAT**BCGGCCGC**CACC**ATG**AAGACCATCATTGCCCTGAGCTACATCCTGTGCCTGGTGTTCG
CCCAGAAGATCCCCGGCAACGACAACAGCACCGCCACCCTGTGTCTGGGCCACCACGCCGTGCC
CAACGGCACCATCGTGAAAACCATCACCAACGACCGGATCGAAGTGACCAACGCCACCGAGCTG
GTGCAGAACAGCAGCATCGGCCGAGATCTGCGACAGCCCCACCAGATCCTGGACGGCGAGAACT
GCACCCTGATCGACGCCCTGCTGGGCGACCCCTCAGTGCGACGGCTTCCAGAACAAGAAATGGGA
CCTGTTCTGTGGAAAGAAGCAAGGCCCTACAGCAACTGCTACCCCTACGACGTGCCCGACTACGCCA
GCCTGAGAAGCCTGGTGGCCAGCAGCGGCACCCTGGAGTTCAACAACGAGAGCTTCAACTGGAC
CGGCGTGACCCAGAACGGCACCAGCAGCGCCTGCATCAGGCGGAGCAGCAGCAGCTTCTTCAGC
AGACTGAACTGGCTGACCCACCTGAACTACACCTACCCCGCCCTGAACGTGACCATGCCCAACAA
CGAGCAGTTCGACAAGCTGTACATCTGGGGCGTGACCAACCCCGGCACCGATAAGGACCAGATCT
TCCTGTACGCCCAGAGCAGCGGCCGGATCACCGTGTCCACCAAGAGAAGCCAGCAGGCCGTGAT
CCCCAACATCGGCAGCCGGGCCAGAATCCGGGACATCCCAGCCGGATCAGCATCTACTGGACA
ATCGTGAAAGCCCGGCGACATCCTGCTGATCAACTCCACCGGCAACCTGATCGCCCCCAGAGGCTA
CTTCAAGATCAGAAGCGGCAAGAGCAGCATCATGCGGAGCGACGCCCCCATCGGCAAGTGCAAG
AGCGAGTGCATACCCCCAATGGCAGCATCCCCAACGACAAGCCCTTCCAGAACGTGAACCGGA
TCACCTACGGCGCCTGCCCCAGATACGTGAAGCACAACACCCTGAAGCTGGCCACCGGCATGCG
GAACGTGCCCGAGAAGCAGACCCGGGGCATCTTCGGCGCCATTGCCGGCTTCATCGAGAACGGCT
GGGAGGGCATGGTGGACGGGTGGTACGGCTTCGGGCACCAGAACAGCGAGGGCAGAGGACAGG
CCGCCGACCTGAAGTCTACCCAGGCCGCCATCGACCAGATCAACGGCAAGCTGAACCGGCTGAT
CGGCAAGACCAACGAGAAGTTCCACCAGATCGAGAAAGAATTTCCGAGGTGGAAGGCCGCATC
CAGGACCTGGAAAAGTACGTGGAAGATACCAAGATCGACCTGTGGTCCTACAACGCCGAGCTGCT
GGTGGCCCTGGAAAACCAGCACACCATCGACCTGACCGACAGCGAGATGAACAAGCTGTTCGAA
AAGACCAAGAAGCAGCTGCGCGAGAACGCCGAGGACATGGGCAACGGCTGCTTTAAGATCTACC
ACAAGTGCGACAACGCCTGCATCGGCAGCATCCGGAACGGCACCTACGACCACAACGTGTACCG
GGACGAGGCCCTGAACAACAGATTCCAGATCAAGGGCGTGGAAGTGAAGTCCGGCTACAAGGAC
TGGATTCTGTGGATCAGCTTCGCCATCAGCTGCTTTCTGCTGTGTGTGGCTCTGCTGGGCTTCATCAT
GTGGGCCTGCCAGAAAGGCAACATCCGGTGCAACATCTGCATC**TGAT**GATATC**GAATTC**AAGGCC
TAGCTGGGCCTCATGGGCCTTCCTTCACTGCCCGCTTCCAG

NotI/EcoRI recognition sites

Start/stop codons

A/Hong Kong/5738/2014 CLEARFLU version 1 (human codon-optimised)

ATAAGAAT**BCGGCCGC**CACC**ATG**AAGACCATCATTGCCCTGAGCTACATCCTGTGCCTGGTGTTCG
CCCAGAAGATCCCCGGCAACGATAATAGCACCGCCACACTGTGTCTGGGACACCACGCTGTGCCT
AACGGCACCATCGTGAAAACCATCTGCAACGACCGGATCGAAGTGACCAATGCCACCGAGCTGG
TGCAGAATAGCAGCATCGGCGAGATCTGCGACAGCCCTCATCAGATCCTGGACGGCGAGAACTGT
ACCCTGATTGATGCCCTGCTGGGCGACCCTCAGTGTGACGGCTTTCAGAACAAGAAGTGGGACCT
GTTCGTCGAGCGGAGCAAGGCCTACAGCAACTGCTTCCCCTACGATGTGCCTGACTACGCCAGCC
TGAGAAGCCTGGTGGCCTCTTCTGGCACCCCTCGAGTTCAACAACGAGAGCTTCAACTGGACCGGC
GTGACCCAGAATGGCACAAGCAGCGCCTGTATCAGACGGTCCAGCAGCAGCTTCTTCAGCAGACT
GAATTGGCTGACCCACCTGAACTACACATACCCCGCTCTGAACGTGACCATGCCTAACAACGAGC
AGTTCGACAAGCTGTACATCTGGGGCGTGACCATCCTGGCACCGACAAGGATCAGATCTTCCTGT
ATGCCCAGAGCAGCGGCGAGAATCACCGTGTCCACCAAGAGAAGCCAGCAGTGCGTGATCCCTTG
CATCGGCAGCAGACCCAGGATCAGAGACATCCCCAGCCGGATCAGCATCTACTGGACAATCGTG
AAGCCCGGCGACATCCTGCTGATCAACAGCACCGGAAACCTGATCGCCCCCTCGGGGCTACTTTAA
GATCAGAAGCGGCAAGAGCAGCATCATGCGGAGCGACGCCCTATCGGCAAGTGCAAGTCTGAG
TGCATCACCCCAAACGGCAGCATCCCCAACGACAAGCCCTTCCAGAACGTGAACAGGATCACCT
ACGGCGCCTGTCTAGATACGTGAAGCACAAACACCCTGAAGCTGGCCACCGGCATGAGAAATGT
GCCCCGAGAAGCAGACACAGCAAGGCTTTGGAGCCATTGCCGGCTTCATCGAAAATGGCGCCGAA
GGCATGGTGGATGGCGCCTATGGCTTCAGACACCAGAATAGCGAAGGCAGAGGACAGGCCGCTG
ACCTGAAATCTACACAGGCCGCCATCGACTGCATCAACGGCAAGCTGAACAGACTGATCGGCAA
GACCAACGAGAAGTTCCACCAGATCGAGAAAGAGTTCAGCGAGGTCGAGGGCAGAATCCAGGAC
CTCGAGAAATACGTGGAAGATACCAAGATCGACCTGTGGTCCTACAACGCCGAAGTGTGGTGGC
CCTGGA AAAACAGCACACCATCGATCTGACCGACAGCGAGATGAACAAGCTGTTGAAAAGACC
AAGAAGCAGCTGCGCGAGAACGCCGAGGATATGGGCAACGGCTGCTTCAAGATCTACCACAAGT
GCGACAACGCCTGCATCGGCTCCATCAGAAACGGCACCTACGACCACAACGTGTACAGAGATGA
GGCCCTGAACAACAGGTTCCAGATCAAAGGCGTGGAAGTGAAGTCCGGCTACAAGGATTGGATT
TGTGGATCAGCTTCGCCATCAGCTGCTTTCTGCTGTGTGTGGCTCTGCTGGGCTTCATCATGTGGGCC
TGCCAGAAAGGCAACATCCGGTGCAACATCTGCAT**TGAT**GATATC**GAATTC**AAGGCCTAG

NotI/EcoRI recognition sites

Start/stop codons

A/Hong Kong/5738/2014 CLEARFLU version 2 (human codon-optimised)

ATAAGAAT**BCGGCCGC**CACC**ATG**AAGACCATCATTGCCCTGAGCTACATCCTGTGCCTGGTGTTCG
CCCAGAAGATCCCCGGCAACGATAATAGCACCGCCACACTGTGTCTGGGACACCACGCTGTGCCT
AACGGCACCATCGTGAAAACCATCTGCAACGACCGGATCGAAGTGACCAATGCCACCGAGCTGG
TGCAGAATAGCAGCATCGGCGAGATCTGCGACAGCCCTCATCAGATCCTGGACGGCGAGAAGTGT
ACCCTGATTGATGCCCTGCTGGGCGACCCTCAGTGTGACGGCTTTCAGAACAAGAAAGTGGGACCT
GTTCGTCGAGCGGAGCAAGGCCTACAGCAACTGCTTCCCCTACGATGTGCCTGACTACGCCAGCC
TGAGAAGCCTGGTGGCCTCTTCTGGCACCCCTCGAGTTCAACAACGAGAGCTTCAATTGGACCGGC
GTGACCCAGAATGGCACCTCTAGCGCCTGTATCAGACGGTCCAGCAGCAGCTTCTTCAGCAGACT
GAATTGGCTGACCCACCTGAACTACACATACCCCGCTCTGAACGTGACCATGCCTAACAACGAGC
AGTTCGACAAGCTGTACATCTGGGGCGTGACCATCCTGGCACCGACAAGGATCAGATCTTCCTGT
ATGCCCAGAGCAGCGGCGAGAATCACCGTGTCCACCAAGAGAAGCCAGCAGTGCCTGATCCCTTG
CATCGGCAGCAGACCCAGGATCAGAGACATCCCCAGCCGGATCAGCATCTACTGGACAATCGTG
AAGCCCGGCGACATCCTGCTGATCAACAGCACCGGAAACCTGATCGCCCCCTCGGGGCTACTTTAA
GATCAGAAGCGGCAAGAGCAGCATCATGCGGAGCGACGCCCTATCGGCAAGTGCAAGTCTGAG
TGCATCACCCCAAACGGCAGCATCCCCAACGACAAGCCCTTCCAGAACGTGAACAGGATCACCT
ACGGCGCCTGTCTAGATACGTGAAGCACAAACACCCTGAAGCTGGCCACCGGCATGAGAAATGT
GCCCCGAGAAGCAGACACAGCAAGGCTTTGGAGCCGGCGCTGCCTTTATCGAGAATGGCTGGGAA
GGCATGGTGGACGGATGGTACGGCTTCAGACACCAGAATAGCGAAGGCAGAGGACAGGCCGCTG
ACCTGAAATCTACACAGGCCGCCATCGACTGCATCAACGGCAAGCTGAACAGACTGATCGGCAA
GACCAACGAGAAGTTCCACCAGATCGAGAAAGAGTTCAGCGAGGTCGAGGGCAGAATCCAGGAC
CTCGAGAAATACGTGGAAGATACCAAGATCGACCTGTGGTCCTACAACGCCGAAGTGTGGTGGC
CCTGGA AAAACAGCACACCATCGATCTGACCGACAGCGAGATGAACAAGCTGTTGAAAAGACC
AAGAAGCAGCTGCGCGAGAACGCCGAGGATATGGGCAATGGCTGCTTCAAGATCTACCACAAGT
GCGACAACGCCTGCATCGGCTCCATCAGAAACGGCACCTACGACCACAACGTGTACAGAGATGA
GGCCCTGAACAACCGTTCCAGATCAAAGGCGTGGAAGTGAAGTCCGGCTACAAGGACTGGATC
CTGTGGATCAGCTTCGCCATCAGCTGCTTTCTGCTGTGTGTGGCTCTGCTGGGCTTCATCATGTGGGC
CTGCCAGAAAGGCAACATCCGGTGCAACATCTGCATCTGATGATATC**GAATTC**AAGGCCTAG

NotI/EcoRI recognition sites

Start/stop codons

A/Guangdong/17SF003/2016 CLEARFLU version 2 (human codon-optimised)

ATAAGAAT**BCGGCCGC**CACC**ATG**AACACCCAGATCCTGGTGTGGCCCTGATCGCCATCATTCCCA
CCAACGCCGATAAGATCTGTCTGGGCCACCACGCCGTGTCCAACGGCACAAAAGTGAACACACT
GTGCGAGCGCGGCGTGGAAGTGGTCAATGCCACAGAGACAGTGGAACGGACCAACACACCCCG
GATCTGCAGCAAGGGCAAGAGAACAGTGGATCTGGGCCAGTGTGGCCTGCTGGGAACAATTACA
GGCCCTCCTCAGTGCAGACCAGTTCTGGAATTTCCGCCGACCTGATCATCGAGCGGAGAGAAGG
CAGCGACGTGTGCTTCCCTGGCAAGTTCGTGAACGAGGAAGCCCTGAGACAGATCCTGAGAGAGA
GCGGCGGCATCGACAAAGAACCCATGGGCTTCACCTACAACGGCATCAGGACCAATGGCGTGAC
CAGCGCCTGTAGAAGAAGCGGCAGCAGCTTCTACGCCGAGATGAAGTGGCTGCTGAGCAACACC
GACAACGCCGCTTTCCACAGATGACCAAGAGCTACAAGAACACCAAAGAGTCCCCAGCCATCA
TCGTGTGGGGCATCCACCATTCTGTGTCTACCGCCGAGCAGACCAAGCTGTACGGCTCTGGCAACA
AGCTGGTCAACGTGGGCGAGCAGCAATTACCAGCAGTGCTTTGTGCCCTGTCTGGCGCTAGACCTC
AAGTGAATGGCCAGAGCGGCAGAAATCGACTTCCACTGGCTGATTCTGAACCCCAACGACACCGTG
ACCTTCAGCTTCAACGGCGCCTTTATCGCCCCTGACAGAGCCAGCTTTCTGAGAGGGCAAGAGCATG
GGCATCCAGAGCAGAGTGCAGGTCGACGCCAATTGCGAGGGCGATTGCTATCACTCTGGCGGCAC
CATCATCAGCAACCTGCCTTTCCAGAACATCGACAGCAGAGCCGTGGGCAAGTGCCCCAGATACG
TGAAACAAAGAAGCCTGCTGCTGGCCACCGGCATGAAGAATGTGCCTGAGATCCCTAAGGGCCA
GCAAGGATTGGAGCCGGCGCAGCCTTTATTGAGAATGGCTGGGAGGGACTGATCGACGGATGGT
ACGGCTTCAGACACCAGAATGCCCAAGGCGAGGGAACAGCCGCCGATTACAAAAGCACACAGA
GCGCCATCGACTGCATCACCGGCAAGCTGAACAGACTGATTGCCAAGACCAACCAGCAGTTCAA
GCTGATCGACAACGAGTTCAACGAGGTGGAAAAGCAGATCGGCAACGTGATCAACTGGACCCGG
GACAGCATCACCGAAGTGTGGTCTTACAATGCCGAGCTGCTGGTGGCCATGGAAAACCAGCACAC
CATCGATCTGGCCGACAGCGAGATGGACAAGCTGTATGAGAGAGTGAAGAGACAGCTGCGCGAG
AACGCCGAGGAAGATGGCACAGGCTGCTTCGAGATCTTCCACAAGTGCGACGACGACTGCATGG
CCAGCATCCGGAACAACACCTACGACCACCGGAAGTACCGGGAAGAAGCCATGCAGAACAGAA
TCCAGATCGACCCCGTGAAGCTGAGCAGCGGCTACAAGGATGTGATCCTGTGGTTCAGCTTCGGC
GCCAGCTGTTTCATCCTGCTGGCTATCGTGATGGGCCTCGTGTTTCATCTGCGTGAAGAACGGCAAC
ATGCGGTGCACCATCTGCATC**TGAT**GATATC**GAATTC**AAGGCCTAG

NotI/EcoRI recognition sites

Start/stop codons

A/Hong Kong/125/2017 wildtype hemagglutinin (human codon-optimised)

ATAAGAAT**CGGGCC**GCCACC**ATG**AACACCCAGATCCTGGTGTGGCCCTGATCGCCATCATTCCCA
CCAACGCCGATAAGATCTGTCTGGGCCACCAACGCCGTGTCCAACGGCACAAAAGTGAACACACT
GACCGAGCGCGGCGTGGAAAGTGGTCAATGCCACAGAGACAGTGGAACGGACAAACATCCCCAG
AATCTGCAGCAAGGGCAAGAGAACCGTGGATCTGGGACAGTGTGGCCTGCTGGGAACAATTACA
GGCCCTCCTCAGTGCAGACAGTTCTGGAATTTCCGCCGACCTGATCATCGAGCGGAGAGAAGG
CTCCGATGTGTGCTACCCCGGCAAGTTCGTGAACGAGGAAGCCCTGAGACAGATCCTGAGAGAGA
GCGGCGGCATCGACAAAGAAACCATGGGCTTCACCTACAACGGCATCAGGACCAATGGCGTGAC
CAGCGCCTGTAAAAGAAGCGGCAGCAGCTTCTACGCCGAGATGAAGTGGCTGCTGAGCAACACC
GACAACGCCGCGCTTTCCACAGATGACCAAGAGCTACAAGAACACCCGCAAGAGCCCCGCCATCA
TCGTGTGGGGAATCCACCATTCTGTGTCCACCGCCGAGCAGACAAAGCTGTACGGCTCTGGCAAC
AAGCTGGTCACCGTGGGCAGCAGCAATTACCAGCAGAGCTTTGTGCCAAGTCCAGGCGCCAGACC
TCAAGTGAATGGCCTGAGCGGCAGAATCGACTTCCACTGGCTGATTCTGAACCCCAACGACACCG
TGACCTTCAGCTTCAACGGCGCCTTTATCGCCCCTGACAGAGCCAGCTTTCTGAGAGGGCAAGAGCA
TGGGCATCCAGTCTGGCGTGCAAGTGGATGCCAATTGCGAGGGCGATTGCTACCACTCTGGCGGC
ACCATCATCAGCAACCTGCCTTTCCAGAACATCGACAGCAGAGCCGTGGGCAAGTGCCCCAGATA
CGTGAAACAAAGAAGCCTGCTGCTGGCCACCGGCATGAAGAATGTGCCTGAGATCCCTAAAGGC
AGAGGCCCTGTTTGGCGCCATTGCCGGCTTTATCGAGAATGGCTGGGAGGGACTGATCGACGGATG
GTACGGCTTCAGACACCAGAATGCCCAAGGCGAGGGAACAGCCGCCGATTACAAAAGCACACA
GAGCGCCATCGACCAGATCACCGGCAAGCTGAACAGACTGATTGCCAAGACCAACCAGCAGTTC
GAGCTGATCGACAACGAGTTCAACGAGGTGGAAAAGCAGATCGGCAACGTGATCAACTGGACCC
GGGACAGCATCACCGAAGTGTGGTCTTACAACGCCGAGCTGCTGATCGCTATGGAAAACCAGCAC
ACCATCGACCTGGCCGACAGCGAGATGGACAAGCTGTATGAGAGAGTGAAGAGACAGCTGCGCG
AGAACGCCGAGGAAGATGGCACAGGCTGCTTCGAGATCTCCACAAGTGCAGACGACGACTGCAT
GGCCAGCATCCGGAACAACACCTACGACCACCGGAAGTACCGGGAAGAAGCCATGCAGAACAG
AATCCAGATCGACCCCGTGAAGCTGAGCAGCGGCTACAAGGATGTGATCCTGTGGTTTAGCTTCGG
CGCCAGCTGCTTCATCCTGCTGGCTATTGTGATGGGCCTCGTGTTTCATCTGCGTGAAGAACGGCAAC
ATGCGGTGCACCATCTGCATC**TGAT**GATATC**GAATTC**AAGGCCTAGGAGCTCGGAGCACAAAGACT
GGCCTCATGGGCCTTCCTTTCACTGCC

[NotI/EcoRI recognition sites](#)

[Start/stop codons](#)

A/Hong Kong/125/2017 CLEARFLU version 2 (human codon-optimised)

ATAAGAAT**BCGGCCGC**CACC**ATG**AACACCCAGATCCTGGTGTGGCCCTGATCGCCATCATTCCCA
CCAACGCCGATAAGATCTGTCTGGGCCACCACGCCGTGTCCAACGGCACAAAAGTGAACACACT
GTGCGAGCGCGGCGTGGAAGTGGTCAATGCCACAGAGACAGTGGAACGGACAAACATCCCCAGA
ATCTGCAGCAAGGGCAAGAGAACCGTGGATCTGGGACAGTGTGGCCTGCTGGGAACAATTACAG
GCCCTCTCAGTGCGACCAAGTTCCTGGAATTTTCCGCCGACCTGATCATCGAGCGGAGAGAAGGC
AGCGACGTGTGCTTCCCTGGCAAGTTCGTGAACGAGGAAGCCCTGAGACAGATCCTGAGAGAGAG
CGGCGGCATCGACAAAGAAACCATGGGCTTACCTACAACGGCATCAGGACCAATGGCGTGACC
AGCGCCTGTAAAAGAAGCGGCAGCAGCTTCTACGCCGAGATGAAGTGGCTGCTGAGCAACACCG
ACAACGCCGCCTTTCCACAGATGACCAAGAGCTACAAGAACACCCGCAAGAGCCCCGCCATCAT
CGTGTGGGGAATCCACCAATTCTGTGTCCACCGCCGAGCAGACAAAGCTGTACGGCTCTGGCAACA
AGCTGGTCAACGTGGGCGAGCAGCAATTACCAGCAGTGCTTTGTGCCCTGTCTGGCGCTAGACCTC
AAGTGAATGGCCTGAGCGGCAGAATCGACTTCCACTGGCTGATTCTGAACCCCAACGACACCGTG
ACCTTCAGCTTCAACGGCGCCTTTATCGCCCCTGACAGAGCCAGCTTTCTGAGAGGGCAAGAGCATG
GGCATCCAGTCTGGCGTGCAAGTGGATGCCAATTGCGAGGGCGATTGCTACCACTCTGGCGGCAC
CATCATCAGCAACCTGCCTTTCCAGAACATCGACAGCAGAGCCGTGGGCAAGTGCCCCAGATACG
TGAAACAAAGAAGCCTGCTGCTGGCCACCGGCATGAAGAATGTGCCTGAGATCCCTAAGGGCCA
GCAAGGATTGGAGCCGGCGCAGCCTTTATTGAGAATGGCTGGGAGGGACTGATCGACGGATGGT
ACGGCTTCAGACACCAGAATGCCCAAGGCGAGGGAACAGCCGCCGATTACAAAAGCACACAGA
GCGCCATCGACTGCATCACCGGCAAGCTGAACAGACTGATTGCCAAGACCAACCAGCAGTTCGA
GCTGATCGACAACGAGTTCAACGAGGTGGAAAAGCAGATCGGCAACGTGATCAACTGGACCCGG
GACAGCATCACCGAAGTGTGGTCTTACAACGCCGAGCTGCTGATCGCTATGGAAAACCAGCACAC
CATCGACCTGGCCGACAGCGAGATGGACAAGCTGTATGAGAGAGTGAAGAGACAGCTGCGCGAG
AACGCCGAGGAAGATGGCACAGGCTGCTTCGAGATCTTCCACAAGTGCGACGACGACTGCATGG
CCAGCATCCGGAACAACACCTACGACCACCGGAAGTACCGGGAAGAAGCCATGCAGAACAGAA
TCCAGATCGACCCCGTGAAGCTGAGCAGCGGCTACAAGGATGTGATCCTGTGGTTCAGCTTCGGC
GCCAGCTGTTTCATCCTGCTGGCTATCGTGATGGGCCTCGTGTTTCATCTGCGTGAAGAACGGCAAC
ATGCGGTGCACCATCTGCATC**TGAT**GATATC**GAATTC**AAGGCCTAG

NotI/EcoRI recognition sites

Start/stop codons

A/Hong Kong/125/2017 CLEARFLU version 3 (human codon-optimised)

ATAAGAAT**CGGGCCGC**CACC**ATG**AACACCCAGATCCTGGTGTGGCCCTGATCGCCATCATTCCCA
CCAACGCCGATAAGATCTGTCTGGGCCACCAACGCCGTGTCCAACGGCACAAAAGTGAACACACT
GTGCGAGCGCGGCGTGGAAGTGGTCAATGCCACAGAGACAGTGGAACGGACCAACATCCCCCGG
ATCTGCAGCAAGGGCAAGAGAACAGTGGATCTGGGCCAGTGTGGCCTGCTGGGAACAATTACAG
GCCCTCTCAGTGCGACCAAGTTCCTGGAATTTTCCGCCGACCTGATCATCGAGCGGAGAGAAGGCT
CCGATGTGTGCTACCCCGGCAAGTTCGTGAACGAGGAAGCCCTGAGACAGATCCTGAGAGAGAG
CGGCGGCATCGACAAAGAAACCATGGGCTTACCTACAACGGCATCAGAACAAATGGCGTGACC
AGCGCCTGTAAGAGAAGCGGCAGCAGCTTCTACGCCGAGATGAAGTGGCTGCTGAGCAACACCG
ACAACGCCGCCTTTCCACAGATGACCAAGAGCTACAAGAACACCCGCAAGAGCCCTGCCATCAT
CGTGTGGGGAATCCACCACTCTGTGTCTACGCCGAGCAGACAAAGCTGTACGGCTCTGGCAACA
AGCTGGTCAACGTGGGCAGCAGCAATTACCAGCAGTGCTTTGTGCCCTGTCTGGCGCTAGACCTC
AAGTGAATGGCCTGAGCGGCAGAATCGACTTCCACTGGCTGATTCTGAACCCCAACGACACCGTG
ACCTTCAGCTTCAACGGCGCCTTTATCGCCCCTGACAGAGCCAGCTTTCTGAGAGGGCAAGAGCATG
GGCATCCAGTCTGGCGTGCAAGTGGATGCCAATTGCGAGGGCGATTGCTACCACTCTGGCGGCAC
CATCATCAGCAACCTGCCTTTCCAGAACATCGACAGCCGGGCCGTGGGCAAGTGCCCCAGATACG
TGAAACAGAGAAGCCTGCTGCTGGCCACCGGCATGAAGAATGTGCCTGAGATCCCTAAGGGCCA
GCAAGGATTGGAGCCGGCGCTGCCTTTATTGAGAATGGCTGGGAGGGGACTGATCGACGGATGGT
ACGGCTTCAGACACCAGAATGCCCAAGGCGAGGGAACAGCCGCCGATTACAAAAGCACACAGA
GCGCCATCGACTGCATCACCGGCAAGCTGAACAGACTGATTGCCAAGACCAACCAGCAGCCTGA
GCTGATCGACAACGAGCCCAACGAGGTGGAAAAGCAGATCGGCAACGTGATCAACTGGACCCGG
GACAGCATCACCGAAGTGTGGTCTTACAATGCCGAGCTGCTGATCGCCATGGAAAACCAGCACAC
CATCGATCTGGCCGACAGCGAGATGGACAAGCTGTATGAGAGAGTGAAGAGACAGCTGCGCGAG
AACGCCGAGGAAGATGGCACAGGCTGCTTCGAGATCTTCCACAAGTGCGACGACGACTGCATGG
CCAGCATCCGGAACAACACCTACGACCACCGGAAGTACCGGGAAGAAGCCATGCAGAACAGAA
TCCAGATCGACCCCGTGAAGCTGAGCAGCGGCTACAAGGATGTGATCCTGTGGTTCAGCTTCGGC
GCCAGCTGTTTCATCCTGCTGGCTATCGTGATGGGCCTCGTGTTTCATCTGCGTGAAGAACGGCAAC
ATGCGGTGCACCATCTGCATC**TGAT**GATATC**GAATTC**AAGGCCTAG

Truncated A/Hong Kong/125/2017 CLEARFLU version 3 segment in E2B4 and H2H5

AGCAAAAGCAGGGGAAAATAAAAACAACCAAATAGAAGGCCAAACCTACTGGTCCTGTTACGTGC
ACTTGCAGCTGCAGTAGC**CGGGCCGCCACC**-1620nt deletion including original start codon-
GTGATGGGCCTCGTGTTTCATCTGCGTGAAGAACGGCAACATGCGGTGCACCATCTGCATCTGATGA
TATCGAATTCGGAAAAGTGTAAAGAAATGGGACTTATGATTATCCCAAATATTCAGAAGAGTCAAAGT
TGAACAGGGAAAAGGTAGATGGAGTGAAATTGGAATCAATGGGGATCTATCAGATTCTGGCGATC
TACTCAACTGTGCGCCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTT
TAATGGATCTTTGCAGTGCAGAATATGCATCTGAGATTAGAATTTAGAAATATGAGGAAAAACAC
CCTTGTTTCTACT

Uni12/13 primers

NotI/EcoRI recognition sites

Start/stop codons

CLEARFLU sequence

Alignment of full length and truncated A/Hong Kong/125/2017 CLEARFLU version 3 viral segments found in round 6 clones

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1  AGCAAAAGCAGGGGAAAATAAAAAACAACCAAATAGAAGGCAAACCTACTGGTCCTGTTAC 60
   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1  AGCAAAAGCAGGGGAAAATAAAAAACAACCAAATAGAAGGCAAACCTACTGGTCCTGTTAC 60

61  GTGCACTTGCAGCTGCAGTAGCGCGGCCGCCACCATGAACACCCAGATCCTGGTGTGTTGC 120
   |||||||||||||||||||||||||||||||||||||||||||
61  GTGCACTTGCAGCTGCAGTAGCGCGGCCGCCACC----- 94

                                start
121  CCTGATCGCCATCATTCCCACCAACGCCGATAAGATCTGTCTGGGCCACCACGCCGTGTC 180
94  ----- 94

181  CAACGGCACA AAAAGTGAACACACTGTGCGAGCGCGGCGTGGAAGTGGTCAATGCCACAGA 240
94  ----- 94

241  GACAGTGGAACGGACCAACATCCCCCGGATCTGCAGCAAGGGCAAGAGAACAGTGGATCT 300
94  ----- 94

301  GGGCCAGTGTGGCCTGCTGGGAACAATTACAGGCCCTCCTCAGTGCGACCAGTTCCTGGA 360
94  ----- 94

361  ATTTTCCGCCGACCTGATCATCGAGCGGAGAGAAGGCTCCGATGTGTGCTACCCCGGCAA 420
94  ----- 94

421  GTTCGTGAACGAGGAAGCCCTGAGACAGATCCTGAGAGAGAGCGGCGGCATCGACAAAGA 480
94  ----- 94

481  AACCATGGGCTTCACCTACAACGGCATCAGAACAAATGGCGTGACCAGCGCCTGTAAGAG 540
94  ----- 94

541  AAGCGGCAGCAGCTTCTACGCCGAGATGAAGTGGCTGCTGAGCAACACCGACAACGCCGC 600
94  ----- 94

601  CTTTCCACAGATGACCAAGAGCTACAAGAACACCCGCAAGAGCCCTGCCATCATCGTGTG 660
94  ----- 94

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661 GGG AATCCACCACTCTGTGTCTACCGCCGAGCAGACAAAGCTGTACGGCTCTGGCAACAA 720
94 ----- 94

721 GCTGGTCACCGTGGGCAGCAGCAATTACCAGCAGTGCTTTGTGCCCTGTCTGGCGCTAG 780
94 ----- 94

781 ACCTCAAGTGAATGGCCTGAGCGGCAGAATCGACTTCCACTGGCTGATTCTGAACCCCAA 840
94 ----- 94

841 CGACACCGTGACCTTCAGCTTCAACGGCGCCTTTATCGCCCCCTGACAGAGCCAGCTTTCT 900
94 ----- 94

901 GAGAGGCAAGAGCATGGGCATCCAGTCTGGCGTGCAAGTGGATGCCAATTGCGAGGGCGA 960
94 ----- 94

961 TTGCTACCACTCTGGCGGCACCATCATCAGCAACCTGCCTTTCCAGAACATCGACAGCCG 1020
94 ----- 94

1021 GGCCGTGGGCAAGTGCCCNAGATACGTGAAACAGAGAAGCCTGCTGCTGGCCACCGGCAT 1080
94 ----- 94

1081 GAAGAATGTGCCTGAGATCCCTAAGGGCCAGCAAGGATTTGGAGCCGGCGCTGCCTTTAT 1140
94 ----- 94

1141 TGAGAATGGCTGGGAGGGACTGATCGACGGATGGTACGGCTTCAGACACCAGAATGCCCA 1200
94 ----- 94

1201 AGGCGAGGGGAACAGCCGCCGATTACAAAAGCACACAGAGCGCCATCGACTGCATCACCGG 1260
94 ----- 94

1261 CAAGCTGAACAGACTGATTGCCAAGACCAACCAGCAGCCTGAGCTGATCGACAACGAGCC 1320
94 ----- 94

1321 CAACGAGGTGGAAAAGCAGATCGGCAACGTGATCAACTGGACCCGGGACAGCATCACCGA 1380
94 ----- 94

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1381 AGTGTGGTCTTACAATGCCGAGCTGCTGATCGCCATGGAAAACCAGCACACCATCGATCT 1440
94  ----- 94

1441 GGCCGACAGCGAGATGGACAAGCTGTATGAGAGAGTGAAGAGACAGCTGCGCGAGAACGC 1500
94  ----- 94

1501 CGAGGAAGATGGCACAGGCTGCTTCGAGATCTTCCACAAGTGCGACGACGACTGCATGGC 1560
94  ----- 94

1561 CAGCATCCGGAACAACACCTACGACCACCGGAAGTACCGGGAAGAAGCCATGCAGAACAG 1620
94  ----- 94

1621 AATCCAGATCGACCCCGTGAAGCTGAGCAGCGGCTACAAGGATGTGATCCTGTGGTTTCTAG 1680
94  ----- 94

1681 CTTGGGCGCCAGCTGTTTCATCCTGCTGGCTATCGTGATGGCCTCGTGTTTCATCTGCGT 1740
94  -----GTGATGGCCTCGTGTTTCATCTGCGT 120
                                new start
                                |||||
                                |||||

1741 GAAGAACGGCAACATGCGGTGCACCATCTGCATCTGATGATATCGAATTCGGAAAGTGTA 1800
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121  GAAGAACGGCAACATGCGGTGCACCATCTGCATCTGATGATATCGAATTCGGAAAGTGTA 180

1801 AGAAATGGGACTTATGATTATCCCAAATATTCAGAAGAGTCAAAGTTGAACAGGGAAAAG 1860
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181  AGAAATGGGACTTATGATTATCCCAAATATTCAGAAGAGTCAAAGTTGAACAGGGAAAAG 240

1861 GTAGATGGAGTGAAATTGGAATCAATGGGGATCTATCAGATTCTGGCGATCTACTCAACT 1920
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241  GTAGATGGAGTGAAATTGGAATCAATGGGGATCTATCAGATTCTGGCGATCTACTCAACT 300

1921 GTCGCCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTTCT 1980
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301  GTCGCCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTTCT 360

1981 AATGGATCTTTGCAGTGCAGAATATGCATCTGAGATTAGAATTTTCAGAAATATGAGGAAA 2040
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361  AATGGATCTTTGCAGTGCAGAATATGCATCTGAGATTAGAATTTTCAGAAATATGAGGAAA 420

2041 AACACCCTTGTTTCTACT 2058
    ||||||||||||||||
421  AACACCCTTGTTTCTACT 438

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X31 wildtype hemagglutinin from Hooper & Bloom, 2013

ATGAAGACCATCATTGCTTTGAGCTACATTTTCTGTCTGGCTCTCGGCCAAGACCTTCCAGG
AAATGACAACAGCACAGCAACGCTGTGCCTGGGACATCATGCGGTGCCAAACGGAACA
CTAGTGAAAACAATCACAGATGATCAGATTGAAGTGACTAATGCTACTGAGCTAGTTCAG
AACTCCTCAACGGGGAAAATATGCAACAATCCTCATCGAATCCTTGATGGAATAAACTGC
ACACTGATAGATGCTCTATTGGGGGACCCTCATTGTGATGTTTTTCAAATGAGAAATGGG
ACCTTTTCGTTGAACGCAGCAAAGCTTTCAGCAACTGTTACCCTTATGATGTGCCAGATTAT
GCCTCCCTTAGGTCAGTGTGCTCGTCAGGCACTCTGGAGTTTATCAATGAGAGTTTCAA
TTGGACTGGGGTCACTCAGAATGGGACAAGCTCAGCTTGCAAAAGGGGACCTAATAGCA
GTTTTTTCAGTAGACTGAACTGGTTGACCAAATCAGGAAGCACATATCCAGTGCTGAACGT
GACTATGCCAAACAATGACAATTTTGACAACTATACATTTGGGGGATTACCCACCCGAG
CACGAACCAAGAACAACACCAGCCTGTATGTTCAAGCATCAGGGAGAGTCACAGTCTCTA
CCAGGAGAAGCCAGCAAACCTATAATCCCGAATATCGGGTCCAGACCCTGGGTAAAGGGGT
CTGTCTAGTAGAATAAGCATCTATTGGACAATAGTTAAGCCGGGAGACGTAAGTGAATTA
ATAGTACTGGGAACCTAATCGCTCCTCGGGGTATTTCAAAATGCGCACTGGGAAAAGCT
CAATAATGAGGTCAGATGCACCTATTGATACCTGTATTTCTGAATGCATCACTCCAAATGG
AAGCATTCCCAATGACAAGCCCTTTCAAACGTAACAAGATCACATATGGAGCATGCC
CCAAGTATGTTAAGCAAAACACCCTGAAGTTGGCAACAGGGATGCGGAATGTACCAGAG
AAACAACTAGAGGCCTATTCGGCGCAATAGCAGGTTTCATAGAAAATGGTTGGGAGGG
AATGATAGACGGTTGGTACGGTTTCAGGCATCAAAATTCTGAGGGCACAGGACAAGCAG
CAGATCTTAAAAGCACTCAAGCAGCCATCGACCAAATCAATGGGAAATTGAACAGGGTA
ATCGAGAAGACGAACGAGAAATTCCATCAAATCGAAAAGGAATTCTCAGAAGTAGAAGG
GAGAATTCAGGACCTCGAGAAATACGTTGAAGACACTAAAATAGATCTCTGGTCTTACAA
TGCGGAGCTTCTTGTCGCTCTGGAGAATCAACATACAATTGACCTGACTGACTCGGAAATG
AACAAAGCTGTTTGA AAAAACAAGGAGGCAACTGAGGGAAAATGCTGAAGACATGGGCA
ATGGTTGCTTCAAATATACCACAAATGTGACAACGCTTGATAGAGTCAATCAGAAATG
GGACTTATGACCATGATGTATACAGAGACGAAGCATTAAACAACCGGTTTCAGATCAAAG
GTGTTGAACTGAAGTCTGGATACAAAGACTGGATCCTGTGGATTTCCCTTGCCATATCATGC
TTTTTGCTTTGTGTTGTTTTGCTGGGGTTCATCATGTGGGCCTGCCAGAGAGGCAACATTAGG
TGCAACATTTGCATT**TGA**

Start/stop codons

X31 passage mutant hemagglutinin (X31-PM) from Hooper & Bloom, 2013

ATGAAGACCATCATTGCTTTGAGCTACATTTTCTGTCTGGCTCTCGGCCAAGACCTTCCAGG
AAATGACAACAGCACAGCAACGCTGTGCCTGGGACATCATGCGGTGCCAAACGGAACA
CTAGTGAAAACAATCACAGATGATCAGATTGAAGTGACTAATGCTACTGAGCTAGTTTCA
AACTCCTCAACGGGGAAAATATGCAACAATCCTCATCGAATCCTTGATGGAATAAACTGC
ACACTGATAGATGCTCTATTGGGGGACCCTCATTGTGATGTTTTTCAAATGAGAAATGGG
ACCTTTTCGTTGAACGCAGCAAAGCTTTCAGCAACTGTTTTCCTTATGATGTGCCAGATTAT
GCCTCCCTTAGGTCACCTAGTTGCCTCGTCAGGCACTCTGGAGTTTATCAATGAGAGTTTCAA
TTGGACTGGGGTCACTCAGAATGGGACAAGCTCAGCTTGCAAAAGGGGACCTAATAGCA
GTTTTTTCAGTAGACTGAACTGGTTGACCAAATCAGGAAGCACATATCCAGTGCTGAACGT
GACTATGCCAAACAATGACAATTTTGACAAACTATACATTTGGGGGATTTTCCACCCGAGC
ACGAACCAAGAGCAAACCAGCGCGTATGTTCAAGCATCAGGGAGAGTCACAGTCTCTAC
CAGGAGAAGCCAGCAAACCTATAATCCCGAATATCGGGTCCAGAAGAATAAGCATCTATT
GGACAATAGTTAAGCCGGGAGACGTAAGTAAATAGTACTGGGAACCTAATCGCTC
CTCGGGGTATTTCAAAATGCGCACTGGGAAAAGCTCAATAATGAGGTCAGATGCACCTA
TTGATACCTGTATTTCTGAATGCATCACTCCAAATGGAAGCATTCCCAATGACAAGCCCTTT
CAAAACGTAAACAAGATCACATATGGAGCATGCCCAAGTATGTAAAGCAAAACACCCT
GAAGTTGGCAACAGGGATGCGGAATGTACCAGAGAAACAACCTAGAGAACTATTCGGCG
CAATAGCAGGTTTCATAGAAAATGTTGGGAGGGAATGATAGACGGTTGGTACGGTTTCA
GGCATCAAAATTCTGAGGGCACAGGACAAGCAGCAGATCTTAAAAGCACTCAAGCAGCC
ATCGACCAAATCAATGGGAAATTGAACAGGGTAATCGAGAAGACGAACGAGGAATTCCA
TCAAATCGAAAAGGAATTCTCAGAAGTAGAAGGGAGAATTCAGGACCTCGAGAAATACG
TTGAAGACACTAAAATAGATCTCTGGTCTTACAATGCGGAGCTTCTTGTGCTCTGGAGAA
TCAACATACAATTGACCTGACTGACTCGGAAATGAACAAGCTGTTTGAAAAACAAGGA
GGCAACTGAGGGAAAATGCTGAAGACATGGGCAATGGTTGCTTCAAATATACCACAAA
TGTGACAACGCTTGCATAGAGTCAATCAGAAATGGGACTTATGACCATGATGTATACAGA
GACGAAGCATTAACAACCGGTTTCAGATCAAAGGTGTTGAACTGAAGTCTGGATACAAA
GACTGGATCCTGTGGATTTCTTTGCCATATCATGCTTTTTGCTTTGTGTTGTTTGGCTGGGGT
TCATCATGTGGGCCTGCCAGAGAGGCAACATTAGGTGCAACATTTGCATT**TGA**

Start/stop codons

Neuraminidase & eGFP sequences

N1 A/Puerto Rico/8/1934 for subcloning into pPol and generating S-Flus

GCTCTCAGGGAGCAAAAGCAGGAGTTTAAAT**ATG**AATCCAAATCAGAAAATAATAACCATTGGAT
CAATCTGTCTGGTAGTCGGACTAATTAGCCTAATATTGCAAATAGGGAATATAATCTCAATATGGAT
TAGCCATTCAATTCAAACCTGGAAGTCAAACCATACTGGAATATGCAACCAAAACATCATTACCTA
TAAAAATAGCACCTGGGTAAAGGACACAACCTTCAGTGATATTAACCGGCAATTCATCTCTTTGTCC
CATCCGTGGGTGGGCTATATACAGCAAAGACAATAGCATAAGAATTGGTTCCAAAGGAGACGTTTT
TGTCTAAGAGAGCCCTTTATTTTCATGTTCTCACTTGGAATGCAGGACCTTTTTCTGACCCAAGGTG
CCTTACTGAATGACAAGCATTCAAGTGGG**A**CTGTTAAGGACAGAAGCCCTTATAGGGCCCTAATGA
GCTGCCCTGTCGGTGAAGCTCCGTCCCCGTACAATTCAAGATTTGAATCGGTTGCTTGCTGAGCAA
GTGCATGTCATGATGGCATGGGCTGGCTAACAATCGGAATTCAGGTCCAGATAATGGAGCAGTGG
CTGTATTAATAACAACGGCATAATAACTGAAACCATAAAAAGTTGGAGGAAGAAAATATTGAGG
ACACAAGAGTCTGAATGTGCCTGTGTAAATGGTTCATGTTTTACTATAATGACTGATGGCCCGAGTG
ATGGGCTGGCCTCGTACAAAATTTCAAGATCGAAAAGGGGAAGGTTACTAAATCAATAGAGTTGA
ATGCACCTAATTCTCACTATGAGGAATGTTCTGTTACCCTGATACCGGCAAAGTGATGTGTGTGTG
CAGAGACAATTGGCATGGTTCGAACCGGCCATGGGTGTCTTTGATCAAAACCTGGATTATCAAAT
AGGATACATCTGCAGTGGGGTTTTCGGTGACAACCCGCGTCCCGAAGATGGAACAGGCAGCTGTG
GTCCAGTGTATGTTGATGGAGCAAACGGAGTAAAGGGATTTTCATATAGGTATGGTAATGGTGTGTTG
GATAGGAAGGACCAAAAGTCACAGTTCAGACATGGGTTTGAGATGATTTGGGATCCTAATGGATG
GACAGAGACTGATAGTAAGTTCTCTGTGAGGCAAGATGTTGTGGCAATGACTGATTGGTCAGGGTA
TAGCGGAAGTTTCGTTCAACATCCTGAGCTGACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTT
GAATTAATCAGGGGACGACCTAAAGAAAAACAATCTGGACTAGTGCGAGCAGCATTTCTTTTTGT
GGCGTGAATAGTGATACTGTAGATTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCAGCATTGAC
AAG**AG**TCTGTTGAAAAAACTCCTTGTCTACTAATA**GAAGAGC**

SapI/BspQI recognition sites

Start/stop codons

AWT → G_{T148A} mutation site

eGFP in S-Flu expression cassette

AGCAAAAGCAGGGGGAAAATAAAAAACAACCAAATAGAAGGCCAAACCTACTGGTCCTGTTACGTGC
ACTTGCAGCTGCAGTAGC**BCGGCCGCC**ACC**ATG**GTTTCTAAGGGCGAAGAACTGTTACCGGCGT
GGTGCCTATCCTGGTGGAAGTGGATGGGGATGTGAACGGCCACAAGTTCTCTGTGTCTGGCGAAGG
CGAAGGGGATGCCACATACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGAAAGCTGCCC
GTGCCTTGGCCTACACTGGTCACAACACTGACCTACGGCGTGCAGTGCTTCAGCAGATACCCCGA
CCACATGAAGCAGCACGATTTCTTCAAGAGCGCCATGCCTGAGGGCTACGTGCAAGAGCGGACC
ATCTTCTTCAAGGACGACGGCAACTACAAGACCAGAGCCGAAGTGAAGTTCGAGGGCGACACCC
TGGTCAACAGAATCGAGCTGAAGGGCATCGACTTCAAAGAGGATGGCAACATCCTGGGCCACAA
GCTCGAGTACAACTACAACAGCCACAACGTGTACATCATGGCCGACAAGCAGAAGAACGGCATC
AAAGTCAACTTCAAGATCCGGCACAACATCGAGGACGGCTCTGTGCAGCTGGCCGACCACTATCA
GCAGAACACACCTATCGGAGATGGCCCCGTGCTGCTGCCCGATAATCACTACCTGTCTACACAGA
GCGCCCTGAGCAAGGACCCCAACGAGAAAAGGGATCACATGGTGCTGCTGGAATTTGTGACCGC
CGCTGGCATCACACTCGGCATGGACGAGCTGTACAAAT**TGA****GAATC**GGAAAGTGTAAGAAATGGG
ACTTATGATTATCCCAAATATTCAGAAGAGTCAAAGTTGAACAGGGAAAAGGTAGATGGAGTGAA
ATTGGAATCAATGGGGATCTATCAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCAGTGGTGCTT
TTGGTCTCCCTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGATCTTTGCAGTGCAGAATATGCAT
CTGAGATTAGAATTCAGAAATATGAGGAAAAACAC**CCTTGTCTTCTACT**

Uni12/13 primers

NotI/EcoRI recognition sites

Start/stop codons

Ebola glycoprotein sequences

Ebola glycoprotein, Sudan (Gulu)

ATGGGCGGCCTGAGCCTGCTGCAGCTGCCCCGGGACAAGTTCGGAAAGTCCAGCTTCTTCGTGTGG
GTGATCATCCTGTTCCAGAAAGCCTTCAGCATGCCCCTGGGCGTGGTGACAAACAGCACCCCTGGA
AGTGACCGAGATCGACCAGCTCGTGTGCAAGGACCACCTGGCCAGCACCGACCAGCTGAAGTCC
GTGGGCCTGAACCTGGAAGGCAGCGGCGTGTCCACCGACATCCCCAGCGCCACCAAGAGATGGG
GCTTCAGATCCGGCGTGCCCCCAAGGTGGTGTCTATGAAGCCGGCGAGTGGGCCGAGAACTGC
TACAACCTGGAAATCAAGAAGCCCGACGGCAGCGAGTGCCTGCCCCCTCCTCCTGATGGCGTGCG
GGGCTTCCCCCGGTGCAGATACGTGCACAAGGCCACGGGCCCTGTCTGGCGACTAC
GCCTTCCACAAGGACGGCGCCTTTTTCTGTACGACCGGTGGCCTCCACCGTGATCTACGGGGG
GTGAACTTCGCCGAGGGCGTGATCGCCTTCCTGATCCTGGCCAAGCCCAAAGAGACATTCTGCA
GAGCCCCCCCATCCGCGAGGCCGTGAACTACACCGAGAACACCAAGCAGCTACTACGCCACCAGC
TACCTGGAATACGAGATCGAGAACTTCGGCGCCCGACAGCACACCACCTGTTCAAGATCGACAA
CAACACCTTCGTGCGGCTGGACCGGCCCCACACCCCCAGTTTCTGTTCCAGCTGAACGACACCA
TCCATCTGCATCAGCAGCTGAGCAACACCACCGGCAGACTGATCTGGACCCTGGACGCCAACATC
AACGCCGACATCGGAGAGTGGGCCTTTTGGGAGAACAAGAAGAACCTGAGCGAGCAGCTGCGGG
GCGAGGAAGTGAAGTTCGAGGCCCTGAGCCTGAACGAGACAGAGGACGACGACGCCGCCAGCA
GCCGGATCACCAGGGCAGAAATCAGCGACCGGGCCACCCGGAAGTACAGCGACCTGGTGCCCA
AGAACAGCCCCGGCATGGTGCCCTGCACATCCCCGAGGGCGAGACAACCCTGCCAGCCAGA
ACAGCACCGAGGGGCGCAGAGTGGGCGTGAACACCCAAGAAACCATCACCGAGACAGCCGCCA
CAATCATCGGCACCAACGGCAACCACATGCAGATCAGCACCATCGGCATCCGGCCCAGCAGCAG
CCAGATCCCTAGCAGCAGCCCTACCACCGCCCCTAGCCCTGAGGCCCCAGACCCCTACCACACAC
ACCAGCGGCCCTAGCGTGATGGCCACCGAGGAACCTACCACCCCTCCTGGCAGCAGCCCAGGCC
CTACCACAGAGGCCCTACCCTGACCACCCCCGAGAACATCACCAACCGCCGTGAAAACCGTGCT
GCCCCAAGAAAGCACCAGCAACGGCCTGATCACAGCACCGTGACCGGCATCCTGGGCAGCCTG
GGCCTGCGGAAGAGAAGCAGACGGCAGACCAACACCAAGGCCACCGGCAAGTGCAACCCCAAC
CTGCACTACTGGACCGCCCAAGAACAGCACAAACGCCGCTGGAATCGCCTGGATTCCCTACTTCGG
CCCTGGCGCCGAGGGCATCTACACCGAGGGCCTGATGCACAACCAGAACGCCCTCGTGTGCGGC
CTGCGGCAGCTGGCCAATGAGACAACCCAGGCCCTGCAGCTGTTCTGCGGGCCACCACCGAGC
TGCGGACCTACCATCCTGAACAGAAAGGCCATCGACTTTCTGCTGCGGAGATGGGGCGGCACC
TGTAAGATCCTGGGCCCCGACTGCTGCATCGAGCCCCACGACTGGACCAAGAATATCACCGACAA
GATCAACCAGATCATCCACGACTTCATCGACAACCCCTGCCCAACCAGGACAACGACGACAAC
TGGTGGACCGGCTGGAAGCTGGAATCCATGGGCATCTACCAGATTCTGGCCATCTACAGCACCGT
GGCCAGCTCCCTGGTGCTGCTGGTGTCCCTGGGCGCCATCAGCTTTTGGATGTGCAGCAACGGCAG
CCTGCAGTGCCGGATCTGCATC**TGA**

Start/stop codons

Ebola glycoprotein, Zaire (Makona)

ATGGGCGTGACCGGAATCCTGCAGCTGCCAGAGACAGGTTCAAGCGGACCAGCTTCTTCCTGTG
GGTGATCATCTGTTCCAGCGGACCTTCAGCATCCCTCTGGGCGTGATCCACAACAGCACCCCTGCA
GGTCTCCGACGTGGACAAGCTCGTGTGCCGGGACAAGCTGAGCAGCACCAACCAGCTGCGGAGC
GTGGGCCTGAACCTGGAAGGCAACGGCGTGGCCACCGATGTGCCAGCGCCACCAAGAGATGGG
GCTTCAGATCCGGCGTGCCACCCAAGGTGGTGAACCTACGAAGCCGGCGAGTGGGCCGAGAACTG
CTACAACCTGGAAATCAAGAAGCCCCGACGGCAGCGAGTGCCTGCCTGCCGCTCCTGATGGCATCC
GGGGCTTCCCCAGATGCAGATACGTGCACAAGGTGTCCGGCACCGGCCCTGTGCTGGCGACTTC
GCCTTTCACAAAGAGGGGCGCCTTTTTCTGTACGACCGGCTCGCCAGCACCGTGATCTACCGGGGC
ACCACCTTTGCCGAGGGCGTGGTGGCCTTCCTGATCCTGCCCCAGGCCAAGAAGGACTTCTTCAGC
AGCCACCCTCTGCGCGAGCCCGTGAACGCCACAGAAGATCCCAGCAGCGGCTACTACAGCACCA
CCATCAGATACCAGGCCACCGGCTTCGGCACCAACGAGACAGAGTACCTGTTTCGAGGTGGACAA
CCTGACCTACGTGCAGCTGGAAGCCGGTTCACCCCTCAGTTTCTGCTGCAGCTGAACGAGACAAT
CTACGCCAGCGGCAAGCGGAGCAACACCACCGGCAAGCTGATCTGGAAGTGAACCCCGAGATC
GACACCACAATCGGAGAGTGGGCCTTCTGGGAGACAAAGAAGAACCTGACCCGGAAGATCAGAA
GCGAGGAACTGAGCTTCACCGCCGTGTCCAACGGCCCCAAGAACATCAGCGGCCAGAGCCCCGC
CAGAACCAGCAGCGACCCCGAGACAAACACCACCAACGAGGACCACAAGATCATGGCCAGCGA
GAACAGCAGCGCCATGGTGCAGGTCCACAGCCAGGGCAGAAAGGCCGCCGTGTCTCACCTGACC
ACCCTGGCCACCATCAGCACCAGCCCTCAGAGCCTGACCACCAAGCCTGGCCCCGACAACTCCA
CCCACAACACCCCTGTGTACAAGCTGGACATCAGCGAGGCCACCCAAGTGGGACAGCACACAG
ACGGGCCGACAACGACAGCACCGCCAGCGATACCCCTCCAGCCACAACAGCCGCCGGACCCCT
GAAGGCCGAGAACACCAACACCAGCAAGAGCGCCGACAGCCTGGATCTGGCCACCACCACAG
TCCTCAGAACTACTCCGAGACAGCCGGCAACAACAACACCCACCACCAGGACACCGGCGAGGA
AAGCGCCAGCTCTGGCAAGCTGGGCCTGATCACCAACACAATCGCCGGCGTGGCCGGACTGATC
ACCGGAGGCAGACGGACCAGACGGGAAGTGATCGTGAACGCCAGCCCAAGTGCAACCCCAAC
CTGCACTACTGGACCACCCAGGACGAGGGCGCTGCTATCGGCCTGGCCTGGATTCTTACTTCGGC
CCTGCCGCCGAGGGCATCTACACCGAGGGCCTGATGCACAACCAGGACGGCCTGATCTGCGGCC
TGCGGCAGCTGGCCAATGAGACAACCCAGGCCCTGCAGCTGTTCTGCGGGCCACCACCGAGCT
GCGGACCTTCTCCATCCTGAACAGAAAGGCCATCGACTTTCTGCTGCAGCGCTGGGGAGGCACCT
GTCACATCCTGGGCCCCGACTGCTGCATCGAGCCCCACGACTGGACCAAGAATATCACCGACAA
GATCGACCAGATCATCCACGACTTCGTGGACAAGACCCTGCCCCAGCAGGGCGACAACGATAAC
TGGTGGACCGGCTGGAAGCTGGAATCCATGGGCATCTACCAGATCCTGGCCATCTACAGCACCGT
GGCCAGCAGCCTGGTCCTGCTGGTGTCTCTGGGCGCCATCAGCTTTTGGATGTGCAGCAACGGCAG
CCTGCAGTGCCGGATCTGCATC**TGA**

Start/stop codons