

Division of Investigative Sciences
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Strategies for influenza vaccines

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

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Abstract

The high mutation rate of influenza virus results in antigenic drift, meaning that each of the three components in the trivalent influenza vaccine are updated regularly so that the vaccine antigen closely matches the predominant or emerging strain. The production of influenza vaccines from the chosen seed has relied on embryonated chickens eggs for more than 40 years. Recent technological advances have resulted in the evaluation of several cell lines as alternative substrates for influenza vaccine production. Reverse genetics of influenza viruses allows the creation of viruses at will from cDNA. However, licensed cell lines have so far proved unpermissive for virus rescue and permissive lines remain largely unlicensed. A reverse genetics system for the production of influenza vaccines in PER.C6 cells has been developed and optimised. Many recent clinical isolates do not grow in eggs and hence require reassortment with a high growth, egg permissive backbone. Adventitious agents aside, cell lines able to support growth of clinical isolates could be used directly included in the vaccine, without the need for reassortment. However, this could lead to year on year variation in the quality and characteristics of the vaccine. Particularly pertinent, is the variation in the amount of IFN a clinical isolate can induce, which could severely limit yields. Yields and effects of IFN for a panel of high and low inducing viruses are investigated in a number of potential vaccine cell lines. The mechanisms behind virus IFN induction have been investigated. Using a panel of high and low inducing viruses the differences in growth and viral protein expressions, and activation of PRR has been analysed. The IFN response in PER.C6 cells has been characterised. Through an improved understanding of the mechanisms of IFN induction and inhibition, antagonists could be introduced into the vaccine cell line to improve yields.

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Abbreviations

β-gal	β-galactosidase
4196	A/England/41/96
5'3P	5' triphosphate
919/99	A/England/919/99
Ab	antibody
ADCC	Ab-dependent, cell-mediated cytotoxicity
AEM	Adenovirus Expression Medium
Ag	Antigen
Amp	Ampicillin
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca	Cold adapted
CARD	Caspase recruitment domain
Cardif	CARD adaptor inducing IFN β
CAT	Chloramphenicol acetyltransferase
CBP	Cap binding protein
CBP	CREB-binding protein
CCLR	Cell culture lysis reagent
cDC	Conventional dendritic cells
cDNA	Copy deoxyribonucleic acid
CEF	Chick embryo fibroblasts
CEK	Chick embryo kidney
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
CP	Coat protein
CPSF	Cleavage and polyadenylation specificity factor
CRM	Chromosomal region maintenance
CTL	Cytotoxic lymphocyte
DABCO	1,4-diazabicyclo[2.2.2]octane

DAPI	4',6-diamidino-2-phenylindole
DI	Defective interfering
DIG	Digoxigenin
DMEM	Dulbecco's modified eagles media
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	2' deoxynucleoside 5'-triphosphates
dsRNA	Double stranded RNA
DSS	Dioctylsulfoctinate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent assay
EMCV	Encephalomyocarditis virus
EU	European union
FAC	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
FISH	Fluorescent in situ histochemistry
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK	Glaxo Smithkline
HA	Haemagglutinin
HAE	Human airway epithelial
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPAI	High pathogenic avian influenza
HRP	Horse radish peroxidase
Hrs	Hours
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IF	Immunofluorescence
IFN	Interferon
IFNAR	Interferon α receptor
Ig	Immunoglobulin
IKK	Inducible κ B kinase
IL	Interleukin
ILL	Influenza like illness
IPS-1	IFN- β promoter stimulator
IRAK	Interleukin-1 receptor-associated kinase
IRAK	Interleukin-1-receptor (IL-1R)-associated kinase-4
IRES	Internal ribosome entry site
IRF	Interferon responsive factor
ISCOM	Immuno stimulating complex
ISG	IFN stimulated gene
ISGF	IFN stimulated gene factor
ISRE	IFN stimulated response element
JAK	Janus kinase
Kan	Kanamycin
KLH	Keyhole limpet haemocyanin
LAIV	Live attenuated influenza vaccine
LB	Luria broth
LMB	Leptomycin B
LPAI	Low pathogenic avian influenza
LGP	Laboratory of genetics and physiology
LRR	Leucine rich repeat
luc	Luciferase
M	Matrix protein
MAb	Monoclonal Ab
MAVS	Mitochondrial antiviral signalling molecule
MDA-5	Melanoma differentiation
MDCK	Madin-Darby canine kidney
MDCK-N	MDCKs expressing BVDV N protein

pro	
MDCK-V	MDCKs expressing paramyxovirus V protein
MEF	Mouse embryo fibroblast
MEM	Modified eagles medium
mins	Minutes
M-MLV	Moloney murine leukaemia virus
MOI	Multiplicity of infection
MyD	Myeloid differentiation primary response gene
NA	Neuraminidase
NCR	Non coding region
NDV	Newcastle disease virus
NEMO	NF- κ B essential modifier
NEP	Nuclear export protein
NES	Nuclear export signal
NF κ B	Nuclear factor κ B
NIBSC	National Institute for Biological Standards and Control
NICE	National institute for Clinical Excellence
NK	Natural killer
NLS	Nuclear localisation signal
NP	Nucleoprotein
NS	Non structural protein
OMPC	Outer membrane protein complex
ONPG	Ortho-Nitrophenyl- β -galactoside
p.i.	Post infection
PA	Polymerase subunit A
PABP	Poly(A)-binding protein
PAMP	Pattern associated molecular pattern
PapMV	Papaya mosaic virus
PB1	Polymerase subunit B1
PB2	Polymerase subunit B2
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline 0.1% tween

PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
Pen/Strep	Penicillin/streptomycin
PI3K	Phosphatidylinositol-3 kinase
PIV5	Parainfluenza 5
PKR	Protein kinase R
PLG	Poly-lactide co-glycosides
poly IC, pIC	Poly inositol choline
PR8	A/Puerto Rico/8/34
PRR	Pattern recognition receptor
qRT PCR	Quantitative real time PCR
RBC	Red blood cells
rHA	Recombinant HA
RIG-I	Retinoic inducible gene I
RIP	Receptor-interacting protein
RIPA	Radio-immuno precipitation assay
RLR	RIG-like receptor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPM	Revolutions per minute
RSV	Respiratory syncytial virus
RT	Room temperature, reverse transcription, reverse transcriptase
SARS	Severe acute respiratory syndrome
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
Secs	Seconds
SeV	Sendai virus
SFV	Semliki forest virus
SIAT	Sialtransferase
SOCS	Suppressor of cytokine signalling

SOP	Standard operating procedure
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
STAT-1P	Phosphorylated signal transducers and activators of transcription-1
STING	Stimulator of interferon genes
SV	Splice variant
SV5	Simian virus 5
Syd	A/Sydney/5/97
TAB	TAK1-binding proteins
TAE	TRIS acetate EDTA
TAK	Transforming growth factor β -activated kinase
TANK	NK- κ B activator
TB	Tuberculosis
TBK-1	TANK-binding kinase
TCID ₅₀	Tissue culture infectious dose 50
Th	T helper
TICAM	TIR domain-containing adapter protein
TIR	Toll/IL-1 receptor
TIV	Trivalent inactivated vaccine
TLR	Toll like receptor
TM	Transmembrane
TNF- α	Tumour necrosis factor α
TRAF	TNF α Receptor Associated Factor
TRIF	TIR domain-containing adapter inducing IFN- β
TRIM25	Tripartite motif-containing 25
TRIS	Tris(hydroxymethyl)aminomethane
TYK	Tyrosine kinase
UTR	Untranslated region
UV	Ultraviolet
V	Volts
Vic	A/Victoria/3/75

VISA	Virus-induced signalling adaptor
VLP	Virus like particle
VP-SFM	Virus production serum free medium
vRNA	Viral ribonucleic acid
WHO	World health organisation
WT	Wild type

Amino acid abbreviations

Amino Acid	3 Letter	1 Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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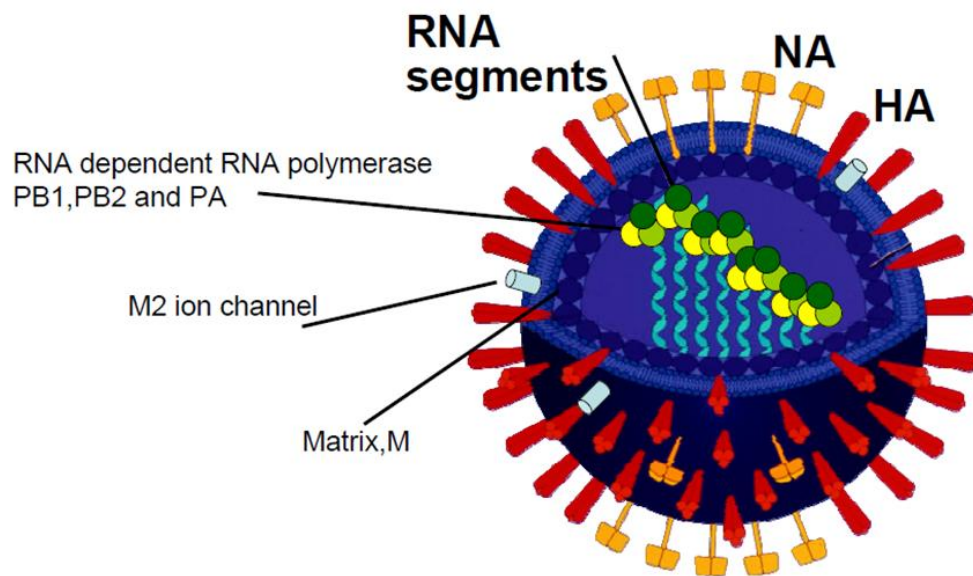
Chapter 1 Introduction

1.1 General Introduction

Influenza is a highly contagious, acute respiratory disease, which causes significant mortality and morbidity worldwide. According to the World Health Organisation (WHO) it causes between three and five million cases of severe illness and 250,000 to 500,000 deaths every year around the world (<http://www.who.int/mediacentre/factsheets/fs211/en/>). The elderly, young children and those with chronic medical disorders such as asthma, diabetes and the immune-compromised are most at risk. It has a range of symptoms including rhinitis, cough, weakness, febrile illness and headache. Most infections are self-limiting however, they can occasionally progress to pneumonia and rare complications include encephalopathy, myocarditis, and myositis.

Influenza is caused by infection with a single stranded, negative sense, RNA virus with a segmented genome (figure 1.1.1). The virus encodes 12 known proteins, encoded on 8 segments. The viral envelope contains the glycoproteins haemagglutinin (HA) and neuraminidase (NA) and matrix protein (M2) in host derived lipids. This encloses a matrix of M1 protein, around the virion core containing nuclear export protein (NEP) and each of the 8 RNA segments. Each segment is coated with nucleoprotein (NP) and complexed with the RNA-dependent RNA polymerase (RdRp), composed of two polymerase basic and one polymerase acidic subunits (PB1, PB2, and PA), and is known as an RNP. NS1 and PB1-F2 are non-structural proteins that help the virus control the host cell immune responses and N40 is a recently described N terminal product of the PB1 open reading frame which affects replication (Wise, Foeglein et al. 2009).

Figure 1.1.1 Schematic of an influenza virion



HA mediates virus binding to the host cell receptor, sialic acid. The virus is internalised by receptor mediated endocytosis (Lakadamyali, Rust et al. 2004). Internalized viruses are trafficked along the endocytic pathway to late endosomes. Exposure to the low pH results in a conformational change in the HA which exposes the HA2 fusion peptide and triggers HA-mediated fusion between the viral and endosomal membranes (Skehel and Wiley 2000). M2 forms an ion channel which results in the acidification of the virus interior, triggering disassembly of the core and release the vRNPs from the matrix proteins (reviewed in (Betakova 2007)). vRNPs are imported into the nucleus via nuclear localisation signals which direct cellular proteins to import the RNPs and viral proteins into the host cell nucleus (Wu, Sun et al. 2007).

RdRp synthesizes viral mRNA and cRNA (copy RNA) from the same vRNA (viral RNA) template (figure 1.1.2). The 3' and 5' non-coding regions are highly conserved among each of the segments and partially complementary (Desselberger, Racaniello et al. 1980). They base pair to form a helical hairpin panhandle (figure 1.1.3), with which the RdRp associates (Gonzalez and Ortin 1999) The conserved non coding nucleotides function as the promoter (Luytjes et al. 1990). The replication of vRNA is primer-independent and is terminated by run-off transcription of the vRNA template to form a full-length cRNA, an exact positive sense copy of the genome, which acts as a template for the synthesis of subsequent vRNA. Initiation of viral mRNA synthesis requires capped RNA fragments derived from host mRNA. PA has endonuclease activity and “steals” or “cap snatches” 5' capped primers from cellular RNA polymerase II (Pol II) activity (Dias, Bouvier et al. 2009). PB2 subunit then binds the 5' cap and is responsible for the initiation of transcription (Guilligay, Tarendeau et al. 2008). PB1 subunit plays a central role in polymerase elongation (Biswas and Nayak 1994). The non-coding regions also include the mRNA polyadenylation signal and termination occurs following reiterative copying of a sequence of uridine residues to generate a poly(A) tail (Luo, Luytjes et al. 1991). 5' capped, poly (A) mRNA can be exported and translated

Figure 1.1.2 Schematic of influenza replication, depicting v, c and mRNA synthesis, and 3' and 5' non coding regions.

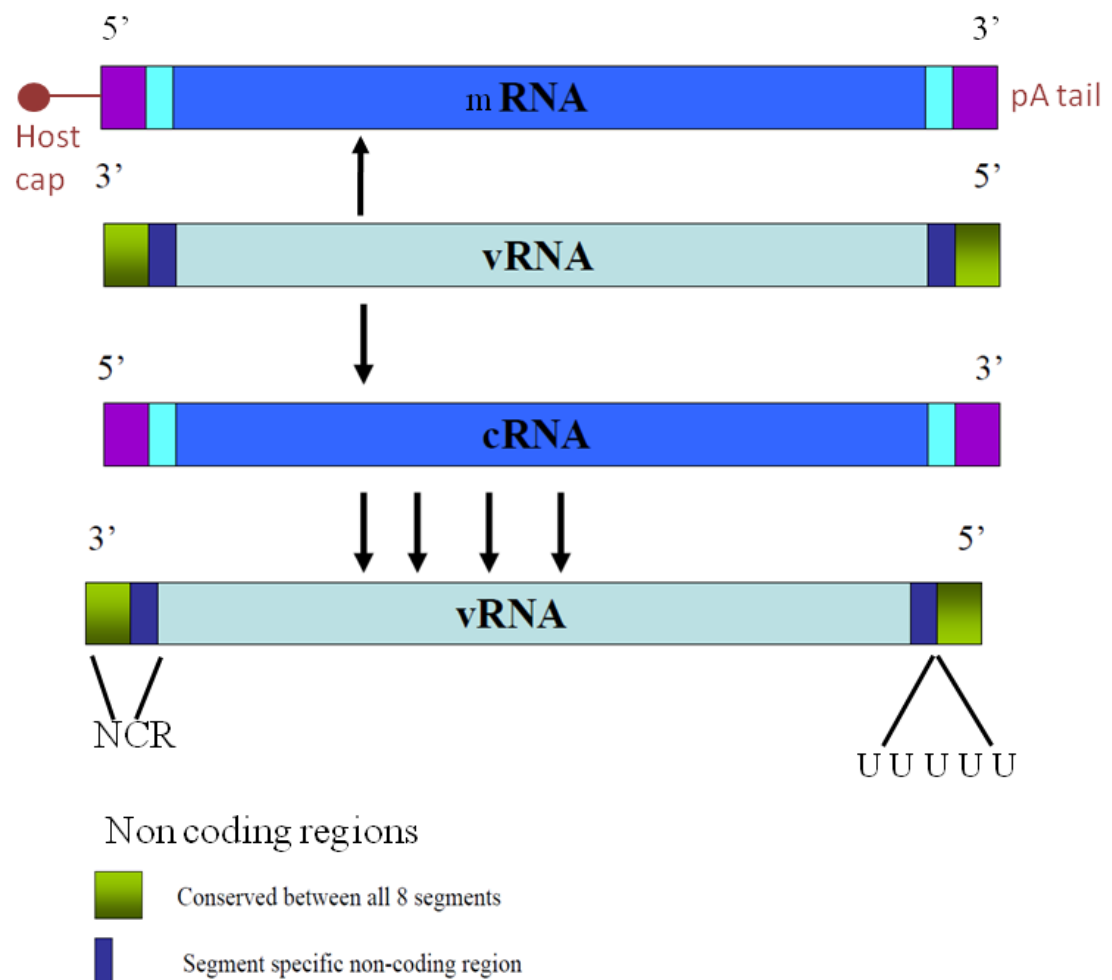
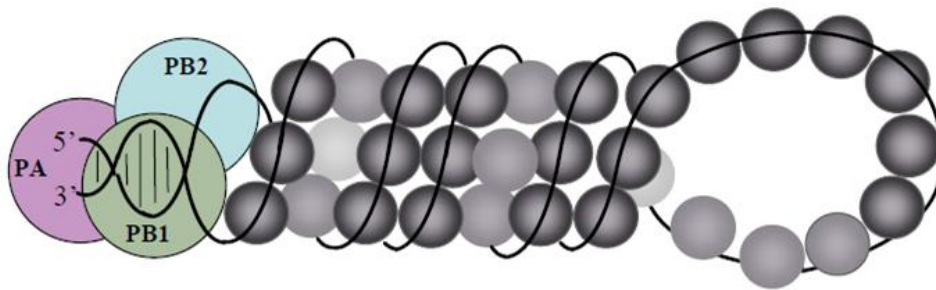


Figure 1.1.3 Schematic of influenza virus vRNP, depicting vRNA complexed with NP and RdRp (PB2, PB1 and PA).



like host mRNA. Nuclear export of vRNA segments is mediated by the viral proteins M1 and NEP via a CRM1-dependant pathway (Neumann, Hughes et al. 2000; Elton, Simpson-Holley et al. 2001; Watanabe, Takizawa et al. 2001). The mechanism controlling the synthesis of mRNA or cRNA from the same template has been long debated. Most recently Brownlee and co-workers have shown that both products are generated at the same time during infection but that cRNA is unstable in the absence of associating NP and polymerases and therefore only accumulates later in infection when intracellular viral protein levels are high (Vreede and Brownlee 2007). This hypothesis is in line with a much older theory that NP accumulation triggers the switch from transcription to replication (Beaton and Krug 1984).

The envelope proteins HA, NA, and M2 accumulate at the cell membrane (Nayak, Hui et al. 2004). M1 is thought to chaperone the RNPs to the cell membrane for packaging, where it interacts with cytoplasmic tails of envelope proteins HA, NA and M2, leading to assembly and budding of virions (Chen, Leser et al. 2008; Nayak, Balogun et al. 2009). Virus budding may be initiated by the accumulation of M1 matrix protein at the cytoplasmic side of the lipid bilayer. The HA spikes continue to bind the virions to the sialic acid on the cell surface until virus particles are released by the sialidase activity of the NA protein (Barman, Adhikary et al. 2004).

The incorporation of the eight segments that comprise the genome into virus particles has also long been a matter of debate. There is evidence for random packaging whereby virions could contain more than eight segments and a proportion would have the correct combination. However, recent elegant work from Kawaoka has shown both genetically and by electron microscopy that there are eight RNPs per virion because of the existence of segment specific packaging signals that extend across both non-coding and coding regions of each of the eight RNA segments (Fujii, Fujii et al. 2005; Noda, Sagara et al. 2006). Interestingly this knowledge may lead to new strategies to generate attenuated viruses suitable for vaccines (Gao, Brydon et al. 2008).

There are three serologically distinct types of the virus; A, B, and C- of which A and B cause widespread outbreaks. Influenza A virus causes most severe disease, whilst B causes significant epidemic outbreaks approximately every three years. Influenza A viruses are further classified into subtypes based on the surface glycoproteins; there are 16 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes. Three subtypes are known to infect and transmit within the human population (H1N1, H2N2 and H3N2). Both, but particularly HA, are major antigenic determinants and antibodies directed against HA and NA, are crucial in current vaccine strategy.

New strains appear from gradual accumulation of point mutations in the surface glycoproteins. This “antigenic drift” allows the virus to evade immune recognition and forms the basis for the seasonal epidemics which occur each year. Because influenza A is able to infect multiple organisms, occasionally a “new” virus emerges, which is unrelated to the circulating strains and arises not from mutation, but from reassortment between 2 different subtypes of the virus, one of which was previously circulating only in an animal reservoirs. This is called antigenic shift and can result in strains to which most adults do not have pre-existing immunity. Antigenic shift can lead to influenza pandemics such as the infamous 1918 “Spanish flu”- which killed 40 million, the Asian flu 1957, and Hong Kong flu 1968, and recently the swine-origin 2009 H1N1 pandemic. Despite the devastating effects of a pandemic, the cumulative deaths during intervening years have been far greater than those associated with pandemics. Influenza B virus infects only humans and therefore is only able to undergo antigenic drift. Although most influenza virus infections are self-limiting, few other diseases have such an effect on absenteeism, hospital admission and economic loss.

1.2 Influenza Vaccines

Vaccination is the primary measure available to control influenza. The most widely available vaccines are based on technology over 50 years old and give inadequate protection to the most in need- the young and the elderly. Current vaccine production could be considered somewhat archaic- involving manufacture in chicken's eggs. With the threat of highly pathogenic avian influenza (HPAI) gaining the ability to transmit between humans, the need to develop new vaccine strategies is essential. Vaccine manufacturers need to be able to respond quickly to new emerging strains, which will probably necessitate an egg free system such as cell culture. Depending on the viruses included in the vaccine and the method of vaccine generation, egg based production may in any case be inappropriate for avian virus based vaccines which could be pathogenic to eggs. New vaccines will need to be highly immunogenic and provide protection to all age groups. This may demand the addition of adjuvants, few of which are currently licensed. The first part of this introduction will discuss at the current state of influenza vaccination and new advances in the field.

1.2.1 *Antiviral drugs*

In the case of an influenza outbreak, there are two classes of antiviral drugs available. Amantadine and rimantadine (only effective against influenza A) block M2 ion channels, which regulate the internal pH of the virion and are crucial during early virus replication. Amantadine has several adverse side effects including insomnia, headaches and dizziness which can lead to falls especially in the elderly. Although rimantadine has fewer side effects, it is not widely available. Furthermore, viruses can rapidly acquire resistance to both these drugs through single non-synonymous nucleotide changes in the membrane spanning domain of M2.

The other antiviral agents available are NA inhibitors. These drugs are effective against both influenza A and B. Zanamivir (Relenza) is administered by inhalation (which is less desirable for elderly use but more convenient for children), and may cause bronchospasm. Oseltamivir (Tamiflu) is administered orally and can induce vomiting if not taken after food. These drugs can reduce infection on average between 0.8-1.3 days. Resistance can occur but seems to be strain and subtype specific (Meijer, Lackenby et al. 2009).

The National institute for Clinical Excellence (NICE) has issued guidance on the use of antivirals; Amantadine is not recommended, whilst Zanamivir and Oseltamivir are not recommended in adults or children unless they are at risk, can start therapy within 48 hrs of the onset of symptoms, and it is known that influenza is circulating. There is clearly scope for improvement to available anti-influenza treatments, and effective vaccination should be both maintained and improved in order to deal with the threat of an avian pandemic and to alleviate morbidity in the interpandemic period.

1.2.2 Current Vaccines

1.2.2.1 Vaccine content

The emergence of new strains by antigenic drift demands the annual reformulation of the vaccines. Retrospective analysis of variants from past major epidemics shows that typically only 3 or 4 mutations in HA molecule are all that is required for a new epidemic strain to emerge (Smith, Lapedes et al. 2004). New variants are identified from epidemiological surveillance studies coordinated by WHO. A panel convenes in February in the northern hemisphere, September in the south, to predict the circulating strains likely to give most the most protection in the coming influenza season. Currently, two influenza A virus strains, representing both subtypes that currently circulate in humans (H1N1, H3N2) and one B strain are selected for inclusion in the

seasonal vaccine. Vaccine manufacturers then have approximately 6 months to produce and register the vaccine. Approximately 50 countries have government funded immunisation programmes (Nicholson, Wood et al. 2003) and approximately 350 million doses are available to some 1.2 billion people worldwide who are at risk

(http://www.who.int/vaccine_research/diseases/ari/en/index.html).

The industry is dominated by only a few manufacturing companies. In 2007, Sanofi Pasteur supplied 180 million doses of seasonal influenza vaccine, an estimated 40 percent of the world influenza vaccine market.

Vaccination results in a 30% reduction of mortality among elderly persons during influenza epidemics (Groenwold, Hoes et al. 2009). Recommendations for vaccination vary; and tend to be founded on rates of morbidity and mortality. Therefore annual vaccination of at risk groups; the elderly, those with chronic medical disorders, asthma, diabetes, immune-suppressed and carers is generally recommended (Zimmerman and Middleton 2007).

1.2.2.2 Inactivated vaccines

The segmented nature of the influenza genome means a vaccine seed can be generated from the reassortment of the WT epidemic strain and a high yield, laboratory adapted strain such as A/Puerto Rico/8/34 (PR8). The reassortant generally contains the six internal genes from PR8 and the surface antigen genes, HA and NA, from the selected vaccine strains. PR8 has been used as the recipient genetic backbone of the influenza A virus vaccine for more than 30 years (Kilbourne 1969).

No such lab adapted high growth master strains exists for influenza B viruses and these viruses are simply strain selected each year. Often yields of influenza B virus are lower than for influenza A as a result.

Trivalent inactivated vaccine (TIV) is the oldest licensed vaccine, its effectiveness was first evaluated in 1938 (Francis 1953) and contains partially purified HA and NA, from formaldehyde or β -propiolactone inactivated virions, detergent treated to give a split product. It is administered by intramuscular injection. When there is a good match between the selected vaccine strains and the circulating strains, the vaccine can confer up to 80% immunity in healthy adults less than 65 years of age (Nicholson, Wood et al. 2003; Subbarao and Katz 2004) and is usually well tolerated with very few adverse side effects. Protection is largely mediated by induction of neutralising serum Ab against HA. Substantial but not complete protection is seen in the elderly population. In young, naïve children, multiple injections are required for high immunity. The split vaccines, delivered intramuscularly, are poor inducers of local IgA in respiratory secretions and of cell mediated immunity, important in protection against airborne pathogens and recovery respectively.

1.2.2.3 Live attenuated vaccines

Whole, live vaccine, administered intranasally is potentially a better alternative to the split, intramuscular vaccine, as it should induce superior immunity more analogous to a natural virus infection. To reduce virulence these vaccines are designed to have limited replication in the upper respiratory tract. As with TIV, a master backbone strain is generated. However, in the case of live attenuated influenza vaccine (LAIV) the backbone confers an attenuated phenotype from internal genes. Vaccines are made from 6:2 reassortants of attenuated backbone and circulating HA and NA. There have been several strategies used to attenuate the backbone strains (Reviewed in (Subbarao and Katz 2004)).

Some non-human viruses are naturally attenuated in humans, for example, some avian viruses have restricted replication in primates and master backbone strains have been developed from these (Snyder, Clements et al. 1986). This overcomes the need for derivation or passage to attain attenuation.

A/Mallard/New York/6750/78 (H3N2) exhibited restricted replication in both nonhuman primates and human adults and was shown to be safe, genetically stable and immunogenic. However, in further clinical trials of vaccine strains based on avian master backbones possessed some residual virulence in infants and children, so use of host restricted attenuation has not been pursued (Steinhoff, Halsey et al. 1991).

Another approach was to generate temperature sensitive (*ts*) viruses in presence of the mutagen 5-fluorouracil, which showed restricted growth at 37°C (Mills, Chanock et al. 1971). They consequently have restricted replication in the lower respiratory tract of animals (37-38°C) but are able to replicate in the upper respiratory tract (32-33°C) and induce a humoral response that protects against lethal virus challenge in mice. Two master strains were developed but these proved genetically unstable and reverted to virulent phenotypes in clinical trials (Tolpin, Massicot et al. 1981).

The last method involves adaptation of the virus for growth at sub-optimal temperatures resulting in decreased virulence. This strategy has been used independently by both American and Russian investigators, to generate highly stable master strains, which have proved good candidates for LAIV.

In the US, A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 were used as master strains (Maassab 1967). Viruses were isolated on chick kidney cells and then adapted for growth at 25°C by 32 stepwise passages at successively lower temperatures. They replicate efficiently between 25°C and 33°C but not 37°C, thus they not only have a cold adapted (*ca*) but also a *ts* phenotype. In ferrets, there is no replication in the lungs and attenuation in the upper respiratory tract, with lower titres and decreased infection times compared to WT. A dose of 10^7 TCID₅₀ administered intranasally, is typically safe and immunogenic in all age groups.

In Russia, A/Leningrad/134/57 (H2N2) was passaged 20 times in eggs followed by a further 17 or 47 passages at reduced temperature 25-26°C (Ghendon, Klimov et al. 1981). The mutants again, are *ts* as well as *ca*, being unable to replicate in eggs at 37°C. A/Leningrad/134/17/57 is used in vaccines for adults, whilst A/Leningrad/134/47/57 is used for children 3-14 yrs, in a trivalent vaccine formation with B/USSR/60/69.

US and Russian strains have similar rates of infectivity in humans (Nicholson, Tyrrell et al. 1987). The attenuating mutations (*ca*) are found mostly in NP and the polymerase subunits, PB1 PB2 and PA (Jin, Lu et al. 2003; Hoffmann, Mahmood et al. 2005; Chen, Aspelund et al. 2006) (see table 1.2.1).

Table 1.2.1 Attenuating mutation in cold adapted viruses.

Adapted from (Subbarao and Katz 2004). For B/Ann Arbor/1/66 only cold adapting mutations are shown

Gene Product	A/Ann Arbor/6/60			A/Leningrad/137/57				B/ Ann Arbor/1/66		
	Amino acid Residue	WT	Ca	Amino acid Residue	WT	Ca 17	Ca 47	Amino acid Residue	WT	Ca
PB2	265	N	S	478	V	L	L	630	S	R
				490	S	-	R			
PB1	391	K	E	265	L	N	N	-		
	457	E	D	317	M	-	I			
	581	E	G	591	V	I	I			
	661	A	T							
PA	613	K	E	28	L	P	P	431	V	M
	715	L	P	341	V	L	L	497	Y	H
NP	23	T	N	341	I	-	I	114	V	A
	34	N	G					410	P	H
								509	A	T
M1	-			15	I	V	V	159	H	Q
								183	M	V
M2	86	A	S	86	A	T	T			
BM2	-			-				-		
NS1	153	A	T	-						
NS2	-			100	M	I	I			

LAIVs have been in use in Russia >50 years. MedImmune Vaccines Inc licensed and marketed Flumist in US in 2002. There have been several evaluations of the efficacy of LAIV (Palker, Kiseleva et al. 2004; Huber and McCullers 2006; Belshe, Ambrose et al. 2008) and comparisons with inactivated vaccine. Serum antibody titres are generally lower and mucosal IgA elevated, although in some trials protection was conferred without detectable serum or nasal antibody implying a CTL response. Generally, trials indicate that in adults LAIV is as effective at eliciting a protective response as inactivated vaccine. In the elderly it can be less immunogenic, possibly due to the limited replication of the vaccine virus, which may be a result of higher levels of pre-existing antibodies to influenza (Powers, Fries et al. 1991). Conversely, in naïve children a substantial level of protection can be elicited after just one dose (Arvin and Greenberg 2006) and children experienced more than 50% fewer cases of influenza illness when vaccinated with LAIV compared to TIV (Ashkenazi, Vertruyen et al. 2006; Belshe, Edwards et al. 2007; Rhorer, Ambrose et al. 2009). However in younger children 6–11 months, a small increase in the number of hospitalizations (1% increase) and wheezing (2.1%) were noted weeks to months after receiving the live vaccine. Although these are not thought to be related specifically to influenza or temporally related to LAIV, LAIV is not approved for use in children <2 years of age (Belshe, Ambrose et al. 2008).

Since LAIV contains live virus there exists the possibility of reassortment with native virus to produce new strains with unpredictable traits. Reassortants from avirulent strains have previously been able to produce pathogenic strains (Scholtissek, Vallbracht et al. 1979). Concerns have also been raised about the potential for the virus to transmit from vaccinated to non-vaccinated individuals and contact with immune-suppressed individuals is not advised. A recent study investigated the duration and extent of virus shedding following vaccination (Block, Yogev et al. 2008) and concluded that the amount of shedding depended on the specific vaccine strains and the level of pre-existing immunity in vaccine recipients. However, transmission was possible particularly

from younger children (<9 years old). The findings support the Advisory Committee on Immunization Practices recommendation that LAIV recipients should avoid contact with severely immunosuppressed. Despite this, ferrets immune compromised by treatment with dexamethasone and cytarabine, elicited improved responses (Huber and McCullers 2006) and a study in HIV infected children showed equivalent responsiveness between groups with different immunological status (Levin, Song et al. 2008). Also to be considered are the benefits of herd immunity due to virus shedding and transmission, which could increase the benefits of LAIV. Due to higher acquisition costs, LAIV costs more per dose than TIV; however, as a result of increased protection and reduced burden of disease, LAIV vaccination of children results in a net saving (Luce, Nichol et al. 2008).

1.2.3 The cell substrate

Although the existing vaccines are comparatively successful in inducing immune responses, there is evidently scope for improvement. Importantly, dependence on eggs is a major limitation for current production procedure. It takes between 3 and 6 months to develop the vaccines in eggs and efficiency is low, requiring 1-2 eggs per dose of vaccine. Careful planning is needed each year to ensure millions of fertile, embryonated eggs are available for vaccine production, and this is often unable to take into account calls for increased production. Furthermore, diseases in laying flocks can affect availability and variation in the quality of the embryos can effect vaccine production. Some highly pathogenic avian influenza (HPAI) strains such as H5N1 are lethal to chicken embryos, and insufficient yields are obtained, making eggs an inadequate method for production of vaccines against these viruses. Furthermore, in the event of a pandemic many birds may be killed by the virus or culled to limit the spread.

The SARS outbreak demonstrated that with increased air travel and globalisation, a pandemic could spread in weeks or days and highlights the

need for a system which can respond rapidly, should HPAI gain the ability for human-human transmission. Cell lines are the obvious alternative to egg based vaccine production. Cell cultures can be used to manufacture vaccine at any time of year, pertinent during the current H1N1 pandemic which emerged outside of the typical winter influenza season in the northern hemisphere. Since they are maintained in a strictly monitored and sterile environment, the risk of contamination, and thus bio burden and end toxin content, is greatly reduced. Consequently, the use of preservatives like thiomersal, which can cause potential side effects, can be decreased. Cell lines are strictly monitored by WHO, and licensed lines are free from contaminants. Moreover, cells can be grown in serum free media, eliminating the potential for allergic reaction to serum or chick embryo proteins. This is particularly relevant to whole, live vaccine production, which does not involve the complex purification process used for inactivated vaccine, and the virus is not purified away from cell proteins. Egg derived vaccines are contraindicated for those with egg allergies.

Many recent clinical isolates, while becoming more adapted to the human host exhibit altered receptor binding characteristics compared to strains that circulated shortly after the emergence of the H3 or H1 subtype more than 40 years ago (Medeiros, Escriou et al. 2001; Mochalova, Gambaryan et al. 2003; Thompson, Barclay et al. 2004). Since the receptor used for cell entry in eggs differs from that in the human respiratory tract, this has the consequence that fewer isolates are able to grow in eggs, and unless they acquire mutations in HA (Schild, Oxford et al. 1983). These can, confer changes to glycosylation patterns (Chen, Aspelund et al. 2008) and can give rise to mismatches in antigenicity between the egg grown human influenza strains and the circulating clinical virus (Mochalova, Gambaryan et al. 2003).

Cell cultures are easy to handle and scale-up and readily adjust to market needs. However, licensed cell lines have largely proved unpermissive for production of high yield influenza virus product, and many permissive lines used in the laboratory are refractory to licensing.

The efficiency of a cell culture depends upon its ability to generate high yields, and requires a well-optimised purification process. In the case of LAIV the capacity of cell line to produce infectious particles is also very important.

The most common cell lines used in the laboratory for the amplification and isolation of influenza viruses are Madin-Darby canine kidney (MDCK) or Vero (African Green Monkey Kidney). These are adherent cell lines and can be expanded for large scale manufacturing in a number of ways including fixed bed reactors, fluidised bed reactors and microcarrier culture.

1.2.3.1 MDCK cells

Although in most published comparisons fluid from embryonated eggs yields the highest virus titres (e.g. 3.9×10^9 PFU/ml (Tree, Richardson et al. 2001), yields from MDCKs remain respectable ranging from 3 to 10 fold less (Tree, Richardson et al. 2001; Audsley and Tannock 2005). Often these comparisons are carried out using the laboratory strain PR8 which has been specifically adapted to be high yield in eggs. If a cell culture adapted backbone strain was generated, equivalent yields could conceivably be achieved. Indeed, MDCKs may well prove a better substrate for more recent clinical isolates which grow less well in eggs. MDCK derived split vaccines have been tested in clinical trials for efficacy. There were no statistical differences between the immunogenicity (e.g. HAI titres) and post immunisation symptoms between subjects who were immunised with either egg and cell derived vaccine (Halperin, Nestruck et al. 1998). As mentioned above recent clinical isolates have a decreased tropism for egg propagation. In 2003/04 season the strain A/Fujian/411/02 was selected for inclusion in the trivalent vaccine. However, following generation of the vaccine seed the reassortant was unable to grow in eggs and an alternative had to be sought, resulting in delays to vaccine issue. Recent research shows that MDCK cells would have supported growth of the Fujian strain (Makizumi,

Kimachi et al. 2008). In terms of virus yield and vaccine efficacy, MDCKs pose a truly viable alternative to eggs. However, there are concerns about the safety of MDCKs due to the non-human origin, lack of documentation about their transformation history and oncogenic potential. The line has consequently been extensively characterised, shown to be free from detectable infectious agents (viral or microbial) and highly unlikely to induce tumours in humans (Kistner, Barrett et al. 1998; Genzel, Olmer et al. 2006). An assessment of the risk of inclusion of adventitious agents in the vaccine product demonstrated that MDCK cells are restricted in the number of mammalian viruses for which they support growth (Gregersen 2008), and testing by infection with >20 virus families and other pathogens, such as Mycoplasma and Chlamydia has shown the manufacturing process capable of reducing adventitious agents to non infectious level (Gregersen 2008). As such, Novartis, Solvay, GlaxoSmithKline and MedImmune have all developed and characterised proprietary cell banks. Moreover, Novartis and Solvay have extensively tested and characterized their cell banks according to USA, EU and ICH requirements. These vaccines are not inferior to those generated in eggs and some have been taken to clinical trials and licensed. In 2001 Influvac-TC, Solvay Pharmaceuticals, was approved in the Netherlands; Novartis have developed an MDCK based vaccine that is now on the market (Gregersen 2008; Doroshenko and Halperin 2009). The high permissivity of MDCKs makes the cell line an attractive prospect for vaccine manufacture. However, MDCKs are difficult to transfect; as such, they are less expedient for the generation of reverse genetics vaccines (see section 1.2.4.1). They have also been evaluated for generation of reassortant vaccine seeds and fell short of the yields attained in eggs. MDCK cells have also been shown to support the growth of an efficacious LAIV (Ghendon, Markushin et al. 2005; Liu, Hossain et al. 2008), an inactivated H5N1 vaccine (Hu, Weng et al. 2008) and an equine vaccine (Genzel, Olmer et al. 2006).

1.2.3.2 Vero cells

Vero cells are an African green monkey kidney cell line, which are already licensed for a range of other human vaccines including poliovirus, rabies and even an inactivated influenza vaccine from Baxter healthcare corp. The influenza vaccine generated in Vero cells is fully immunogenic (Youil, Su et al. 2004; Audsley and Tannock 2005; Ghendon, Markushin et al. 2005). A whole, formalin and UV inactivated, H5N1 vaccine has been developed from wt virus, which has proved immunogenic in both mice and guinea pigs (Kistner, Howard et al. 2007; Howard, Kistner et al. 2008). In clinical trials (against A/Vietnam/1203/2004), a relatively high dose (7.5-15 μ g) was able to induce neutralising and cross protecting antibodies to clades 1, 2 and 3 (Ehrlich, Muller et al. 2008). The addition of alum adjuvant did not induce a dose sparing effect. Generally, titres are at least a log lower than MDCKs, and maximum yields are achieved in 4 rather than 2 days. A key advantage in the use of Vero cells is their primate origin; since the cells are more human the number of cell line specific mutations are reduced (Romanova, Katinger et al. 2003). Furthermore, glycosylation of HA has been implicated in the antigenicity and immunogenicity of viral glycoproteins (Skehel, Stevens et al. 1984), and the infectivity of macrophages (Reading, Miller et al. 2000), this could be particularly relevant for LAIV and could decrease the efficacy of a vaccine.

The UK government has a sleeping contract with Baxter (now active) for 72 million doses of a whole, inactivated pandemic vaccine (Kistner, Howard et al. 2007) which will be produced in Vero cells (<http://www.parliament.uk/documents/upload/postpn331.pdf>).

An LAIV was developed that is attenuated by inclusion of a truncated NS-1. This vaccine is attenuated in most cell culture substrates via the induction of IFN- β (Steel, Lowen et al. 2009). However, since Vero cells are IFN

incompetent IFN response they would represent a suitable cell line for vaccines of this type.

1.2.3.3 PER.C6 cells

For commercial interest, a cell line to needs to be well characterised. Developed by the Dutch company Crucell N.V., PER.C6 cells are a recently derived cell line originating from the primary culture of foetal human retinoblasts immortalised with an E1A minigene from type 5 adenovirus. They can be grown in suspension, which eliminates the need for microcarriers, making scale-up easier than for adherent MDCK and Vero cells (Fallaux, Bout et al. 1998). PER.C6 cells support growth of a variety of influenza A and B strains and produce high titres (10^{10} TCID₅₀/ml) of virus in 4 days, in roller bottles and bioreactors (Pau, Ophorst et al. 2001). Preliminary data, suggests that passage of virus could result in higher yields of HA antigen. PER.C6 cells are licensed for the use of a variety of vaccines and therapeutics, including a West Nile virus vaccine (Samina, Havenga et al. 2007), a rabies vaccine (Marissen, Kramer et al. 2005), recombinant adenovirus vectors for gene delivery (Subramanian, Kim et al. 2007), production of recombinant proteins such as an HIV-1 gag protein vaccine (Ledwith, Lanning et al. 2006) and neutralizing monoclonal antibodies, e.g. SARS-CoV (SARS-AB) (Havenga, Holterman et al. 2008). Sanofi Pasteur purchased an exclusive license from Crucell N.V. in 2003 to research, develop, manufacture and market cell-based human influenza vaccines using PER.C6 cell line. Crucell retains the commercial rights for human vaccines in Japan and pandemic vaccine.

1.2.3.4 PBS-1

A recently developed cell line derived from 11 day old immortalized chick embryo cell line, termed PBS-1, is especially novel because it can support the

replication of influenza in the absence of exogenous trypsin. PBS-1 cells support the growth of recent isolates of human influenza A and B viruses as well as avian viruses to high titres (up to $10^{7.5}$ PFU/ml). The authors claim that PBS-1 cells outperformed other licensed cell lines: CEK cells, MDCK cells and Vero (Smith, Colvin et al. 2008). PBS-1 cells are free of exogenous agents, are non-tumorigenic, and are readily adaptable to a variety of culture conditions, including growth on microcarrier beads.

1.2.4 Vaccine content

1.2.4.1 The use of reverse genetics to generate the vaccine seed

The strains of virus used in the vaccine are updated every year. High growth 6:2 reassortants with the backbone strain need to be generated and selected. The process can be time consuming and unpredictable. In 1990 a technique to generate designer influenza viruses was developed. At this time, the ‘reverse genetic’ technique used helper viruses and exchanged synthetic segments derived from cDNAs one at a time into the influenza genome. RNA was transcribed *in vitro* from cDNA and contained RNP proteins purified from virus particles. The reconstituted RNPs were transfected into cells simultaneously infected with ‘helper’ virus. Reassortants containing the novel segment were then selected from the progeny which also contained abundant titres of helper virus progeny. Even with the use of tight selection markers such as antigenic differences or *ts/ca* mutations, the technique was laborious. Still, some candidate vaccines were generated using this technique (Li et al 1997).

Two laboratories independently came up with a procedure to “rescue” whole virus genomes entirely from viral cDNAs (Fodor, Devenish et al. 1999; Neumann, Watanabe et al. 1999). Cells are co-transfected with 8 plasmids encoding each of the viral genomic RNAs and 4 helper plasmids directing expression of viral mRNAs (fig 1.2.1). The 8 viral RNAs are under the control

of an RNA polymerase I (pol I) promoter; a hepatitis delta virus (HDV) genomic ribozyme or a polymerase terminator, downstream of the vRNA-coding region ensures that RNA is processed correctly from 3' end of the vRNA. The helper plasmids typically express proteins from a viral promoter such as that of CMV, which directs transcription by the host pol II apparatus, a poly A tail is added via a 3' polyadenylation site. The helper plasmids encode the three subunits of the viral RNA-dependent RNA polymerase complex (PB1, PB2, and PA) and the nucleoprotein (NP), and comprise the minimal set of viral proteins required for encapsidation, transcription and replication of the viral genome (Parvin, Palese et al. 1989). More recently, an 8 plasmid expression system was developed. The cDNA of each of the 8 influenza virus segments was inserted between the pol I promoter and the pol I terminator. The pol I transcription unit is flanked by the pol II promoter of the human cytomegalovirus and the polyadenylation signal of the gene encoding bovine growth hormone. After transfection of the eight expression plasmids, two types of molecules are synthesized. From the human pol I promoter, negative-sense vRNA is synthesized by cellular pol I. The synthesized vRNA contains the noncoding regions (NCR) at the 5' and 3' ends. Transcription by pol II yields mRNAs with 5' cap structures and 3' poly (A) tails; these mRNAs are translated into viral proteins. The ATG of the viral cDNA is the first ATG downstream of the pol II transcription start site. The orientation of the two transcription units allows the synthesis of both negative-sense viral RNA and positive-sense mRNA from one viral cDNA template (Hoffmann, Krauss et al. 2002). More recently still all the viral cDNAs have been combined onto a single plasmid for ease of transfection (Neumann, Fujii et al. 2005). The efficiency of recovery of virus may be enhanced by reducing the number of plasmids required for the transfection.

Due to the species specificity of the pol I promoter the earliest reverse genetics systems were ineffective in MDCK cells. Two labs cloned the canine RNA polymerase I (pol I) promoter from MDCK cells and exchanged the promoter regions of the respective reverse genetics systems; a human pol I promoter in a

Figure 1.2.1 Schematic of reverse genetics systems.

There are several reverse genetics systems available. Plasmids from each system are transfected into cells and virus recovered.

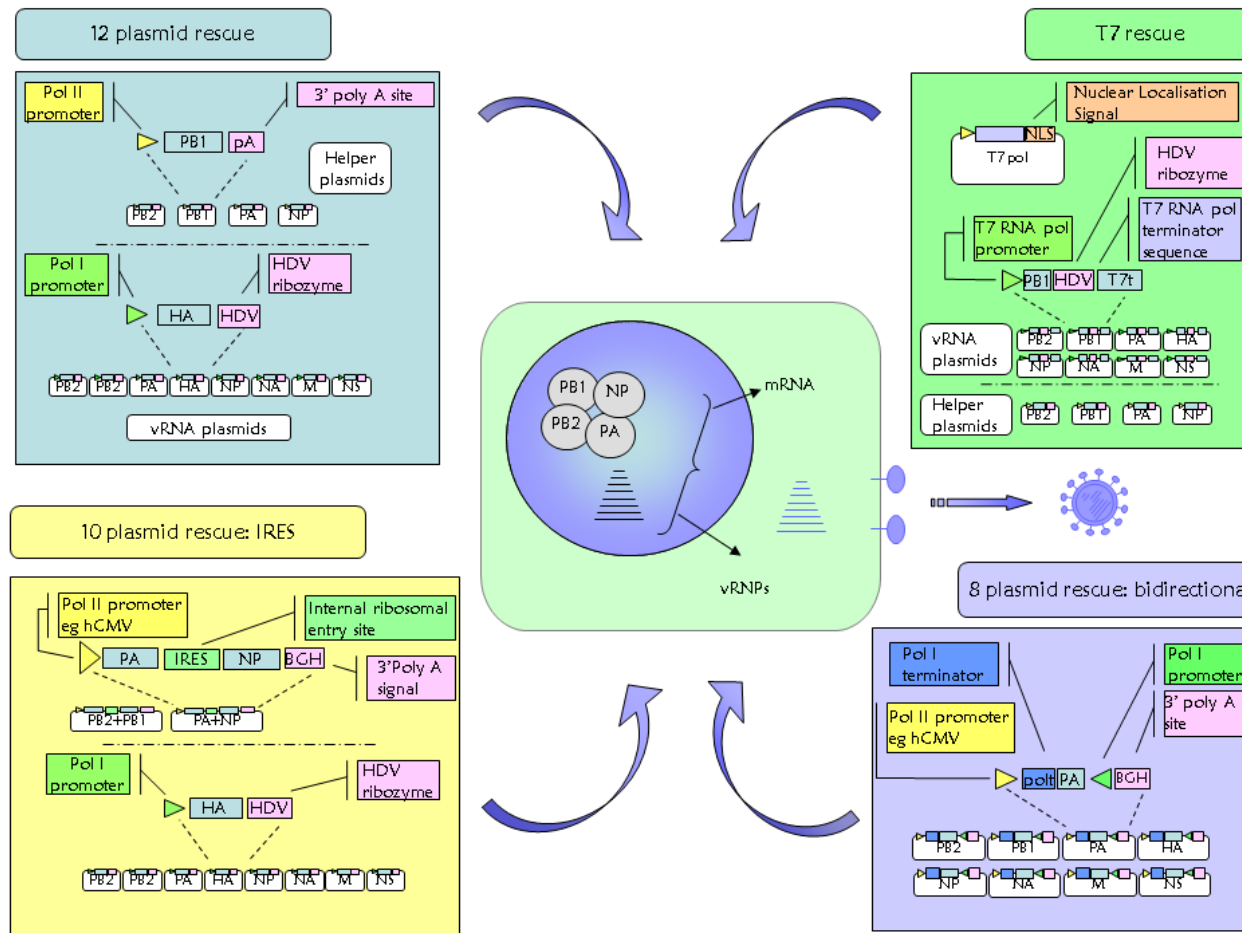
In the 12 plasmid system 8 plasmids encode each of the viral genomic RNAs and 4 helper plasmids directing expression of viral mRNAs of the minimal set of virus proteins required to drive virus replication (PB2, PB1, PA and NP). The viral RNAs are under the control of an RNA polymerase I (pol I) promoter; a hepatitis delta virus (HDV) genomic ribozyme or a polymerase terminator, downstream of the vRNA-coding region ensures that RNA is processed correctly from 3' end of the vRNA. The helper plasmids typically express proteins from a viral promoter such as that of CMV, which directs transcription by the host pol II apparatus. A poly A tail is added via a 3' polyadenylation site. This system has been adapted for use in both human and canine cells (MDCK) by using a species specific pol I promoters.

The 10 plasmid system (described in chapter 3) encodes the helper plasmids on just two plasmids, one carrying PB1 and PB2 separated by an internal ribosomal entry site (IRES) sequence of EMCV (Clontech) and another carrying NP and PA also separated by an IRES sequence. These double constructs are cloned between a CMV promoter and BGH polyadenylation site. The vRNA plasmids are of a similar design to the 12 plasmid rescue system.

The T7 system directs vRNA transcription from a T7 promoter. The T7 RNA polymerase is co-transfected with 8 vRNA plasmids.

The 8 plasmid system is bidirectional, the human pol I promoter, synthesizes vRNA. Proteins are expressed from a pol II promoter.

Figure 1.2.1 Schematic of reverse genetics.



cold adapted backbone (Wang and Duke 2007) or the chicken pol I promoter in a PR8 backbone (Murakami, Horimoto et al. 2008). Thus, providing MDCK cells can be transfected efficiently, they can be used to generate the vaccine seed, and represent a single cell suitable for all stages of vaccine manufacture. Both these labs report rescue titres in the range of 10^6 PFU/ml, equivalent to titres achieved by co-culture with 293T cells.

An alternative reverse genetics system based on the T7 promoter rather than pol I has also been described (de Wit, Spronken et al. 2007). The vector contains the T7 RNA polymerase promoter, hepatitis delta virus ribozyme sequence and T7 RNA polymerase terminator sequence. The system is not species specific since the T7 RNA polymerase is provided in the form of plasmid DNA that is co-transfected with the vRNA and has been used in cell lines from a variety of species including 293T, MDCK and QT6 cells. vRNA is transcribed from the T7 promoter in a negative-sense orientation and efficiency is improved when a T7 RNA polymerase with the inclusion of a nuclear-localization signal was used.

1.2.4.2 Novel reverse genetics strategies

Reverse genetics not only permits the generation of 6:2 reassortants but also has the potential for easy genetic modification of viruses. Individual segments can be manipulated to investigate the role of viral proteins on replication and dissemination, to generate higher growth, higher yielding viruses or perhaps viruses more immunogenic to humans, all of which have scope for improving vaccine production and efficacy. It also makes possible the generation of safer vaccine strains from HPAI strains by removing known pathogenicity determinants by genetic engineering. For example, the replication cycle of the virus requires cleavage of HA by host proteases to activate fusion and infect cells. Natural cleavage of the HA protein is performed by the trypsin-like enzyme Clara trypsin which is secreted from the Clara cells of the respiratory

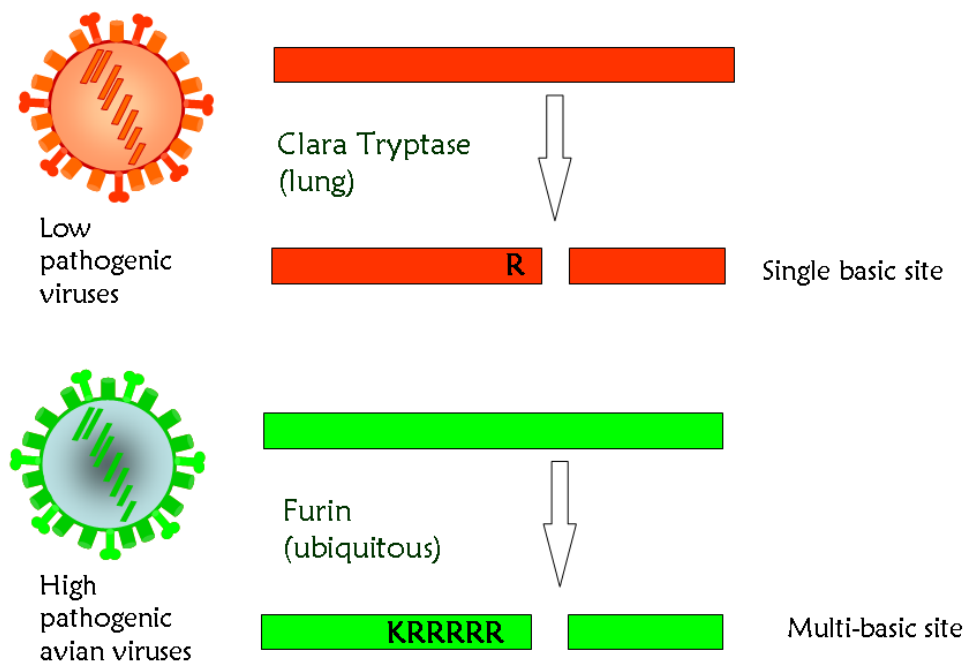
tract. This restricts virus replication to sites in the host where such enzymes are found. HPAI viruses possess multiple, basic amino acids (arginine and lysine) at their cleavage sites and these are cleavable by a ubiquitous protease, furin (fig 1.2.2) These viruses are thus able to replicate throughout the host, damaging vital organs and tissues causing more severe disease and death. These highly pathogenic HA are unsuitable for inclusion in a vaccine strain since they may confer pathogenicity to the backbone strain and pose the risk of recombination with other circulating human strains. For H5 and H7 vaccines, designed to protect against HPAI, viruses bearing HAs with the multibasic site deleted have been constructed (Stieneke-Grober, Vey et al. 1992; Li, Liu et al. 1999; Subbarao, Chen et al. 2003; Subbarao, Chen et al. 2003; Horimoto, Takada et al. 2006; Whiteley, Major et al. 2007).

A whole, live HA attenuated vaccine has been designed in which the HA cleavage site has been modified to be dependent on elastase for cleavage (Stech, Garn et al. 2005). The virus is a conditional mutant, attenuated *in vivo* where elastase like enzymes are not present naturally, but able to grow as well as wild type *in vitro*, in the presence of exogenous elastase. This means an epidemic strain can be converted to a live attenuated vaccine. The chief difference between this live attenuated vaccine strategy and the cold adapted strains is the self-limiting replication. A virus can cause shedding up to 11 days pi which increases the probability of reversion and recombination.

Initial elastase dependant mutants were made in a lab-adapted virus, A/WSN/33 (WSN). Replication was restricted to very few cycles and there were no revertants after five passages. Inactivation of elastase dependant mutants (WSN-E) with formalin abrogated the ability of the vaccine to protect against a wt challenge, so minimal replication of the WSN-E mutant is essential for protection. One intranasal dose was sufficient to generate HA inhibiting titres, serum IgG and mucosal IgA. However a high dose of WSN-E (10⁶ PFU) is required to protect against challenge.

Figure 1.2.2 Schematic of multibasic cleavage.

High pathogenic avian influenza (HPAI) viruses possess multiple, basic amino acids (arginine and lysine) at their cleavage sites and these are cleavable by a ubiquitous protease, furin.



Reverse genetics has also been used to develop an attenuated virus which could serve as a LAIV against classical swine influenza virus (SIV) (Masic, Babiuk et al. 2009). The HA cleavage site was mutated from arginine to valine at amino acid 345, which like the WSN-E mutant removes sensitivity to trypsin cleavage but retains elastase sensitivity. The resultant virus was attenuated in both mice and swine.

Reverse genetics has been used to assess growth determinants of the PR8 backbone in eggs compared to MDCK cell culture (Murakami, Horimoto et al. 2008). There are several different strains of PR8 maintained in laboratories across the world. Certain strains contain amino acids which confer higher growth characteristics in MDCKs which could be important if this cell line were used for the manufacture of vaccines. Two amino acid positions were found to be important for the MDCK high yield phenotype, S360 on PB2 and E55 on NS1. Only the PR8 (Cambridge) strain possesses S360 in PB2, all the others strains possess Y360. E55 NS1 is expressed in several strains and seems to confer improved IFN antagonism.

1.2.4.3 Baculovirus expression systems

Cell culture has been used for the production of whole virus, with similar inactivation and downstream processing steps, effectively replacing eggs directly with cell culture; however, this still requires generation of high yield reassorted virus. Novel approaches to vaccine manufacture, presentation and delivery are also being developed. Recombinant protein technology, the cloning of virus subunits into DNA vectors, enables the production of pure viral antigens such as HA and NA, in cell culture, as the active ingredient in vaccine (Wang, Holtz et al. 2006). Recombinant DNA vaccines have been shown to be highly effective inducers of both humoral and cellular immunity, most notably in the development of HIV vaccines (Barouch, Santra et al. 2000; Barouch, Santra et al. 2001). Several have been tested in clinical trials, and

indications are that they are safe to use, immunogenic and may confer more cross-reactivity than inactivated viruses.

Indeed a plethora of biotech companies have been investing in developing different cell lines, vectors, influenza strains and fusion proteins (Crawford, Wilkinson et al. 1999; Treanor, Wilkinson et al. 2001; de Wit, Munster et al. 2005; Wang, Holtz et al. 2006; Huber and McCullers 2008; Huber, Thomas et al. 2009). The protein-based approach represents a safe alternative to egg-based technology for pandemic vaccines; since it uses HA proteins as antigens, it does not require the large-scale production of potentially dangerous live virus. Purification is rapid- fermentation to 95% purity can be achieved in 6 hrs, with a yield of 57% (Wang, Holtz et al. 2006). Following the H5 influenza outbreak in Hong Kong in 1997, a vaccine based on a recombinant baculovirus, was prepared 4 months after isolation of etiological virus and was ready for trial before any other vaccine candidates (Treanor, Wilkinson et al. 2001).

Baculovirus DNA vectors have been applied to the development of influenza vaccines production and have been particularly employed in development of HPAI H5 vaccines. They can also be fused with other proteins with adjuvant or immune stimulatory effects.

A trivalent epidemic vaccine of full length, recombinant HA (rHA) H1N1, H3N2 and a flu B, marketed as FluBIØk has been developed by Protein Sciences Corp and UMN Pharma Inc (Huber and McCullers 2008). It has been developed in a high-yield SF9-derived insect cell line, expresSF+, in serum-free conditions (McPherson 2008). FluBIØk has recently been through phase III clinical trials which are awaiting publication. Phase II clinical trials showed that although high concentrations of protein were required (45 µg) to induce serum antibody response, even higher doses were well tolerated and were more immunogenic than the H3 component of an inactivated TIV, 15 µg Fluzone (Sanofi Pasteur) (Treanor, Schiff et al. 2006).

Chickens were effectively protected against H5N1 (Crawford, Wilkinson et al. 1999) and mice against H7N7 (de Wit, Munster et al. 2005) following inoculation with rHA vaccines, and these to some extent also conferred heterosubtypic immunity. The main drawback to rHA is the high doses required to induce neutralising antibody (two 90 μ g doses) and indeed thus far have proved less immunogenic than conventional virus based vaccines (Treanor, Wilkinson et al. 2001).

This has partly been explained by the conformation of the recombinant HA (rHA). HAs derived from insect cells are monomeric, whereas they naturally exists as trimers. Development of a trimeric and oligomeric HA has improved the immunogenicity (Wei, Xu et al. 2008).

A vaccine strategy combining rHA with LAIV, in a prime-boost approach shows promise for wide cross protection (Huber, Thomas et al. 2009). Priming with rHA followed by an LAIV boost, strengthened and broadened the antibody response, such that it elicited immunity against multiple strains within the H3 subtype. Although not a universal vaccine, since protection is limited to circulating viruses within a two-decade period, full protection within a subtype may be possible with the inclusion of multiple HAs from current and predicted future influenza strains.

rHA can be stored in PBS without the need for preservatives such as thiomersal (a mercury derivative which is required in egg-based vaccines), or antibiotics. The vaccine contains no live virus, and so does not require any biocontainment, nor chemical inactivating treatments, and could be the reason for decrease in observed in side effects compared with licensed vaccines (Wang, Holtz et al. 2006). If the problems with immunogenicity can be resolved, perhaps by delivery with adjuvants, this vaccine strategy could be a reliable, affordable and effective alternative.

1.2.4.4 Recombinant viruses

A number of other recombinant viruses have been used to direct expression of HA and generate live attenuated vaccines for influenza.

Parainfluenza 5 (PIV5) (formerly SV5) infects humans but is not associated with any known human illness. The HA gene from A/Udorn/72 (H3N2) was inserted into the PIV5 genome, as an extra gene between the haemagglutinin-neuraminidase (HN) gene and the large (L) polymerase gene. Infection with this recombinant was able to protect mice against infection with WSN-H3, a 7:1 reassortant virus containing all of the genome segments from A/WSN/33 virus except the HA from A/Udorn/72 (Tompkins, Lin et al. 2007). Vesicular stomatitis virus (VSV) expressing H5 has been used as an experimental vaccine vectors (Schwartz, Buonocore et al. 2007). A recombinant fowlpox virus co-expressing HA and NA (H5N1) genes was evaluated for its ability to protect chickens against intramuscular challenge with a lethal dose of HPAI (Qiao, Yu et al. 2003).

An adenovirus vector with E1/E3 deletion, expressing the HA gene from A/Vietnam/1203/2004, protected mice and chickens from wt H5N1 with broad and potent HA specific humoral and cellular immune response (Gao, Soloff et al. 2006), and pigs were protected from swine influenza virus by immunisation with an adenovirus H3N2 (Wesley, Tang et al. 2004). Studies in pigs and mice reveal adenovirus vaccines can afford cross protection in the absence of neutralising humoral immunity. Phase 1 clinical trials indicate Adenovirus vaccines to be safe (Van Kampen, Shi et al. 2005).

An important consideration for the use of vector based vaccines is vector-specific immunity, which could reduce the overall efficiency of a vaccine and poses a major drawback for the use of vectors in vaccination. However, in spite of the presence of pre-existing anti-adenovirus antibodies, these vaccines

have still proved efficient, suggesting vector directed immunity can be overcome. Should vector immunity become an issue, a wide range of human and simian adenovirus serotypes are being developed as alternative vectors (Bangari and Mittal 2006).

1.2.4.5 Influenza M2 protein

Since the origin of the next human pandemic is impossible to predict, the holy grail of the vaccine industry would be to produce a universal vaccine which cross-protects against all strains of influenza (Kaiser 2006; Hellemans 2008). The vaccines discussed above, still require sufficient homology between circulating and selected strains to give adequate protection. In 2003-4 season the vaccine strain A/Panama/2007/99 did not match circulating strain A/Fujian/411/2002, and vaccine protection was decreased by 50% (De Filette, Min Jou et al. 2005).

HA and NA are highly immunogenic, but antigen drift means new epidemic strains arise every 1-2 years and any HA based vaccine will need to be updated in response. An ideal vaccine candidate is one which gives full heterosubtypic protection between strains of influenza. The membrane protein, M2, is an integral membrane protein like HA and NA but is much smaller- only 97 amino acids. The M2 extracellular domain (M2e) is extremely highly conserved among human viruses, although there is more variation among the avian strains sequenced (Buckler-White, Naeve et al. 1986; Obenauer, Denson et al. 2006). Amino acid changes have been recorded in only two human influenza viruses sequenced since the first virus sequence in 1933 (A/WSN/33- H1N1)- 1 amino acid in PR8, 2 amino acids in A/Fort Monmouth/1/47 (Lamb and Lai 1981). Segment 7 encodes for both M1 and M2. Since M2 shares its first 9 amino acids directly with M1, and the next 15 in a different reading frame, this could constrain possible mutations and maintain conservation. M2 tetramers are present in low abundance on the virus particle and since it has a small

external domain may be shielded from interaction with antibodies and effector cells (Black, Rota et al. 1993).

The most prominent M2 vaccine is a recombinant vaccine derived from M2e fusion to hepatitis B core protein (M2eHBc) designed by Walter Fiers at Ghent University, Belgium (Neirynck, Deroo et al. 1999) which has recently been tested in phase I clinical trials as ACAM-FLU-ATM, in collaboration with Acambis, Cambridge, although the results are yet to be published. The authors claim it is highly immunogenic and provides protection against a potentially lethal virus challenge in mice (Neirynck, Deroo et al. 1999), especially in the presence of adjuvants and confers full protection when administered parenterally or intranasally (De Filette, Min Jou et al. 2005). The mechanism of protection from M2e vaccines is still unclear, but since protective immunity is transferred in serum it suggests that the humoral response confers protection (Neirynck, Deroo et al. 1999).

However, despite the apparent success of M2e vaccines in mice there has been limited evidence of testing in other animal models. An M2eHBc vaccine was tested in pigs. Surprisingly no protection was conferred when challenged with classical swine influenza virus (Heinen, de Boer-Luijtz et al. 2001; Heinen, Rijsewijk et al. 2002). Clinical signs and virus excretion were not reduced in vaccinated group compared to the control and vaccinated pigs showed more severe clinical signs, suggesting that M2e antibodies may exacerbate disease. Side by side testing of two M2 vaccine in mice provided equivalent protection (Fan, Liang et al. 2004), however differences in the level of protection were seen in rhesus monkeys (Fu, Grimm et al. 2009). Pigs are a natural reservoir for influenza and the pathology of disease in pigs and monkeys is more similar to humans than the pathology in mice. This highlights the need for stringent analysis and the use of adequate controls in assessing vaccine efficacy.

The M2e is currently well conserved in human influenza however this could be partly due to a poor M2e-specific Ab response and thus the absence of pressure

for change. Evolutionary pressure from vaccination against could induce mutations, rendering the vaccine ineffective. SCID mice were infected in the presence or absence of passive M2e-specific monoclonal antibodies (mAbs) (Zharikova, Mozdzanowska et al. 2005). Escape mutants emerged following weekly treatment with M2e mAbs, suggesting that M2e may not prove to be the universal vaccine its creator's desire.

1.2.5 Adjuvants

Intramuscular injection of inactivated vaccine does not elicit a strong immune response and nasopharyngeal administration of soluble antigen to mucosal surfaces is inefficient. Vaccination often result in a poor response in those with poor susceptibility, particularly the elderly who have had been repeatedly infected or vaccinated with influenza. The decline of the immune system related to impaired immunity in elderly is a growing problem with the global rise in the ageing population. Early H5N1 vaccine trials required 2 high doses (95 μ g compared to 15 μ g of annual trivalent vaccines to elicit an adequate serum antibody response (Treanor, Campbell et al. 2006). This could in part be due to immunological naivety of humans to avian subtypes of influenza. The newer generation of vaccines such as recombinant proteins and synthetic peptides, yield purer products which although considered safer, often prove less immunogenic. Unsurprisingly, attention has focused on ways to improve the immunogenicity and enhance the immune response to these vaccines. In the event of a pandemic, large quantities of vaccine need to be made available, and dose-sparing measures will greatly facilitate vaccine distribution (Radošević, Rodríguez et al. 2008). Recent developments involving the use of adjuvants has generated increased confidence in the ability to vaccinate against pandemic influenza.

Adjuvants have an important role to play in the efficacy of vaccines. Many substances have adjuvant properties, although their mechanisms of action are often still unknown and most are unlicensed due to their potential toxicity and

side effects. The type of adjuvant employed depends upon a number of factors ranging from the target species, type of antigen presented, route of inoculation, and type and duration of immune response required. Hence, there is currently no “universal” adjuvant, able to fulfil all requirements.

1.2.5.1 Aluminium hydroxide

Aluminium hydroxide generically called alum, was first described as an adjuvant 1926 by Glenny et al (as cited in (Singh, Ugozzoli et al. 2006). Aluminium hydroxide and aluminium phosphate have since been used as the preferred adjuvant in a number of human vaccines, although this tendency is perhaps more a result of its long-standing history, than its suitability. Alum is a good adjuvant for bacterial toxins e.g. diphtheria and tetanus, but has had limited success with newer vaccine antigens. It stimulates IgE, a Th₂ dependant response and is a poor inducer of Th₁, MHC class 1 restricted, cellular immune responses, in influenza vaccination this corresponds to high IgG1 levels and a low number of IFN- γ producing T cells. Th₁ responses are favourable for most infectious diseases (e.g. TB, HIV, HCV and influenza) which benefit from IL-2 induction and antiviral IFN activity. Mice vaccinated with inactivated vaccine plus alum adjuvant had higher antibody titres than unadjuvanted vaccinated mice. However, they suffered more severe weight loss and had significantly higher virus loads in their lung tissue than mice receiving TIV alone (Bungener, Geeraedts et al. 2008). The major difference between these groups of mice was the type of immune response induced, Th₂ instead of Th₁, indicating that a Th₁ response plays a major role in viral clearance. Alum adjuvants are therefore not well suited to the new generation of influenza vaccines, and yet they continue to be used (e.g. (Ehrlich, Muller et al. 2008; Nolan, Richmond et al. 2008; Cox, Madhun et al. 2009).

1.2.5.2 MF59

Emulsions have proved highly effective adjuvants however many are not well tolerated. A proprietary squalene based oil in water emulsion adjuvant MF59 (4.3% squalene, 0.5% tween, 0.5% span 85), has been developed by Novartis (Podda 2001). MF59 has been licensed for use in an influenza vaccine, FLUAD (Novartis), in 20 countries. In clinical trials it has been shown to be safe and tolerable, and stimulates both humoral and cellular immune responses (Gupta and Siber 1995). The systemic reaction between adjuvanted and non adjuvanted vaccines were similar, there were reports of pain, erythema and induration, at site of injection but these were short lived and self limiting and in most cases described as mild.

Trials comparing adjuvanted and non-adjuvanted licensed vaccines found both vaccines to be effective. In one trial (Frey, Poland et al. 2003) the adjuvanted vaccine gave rise to a fourfold increase in haemagglutination titres against the influenza B antigen component of the vaccine after 28 days and a fourfold increase in the H3N2 component titres 28 days after the second dose, although this effect was minimal after 180 days. Importantly the adjuvanted vaccine was more immunogenic in elderly recipients (≤ 75 years of age (Squarcione, Sgricia et al. 2003) (<64 years of age (Puig-Barbera, Diez-Domingo et al. 2004).

As mentioned previously avian viruses are poor immunogens in humans (Nicholson, Colegate et al. 2001) and have even proved poor in vaccination of poultry (Stephenson, Nicholson et al. 2003). Antibody responses in a number of cases to vaccines containing avian derived HA have been low. As an example, responses to A/Duck/Singapore (H5N3) were poor, even with two 30 μ g HA doses; but when adjuvanted with MF59, two 7.5 μ g doses were sufficient to give levels of antibody associated with protection. In further trials in humans (Stephenson, Bugarini et al. 2005), adjuvanted A/Duck/Singapore

(H5N3) induced antibodies against H5N1 viruses, having significant implications for potential avian influenza outbreaks.

Addition of MF59 to a subunit H5N1 vaccine A/Vietnam/1203/2004(H5N1) was able to significantly reduce the dose required, 15 μ g of adjuvanted vaccine elicited a better response than 45 μ g vaccine alone (Bernstein, Edwards et al. 2008). Two 7.5 μ g doses of a subunit H5N1 vaccine (A/Vietnam/1194/2004) (clade 1) elicited high antibody titres and induced heterologous Abs to a clade 2 virus, H5N1/turkey/Turkey/05 (Banzhoff, Gasparini et al. 2009). This response is sustained, priming subjects with MF59-adjuvanted H5 vaccine induces cross reactive B-cell memory responses that can be rapidly mobilized many years later by a single booster dose (Galli, Hancock et al. 2009). The data is encouraging for the priming strategy currently being considered by the WHO, which have previously been dismissed due to the lack of cross-reactivity of antibodies.

Use of MF59 is not limited to H5N1; it has also been shown to improve antigenicity of an H9N2 vaccine (Radosevic, Rodriguez et al. 2008). Much publicity surrounds the pandemic potential of H5N1, yet other influenza viruses circulate in bird or swine populations have equal potential for zoonosis as recently seen with pandemic H1N1 2009.

Senescence of the immune system means the ability to mount novel immune responses may be compromised in the elderly who often don't respond well to vaccination (Puig-Barbera, Diez-Domingo et al. 2004). The ability of MF59 to improve the immune responses of the elderly has also been widely tested (de Bruijn, Meyer et al. 2007). The addition of MF59 increased the immunogenicity of the influenza vaccines in the elderly but was particularly pronounced in those with low pre-vaccination titres, who are at greatest risk of developing severe influenza disease and vaccine failure (De Donato, Granoff et al. 1999; Banzhoff, Nacci et al. 2003). Systemic reactions were more common

in those receiving the adjuvanted vaccine, however, these were predominantly mild and transient, and none were serious.

MF59 adjuvanted vaccine (FLUAD, Novartis) also exhibited better immunogenicity in HIV patients than non adjuvanted vaccine (Agrimipal, Novartis) in an analysis that compared the geometric mean antibody titres (Durando, Fenoglio et al. 2008).

1.2.5.3 AS03

GSK has also produced a proprietary oil in water emulsion adjuvant (5% DL- α -tocopherol and squalene, 2% Tween 80 in the aqueous phase) as an antigen-sparing strategy (Leroux-Roels, Borkowski et al. 2007). Two doses of an inactivated split NIBRG-14 (A/Vietnam/1194/2004) vaccine were well tolerated and no serious adverse events were reported during the trial. There were no more participants reporting unsolicited symptoms in adjuvanted or non-adjuvanted vaccine groups. Adjuvantation substantially improved both seroconversion and seroprotection rates; after the second dose 86% of participants administered 3.8 μ g HA adjuvanted vaccine had neutralising antibody responses compared to only 65% of participants vaccinated with the highest dose of unadjuvanted vaccine (30 μ g) (Leroux-Roels, Borkowski et al. 2007; Sambhara and Poland 2007). The vaccine also induced heterosubtypic protection; the neutralising seroconversion rate was 77% after the second dose of adjuvanted vaccine, whereas in the non-adjuvanted groups, rates were just 9%. Interestingly, the two dose prime-boost regime was found to be more effective when the second dose was administered 6 months after the first rather than the typical 21 days (Schwarz, Horacek et al.).

Vaccine adjuvanted with AS03 was approved for use in Europe in May 2008 as Prepandrix and Pandemrix, to be administered as pre-pandemic and pandemic vaccines respectively (Jones 2009). Furthermore, 60 million doses of 2009

pandemic swine origin influenza vaccine being manufactured as a result of a sleeping contract between the government and GSK (<http://www.parliament.uk/documents/upload/postpn331.pdf>) is adjuvanted with AS03.

1.2.6 Conclusions

In the current swine-origin H1N1 2009 influenza pandemic, vaccines are being manufactured in both eggs and cell culture. Vaccine seeds have been produced by classical reassortment and reverse genetics (http://www.who.int/immunization/sage/4.Zhang_SAGE_vaccine_virus_reagents_-_Final.pdf). In the UK, the government has activated two sleeping contracts with Baxter and GSK (<http://www.parliament.uk/documents/upload/postpn331.pdf>). These are for 72 million doses from Baxter of a whole, inactivated Vero derived vaccine, and 60 million doses from GSK of egg grown, split vaccine.

There are many new adjuvants being developed, those mentioned here are a small cross-section. An adjuvant of some kind is certainly required to enhance the immunogenicity of split pandemic and recombinant vaccines, to stretch the number of doses available, and to enhance the immunogenicity.

The vaccine industry has the tools and techniques available to update the antiquated way in which influenza vaccines are made. Cell culture is a convenient alternative to eggs, and easy scale-up means the number of doses can be controlled as required. Reverse genetics means vaccines can be designed as and when new strains arise. The current pandemic has seen the fast tracking of new vaccines (<http://www.emea.europa.eu/pdfs/human/press/pr/46856809en.pdf>) and the use of reverse genetics, which is not covered by IP in the event of a pandemic, to generate vaccine seeds

(http://whqlibdoc.who.int/hq/2009/WHO_IVB_09.05_eng.pdf). Hopefully, the experience will leave us well prepared in the event of a more pathogenic pandemic in the future.

1.3 The innate immune response to influenza viruses

Multiple cytokines and chemokines are produced in response to a viral infection. The principal cytokines involved in the antiviral response are the interferons (IFN), a family of secreted cytokines which are grouped according to amino acid sequence into three classes; I, II and III. Type I IFNs comprising IFN- α and IFN- β were discovered in 1957 by Isaacs and Lindemann (Isaacs and Lindenmann 1957) in classic experiments performed at Mill Hill in London using influenza virus infected embryonated chicken eggs. There are multiple isoforms of IFN- α and between one and three forms of IFN- β plus other members such as - ϵ , - κ , - ω ; type I IFNs are ubiquitously expressed by all nucleated cells in response to infection. IFN- γ is the only type II IFN isoform and is secreted by mitogenically activated T cells and natural killer (NK) cells. The type III IFN group (IFN- λ) have been described quite recently and were previously known as IL-29, IL-28A and IL-28B (Sommereyans, Paul et al. 2008). IFN- λ expression is prominent in the stomach, intestine and lungs but less in other organs, suggesting that IFN- λ may contribute to the prevention of viral invasion through skin and mucosal surfaces.

IFN- α and - β activate a ubiquitously expressed heterodimeric type 1 IFN receptor, IFNAR, to initiate a signalling cascade which results in the induction of transcription of an assortment of IFN stimulated (IS) genes which set up an antiviral state in the cell.

1.3.1 JAK/STAT positive feedback

Activation of IFNAR induces the expression of antiviral response gene signals (Samuel 2001). STAT (signal transducer and activator of transcription) proteins are transcription factors that reside in the cytoplasm and become phosphorylated by JAK-1 (Janus kinase) and TYK-2 (tyrosine kinase) after IFNAR activation. Phosphorylated STAT-1 and -2 recruit IRF-9 (IFN response

factor) and translocate to the nucleus in a trimeric complex known as ISGF-3 (IFN stimulated gene factor). This binds to the cis-acting DNA element designated the IFN stimulated response element (ISRE promoter) which up-regulates the expression of many genes e.g. PKR (protein kinase R), Mx.

Cells also express SOCS (suppressor of cytokine signalling) proteins which are potent endogenous inhibitors of JAK/STAT signalling. SOCS-1 and -3 levels were elevated in influenza virus infection and SOCS deficient cells had elevated expression of ISRE genes and reduced viral titres (Pauli, Schmolke et al. 2008; Pothlichet, Chignard et al. 2008). The mechanism of SOCS induction by influenza virus is still unclear since two groups have described different pathways. (Pauli, Schmolke et al. 2008) show that SOCS-3 but not SOCS-1 mRNA induction is dependent upon NF- κ B and is independent of IFN. (Pothlichet, Chignard et al. 2008) demonstrated that SOCS-1 and SOCS-3 are up-regulated during influenza infection in a manner independent of both the TLR-3, and of the RIG-I/MAVS pathways. They also show that SOCS-1 and SOCS-3 differentially modulate inflammatory signalling pathways.

1.3.2 Pattern Recognition Receptors

To mediate an antiviral response, a cell must first detect the virus or viral antigen in the cell. Cellular pattern-recognition receptors (PRRs) recognise specific molecular structures on viral particles or products of viral replication known as pathogen associated molecular patterns (PAMPs) (Mogensen and Paludan 2005).

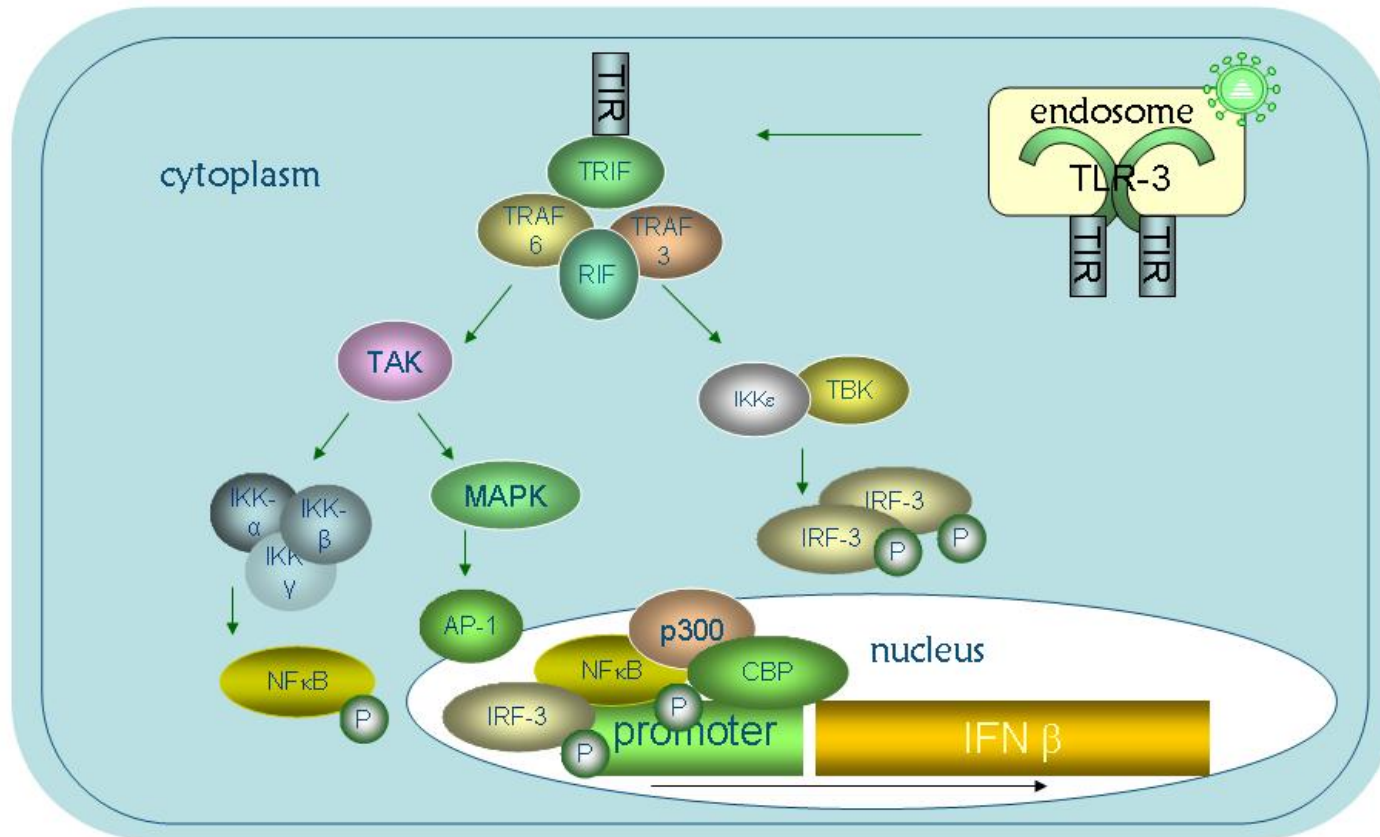
Toll receptors were first identified in *Drosophila melanogaster* as the major PRR involved in the innate immune response and this subject is comprehensively reviewed in (Koyama, Ishii et al. 2008; Randall and Goodbourn 2008). The mammalian homologue Toll-like receptors (TLRs) are membrane-bound PRRs and are extensively expressed on antigen presenting cells such as macrophages and dendritic cells. There are at least 11 TLRs which

are characterised by a Toll/IL-1 receptor (TIR) domain in the cytoplasmic tail and leucine rich repeats (LRR) in the extracellular domain which are believed to interact directly with their cognate ligands. TLRs are widely distributed on and in the immune cells and recognize and respond to a diverse range of molecules such as lipids, proteins, and nucleic acids derived from pathogens and damaged host cells (Ishii, Coban et al. 2005). Many TLRs are expressed on the cell surface and so are not involved in virus recognition. TLR-3, -7, -8 and -9 are expressed in endosomes. TLR-3 is able to detect dsRNA, TLR-7 recognises ssRNA and TLR-9 recognizes unmethylated DNA with a CpG motif (CpG-DNA).

1.3.3 TLR-3 signalling

Tyrosine phosphorylation and dimerisation of TLR-3 following exposure to dsRNA initiates two distinct pathways (Sarkar, Peters et al. 2004) (fig 1.3.1). Upon TLR-3 activation the TRIF (TIR (Toll-interleukin 1 receptor) domain-containing adapter inducing IFN- β) (also known as TIR domain-containing adapter protein TICAM-1) is recruited to the receptor and associates with TRAF-3, TRAF-6 (tumour necrosis factor (TNF) receptor-associated factors -3 and -6) and RIP-1 and -3 (receptor interacting protein) (Yamamoto, Sato et al. 2002; Meylan, Burns et al. 2004). Oligomerisation of TRAF-6 initiates its ubiquitin E3 ligase activity which polyubiquitinates itself and RIP-1. The polyubiquitin chains are recognized by TAB-2 and TAB-3 (TAK1-binding proteins -2 and -3) (Kanayama, Seth et al. 2004), which recruit TAK-1 (transforming growth factor β -activated kinase 1) to the complex (Wang, Deng et al. 2001). Polyubiquitinated RIP-1 is also recognised by a subunit of IKK, NEMO (NF- κ B essential modifier) (Ea, Deng et al. 2006; Li, Kobayashi et al. 2006) which recruits IKK (Wu, Conze et al. 2006). TAK-1 is then able to phosphorylate the IKK β subunit of the IKK complex (Wang, Deng et al. 2001), leading to the downstream phosphorylation of I κ B. Following subsequent ubiquitination and degradation, NF- κ B is translocated to the nucleus.

Figure 1.3.1 Schematic of TLR-3 signalling



The alternative signalling cascade involves the interaction of TRAF-3 with TANK (TRAF family member-associated NK- κ B activator) which associates with TBK-1 (TANK-binding kinase 1) and inducible I κ B kinase (IKK-i) (Sato, Sugiyama et al. 2003), which are able to phosphorylate IRF-3. Phosphorylated IRF-3 homodimerises, and translocates to the nucleus. This recruits the transcriptional co-activators p300 and CBP (CREB-binding protein) and the synthesis of IFN- β is induced. IFN- β triggers the expression of IRF-7, which up-regulates the synthesis of IFN- α .

Phosphorylation of TLR-3 also recruits and activates PI3K (phosphatidylinositol-3 kinase) (Sarkar, Peters et al. 2004), activating the downstream kinase Akt leads to full phosphorylation and dimerisation of IRF-3.

TLR-3 is more widely expressed than the other TLRs, being found in conventional dendritic cells (cDCs), macrophages and non-immune cells such as fibroblasts and epithelial cells. It recognises dsRNA, a predicted replication intermediate for ssRNA viruses, although it has yet to be detected in influenza virus infected cells (Weber, Wagner et al. 2006).

1.3.4 TLR-7 signalling

TLR-7 recognises ssRNA and signals through a TIR domain-containing adapter, MyD88 (myeloid differentiation factor 88). Upon exposure to its ligand, MyD88 forms a complex with IRAK-4 (interleukin-1-receptor (IL-1R)-associated kinase-4), IRAK-1, TRAF-3, TRAF-6, I κ k α and IRF-7 (Honda, Yanai et al. 2004; Kawai, Sato et al. 2004; Uematsu, Sato et al. 2005). TRAF-6 can then initiate the NF- κ B signalling pathway described for TLR-3. Alternatively IRF-7 can be bound and ubiquitinated by TRAF-6 and then interacts with RIP-1 (Huye, Ning et al. 2007). Recruited IRF-7 is phosphorylated by IRAK-1 and also possibly I κ K α (Hoshino, Sugiyama et al. 2006), and is then able to translocate into the nucleus (still in association with MyD88, TRAF-6 and IRAK-1), where it binds to

the ISRE. Expression of TLR-7 is however, restricted to plasmacytoid dendritic cells (pDCs), thus their involvement in influenza antigen recognition is limited.

1.3.5 RIG-Like Receptors

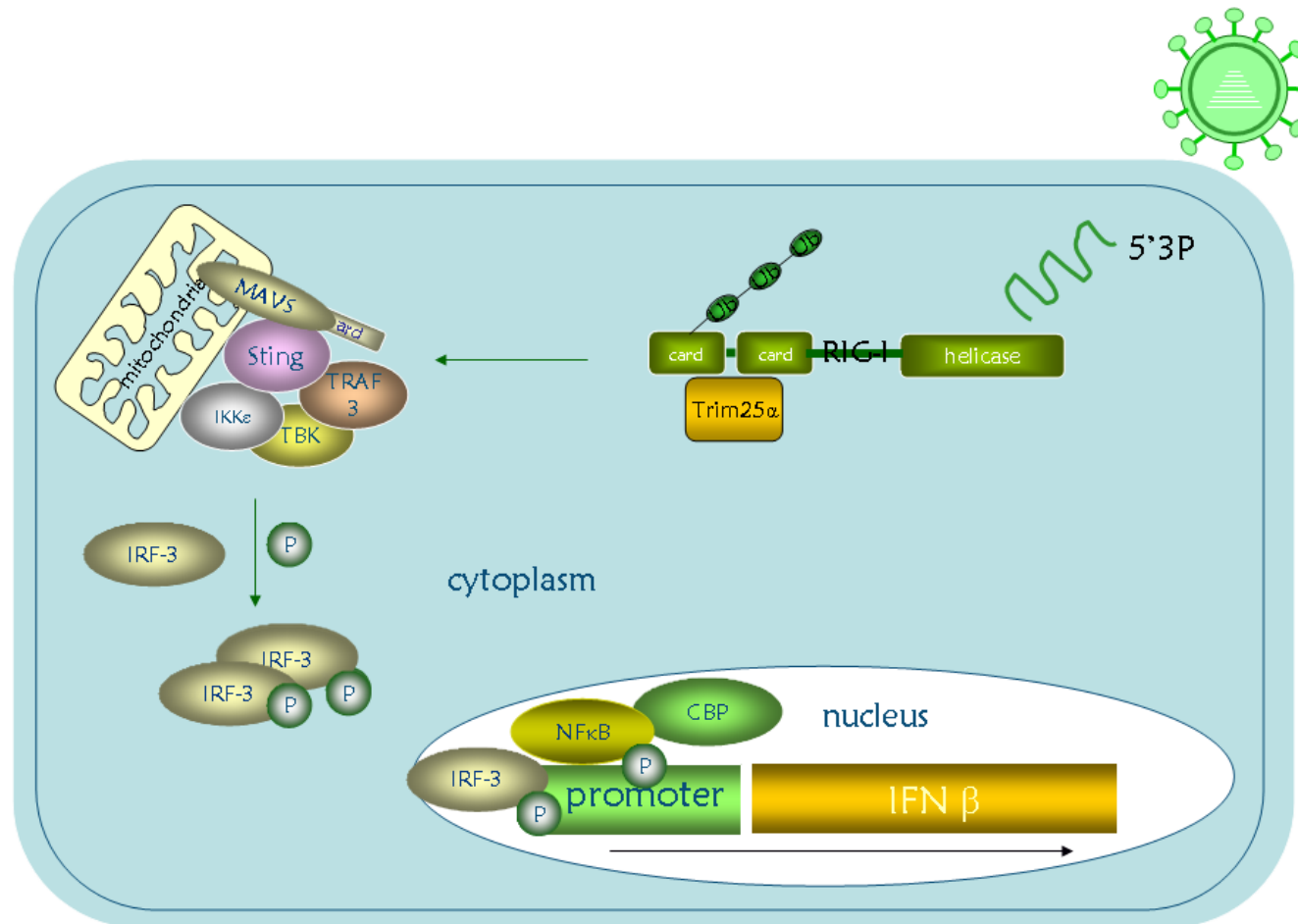
Respiratory viruses, such as influenza, target airway epithelial cells which have limited expression of TLRs. These cells instead encode other PRR, such as the intracellular RNA helicases RIG-I (retinoic inducible gene I (Yoneyama, Kikuchi et al. 2004) and MDA-5 (melanoma differentiation-associated gene-5 (Andrejeva, Childs et al. 2004) and the negative regulator, LGP2 (laboratory of genetics and physiology 2 (Murali, Li et al. 2008).

LGP2 has homology to RIG-I and MDA-5 but lacks a caspase recruitment domain (CARD), and binds dsRNA but not ssRNA with a higher affinity (Murali, Li et al. 2008). LGP2 inhibits virus induction of ISRE genes and NF- κ B-dependent pathways, and as such acts as a dominant negative regulator of RIG-I/ MDA-5 signalling through competitive sequestering of dsRNA, suggesting a role in negative feedback (Rothenfusser, Goutagny et al. 2005; Yoneyama, Kikuchi et al. 2005; Komuro and Horvath 2006)

The signalling pathways of RIG-I (figure 1.3.2) and MDA-5 are less well characterised than those of TLRs. Both RIG-I and MDA-5 have two N-terminal CARDs and a C-terminal DExD/H box RNA helicase, also known as the regulatory domain (RD).

The helicase activity is essential for MDA-5 and RIG-I but not LGP-2 indicating a differential mode of action (Bamming and Horvath 2009). A single-point mutation (K270A) in the ATPase site rendered RIG-I inactive in antiviral signalling, despite retention of RNA binding. Helicases unwind duplex nucleic acids by translocating along one of the strands of nucleic acid by ATP

Figure 1.3.2 Schematic of RIG-I signalling



hydrolysis (Myong, Cui et al. 2009). This suggests that the ATP-dependant dsRNA translocation activity is linked to sensing of the PAMPs.

Recently RIG-I has been the focus of intense research and the mechanisms of downstream activation are now more clearly defined than for MDA-5. RIG-I has been shown to associate with the actin cytoskeleton via the CARDs although the significance has yet to be determined (Mukherjee, Morosky et al. 2009). Engagement of the PAMP is thought to induce a conformational change (Yoneyama, Kikuchi et al. 2004), which exposes the CARDs and promotes the formation of homodimers (Childs, Andrejeva et al. 2009). Yeast 2-hybrid experiments showed no self-interaction suggesting that dimer formation does not occur via direct interaction and requires the binding of RNA to regulatory domain.

Following activation, RIG-I is ubiquitinated by two proteins; ubiquitination is not required for MDA-5 activation (Gack, Shin et al. 2007). RIG-I CARDs interact with tripartite motif protein 25 (TRIM25), an E3 ubiquitin ligase which contains a RING finger domain. The first and second card domains have distinct roles in RIG-I signalling. TRIM25 binds the first CARD (Gack, Kirchhofer et al. 2008), the C-terminal SPRY domain of TRIM25 then delivers K63-linked ubiquitin moieties to the second CARD (Gack, Shin et al. 2007). Mutation of T55I in the first CARD abolishes TRIM25 interaction, whereas mutation of K172R in the second CARD eliminates polyubiquitin attachment. TRIM25 expression did not result in ubiquitination of MDA-5 CARD. Polyubiquitination may facilitate the interaction of RIG-I with MAVS in a similar fashion to RIP and NEMO in TLR-3 signalling.

Another E3 ubiquitin ligase, Riplet (RING finger protein leading to RIG-I activation), shares 60% sequence homology to TRIM25, but ubiquitinates the C-terminal RD of RIG-I via a PRY domain (Oshiumi, Matsumoto et al. 2009). Since RIG-I C-terminal RD region is a negative regulator of CARD activity, it is suggested that ubiquitination by Riplet stabilizes RIG-I by inhibiting conversion

from the active to inactive conformation and providing sufficient structure for RIG-I to maintain the accessibility to TRIM25.

Upon ubiquitination of the CARD, RIG-I binds to MAVS (mitochondrial antiviral signalling molecule (Seth, Sun et al. 2005) (also known as IPS-1 (IFN- β promoter stimulator (Kawai, Takahashi et al. 2005)), VISA (virus-induced signalling adaptor (Xu, Wang et al. 2005) and Cardiff (CARD adaptor inducing IFN β (Meylan, Curran et al. 2005). MAVS localises to the mitochondrial membrane via its C-terminal, transmembrane domain. This localisation is essential for its function. The transmembrane domain mediates the dimerisation of MAVS which mediates association and subsequent activation of the E3 ligase, TRAF-3 (Tang and Wang 2009). MAVS N-terminal CARD is required for the interaction with RIG-I, CARD-CARD interactions may allow the stabilisation and accumulation of active MAVS homodimers which interact with TRAF-3.

Expression of a RIG-I splice variant (SV) is up-regulated upon viral infection and IFN- β pre-treatment. It carries a short deletion (amino acids 36–80) within the first CARD which renders it unable to bind TRIM25; CARD ubiquitination and downstream signalling ability is ablated. The RIG-I SV is detectable in a number of cell lines upon IFN- β treatment (Gack, Kirchhofer et al. 2008), and maintains the ability to bind MAVS, so competitively inhibits downstream RIG-I signalling.

Two novel proteins have recently been shown to interact with MAVS. NLRX1 interacts with the CARD domain of MAVS, and may compete with RIG-I as a negative regulator of IFN induction (Moore, Bergstralh et al. 2008). STING (Bowzard, Ranjan et al. 2009) interacts with both MAVS and RIG-I and is essential for IFN induction. It is proposed that STING recruits TBK-1 to the mitochondria, where it phosphorylates IRF-3. IRF-3 can also be phosphorylated by another I κ B kinase- IKK ϵ (Fitzgerald, McWhirter et al. 2003). IKK ϵ and TBK1 differ functionally in DNA virus-mediated IFN responses;

however, they are redundant in RNA virus-mediated IFN responses (Miyahira, Shahangian et al. 2009).

Alternative splicing of TBK-1 pre-mRNA which removes the kinase domain is induced by Sendai virus infection or priming with IFN- β (Deng, Shi et al. 2008) and results in negative regulation of IFN- β induction. Since binding to IRF-3 is maintained, an interaction with RIG-I that disrupts the subsequent interaction with MAVS is thought to occur, as opposed to prevention of IRF-3 activation.

Ultimately RIG-I signalling results in the activation of IRF-3 by phosphorylation and homodimerisation. The dimers translocate to the nucleus and initiate the synthesis of IFN- β as described for TLR-3 signalling by recruitment of the transcriptional co-activators p300 and CBP.

1.3.6 Pathogen Associated Molecular Patterns

As cytoplasmic RLRs, RIG-I and MDA-5 seem to maintain both unique and redundant roles of RIG-I and MDA-5 in signalling (Loo, Fornek et al. 2007). Their signalling pathways converge on MAVS activation which is essential for signalling via IRF-3 activation by both RLRs.

RIG-I is essential for IFN induction by negative sense single stranded RNA viruses such as influenza virus A and B, human respiratory syncytial virus (RSV), and Ebola virus. Whilst MDA-5 is required for the detection of PAMPs made by positive sense single stranded Picornaviridae such as Encephalomyocarditis (EMCV) (Gitlin, Barchet et al. 2006) and Paramyxoviruses (PIV5) (Childs, Stock et al. 2007). However, since both MDA-5 and RIG-I can transduce IFN triggered by dsRNA Reoviruses and the positive sense ssRNA Flavivirus, Dengue virus; it is not simply the species of RNA. Indeed the nature of the PAMPs still remains somewhat elusive.

The PAMP in an influenza infection was historically considered to be dsRNA, a predicted intermediate of viral replication, however detectable levels of dsRNA have yet to be observed in influenza infected cells by immunofluorescence or FACs (Pichlmair, Schulz et al. 2006; Weber, Wagner et al. 2006). One group (Kato, Takeuchi et al. 2006) suggested MDA-5 and RIG-I are differentially activated by different species of dsRNA; MDA-5 recognizing the dsRNA analogue, poly IC whilst RIG-I detects *in vitro* transcribed dsRNA. MDA-5 has been shown to be more readily activated by lower concentrations of poly IC than RIG-I in *in vitro* assays (Childs, Andrejeva et al. 2009), suggesting MDA-5 may be more sensitive to poly IC ligand.

RIG-I is activated by 5' triphosphate (5'3P) ssRNA in influenza infection (Pichlmair, Schulz et al. 2006). ssRNA lacking a 5'3P can bind RIG-I *in vitro* but is not able to activate signal transduction (Ranjith-Kumar, Murali et al. 2009) and treatment with calf intestinal phosphatase (CIP) to remove the 5'3P completely abrogated ssRNA IFN induction (Pichlmair, Schulz et al. 2006). More recent research suggests that short doubled stranded secondary structures may form within 5'3P ssRNA, which are important for RIG-I detection of the PAMP (Schlee, Roth et al. 2009; Schmidt, Schwerd et al. 2009). RIG-I α MEFs (Kato, Takeuchi et al. 2006; Loo, Fornek et al. 2007), dominant negative RIG-I (Matikainen, Siren et al. 2006; Siren, Imaizumi et al. 2006) and small interfering RNA (siRNA) knockdown of RIG-I (Guo, Chen et al. 2007; Mibayashi, Martinez-Sobrido et al. 2007) abrogate the response to influenza viruses suggesting that IFN induction by influenza PAMP is RIG-I dependant.

Ligand specificity may also be a result of length, since poly IC can be converted to a RIG-I ligand following shortening (Kato, Takeuchi et al. 2008). Thus, RIG-I and MDA-5 may be differentially activated depending on the PAMP length, and RIG-I and MDA-5 may selectively recognize short and long dsRNAs, respectively.

RIG-I is also the RLR involved in the detection of dsRNA lacking 5'3P in infection with Vesicular stomatitis virus (Kato, Takeuchi et al. 2008) and SeV (Hausmann, Marq et al. 2008). It seems that RIG-I interacts with poly IC and dsRNA in different ways, indeed poly IC has been shown to activate RIG-I variants which lack the C-terminal regulatory domain (Hausmann, Marq et al. 2008).

At the moment there exists a certain amount of discrepancy in the field detailing the precise nature of the PAMPs and the redundancy and differential roles of each RLR.

1.3.7 NS1 as an antagonist of IFN- β

Many viruses encode proteins which can negatively regulate the IFN response. Influenza encodes the non-structural protein NS1, on the smallest influenza segment (segment 8) whose mRNA upon splicing also encodes nuclear export protein (NEP, previously known as NS2). The numerous functions of NS1 are extensively reviewed in (Hale, Randall et al. 2008). An influenza virus with the NS1 protein knocked out induces very high levels of IFN, the growth of these viruses is attenuated in most cell lines but restored in the IFN incompetent cell line Vero, although still reduced compared to WT virus. The ability of NS1 to antagonise IFN has been shown to be strain specific (Hayman, Comely et al. 2006; Kochs, Garcia-Sastre et al. 2007).

NS1 is a small protein of ~26 KD, which varies in length from 230 to 237 amino acids, although some strains may encode further C-terminal deletions. There are two functional domains, an N-terminal RNA-binding domain (residues 1–73) and a C-terminal effector domain (residues 74–230) which mediates the interactions with host-cell proteins, and also stabilizes the RNA-binding domain. NS1 has been shown to form homodimers (Xia, Monzingo et al. 2009).

NS1 contains one or two nuclear localisation signals (NLS) depending on strain, and is found predominantly in the nucleus although again this is strain dependant and can depend upon the time post infection. The N terminal NLS 1 (residues 35-41) (Melen, Kinnunen et al. 2007) also contains the RNA binding domain (Arg-35, Arg-38 and Lys-41). Not all strains of influenza contain the second NLS in the C terminus (Lys-219, Arg-220, Arg-231 and Arg-232). There is also a third potential nucleolar localisation signal (Arg-224 and Arg-229).

Pre transcriptional regulation, suppressing the activation of IRF-3 dependant signalling is dependent on the RNA binding domain of NS1 (Arg-35, Arg-38 and Lys-41) and NS1 has been shown to interact with the dsRNA (Donelan, Basler et al. 2003) (Hatada and Fukuda 1992) (Wang, Riedel et al. 1999). Initially it was proposed that NS1 interacted with dsRNA sequestering it from PRR, but dsRNA has yet to be detected in infected cells (Weber, Wagner et al. 2006). NS1 co-precipitates with the PRR, RIG-I (Pichlmair, Schulz et al. 2006), and the stability of the interaction is enhanced by the inclusion of RNA (Mibayashi, Martinez-Sobrido et al. 2006). Residues E96/E97 of NS1 mediate an interaction with the coiled-coil domain (CCD) of TRIM25, and so block TRIM25 multimerisation and subsequent ubiquitination of the RIG-I CARD (Gack, Albrecht et al. 2009) which binds to the SPRY domain of TRIM25 (Gack, Shin et al. 2007). Thus NS1 and RIG-I do not compete for binding to TRIM25, instead the data suggest that a triple complex is formed in influenza virus-infected cells. The RNA binding residues R38 and K41 are important for the interaction with TRIM25, since the mutations R38A/K41A lack TRIM25 binding perhaps as a result of changes to NS1 conformation that affects its dimerisation or stability.

NS1 also prevents nuclear post-transcriptional processing and inhibits the nuclear export of mRNAs. The CPSF30 (C-terminal effector domain binds to cleavage and polyadenylation specificity factor) (Nemeroff, Barabino et al. 1998) mediated by residues P103, M106, and interacts with PABPII (poly(A)-binding protein II) via residues 223–237aa (Chen, Li et al. 1999). These

interactions interfere with CPSF30 binding of cellular pre-mRNAs; and thus inhibit the cleavage and polyadenylation of the 3'-end of host-cell mRNAs (Nemeroff, Barabino et al. 1998). Since the polyadenylation of influenza A virus mRNAs is independent of cellular 3'-end processing factors, viral mRNAs are not affected by CPSF30 inhibition. NS1 also forms an inhibitory complex with the host mRNA nuclear export machinery, NXF1/TAP, p15/NXT, Rae1/mrnp41, and E1B-AP5, which direct mRNAs through the nuclear pore complex (Satterly, Tsai et al. 2007) abrogating the export of cellular mRNAs, including those that encode anti-viral genes.

NS1 is also involved in splicing of vRNAs and the temporal regulation of vRNA synthesis and translation. NS1 protein can enhance transcription or translation of viral RNAs although this may be strain specific. NS1 also interacts with the IFN induced protein, PKR (protein kinase R) (Wang, Riedel et al. 1999; Salvatore, Basler et al. 2002).

1.3.8 Conclusion

Incongruent induction of IFN may play an important role in pathogenesis. Infection with HPAI viruses (such as H5N1 and 1918) can elicit hypercytokaemia or “cytokine storm” (Cheung, Poon et al. 2002; Heui Seo, Hoffmann et al. 2002; Zambon and Barclay 2002; R Deng, M Lu et al. 2008). It has been suggested that this contributes to the highly pathogenic phenotype of these viruses. Unrestricted viral replication in the face of cytokine induction leads to exponential cytokine secretion and corresponds with acute respiratory distress, lymphopaenia, haemophagocytosis and eventually multiple organ failure. However, recent publications suggest that lack of hypercytokaemia in transgenic mice did not alter the pathogenicity of these viruses (Droebner, Reiling et al. 2008). The contribution of individual inflammatory cytokines to the morbidity and mortality was not apparent, and they may either not be involved in the destructive cytokine response or are redundant to other factors elicited during such a response (Salomon, Hoffmann et al. 2007).

Understanding the balance between cytokine induction and viral antagonism, and the mechanism of induction, will provide insight into their effects on both virus and host, including their role in pathogenicity and severity of infection. Importantly, a better understanding of IFN induction by influenza viruses may reveal further targets for drug use and vaccines.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Plasmids

Construct	Description	Source
pPol I	pPol I vector for reverse genetics containing human pol I promoter and hepatitis delta virus ribozyme	Dr T. Zurcher GlaxoSmithKline
pPol I PR8 PB2	pPol I vector containing PR8 Segment 1 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 PB1	pPol I vector containing PR8 Segment 2 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 PA	pPol I vector containing PR8 Segment 3 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 HA	pPol I vector containing PR8 Segment 4 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 NP	pPol I vector containing PR8 Segment 5 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 NA	pPol I vector containing PR8 Segment 6 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 M	pPol I vector containing PR8 Segment 7 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)
pPol I PR8 NS	pPol I vector containing PR8 Segment 8 cDNA FluPAN backbone	(Whiteley, Major et al. 2007)

pPol I PR8 PB2 Crucell	pPol I vector containing PR8 Segment 1 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)
pPol I PR8 PB1 Crucell	pPol I vector containing PR8 Segment 2 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)
pPol I PR8 PA Crucell	pPol I vector containing PR8 Segment 3 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)

Construct	Description	Source
pPol I PR8 HA Crucell	pPol I vector containing PR8 Segment 4 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)
pPol I PR8 NP Crucell	pPol I vector containing PR8 Segment 5 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)
pPol I PR8 NA Crucell	pPol I vector containing PR8 Segment 6 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)
pPol I PR8 M Crucell	pPol I vector containing PR8 Segment 7 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)
pPol I PR8 NS Crucell	pPol I vector containing PR8 Segment 8 cDNA Crucell backbone	(Koudstaal, Hartgroves et al. 2009)

pPol I/pPol II PR8 PB2	pPol I/pPol II vector containing PR8 Segment 2 cDNA	(Whiteley, Major et al. 2007)
pPol I/pPol II PR8 PB1	pPol I/pPol II vector containing PR8 Segment 3 cDNA	(Whiteley, Major et al. 2007)
pPol I/pPol II PR8 PA	pPol I/pPol II vector containing PR8 Segment 4 cDNA	(Whiteley, Major et al. 2007)
pPol I/pPol II PR8 NP	pPol I/pPol II vector containing PR8 Segment 5 cDNA	(Whiteley, Major et al. 2007)

pPol I Vic PB2	pPol I vector containing A/Victoria/3/75 segment 1 cDNA	Dr T Zurcher GlaxoSmithKline
pPol I Vic PB1	pPol I vector containing A/Victoria/3/75 segment 2 cDNA	Dr T Zurcher GlaxoSmithKline
pPol I Vic PA	pPol I vector containing A/Victoria/3/75 segment 3 cDNA	Dr T Zurcher GlaxoSmithKline
pPol I Vic HA	pPol I vector containing A/Victoria/3/75 segment 4 cDNA	Dr T Zurcher GlaxoSmithKline
pPol I Vic NP	pPol I vector containing A/Victoria/3/75 segment 5 cDNA	Dr T Zurcher GlaxoSmithKline

pPol I Vic NA	pPol I vector containing A/Victoria/3/75 segment 6 cDNA	Dr T Zurcher GlaxoSmithKline
pPol I Vic M	pPol I vector containing A/Victoria/3/75 segment 7 cDNA	Dr T Zurcher GlaxoSmithKline
pPol I Vic NS	pPol I vector containing A/Victoria/3/75 segment 8 cDNA	Dr T Zurcher GlaxoSmithKline

Construct	Description	Source
pPol II Vic PB2	pPol II vector containing A/Victoria/3/75 segment 1 cDNA	Dr T Zurcher GlaxoSmithKline
pPol II Vic PB1	pPol II vector containing A/Victoria/3/75 segment 2 cDNA	Dr T Zurcher GlaxoSmithKline
pPol II Vic PA	pPol II vector containing A/Victoria/3/75 segment 3 cDNA	Dr T Zurcher GlaxoSmithKline
pPol II Vic NP	pPol II vector containing A/Victoria/3/75 segment 5 cDNA	Dr T Zurcher GlaxoSmithKline

pPol II WSN PB2	pPol II vector containing WSN segment 1 cDNA	Ervin Fodor, Oxford University
pPol II WSN PB1	pPol II vector containing WSN segment 2 cDNA	Ervin Fodor, Oxford University
pPol II WSN PA	pPol II vector containing WSN segment 3 cDNA	Ervin Fodor, Oxford University
pPol II WSN NP	pPol II vector containing WSN segment 5 cDNA	Ervin Fodor, Oxford University

pPol I/pPol II PR8 PB2	pPol I/pPol II vector containing PR8 Segment 2 cDNA	(Koudstaal, Hartgroves et al. 2009)
pPol I/pPol II PR8 PB1	pPol I/pPol II vector containing PR8 Segment 3 cDNA	(Koudstaal, Hartgroves et al. 2009)
pPol I/pPol II PR8 PA	pPol I/pPol II vector containing PR8 Segment 4 cDNA	(Koudstaal, Hartgroves et al. 2009)
pPol I/pPol II PR8 NP	pPol I/pPol II vector containing PR8 Segment 5 cDNA	(Koudstaal, Hartgroves et al. 2009)

pPol I B PB2	pPol I vector containing B/Beijing/87 segment 1 cDNA	(Jackson, Cadman et al. 2002)
pPol I B PB1	pPol I vector containing B/Beijing/87 segment 2 cDNA	(Jackson, Cadman et al. 2002)
pPol I B PA	pPol I vector containing B/Beijing/87 segment 3 cDNA	(Jackson, Cadman et al. 2002)
pPol I B HA	pPol I vector containing B/Beijing/87 segment 4 cDNA	(Jackson, Cadman et al. 2002)
pPol I B NP	pPol I vector containing B/Beijing/87 segment 5 cDNA	(Jackson, Cadman et al. 2002)
pPol I B NA	pPol I vector containing B/Beijing/87 segment 6 cDNA	(Jackson, Cadman et al. 2002)

Construct	Description	Source
pPol I B M	pPol I vector containing B/Beijing/87 segment 7 cDNA	(Jackson, Cadman et al. 2002)
pPol I B NS	pPol I vector containing B/Beijing/87 segment 8 cDNA	(Jackson, Cadman et al. 2002)
pPol II B PB2	pPol II vector containing B/Beijing/87 segment 1 cDNA	(Jackson, Cadman et al. 2002)
pPol II B PB1	pPol II vector containing B/Beijing/87 segment 2 cDNA	(Jackson, Cadman et al. 2002)
pPol II B PA	pPol II vector containing B/Beijing/87 segment 3 cDNA	(Jackson, Cadman et al. 2002)
pPol II B NP	pPol II vector containing B/Beijing/87 segment 5 cDNA	(Jackson, Cadman et al. 2002)

pPol I A/Sydney/97 HA	pPol I vector containing A/Sydney/97/5 segment 4 cDNA	(Hayman, Comely et al. 2006)
pPol I A/Sydney/97 NA	pPol I vector containing A/Sydney/97/5 segment 6 cDNA	(Hayman, Comely et al. 2006)
pPol I A/Sydney/97 NS	pPol I vector containing A/Sydney/97/5 segment 8 cDNA	(Hayman, Comely et al. 2006)

pPol I Panama HA	pPol I vector containing A/Panama/ segment 4 cDNA	Dr Alison Whiteley, Reading University
pPol I Panama NA	pPol I vector containing A/Panama/ segment 6 cDNA	Dr Alison Whiteley, Reading University
pPol I New Caledonia HA	pPol I vector containing A/New Caledonia/20/99 segment 4 cDNA	Isabelle Legastalois, Sanofi Pasteur
pPol I New Caledonia NA	pPol I vector containing A/New Caledonia/20/99 segment 6 cDNA	Isabelle Legastalois, Sanofi Pasteur

pPol I RD3 HA	pPol I vector containing A/Chicken/Italy/1347/99 segment 4 cDNA	(Whiteley, Major et al. 2007)
pPol I RD3 NA	pPol I vector containing A/Chicken/Italy/1347/99 segment 6 cDNA	(Whiteley, Major et al. 2007)
pPol I HK/97 HA	pPol I vector containing A/Hong Kong/156/97 segment 4 cDNA	(Koudstaal, Hartgroves et al. 2009)
pPol I HK/97 NA	pPol I vector containing A/Hong Kong/156/97 segment 6 cDNA	(Koudstaal, Hartgroves et al. 2009)

Construct	Description	Source
pPol I DI 220	pPol I vector containing 225 ntd of 3' and 220 ntd of 5' from PB2 A/equine/Newmarket/7339/79	(Duhaut and Dimmock 2002)
pPol I DI 317	pPol I vector containing 268 ntd of 3' and 317 ntd of PB2 from A/chicken/Dobson/27	(Duhaut and Dimmock 2002)

pCAGG A/Victoria/75 NS	pCAGG vector containing A/Victoria/3/75 segment 8 cDNA	(Hayman, Comely et al. 2006)
pCAGG A/England/24/00 NS	pCAGG vector containing A/England/24/00 segment 8 cDNA	Dr. Anna Hayman, Reading University
pCAGG A/England/492/95 NS	pCAGG vector containing A/England/492/95 segment 8 cDNA	Dr. Anna Hayman, Reading University
pCAGG A/Equine/Sussex/89 NS	pCAGG vector containing A/Equine/Sussex/89 segment 8 cDNA	Sarah Linton, Reading University

IFN- β luc	Fire fly luciferase reporter gene under the control of IFN- β promoter	Steve Goodbourn, St. Georges, London
ISRE LUC	Fire fly luciferase reporter gene under the control of ISRE promoter	Steve Goodbourn, St. Georges, London
β -gal	β -galactosidase reporter with a rat actin promoter and a 3' SV40 T Ag splice /polyadenylation site	Steve Goodbourn, St. Georges, London
Up-luc	A/Victoria/75/3 segment 7 with fire fly luciferase reporter gene under the control of 358 up promoter	Dan Brookes, ICL
pEGFP	Green fluorescent protein	Clontech

2.1.2 Primers

Primer Name	Sequence	Description
PB2-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GTC 3'	cloning segment 1
PB2-BsmBI R	5' ATA TCG CGT CTC TTA TTA GTA GAA ACA AGG TCG TTT 3'	cloning segment 1
PB1-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GCA 3'	cloning segment 2
PB1-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG CAT TT 3'	cloning segment 2
PA-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GTA C 3'	cloning segment 3
PA-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG TAC TT 3'	cloning segment 3
HA-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GGG 3'	cloning segment 4
HA-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG GTG TTT T 3'	cloning segment 4
NP-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GGT A 3'	cloning segment 5
NP-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG GTA TTT TT 3'	cloning segment 5
NA-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GAG T 3'	cloning segment 6
NA-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG AGT TTT TT 3'	cloning segment 6
M-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GTA G 3'	cloning segment 7
M-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG TAG TTT TT 3'	cloning segment 7
NS-BsmBI F	5' TAT TGG CGT CTC ACC CAG CGA AAG CAG GGT G 3'	cloning segment 8
NS-BsmBI R	5' ATA TGC CGT CTC TTA TTA GTA GAA ACA AGG GTG TTT T 3'	cloning segment 8
Universal primer 4a	5' TAT TGG CGT CTC ACC CAG CGA AAG 3'	cDNA RT PCR
Universal primer 4g	5' TAT TGG CGT CTC ACC CAG CGA AAA 3'	cDNA RT PCR

Primer Name	Sequence	Description
T7 Flu G F	5' GCA TCC TAA GAC TCA CTA TGG AGC GAA GAC AGG 3'	dsRNA
T7 Flu R	5' GGA TCC TAA TAC GAC TCA CTA TGG AGT AGA AAC AAG 3'	dsRNA

RIG-I F	5' TGT TTC CAG GGA TCC CAG CAA TGA 3'	RT PCR
RIG-I R	5' ACT TCA CAT GGA TCC CCC AGT CAT GGC 3'	RT PCR
MDA-5 F	5' GCT CAC AGT GGA TCC GGA GTT ATC AA 3'	RT PCR
MDA-5 R	5' CAC CAT CAT GGA TCC CCA AGC CTG GCC 3'	RT PCR
SOCS-3 F	5' TCA CCC ACA GCA AGT TTC CCG C 3'	RT PCR
SOCS-3 R	5' GTT GAC GGT CTT CCG ACA GAG ATG C 3'	RT PCR

Primers were synthesised by eurofins MWG operon.

2.1.3 Virus Preparations

Virus	Comments	Source
A/PR8/34	Wild type virus H1N1	Dr Alison Whiteley, University of Reading
A/Victoria/3/75	Wild type virus H3N2	Dr Wendy Barclay, Reading University
A/Sydney/5/97	Wild type virus H3N2- high inducer	Dr. Maria Zambon, HPA CfI
A/England/41/96	Wild type virus H3N2- high inducer	Dr. Maria Zambon, HPA CfI
A/England/919/99	Wild type virus H3N2- low inducer	Dr. Maria Zambon, HPA CfI
A/England/370/02	Wild type virus H3N2- high inducer	Dr. Maria Zambon, HPA CfI

Vic +Syd NS	7:1 Recombinant virus A/Victoria/3/75 + A/Sydney/5/97 NS	(Hayman, Comely et al. 2006)
Vic +Syd HA	7:1 Recombinant virus A/Victoria/3/75 + A/Sydney/5/97 HA	(Hayman, Comely et al. 2006)
Vic +Syd NA	7:1 Recombinant virus A/Victoria/3/75 + A/Sydney/5/97 NA	(Hayman, Comely et al. 2006)
Vic +Syd HA +NS	6:2 Recombinant virus A/Victoria/3/75 + A/Sydney/5/97 HA +NS	(Hayman, Comely et al. 2006)

PR8P	6:2 Recombinant virus PR8 A/Panama HA NA	(Hayman, Comely et al. 2006)
PR8 +Syd HA NA	6:2 Recombinant virus PR8 + A/Sydney/5/97 HA NA	(Hayman, Comely et al. 2006)
PR8 +Syd NS	7:1 Recombinant virus PR8 + A/Sydney/5/97 NS	(Hayman, Comely et al. 2006)
PR8P +Syd HA +NS	5:2:1 Recombinant virus PR8 + A/Panama NA + A/Sydney/5/97 HA +NS	(Hayman, Comely et al. 2006)
PR8P +Syd HA	5:2:1 Recombinant virus PR8 + A/Panama NA + A/Sydney/5/97 HA	(Hayman, Comely et al. 2006)
PR8P +Syd NA	5:2:1 Recombinant virus PR8P + A/Panama HA + A/Sydney/5/97 HA NA	(Hayman, Comely et al. 2006)

Virus	Comments	Source
PR8P +Syd NS	5:2:1 Recombinant virus PR8P + A/Sydney/5/97 NS	(Hayman, Comely et al. 2006)
RD3	6:2 A/PR/8/34 + A/Chicken/Italy/1347/99 HA & NA H7N1	(Koudstaal, Hartgroves et al. 2009)
RG14	6:2 A/PR/8/34 + A/Hong Kong/156/97 HA & NA H5N1	(Koudstaal, Hartgroves et al. 2009)

2.1.4 Cell Lines

Cell line	Description	Source
PER.C6	Human retina cell line immortalised with adenovirus	Crucell N.V.
Vero	African Green Monkey Kidney cell line	ATCC
MDCK	Madin Darby canine kidney cell	Glaxo Smithkline
MDCK-SIAT	Madin Darby canine kidney cell over- expressing 2-6 sialidase	HPA, (Matrosovich, Matrosovich et al. 2003)
293 T	Human embryonic kidney cell line	Glaxo Smithkline
Huh-7	Human hepatoma cell line	Dr. Michael McGarvey, IC
Huh-7.5	Human hepatoma cell line containing a point mutation in RIG-I TRIM25 binding domain	Dr. Michael McGarvey, IC
A549	Human lung cell line	ATCC
A549 IFN-luc	Human lung cell line stably expressing Fire fly luciferase reporter gene under the control of IFN- β promoter	(Hayman, Comely et al. 2006)

2.1.5 Antibodies

Antibody	Description	Dilution for Western blot	Dilution for IF	Source
α -NP	Mouse monoclonal antibody raised against Influenza A H7 NP clone 2F6.C9	1/300	1/300	Dr. Maria Zambon, HPA Cfl
α -NS1	Rabbit polyclonal raised against A/PR/8 NS1 clone V29	1/300	NA	Dr. Paul Digard, Cambridge University
α -M1	Mouse monoclonal antibody raised against Influenza A matrix protein	1/1000	NA	Oxford Biotechnologicals
α -IRF3	Rabbit polyclonal raised against IRF-3	NA	1/300	Santa Cruz
α -Stat-1 P	Goat polyclonal antibody raised against Stat-1 Tyr 701	1/500	NA	Santa Cruz
α -vinculin	Goat polyclonal antibody raised against vinculin	1/1000	NA	Santa Cruz
Goat α -rabbit FITC	FITC conjugated antibody used in confocal microscopy and flow cytometry	NA	1/500	Immunologicals Direct
Goat α -mouse Texas Red	Texas Red conjugated antibody used in confocal microscopy	NA	1/500	Oxford Biotechnologicals
Sheep α -rabbit HRPO	Horse radish peroxidase conjugated antibody	1/1000	NA	AbD Serotec
Goat α -mouse HRPO	Horse radish peroxidase conjugated antibody	1/1000	NA	AbD Serotec
donkey α -sheep/goat HRPO	Horse radish peroxidase conjugated antibody	1/1000	NA	AbD Serotec
Goat α -mouse β -gal	β -galactosidase conjugated antibody, used in blue cell assay	NA	1/400	AbD Serotec

2.1.6 Buffers, diluents and culture media

Unless otherwise stated, all reagents were obtained from Sigma

2.1.7 Virus and cell culture reagents

2.1.7.1 Virus diluent

3% FCS (Bio Sera International)

0.1% Pen/Strep (Gibco)

PBS

2.1.7.2 Plaque assay overlay

0.7x EMEM (Gibco)

0.303 % BSA (fraction V) (Gibco)

0.7xL-Glutamine (Gibco)

0.2% NaHCO₂ (Gibco)

0.7M HEPES (Gibco)

0.007% dextran DEAE (Sigma)

0.7x Pen/Strep (Gibco)

2.1.7.3 Agarose for plaque assay

2% purified agar in ddH₂O, Oxoid Ltd

2.1.7.4 RIPA buffer

50 mM Tris HCl pH 8

150 mM NaCl

1% NP-40

0.5% sodium deoxycholate

0.1% SDS

10% sodium deoxycholate stock solution (protected from light)

5 mM Iodoacetamide

2.1.8 Bacterial culture and cloning

2.1.8.1 LB

1% Oxoid tryptone

0.5% Oxoid yeast extract

0.5% NaCl

0.1% glucose

2.1.8.2 SOCS

2% Oxoid tryptone

0.5% Oxoid yeast extract

10 mM NaCl

2.5 mM KCl

10 mM MgCl_2

10 mM MgSO_4

20 mM glucose

2.1.8.3 TAE Buffer

4 mM Tris acetate

1 mM EDTA

2.1.8.4 6 × DNA loading buffer

0.25% bromophenol blue

30% glycerol

2.1.9 Assays

2.1.9.1 6 x SDS sample buffer

300 mM Tris HCl

600 mM DTT

2% SDS

0.1% bromophenol blue

50% glycerol

2.1.9.2 Tris-glycine SDS-Polyacrylamide Gel running buffer (10X)

25 mM Tris base

250 mM Glycine

0.1% SDS

pH 8.3

2.1.9.3 Transfer buffer

0.037 % (w/v) SDS

48 mM Tris

39 mM Glycine

20% (v/v) Ethanol added just before use

2.1.9.4 X-gal

10% in DMF

2.1.9.5 10X CN buffer

3 mM Potassium Ferricyanide
3 mM Potassium Ferrocyanide
1 mM MgCl_2
PBS

2.1.9.6 X-gal substrate

8.75 ml PBS
1 ml CN buffer
0.25 ml 10% X-gal

2.1.9.7 Molviol

6g glycerol
2.4g Molviol 4-8 Hoechst
6 ml ddH_2O
12 ml 0.2M TRIS pH 8
DAPI 1000x stock
DABCO final concentration 2.5%

Glycerol, Molviol and ddH_2O were dissolved O/N rotating. Tris was added and allowed to dissolve for 2-3 hours alternating between 50°C and rotation. Following centrifugation 2500 RPM x 5 minutes, the supernatant was transferred, DAPI and DABCO were added as required, aliquotted and stored - 20°C up to 6 months.

2.1.9.8 Lac Z buffer

60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

10 mM KCl

1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

2.2 Methods

2.2.1 Viral and cell culture techniques

2.2.1.1 Standard cell culture

Cells were passaged in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% FCS (v/v) and 1% penicillin streptomycin and were maintained at 37°C, 5% CO₂.

Media for A549 luc cells was supplemented with 2 mg/ml Genetcin (G418).

Media for MDCK-SIAT cells was supplemented with 0.4 mg/ml Genetcin (G418).

2.2.1.2 PER.C6 cell culture

PER.C6 cells were passaged in DMEM +10% FCS, 1% penicillin streptomycin and 10 mg/ml MgCl₂ and were maintained at 37°C, 10% CO₂.

2.2.2 Production of virus stocks

Viruses were grown in MDCK or MDCK-SIATs cells. All viruses were propagated at the 33°C, except A/Victoria/3/75 and A/Sydney/5/97, at 37°C. Cells were infected at an MOI of 0.01 in serum free DMEM (SF DMEM) for 1 hour. Media was replaced with SF DMEM containing 2.5µg/ml of porcine trypsin (Sigma). Viruses were harvested 2-3 days post infection by collecting supernatants. These were clarified by centrifugation at 2,500 RPM for 10 minutes, aliquotted and stored at -80°C.

2.2.3 Infection of cell lines with influenza viruses

PER.C6, Huh-7, Huh-7.5 and A549 cell lines were infected with influenza virus at the relevant MOI in SF DMEM. After 1 hour incubation at 34°C or 37°C the media was removed and cells washed with PBS, the media was replaced with 2% DMEM.

2.2.4 Haemagglutinin assay

Haemagglutination assays were performed by mixing two-fold dilutions of virus in PBS with equal volumes of 0.5% chicken, turkey or guinea pig red blood cell suspensions in PBS in 96 well V-bottom plates. These were incubated for 1 hour, at RT. The HA titre of the virus was determined as the well prior to the first well displaying a red blood cell pellet.

2.2.5 Plaque assay

Plaque assays were carried out in 12 well plates. Serial 10-fold dilutions of samples were created. 200 µl of diluted virus was incubated on MDCK monolayers for 1 hour at 34°C or 37°C depending on the virus. Media was then removed and 1 ml semisolid agar overlay media (17.5 ml plaque assay overlay, 2.1.7.2 plus 7.5 ml Oxoid agar, 2.1.7.3) containing 0.4 µg/ml trypsin (Worthington Biochemical Corporation) was added to each well. These were incubated at 34°C or 37°C for 3 to 4 days and then visualised by staining with crystal violet stain. Plaques were counted and used to determine viral titres in terms of PFU/ml.

2.2.6 Transfections

2.2.6.1 PER.C6 cell transfection

Adherent PER.C6 cells in a 6 well plate in 2% DMEM were transfected with at a 2:1 ratio reagent (μ l): μ g DNA, poly IC or RNA. Generally 0.5 μ g plasmid (e.g. IFN luc) and 0.25 μ g b-gal was used. Lipofectamine was added to 250 μ l Optimem and the plasmids added to 250 μ l Optimem. These were incubated 5 minutes RT, combined and incubated a further 20 mins, then added drop wise to cells, and incubated for 24-48 hours. Volumes and concentrations were scaled appropriately for smaller wells.

2.2.6.2 A549 cell transfection

A549 cells in a 6 well plate in 1.5 ml/well of 2% DMEM media were transfected with plasmids. Generally 0.5 μ g LUC reporter and 0.25 μ g b-gal reporter. These were combined in a total volume of 100 μ l/reaction of serum free DMEM, in a 1.5 ml Eppendorf. 12 μ l of Polyfect (QIAGEN) was added, vortexed for 15 seconds, and incubated for 10 minutes at RT. 600 μ l of 10% DMEM added to the Eppendorf and pipetted gently twice. The transfection mix was added directly to each well and incubated at 37°C for 24-48 hours. Volumes and concentrations were scaled appropriately for smaller wells.

2.2.6.3 Huh-7 cell transfection

Huh-7 cells were transfected following the same protocol used for PER.C6 cells.

2.2.7 Virus rescue

2.2.7.1 Co-culture virus rescue

293T cells in DMEM+ 3% FCS in 12 well plates, 70-80% confluent were transfected with 12 plasmids for virus rescue and a ratio of 3:1 Fugene reagent: DNA. 20 μ l Fugene was added to 200 μ l SF DMEM. Incubated 5 minutes, RT. Plasmids required for virus rescue as below were added and incubated a further 15 mins, then added drop wise to cells. These were incubated overnight at 37°C. Confluent MDCK cells were detached using trypsin and cells resuspended in 20 ml DMEM +10% FCS + pyruvate/75 cm² flask. 4 ml MDCK cell suspension was transferred to a 25 cm² flask. Transfected 293T cells were washed in PBS and detached by adding 5 drops of PBS, EDTA, trypsin. 1 ml MDCK cell suspension was added to the detached 293T cells, mixed and transferred to 25 cm² flask. These were allowed to adhere for 6 hours at 37°C. Cells were washed in serum-free DMEM and then incubated 5 ml serum-free media + pyruvate with 2.5 μ g/ml trypsin, 37°C (A/Victoria/75 backbone viruses) or 33°C (A/PR/8 backbone viruses and influenza B) until cytopathic effect was observed. Samples were clarified by centrifugation, 3000 RPM for 3 minutes, and stored -80°C.

The amounts of each plasmid used for the rescue are as follows:

vRNA Plasmids:

pPol I PB1	0.5 μ g
pPol I PB2	0.5 μ g
pPol I PA	0.5 μ g
pPol I HA	0.5 μ g
pPol I NP	0.5 μ g
pPol I NA	0.5 μ g

pPol I M	0.5 μ g
pPol I NS	0.5 μ g

Helper Plasmids:

pCMV PB1	0.5 μ g
pCMV PB2	0.5 μ g
pCMV PA	0.05 μ g
pCMV NP	1 μ g

2.2.7.2 PER.C6 virus rescue

For rescue in PER.C6, adherent cells in DMEM +2% FCS in 6 well plates were transfected with 12 plasmids for virus rescue. Lipofectamine 2000 2:1 ratio reagent: DNA. 26 μ l Lipofectamine was added to 250 μ l Optimem and double the amount of the plasmids described above added to 250 μ l Optimem. These were incubated 5 mins RT, combined and incubated a further 20 minutes, then added drop wise to cells. The transfection reagent was removed after 3 hrs and cells resuspended in 2 ml virus growth media (2/3 adenovirus expression media-AEM, 1/3 VPSF) +3 μ g/ml trypsin. Cells were then shaken 80 RPM (Shaker flask table IKA KS 260). Samples were clarified by centrifugation, 3000 RPM for 3 minutes, and stored -80°C.

2.2.8 Molecular biology techniques

2.2.8.1 vRNA extraction

vRNA was extracted using the QIAamp viral RNA mini kit (QIAGEN) or TRIzol LS (Invitrogen) according to manufacturer's instructions. For the QIAamp kit 280 μ l of virus stock was lysed using the AVL buffer containing Raisin

(Promega). This was bound to silica membrane, washed and eluted in 30 μ l of AVE elution buffer. vRNA was stored at -20°C. For TRIzol LS protocol 250 μ l virus preparation was combined with 750 μ l TRIzol LS. 200 μ l of chloroform was added, shaken 15 seconds, incubated RT for 2-3 minutes and centrifuged at 12,000g, 4°C, for 15 minutes. The supernatant was removed using RNase free tips, and transferred to a clean Eppendorf. An equal amount of isopropanol was added and incubated RT for 10 minutes, then centrifuged 12,000 RPM, 4°C, for 15 minutes. The supernatant was removed and the pellet washed in pre-chilled -20°C 75% ethanol, and centrifuged 7,500g, 4°C, for 5 mins. The pellet was air dried for 10 minutes and dissolved in 50-100 μ l nuclease free water.

RNA was quantified by spectrophotometry by measurement of absorbance at 260 nm. Concentration (μ g/ml) = $A_{260} \times \epsilon$ where for RNA ϵ = 44.19

2.2.8.2 RNA extraction

Total cellular RNA was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. Cells were washed with PBS, lysed in 1 ml TRIzol, transferred to an RNase free Eppendorf tube. Following addition of 200 μ l of chloroform, the protocol is the same as for TRIzol LS RNA extraction.

2.2.8.3 Reverse Transcription

Generation of cDNA by reverse transcription of RNA was performed using Promega reagents. A reaction mix consisting of 2 μ g of RNA, 0.5 μ g of oligo dT primers (Promega), nuclease free water to 15 μ l. The mix was incubated 70°C x 5 minutes, then 5 μ l 5x RT buffer, 1.25 μ l of 10 mM dNTP mix (Promega), 200 units M-MLV RT 200 U/ μ l (Promega), 25 units RNasin (Promega) was added

and mixed by flicking. This was incubated 42°C for 1 hour, then stored -20°C until required for PCR.

2.2.8.4 PCR amplification

PCR was performed with KOD Taq (Novagen) or GoTaq green master mix (Promega). For cDNA amplification, 2 µl was used, for plasmid amplification 200 ng was used.

For KOD, a 25 µl PCR mix was made consisting of 2.5 µl 10X Buffer for KOD DNA Polymerase, 0.4 µM forward and reverse primers, 2.5 µl dNTPs 2 mM, 0.2µl KOD polymerase (2.5 U/µl), nuclease free water to 25 µl.

30 cycles of: 95° C for 15 seconds, optimised annealing temperature for 5 seconds/ 1 Kb DNA, 72° C for 30 seconds.

For GoTaq, 12.5 µl 2X GoTaq® Green Master Mix, forward and reverse primers, 0.25 µM, nuclease free water to 25 µl. 25 cycles was used for semi-quantitative RT-PCR

Cycling conditions were: 95° C for 2 minutes, 30 cycles of 95° C for 30 seconds, optimised annealing temperature for 1 minute/ Kb DNA, 72°C for 30 seconds, followed by a final extension of 72° C 5 minutes

2.2.8.5 *In vitro* transcribed dsRNA synthesis

In vitro transcribed dsRNA was synthesised using T7 RiboMAX Express RNAi System (Promega). Two complementary RNA strands were synthesized from DNA templates, in this instance a plasmid encoding an influenza DI (kindly provided by Nigel Dimmock). T7 RNA polymerase promoters were added at the 5' ends of both DNA strands with amplification by PCR with primers containing the T7 promoter sequence at the 5' end. Generating the two DNA

templates requires two separate PCR amplifications, each containing one gene specific primer and the converse T7 primers. PCR was performed with KOD as section 2.2.8.4; the first 10 cycles were performed at an annealing temperature 5°C above the melting temperature of the gene-specific sequences, followed by 25 cycles of annealing approximately 5°C above the melting temperature of the entire primer, including the T7 promoter. A 20 µl reaction was set up at RT included: Express T7 2X Buffer 10, linear DNA template (~1 µg total; 1-8 µl, nuclease free water 0-7 µl, T7 Express Enzyme Mix, 2 µl to a final Volume of 20 µl. the reaction was mixed gently and incubated at 42°C, since the RNA contains a high level of secondary structure, for 30 minutes. The RNA strands were annealed by mixing equal volumes of complementary RNA reactions together and incubated at 70°C for 10 minutes, then slowly cooled to room temperature (~20 minutes). The DNA template was removed by digestion with RQ1 RNase-Free DNase. The RNase Solution was diluted 1:200 in nuclease free water. 1 µl freshly diluted RNase Solution was added to 1 µl RQ1 RNase-Free DNase per 20 µl reaction volume, and incubated for 30 minutes at 37°C. 0.1 volume of 3M Sodium Acetate (pH 5.2) and 1 volume of isopropanol was added and incubated on ice for 5 minutes, then centrifuged for 10 minutes, 13,000 RPM. The supernatant was aspirated and the pellet washed with 2 volumes of cold 70% ethanol. The pellet was air dried for approximately 15 minutes RT, and resuspended in nuclease free and stored -20°C.

2.2.8.6 Site directed mutagenesis

Site directed mutagenesis was performed using the Quikchange XL site directed mutagenesis kit (Stratagene), following the manufacturers protocol. The PCR reaction mixtures comprised of 10 ng of DNA template, 125 ng of each of forward and reverse primers, 1 µl 10 µM dNTPs, 1x reaction buffer, 3 µl Quikchange solution, 2.5 units of Pfu turbo DNA polymerase and made up to 50 µl with sterile water. The PCR was carried out using an initial denaturation

stage of 1 minute at 95°C, followed by 18 cycles of denaturation at 95°C for 50 seconds, annealing at 60°C for 50 seconds and elongation at 68°C for 8 minutes. The final elongation step occurred at 68°C for 7 minutes. The PCR products were digested with 10 U of DpnI restriction enzyme to remove the methylated parental (non-mutated) DNA prior to transformation. Plasmids were transformed into XL10-Gold ultracompetent cells (Stratagene) following manufacturers' instructions, and plated out onto L Agar + 100 µg/ml ampicillin plates.

2.2.8.7 Restriction digest

Restriction enzymes were from New England Biosciences (NEB) unless otherwise stated. They were used according to manufacturers' instructions in the buffers suggested. pPol I constructs were digested with EcoRI and HindIII.

2.2.8.8 Transformation

Plasmids were transformed into One Shot TOP 10 chemically competent cells (Invitrogen) according to manufacturers' instructions. 100 µl of cells were plated onto LB plates containing 100 µg/ml ampicillin and incubated inverted overnight at 37°C.

2.2.8.9 Small scale plasmid purification

A single bacterial colony was picked from selection plates and grown up overnight in LB + 100 µg/ml ampicillin at 37°C, shaking. Bacteria were pelleted from 1.5 ml of the bacterial suspension in a 1.5 ml Eppendorf using a microcentrifuge at 13,000 RPM for 3 minutes. The supernatant was discarded, 1.5 ml fresh bacterial suspension added and the process repeated. Plasmids were recovered using the QIAprep spin miniprep kit (QIAGEN) according to

manufacturer's instructions. This involves alkaline lysis of bacterial cells followed by neutralisation and binding of plasmid DNA to a silica membrane. The membrane is then washed and plasmids eluted in 30 μ l of sterile water and stored at -20°C. 300 μ l of the original suspension were mixed with 300 μ l of sterile glycerol and stored at -80°C as a glycerol stock.

2.2.8.10 Large scale plasmid purification

A single bacterial colony was picked from selection plates and grown in LB plus 100 μ g/ml ampicillin at 37°C for 8 hours, shaking. 100 μ l of this was inoculated into 100 ml of LB plus 100 μ g/ml ampicillin. This was grown overnight at 37°C, shaking. Plasmids were recovered using the high-speed plasmid purification maxi kit (QIAGEN) according to the manufacturer's instructions. Briefly, bacterial cells were pelleted by centrifugation at 6000 RPM for 15 minutes at 4°C using a Sorval GSA rotor. The supernatant was discarded and cells resuspended in 10 ml of resuspension buffer containing RNaseA. Alkaline lysis buffer containing SDS was added to lyse the cells and denature the chromosomal DNA. The suspension was neutralised, precipitating contaminants, and passed through a resin containing column that binds plasmid DNA. The columns were washed and plasmid DNA eluted. Isopropanol was added to desalt and precipitate plasmid DNA, which was then ethanol washed and eluted in 500 μ l of nuclease free water. Plasmid preparations were stored at -20°C.

2.2.8.11 Agarose gel electrophoresis

DNA fragments were separated on 1% agarose gels diluted with TAE and supplemented with 1x gel red (Cambridge Bioscience). Gels were made and run in TAE buffer. Samples were run alongside commercially available DNA size markers at 80-100V until the bands had sufficiently separated. DNA was

visualised using a UV transilluminator. Where required bands were purified using a QIAquick gel extraction kit (QIAGEN), following manufacturer's instructions. PCR products were eluted in 30 μ l nuclease free water and stored at -20°C.

2.2.8.12 DNA Sequencing

Constructs were sequenced using the in-house sequencing service.

Reactions were sequenced using the ABI Prism 3100 (ABI), a fluorescence based automated capillary electrophoresis system.

2.2.9 Assays

2.2.9.1 Blue cell assay

Cells were infected with virus at serial dilutions incubated at 37°C for 1 hour. The infection media was replaced with DMEM plus 2% FCS for 8 hours 37°C. Cells were fixed and permeabilised using ice-cold methanol: acetone (50:50 v/v) for 10 minutes, RT. NP was detected by incubation with mouse α -NP antibody (1/300) for 1 hour at room temperature, washed 3 times with PBS, followed by goat α -mouse antibody conjugated to β -galactosidase (1/400) for 1 hour RT. Infected cells were visualised by incubation with CN buffer (X-Gal substrate) which changes from yellow to blue in the presence of β -galactosidase, for 2 hours- overnight at 37°C depending on cell type.

2.2.9.2 SDS-PAGE

A549 or Huh-7 cells were infected with virus at a relevant MOI or left uninfected for 8 hours. Cells were washed in PBS, then lysed in an appropriate

volume of RIPA buffer for 10 minutes, centrifuged at 1000 RPM, for 1 minute, and stored -80°C. Lysates were mixed with 6x loading buffer containing 2% β -mercapto-ethanol. Samples were heated at 55°C for 5 minutes. Proteins were separated by denaturing SDS-PAGE on polyacrylamide gels at 100V for 15 mins, followed by 180V.

2.2.9.3 Western blotting

Proteins were transferred onto ethanol activated PVDF at 200 mA for 1 hour. Membranes were blocked in blocking solution: 5% Marvel milk powder in PBS + 0.1% Tween20 (PBST) except NS1 antibody 5% Marvel milk powder, 10% horse serum, in PBS, for 1 hour, RT rocking. Membranes were incubated primary antibodies at appropriate dilution (see 2.1.5) in blocking solution for 1 hour at RT. Membranes were washed 3 times 5 minutes in PBST, then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 hour at RT. Membranes were washed 3 times as before then incubated with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) according to manufacturer's instructions for 1 minute and developed using autorad film (Kodak).

2.2.9.4 Immunofluorescence

A549 cell monolayers grown on poly-L-lysine coated coverslips, were infected with influenza virus at the relevant MOI in serum free DMEM for 1 hour, then replaced with 2% DMEM for the desired time. Coverslips were washed with PBS and fixed with freshly made 4% paraformaldehyde for 20 minutes at RT. After fixing, cells were blocked with blocking buffer (3% BSA in PBS, 0.02% azide) for 30 minutes. cells were permeabilised in 0.1% Triton, PBS for 30 mins. Antibodies were diluted in blocking buffer and incubated for 1 hour, RT, rocking, followed by 3 washes with PBS. Coverslips were washed in water and

mounted with Molviol. Cells were visualised using a Pascal confocal microscope and analysed using Leica software.

2.2.9.5 Flow cytometry

Cells were transfected with eGFP for 8 hrs, washed once with PBS, suspended in PBS containing 2% FCS and 0.02% Azide and analysed by flow cytometry using a Becton Dickinson flow cytometer.

2.2.9.6 Mini replicon assay

Cells were transfected with 0.5 μ g of PB2 pol II, PB1 and PA, 1 μ g of NP and a reporter construct: CAT (chloramphenicol acetyltransferase) reporter construct, or M-luc (segment 7 UTRs flanking a luciferase reporter). After 48 hours, cells were washed with PBS. For CAT, production was quantified using a CAT ELISA kit (Roche) following manufacturers' instructions. Briefly, cells were lysed and diluted appropriately in sample buffer provided. Samples and standards were loaded onto a 96 well plate pre-coated with α -CAT antibodies and incubated at 37°C for 1 hour. Wells were washed and incubated with a DIG labelled α -CAT antibody for 1 hour at 37°C. Following washing, wells were incubated with an α -DIG antibody conjugated to peroxidase, followed by washing and visualisation using the peroxidase substrate ABTS. The absorbance at both 405 and 490 nm was measured and the value for 405 nm subtracted from the value at 490 nm. The levels of CAT present in the samples were determined by reading off a standard curve. For Luciferase reporter, cells were washed in PBS, lysed in CCLR. Luciferase was measured using a luminometer.

2.2.9.7 IFN Luciferase assays

Cells transiently or stably expressing IFN luc reporter construct were infected with influenza virus at MOI 2 (unless otherwise stated) in SF DMEM. After 1 hour incubation at 34°C or 37°C the media was removed, cells were washed with PBS, and the media replaced with 2% DMEM. After 8 hours or appropriate incubation cells were lysed in CCLR for 5 minutes, centrifuged 1,000 RPM for 1 minute, supernatants were transferred to clean Eppendorf and stored -20°C. Luciferase was measured using a luminometer.

2.2.9.8 ISRE Luciferase assays

Cells transiently expressing ISRE luc reporter construct were treated with 1U/ml IFN for 16 hours at 37°C media. Cells were lysed in CCLR for 5 minutes, centrifuged 1000 RPM for 1 minute transferred to clean Eppendorf. Luciferase was measured using a luminometer.

2.2.9.9 Up luc assay

Cells transiently expressing the up luc reporter construct were infected with influenza virus at the MOI 2 (unless otherwise stated) in SF DMEM. After 1 hour incubation at 34°C or 37°C media was removed and cells washed with PBS, replaced with 2% DMEM. After 8 hours or appropriate incubation cells were lysed in CCLR for 5 minutes, centrifuged 1000 RPM for 1 minute transferred to clean Eppendorf. Luciferase was measured using luminometer.

2.2.9.10 β -galactosidase assay

In a 96 well plate CCLR lysates (20 μ l PER.C6, 2 μ l 293T, 20 μ l A549, 20 μ l Huh-7), plus 800 μ l lacZ buffer and 200 μ l ONPG (4 mg/ml) were incubated 37°C until lemon yellow. Absorbance measured at 420 nm. If reactions are very fast (2 hours or less), stopped by addition of 500 μ l of 1M Na₂CO₃.

Chapter 3 Rescue of recombinant influenza virus

in PER.C6 cells

3.1 Introduction

Influenza vaccines of the future will be manufactured in cell cultures rather than eggs. Future influenza vaccines may be generated from recombinant proteins purified from insect cells infected with engineered baculoviruses (Cox, Patriarca et al. 2008; He, Madhan et al. 2009), or from mammalian cells infected with other viral vectors (adenovirus (Hoelscher, Singh et al. 2008; Holman, Wang et al. 2008), modified vaccinia virus Ankara (MVA) (Kreijtz, Suezer et al. 2009; Rimmelzwaan and Sutter 2009)). However, it is likely that the bulk of doses manufactured during the next few decades will be derived from whole influenza viruses, as they are today. Thus, whether further processed to become split, subunit or whole virion inactivated product, or administered as a live attenuated vaccine, most influenza vaccines will continue to be made from a live influenza virus. Many cell culture systems that might be used to generate influenza vaccines have low permissivity to influenza infection and this is a serious problem for production and yield. The most permissive cell culture system for amplification of influenza viruses in the laboratory are Madin Darby Canine Kidney (MDCK) cells. Solvay has successfully licensed a vaccine grown in MDCK cells. The product is known as Influvac®TC (Medema, Wijnands et al. 2004). Licensing required rigorous testing to prove that the final purified product would not contain any oncogenic or other adventitious agents.

Since African Green Monkey Vero cells have been used for many years for the production of vaccines for other viruses such as polio, their use for influenza vaccines requires less validation to be licensed. However, Vero cells show relatively low permissivity for influenza viruses, although Baxter describe Vero cell clones that can yield higher titres of virus under suspension growth

conditions (Kistner, Barrett et al. 1998; Audsley and Tannock 2004; Youil, Su et al. 2004) and they are the substrate for one of the vaccine for the current swine H1N1 pandemic.

An important requirement of the cell line in which influenza vaccine is produced is that it supports faithful virus replication, without inducing adapting mutations. After all, the vaccine administered should be a close match to strains that circulate in humans. The propagation of influenza viruses in eggs is associated with changes in the HA protein which can alter antigenicity. Therefore human virus HA sequences were analysed following propagation in the Vero cell line (Romanova, Katinger et al. 2003), all were identical to that of the original clinical isolates. Meanwhile, viruses passaged in MDCK cells acquired several different mutations within the HA. Two of the viruses, despite having the same sequence as the clinical isolates, displayed differential abilities to agglutinate chicken red blood cells suggesting that cell type specific post-translational modifications can also occur. In this instance, all of the viruses passaged in eggs acquired mutations located within the receptor binding site. The MDCK-SIAT cell line which over-expresses $\alpha 2-6$ sialic acid, although not currently used for vaccine manufacture, are routinely used for the isolation of clinical viruses and have been shown to faithfully replicate influenza viruses (Oh, Barr et al. 2008).

Sanofi Pasteur, the vaccine arm of Sanofi Aventis, is the largest manufacturer of influenza vaccine in the world. Currently, their principal product is a beta-propiolactone inactivated, split virion influenza vaccine; marketed in the UK as Vaxigrip. Sanofi Pasteur manufactured 170 million doses of Vaxigrip in 2008 by propagating influenza virus in eggs. Like any large biopharma company, Sanofi Pasteur is investing in research and development that will allow them to keep abreast of new developments in their relevant market place. This includes the investigation of the use of cell lines for influenza vaccine production.

Crucell N.V. a Dutch company, have developed a human cell line, PER.C6, originating from the primary culture of foetal human retinoblasts immortalised

with the oncogenic Adenovirus 5 early region 1 (AdE1) gene. These cells have been rigorously documented since their conception and comply with all regulatory requirements; therefore, they are suitable for the production of human biopharmaceuticals. PER.C6 cells can be grown in suspension, which eliminates the need for microcarriers and makes scale-up easier (Fallaux, Bout et al. 1998). These human cells support growth of a variety of influenza A and B strains and can produce high titres (10^{10} TCID₅₀/ml) of virus in 4 days following infection in roller bottles or bioreactors. Preliminary data suggests that passage of virus to adapt it to this cell line could result in even higher yields of HA antigen (Pau, Ophorst et al. 2001).

Sanofi Pasteur has licensed the PER.C6 cell line from Crucell for manufacture of human influenza vaccine in Europe and the Americas. The aim of work described in the first part of this thesis is to investigate the suitability of PER.C6 cells for production of influenza vaccines. Since it is likely that future influenza vaccines will be generated by the reverse genetics procedure, the first part of this work describes the evaluation of PER.C6 cell line to support recombinant influenza virus rescue from cDNA.

Although it is not absolutely necessary that the vaccine seed strain be produced in the same cell line as the vaccine product, using a single cell line would simplify the procedures and regulatory requirements for vaccine production. Recognizing this, several groups have already described the application of the other two cell lines, MDCK and Vero for recombinant influenza virus rescue (Ozaki, Govorkova et al. 2004; Nicolson, Major et al. 2005; Murakami, Horimoto et al. 2008).

Thus, a reverse genetics system for use in canine cells, has been developed (Murakami, Horimoto et al. 2007). This adaptation from the standard reverse genetics systems used widely in research laboratories has required the delineation and cloning of the species-specific canine polymerase I promoter to drive *in situ* synthesis of influenza viral RNA in the MDCK cell nuclei. Although

MDCK cells are notoriously difficult to transfect, reported titres of rescued influenza viruses are typically 10^{3-4} PFU/ml up to a maximum of 10^6 PFU/ml.

The use of Vero cells for influenza virus rescue has been widely described but because of the generally low permissivity of Vero cells for influenza amplification, the success of this approach relies on passage of transfection products through eggs (Nicolson, Major et al. 2005), or chick embryo fibroblasts (CEFs) (Whiteley, Major et al. 2007). Further adaptation of the plasmids used for influenza virus rescue has allowed this procedure to be performed in chicken cells such as CEFs (Massin, Rodrigues et al. 2005). However, virus rescue in CEFs is difficult, inconsistent and unreliable, and therefore is unlikely to be utilized routinely for generation of vaccine seeds.

3.2 Establishing a reverse genetics virus rescue system in PER.C6

The development of a reverse genetics system in which influenza viruses can be rescued from cloned cDNAs was first described by Palese and co-workers in 1989 (Luytjes, Krystal et al. 1989). However, in 1999, the Palese group (Fodor et al. 1999) refined their methodology and a second group (Neumann, Fujii et al. 2005) described a similar approach that enhanced our ability to generate recombinant influenza viruses at will. This more recent method is likely to be the route for vaccine seed generation in the future. In this procedure (figure 1.1.1), cells are co-transfected with 8 plasmids encoding each of the viral genomic RNAs under the control of an RNA polymerase I (pol I) promoter and 4 plasmids directing expression of viral mRNAs from a host cell pol II promoter. The pol II plasmids encode the three subunits of the viral RNA-dependent RNA polymerase complex (PB1, PB2, and PA) and the nucleoprotein (NP), and are referred to later in this chapter as 'helper plasmids'. They comprise the minimal set of viral proteins required for encapsidation, transcription and replication of the viral genome (Parvin, Palese et al. 1989). In the nucleus of transfected cells, the viral promoters of the

negative sense vRNAs produced in situ are recognized by the polymerase complex, and then amplified and expressed. Viral gene products and newly synthesized genome segments then assemble to form *de novo* infectious influenza virus whose genome sequence has been predetermined by the cDNAs that were originally transfected. In order to be a suitable cell line for influenza vaccine seed generation, PER.C6 cells must be able to support the procedure to generate infectious influenza virus from cDNA.

3.2.1 PER.C6 cells support the rescue of recombinant influenza virus

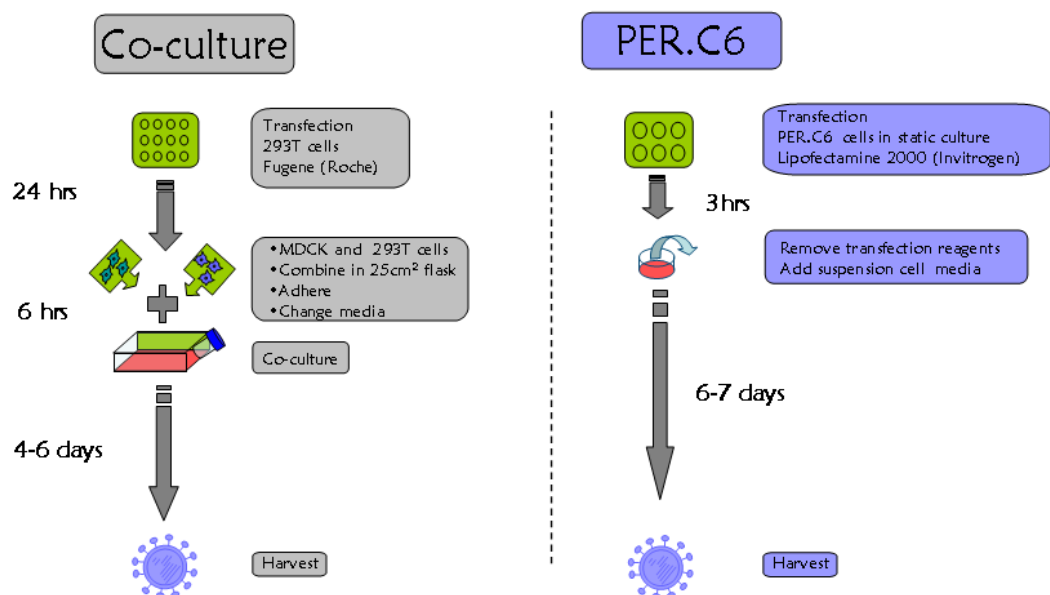
The first step in assessing the suitability of PER.C6 cells for the generation of influenza viruses by reverse genetics was to determine how efficiently they could be transfected. Cells were transfected with an expression plasmid encoding the reporter gene, eGFP, under a variety of conditions and reagents according to the manufacturers' instructions. Transfection efficiency was measured by monitoring the number of fluorescent cells using fluorescence microscopy and the percentage positive cells using FACs (data not shown). Adherent PER.C6 cells are readily transfectable. 13.7% PER.C6 cells were positive for GFP expression compared to 12.5% achieved with the optimized protocol used in the laboratory to transfect the 293T cell line that is routinely used for recombinant influenza virus rescue. In contrast, suspension PER.C6 cells were found not to be transfectable by lipofection.

The optimum transfection conditions established for transfection of a single plasmid into adherent PER.C6 cells were achieved using Lipofectamine 2000 (Invitrogen) at a ratio of 2 μ l of reagent: 1 μ g DNA. Cells were most transfectable when $\sim$ 95% confluent. This correlates to 2×10^6 adherent cells in a well of a 6 well plate. To achieve this, 1×10^6 cells were plated the day prior to transfection. A 95% confluent monolayer of adherent PER.C6 cells from a T80 cm² flask split at a 1:2.5 ratio will realize this.

Figure 3.2.1 Schematic representing virus rescue procedure by either co-culture or PER.C6

For co-culture: 293T cells in 12 well plates are transfected with the 12 plasmids for virus rescue using Fugene (Roche). Following an overnight incubation, cells are mixed with an MDCK cell suspension in T25 cm² flask. These are allowed to adhere for 6 hours at 37°C in 10% DMEM. Media is then replaced with serum-free DMEM containing 2.5 µg/ml trypsin, and incubated until cytopathic effect was observed (4-6 days).

For rescue in PER.C6 cells: adherent PER.C6 cells in 6 well plates are transfected with the 12 plasmids for virus rescue using Lipofectamine 2000 (Invitrogen). The transfection reagent was removed after 3 hrs and cells resuspended in 2 ml virus growth media (2/3 adenovirus expression media-AEM, 1/3 VPSF) +3 µg/ml trypsin. Cells were then shaken 80 RPM (Shaker flask table IKA KS 260). Virus was harvested 6-7 days post transfection.



In the laboratory, the established procedure for rescue of recombinant influenza virus for research purposes involves the transfection of 293T cells with the 12 plasmids for virus rescue, followed by co-culture with MDCK cells the following day. Since PER.C6 cells in suspension are more permissive to virus growth but are resistant to transfection, a two-step procedure was developed. The ratios determined above were applied to the 12 plasmid virus rescue in PER.C6 cells. The transfection of 12 plasmids compared to a single plasmid means higher concentrations of DNA and accordingly larger volumes of reagents will be applied to the transfected cells. Higher quantities of transfection reagents are associated with increased cytotoxicity; however, PER.C6 cells tolerated these higher concentrations.

Adherent cells were transfected with the 12 plasmids and incubated for 3 hours, 37°C, 10% CO₂. This was deemed sufficient time to allow transfection to occur. The media was then removed and cells resuspended in virus growth media (2/3 AEM, 1/3 VPSF, Pen/Strep), 1x10⁶ cells/ml (i.e. 2 ml). Using this procedure, recombinant influenza virus of PR8 vaccine strain was rescued from cDNA and titres of 1x10⁶ PFU/ml were achieved 6-7 days after transfection (fig 3.2.1). This protocol was written as an SOP for use by national and international reference laboratories for the generation of vaccine seeds in the PER.C6 cell line.

3.3 Optimising virus rescue conditions

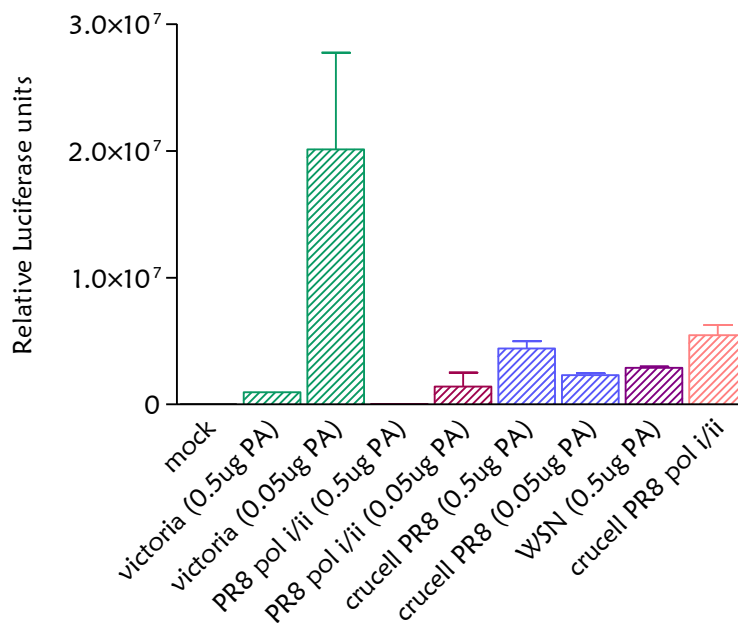
3.3.1 'Helper' plasmids derived from A/Victoria/3/75 virus drive replication most efficiently

Having established that the PER.C6 cell line can support virus rescue, it was next investigated whether rescue efficiency could be improved by adapting rescue conditions. Virus rescue requires transfection of 4 'helper' plasmids (PB1,

PB2, PA and NP) which express the minimal set of viral proteins required for virus RNA replication, (Parvin, Palese et al. 1989). The viral origin of the helper plasmids may influence the efficiency of virus rescue because of differences in the processivity of the viral polymerases they encode. For example, the polymerase of the H3N2 virus A/Victoria/3/75 has been used in the lab to efficiently drive minireplicon expression and recombinant virus rescue (Elleman and Barclay 2004). Others have described sets of helper plasmids derived from influenza PR8 vaccine strain (Schickli, Flandorfer et al. 2001; Whiteley, Major et al. 2007; Koudstaal, Hartgroves et al. 2009) or the laboratory adapted A/WSN/33 virus (Pleschka, Jaskunas et al. 1996; Fodor, Devenish et al. 1999). It is important to emphasise that, since the helper plasmids only encode viral proteins, and not vRNA, at no point will the sequences encoding the helper polymerases form the genome of the rescued virus. To compare efficiency of rescue using different helper polymerases, helper plasmid sets were transfected into cells and their ability to drive the replication of an influenza-like minireplicon RNA, encoding the non-coding regions of segment 8 flanking a luciferase reporter was assessed. Some influenza A virus strains encode a PA protein which can be toxic to cells, so a range of concentrations of PA were tested. Five helper plasmids sets were compared in their ability to drive influenza minireplicon expression (figure 3.3.1), including the polymerases cloned from A/Victoria/75, and polymerases cloned from a number of different sources of the vaccine strain of influenza A virus, PR8. PR8 polymerases cloned from a virus supplied by NIBSC were expressed from developed bi-directional pol I/II plasmids (Whiteley, Major et al. 2007); these direct production in transfected cells of both proteins and vRNA and are used in transfection systems described by (Hoffmann, Krauss et al. 2002). A set of plasmids encoding PR8 polymerase proteins were also independently cloned by Wouter Koudstaal of Crucell N.V. and supplied for testing in this experiment and either as 4 separate plasmids or just 2 plasmids expressing proteins separated by and IRES (fig 1.1.1 (Koudstaal, Hartgroves et al. 2009).

Figure 3.3.1 “Helper” plasmids derived from A/Victoria/3/75 are the most efficient at driving replication for influenza virus rescue

The efficiency of different helper plasmid sets was measured by their ability to drive the replication of an influenza-like minireplicon RNA, encoding the non-coding region of segment 8 flanking a luciferase reporter. PER.C6 cells were transfected with helper plasmid constellations cloned from A/Victoria/75 and A/WSN/33, bi-directional pol I/ pol II A/PR/8/34 and IRES/BGH A/PR/8/34, and the luciferase reporter. Since PA from certain strains of influenza can be toxic, two concentrations were tested for A/Victoria/75 and A/PR/8 plasmid sets. 42 hrs post transfection cells were washed with PBS, lysed in CCLR and luciferase activity measured. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments.



Finally, polymerase expression plasmids for the virus strain A/WSN/33 that are routinely used at NIBSC to generate vaccine seeds in Vero cells were provided by Ervin Fodor, (SWDSOP, Oxford). The data (figure 3.3.1) shows that the most efficient expression of the minireplicon luciferase reporter was driven following transfection of helper plasmid encoding the polymerase complex cloned from A/Victoria/3/75 virus and 0.05 μ g of PA. These are the conditions previously optimised for rescue in co-culture and therefore fortuitously the conditions used to establish the virus rescue procedure described for PER.C6 cells above; thus no alterations to this part of the procedure were proscribed.

3.3.2 A single step rescue transfection procedure is most efficient for virus rescue in the PER.C6 cell line

Rather than transfecting all 12 plasmids at once, a variation to the procedure was made whereby helper plasmids were added on day 1, to allow strong expression of viral polymerase proteins before transfection of plasmids encoding viral RNAs (figure 3.3.2). Transfection of all plasmids at once resulted in >3 logs more virus than a two step procedure (figure 3.3.3). A single step transfection is the most efficient and also the most straightforward protocol for virus rescue in PER.C6 cells.

Figure 3.3.2 Schematic representing rescue procedures for one- or two- step transfection protocol

In the one-step procedure cells were transfected with all 4 Victoria helper plasmids and 8 RD3 (6:2 PR8 +HA and NA A/Chicken/Italy/1347/99 H7N7) rescue plasmids. 3 hours later the transfection reagent was removed and the cells resuspended in virus growth media (2/3 AEM, 1/3 VPSF with pen/strep). In the two-step procedure cells were transfected with 4 Victoria helper plasmids. 16 hours post transfection the media was removed and 8 RD3 rescue plasmids were transfected, 3 hours later the transfection reagent was removed and the cells resuspended in virus growth media (2/3 AEM, 1/3 VPSF with pen/strep). Virus was harvested and clarified 7 days following the first transfection.

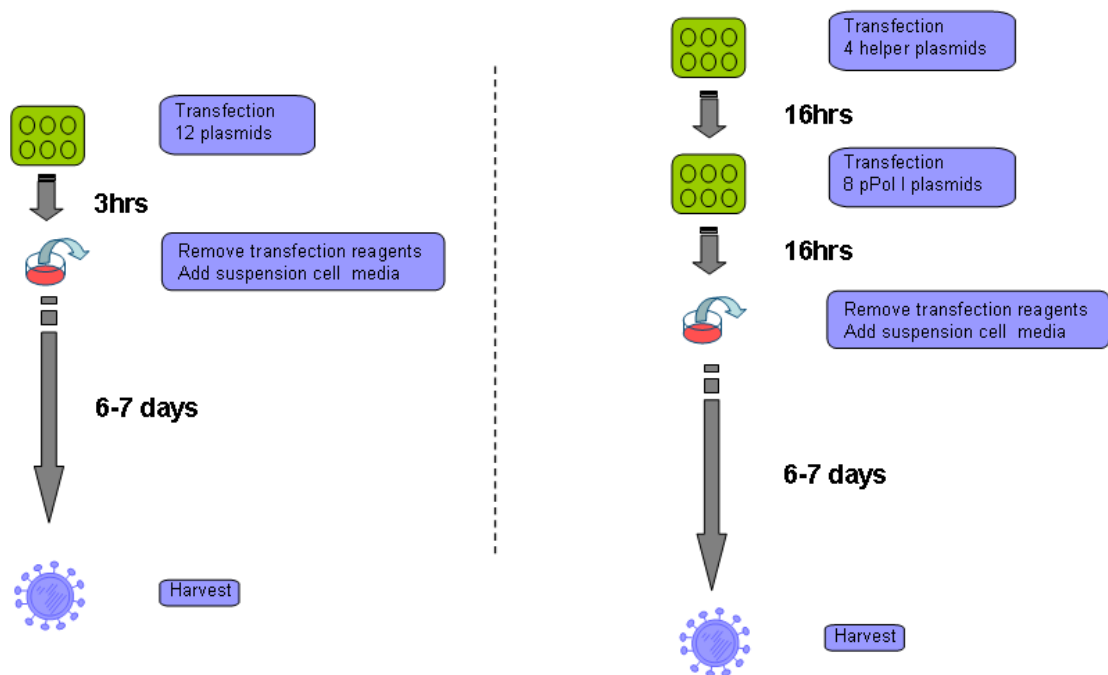
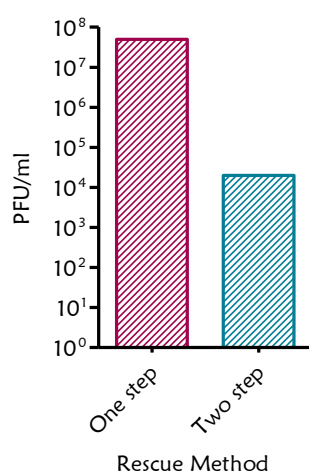


Figure 3.3.3 One-step virus rescue is the most efficient procedure

The efficiency of one- and two- step rescue procedures of the vaccine strain RD3 (6:2 A/PR/8 internal gene segments plus HA and NA from A/Chicken/Italy/1347/99) were compared in PER.C6 cells. In the one-step procedure cells were transfected with all 4 Victoria helper plasmids and 8 RD3 rescue plasmids and incubated 37°C for 3 hours. The transfection reagent was removed and the cells resuspended in virus growth media (2/3 AEM, 1/3 VPSF with pen/strep). In the two-step procedure cells were transfected with 4 Victoria helper plasmids incubated 37°C. 16 hours post transfection the media was removed and 8 RD3 rescue plasmids were transfected, incubated 37°C x16 hours. The transfection reagent was removed and the cells resuspended in virus growth media (2/3 AEM, 1/3 VPSF with pen/strep). Virus was harvested and clarified 7 days following the first transfection. Virus titres were assayed by plaque assay. This assay is representative of two repeat experiments.

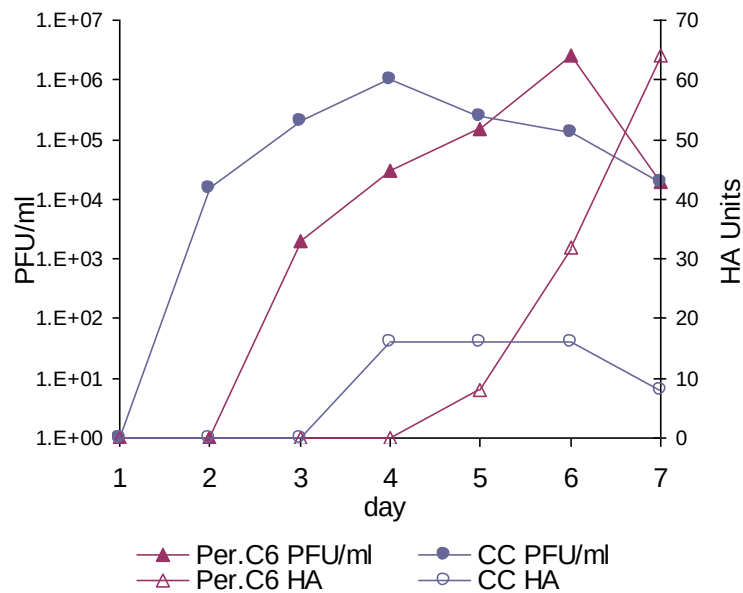


3.3.3 *The kinetics of virus rescue in the PER.C6 cells*

In order to establish the optimum day post-transfection to yield the highest titre of infectious influenza virus in PER.C6 cells supernatants were taken over the course of the rescue. Following the SOP, the 12 plasmids that direct the rescue of A/PR/8/34 were transfected on day 0. Cell supernatants were taken daily from day 1, clarified by centrifugation (3000 RPM for 3 minutes) and stored at -80°C until all samples were collected and could be assayed simultaneously for the presence of virus by HA assay using CRBC and plaque assay on MDCK cells. The procedure was performed in parallel in PER.C6 cells or in 293T/MDCK cell co-culture. Virus was detected on day 2 post transfection using the co-culture method or day 3 using PER.C6 cells (figure 3.3.4). Peak recovery of virus was obtained 6 days after transfection of PER.C6 cells. Peak titres determined by plaque assay, were similar for each method (approximately 1×10^6 PFU/ml). In PER.C6 an increase in HA units consistently occurred towards the end of infection despite a decrease in infectious plaque forming units. The reasons for this have not been determined.

Figure 3.3.4 Kinetics of virus rescue

Following the SOP for PER.C6 or standard lab procedure for co-culture, cells were transfected on day 0 with the 12 plasmids that direct the rescue of A/PR/8/34 were. Cell supernatants were taken daily from day 1, clarified by centrifugation (3000 RPM x3 min) and stored at -80°C. Samples were assayed for the presence of virus by HA assay using CRBC and plaque assay on MDCK cells. This data is representative of 2 repeat experiments.



3.3.4 PER.C6 cells support rescue of a range of backbones and subtypes

Next, the ability of PER.C6 cells to support the rescue a range of influenza viruses and subtypes was assessed. Two complete reverse genetics systems with plasmids for the rescue of influenza A/PR/8/34 (H1N1) or A/Victoria/3/75 (H3N2) were tested. PR8 has been used as the recipient genetic backbone of the influenza A virus vaccine for more than 30 years (Kilbourne 1969), and was developed particularly for high yields in eggs. A/Victoria/3/75 (H3N2) is more representative of a typical human influenza strain. PER.C6 cells were able to support the rescue of both PR8 and A/Victoria/3/75 viruses. Moreover, PR8 based 6+2 reassortants with a wide variety of HA and NA subtypes, representing typical vaccine seed strains were reliably rescued in these cells (table 3.3.1), yields can be low but are recovered following subsequent passage. In general, yields were higher and more reliable in PER.C6 than in the licensed alternatives- rescue in Vero cells co-cultured with chick embryo fibroblasts. Interestingly rescue of the A PR8-HK97 virus was rather inefficient in PER.C6 cells, even though the rescued virus was amplified during one additional passage in PER.C6 cells and eventually a model vaccine was prepared from this strain (Koudstaal, Hartgroves et al. 2009).

Table 3.3.1 PER.C6 cells are able to support the rescue of range of virus backbones and subtypes

PER.C6 cells were transfected according to the SOP and assayed 6 days post transfection by plaque assay.

Virus	Subtype	Titre (PFU/ml)
A/PR/8/34	H1N1	5x10 ⁶ - 2x10 ⁶
A/Victoria/3/75	H3N2	8x10 ³
B/Beijing/87	B	1x10 ⁴
6:2 A/PR/8/34 + A/New Caledonia/20/99 HA & NA	H1N1	1x10 ²
6:2 A/PR/8/34 + A/Victoria/3/75 HA & NA	H3N2	2x10 ⁵
6:2 A/PR/8/34 + A/Panama/2007/99 HA & NA	H3N2	19
6:2 A/PR/8/34 + A/Wisconsin/67/2005 HA & NA	H3N2	5x10 ²
6:2 A/PR/8/34 + A/Chicken/Italy/1347/99 HA & NA ^a	H7N1	5x10 ⁷
6:2 A/PR/8/34 + A/Viet Nam/1194/2004 HA & NA ^a	H5N1	9x10 ⁶
6:2 A/PR/8/34 + A/Hong Kong/156/97 HA & NA ^a	H5N1	<10

^a Sequence of the HA cleavage site modified to remove pathogenic traits

3.3.5 Virus rescue can be performed from a single PER.C6 suspension cell line

Initially the procedure for rescue of influenza virus was established in adherent PER.C6 cells because suspension cells were reported to show very low transfection efficiency that may preclude virus rescue. On the other hand, virus propagation is most efficient in PER.C6 suspension cells (Pau et al. 2005). For vaccine generation and production this would require maintenance of both adherent and suspension cell lines. It would be preferable to maintain a single cell line which could produce recombinant vaccine seed and high titre vaccine manufacture. The addition of serum in the growth media rapidly drives PER.C6 suspension cells to adherence, thus one solution to this would be the generation of recombinant virus by transfection in adherent cells that had only recently been derived from a suspension culture (fig 3.3.5). The health agencies responsible for seed generation (e.g. NIBSC or CDC) would then be required to maintain only PER.C6 cells in suspension. Once the rescued virus had been generated, it could then be propagated in the same suspension cells from which the adherent cells were derived.

We were provided with a line of PER.C6 suspension cells that have been adapted by Sanofi Pasteur for influenza propagation and represent the cell line they use for vaccine manufacture. In parallel, suspension cells were derived from the adherent cell stock originally supplied from Crucell, which has been used throughout for the development of virus rescue. To generate the suspension cells, the adherent cells were passaged a minimum of six times in suspension cell media (AEM +L-glutamine), until stable growth characteristics were observed. One day before transfection, the cells were returned to adherent state by transfer to serum containing media as before, at a seeding density of 2×10^6 cells. Following the standard SOP, the 12 plasmids that direct rescue of PR8 virus were transfected into duplicate dishes of either the suspension cells of Sanofi Pasteur or the derived cells of Crucell. Many cells

Figure 3.3.5 Schematic representing virus rescue from a single cell line

Adherent PER.C6 cells were reported to show very low transfection efficiency that may preclude virus rescue, whilst virus propagation is most efficient in PER.C6 suspension cells. It would be preferable to maintain a single cell line which could produce both recombinant vaccine seed and high titre vaccine. The addition of serum to the growth media rapidly drives PER.C6 suspension cells to adherence, so virus can be rescued from adherent cells that have only recently been derived from a suspension culture. Rescued virus is then propagated in the same suspension cell stock from which the adherent cells were derived.

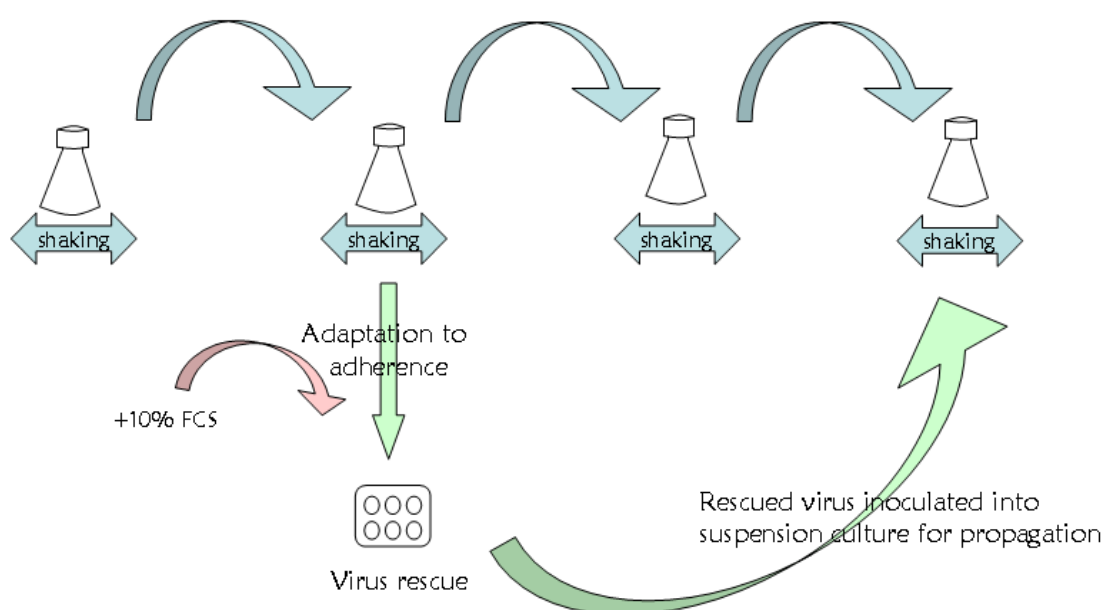


Table 3.3.2 Virus rescue can be performed in single PER.C6 cell line in suspension

PER.C6 suspension cells were driven to adherence by the addition of serum to the growth media. PER.C6 suspension cells adapted by Sanofi Pasteur for influenza propagation were compared to suspension cells derived from the original adherent PER.C6 cell stock supplied from Crucell, by passage in suspension cell media (AEM +L-glutamine) with adherent PER.C6 cells as a rescue control. Cells were transfected with 12 plasmids that drive rescue of PR8 virus, following the SOP. Virus titres 7 days post transfection were determined by TCID₅₀/ml in MDCK cells.

Cell origin	#	TCID ₅₀	HA
Adherent: Crucell	1	3.2x10 ⁴	64
	2	3.2x10 ¹	0
Suspension: Crucell	1	3.2x10 ³	0
	2	5.6x10 ⁴	32
Suspension: Sanofi	1	1.3x10 ⁴	16
	2	4.2x10 ⁵	32

looked granulated and were not well adhered to the well, nonetheless, when titres of recombinant viruses at day 7 were compared by TCID₅₀ assay in MDCK cells and HA of chicken RBC (table 3.3.2) they were comparable to those achieved from rescue in cells fully adapted to adherent growth.

By increasing the total number of cells undergoing transfection, yields might be further increased; therefore, cells were also allowed to adhere over night then trypsinised and reseeded on day 2 (P1). This did not affect rescue titres (data not shown).

3.3.6 Comparison of PR8 backbones

Although the PR8 strain was rescued and propagated efficiently in PER.C6 cells, this vaccine backbone has been adapted for high growth in eggs. It may be that PR8 is not well adapted for growth in the human cell line and that by further passage of a PR8 stock, a derivative could be generated that would more efficiently be rescued. The stock of PR8 virus originally obtained from NIBSC was passaged 11 times in suspension PER.C6 by Wouter Koudstaal at Crucell. The P11 virus did indeed show enhanced growth in the PER.C6 line, and whole genome sequencing revealed changes in three viral gene segments, HA, NA and NP (L37F and I349F in HA, S349N in NA, I33T and F346S in NP, Wouter Koudstaal personal communication). The entire eight segments of P11 PR8 virus were cloned into polymerase I rescue plasmids for generation of recombinant virus by Wouter Koudstaal. The ability of this set of P11 plasmids to direct virus rescue was compared with those generated in the Barclay laboratory as part of the FLUPAN EU funded project (Whiteley, Major et al. 2007). The rescue efficiency was compared in either PER.C6 or 293T/MDCK co-culture rescue. The rescue of three different vaccine strain viruses were assessed; these contained plasmids encoding the 6 internal gene segments from either P11 or FLUPAN PR8 virus, together with plasmids encoding the surface antigens, HA and NA, from either wt PR8 or avian influenza viruses A/HK/97 (H5N1) or A/Chicken/Italy/1347/99 (RD3/ H7N1).

Table 3.3.3 Comparison of virus rescue backbones

A backbone was generated in house in the Barclay laboratory as part of the FLUPAN EU funded project. Another backbone was derived from a stock of PR8 virus originally obtained from NIBSC passaged 11 times in suspension PER.C6 by Wouter Koudstaal at Crucell. Rescue by co-culture and PER.C6 were compared. Cells were transfected with either backbone according to the standard SOP, virus titres were assayed by plaque assay in MDCK cells.

backbone	transfection method	PR8	H5N1	H7N1
FluPAN	Co-culture	3.0×10^7	1.9×10^7	1.5×10^5
Crucell	Co-culture	3.1×10^7	2.5×10^6	1.5×10^5
FluPAN	PER.C6	3.0×10^5	ND*	5.0×10^7
Crucell	PER.C6	1.2×10^5	ND*	ND*

*ND- not detected

Wt A/PR8/34 virus of both P11 and FLUPAN derived backbone was rescued using either set of cells (table 3.3.3). Interestingly, RD3 H7N1 6:2 recombinant virus was only rescued in conjunction with the FLUPAN plasmids. This might imply that the sequence changes in the P11 NP gene are incompatible with the H7 or N1 genes. The 6:2 PR8 -HK97 H5N1 recombinant virus was not rescued under any conditions in PER.C6, although it was rescued using the 293T/MDCK co-culture protocol. Further work has shown that upon passage of transfected cell supernatants through PER.C6 cells the PR8 -HK97 virus can be propagated and indeed a vaccine batch has been prepared and tested in mice for this strain (Koudstaal, Hartgroves et al. 2009). The data suggests the co-culture protocol is more efficient for virus rescue, and that certain combination of internal and surface antigens may be more compromised than others for rescue and propagation in PER.C6 cells.

3.4 Discussion

Adherent PER.C6 cells are easily transfectable. Furthermore, they support the rescue of recombinant viruses by reverse genetics. A rescue procedure has been developed and optimised and an SOP has been supplied to NIBSC for generation of seed virus for vaccine production in PER.C6.

The procedure has been optimised using the available reverse genetics backbone and the current vaccine donor strain PR8, which was originally adapted for high growth in eggs. Yields in PER.C6 cells could be increased by adapting or improving the backbone of the virus. H5N1 viruses have proved difficult to grow to sufficiently high titres. A PR8 based backbone containing a chimeric NA was able to increase the yields for a prototype H5N1 vaccine (Adamo, Liu et al. 2009).

Following the generation of reassortants for the 2006/7 vaccine season, a reassortant of the H3N2 strain, A/Wisconsin/67/2005, was selected (X116)

which did not grow well in eggs. An alternative reassortant was then selected (X116B) which replicated well in eggs. The passage of these viruses in PER.C6 cells resulted in the opposite growth kinetics to eggs; X116 grew to high titres whilst X116B grew to poor titres. Whilst a 6:2 reassortant is ideally required, only the incorporation of surface antigens (HA and NA) and segment 7 into the vaccine seed are routinely screened. Subsequent sequencing of both reassortants revealed that X116 contained only segment 7 from PR8 and all other 7 segments from Wisconsin, whilst X116B was a true 6:2 reassortant. This suggests that an H3N2 backbone may be better suited for higher yields of virus in PER.C6 cells. However, titres of rescued virus were not higher when the A/Victoria/75 (H3N2) backbone was used. A/Victoria/75 is a lab adapted older strain of virus; a more modern H3N2 might represent a more suitable donor strain for rescue of viruses PER.C6.

PR8 virus has been serially passaged in PER.C6 cells by both Sanofi and Crucell in order to adapt the virus to the cell line. Eleven serial passages by Crucell resulted in amino acid changes in HA and NA and NP; the mutations in the surface antigens are likely to improve the efficiency by which the virus is able to enter cells but would confer no additional benefits to PR8 as a vaccine donor strain, since only the internal genes are donated. The mutation to NP did not seem to increase the rescue efficiency (section 3.3.6). Alternatively, ten serial passages by Sanofi resulted in a log increase in virus with yields (Sanofi personal communication). Sequencing of the virus revealed two mutations in segment 7 (M). Segment 7 encodes two matrix proteins- M1 and M2. M1 is involved in assembly and release of the virus. Whilst alternative splicing encodes M2 a small ion channel protein, which alters pH of vesicles allowing conformational change of HA (Elleman and Barclay 2004) and has also been associated with the formation of infectious viral particles (McCown and Pekosz 2006). One mutation is located in M1, H175D, and the second in M2, V92A. However, although inclusion of PER.C6 adapted segment 7 resulted in the detection of virus a day earlier than rescue with wt PR8 segment 7 (day 2 compared to day 3), there was no increase in the final titres of rescued virus

(data not shown). Although the addition of PER.C6 adapted segment 7 confers no increase in rescued virus titres its inclusion may benefit difficult to rescue viruses, and may confer higher titres when propagating the vaccine seed.

PR8 is the approved donor strain currently used in vaccine manufacture. Although increases in yields could potentially be obtained with adapted viruses and other backbones these will require validation and safety approval. The continued use of PR8 as a backbone means seed viruses can be generated by reverse genetics in PER.C6 and used for vaccine manufacture in both eggs and PER.C6 cell culture.

Chapter 4 Rapid generation of a well-matched vaccine seed from a modern influenza A virus primary isolate without recourse to eggs

4.1 Introduction

The production of influenza vaccines from seed reassortants has relied on embryonated chickens eggs for large-scale viral growth for more than 40 years. However, the receptor used for cell entry in eggs differs from that in the human respiratory tract. As a consequence of host adaptation combined with antigenic drift, many recent isolates of influenza A virus have developed altered receptor binding characteristics compared to strains that circulated shortly after the emergence of the H3 or H1 subtype as pathogens in man more than 40 years ago (Medeiros, Escribe et al. 2001; Mochalova, Gambaryan et al. 2003; Thompson, Barclay et al. 2004) and now show decreased potential to propagate in eggs.

It has long been known that for many human influenza isolates, propagation in eggs selects for mutations (Schild, Oxford et al. 1983) some of which give rise to a mismatch in antigenicity between the egg-grown virus and the naturally circulating virus (Mochalova, Gambaryan et al. 2003). For influenza B viruses, this often manifests as changes in the glycosylation patterns of HA during egg adaptation that may additionally affect the antigenicity (Schild, Oxford et al. 1983; Chen, Aspelund et al. 2008). Indeed the positions of egg-adapting mutations are key to whether the antigenicity of the vaccine would be affected. Egg passage of the H3N2 virus A/Memphis/7/90 resulted in two different egg-adapted mutants, containing HA mutations at either G156K or S186I. Although the S186I virus induced a protective antibody profile similar to that of the wt virus, antibodies induced by the virus with G156K mutation

were cross-reactive but did not protect against challenge with a lethal dose of unadapted A/Memphis/7/90 virus (Widjaja, Ilyushina et al. 2006). Thus, strains chosen for inclusion in the vaccine should be monitored for the occurrence of antigenic mismatches. Moreover, for some viruses, propagation in eggs is impossible altogether and in this case, reliance on eggs for vaccine manufacture may result in the exclusion of the most suitable strain from incorporation into the vaccine or a delay in vaccine manufacture. A recent example is the H3N2 influenza A/Fujian/411/02 virus that was predicted to be the dominant strain in the 2003/04 season. Since this virus did not grow in eggs and an alternative seed had to be sought (A/Wyoming/3/2003) resulting in delays to the delivery of the vaccine seed. Subsequent molecular analysis revealed that mutations at G186V and V226I were sufficient to confer egg adaptation to Fujian and that this had little or no effect on the antigenicity of the strain (Lu, Zhou et al. 2005). The G186V mutation alone was also able to confer egg adaption to other H3 viruses unable to grow in eggs (Lu, Zhou et al. 2006).

Several cell lines are currently being explored as alternative substrates for influenza vaccine production. These include the MDCK, Vero and PER.C6 cell lines (Halperin, Nestruck et al. 1998; Kistner, Barrett et al. 1998; Brands, Visser et al. 1999; Pau, Ophorst et al. 2001; Audsley and Tannock 2005). Using cell culture systems would add substantial flexibility to the influenza vaccine production process, for example in times requiring rapid scale up, such as onset of a pandemic. Furthermore, the use of mammalian cell substrates for propagation of vaccine strains reduces the likelihood of variants with altered antigenicity occurring during propagation since HA mutations are not necessarily selected for during replication in cell culture (Minor, Engelhardt et al. 2009).

The generation of vaccine seed strains on cell culture has been inhibited by concerns about the increased potential for carryover of adventitious agents from clinical specimens. Egg propagation has been previously been thought of

as a filter for such agents. However, reverse genetics technology (Fodor, Devenish et al. 1999; Neumann, Watanabe et al. 1999), allows for the generation of recombinant influenza viruses entirely from cDNA, and thus eliminates this risk. Moreover, by removing the random aspect of the reassortment process, reverse genetics makes it possible to create vaccine seed strains at unprecedented speed and even to incorporate desirable safety and growth characteristics (Stieneke-Grober, Vey et al. 1992; Li, Liu et al. 1999; Subbarao, Chen et al. 2003; Stech, Garn et al. 2005; Horimoto, Takada et al. 2006).

Technological advances in DNA synthesis mean that long sequences can be synthesised *de novo*, introduced directly into the appropriate plasmids for use in reverse genetics, and rescued in a suitable backbone strain. If rescue and subsequent propagation does not introduce mutations, this approach should provide the best possible antigenic match between vaccine and circulating strains.

Previously we showed that PER.C6 cells are suitable for the recovery of recombinant influenza viruses by the reverse genetic technique and subsequent production of vaccine (chapter 3, (Koudstaal, Hartgroves et al. 2009). Here we assess whether the PER.C6 cell line faithfully supports replication of recently isolated human influenza strains and facilitates production of appropriate vaccine seeds based on their sequence.

However, current manufacturing capacity for the production of influenza vaccines on cell culture is insufficient to meet vaccine demand, and some proportion of the vaccine quota will require propagation in eggs. Therefore, at least for the immediate future, an ideal seed virus should replicate in both cell culture and eggs.

Here we investigated whether it was possible to generate a universal seed appropriate for both egg and cell culture derived vaccine manufacture for a

wild type strain that did not replicate in eggs. As a representative strain to demonstrate proof of principle we used A/Eng/611/07 (E611) that is antigenically similar to the vaccine strain A/Brisbane/10/07 (the H3 component of the 2008/09 vaccine) but whose suitability as a vaccine seed would have been precluded by its inability to grow in eggs. Since the G186V mutation has been shown to confer egg growth to a number of strains from the first part of this decade (2000-2005), we engineered G186V into E611 HA. Additionally, we found a novel mutation in the E611 HA gene, L194P, was selected during egg passage of the recombinant virus. Therefore, we also directly engineered this mutation into the wild type E611 HA gene and compared its ability to adapt a virus with E611 HA for growth in eggs with the G186V mutation. We found that combination of the wild type HA gene into the PR8 vaccine backbone allowed for the generation in PER.C6 cells of a vaccine seed that would be capable of efficient propagation in both cell substrates and eggs.

4.2 PER.C6 cells support the faithful propagation of recent clinical isolates

Contemporary circulating strains of human influenza A virus are frequently isolated in surveillance laboratories on the MDCK-SIAT cell line. These cells have been genetically manipulated to over-express the $\alpha 2$ -6 sialyltransferase enzyme and consequently display a higher level of $\alpha 2$ -6 linked SA at the cell surface than do unmanipulated MDCK cells (Matrosovich, Matrosovich et al. 2003). Recent strains of human influenza A virus that tend to display low SA receptor binding affinity are isolated in these cells more efficiently than in standard MDCK cells (Oh, Barr et al. 2008). In contrast, inoculation of eggs with recent clinical isolates results in sporadic recovery of infectious virus. To assess whether recent human influenza viruses were faithfully propagated in PER.C6 cells, a small panel of isolates of human influenza A (H3N2 and H1N1) and B viruses isolated from clinical cases of influenza like illness in the winters

of 2003/04 and 2004/05 were obtained from the Health Protection Agency Centre for Infections and had been isolated in the MDCK-SIAT cell line.

The viruses were passaged again once in either MDCK-SIAT cells, in PER.C6 cells or inoculated into eggs. After 3 days incubation, cell supernatants and allantoic fluids were harvested and virus was detected by haemagglutination assay using either chicken or turkey red blood cells. Since the 1999/2000 influenza season, turkey red blood cells have been more sensitive for the detection of clinical strains of human influenza viruses than the traditional chicken red blood cells. This is most likely because the density of SA on turkey RBC is higher than on chicken RBC, and viruses whose HA proteins have low affinity for SA maintain the ability to agglutinate them (Kumari, Gulati et al. 2007). An inability to agglutinate either type of red blood cell was taken as absence of virus growth and this was confirmed by plaque assay of undiluted samples on both MDCK cells and MDCK-SIAT cells. Table 4.2.1 shows that the only viruses to be propagated efficiently in eggs were two strains of influenza B virus, B/E/216/05 and B/E/429/05. These isolates had a strong HA titre measured with chicken RBC even before passage through eggs suggesting they retained a receptor binding phenotype typical of older influenza virus isolates. For all the other H3N2, H1N1 and influenza B viruses analysed, no virus growth in eggs was detected. Moreover, following passage through either PER.C6 cells or SIAT-MDCK cells, this group of viruses did not acquire the ability to agglutinate chicken RBCs suggesting that receptor binding profile of the original isolate was faithfully maintained. The entire HA gene of a representative H3N2 virus (A/Eng/26/05) passaged in PER.C6 and MDCK-SIAT cells was sequenced and found to be identical from both cell substrates.

Table 4.2.1 PER.C6 cells support the faithful propagation of recent clinical isolates

Clinical isolates from the 2004/2005 influenza season were previously passaged once on MDCK-SIATs. PER.C6 cells in suspension, MDCK-SIAT cells, or 10 day old embryonated chicken eggs were inoculated with virus inoculum at equivalent HA units. Supernatants were harvested after 4 days for cell lines and 3 days for eggs. Receptor binding characteristics of clarified supernatants or allantoic fluid were determined by HA assay with chicken and turkey RBC to analyse.

Strain name	Virus type	SIAT		PER.C6		Egg	
		TRBC	CRBC	TRBC	CRBC	TRBC	CRBC
B/E/216/05	B	4	>8	4	>8	32	>32
B/E/429/05		4	>8	4	>8	16	>32
B/E/319/05		8	>8	4	2	<	>32
B/E/354/05		4	>8	4	2	<	<
B/E/426/05		4	>8	4	2	<	<
A/E/67/04	H1N1	8	<	4	<	<	<
A/E/82/05		8	<	4	<	<	<
A/E/48/04		4	<	4	<	<	<
A/E/204/05		4	<	4	<	<	<
A/E/208/05		4	<	4	<	<	<
A/E/280/05		4	<	4	<	<	<
A/E/180/05	H3N2	8	<	4	<	<	<
A/E/81/05		8	<	4	<	<	<
A/E/26/05		4	<	4	<	<	<
A/E/13/05		4	<	4	<	<	<
A/E/29/05		4	<	4	<	<	<

4.3 A/Eng/611/07 is typical of current circulating strains

The current vaccine strain selection procedure requires growth of viruses in eggs before they are put forward for consideration for inclusion in the new season's vaccine. Since many circulating strains do not grow in eggs, the best vaccine candidates may potentially be excluded. In addition, unpredictable delays occur while a strain that does grow in eggs is found. Therefore, we wanted to assess the feasibility of generating a vaccine seed with the HA antigen of a typical circulating influenza A virus that did not grow in eggs. This approach is facilitated by two novel technologies: firstly, long sequences of DNA can now be generated de-novo and secondly, influenza viruses can be generated directly from cDNA. Using this strategy, we reasoned that recombinant viruses displaying the surface antigen of circulating viruses could be generated directly from sequenced viruses without the need for virus propagation in eggs or in cell culture.

From the clinical isolates available through the surveillance programme at the HPA, we obtained a sample of a recent H3N2 influenza A virus and deduced the complete nucleotide sequence of the HA gene. HAI tests with ferret antisera showed that the specific isolate, A/Eng/611/07 (E611), is antigenically like A/Brisbane/10/07 (Brisbane), the virus included in the trivalent seasonal vaccine for 2008/9 (data not shown). Sequence analysis revealed that the HA of E611 differs from that of Brisbane by only two amino acids, one in HA1 at residue 194 where E611 has leucine and Brisbane has proline, and the other in HA2 at residue 418 where E611 has valine and Brisbane has isoleucine. E611 was isolated and propagated on MDCK-SIAT cells and like other very recent influenza A H3N2 strains, E611 agglutinated guinea pig RBC but did not agglutinate turkey or chicken RBC. Unlike Brisbane virus however, it was not possible to attain a primary egg isolate of E611, as this virus did not acquire the ability to grow in eggs, even following two blind passages. Absence of virus growth in eggs was confirmed by the inability of allantoic fluid to agglutinate

guinea pig RBC, and the absence of cytopathic effect or expression of viral antigens in MDCK-SIAT cells after inoculation with undiluted allantoic fluid. Taken together these data show that E611 is a typical contemporary human influenza virus that has highly-adapted human receptor binding characteristics and may also be restricted for egg growth by incompatibilities within the internal viral genes. Despite the fact that E611 is an antigenic representative of the majority of H3N2 clinical isolates of the 2007/08 influenza season, it would not have been put forward as a vaccine candidate because the wild type E611 virus did not grow in eggs.

4.4 Recombinant viruses with the HA of a recent clinical strain in a PR8 backbone can acquire the ability to grow in eggs

The cDNA representing a full length HA gene from E611 flanked at the 3'UTR by a HDV ribozyme and at the 5'UTR by a human polymerase I promoter was synthesised de novo (GENEART) and directly cloned into the pUC19 vector. Using our previously described protocols (Koudstaal, Hartgroves et al. 2009), recombinant influenza viruses bearing the E611 HA gene in combination with the A/Puerto Rico/8/34 (PR8) genetic backbone were rescued in either 293T cells with MDCK co culture, or directly in PER.C6 cells. Two different recombinant viruses were rescued, combining the E611 HA gene with either a modern N2 NA cloned from the H3N2 vaccine strain, A/Sydney/97, or with the NA gene of the PR8 vaccine strain. Both viruses were readily rescued in both cell systems. The virus containing PR8 NA grew to a high enough titre to agglutinate guinea pig RBCs but retained the wt E611 phenotype, being unable to agglutinate chicken RBCs (Table 4.4.1). Even passage of rescued viruses in MDCK-SIATs, increasing titres above the limit of detection did not affect the wild type E611 phenotype.

Table 4.4.1 A Synthetic A/Eng/611 HA was rescued by co-culture of 293Ts with MDCK-SIATs in a A/PR/8 backbone with NA derived from either A/PR/8 or A/Sydney/97

A Synthetic A/Eng/611 HA was rescued by co-culture of 293Ts with MDCK-SIATs in a A/PR/8 backbone with NA derived from either A/PR/8 or A/Sydney/97. Viruses were passaged once in MDCK-SIATs. Virus titres of recombinant influenza viruses were determined by plaque assay in MDCK-SIAT cells. Receptor binding characteristics of the recombinant viruses were inferred by HA assay with either chicken or guinea pig RBC.

		Passage status					
		Rescue			Passage 1- SIATs		
HA	NA	CRBC	GPRBC	PFU/ml	CRBC	GPRBC	PFU/ml
611	PR8	<	16	3.5×10^5	<	256	5.8×10^7
	Syd	<	<	2.5×10^4	<	256	7.5×10^7
< Not detectable							

Table 4.4.2 Recombinant viruses with the HA of a recent clinical strain in a PR8 backbone can acquire the ability to grow in eggs

Rescued recombinant influenza viruses with the HA of A/Eng/611, the NA of either PR8 or A/Sydney/97 (Syd) in a A/PR/8 backbone were inoculated into the allantoic cavity of three 10 day old embryonated chicken eggs with equivalent HA units and incubated for 3 days at 37°C. Allantoic fluid was clarified by centrifugation at 2500 RPM for 10 mins, 4°C and stored at -80°C. Growth and adaptation in eggs were determined by ability to agglutinate chicken and turkey RBC. Virus titres of recombinant influenza viruses were determined by immuno-staining of plaques in MDCK-SIAT cells

HA	NA	TRBC	CRBC	PFU/ml
E611	PR8	16	4	8.8x10 ⁴
		4	4	8.8x10 ⁴
		8	<	5x10 ³
E611	Syd	<	<	9x10 ³
		<	<	5.5x10 ⁴
		32	32	2x10 ⁵

< Not detectable

Therefore, the rescued viruses retain the receptor binding characteristics of wt E611. The rescued E611 HA virus containing A/Sydney/97 NA grew to a titre below the threshold of detection by HA assay but was detected by immunostaining of plaques in MDCK cells. The ability to agglutinate guinea pig RBCs was recovered following passage in MDCK-SIATs.

These two recombinant viruses were subsequently inoculated in triplicate in 10-day-old embryonated chicken eggs. The E611 HA: PR8 NA virus grew to high titre in eggs. However, egg grown E611 HA virus displayed an altered receptor binding phenotype inferred by the agglutination of chicken and turkey RBCs (Table 4.4.2) and attributable to mutation in the HA at L194P. Interestingly, proline is the amino acid present at residue 194 in the HA gene of Brisbane/10/07 virus, which also grows in eggs. Virus propagated in one of the three eggs inoculated with the E611 HA: A/Sydney/97 NA virus also acquired the L194P mutation, concurrent with the ability to agglutinate chicken RBCs. Although we cannot exclude the possibility that the viruses which do not agglutinate chicken RBCs are below the limit of detection since they have lower titres.

4.5 G186V is less efficient adapting the growth of more in eggs

We had demonstrated that the introduction of the E611 HA gene into a virus background that facilitates replication in eggs had increased the propensity for adaptive mutations in the HA gene to be selected. In this genetic background, all of the mutations that conferred egg growth were the same, L194P. However, it has previously been shown that another mutation, G186V, can confer egg adaptation to a range of recent influenza H3N2 viruses so we were surprised that this mutation had not arisen in at least some of the viruses with egg-adapted E611 HA. We introduced either the G186V or the L194P mutations into the synthetic E611 HA cDNA. Viruses containing each of the HA variants

Table 4.5.1 A/Eng/611 HA containing mutations thought to improve yields in eggs, L194P or G186V, were rescued by co-culture in a A/PR/8 backbone

Amino acid mutations L194P or G186V were introduced into synthetic A/Eng/611 HA by QuikChange XL site directed mutagenesis kit (Stratagene). Recombinant viruses were rescued by co-culture in a PR8 backbone. Receptor binding characteristics of the recombinant viruses were inferred by HA assay with either chicken or guinea pig RBC.

HA	CRBC	GPRBC
E611 wt	<	512
E611 G186V	<	512
E611 L194P	64	512
< Not detectable		

Table 4.5.2 G186V is less efficient adapting more modern viruses for growth in eggs

Recombinant influenza viruses containing either the wild type HA of A/Eng/611, or G186V or L194P mutation, in a PR8 backbone (including PR8 NA) were inoculated into the allantoic cavity of three 10 day old embryonated chicken eggs with equivalent HA units and incubated for 3 days at 37°C. Allantoic fluid was clarified by centrifugation at 2500 RPM for 10 minutes, 4°C and stored at -80°C. Adaptation in eggs was determined by ability to agglutinate chicken RBC. Virus titres of recombinant influenza viruses were determined by plaque assay in MDCK-SIAT cells.

HA	CRBC	GPRBC	PFU/ml
E611 wt	1,024	64	3x10 ⁵
	1,024	64	2x10 ⁵
E611 G186V	<	16	1.4x10 ⁵
	<	<	8x10 ⁴
E611 L194P	1,024	256	3x10 ⁷
	256	128	6.3x10 ⁶

< Not detectable

were rescued in a PR8 backbone in conjunction with the PR8 NA gene (7:1 recombinants) (Table 4.5.1). All viruses were rescued regardless of whether the 293T MDCK co-culture system or direct rescue in PER.C6 cells was employed. Viruses grew in cell culture to equivalent HA titres measured by HA assay with guinea pig RBCs. Only the L194P mutation conferred the ability to agglutinate chicken RBCs on viruses with E611 HA. Contrary to the implications of previous studies, G186V did not confer the ability to agglutinate chicken RBCs. Sequencing of the HA genes of each virus confirmed the presence of the engineered mutations, and that no other mutations were present.

Eggs were inoculated in duplicate with each of the recombinant viruses (Table 4.5.2). As before, eggs inoculated with a PR8 based recombinant virus with wt E611 HA supported virus replication and the propagated virus gained the ability to agglutinate chicken RBCs. HA gene sequencing revealed the acquisition of the L194P mutation but not the G186V change. Viruses with the G186V mutation engineered into the E611 HA gene grew to slightly lower titres in eggs than those with the L194P mutation and did not agglutinate chicken RBCs, although we cannot differentiate whether this was due to insufficient virus titre or to inability of the HA to bind SA on chicken RBCs. Although the recombinant virus with wt E611 HA gained the ability to agglutinate chicken RBCs, and the L194P mutation, it grew to lower titres than the virus engineered with the same mutation. However, this could be expected, since the virus had to acquire this mutation over multi-cycle growth. Of the two eggs inoculated with the L194P, the HA titres measured with chicken RBCs differed appreciably (1024 vs. 256). Interestingly, the HA gene of the virus with the higher chicken RBC HA titre contained an additional mutation G188D that may have increased the HA affinity for chicken RBCs. To ensure that these virus are still antigenically similar to the clinical isolate, and the mutations do not alter the antigenicity and therefore the protection provided if they were to be used as vaccines, in the future haemagglutinin inhibition assays (HAI) could be carried out with sera raised against the clinical isolate. However, as Brisbane, which

has the L194 mutation, has been included in the seasonal trivalent vaccine, this mutation is not expected to change the antigenicity.

4.6 PER.C6 cells faithfully support the growth of recombinant viruses containing the HA of a recent clinical isolate A/England/611/07

A universal vaccine seed must be able to grow in both eggs and cell culture. PER.C6 cells have proven suitable for the rescue a number of subtypes of viruses (Koudstaal, Hartgroves et al. 2009) and we have shown here that they faithfully support the growth of recent clinical isolates. To ensure that PER.C6 cells could support the rescue and propagation of proposed universal vaccine seeds, E611 wt, G186V and L194P HAs were rescued in the PR8 backbone, and passaged through PER.C6 cells. The viruses grew to high titres as measured by TCID₅₀ in MDCK cells (Table 4.6.1). The resultant viruses were sequenced, each virus was replicated accurately and no sequence changes were observed suggesting that each virus was replicated accurately.

Table 4.6.1 PER.C6 cells faithfully support the growth of recombinant viruses containing the HA of a recent clinical isolate A/England/611/07

Recombinant influenza viruses were rescued in PER.C6 cells, containing either the wild type HA of A/Eng/611, or G186V or L194P mutation, in a PR8 backbone (including PR8 NA). Virus was harvested 7 days post transfection and passaged once on PER.C6 cells. Titres of recombinant influenza viruses were determined by TCID₅₀ in MDCK cells.

HA	TCID ₅₀ /ml
E611 wt	3.9x10 ⁸
E611 G186V	4.4x10 ⁷
E611 L194P	1.9x10 ⁷

4.7 Discussion

The application of cell culture for the generation of influenza vaccine is widely being investigated as a supplement or replacement for egg grown vaccine (Minor, Engelhardt et al. 2009). Of particular consequence will be the ease of scale-up to produce more vaccine should the need arise (e.g. during an epidemic or pandemic). Furthermore, over the last decade, human influenza viruses have evolved a decreased capacity for growth in eggs to the extent that many recent clinical isolates are unable to grow. Here we show that the PER.C6 cell line faithfully supports the growth of recent influenza virus isolates, many of which were unable to grow in eggs. Receptor binding changes in the HA gene were not incurred during amplification of clinical isolates in PER.C6 cells. Sequencing corroborated the absence of mutations in PER.C6 passaged material. Thus, PER.C6 cells represent a suitable media for the propagation of vaccine viruses bearing authentic HA derived from clinical isolates, including those which are unable to grow in eggs.

However, since the use of cell culture for influenza vaccine is still in development, there is not yet the capacity to comprehensively supply the vaccine market with cell grown influenza vaccine. Therefore, a lag period is foreseeable, in which both cell culture and egg vaccine are in production. Rather than considering the generation of two disparate vaccine seeds, in the current study we investigated whether it was possible to generate a seed virus appropriate for both egg and cell culture derived vaccine manufacture for a typical influenza A virus H3N2 strain that originally did not replicate in eggs. We approached this using two different strategies: Firstly, we generated the recombinant vaccine virus and passaged it through eggs to assess whether egg-adapting mutations would be generated. In the second approach we pre-empted the 'random' generation of egg-adapting mutations by engineering mutations into the HA gene before rescue as recombinant virus. For this purpose, we tested two different mutations close to the receptor-binding site of

the H3 HA protein to see if they would confer egg adaptation to a virus with the H3 HA from a 2007/8 season clinical isolate. The first mutation, G186V, was proposed by others as a universal mutation to confer growth in eggs (Lu, Zhou et al. 2005) and was certainly able to do so when engineered into the HA gene of viruses from the early half of this decade (Lu, Zhou et al. 2006). However, the G186V mutation did not fully confer egg adaptation to the HA gene of our very recent (2007) clinical isolate, A/England/611/07 (E611). The second mutation we engineered was at position 194 in HA1. This change was present in the closely related vaccine strain A/Brisbane/10/07 and was also selected during passage of the 7:1 PR8/E611 HA recombinant virus in eggs. As expected, the L194P mutation was able to confer egg adaptation to the E611 HA protein. Thus, it would appear that the very recent human influenza isolates of the H3 HA subtype have an altered receptor-binding site in comparison with viruses isolated even just 5 years previously, a finding borne out by their inability to agglutinate turkey red blood cells in comparison to viruses of the Fujian/02 era. It may be difficult to find a universal egg adapting mutation for the H3N2 human influenza viruses.

It is noteworthy that the wild type E611 clinical isolate was not propagated in eggs even on serial passage. This suggests that its growth in eggs might be limited by incompatibilities within several viral genes. On the other hand, when the HA gene in isolation was conferred to the egg-adapted PR8 genetic backbone, mutants of the 7:1 recombinant virus were readily selected upon single passage in eggs. Thus, many clinical isolates whose HAs might be very suitable as components of the annual vaccine would not be considered due to limitations in the egg growth conferred by internal genes that are irrelevant to the vaccine process.

The combination of the HA gene of E611 with the NA gene from the PR8 vaccine strain may also account for the ability of the recombinant virus to grow sufficiently in eggs for adapting mutations to be selected. Influenza virus adaption to new hosts can be mediated by mutations in NA (Mitnaul,

Matrosovich et al. 2000; Wagner, Matrosovich et al. 2002). The NA enzyme activity and/or specificity of recent human influenza viruses may be insufficient to accommodate growth in eggs. Indeed, Fujian/02 and later strains acquired growth in eggs either with the G186V mutation in HA mentioned above (Lu, Zhou et al. 2006) but also through mutations in NA that affect the enzyme activity and consequently the balance between HA:SA affinity and receptor destroying activity. The most significant of the NA mutations for egg-adaption of Fujian-like strains was E119Q, but Q136K and H374Y were also noted (Lu, Zhou et al. 2005). Neither E611 nor Brisbane NA genes possess any of those key egg-adapting NA mutations. It is possible that the Brisbane NA gene that supports growth in eggs of viruses with Brisbane HA contains its own unique egg-adapting motifs. There are seven amino acid differences between the NA proteins of A/Eng/611/07 virus and the vaccine strain A/Brisbane/10/07 at positions 26, 45, 126, 147, 212, 267 and 312. Whether any of these accounts for the high growth properties of viruses with Brisbane HA in eggs has not been assessed. As for HA, it may be difficult to elucidate universal mutations to be introduced to wild type NA genes for egg-adaptation.

Incorporation of the E611 HA gene into the PR8 backbone and combination with PR8 NA was sufficient to allow a virus to acquire egg adaptation, and therefore supersedes the need for prior knowledge of the identity of the favoured mutation of any virus lineage. This suggests that PR8 NA, which shows only 42% identity with NAs of currently circulating viruses such as Brisbane, is well adapted to growth in eggs, and that matching HA and NA activity for high yield in eggs is not easily predicted.

This approach would mean that vaccines would not be updated with respect to the NA component. However, vaccine efficacy is measured by haemagglutination inhibition assay (HAI), which only measures the haemagglutinin specific antibodies and, vaccine content is largely described in quantities of HA. Moreover, recombinant baculovirus vaccines are being developed which express HA alone (Treanor, Wilkinson et al. 2001; Wang,

Holtz et al. 2006; Huber and McCullers 2008); these have proved to be immunogenic and protective. Furthermore, a 7:1 vaccine strategy has been described for the generation of H5N1 vaccine and H5 vaccine viruses with PR8 NA showed improved growth in eggs (Horimoto, Murakami et al. 2007). Thus matching with PR8 NA removes potential delays and facilitates timely and efficient vaccine production. To determine the contribution of PR8 NA, virus could be rescued in the future as a true 6:2 recombinant PR8: E611.

The quantity of HA gene sequences available has grown exponentially over recent years. Synthesis of HA genes using the data from sequences derived from clinical isolates allows the rapid generation of recombinant vaccine seeds from circulating viruses which otherwise might have been excluded from consideration as potential vaccine strains due to their inability to grow in eggs, and simultaneously eliminates the potential for transfer of adventitious agents to the vaccine seed. A 7:1 recombinant virus in a PR8 backbone generates a single vaccine seed which is suitable for influenza vaccine culture in both cell lines and eggs.

PER.C6 cells are able to support the rescue of vaccine seeds derived from any strategy. Furthermore, virus propagation in PER.C6 cells induces no adapting mutations, so they provide a highly suitable substrate to produce both vaccine seeds and large-scale vaccine which is an exact antigenic match for the chosen clinical isolates.

Chapter 5 Characterisation of PER.C6 IFN- β response

5.1 Introduction

The type I IFN response is a major element of the innate immune system's antiviral defence (reviewed by (Haller, Kochs et al. 2006), (Randall and Goodbourn 2008), and chapter 1). Virus infected cells synthesise and secrete type I IFN (IFN- α and - β), these cytokines alert the immune system to the invading pathogens and cause cells to express antiviral proteins which inhibit viral replication and dissemination. Upon detection of virus by pattern recognition receptors (PRR), such as intracellular RNA helicases retinoic inducible gene I (RIG-I (Yoneyama, Kikuchi et al. 2004) and melanoma differentiation-associated gene-5 (MDA-5 (Andrejeva, Childs et al. 2004), IRF-3 and NF κ B are activated and this leads to the induction of IFN- β (Sato, Tanaka et al. 1998; Sato, Suemori et al. 2000).

The induction of IFN- β by influenza viruses could have an adverse effect on the ultimate yield of influenza vaccine propagation under multicycle growth conditions. African green monkey kidney (Vero) cells, which are licensed for a range of human vaccines including poliovirus, rabies and an inactivated influenza vaccine from Baxter healthcare corp. vaccines (Kistner, Howard et al. 2007), are able to respond to IFN- β but lack a functional IFN- β gene and therefore do not secrete it. This may contribute to their success as a vaccine substrate. The IFN- β response of PER.C6 has yet to be characterised and it is unknown whether it is complete and functional.

PER.C6 cells have been shown to support the growth of wild type clinical strains of influenza viruses (chapter 3, (Pau, Ophorst et al. 2001). Since growth in cell culture does not require the high growth backbone that is needed for

vaccine manufacture in eggs, pharmaceutical companies may consider the generation of vaccines directly from wild type clinical isolates. In this event, with no backbone donor, the internal genes of vaccine strains will vary. Influenza viruses have been shown to induce strain dependant levels of IFN- β in IFN competent cells lines (e.g. A549 cells (Hayman, Comely et al. 2006), pig macrophages (Seo, Hoffmann et al. 2004), and certain strains such as A/Sydney/97, a chosen vaccine strain, induce particularly high levels. Accordingly, the use of wt viruses as vaccine seeds will be significantly less predictable than recombinant or reassortant viruses and the IFN- β induction could limit virus propagation.

Also pertinent is the novel strategy for the generation of LAIV from recombinant viruses attenuated through the truncation or deletion of the influenza encoded IFN antagonist, NS1. Deletion of NS1 results in high IFN- β induction by the recombinant virus (Egorov, Brandt et al. 1998; Garcia-Sastre, Egorov et al. 1998). LAIV has been developed with either truncated or deleted NS1 proteins, that are highly attenuated in both immunologically mature, embryonated eggs and mice (Talon, Salvatore et al. 2000; Romanova, Krenn et al. 2009). These viruses have been rescued in Vero cells which as stated above, lack the ability to secrete IFN- β . They are likely to be attenuated in IFN competent cell lines. The strategy of NS1 mutation has also been used to make H5N1 vaccine. These were highly attenuated in mice, and reduced growth correlated with high levels IFN- β . These viruses protected against challenge with HPAI H5N1 viruses in poultry, ferrets and macaques and hold the potential for use in mammalian hosts (Romanova, Krenn et al. 2009; Steel, Lowen et al. 2009). Vaccines of this design will require propagation in IFN incompetent cell lines.

It has already been demonstrated that cell lines which lack an IFN response give rise to higher yields for other virus vaccines (Young, Andrejeva et al. 2003) and this general strategy could be applied to the manufacture of

influenza vaccines. Hepatitis C virus (HCV) has been notoriously difficult to research due to the lack of permissive cell lines. It was established that a hepatoma cell line, Huh-7, could support the replication of HCV replicons (Lohmann, Ouml et al. 1999; Blight, McKeating et al. 2002). A subline of Huh-7, Huh-7.5, showed much increased susceptibility and this has been attributed to a deficiency in the induction of IFN signalling as a result of a point mutation in RIG-I (Sumpter, Loo et al. 2005) in the TRIM25 binding domain (Gack, Albrecht et al. 2009). Cells lines have been developed which have artificially abrogated IFN responses by transduction with viral antagonists. Many viruses encode proteins which antagonise IFN induction. Paramyxovirus 5 encodes V protein, which interacts with MDA-5 and targets STAT-1 for degradation, thus blocking IFN- α , - β and - γ signalling within infected cells (Didcock, Young et al. 1999; Andrejeva, Childs et al. 2004). Bovine Viral Diarrhoea virus (BVDV) encodes N-pro, which blocks signalling of IRF-3 by preventing translocation to the nucleus and targeting it for degradation (Hilton, Moganeradj et al. 2006). MDCK and A549 cell lines have been developed by selection of stable transfection (Young, Andrejeva et al. 2003; Hayman, Comely et al. 2006; Hayman, Comely et al. 2007)) and more recently infection with lentivirus encoding these viral proteins (Hale personal communication). The development of several negative-strand RNA virus recombinant vaccines that lack IFN antagonists, such as respiratory syncytial virus (RSV) and Bunyamwera virus, has been compromised by the inability of these viruses to grow to high titres in susceptible cell types which are IFN competent. MRC5 and HEp2 cell lines were transduced with the V protein of PIV5 (Young, Andrejeva et al. 2003). The most significant results were seen in the HEp2/SV5-V cells. In this cell line there was a 4,000-fold increase in the yield of Bunyamwera Δ NS virus compared to wt HEp2 cells. Similarly, there was a $\sim$ 200-fold increase in the yield of RSV Δ NS1 and RSV Δ NS2 in HEp2/SV5-V cells compared to the wt cell line. Only a 35-fold increase was seen in Vero cells for the RSV Δ NS viruses. Evidently, IFN competent cells have the potential to severely limit viral yields. If PER.C6 cells have a vigorous IFN response, then antagonism of the response by strategies that ablate the activity of genes involved in IFN activation may

help to further improve influenza vaccine yields that may naturally vary in the extent to which they trigger an IFN- β response.

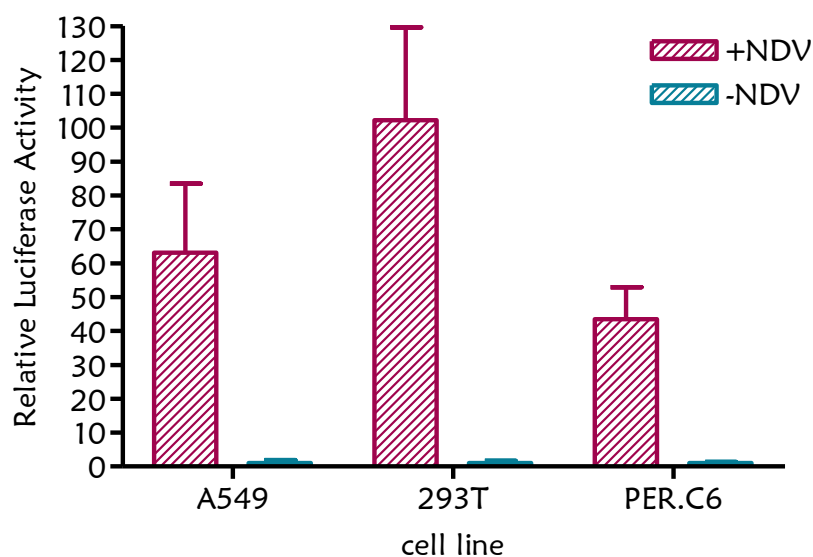
The aim of this chapter is therefore to characterise the IFN response in PER.C6 cells to establish whether antagonism of the IFN response is appropriate and whether PER.C6 are a suitable cell line in which to prepare vaccines from clinical isolates.

5.2 PER.C6 cells make IFN- β in response to Newcastle disease virus

To ascertain whether PER.C6 cells produce IFN- β in response to virus infection, cells were transiently transfected with a reporter construct in which luciferase gene expression is driven by IFN- β promoter (IFN-luc). Newcastle disease virus (NDV) is a negative sense RNA virus which replicates in the cytoplasm and is a potent activator of IFN in mammalian cells (figure 5.2.1). In IFN- β luc transiently transfected PER.C6 cells, NDV infection resulted in approximately a 40-fold activation of the IFN- β promoter. This level was similar to that seen in other IFN competent cell lines such as A549s (human lung epithelial cells) and 293T cells (human epithelial kidney cells). PER.C6 cells must therefore possess the upstream components of the IFN signalling pathway to activate the IFN- β promoter.

Figure 5.2.1 IFN- β promoter is activated in PER.C6 cells following Newcastle disease virus infection

Infection with Newcastle disease virus (NDV) induces IFN- β in PER.C6 cells. PER.C6 and 293T cells were transiently transfected with IFN- β luc reporter plasmid. An A549 cell line stably expressing IFN- β luc was previously generated (Hayman, Comely et al. 2006). Cells were infected with NDV (48 hrs post transfection for PER.C6 and 293T) for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hours at 37°C. Cells were washed PBS, lysed in CCLR, clarified 1000 RPM for 1 min and luciferase measured. Data is represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments.



5.3 Influenza viruses poorly activate the IFN- β promoter in PER.C6 cells

NDV replicates in the cytoplasm, whilst influenza replicates in the nucleus; the IFN response may therefore differ for these viruses. Most influenza viruses are able to suppress the IFN response; however, Hayman et al (Hayman, Comely et al. 2006) described significant variation in the IFN response induced by different strains of influenza in A549 cells. The strain A/Sydney/97 (Syd) typified an influenza virus with relatively high IFN- β inducing properties. A/Victoria/3/75 (Vic) is an older H3N2 strain which induces low levels of IFN- β in A549 cells.

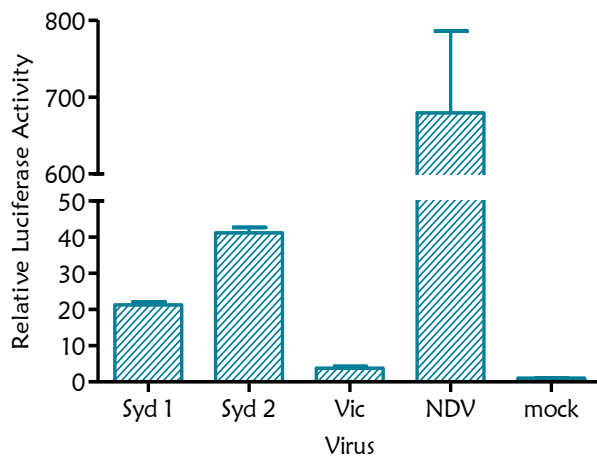
Tested on several occasions with different preparations of virus, A/Sydney/97 did not activate the IFN- β promoter in PER.C6 cells. In figure 5.3.1, two separate preparations of A/Sydney/97 were compared to A/Victoria/75. As seen previously in A549 IFN- β luc cells (figure 5.3.1A), A/Sydney/97 activated the IFN- β promoter by 20-40 fold. In PER.C6 cells (figure 5.3.1 B), A/Sydney/97 activated the IFN- β promoter to insignificant levels similar to the negligible activation by A/Victoria/3/75. Even at a higher MOI A/Sydney/97 did not activate the IFN- β promoter to high levels in PER.C6 (figure 5.3.2). A panel of recent clinical isolates from 04/05 season was also tested in this assay, these included H1N1 viruses (A/Eng/48/04, (A/Eng/208/05, A/Eng/280/05, A/Eng/204/05), H3N2 viruses (A/Eng/26/05, A/Eng/29/05, A/Eng/81/05, A/Eng/180/05), influenza B viruses (B/Eng/216/05, B/Eng/319/05). None of these viruses activated the IFN- β promoter to significant levels in PER.C6 (figure 5.3.3).

It was possible that the low IFN- β induction in PER.C6 cells reflected a low infectivity of influenza viruses in these cells. This was deemed unlikely since it has been shown that influenza viruses grow to high titres in PER.C6 cells (chapters 3&4). Moreover, immuno-staining of cells infected with virus (figure

Figure 5.3.1 A/Sydney/97 does not induce high levels of IFN- β in PER.C6

A549 cells stably transfected with IFN- β luc (A) were compared to PER.C6 cells transiently transfected IFN- β luc (B). 48 hours post transfection, cells were infected with two different A/Sydney/97 preparations, or A/Victoria/3/75 MOI 2, for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hrs at 37°C. Cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. Error bars represent standard error.

A)



B)

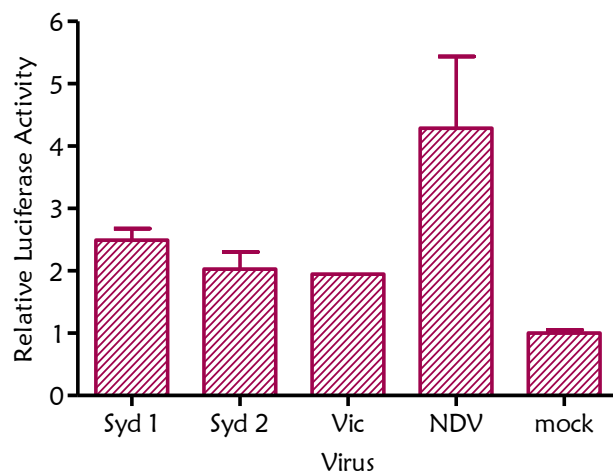


Figure 5.3.2 A high MOI of virus does not activate the IFN- β promoter in PER.C6 cells

PER.C6 cells were transiently transfected IFN- β luc and compared to A549 cells stably transfected with IFN- β luc. 48 hours post transfection cells were infected with virus MOI 5 A/Sydney/97 (Syd) or A/Victoria/3/75 (Vic), for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hrs at 37°C. Cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments. Error bars represent standard error.

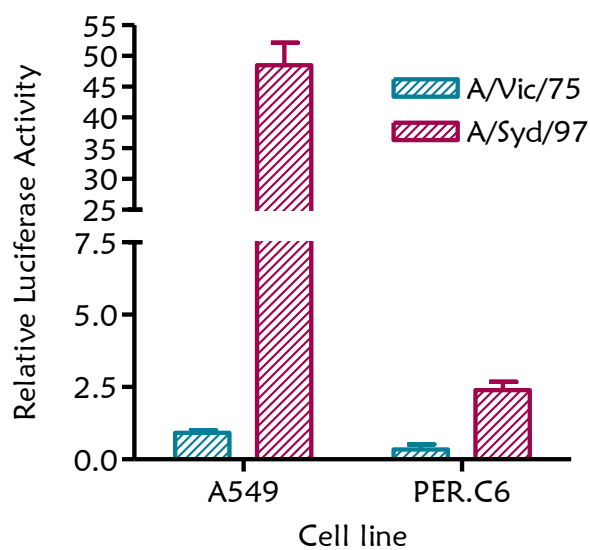
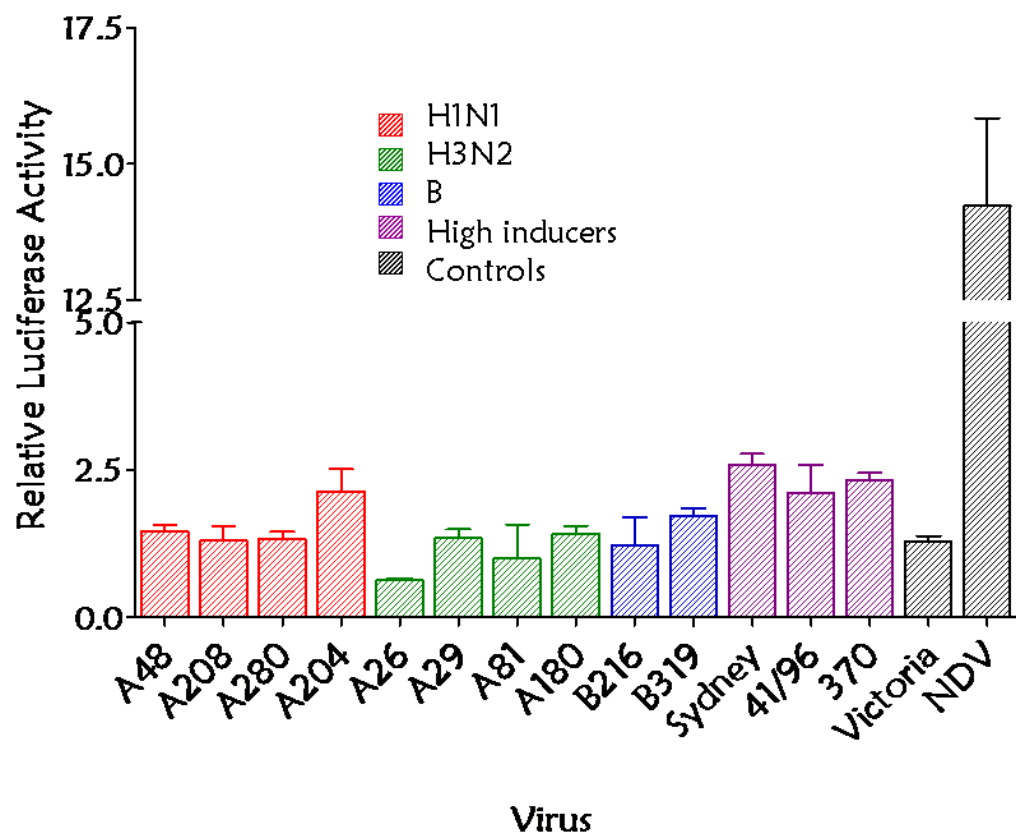


Figure 5.3.3 Recent seasonal influenza isolates do not activate the IFN- β promoter in PER.C6 cells

PER.C6 cells were transiently transfected IFN- β luc. 48 hrs after transfection cells were infected with virus MOI 2, for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hours at 37°C. Cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of two repeat experiments. Error bars represent standard error.

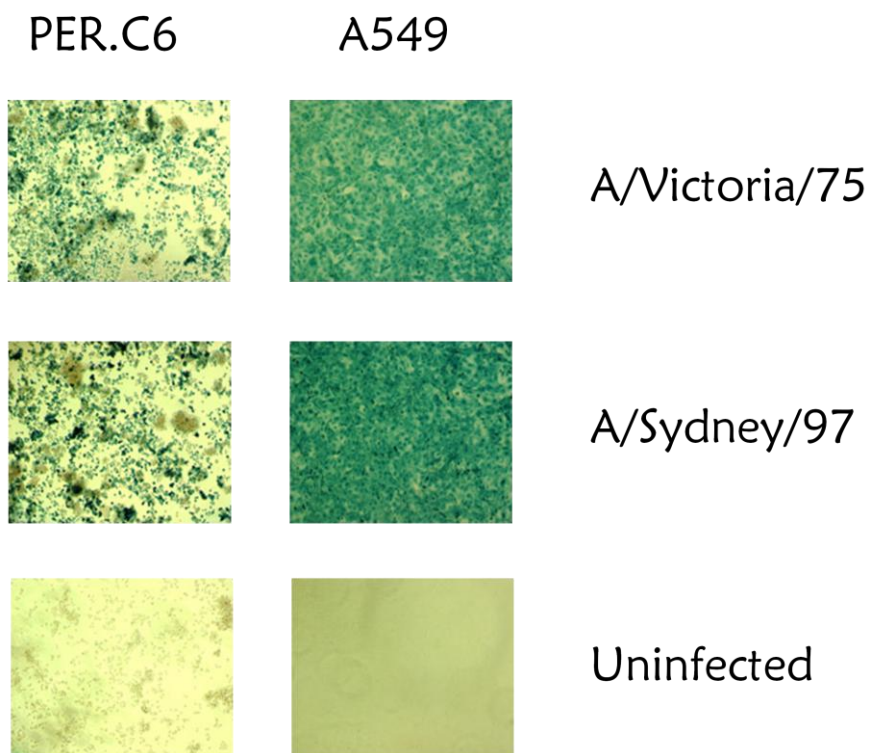


5.3.4A) in parallel with the luciferase assay (figure 5.3.2) demonstrate that the low IFN- β induction in PER.C6 was not a result of an unproductive infection, since most cells express NP. Despite coating the plates with poly-L-lysine, the PER.C6 cells do not withstand the repeated wash steps and become easily detached, resulting in gaps in the monolayer. The cells also form clumps which do not stain well; the clumps are possibly inaccessible to either the virus or the antibody. These effects may arise because PER.C6 cells are essentially a suspension cell line which can be driven to adherence by the addition of serum to the media. To use an alternative method for assessing infection of PER.C6 cells by influenza, the generation of vRNA was measured using qRT PCR to determine the number of copies of segment 7 (M) vRNA/ml. This data also showed that viral replication was occurring to at least equivalent levels in PER.C6 cells and A549 cells (figure 5.3.4B). In accordance with data to be presented in chapter 6, following infection with A/Sydney/97 in either cell line more vRNA is generated than infection with A/Victoria/75, suggesting faster replication. Furthermore, both viruses generate more vRNA in PER.C6 cells suggesting that PER.C6 cells are more permissive for RNA replication than A549 cells. Thus, the low IFN- β induction in PER.C6 cells is not a result of either reduced viral infection or replication.

The luciferase assay does not measure secreted IFN- β , rather the activation of the IFN- β promoter. A complete and functioning set of upstream signalling proteins will activate the IFN- β promoter but may not correlate with synthesis of IFN- β . However, the induction of the IFN- β promoter measured by the luciferase reporter assay correlated with IFN- β RNA measured by qRT PCR (figure 5.3.5). In A549 cells A/Sydney/97 induced very high levels IFN- β mRNA which correlate to ~65 fold induction over A/Victoria/75, in line with results seen using the reporter assay. In PER.C6 cells neither A/Victoria/75 nor A/Sydney/97 virus infection results in induction of IFN- β mRNA. An IFN- β ELISA (R&D systems) was used to determine whether luciferase expression corresponded to secreted IFN- β .

Figure 5.3.4 The low IFN- β induction by influenza viruses in PER.C6 cells is not a result of unproductive infection

A) A549 cells and PER.C6 cells were infected with A/Victoria/75 or A/Sydney/97 virus at an MOI 5 for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hours at 37°C. Cells were fixed and permeabilised using ice-cold methanol: acetone (50:50 v/v) for 10 minutes. NP was detected by incubation with mouse α -NP antibody (1/300) for 1 hour at RT, washed 3x PBS, followed by goat α -mouse antibody conjugated to β -galactosidase (1/400) for 1 hour at RT. Infected cells were visualised by incubation overnight at 37°C with CN buffer (X-Gal substrate) which changes from yellow to blue in the presence of β -galactosidase.



B) A549 cells and PER.C6 cells were infected with A/Victoria/75 or A/Sydney/97 virus at an MOI 2 for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hours at 37°C. RNA was extracted with TRIzol, and cDNA generated with QuantiTect RT (Qiagen) using random hexamer primers. cDNA was amplified by qRT PCR, using primers specific for H3N2 influenza matrix protein. Copies/ml were normalised to GAPDH

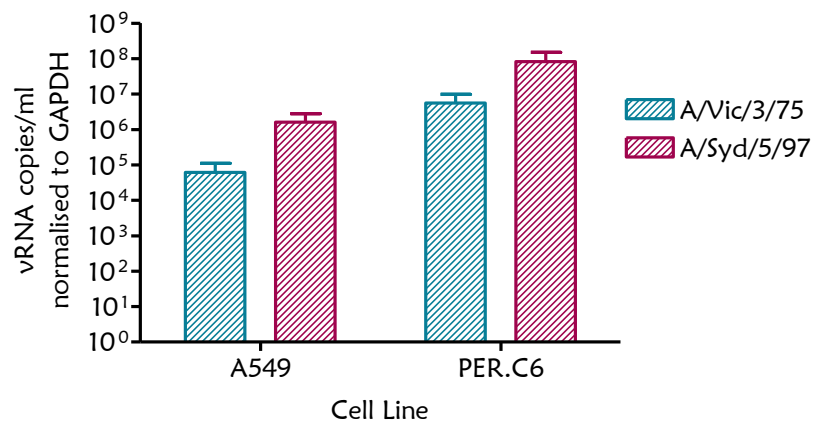
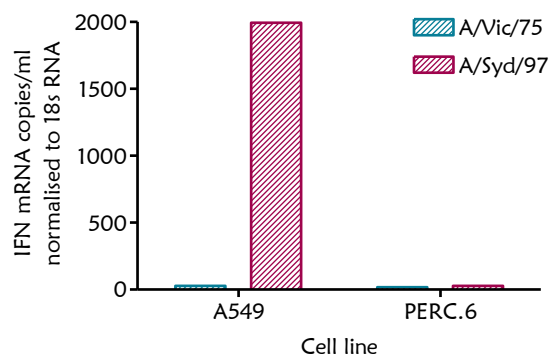


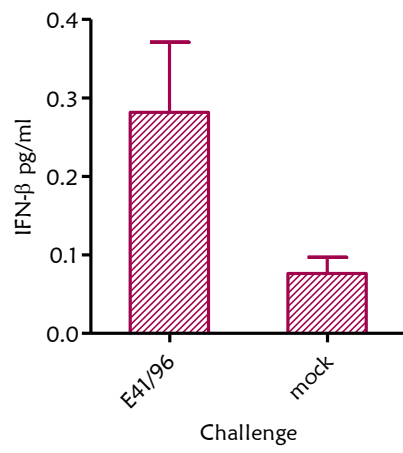
Figure 5.3.5 Luciferase expression correlates with IFN- β expression and secretion

A) A549 cells and PER.C6 cells were infected with A/Victoria/75 or A/Sydney/97 virus at an MOI 2 for 1 hour, washed in PBS, then incubated 2% DMEM, for 7 hours at 37°C. RNA was extracted with TRIzol, and cDNA generated with QuantiTect RT kit, Qiagen. cDNA was amplified by qRT PCR, using primers specific for human IFN- β . Copies/ml were normalised to 18s ribosomal RNA. Overleaf, A549 cells (B) and PER.C6 cells (C) were infected with influenza virus A/England/41/96 (4196) at an MOI 2, and PER.C6 cells with NDV as a positive control, for 16 hours. Supernatants were removed, clarified by centrifugation 13,000 RPM for 1 min and then applied to an ELISA which employs streptavidin-conjugated horseradish peroxidase (HRP) and Tetramethyl-benzidine (TMB) as the substrate, to measure IFN- β secretion. This assay is representative of two repeat experiments.

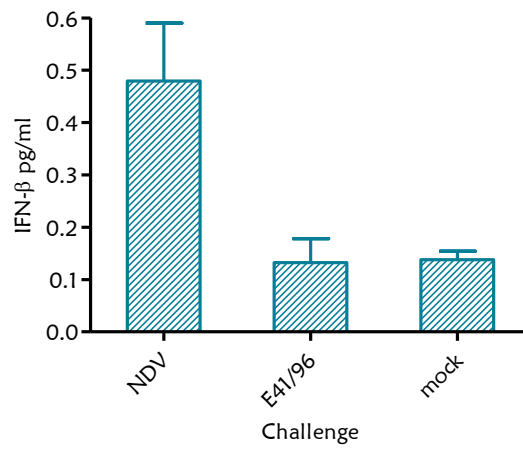
A)



B)



C)



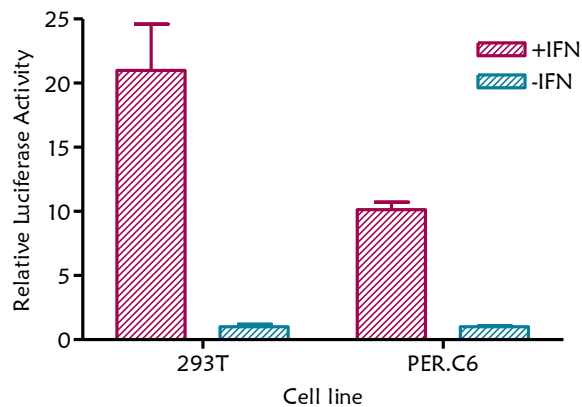
Cells were infected with A/England/41/96 (4196) an alternative high IFN- β inducing strain of influenza (Hayman, Comely et al. 2006), which resulted in IFN- β secretion in A549 cells (figure 5.3.5B). In contrast, infection of PER.C6 cells with A/England/41/96 virus did not result in secretion of IFN- β . This correlated with both the activation of the IFN- β promoter measured by the luciferase assay and the number of RNA copies measured by qRT PCR (figure 5.3.4C). However, infection with NDV does result in the secretion of IFN- β in PER.C6 cells, albeit to a low level. Taken together the data suggests that the mechanisms involved in IFN- β induction by influenza viruses may be defective in PER.C6 cells.

5.4 PER.C6 cells respond to IFN- β

Paracrine IFN signalling results in the formation of signalling complexes which translocate to the nucleus and bind to the IFN stimulated response element (ISRE promoter) leading to the expression of many antiviral genes. Infection of Vero cells, which lack a functional IFNAR, suggest that positive feedback enhances IFN- β induction by influenza viruses (Johnson et al manuscript in preparation). The ISRE promoter has been cloned upstream of a luciferase gene by Steve Goodbourn (St. Georges, University of London) to provide a simple quantification of ISRE promoter activation. To assess whether PER.C6 cells were able to respond to IFN- β they were transiently transfected with an IFN stimulating response element upstream of a luciferase reporter gene (ISRE-luc) and stimulated with 1U/ml of IFN- β for 16 hours (figure 5.4.1). Priming of PER.C6 ISRE-luc cells resulted in a 10-fold activation of the ISRE promoter. Although the response was not as marked as in 293T ISRE-luc cells transfected with the same reporter, this could be due to a less efficient IFN- β induction in PER.C6 or less transfected cells since β -gal assay was not performed for this assay. However, data shown in chapter 3 indicates little difference in the transfection efficiency between PER.C6 and 293T cell lines (section 3.2.1)

Figure 5.4.1 PER.C6 cells respond to IFN- β

293T cells and PER.C6 cells were transiently transfected with ISRE-luc. 24 hours post transfection cells stimulated with 1U/ml IFN- β for 16 hours. Cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments. Error bars represent standard error.



In summary, PER.C6 cells respond to IFN- β and secrete it following NDV infection, but respond only poorly to influenza viruses.

5.5 IFN- β promoter is activated in PER.C6 cells in response to poly IC but not to *in vitro* transcribed viral-like dsRNA

It is intriguing that PER.C6 cells do not secrete IFN- β in response to infection by influenza virus. It is possible that they lack the ability to detect the influenza RNA PAMP. In order to identify the possible PAMPs that PER.C6 cells can recognise and thus further characterise the IFN response in PER.C6, cells were stimulated with three different RNA based PAMPs. Poly IC is a synthetic dsRNA analogue thought to activate the MDA-5 pathway (Kato, Takeuchi et al. 2006). Poly IC was obtained from a commercial source (GE healthcare, formerly Pharmacia). *In vitro* transcribed dsRNA with 5' triphosphate is thought to activate the RIG-I pathway. A particularly relevant dsRNA would contain influenza sequences. Influenza-like dsRNA was synthesised from plasmids encoding short defective viral RNAs. These short RNAs result from spontaneously occurring deletions in full-length viral RNAs during RNA synthesis. The short RNAs are incorporated into virions, often called defective interfering particles (DIs) which are able to infect cells, but are unable to replicate without the assistance of an intact helper virus. dsRNA representing influenza DI RNA was generated by *in vitro* transcription from appropriate cDNA that contained a T7 promoter upstream of influenza sequences (figure 5.5.1). RNPs are the packaged state of influenza viral genomes and consist of influenza viral RNA complexed with nucleoproteins. RNPs represent a form of viral RNA which is known to exist in cells during viral replication, although it has yet to be determined if RNPs induce IFN- β . RNPs were obtained by purifying the NP rich fraction of egg derived virus particles through CsCl gradient ultracentrifugation purification (Parvin, Palese et al. 1989)

Fig 5.5.1 Schematic representation of *in vitro* transcribed dsRNA synthesis

Influenza-like dsRNA was synthesised from plasmids encoding defective interfering RNAs. T7 RNA polymerase promoters are added at the 5' ends of both DNA strands with amplification by PCR with primers containing the T7 promoter sequence at the 5' end. Generation of the two DNA templates requires two separate PCR amplifications, each containing one gene specific primers, and the converse T7 primers. Two complementary RNA strands are then synthesized from the DNA templates using T7 RiboMAX Express RNAi System (Promega). The resulting RNA strands are annealed to form dsRNA.

Figure 5.5.1 Schematic representation of *in vitro* transcribed dsRNA synthesis

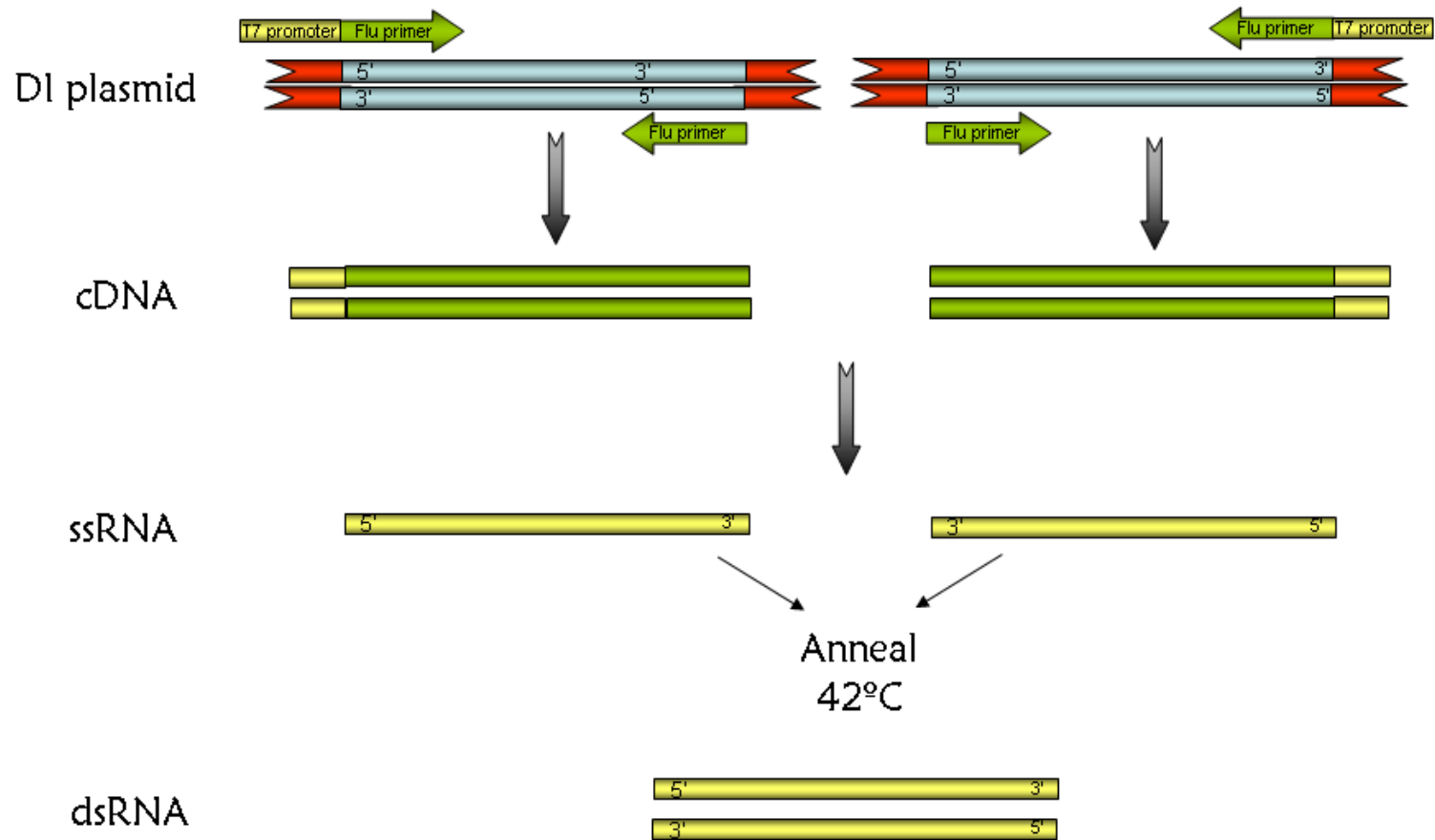
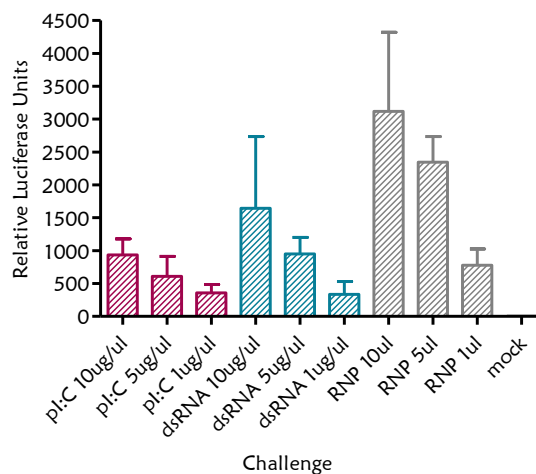


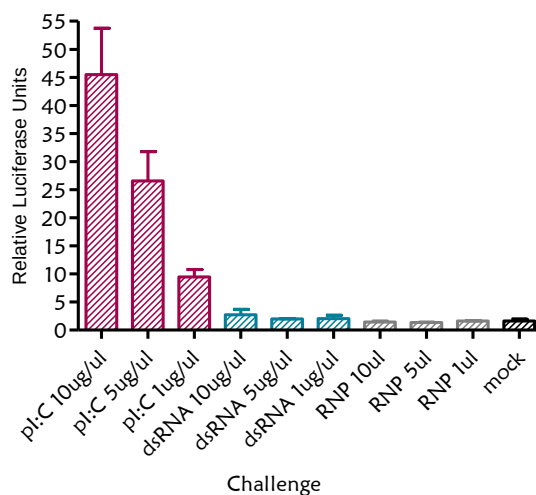
Figure 5.5.2 IFN- β promoter is activated in PER.C6 cells in response to poly IC but not dsRNA

A549-luc cells (A) or PER.C6 cells transiently transfected with IFN- β luc (B) were stimulated with a panel of stimuli. Poly IC (pl:C), *in vitro* transcribed dsRNA and RNPs were transfected into cells 48 hours post IFN- β luc transfection. 6 hours post transfection cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of two repeat experiments. Error bars represent standard error.

A)



B)



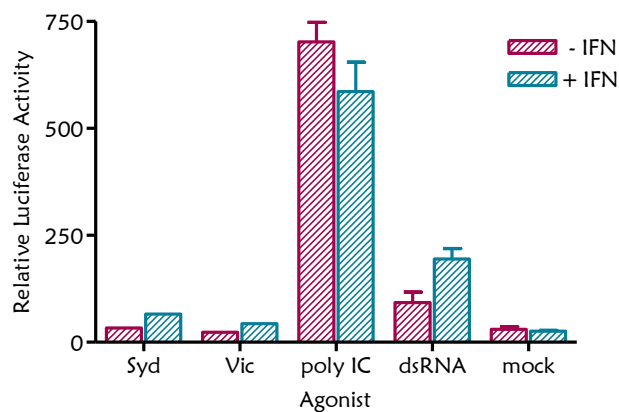
A549s stably expressing the IFN-luc reporter or PER.C6 cells transiently transfected with IFN-luc reporter plasmid, were stimulated with poly IC, *in vitro* transcribed dsRNA, RNPs or NDV (figure 5.5.2). All three RNA based PAMPs activated the IFN- β promoter in A549 cells (figure 5.5.2 A). However, only poly IC was able to activate the IFN- β promoter in PER.C6 cells (figure 5.5.2 B). This implies that PER.C6 cells have an intact MDA-5 pathway but are impaired in RIG-I signalling. Alternatively poly IC may be detected by TLR-3 and both MDA-5 and RIG-I could be defective in PER.C6 cells.

5.6 PER.C6 cells respond to IFN- β priming

RIG-I and MDA-5 are IFN stimulated genes (ISGs), their expression is upregulated by IFN- β (Yoneyama, Kikuchi et al. 2004; Siren, Imaizumi et al. 2006). In order to test whether IFN- β primed PER.C6 cells would respond more robustly to challenge with influenza viruses or viral like dsRNA, after up-regulation of PRRs, cells were transiently transfected with IFN- β luc plasmid and pre-treated with exogenous IFN- β overnight. Cells were stimulated as before with influenza virus, poly IC or dsRNA (figure 5.6.1). Following stimulation with virus and dsRNA a two-fold greater activation of IFN- β promoter is seen in IFN- β primed cells than in naïve cells. This could be the result of up-regulation of RIG-I following IFNAR signalling. IFN- β promoter activation by poly IC transfection was unaffected by IFN- β pre-treatment. Poly IC IFN- β activation was much greater than for other stimuli. The amount of poly IC added could be in excess and may have already saturated the response. Alternatively, the high levels of IFN- β could be a result of very high or even constitutive expression of the poly IC PRR.

Figure 5.6.1 PER.C6 cells respond to IFN- β priming

PER.C6 cells were transiently transfected with IFN- β luc plasmid. 48 hours post transfection cells were primed with 0.5 U/ml IFN- β , for 16 hours. Cells were subsequently infected with virus MOI 2 A/Sydney/97 (Syd) and A/Victoria/3/75 (Vic), or transfected with 10 μ g/ml poly IC or dsRNA. 7 hours post transfection/infection cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of two repeat experiments. Error bars represent standard error.

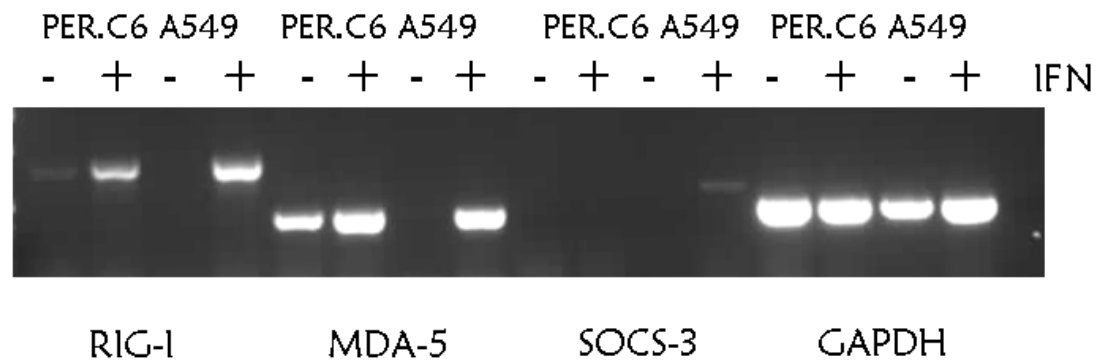


5.7 Expression of PRR differs in PER.C6 cells to IFN- β competent A549 cells

Semi-quantitative RT-PCR was used to determine the levels of expression of MDA-5 and RIG-I mRNAs, pre and post-IFN- β treatment. Cells were primed with 0.5U/ml IFN overnight and lysed in TRIzol according to manufacturer's instructions. Total cellular RNA was quantified by spectrophotometer. Equivalent amounts of RNA were used to generate cDNA and then equal volumes of cDNA were used in the PCR reaction to render the PCR quantitative. The PCR was limited to 25 cycles to maintain the proportional relationship between amplification and input RNA. Expression of RIG-I, MDA-5, SOCS3 and GAPDH mRNA as a loading control, were evaluated (figure 5.7.1). As expected in A549 cells RIG-I and MDA-5 mRNAs are present in low amounts in untreated cells, and were up-regulated following priming with IFN- β (Siren, Imaizumi et al. 2006). SOCS3 is moderately upregulated. Although total signals for SOCS3 are low, SOCS3 expression has been shown to peak 3 hours p.i. in A549 cells (Pauli, Schmolke et al. 2008), therefore it is possible that expression of SOCS3 had already peaked after overnight IFN- β treatment. In a human bronchial epithelial cell line, BEAS-2B SOCS3 expression peaks after 14 hours (Pothlichet, Chignard et al. 2008). In PER.C6 cells both RIG-I and MDA-5 were upregulated following IFN- β pre-treatment. Interestingly, MDA-5 was constitutively expressed prior to IFN- β treatment; this may contribute to the high reactivity to poly IC. Since no standards were used, it is not possible to compare the levels of expression from gene to gene; the levels of MDA-5 might not be higher, rather its primers may be more efficient than those of SOCS-3 for example. If either PRR are functionally competent in un-primed cells, their residual expression could certainly have an adverse effect vaccine yields for viruses that are IFN- β sensitive.

Figure 5.7.1 Expression of PRR differs in PER.C6 cells to IFN- β competent A549 cells

Cells were primed with IFN- β for 16 hours. Cells were lysed in TRIzol (Invitrogen) and RNA extracted according to instructions. 2 μ g RNA was used in an RT reaction to synthesize cDNA. 2 μ l of this reaction was used in a 25 μ l PCR reaction using primers against RIG-I, MDA-5, SOCS-3, GAPDH was used as loading control.

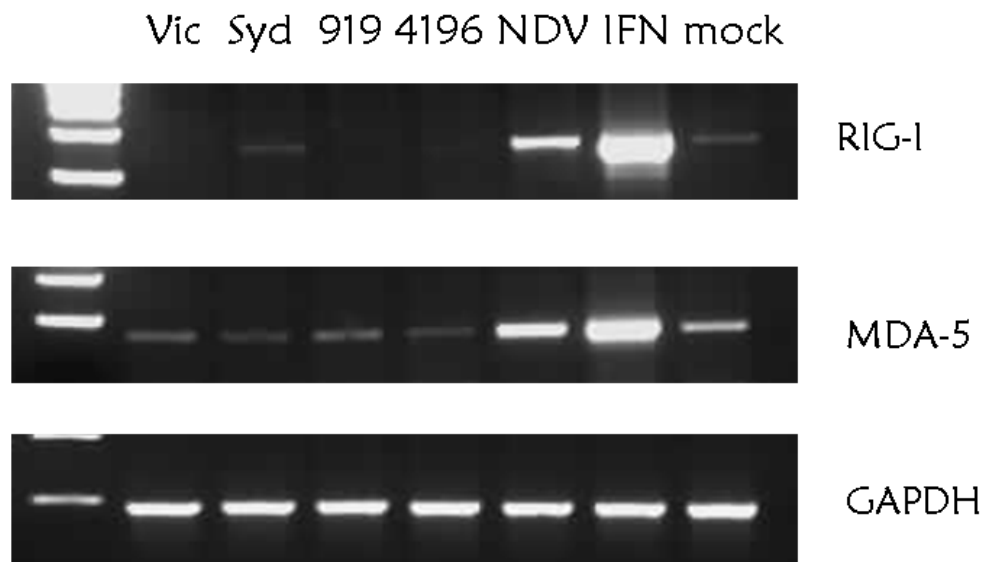


5.8 Viruses do not upregulate PRR RIG-I and MDA-5

IFN- β priming moderately enhanced the sensitivity of PER.C6 cells to PAMPs, but this would only be relevant to vaccine production if it were to occur during multicycle virus growth in response to virus infection. Therefore, expression levels of PRRs were also analysed following infection with a panel of high inducing viruses A/Sydney/97 (Syd) and A/England/41/96 (4196), or low inducing viruses (A/Victoria/3/75 (Vic) and A/England/919/99 (919) (figure 5.8.1). Cells were infected with the viruses and treated as previously described (section 5.7). As before, MDA-5 expression is constitutive in PER.C6 cells and priming with IFN- β or infection with NDV both up-regulated expression of the PRR mRNAs in both A549s cells (data not shown) and PER.C6 cells. However, infection with influenza virus did not elicit induction of PRR expression.

Figure 5.8.1 Viruses do not upregulate PRR RIG-I and MDA-5

PER.C6 cells were infected with influenza virus for 8 hours MOI 2 A/Victoria/3/75 (Vic), A/Sydney/97 (Syd) A/England/919/99 (919) and A/England/41/96 (4196), or infected with NDV for 8 hours, or were primed with IFN- β for 16 hours. Cells were lysed in TRIzol (Invitrogen) and RNA extracted according to instructions. 2 μ g RNA was used in an RT reaction to synthesize cDNA. 2 μ l of this reaction was used in a 25 μ l PCR reaction. Screened with primers against MDA-5, RIG-I and GAPDH as a loading control.



5.9 Discussion

Vaccine manufacturers are investigating the potential of cell culture for the next generation of influenza vaccines. Most laboratory based cell lines available are unpermissive, unlicensable or low yielding, hence the need to test new lines such as PER.C6. PER.C6 cells are also able to support the growth of both clinical isolates and vaccine strains (chapter 3 and chapter 4). There exists great variation in the levels of IFN- β induction from clinical isolates from year to year and even within a single season, which could lead to inconsistency in yields (Hayman, Comely et al. 2006).

PER.C6 cells appear to be IFN- β competent; the IFN- β promoter is activated and IFN- β protein is secreted in response to poly IC or NDV. They also respond to exogenous IFN- β by activation of the IFN- β responsive promoter, ISRE. However, infection of PER.C6 cells by influenza virus results only in low activation of IFN- β promoter, suggesting a defect in either the detection of the influenza PAMP or the downstream signalling pathway. As such, PER.C6 cells represent a good cell line for the propagation of clinical influenza isolates which may vary in their ability to induce IFN- β .

The key PRRs involved in detection of RNA PAMPs are MDA-5 and RIG-I. Previously MDA-5 has been associated with detection of poly IC and positive sense RNA viruses whilst RIG-I has been associated with influenza virus (Kato, Takeuchi et al. 2006) and in particular the presence of 5' triphosphate RNA (Pichlmair, Schulz et al. 2006). However, recent research suggests that rather than being related to the nature of the nucleic acids, the recognition by each PRR is associated to its length; RIG-I and MDA-5 selectively recognize short (<1kb) and long (>1kb) dsRNAs, respectively (Kato, Takeuchi et al. 2008). The DI dsRNAs used here (DI 316) was 585 nucleotides long (Duhaut and Dimmock 2002) and the poly IC (GE healthcare (formerly Pharmacia) ranges between

4,000 and 6,000 nucleotides. These therefore represent distinct activators of RIG-I and MDA-5 respectively.

The constitutive expression of the PRR MDA-5 may explain why PER.C6 cells respond more robustly to poly IC. Alternatively, the response to poly IC in PER.C6 may also be transduced by TLR-3. More accurate and sensitive techniques, such as quantitative RT PCR, would allow comparisons between the levels of gene expression in different cell lines and give further indication of any irregularities in genes associated with IFN induction. Following activation of RIG-I, expression of an inactive splice variant (SV) is up-regulated (Gack, Kirchhofer et al. 2008). Analysis of MDA-5 has yet to be performed but inactive splice variants may also be generated following IFN induction. Full length RT PCR of PRRs could determine potential defects in PRR splice variant expression in PER.C6. Furthermore, application of a micro-array would detect difference in expression of the full range of proteins involved in IFN signalling and clarify precisely if the induction or expression of any proteins is defective. Sequencing of both MDA-5 and RIG-I, and proteins flagged in the micro array might indicate where the defects lie. However, since PER.C6 cells are able to support the propagation of influenza virus, further characterisation of these genes may not be necessary.

On the other hand, antagonism of the residual IFN response albeit low may increase yields further. Cell lines expressing virally encoded antagonists have improved yields for viruses which induce high levels of IFN e.g. MDCK cells that express PIV5 V protein (Young, Andrejeva et al. 2003). No significant increase in yield was noted for viruses which do not induce significant levels of IFN- β (unpublished observation Anna Hayman). However, since very little IFN- β has been detected following infection of PER.C6 with influenza with either vaccine strains made by reverse genetics, clinical isolates or reassortant viruses, no significant benefit is likely to be gained by growth in an IFN antagonised cell line. Furthermore, an altered cell line would also require re-

validation to ensure the antagonism has no ensuing adverse side effects on the safety, composition and efficacy of the vaccine.

The constitutive expression of MDA-5 may be a concern for the propagation and vaccine development for positive sense RNA viruses such as members of the Picornaviridae family, e.g. Encephalomyocarditis (EMCV) (Gitlin, Barchet et al. 2006) or the negative sense paramyxoviruses (PIV5) (Childs, Stock et al. 2007) which are known to induce IFN- β expression in an MDA-5 dependant manner. In this instance, antagonism of the IFN response by transduction of the cells with viral antagonist proteins such as PIV5-V may be appropriate for the propagation of these viruses.

Further characterisation is required to describe the irregularities in IFN response in PER.C6 cells. However, since the secreted IFN- β following infection with influenza is lower than for other IFN competent cell lines and the cell line is able to support the growth of clinical isolates, it may not generate any further constructive data. As such, PER.C6 cells represent a highly suitable cell line for the generation of influenza vaccine.

Chapter 6 Characterising viral IFN induction, **implications for vaccine manufacture**

6.1 Introduction

Comparisons in the induction of IFN- β by a panel of human H1N1 and H3N2 clinical isolates found considerable variation in their ability to induce IFN- β in A549 lung epithelial cells. A number of strains induced high levels of IFN- β , exemplified by the H3N2 strain A/Sydney/5/97. Although the PER.C6 cell line elicits only a very limited IFN- β response to influenza virus infection (chapter 5), other cell lines used in vaccine manufacture may still be IFN competent. Should vaccines be made directly from clinical isolates in IFN competent cell lines, IFN induction may be of consequence. Although high IFN- β induction in immortalised cell culture has yet to be correlated with high cytokine induction *in vivo*, the variation in IFN- β induction by different strains of influenza A viruses is likely to contribute to the biological consequences of infection including the pathology, pathogenicity and outcome of disease.

The concept that the cytokine response, including IFN, might contribute to the disease process following influenza infection is most recognised following infection with H5N1 virus. Since 2003, the H5N1 virus has caused devastating outbreaks in poultry in Asia, Africa and Europe, and resulted in 438 human infections, with 262 fatalities thus an overall case fatality rate of 60%, (according to the WHO 5th Feb. 2009

(http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_08_11/en/index.html). Thankfully, human-to-human transmission so far remains restricted. Post mortem analysis showed elevated levels of the inflammatory cytokines interleukin-6 (IL-6), TNF- α and IFN- γ (To, Chan et al. 2001). This correlates with H5N1-infected ferret lungs, where IFN stimulated genes were

more strongly expressed than in lungs from ferrets infected with the less pathogenic H3N2 subtypes (Cameron, Cameron et al. 2008).

The 1918 “Spanish flu” pandemic was unusually severe, resulting in approximately 50 million deaths. Analysis of the 1918 pandemic virus was not possible until RT-PCR of viral RNA isolated from preserved tissue obtained from formalin-fixed pathology specimens and a cadaver buried in permafrost at the time of the pandemic, allowed the sequencing and subsequent reconstruction of the virus (Taubenberger, Reid et al. 1997; Basler, Reid et al. 2001; Tumpey, Basler et al. 2005; Tumpey, Garcia-Sastre et al. 2005). Infection of macaques with 1918 virus caused an ultimately lethal, highly pathogenic respiratory disease (Kobasa, Jones et al. 2007). Infected animals mounted an immune response, characterized by dysregulation of the antiviral response that was insufficient for protection. Several key cytokines (including IL-6, IL-8), were expressed at a high level in 1918-virus-infected animals but not in low pathogenic H1N1 infected animals. The dysregulation of IFN induction by H5N1 and 1918 have been reviewed by (Basler and Aguilar 2008). The IFN response clearly plays an important role in pathogenesis and understanding the mechanisms will enable more co-ordinated treatment of viral infection.

Some high IFN- β inducing strains of influenza virus, in some cell lines seem to be resistant to the IFN they induce. For example the replication of the H5N1 strain, A/HK/97, in pig epithelial cells was not affected by pre-treatment of cells with a number of cytokines including, IFN- α , IFN- γ or TNF- α . In this instance, resistance was shown to map to glutamic acid at position 92 on the non-structural protein, NS1, replacing the usual aspartic acid (Seo, Hoffmann et al. 2002; Seo, Hoffmann et al. 2004). The seasonal strain A/Sydney/97 which induces a high level of IFN- β , was less affected by the presence of exogenous IFN- β in A549 cells; whilst viruses which induced lower levels of IFN- β , e.g. A/Victoria/75, were more susceptible (Hayman, Comely et al. 2006). Since A/Sydney/97 does not possess the D92E mutation, there must also exist

alternative mechanisms of IFN- β resistance. Furthermore, the mechanisms behind high IFN- β induction are also yet to be established.

Many viruses encode immunomodulatory proteins that interfere with the induction, response to, or action of host cytokines. The influenza NS1 protein antagonises the innate immune response in several ways (Egorov, Brandt et al. 1998; Garcia-Sastre, Egorov et al. 1998; Hale, Randall et al. 2008). NS1 has two domains involved in suppression of the IFN response. The mechanisms are strain-dependent since not all strains retain all functions, and are likely to be determined by the amino acid sequence of NS1. NS1 has been shown to interact with both the PAMP (currently believed to double stranded structures within 5' triphosphate ssRNA (Pichlmair, Schulz et al. 2006) mediated via an RNA binding domain within residues 38 and 41 (Hatada and Fukuda 1992; Wang, Riedel et al. 1999; Donelan, Basler et al. 2003), and PRR such as RIG-I (Garcia-Sastre, Egorov et al. 1998; Talon, Horvath et al. 2000; Wang, Li et al. 2000; Pichlmair, Schulz et al. 2006). It also interacts with many other proteins involved in IFN induction such as protein kinase R (PKR) (Wang, Riedel et al. 1999), and the p85 β regulatory subunit of host-cell phosphoinositide 3-kinase (PI3K) which activates signalling (Hale, Batty et al. 2008). The NS1 from some strains of influenza A virus have been shown to bind to the 30 kDa subunit of the cellular processivity and specificity factor (CPSF-30) and poly (A)-binding protein II (PABII), thus abrogating the export of cellular mRNA, including those that encode anti-viral genes and IFN- β (Nemeroff, Barabino et al. 1998; Chen, Li et al. 1999; Wang, Riedel et al. 1999; Noah, Twu et al. 2003).

As part of the investigation into the role IFN may play in the ultimate yield of vaccine strains and the extent to which antagonism can be beneficial, the mechanism by which viruses induce IFN- β , and the reasons for the disparity in levels of IFN- β induction between virus strains, needs to be understood. This supplements research done by two previous lab members: initially Anna Hayman and subsequently Ben Johnson.

6.2 IFN- β induction does not map to HA, NA, NS

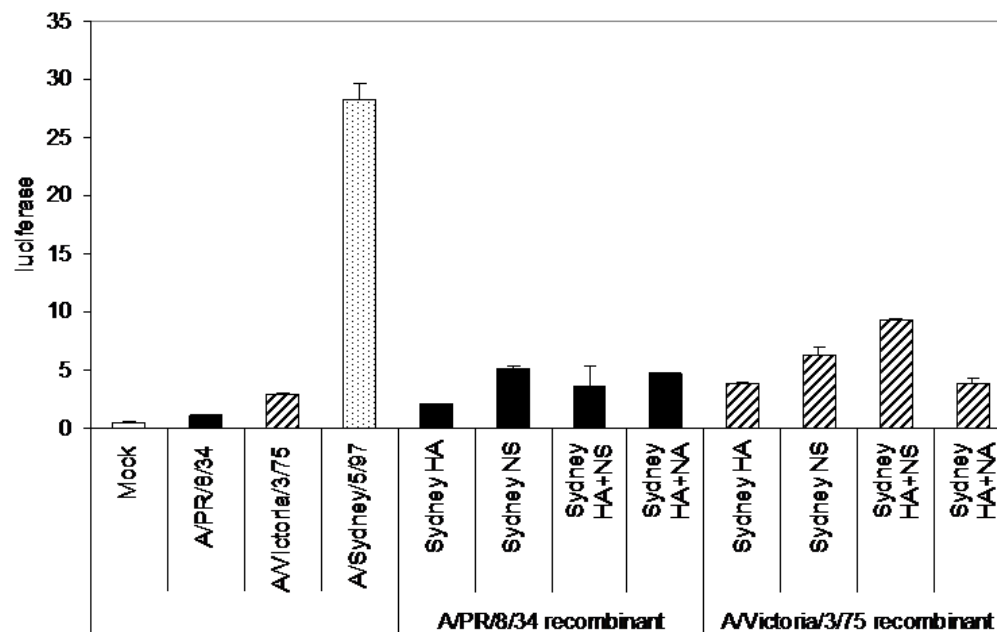
Since the NS1 protein has been shown to antagonise IFN- β induction by multiple mechanisms it is the obvious candidate to account for the variation in IFN- β induction. Using reverse genetics, a panel of recombinant influenza viruses that differed only in the origin of segment 8 RNAs, was generated (Hayman, Comely et al. 2006). Initially this was done in the A/Victoria/75 background (Anna Hayman, figure 6.2.1). To exclude the possibility that an additional gene(s) of A/Victoria/75 was acting to suppress IFN- β induction and thus mask a defect in NS1 function, a selection of viruses were also rescued in PR8P background (a 6:2 recombinant virus with six internal genes of A/PR/8/34 and surface antigens HA and NA of A/Panama/2007/99, representative of a typical vaccine seed). Using the A549 IFN- β reporter cell line, recombinant viruses with NS1s derived from human viruses with both low and high IFN- β inducing phenotypes, all only activated the IFN- β promoter to low amounts, similar to that induced by the parental strains. Moreover, endogenous expression of high inducing virus NS1 proteins in the context of virus infection, were able to control IFN- β induction by Sendai and influenza virus (Johnson et al manuscript in preparation). Thus, the high induction of IFN- β in human cells by wild-type influenza viruses was not accounted for by a deficiency in their NS genes.

Besides NS1, the surface glycoproteins HA and NA of influenza have also been implicated in the high cytokine induction of the 1918 “Spanish” pandemic virus and high TNF- α induction by Sydney in porcine macrophages in vitro (Seo, Webby et al. 2004). For vaccine manufacture, the properties of the surface antigens are the most significant since the vaccine is a 6:2 recombinant. Recombinant viruses in both A/Victoria/75 and PR8P backgrounds, containing constellations of HA, NA and NS derived from A/Sydney/97, all induced low amounts of IFN- β , similar to that induced by the parental strains in the A549 IFN- β reporter cell line (figure 6.2.1), suggesting that high IFN- β induction in

A549 cells is not conferred by either the influenza surface glycoproteins or NS1. If vaccine seeds are 6:2 reassortants derived from a low inducing donor strains then IFN- β induction should not be an issue for vaccine manufacture.

Figure 6.2.1. IFN induction by wild type A/Sydney/97 virus does not map to HA, NA, NS

Figure 6.2 The A549 IFN- β luc reporter cell line was infected with recombinant viruses containing constellations of HA, NA and NS genes from A/Sydney/97 in stable human genetic backgrounds (A/Victoria/3/75 or A/PR8/) at an MOI of 3 PFU/cell. 8 hours post infections cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of two repeat experiments. Error bars represent standard error.



6.3 High IFN- β inducing viruses exhibit dysregulation of protein expression

Although it had been shown that A/Sydney/97 virus NS1 protein was functional and able to antagonise IFN- β induction, it was possible that its expression could be dysregulated in the context of wild type virus infection such that when PAMP is generated insufficient NS1 protein has accumulated to antagonise IFN- β induction. To assess this possibility protein levels over the course of infection were measured by Western blot. Expression of A/Sydney/97 viral proteins were compared to a low IFN- β inducing virus A/Victoria/75. A recombinant virus containing A/Sydney/97 NS in the A/Victoria/75 background was used as a second, low IFN- β inducing virus for comparison.

A dose of virus was established that infected an equal number of cells. The expression of viral proteins, NS1, M1 and NP, were assessed by Western blot (figure 6.3.1). JAK/STAT signalling following IFNAR stimulation by IFN- β results in the phosphorylation of STAT-1, so phosphorylated STAT-1 was used as a marker for the induction of IFN- β .

Infection with A/Sydney/97 was found to be synchronized differently than for A/Victoria/75 based viruses. Viral proteins in A/Sydney/97 infection accumulated much earlier in infection than did proteins expressed by the two low inducing viruses. Viral proteins (NP, M and NS1) were detected as early as 2 hours post infection with A/Sydney/97 and this correlated with earlier STAT-1 phosphorylation. Furthermore, the expression of A/Sydney/97 NS1 protein appeared to lag behind the other viral proteins. Although differences in the affinity of the antibodies to each of the viral proteins cannot be excluded, the data suggests that viral replication resulting in the synthesis of certain viral proteins, and the accumulation of PAMP, occurred earlier during infection with A/Sydney/97 without a corresponding increase in the levels of NS1 to antagonise the IFN- β induction

Viral proteins also accumulated, to a lesser extent, earlier following infection with the recombinant virus containing A/Sydney/97 NS1 in the A/Victoria/75 background, than during infection with wild type A/Victoria/75. However, this was associated with much higher relative levels of NS1. The increased expression of NS1 can antagonise the IFN- β induced by the increased levels of PAMP and thus results in much reduced levels of phosphorylated STAT-1; which do not accumulate until later in infection. The NS1 protein has been shown to mediate the temporal regulation of viral RNA synthesis via interactions with the viral polymerase (Min, Li et al. 2007) and also enhance the rate of translation of certain viral proteins (Enami, Sato et al. 1994; de la Luna, Fortes et al. 1995) and this could in part explain the differences in viral protein expression of the recombinant virus.

Viral proteins associated with infection with A/Victoria/75 accumulated slowly and did not even reach equivalent levels to those expressed by the other two viruses. However, this does not correlate with outcome of infection; A/Victoria/75 is an extremely “fit” virus and can achieve titres of 10^8 PFU/ml following propagation in cell culture whilst A/Sydney/97 usually attains a log less.

Furthermore, the earlier phosphorylation of STAT-1 correlates with early IFN- β induction by A/Sydney/97 virus (figure 6.3.2). A549 cells harbouring the IFN- β luciferase reporter were infected with the same panel of viruses. IFN- β induction was detected as early as 2 hours post infection by A/Sydney/97. Infection with the recombinant virus in the A/Victoria/75 background with A/Sydney/97 NS resulted in a slight induction of IFN- β which correlates to the lower levels of phosphorylated STAT-1 noted by Western blot.

Figure 6.3.1. A/Sydney/97 proteins accumulate early in infection

A/Sydney/97 proteins are synthesised earlier in infection than those of A/Victoria/75. A549 cells were infected with viruses (A/Victoria/75- V, A/Sydney/97- S, and a recombinant containing NS from A/Sydney/97 in the A/Victoria/75 background- NS) at a dose twice that required for every cell to express antigen, cells were lysed in RIPA buffer containing iDOC at 2, 4, 6, and 8 hours post infection. Cell lysates were run on 10% or 15% SDS PAGE gels and Western blotted on to PVDF membrane. Membranes were probed with antibodies and detected with ECL reagent. Vinculin was used as a loading control. (Antibody concentrations were Vinculin 1/1000, Stat-1 1/500, NP 1/400, M 1/1000, NS 1/300, secondary antibodies 1/2000).

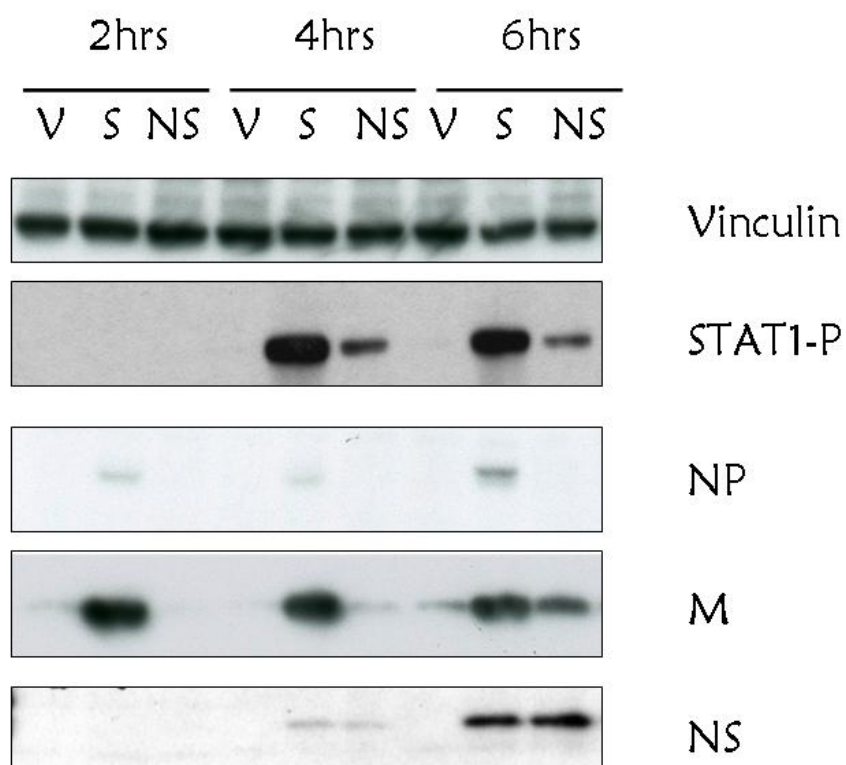
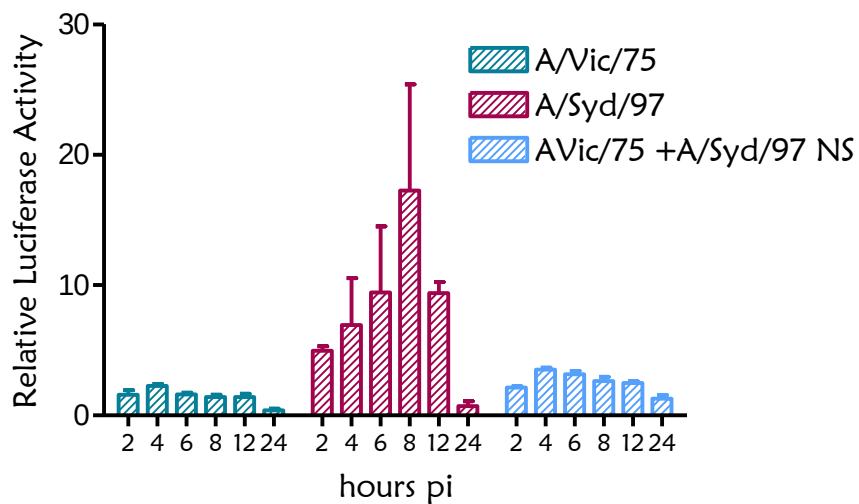


Figure 6.3.2 A/Sydney/97 induces IFN- β early in infection

The A549 IFN- β luc reporter cell line was infected with A/Sydney/97, A/Victoria/75 and a recombinant containing NS from A/Sydney/97 in the A/Victoria/75 background at a dose twice that required for every cell to express antigen. At 2, 4, 6, 8, 12 and 24 hours post infection cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments. Error bars represent standard error.



To normalise infections, the infectivity of viruses in the A549 cell line was measured by “blue cell” assay, where expression of the viral protein, NP was immuno-detected. Interestingly, it was observed that not all viruses expressed the same levels of NP in infected cells. Infection with some strains resulted in cells which stained very weakly whilst for others staining was more intense. The "blue cell" assay standardises the volume of virus required to infect an equal number of cells regardless of intensity. In some instances, Western blots of virus proteins have been used to standardise assays and ensure equivalent levels of infectivity. The results of both the Western blot and the blue cell assay highlight the need to be aware when comparing some virus strains that the levels of virus protein from different strains are not absolute.

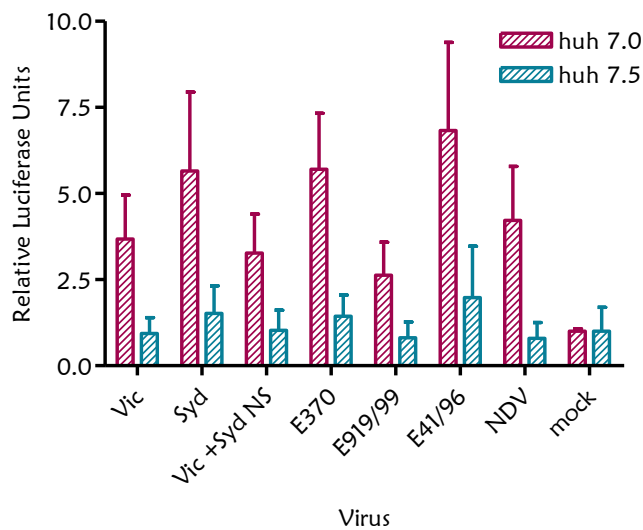
6.4 High IFN- β induction requires expression of RIG-I

Work in section 6.3 suggested that the higher levels of IFN- β induced by A/Sydney/97 may be due to a dysregulation in the levels of PAMP relative to the levels of NS1 protein. Alternatively, A/Sydney/97 may also generate a different kind of PAMP which NS1 is unable to control. The PRR, RIG-I, has been shown to mediate the IFN- β response to influenza viruses (Pichlmair, Schulz et al. 2006; Le Goffic, Pothlichet et al. 2007; Loo, Fornek et al. 2007; Pothlichet, Chignard et al. 2008), however, these experiments have largely been performed with lab strains of viruses which tend to have a low IFN- β inducing phenotype.

Huh-7.5 cells have a point mutation the PRR, RIG-I (Sumpter, Loo et al. 2005) in the TRIM25 binding domain (Gack, Albrecht et al. 2009) and are believed to be unable to respond to RIG-I activating PAMPs. Huh-7.5s were used to determine whether the high IFN- β induction by A/Sydney/97 virus was mediated via the RIG-I dependant PRR pathway.

Figure 6.4.1. High IFN- β induction by influenza viruses is mediated by RIG-I

High IFN induction is mediated by RIG-I. Huh-7 and the RIG-I deficient subline Huh-7.5 cells were transiently transfected with the IFN- β luc reporter construct, and infected 48 hours post transfection at a dose twice that required for every cell to express antigen with a panel of high and low inducing viruses and a recombinant in the A/Victoria/75 background containing A/Sydney/97 NS. 8 hours post infection cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are normalized to β -galactosidase activity for each transfection to account for differences in translation and transfection efficiencies and are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of two repeat experiments. Error bars represent standard error.



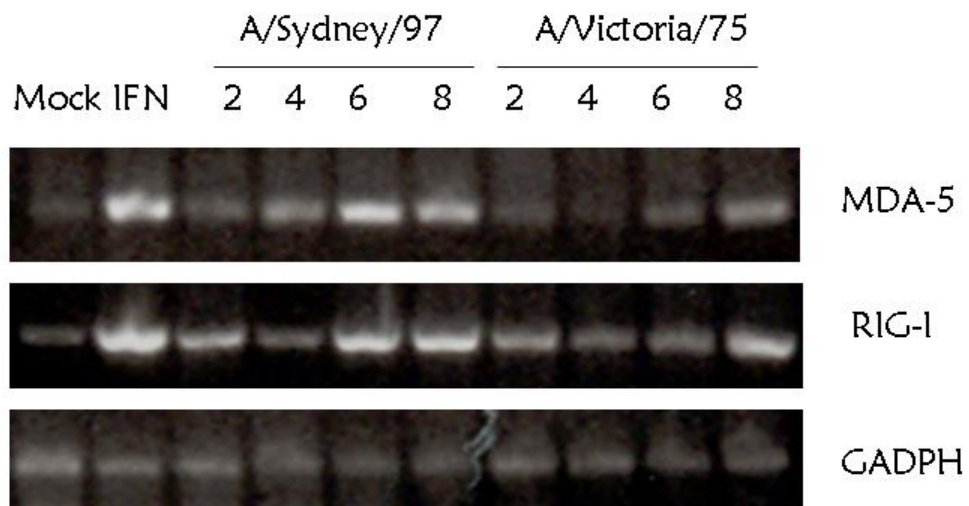
Huh-7 and Huh-7.5 cells were transiently transfected with the IFN- β luc reporter construct, and infected with a panel of high and low inducing WT viruses and the recombinant virus containing A/Sydney/97 NS in the A/Victoria/75 background (figure 6.4.1). Huh-7 cells with intact RIG-I pathway displayed a similar pattern of IFN- β induction upon infection had been seen following as infection of A549 cells. In contrast, only low amounts of IFN- β were induced following infection of Huh-7.5 cells irrespective of the virus strain. This suggests that the high induction of IFN- β by wild-type influenza viruses, such as A/Sydney/97, is mediated via RIG-I.

6.5 RIG-I and MDA-5 mRNAs are upregulated during influenza infection

PRR such as MDA-5 and RIG-I, are IFN stimulated genes (ISGs) which are upregulated by IFN- β induction. To determine whether the high IFN- β induction by some strains of influenza viruses elicited a typical upregulation of ISG, semi-quantitative RT-PCR was used to determine the levels of expression of MDA-5 and RIG-I, in response to virus infection of A549 cells. Total RNA was extracted from infected cells at various times post infection. Equal amounts of RNA, quantified by spectrophotometer, were used to generate cDNA, then equivalent volumes used to generate cDNA. Only 25 cycles of PCR were performed to ensure the reaction was terminated in the exponential phase. mRNA expression of RIG-I, MDA-5 and GAPDH as a loading control, were analysed (fig 6.5.1). In A/Sydney/97 infected A549 cells RIG-I and MDA-5 were up-regulated early in infection; RIG-I mRNA signal increased as early as 2 hours post infection. Both PRR are upregulated by positive feedback in a typical autocrine manner as a consequence of IFN- β induction during infection with A/Sydney/97.

Figure 6.5.1 PRR are upregulated by IFN- β induction by influenza viruses

MDA-5 and RIG-I are upregulated earlier in infection in cells infected with A/Sydney/97. A549 cells were infected with virus at a dose twice that required for every cell to express antigen and lysed in TRIzol (Invitrogen) according to manufacturer's instructions at the given time points. Purified RNA was quantified by spectrophotometer. Equivalent amounts of RNA were used to generate cDNA. Equivalent volumes of cDNA were used in the PCR reaction, for 25 cycles to ensure PCR was in the quantitative phase. Expression of RIG-I, MDA-5, and GAPDH as a loading control, were evaluated.



6.6 High IFN- β inducing viruses are prone to making more defective RNA

UV treatment denatures nucleic acids, but viral proteins are undamaged and the virion particles remain intact. UV treatment abrogated IFN- β induction by influenza viruses including A/Sydney/97 (Johnson et al manuscript in preparation). Therefore, IFN- β induction is not activated by virus cell binding or entry but rather must be reliant on RNA replication. The most systematic way to dissect the mechanisms for high IFN- β induction would be to test the contribution of each RNA segment in the phenotype by generating recombinant viruses containing constellations of segments from high and low inducing viruses; this approach had eliminated HA, NA and NS genes (figure 6.2.1). However, we do not possess a full reverse genetics system for a high inducing virus. While attempting to clone the remaining genes of A/Sydney/97, using primer pairs complementary to the 5' and 3' termini of each viral RNA, a number of shorter products were consistently generated and for some viral RNA segments the full-length band was not even detectable. This occurred frequently during attempts to clone segments from viruses which induced high levels of IFN- β , and although these short products could be PCR artefacts, they were rarely seen in low inducing viruses. A number of these bands were sequenced and had the properties of defective interfering RNAs (figure 6.6.1 and table 6.6.1). Defective RNAs result from spontaneously occurring deletions within the full-length viral RNAs during RNA synthesis. These shorter RNAs retain the 3' and 5' non coding regions which encode the packaging signals. This means they can be incorporated into virions to generate defective interfering particles (DIs) which are able to infect cells but since they lack a complete genome are unable to replicate without the assistance of an intact helper virus. In this event, as a result of the small size of the defective RNAs the shorter RNAs are replicated to much higher levels than full-length segments, so

Figure 6.6.1 Schematic representation of defective interfering RNA

Defective RNAs result from spontaneously occurring deletions within the full-length viral RNAs during RNA synthesis. These RNAs are incorporated into virions to generate defective interfering particles which are able to infect cells but due to the lack of a complete genome are unable to replicate without the assistance of an intact helper virus. As a result of the small size of defective interfering RNA it is replicated to much higher levels than full-length segments, so the cell produces defective virions in place of infectious progeny (Duhaut and Dimmock 2000; Duhaut and Dimmock 2002).

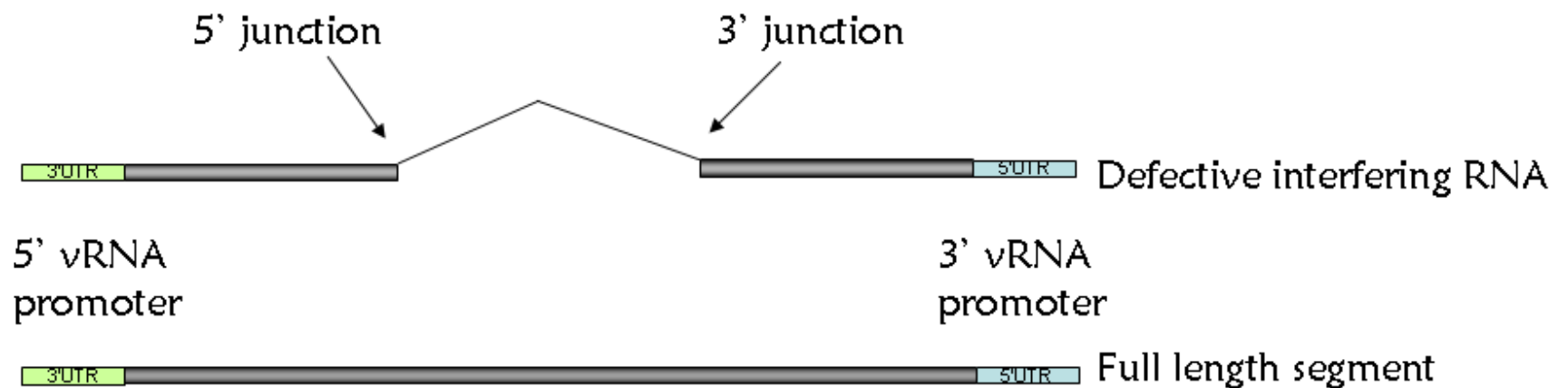


Table 6.6.1 Defective RNAs accumulate during infection with A/Sydney/97 virus

Sequence analysis of shorter PCR products generated from amplification of A/Sydney/97 virus show signatures of defective RNAs. Alignment against the full length sequence allows the junctions of the DI to be determined.

Segment of origin	Approx. PCR product size	Full Length of segment	5' junction	3' junction	Deduced length of RNA
PB2	600	2280	259	2010	529
PB1	900	2274	275	1943	606
PB1	600	2274	204	2025	453
PA	900	2183	115	1586	712

the cell produces defective virions in place of infectious progeny (Duhaut and Dimmock 2000; Duhaut and Dimmock 2002). DIs usually occur late in infection or when a high MOI is used (Odagiri and Tobita 1990), preparations that contain them are known to induce high levels of IFN- β (Johnston 1981). The amplification of short products suggests that A/Sydney/97, and other high IFN- β inducing viruses, have a propensity to make defective RNAs. Primer extension analysis was used to quantify the RNA species in virus infected cells (fig 6.6.2). ³²P labelled oligos complimentary to viral gene segments were used in an extension assay with total RNA as a template. In this case, primers specific to the 3' UTR of vRNA were used. Shorter RNAs were produced during infection with A/Sydney/97 or A/England/370/02 again suggesting that viruses that induce high IFN- β have a propensity to make defective RNAs. Although these short RNAs could be the result of mispriming, this is unlikely because they did not occur when RNA from cells infected with A/Victoria/75. Analysis showed that the primer sequence is unique and specific for the 3'UTR of the PB2 segment 1. Plaque picking removes DIs which accumulate during an infection, because virus isolated from a plaque will be derived only from a single infectious event. Bearing in mind Ben Johnson showed the high IFN- β inducing phenotype was carried over in plaqued picked viruses and was also genetically determined (Johnson et al, manuscript in preparation) this suggests that the DIs are generated spontaneously during infection with viruses which induce high levels IFN- β .

6.7 A/Sydney/97 virus drives replication early in infection

Taken together the data suggested that the high IFN- β inducing phenotype maybe inherent to the polymerase complex. To assess this the rate of viral replication was measured using a luciferase reporter assay. The principal involves transfection of cells with a plasmid encoding the non-coding regions of segment 8 flanking a luciferase reporter gene. The amount of luciferase expression driven by viral polymerase allows replication to be quantified. This

Figure 6.7.1 Schematic of up-luc assay (overleaf)

In a minireplicon assay it is sufficient to transfect cells with the polymerase subunits, PB2, PB1, PA and NP together with a minireplicon reporter construct of viral like RNA containing a luciferase gene flanked by the 5' and 3' UTR of segment 8. However, in virus infection the viral polymerases are unable to drive the replication of such a reporter, mutation of nucleotides 3, 5 and 8 in 3' UTR, to generate a so-called "up-promoter, are able to restore this capacity. Cells are transfected with the up-luc reporter construct, then infected with virus. Viral polymerases drive the amplification and expression of the viral like RNA. Quantification of the luciferase signal allows the rate of virus replication to be determined.

Figure 6.7.1 Schematic of the “up-luc” assay

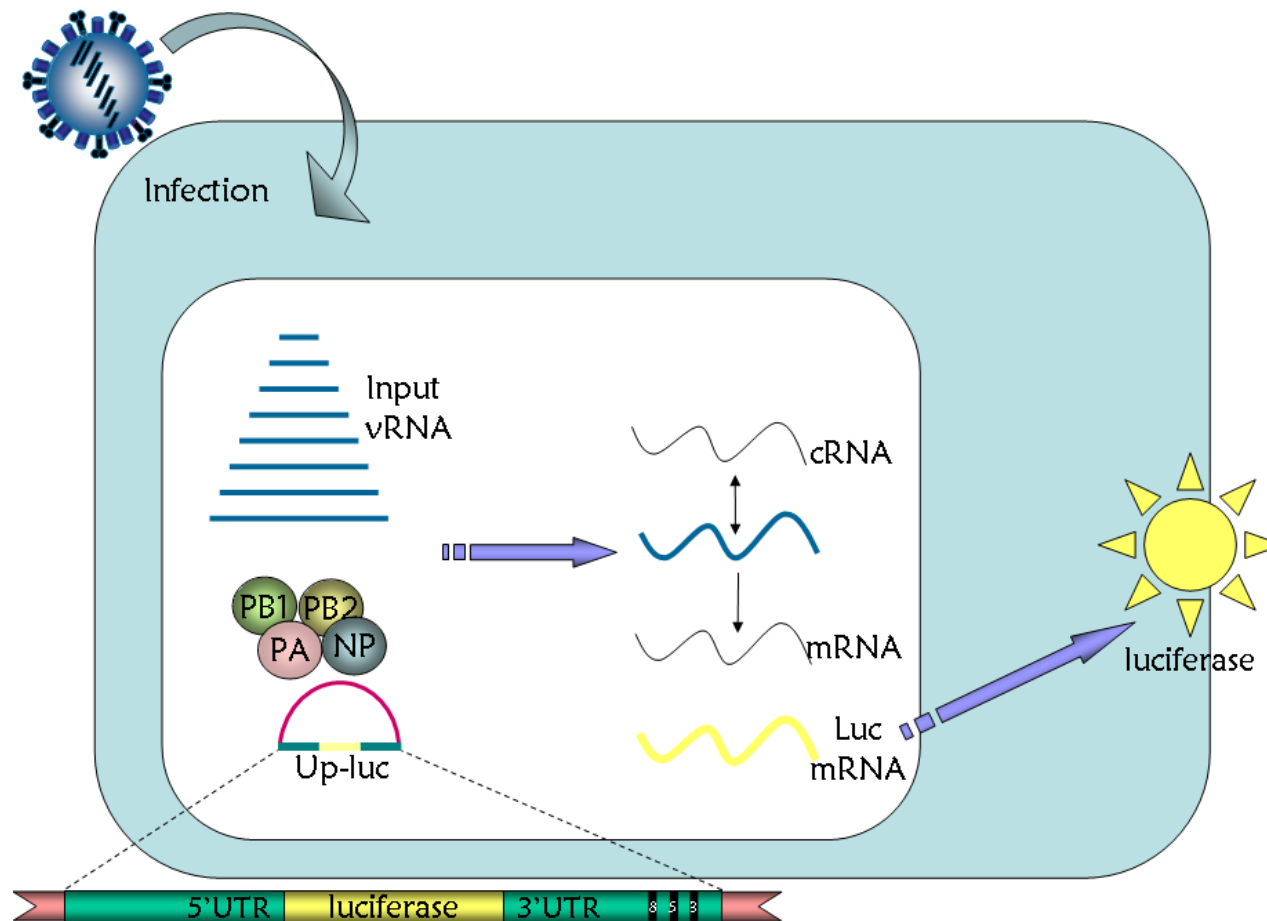
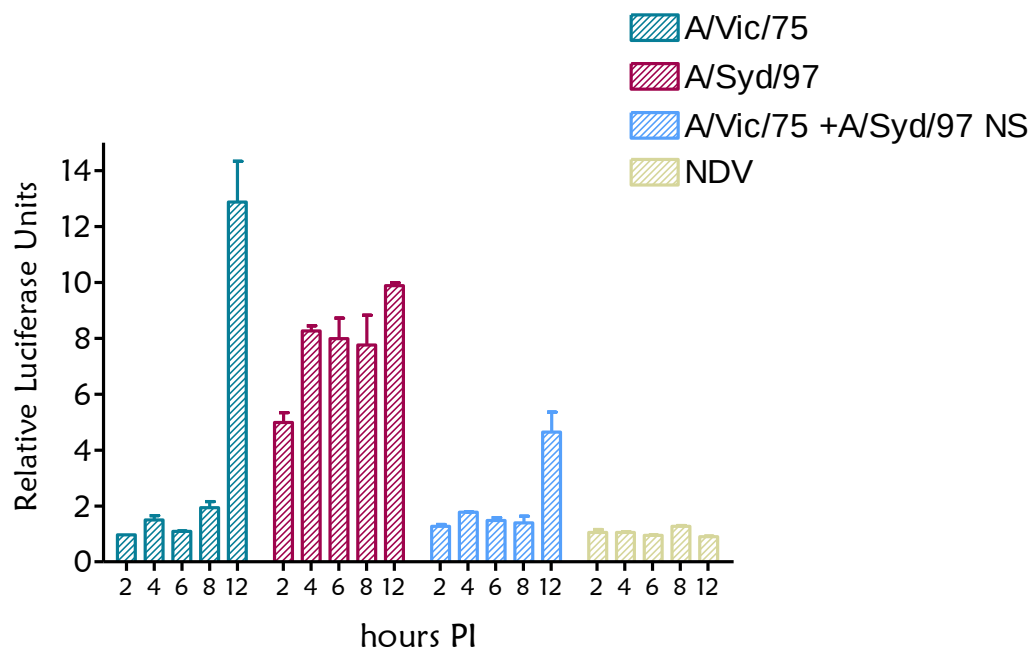


Figure 6.7.2 A/Sydney/97 virus drives replication early in infection

Cells were transfected with the up-luc reporter construct. After 48 hours, cells were infected with virus. 8 hours post infection cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments. Error bars represent standard error.



is apt for minireplicon assays, however, the polymerases, in the context of virus infection, seem unable to recognise and replicate 'naked' vRNA present inside a cell to detectable levels (Lutz et al., 2005) unless three nucleotide changes at positions 3, 5 and 8 in the 3' non-coding region are present. This mutation appears to increase viral polymerase transcription of a synthetic viral-like RNA (Neumann & Hobom, 1995). Therefore these three point mutations were engineered into a minireplicon containing the luciferase reporter gene (Dan Brookes), and function as an 'up-promoter' enhancing recognition of the reporter gene by virus polymerases (here on called the "up-luc" reporter) (figure 6.7.1).

The extent to which the virus polymerase of A/Sydney/97, A/Victoria/75 or recombinant, in the A/Victoria/75 background containing A/Sydney/97 NS drove RNA replication and expression were compared. Huh-7 cells were transiently transfected with the up-luc reporter construct and infected with twice the volume of virus required to infect every cell. Luciferase production was measured at various times post-infection (figure 6.7.2). A/Sydney/97 virus drove expression of the up-luc reporter very early in infection. Luciferase was measured at the earliest time point; just 2 hours post infection. The low inducing viruses A/Victoria/75 or the A/Victoria/75 +A/Sydney/97 NS recombinant eventually attained similar or higher levels of luc expression, but not until much later in infection at 12 hours. These data imply that the polymerases of A/Sydney/97 virus are particularly active and initiated replication very early in infection.

6.8 Total RNA from infected cells induces similar levels of IFN- β irrespective of strain

The increased rate of replication of A/Sydney/97 appears to correlate kinetically with its high IFN- β induction. However, the nature of the PAMP which drives the high IFN- β induction is as yet undetermined. 5' triphosphate

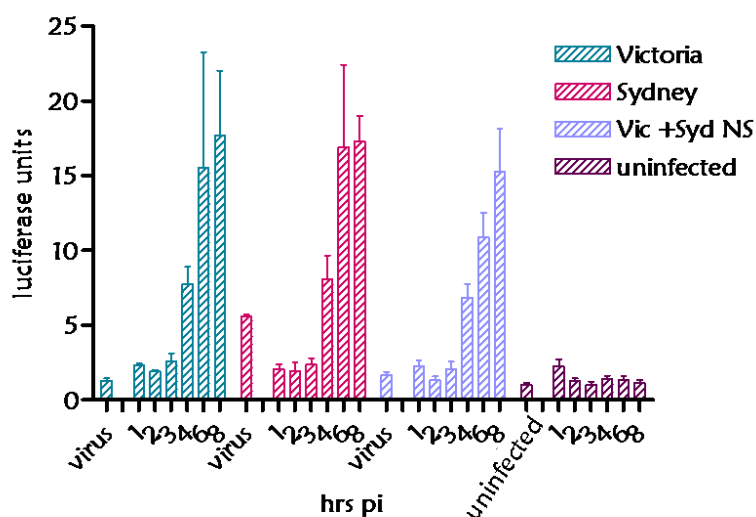
RNA has been shown to mediate IFN- β induction for laboratory strains of influenza virus but this has yet to be associated with the high IFN- β phenotype. The earlier replication observed in A/Sydney/97 virus infection may result in the generation of vRNAs, possessing 5'3P, which accumulate in the infected cells in the absence of sufficient NS1 protein to antagonise IFN- β induction. To investigate the ability of RNAs generated in infected cells to trigger IFN- β induction, total RNA was extracted from cells infected over the course of infection with RNA extracted from uninfected cells serving as a control. The RNA was then applied to the same cell type expressing the IFN- β luc reporter, and its ability to induce activation of the IFN- β promoter was assessed (figure 6.8.1).

RNA from uninfected cells did not activate the IFN- β promoter. RNA from infected cells induced no IFN- β until 4 hours post infection after which up to 20 fold induction of IFN- β was observed, irrespective of strain. This was higher than the 5 fold induction measured by infection with A/Sydney/97 virus and transpired at a later time during infection. The data suggest that virus infection causes accumulation of a PAMP from 4 hours post infection which is not strain dependant, perhaps via a general modification to RNAs present in the cells which renders them immuno-stimulatory.

It is surprising that IFN- β induction by RNA extracted from A/Sydney/97 infected cells was not detected earlier since A/Sydney/97 polymerases were active as early 2 hours post infection (section 6.3 and 6.7) and IFN- β induction was also seen at that time (figure 6.3.2). The assay may not be sufficiently sensitive to respond to the levels of PAMP present in the total RNA extraction at the earlier time points. 500 ng of RNA extracted from virions was able to induce IFN- β promoter induction, yet 100 ng of this RNA could not (data not shown). In the present assay, 250 μ g of total RNA from infected cells was applied to IFN- β luc reporter cells.

6.8.1 RNA from infected cells induces similar levels of IFN- β irrespective of strain

Total cellular RNA from infected cells induces similar levels of IFN- β , irrespective of IFN inducing phenotype of virus. Cells were infected at a dose twice that required for every cell to express antigen. Total cellular RNA was extracted with TRIzol (Invitrogen) according to manufacturer's instructions, from infected Huh-7 cells at given times (1, 2, 3, 4, 6, 7 hours) post infection. 250 μ g RNA was then transfected onto Huh-7 cells transiently transfected with IFN- β luc and β -galactosidase, 48 hours post transfection. After 8 hours post transfection cells were washed PBS, lysed in CCLR and clarified by centrifugation, 13,000 RPM for 1 minute. Luciferase activity was measured and normalized to β -galactosidase activity for each transfection to account for differences in transfection efficiencies. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of two repeat experiments. Error bars represent standard error.



Should the proportion of viral RNA contained within the total RNA fall below the threshold of detection (between 500 ng and 100 ng vRNA), IFN- β induction would not be measured. Increasing the amount of total RNA applied to cells and thereby increasing the proportion of PAMP or increasing the amount of time the RNA is incubated on the cells before taking measurements may improve the sensitivity.

6.9 Leptomycin B, inhibits the export of luciferase mRNA from the nucleus and activates IRF-3

It seemed likely that A/Sydney/97 induced IFN when replicated RNA reached the cytoplasm, where it would be detected by RIG-I. If this were the case it was reasoned that inhibition of the export of viral RNAs from the nucleus would prevent IFN- β induction. Leptomycin B is a specific nuclear export inhibitor, which alkylates and thus inhibits CRM-1 (chromosomal region maintenance) mediated nuclear export of proteins containing nuclear export sequences (NES). Addition of LMB has been shown to prevent the export of viral RNPs (Elton, Simpson-Holley et al. 2001). A549 IFN- β luc reporter cells infected with virus were incubated in the presence of 10 nM LMB. Luciferase activity, reflecting activation of the IFN- β promoter, was measured after 8 hours (figure 6.9.1). IFN- β promoter induction by A/Sydney/97 was greatly reduced although not completely prevented. However, LMB also reduced IFN- β induction by NDV infection. Since NDV replicates in the cytoplasm it is not dependant on nuclear export of its PAMP to mediate an IFN- β response. This result suggests that LMB treatment retains luciferase mRNA in the nucleus and that the luciferase mRNA is also exported from the nucleus in a CRM-1 dependant manner. The abrogation of IFN- β induction by A/Sydney/97 virus was not complete, and some induction of the IFN- β promoter was measured. LMB was shown to be effective at retaining NP in the nucleus when added 3 hours post infection at a concentration of 11 nM (Elton, Simpson-Holley et al. 2001). Other groups have used a similar range of concentrations (2-40 nM)

and addition as late as 6 hours post infection to inhibit export of vRNA and RNPs from the nucleus (Ma, Roy et al. 2001; Watanabe, Takizawa et al. 2001; Wu and Pante 2009). Typical virus infection procedures allows 1 hour for the virus to enter the cells, after which the media is replaced with serum free DMEM. This minimises the carryover of artefacts in the virus preps which are able to induce IFN- β . The LMB was therefore added to the replacement serum free media. Since it has been demonstrated that virus replication and IFN- β induction occurs early during A/Sydney/97 virus infection, the IFN- β luc measured could have been induced during the hour prior to the addition of LMB and whilst the later addition of LMB is appropriate for other virus strains, addition from the onset of infection is required for viruses such as A/Sydney/97 to completely block export from the nucleus.

Because LMB inhibits the export of luciferase messenger RNA from the nucleus, the luciferase reporter assay is not an appropriate assay to determine whether nuclear export is required for influenza mediated IFN- β induction. Immunofluorescence was used as an alternative assay; the activation of the IFN- β promoter can be inferred by the translocation of IRF-3 from the cytoplasm to the nucleus. Cells were infected as above but fixed at 7 hours post infection and immunostained for viral antigen (NP) and IRF-3. Surprisingly, however, even in uninfected cells treated with LMB, IRF-3 was predominantly nuclear (figure 6.9.2 top right panel). Thus, the addition of LMB seems to activate IRF-3 and this precluded an assessment of the relationship between RNP localisation during influenza infection and RIG-I activation. Interestingly, in A/Sydney/97 untreated infected cells treated with LMB, IRF-3 translocation to the nucleus was still more substantial than in infected cells. This again could be due to IRF-3 activation during the first hour of infection when LMB is not present. Alternatively, IFN- β induction by A/Sydney/97 virus could indeed occur in the absence of nuclear export. NP was also more nuclear in LMB treated cells confirming NP required CRM-1 for export from the nucleus, and that inhibition of CRM-1 by LMB had occurred. In conclusion, it is not possible to equivocally establish the importance of vRNP

Figure 6.9.1 Inhibition of nuclear export with Leptomycin B

The A549 IFN- β Luc reporter cell line was infected with a high IFN inducing virus A/Sydney/97, a low inducing viruses A/Eng/99/00 at a dose twice that required for every cell to express antigen and NDV. 10 nM Leptomycin B was added 1 hours p.i. 8 hours post infection cells were washed PBS, lysed in CCLR, clarified by centrifugation, 13,000 RPM for 1 minute and luciferase measured. Data are represented as ratio of induction corrected to unchallenged cells. Means and standard error were calculated for duplicate wells. This assay is representative of three repeat experiments. Error bars represent standard error.

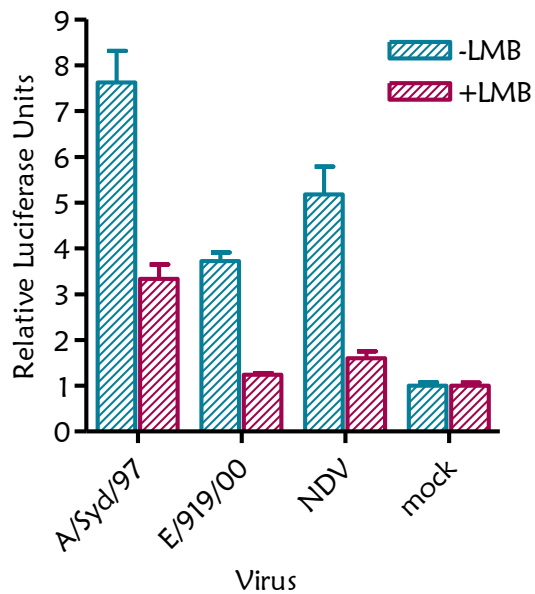
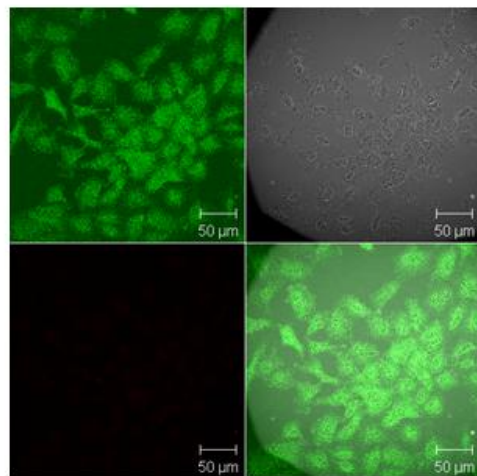


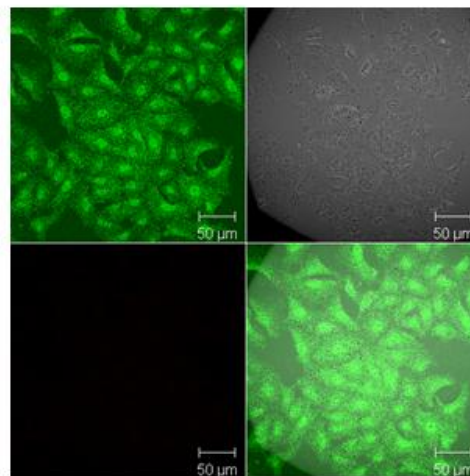
Figure 6.9.2 Leptomycin B immunofluorescence of NP and IRF-3

A549 cells were infected with A/Sydney/97 at a dose twice that required for every cell to express antigen. 10 nM Leptomycin B was added 1 hours post infection. 7 hours post infection cells were washed PBS, then fixed 4% PFA/PBS for 10 minutes, RT. Coverslips were blocked 3% BSA for 30 minutes RT. Proteins were immunodetected (top left panel- goat IRF-3 1/300, anti goat FITC 1/500, bottom left panel- mouse NP 1/300, anti mouse Texas red 1/500, top right panel- phase contrast, bottom right panel- overlay).

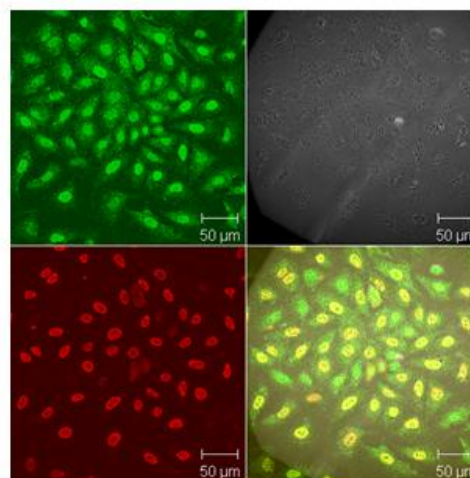
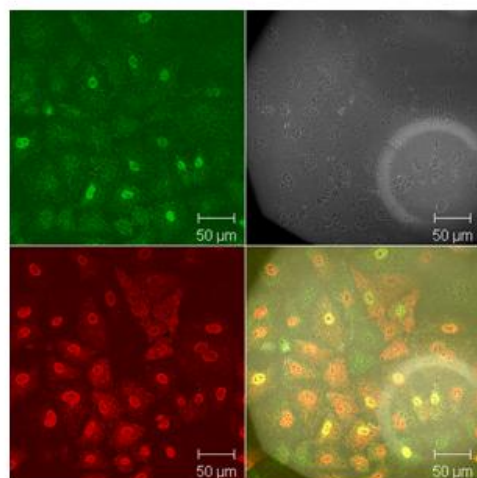
-Leptomycin B



+Leptomycin B



Uninfected



A/Sydney/97

nuclear export in the A/Sydney/97 high IFN- β inducing phenotype because the drug used to inhibit CRM-1 had unexpected albeit interesting effects.

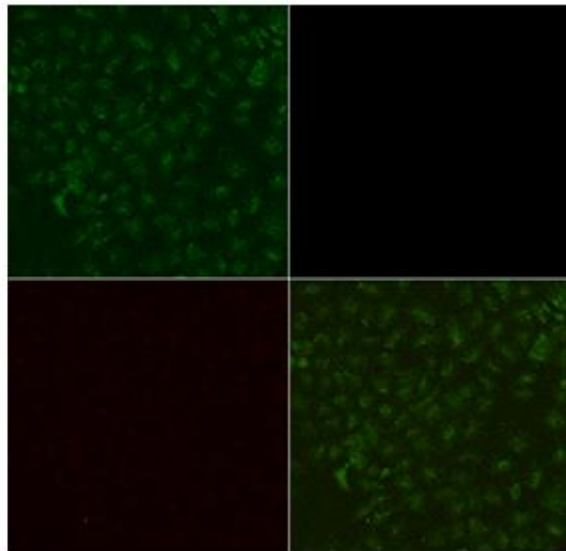
6.10 Cells infected with A/Sydney/97 do not have early nor greater translocation of NP to the cytoplasm

Viral proteins, particularly NEP (Neumann, Hughes et al. 2000; Robb, Smith et al. 2009) and M1 (Liu and Ye 2002) are involved in the export of vRNA and RNPs from the nucleus. The accumulation of viral proteins earlier in A/Sydney/97 (figure 6.3.1) infection may have promoted the export of vRNAs and RNPs from the nucleus before sufficient NS accumulated to antagonise the IFN- β response. It has been noted previously that following infection with A/Sydney/97 virus, a portion of virus infected cells have IRF-3 in the nucleus. Typically such cells showed increased levels of NP (and by inference, RNPs) in the cytoplasm (Hayman, Comely et al. 2006). In this instance, the cells were fixed 8 hours post infection, however, data presented in this chapter (sections 6.3.1 and 6.5.2) indicates that IFN- β promoter activation occurs already at earlier time points. Therefore, the location of NP and IRF-3 in virus infected A549 cells were determined by IF at 4, 5, 6 and 7 hours p.i. (figure 6.6.1). Since IFN- β was induced as early as 2 hours post infection (figure 6.3.2) and STAT-1 was phosphorylated 4 hours post infection (figure 6.3.1), IRF-3 translocation to the nucleus ought to occur early in infection and be detectable by 4 hours post infection. The number of A/Sydney/97 infected cells with nuclear IRF-3 was expected to be higher than in cells infected with either A/Victoria/75 or the recombinant. As previously observed, infection with A/Sydney/97 virus resulted in an increase in the number of cells in which IRF-3 had translocated to the nucleus (figure 6.10.1), corresponding to induction of IFN- β . Activated IRF-3 had translocated to the nucleus even at the earliest time point, 4 hours. At 7 hours post infection, the latest time point examined, 40% of A/Sydney/97 infected cells had IRF-3 in the nucleus, compared to just 14% for the recombinant with A/Victoria/75 with A/Sydney/97 NS and none for WT A/Victoria/75.

Figure 6.10.1 Cells infected with A/Sydney/97 do not have early nor greater translocation of NP to the cytoplasm

A549 cells were infected with A/Victoria/75, A/Sydney/97 or a recombinant A/Victoria/75 with A/Sydney/97 NS at a dose twice that required for every cell to express antigen. A) At the times indicated post infection cells were washed PBS, then fixed 4% PFA/PBS 10 minutes, RT. Coverslips were blocked with 3% BSA for 30 minutes, RT. Proteins were immunodetected (top left panel- rabbit IRF-3 1/300, anti rabbit FITC 1/500, bottom left panel- mouse NP 1/300, anti mouse Texas red 1/500, top right panel- phase contrast, bottom right panel- overlay).

A) On this page uninfected cells, on the following pages virus infected cells at 4, 5, 6 and 7 hours post infection.

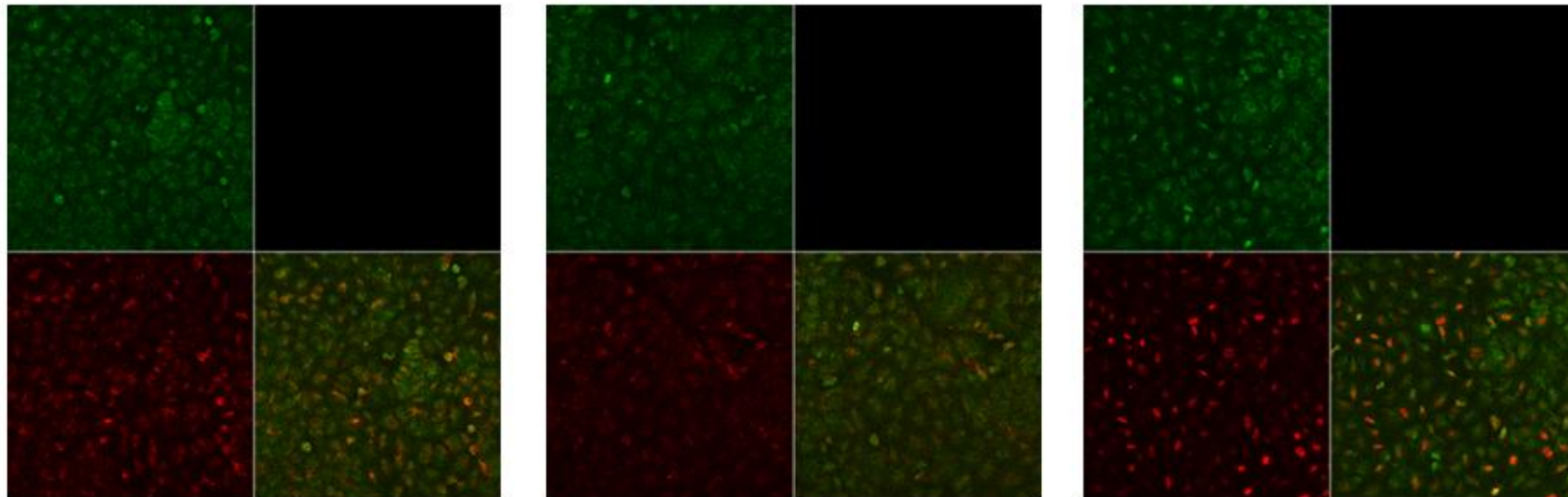


4 hr p.i.

A/Victoria/3/75

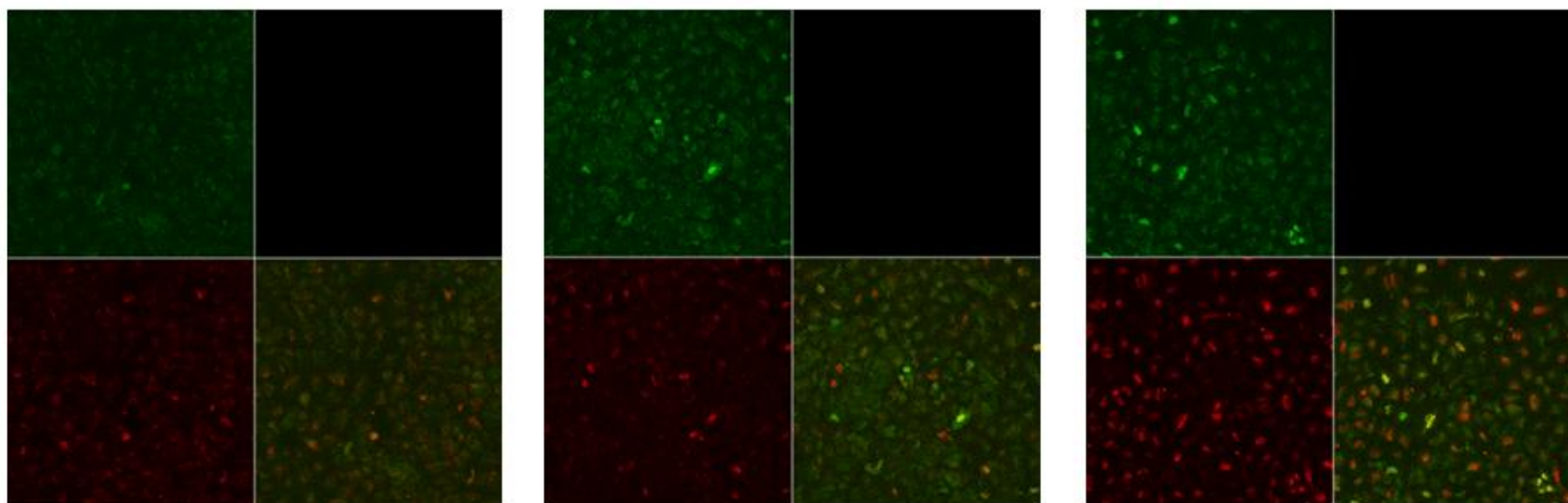
A/Sydney/5/97

A/Victoria/3/75
+A/Sydney/5/97 NS



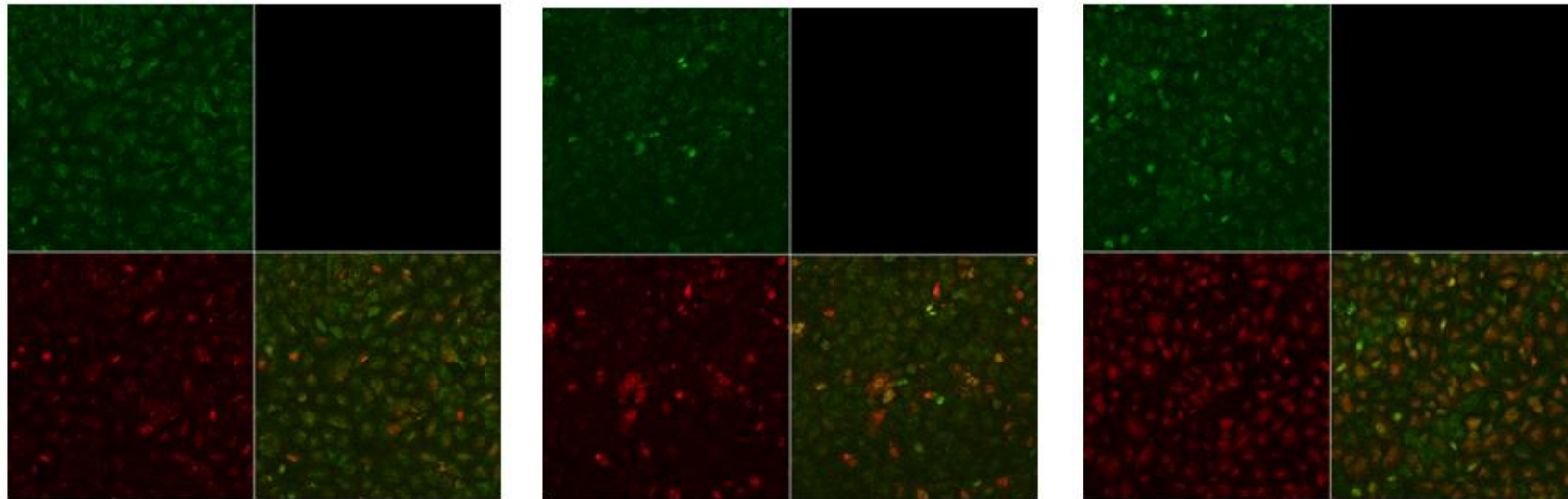
5hr p.i.

A/Victoria/3/75 A/Sydney/5/97 A/Victoria/3/75
+A/Sydney/5/97 NS



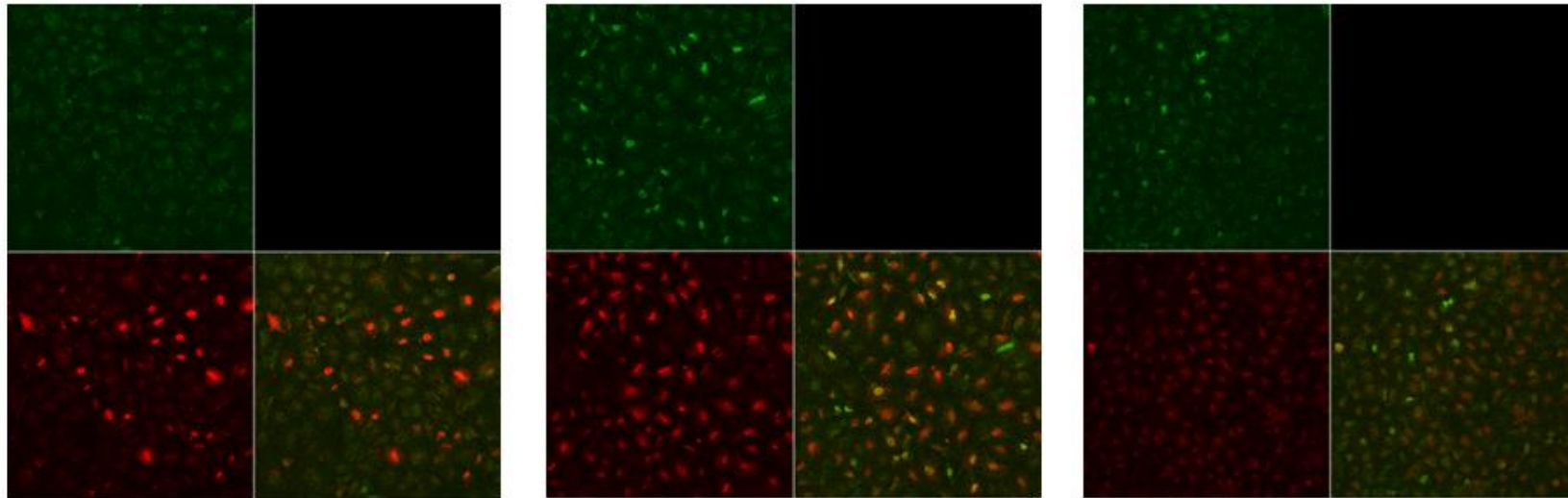
6 hr p.i.

A/Victoria/3/75 A/Sydney/5/97 A/Victoria/3/75
+A/Sydney/5/97 NS

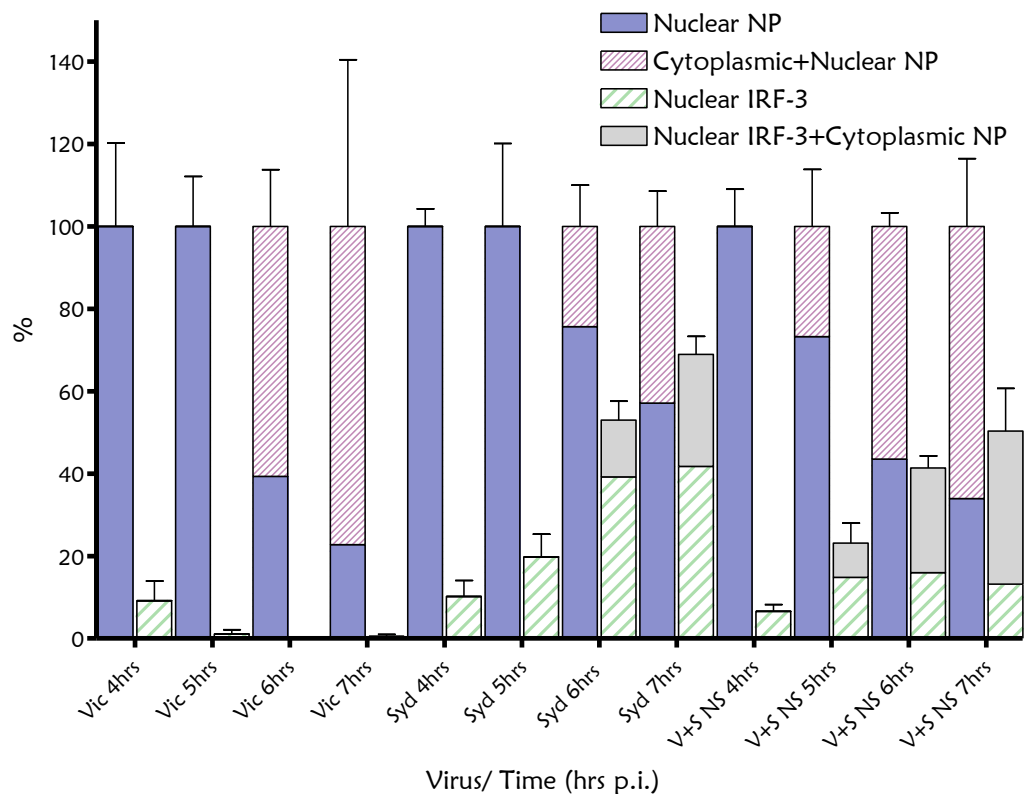


7 hr p.i.

A/Victoria/3/75 A/Sydney/5/97 A/Victoria/3/75
+A/Sydney/5/97 NS



B) Three fields were stained for each virus and each time point. Cells were scored on expression of NP exclusively in the nucleus or expression in both the nucleus and the cytoplasm, and cells with nuclear IRF-3 and those with both nuclear IRF-3 and cytoplasmic NP. Three fields stained for NP were counted, the percentage of cells with nuclear NP are shown by blue bars, the percentage of cells with nuclear and cytoplasmic NP are shown by the pink hashed bars. Three fields stained for IRF-3 were counted, the percentage of cells with nuclear IRF-3 are shown by green hashed bars, for three fields the number of cells with cytoplasmic NP as a percentage of cells with nuclear IRF-3 are shown in grey bars.



However, at the same time point 65% cells infected with either of the low inducing viruses expressed NP in the cytoplasm; compared to only 40% cells infected with A/Sydney/97. Thus, there was no evidence for an early or greater translocation of NP to the cytoplasm in cells infected with A/Sydney/97. Moreover, more of the infected cells in which IRF-3 was nuclear had cytoplasmic NP after infection with the recombinant virus than after A/Sydney/97 infection, 36% compared to 26%). An increase in the expression of NP, based on the intensity of the antibody staining, in A/Sydney/97 infected cells was not observed. The experiment was carried out in the same cell line, with the same virus preparations and equivalent inoculum as the Western blot. Since the Western blots have been repeated a number of times, this suggests that immunofluorescence is a less quantitative technique. At optimised concentrations digitonin can selectively lyse the plasma membrane leaving the nuclear membrane intact allowing the cytoplasm to be separated from the nuclear fraction. Western blots of cell fractions could be used in the future to quantify the amount of NP export from the nucleus. However, it should be noted that IRF-3 translocation is transient and the dimer shuttles in and out of the nucleus (Reich 2002).

6.11 Discussion

Ongoing work in the lab has attempted to establish the mechanisms responsible for the variation in IFN- β induction between influenza strains. This body of work has not been fully resolved the issue but has alluded to the viral genes involved. In human strains of influenza, high IFN- β induction does not map to segments 4, 5 and 8; those of the surface antigens HA and NA nor surprisingly the IFN- β antagonist, NS.

These experiments have shown that the IFN- β induction profile is set very early in induction. Polymerases from high IFN- β inducing viruses initiate RNA synthesis and viral replication within 2 hours of infection. This has a knock on

effect of increased protein expression early in infection. Thus, viral proteins are synthesised early and ultimately accumulate to greater abundance over the course of infection. Surprisingly, this does not correlate with increased viral yields or indeed increased virus fitness in immortalised cell lines. It could however, result in the dysregulation of viral RNA or protein targeting within the infected cells. The experiments designed thus far were not able to demonstrate this. Since the high IFN- β induction requires replication, the early start by A/Sydney/97 could be due to very quick entry and uncoating. The viral inhibitor, Amantadine, inhibits formation of M2 mediated pores in the virion whilst within endosomes and thus prevents virus uncoating. Its use would provide further evidence that input virus does not induce IFN- β . Its addition at staggered times post infection might demonstrate that A/Sydney/97 infects and uncoats earlier in infection than viruses such as A/Victoria/75. Whilst the use of RNA dependant RNA polymerase specific inhibitor such as Ribavirin would corroborate that viral replication is required.

The ideal way to assay the molecular basis for high IFN- β induction would be to employ a reverse genetics system. Recombinant viruses could be used to determine the contribution of each gene segment and subsequently which motifs within these genes are required to elicit the high IFN- β induction. However, difficulties have been encountered trying to obtain full length PCR products. Two laboratories have published the use of a A/Sydney/97 virus reverse genetics system (Parks, Latham et al. 2007). They were approached for access, but unfortunately, did not respond.

The attempt to establish a reverse genetics system revealed further irregularities in the activity of A/Sydney/97 polymerase. RNA analysis and the generation of smaller PCR products amplified while attempting to clone each segment, suggest these viruses have a propensity to make short defective RNAs which are known to induce high levels of IFN- β in virus preparations. Thus far, primer extensions have only been performed using the PB2 gene as a template. Although DIs most frequently occur within the longer segments, using the mid-

range or smaller segments of the influenza genome may infer whether the phenomenon is the result of an unstable polymerase complex for example or a result of genetic encoding within the RNA sequence. A mutation in PA, R638A of A/WSN/33 virus has been shown to drive the synthesis of DIs (Fodor, Mingay et al. 2003). The mutation in PA was proposed to destabilize polymerase-RNA template interactions during elongation which results in the release of the RNA template, the polymerase then reinitiates downstream on the template and completes elongation. These viruses were severely attenuated in MDBK cells, but were rescued by a compensating mutation in PA, C453R (Fodor, Mingay et al. 2003). It is interesting to speculate that the attenuation is due to the IFN- β induction, although this was not investigated. A mutation in NEP, I32T of A/Aichi/2/68 virus was also shown to drive the aberrant synthesis of RNAs derived from the PA vRNA segment (Odagiri and Tobita 1990; Odagiri, Tominaga et al. 1994); thus there seem to be multiple mechanisms which can lead to the generation of DIs. A/Sydney/97 virus does not encode either of the mutations described above (neither R638 in its PA, nor T32 NEP), but it and other high IFN- β inducing virus could encode other mutations which are compensated through mutations which drive resistance to IFN- β induction caused by DIs, rather than mutations which repair the destabilisation of the polymerase.

Pichlmair et al, (Pichlmair, Schulz et al. 2006) showed 5'3P RNA to be involved in influenza virus driven IFN- β induction since CIP treatment of vRNA abrogated the response. This work was all performed using the laboratory strains A/PR/8, and high inducing viruses may make another PAMP which has yet to be determined. RIG-I thus far seems to be the most important transducer of IFN- β induction in influenza infection, and although MDA-5 was found to be upregulated in influenza virus infected cells in an autocrine manner, it is not involved in viral PAMP recognition. Short doubled stranded secondary structures within single stranded RNA, such as the proposed pan handle of influenza vRNA (Brownlee and Sharps 2002), have also been shown important for RIG-I detection of PAMP (Schlee, Roth et al. 2009; Schmidt, Schwerd et al.

2009). In order to understand the nature and localisation of the influenza PAMP, an antagonist of CRM-1 dependant nuclear export, LMB was used. It was hoped this would demonstrate whether the PAMPs presumably produced during viral RNA replication in the nucleus, required transport into the cytoplasm. Unfortunately, LMB also blocked export of the reporter luciferase mRNA and so IFN- β induction could not be measured. Intriguingly, LMB also seemed to activate IRF-3 and this masked the ability to detect influenza activated IRF-3 translocation by immunofluorescence. The situation may be resolved in the future by isolating RNA from nuclear and cytoplasmic extracts from infected cells, to help determine the location of the PAMP.

Increased expression of viral proteins particularly NEP (Neumann, Hughes et al. 2000; Robb, Smith et al. 2009) and M (Liu and Ye 2002) which are involved in nuclear export, could result in dysregulation in protein and RNP targeting. FISH can be used to determine the localisation of influenza RNA species (Hutchinson, Curran et al. 2008), but has yet to be used to investigate differences in RNA localisation between high and low IFN- β inducing viruses. It would be interesting to determine whether high inducing viruses yield more RNAs in the cytoplasm, whether this correlates to all segments or is segment specific and NP localisation. An increase in the export of RNAs or another PAMP, either by increased expression or more efficient protein function, could account for A/Sydney/97 high IFN- β induction phenotype.

The mechanisms and consequences of high IFN induction have yet to be determined. Experiments to investigate the consequences of high IFN induction are to be carried out in the ferret model and in redifferentiated human airway epithelial (HAE) cells. Preliminary experiments in the ferret model, suggest that animals infected with A/Sydney/97 showed more severe symptoms, they had audible respiratory infection and wheezing, they were less interactive and more lethargic (personal communication Kim Roberts). To determine whether this correlates with high IFN- β induction by A/Sydney/97 virus, cytokine levels, measured by qRT-PCR are in progress on both ferret tissue samples and human

airway epithelial cells cultures. Infection of HAE cells resulted in high cytokine induction (personal communication Holly Shelton) and also resulted in more cell death- a phenotype not seen in immortalised cell lines.

Through understanding the mechanisms of high IFN- β induction by A/Sydney/97, improvements and modifications can be made to virus antagonism, prophylaxis and vaccine design. Furthermore, this could have implications for other viruses which induce high levels IFN but have more severe pathology, such as HPAI H5N1.

Chapter 7 General discussion

7.1 Emergence of a new pandemic- swine origin H1N1 influenza

March 2009 saw the emergence of the first pandemic of the 21st century. The work in this thesis has concerned two aspects of influenza research that are pertinent to the emergence of this pandemic. Firstly, the acknowledgment that novel vaccine substrates are required in order to meet the global needs for pandemic vaccination policy. The PER.C6 cell line is one of a number of cell lines that might be part of this new wave of vaccine substrates and it has been important to evaluate their usefulness for the propagation of a range of influenza strains, also as a vehicle in which recombinant influenza vaccine seeds might be generated quickly and efficiently. Secondly, we need to understand how severe the consequence of the new pandemic will be. The viral factors that determine pathogenicity of influenza strains are not completely elucidated but the induction of host cytokine response is one of the foremost mechanisms by which some viruses appear to display increased virulence. The second part of this thesis aimed towards understanding the molecular basis of high cytokine induction by naturally occurring variants of influenza virus and may indeed be directly relevant for predicting the biological outcome of the H1N1 swine influenza viruses.

The new H1N1 2009 pandemic virus originated not from the much anticipated highly pathogenic H5N1 avian influenza, but from a classical swine influenza virus. Thanks to extensive prepandemic planning, the UK has a large stockpile of the antiviral drug oseltamivir (Tamiflu) and despite several isolated cases the virus largely remains sensitive to this drug (<http://www.thelancet.com/H1N1-flu/egmn/0c03a65d>). However, prophylaxis is clearly preferable to treatment. In contrast to previous pandemics, we possess the knowledge and technology to respond quickly and an H1N1 vaccine will hopefully be available before the

winter season (in the northern hemisphere) when the potential for more sustained transmission will increase (Lipsitch and Viboud 2009). The threat of an HPAI pandemic has led to the foundation of sleeping contracts with vaccine manufacturers by many governments worldwide. However, these agreements are complex and may only be activated once WHO pandemic alert level 6 is reached. Furthermore, the ability to deliver the vaccine to a country will be dependent on the number of other countries which activate their contracts and the relative scale of each. This, and the desire to assuage public anxiety, may explain why WHO was reticent to raise the pandemic alert level to 6 despite widespread transmission in several countries on different continents in late May and early June. The UK has two, now active, contracts for 72 million doses from Baxter of a whole, inactivated Vero cell culture derived vaccine, and 60 million doses from GSK of egg grown, split vaccine (<http://www.thelancet.com/H1N1-flu/egmn/0c03a65d>). Both vaccines will be given as a 2 dose regime of a prime followed by boost, since the main target groups (under 60 years of age) are expected to be immunologically naive to the H1 antigen. In addition, the immunogenicity of the vaccines will be enhanced by their formulation. Whole virion preparations have an immunostimulatory effect compared to split vaccine (Geeraedts, Bungener et al. 2008). Otherwise they will be given in conjunction with novel adjuvants such as MF59 (Radošević, Rodríguez et al. 2008; Banzhoff, Gasparini et al. 2009) or AS03 (Leroux-Roels, Borkowski et al. 2007; Sambhara and Poland 2007). In our own work with an H5 vaccine prepared in the PER.C6 cell line, the immune response was greatly enhanced by a proprietary adjuvant (a double oil emulsion of 2% Montanox-80) (Koudstaal, Hartgroves et al. 2009). In human trials with an H7N1 prepandemic vaccine prepared in the PER.C6 cell line, the use of alum adjuvant was essential to enhance the otherwise rather low response in naive volunteers (Cox, Madhun et al. 2009). Influenza vaccines generated in the PER.C6 cell line are still at the clinical trial stage, so the cell line was not certified for vaccine production during the current pandemic. The ability of PER.C6 to generate vaccine seed in the same substrate

as vaccine production and the high vaccine yields, shown in this thesis, would have made them an ideal substrate.

7.2 Swine-origin influenza flu vaccine

Once the severity of the current outbreak became clear, reference labs across the globe began generating vaccine seeds. Five seeds have, as of August 09, been approved by WHO. These have been generated by both classical reassortment (IVR-153, NYMC X-179a) and by reverse genetics, (IDCDC-RG15, NIBRG-121, CBER-RG2)

(http://www.who.int/immunization/sage/4.Zhang_SAGE_vaccine_virus_reagents_-_Final.pdf). NIBSC, the UK institute responsible for generating vaccine seeds for Europe, along with the other labs attempting to make vaccine candidates encountered some technical difficulties with the gene cloning step (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1245138053330). The use of synthetically derived sequences (as discussed in chapter 4) could potentially have overcome the problems encountered with cloning. In the present outbreak, our lab encountered delays in the delivery of ordered sequences due to the sudden high demand for the service, however reference laboratories might in the future hold sleeping contracts with gene synthesis companies, whereby the required sequences can be prioritised, and synthesised within days of submission.

7.3 Poor growth of vaccine seeds

Some of the reassortant and recombinant swine influenza strains vaccine seeds generated (x179a, CBER-RG2, NIBRG-121) were reported to grow poorly in either eggs or cell culture; yields were approximately 50% less than expected for H1N1 component of the seasonal vaccine

(http://www.who.int/immunization/sage/4.Zhang_SAGE_vaccine_virus_reagents_-_Final.pdf). A serial passage derivative of NIBRG-121, NIBRG-121xp, has shown a 4 fold improvement in yields. Since we have shown that vaccine seeds can be generated and propagated in PER.C6 this adaption may not have been required, meaning high growth recombinants might have been available for production pipelines earlier.

The most commonly used donor strain for inactivated influenza vaccine is A/PR/8, which was selected for its high growth characteristics in eggs. Recent clinical trial data (Kistner, Howard et al. 2007) and unpublished data for a wild type swine origin influenza vaccine (A/California/7/09)

(http://www.who.int/immunization/sage/4.Zhang_SAGE_vaccine_virus_reagents_-_Final.pdf) suggest that yields with wild type strains can be higher than 6:2 recombinants in Vero cells. The use of wild type strains for seasonal vaccine manufacture is being investigated. However, these are subject to seasonal variability in growth characteristics, and will also require more rigorous containment. An alternative would be to develop a high growth strain adapted for high growth in cell culture, based either on a cell passaged derivative of A/PR/8 or an entirely different strain. Indeed, experience with PER.C6 cells suggests that an H3N2 virus may be a more suitable backbone (as discussed in chapter 3). During the 2006/7 vaccine season, the reassortant of the H3N2 strain, A/Wisconsin/67/2005 which grew best in PER.C6 cell culture (X116) actually contained 7 genes from the seasonal strain and only one gene, (M), from the A/PR/8 donor. Reverse genetics could be used in the future to map which 'wild type' genes confer the increased growth advantage to X116 in PER.C6 and perhaps stably utilize their genotypes as a novel vaccine backbone. The chief obstacle in developing a cell culture adapted donor strain is the wide selection of cell culture substrates that are being developed. It is unlikely that the reference labs would be willing to generate more than one reference strain, nor the pharma companies willing to incur the costs of having their preferred donor incorporated. Furthermore, regulatory bodies may also be reticent to approve multiple reference strains. However, improvements in vaccine yield

will not only be more cost effective for the manufacturer but also allow more doses of vaccine to be generated. This is still more important during a pandemic, when vaccine is in high demand. The choice of the 'universal backbone' for influenza vaccine seeds in the future will require careful negotiations and open mindedness from manufacturers and regulators alike.

7.4 Pathology of disease

There are now more than 150,000 confirmed cases and more than 1,000 deaths, in 168 countries resulting from infection with swine origin H1N1 virus, and all continents are affected (WHO). The infection is associated with mild or moderate clinical signs but an increase in morbidity has been noted compared to seasonal influenza

([http://www.ecdc.europa.eu/en/files/pdf/Health_topics/090731_Influenza_A\(H1N1\)_Analysis_of_individual_data_EU_EEA-EFTA.pdf](http://www.ecdc.europa.eu/en/files/pdf/Health_topics/090731_Influenza_A(H1N1)_Analysis_of_individual_data_EU_EEA-EFTA.pdf)). Virus shedding in ferrets was equal (Maines, Jayaraman et al. 2009) or slightly higher (1.5 fold) (Munster, de Wit et al. 2009) than seasonal influenza depending on the study. However, in all cases the virus replicated efficiently in both the upper and lower respiratory tract of ferrets. The disease was associated with lesions in the lower respiratory tract and virus was detectable in the lungs (Munster, de Wit et al. 2009) which correlates with a cough as a prevalent symptom in human infection (Novel Swine-Origin Influenza A Virus Investigation Team 2009). Although the virus is currently not as virulent, one of the possible contributions to the severity of the 1918 virus was the tropism to both the upper and lower respiratory tract. Infection of the upper respiratory tract facilitated transmission; it has been suggested lack of replication in the upper respiratory tract is a factor contributing to the restriction of H5N1 virus transmission (Shinya, Ebina et al. 2006; van Riel, Munster et al. 2006). However, replication in the lower respiratory tract has been correlated with higher pathogenicity (Watanabe, Watanabe et al. 2009).

In Britain, the number of cases has increased such that testing of all influenza like illness (ILI) is impossible and deemed unnecessary, and has now been superseded by estimates based upon the numbers of people consulting their GPs. There is no information relating the proportion of pandemic (H1N1) 2009 cases who consult their GP. A telephone helpline The National Pandemic Flu Service (NPF5) will have reduced the proportion of pandemic (H1N1) 2009 cases who consulted their GP. However, it is thought, that this proportion is likely to lie within the range 20% to 50% based on seasonal influenza (http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1248940851283), and a website set up to monitor the activity of ILI in the population through the internet (www.flusurvey.co.uk) also generates estimates within this range. Interestingly reporting levels are still lower than the peak during the winter 08/09.

Bearing this in mind 110,000 new cases per week were estimated in England at the beginning of August 2009. Children and young adults still seem to be the most affected and this is a profile that has been noted for previous pandemics' of the 20th century including the notorious 1918 (Lipsitch and Viboud 2009). To date in the UK, there have been more than 30 deaths, ~800 hospitalised patients. It is too early for information on human pathology, particularly cytokine profiles to be published but initial accounts suggest that swine origin pandemic H1N1 virus is not particularly virulent and does not elicit extreme responses such as the cytokine storm induced by H5N1 and 1918 type viruses. Cytokine arrays are to be performed on hospitalised patients with severe disease; this may provide information on the pathology of the virus, but may also be skewed by variability in patients' immune response. Hospitalisation can be caused by two factors, patients may be either genetically predisposed or have pre-existing conditions such as asthma or pregnancy, which can result in severe illness; alternatively they may become infected with a more virulent strain of the virus. Sequencing is being performed on all cases admitted to St. Mary's hospital, London. Aside from mutations universally occurring in severe cases, virally encoded virulence markers will be anticipated. Of the sequences

available to date, the virus does not contain these markers which could infer the emergence of a more dangerous strain. Using the knowledge acquired from virulent strains, particularly 1918 and HPAI (H5N1, H7N1), as benchmarks key virulence factors have been identified. These viruses often displayed both increased viral titres *in vitro* and *in vivo* (Hatta, Gao et al. 2001; Tumpey, Basler et al. 2005; Watanabe, Watanabe et al. 2009) that have been associated with the polymerase complex (Rolling, Koerner et al. 2009). In H5N1, this has been attributed to the presence of a lysine at position 627 in place of glutamic acid on PB2. Another virulence factor associated with 1918 influenza, is the presence of a full length protein from an alternative reading frame of PB1, PB1-F2, encoding the mutation in N66S (Conenello, Zamarin et al. 2007). 1918 PB1-F2 has also been associated with increased inflammation which rendered the patient more susceptible to secondary bacterial infections which were particularly prevalent in the 1918 pandemic (McAuley, Hornung et al. 2007). Secondary bacterial infection has been proposed as a factor for the increased severity seen in Mexico (Perez-Padilla, de la Rosa-Zamboni et al. 2009). The increased replication of 1918 virus in the lungs, which appears to have been a contributing factor to the pathogenicity of the virus has also been linked to the polymerases (Watanabe, Watanabe et al. 2009). Fortunately, sequencing gazing suggests that the circulating 2009 pandemic H1N1 swine influenza has a very truncated F2, and requires the reversion of 2 stop codons for full length protein expression to be regained, thus the likelihood of it acquiring a more virulent form through mutation alone is low. However, this virus could equally reassort with other circulating human seasonal viruses, and pick up more virulent polymerase genes.

HPAI and 1918 viruses display high virulence associated with hypercytokinemia, (Tumpey, Garcia-Sastre et al. 2005; de Jong, Simmons et al. 2006; Kobasa, Jones et al. 2007; Basler and Aguilar 2008). Although no information relating to cytokine storms and their role in the severity of disease associated with the current pandemic has been published, infection of pigs with swine origin H1N1 influenza suggest that the IFN response may play a role in the pathology

(Barbé, Saelens et al. 2009). Work presented in this thesis suggests that there is a correlation between increased polymerase activity and cytokine induction, and this has also been associated with severe pathology by H5N1 in the avian model (Suzuki, Okada et al. 2009). Although the mechanism have yet to be fully clarified, the data suggests that destabilisation of the polymerase genes may result in the generation of defective RNAs which induce cytokines to which the viruses have evolved resistance.

The mutation in swine origin H1N1 that could have the most impact on our public health policies is the acquisition of resistance to Tamiflu. Previously the resistance acquired through mutation of amino acid 275 from histidine to tryptophan, has been associated with attenuation of the virus (Meijer, Lackenby et al. 2009). However, recent seasonal H1N1 viruses have acquired subsequent mutations which compensate this and they replicate as well as Tamiflu susceptible viruses. Worryingly, although the majority of the swine flu isolates do not have the Tamiflu resistance mutation they do have the associated mutations which suggest that the virus will not be attenuated if they are acquired (Rameix-Welti, Enouf et al. 2008).

These virulence determinants can be picked up by pyrosequencing. However, other unknown mutations may also arise which result in a similar phenotype. The techniques described in this thesis provide a useful panel with which to analyse viruses isolated from severe and fatal cases and determine quickly and easily whether any viruses display a different phenotype. Moreover, the techniques could be adapted to could include analysis of other important cytokines such as TNF- α , IL-6 and those associated with apoptosis such as CCLR5 (RANTES) (To, Chan et al. 2001; Tyner, Uchida et al. 2005; Zhou, Law et al. 2006) and caspase-1 (Allen, Scull et al. 2009). Monitoring of these features may help to reveal whether the virus is evolving and whether this correlates with a shift from the current mild symptoms to more severe pathology.

Use of reverse genetic will allow the contribution of each gene segment to the severity of disease to be assessed, and clarify why this virus is different to either the TRIG (triple reassortant) swine influenza which has circulated in pigs for the last decade (Shinde, Bridges et al. 2009) or other virus which transmitted from pigs to humans (Yassine, Khatri et al. ; Vincent, Swenson et al. 2009) but did not results in sustained human to human transmission.

The pathology of disease can be considered a balance between transmissibility and pathogenicity, a virus that kills its hosts or incapacitates them may not be optimally transmissible (Morens, Taubenberger et al. 2009). Some viruses such as A/Sydney/97 virus are somewhat resistant to the IFN- β they induce (Hayman, Comely et al. 2006), whilst other strains rely on antagonism. These differences could have important implications for the pathology of disease.

The consequences of globalisation; increased air travel and free movement between countries and continents mean viruses are able to spread with increased ease throughout the world. Nevertheless, we are also better equipped than ever before to deal with the effects of a pandemic. It is too early to say whether sufficient vaccine doses will be produced in time or indeed whether the pandemic will reach the scale that has been forecast (Fraser, Donnelly et al. 2009). However, a new era of influenza vaccine production in cell culture is coming to the fore which means together with reverse genetic derived seeds, in the future vaccines will be available more readily and the terrible consequences of previous pandemics may be averted.

Chapter 8 References

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