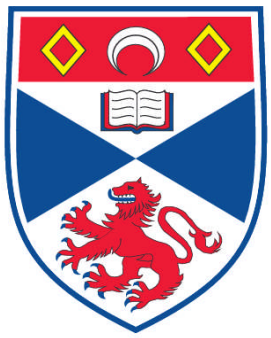
STUDIES ON INTERFERON (IFN) INDUCTION AND ISOLATION OF IFN-INDUCING MUTANT VIRUSES

Shu Chen

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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STUDIES ON INTERFERON (IFN) INDUCTION AND ISOLATION OF IFN-INDUCING MUTANT VIRUSES

Shu Chen

Centre for Biomolecular Sciences

University of St Andrews

A thesis submitted for the degree of Doctor of Philosophy in Molecular Virology

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This thesis is dedicated to my family.

The interferon (IFN) system is a powerful antiviral defense system. Host cell pattern recognition receptors (PRRs) recognise pathogen-associated molecule patterns (PAMPs) which when activated, lead to the transcription of the IFN-β gene. As a consequence IFN is secreted from the cell and activates the JAK-STAT pathway to up-regulate the transcription of IFN-stimulated genes (ISGs). The products of many ISGs inhibit viral replication and cell proliferation. Viruses encode IFN antagonists that dampen down the IFN response, making it less effective. However, within a virus population, there are always likely to be naturally occurring mutant viruses that have lost the ability to circumvent the host IFN response, and if isolated, these viruses would be unlikely to cause severe disease in the host and may therefore be developed as live attenuated virus vaccine candidates.

To develop a methodology to rapidly isolate IFN-inducing mutant viruses, we generated an A549 reporter cell-line in which expression of GFP was driven by the IFN-β promoter. Using this cell-line, we show that the number of cells that became positive for GFP correlated with the amount of IFN secreted by the infected cells and the number of defective interfering (DI) particles within the virus preparations. However, we were unable to isolate IFN-inducing mutant viruses using the A549/pr(IFN-β).GFP cell-line(s). Possible reasons for this may be either that, in cells infected by IFN-inducing mutant viruses, an antiviral state was established independent of IFN that prevented virus replication in the reporter cells in which the IFN-β promoter was activated; or the viruses that activated the IFN-β promoter were DIs only which were not be able to replicate without non-defective helper viruses.

A549/pr(IFN-β).GFP cells are also being used for high throughput assays to screen chemical libraries for compounds that block IFN induction. Such compounds may be potential candidates for anti-inflammatory drugs.

DECLARATIONS

I, **Shu Chen**, hereby certify that this thesis, which is approximately 34,700 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date ……..... Signature of candidate …….....

I was admitted as a research student in October 2007, and as a candidate for the degree of **Doctor of Philosophy (PhD) in Molecular Virology**; the higher study for which this is a record and was carried out in the University of St Andrews between 2007 and 2010.

Date ……..... Signature of candidate …….....

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Doctor of Philosophy in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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Prof. R. E. Randall

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Prof. R. E. Randall

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CONTENTS

^{o}C	degrees Celsius (temperature)
2',5'-OAS	2',5'-oligoadenylate synthase
Ab	antibody
AdV	adenovirus
Ag	antigen
AP	alkaline phosphatase
APC	antigen-presenting cell
ASFV	African swine fever virus
ATF-2	activating transcription factor 2
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair
BVDV	bovine viral diarrhoea virus
C	cytosine
CARD	caspase recruitment domain
CARDIF	CARD adaptor inducing IFN-β
CBP	CREB-binding protein
cDC	conventional DC
CHX	cycloheximide
CID	central interacting domain
CMV	cytomegalovirus
CPE	cytopathic effect
CPI	canine parainfluenza virus
CpG	cytosine followed by guanine in a DNA sequence
CPSF	cleavage and polyadenylation factor
CREB	cAMP-responsive-element-binding protein
CTL	cytotoxic T-lymphocyte
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DDB1	damage-specific DNA-binding protein 1
DI	defective interfering particles
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpi	days post-infection
ds	double-stranded
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eIF	eukaryotic initiation factor
FACS	fluorescence-activated cell sorting
EDTA	ethylene diamine tetra-acetic acid
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum

FADD	Fas-associated death domain
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FLUAV	influenza A virus
FLUBV	influenza B virus
FMDV	Foot-and-mouth disease virus
FSC	forward scatter
g	gram
G	guanosine
GAS	gamma-activated sequence
GFP	green fluorescent protein
GTP	guanosine triphosphate
HA	haemagglutinin
HBV	human hepatitis B virus
HCV	human hepatitis C virus
HeV	Hendra virus
HHV	human herpesvirus
HIV	human immunodeficiency virus
HMG	high mobility group
HMGI(Y)	HMG high mobility group I protein
hpi	hours post-infection
HPIV	human parainfluenza virus
HPV	human papillomavirus
HRP	horseradish peroxidase
HSP40	heat shock protein 40kD
HSV	herpes simplex virus
ICP27	infected cell protein 27
IFN	interferon
IFNAR	IFN-α/β receptor
IFNGR	IFN-γ receptor
IFNLR	IFN-λ receptor
IgG	immunoglobulin G
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
IL-1R	IL-1 receptor
IPS	IFN-β promoter stimulator
IRAK	IL-1 receptor associated kinase
IRES	internal ribosome entry site
IRF	IFN regulatory factor
ISG	IFN-stimulated gene
ISGF3	ISG factor 3
ISRE	IFN-stimulated response element
JAK	Janus/just another kinase

kDa	kilodalton
LCMV	lymphocytic choriomeningitis virus
LTRs	long terminal repeats
LZ	leucine zipper
M	molar
MAVS	mitochondrial antiviral signalling protein
MCMV	murine cytomegalovirus
mDC	myeloidderived dendritic cell
MEF	mouse embryonic fibroblast
MeV	measles virus
MeVEd	MeV of Edmonston vaccine strain
mda-5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
MMR	mumps measles and rubella
MOI	multiplicity of infection
mRNA	messenger RNA
MuV	mumps virus
Mx	myxovirus resistance protein
MyD88	myeloid differentiation primary-response protein 88
NA	neuraminidase
NAK	NF-κB-activating kinase
NAP1	NAK-associated protein 1
NAT	N-acetyl trypsin
NDV	Newcastle disease virus
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NiV	Nipah virus
NK	natural killer cell
NLS	nuclear localisation signal
NP	nucleocapsid protein
Npro	N-terminal protease fragment
nt	nucleotide
pac	puromycin resistant gene
PACT	PKR activator
PAMP	pathogen-associated molecule pattern
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
PFU	plaque-forming units
pH	-log10[H+]
PIAS	protein inhibitors of activated STAT
PIV5	parainfluenza virus 5
PKR	interferon-induced, double-stranded RNA-activated protein kinase
PML	polymorphonuclear leukocyte
polyI:C	polyinosinic: polycytidylic acid
PP2A	protein phosphatase 2A

PRMT1	protein arginine methyltransferase 1
PRR	pattern recongnition receptor
PVDF	polyvinylidene difluoride
RIG-I	retinoic acid-inducible gene product I
RIP	receptor-interacting protein
RNA	ribonucleic acid
RNAP	RNA polymerase
RSV	Respiratory syncytial virus
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	small hydrophobic integral membrane protein
SH2	Src-homology-2
SINV	Sindbis virus
siRNA	small (short) inhibitory RNA
shRNA	small (short) hairpin RNA
SINTBAD	similar to NAP1 TBK1 adaptor
ss	single-stranded
SSC	side scatter
STAT	signal transducer and activator of transcription
SV	Simian virus
TAB	TAK-1 binding protein
TAK	transforming growth factor β-associated kinase
TANK	TRAF-associated NF-kB activator
TBK	TANK-binding kinase
TH	helper T cell
TIR	Toll/Interleukin-1 resistance
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor inducing IFN-β
Tris	Tris-hydroxymethyl-aminomethane
Tyk	tyrosine protein kinase
Ub	ubiquitin
Ud	FLUAV strain A/Udorn/72
UV	ultraviolet
V	volts
V5	epitope tag derived from a small 14-amino acid region of PIV5-V
v/v	volume per volume
VACV	vaccinia virus
VAI RNA	virus-associated RNA I
VISA	virus-induced signalling adaptor
vRNAP	viral RNAP
VSV	vesicular stomatitis virus
w/v	weight per volume
wt	wild-type

1. INTRODUCTION

1.1 Viruses

Viruses are small mobile particles which cause infections in host cells. Viruses that can parasitise virtually every living organism (bacteria, fungus, plants, animals and human), are one of the major causes of infectious diseases leading to significant numbers of deaths every year. Viruses contain the essential 'living' features of reproducibility (replication), inheritability, and mutagenesis. However, because viruses do not have the independent ability of self metabolism, they utilise the hosts' biosynthetic machinery to build up genomic structure during viral replication, and synthesise viral proteins. Viruses consist of a single type of nucleic acid (either DNA or RNA) as its genomic information, capsid proteins and have no cell structure.

1.2 *Paramyxoviridae* family

The *Paramyxoviridae* family of viruses has been comprehensively reviewed in Lamb & Parks, 2006. Paramyxoviruses are a group of negative strand RNA viruses, responsible predominantly for a number of important acute respiratory diseases in humans and animals. The *Paramyxoviridae* family includes some of the most important viruses causing serious infectious diseases in animals and humans. Examples include: measles virus (MeV), one of the most well-known genera, causing acute, contagious viral disease, mainly affecting children; mumps virus (MuV), commonly causes diseases in children and is still a health threat worldwide; respiratory syncytial virus (RSV) causes diseases in the lower respiratory tract in infants and children and can cause bronchiolitis and pneumonia; Newcastle disease virus (NDV) causes diseases in birds and poultry; parainfluenza virus 5 (PIV5), also known as canine parainfluenza (CPI) and Simian virus 5 (SV5), cause diseases in dogs; some newly recognised viruses, Hendra virus (HeV) and Nipah virus (NiV), cause deadly diseases in animals and in humans. Although there is no existing pandemic threat posed by viruses in the *Paramyxoviridae* family, these viruses infect a broad range of host, generate diseases with a significant mortality in humans, and

have a heavy economic impact.

Paramyxoviruses are enveloped, linear, negative sense, single strand RNA viruses. The International Committee on Taxonomy of Viruses (ICTV) defined the *Paramyxorividae* family into two subfamilies – *Paramyxoviridae* and *Pneumovirinae*, which are sub-divided into genera. The *Paramyxoviridae* contains *Respirovirus*, *Rubulavirus*, *Avulavirus*, *Morbillivirus*, and *Henipavirus*. The *Pneumovirinae* contains *Pneumovirus* and *Metapneumovirus*. The classification is based on morphology, genomic structure, and viral protein functions (ICTV, 2005; Lamb & Parks, 2006; Figure 1.2.1).

1.2.1 Replication strategy of paramyxoviruses

Paramyxovirus transcription and replication are carried out in the cytoplasm. As soon as the virus nucleocapsid is released into the cell, the viral RNA-dependent RNA polymerase complex (RNAP) sits on the template, recognises the negative sense RNA genome. Genomic RNA itself is non-infectious, and it has to be transcribed into 5'-capped and 3'-polyadenylated mRNAs by viral RNAP (vRNAP). mRNA transcription terminates and restarts at each gene junction and is then released after polyadenylation and translated into viral proteins. After primary transcription, when there is sufficient unassembled nucleocapsid (N) protein present, viral genome replication starts. vRNAP ignores all the gene junctions and full length complimentary anti-genome (positive strand RNA) copy is replicated and assembled in an encapsidated form. The positive strand RNA then works as a replication intermediate for further viral genomic RNA (negative strand RNA) synthesis. Replicated genomic RNA is either assembled as progeny virus, or used for further replication.

1.2.2 The *Paramyxoviridae* genomes and encoded proteins

The way in which paramyxoviruses adapt to host varies. However, they share genomic structure and functions in common. The virions are enveloped and the

shapes are generally spherical. The virion is composed of an envelope, matrix (M) protein and a nucleocapsid core; the genomic RNA being encapsidated by the nucleocapsid (N) protein (Figure 1.2.2.1). The lipid envelope contains two surface glycoproteins: the tetrameric haemagglutinin-neuraminidase glycoproteins (HN, or H or G) and the trimeric fusion glycoproteins (F), which are inserted into the viral membrane as spikes. The viral matrix protein resides underneath the lipid bilayer between the envelope and the nucleocapsid core and is important for maintaining the virion structure. Inside the virus, associated with the nucleocapsid, are the large polymerase (L) and phosphoprotein (P) proteins and together this complex composes the RNA polymerase complex. For most paramyxoviruses, V protein, a cysteine rich protein, is only present inside infected cells. However, for rubulaviruses, V protein is found as an internal component of the virion (Paterson *et al.*, 1995; Randall & Bermingham, 1996).

The Nucleocapsid Protein

For paramyxoviruses, excluding pneumoviruses, the nucleocapsid (N) protein is the first transcribed gene in the genome. In infected cells, N protein is present in at least two forms: either associated with genomic RNA and antigenomic RNA to form the helical nucleocapsid template which biologically activates these RNAs and protects them from nuclease digestion, or, in an unassembled soluble form termed N^0. This unassembled N^0 is important for encapsidation of nascent RNA during virus replication (Horikami *et al.*, 1992; Curran *et al.*, 1995). The N^0 protein associated with P in a number of viruses, including Sendai virus (Horikami *et al.*, 1992), PIV5 (Precious *et al.*, 1995), human parainfluenza virus 2 (HPIV2) (Nishio *et al.*, 1999), HPIV3 (Zhao & Banerjee, 1995), MeV (Huber *et al.*, 1991; Spehner *et al.*, 1997) and RSV (Garcia-Barreno *et al.*, 1996; Mallipeddi *et al.*, 1996), forms a polymerase complex, and together they function in an encapsidative manner, and play a central role in protecting viral RNA as template for replication.

The Phosphoprotein

Phosphoprotein (P) is important for viral RNA synthesis (Curran *et al.*, 1994; Curran *et al.*, 1995). P protein is a crucial factor for vRNAP enzyme (Hamaguchi *et al.*, 1983) and also facilitates RNA encapsidation in the N^0 nascent chain assembly complex during viral replication. The N-terminal domain of P protein faciliates interactions with unassembled N^0 by preventing N aggregation securing assembly specificity (Horikami *et al.*, 1992; Curran *et al.*, 1995).

The V Protein

The V protein is a protein found in most paramyxoviruses, and plays an important role in viral pathogenesis by targeting host antiviral activities at early infection stages (Chapter 1.5.3). V protein acts as a negative regulator of viral RNA synthesis. V protein shares an N-terminal domain with the P protein, interacts with the unassemblemd soluable N protein - N^0 and inhibits assembly through the formation of a V-N^0 complex. The V-N^0 complex negatively regulates assembly in a number of viruses, including PIV5 (Precious *et al.*, 1995), Sendai virus (Horikami *et al.*, 1996) and MeV (Tober *et al.*, 1998). V protein also binds RNA and inhibits RNA synthesis in the case of MeV V protein (Parks *et al.*, 2006). In addition, V proteins also interact with cellular proteins to antagonise host antiviral proteins. For example, PIV5 V protein interacts with the cellular damage-specific DNA-binding protein 1 (DDB1) to target interferon (IFN) signalling pathway (Andrejeva *et al.*, 2002). The V protein inhibition of the host IFN response is discussed in Chapter 1.5.3.

The Large Protein

The large (L) protein is present in infected cells at low levels (Lamb *et al.*, 1976) and is associated with nucleocapsids and virions, and is an important component of paramyxovirus RNAP. L protein is involved in nucleotide polymerisation, 5'-capping and methylation and 3'-polyadenylation of mRNAs synthesis activity (Gradzelishvili *et al.*, 2005; Hercyk *et al.*, 1988; Ogino *et al.*, 2005).

The Matrix Protein

The Matrix (M) protein is the most abundant protein within the virion. M protein is localised peripherally in the membrane, and is hydrophobic. M protein is essential for virus morphogenesis, due to its contact with the lipidbilayer, nucleocapsid, and membrane proteins. The M protein, inserted in the membrane, interacts specially with F protein and HN glycoprotein tails in Sendai virus (Sanderson *et al.*, 1993a; Sanderson *et al.*, 1993b). M protein interaction with nucleocapsid plays a central role in assisting the egression of daughter viral particles (Peeples, 1991).

Envelope Glycoproteins

Haemaggultinin-Neuraminidase (HN), haemagglutinin (H) and G glycoproteins are responsible for virus adsorption. Haemagglutinin allows viruses to bind to specific sialic acid receptor, whereas neuraminidase helps to prevent self-aggregation of new viral particles through sialic acid cleavage on the cell membrane upon budding.

Fusion protein (F) works as an intermediate to fuse virus envelope with the cell plasma membrane in order to deliver the viral nucleocapsid into the cytoplasm at neutral pH. At later stages of infection, the F protein facilitates fusion between infected cells and neighbouring cells. An example of this is the cytopathic effect (CPE) *in vivo* which allows virus spread. The F proteins are inactive when synthesised and have to be cleaved by a host cell protease to become biologically activated.

1.2.3 Parainfluenza virus 5

PIV5 is a member of the *Rubulavirus* of the *Paramyxioviridae* family which has been routinely used in our laboratory as a model to study virus infections and host cell antiviral responses. PIV5 was originally isolated from cultured primary rhesus monkey cells, although its natural host is the dog in which it causes respiratory diseases. PIV5 has been isolated from a variety of sources, including humans, monkeys and dogs (Chatziandreou *et al.*, 2004). Although PIV5 can infect humans (Hsiung, 1972; Goswami *et al.*, 1984) and has been isolated from human sources,

such as bone-marrow cells (Goswami *et al.*, 1984), there is no convincing evidence that PIV5 infection is associated to human acute respiratory diseases.

The genome of PIV5 is 15,246 nucleotides (nt) (for isolate W3A) in length and contains seven genes that encode eight known viral proteins (NP, P and V, M, F, small hydrophobic integral membrane protein (SH), HN and L) (Figure 1.2.3.1). The V and P proteins are both transcribed from the V/P gene, as the open reading frames of V/P gene are overlapping, therefore resulting in two different gene products. The PIV5 V mRNA is transcribed as a complete copy of the V/P gene, whereas the P mRNA is transcribed by two additional nontemplated guanosine (G) residues. The two structural proteins V and P share the first 164 amino acids at the N-terminus. V and P proteins have unique C-termini which are biologically important for viral functions (Thomas *et al.*, 1988).

1.2.4 PIV5 strain isolates

PIV5 W3, PIV5 VΔC

Two original PIV5 isolates were isolated from rhesus and cynomolgus monkey kidney cell cultures and are referred to as WR and W3A (or W3) as wild-type (wt) viruses (Choppin, 1964; Hull *et al*., 1956). A mutant strain of PIV5 W3 has been isolated from a recombinant PIV5 (rSV5) which has deletions at the V protein specific C-terminal domain (VΔC) and thus lost the ability to target STAT1 for proteasome-mediated degradation (He *et al.*, 2002). STAT1 is a key trascription factor of the Signal Transducers and Activators of Transcription family and plays an important role in upregulating of the induction of IFN stimulated genes (ISGs) (Chapter 1.4.3 and 1.5.3).

PIV5 VΔC (P2) and defective interfering particles (DIs)

PIV5 VΔC (P2) was generated from the original PIV5 VΔC stock by infecting confluent Vero cells at a high MOI in order to increase the number of DIs within the virus population by Mr Dan Young in our laboratory. PIV5 VΔC (P2) was

characterised to be an extremely good inducer of IFN as it contained large amounts of defective interfering particles (DIs). DIs are virus particles which have mutations or deletions in their genome. They do not have the ability to replicate on their own, but require a helper virus to sustain an infection. DIs interfere with the replication of helper virus and compete with their helper virus genomes for the viral replication substrates. Possibly due to their defective nature, DIs were found to be potent inducers of IFN. Some SeV stocks enriched in DIs were reported to be strong inducers of IFN (Strahle *et al.*, 2006). However, current studies in our laboratory showed that, although DI-rich stock of PIV5 VΔC was good inducers of IFN, not all DIs induce IFN. DI-rich virus stocks were used in this project to activate the IFN-β promoter.

CPI+, CPI-

Two canine strains are routinely used in our laboratory as well as PIV5 W3 and PIV5 VΔC. One of the strains, termed CPI+, was isolated from the cerebrospinal fluid of a dog with incoordination and posterior paralysis (Evermann *et al.*, 1980; Evermann *et al.*, 1981) and is antigenically related to a prototype strain of canine parainfluenza virus. The other PIV5 canine strain CPI- was isolated from the brain tissue of a dog experimentally infected with CPI+. This CPI- virus strain establishes persistent infections immediately *in vitro* when compared to the syncytial giant cell-forming CPI+ (Baumgartner *et al.*, 1987; Southern *et al.*, 1991) and fails to block IFN signalling (Chatziandreou *et al.*, 2002). Antigenic studies have also shown that out of 53 antibodies tested, MAb P-k was the only antibody that distinguished between cells infected with CPI + and CPI- (Southern *et al.*, 1991). MAb P-k has been reported as recognising an epitope for both P and V proteins of CPI+ (Thomas *et al.,* 1988) and was not able to recognise either P or V proteins of CPI-. Sequence comparison of the V protein P/V N-terminal domain of CPI+ with CPI- revealed three amino acid substitutions which are responsible for CPI- failure to block IFN signalling (Chatziandreou *et al.*, 2002). Immonoblotting data has also shown that both CPI+ and CPI- were poor IFN inducers at early infection stage, however, from 21h

post-infection (hpi), CPI- became a better IFN inducer than CPI+ (Poole *et al.*, 2002).

1.3 Immune response to viruses

One motivation to study the immune system and how viruses overcome the host immune response is that virus infections cause severe diseases and kill millions of people every year across the world. An example of this is the 1918 Spanish flu outbreak; a third of the world population (~500 million people) were infected in the 1918 Spanish influenza pandemic, and more than 50 million (3% of the world's population at the time) people were estimated to be killed by the virus (Wang & Tao, 2010). Therefore, it is important to understand the strategies that viruses use to subvert the host immune system, and have better solutions in response to emerging viruses, for example, the development of a rapid way of generating vaccines (Chapter 1.6).

The immune system is a complex and integrated group of organs, tissues and cells that differentiates self and non-self providing defence against potentially pathogenic organisms or substances. Immunity is composed of two cooperative defence systems, called non-specific, innate immunity and specific, acquired immunity. The innate system is an immediate primary defence mechanism against infection, while the adaptive system, which exhibits an immunological memory, acts as a second line of defence.

1.3.1 Innate immunity

The innate response is usually activated when microorganisms are identified by specialised sets of pattern recognition receptors (PRRs). The receptors usually recognise components of microorganisms (pathogen-associated molecule patterns, PAMPs) that are not found in cells of the host, e.g. components of bacterial cell wall, bacterial flagella, or viral nucleic acids. PAMP binding to these receptors gives rise to a well-established microbial disposal process, such as phagocytosis of harmful foreign particles, inactivation of viruses and bacteria by macrophages and neutrophils, or the

production of IFNs. Since innate immunity is non-specific, these systems respond to pathogens in a universal manner and this system does not establish long-lasting immunity against a pathogen. Microorganisms which can overcome this system and survive, may cause disease, unless prompt adaptive immune response develops.

The innate immune system can be seen to comprise of four types of defensive barriers: anatomical barriers (e.g. skin - the mechanical barrier preventing entry of microorganism), physical barriers (e.g. temperature, pH), inflammation and cellular barriers depending primarily on phagocytosis.

Acute inflammation

When pathogens overcome epithelial barriers and establish a local infection, the host mobilises its defences to the site of infection. Inflammation is one of the first response processes of the host immune system to injuries or infections. When a host is infected by an injurious agent, phagocytes that reside in all tissues will try to remove these agents. At the same time, host cells respond to infection by the initiation of inflammation through the release of inflammatory mediators, such as leukocytes and cytokines, into the tissues. The prompt arrival of leukocytes in the tissues plays a vital role in killing and removing the invading microorganisms. IFNs are important cytokines involved in protection against viral infections. Once the infectious agent is eliminated, the anti-inflammatory defence is established, the damaged tissue is promptly healed, and the pathogen is cleared, the host would typically recover from infection.

Phagocytosis

Phagocytosis is another important defence mechanism of innate immunity performed by cells called phagocytes. When a pathogen attaches to the host cell, phagocytes engulf that microorganism. The microorganism is then ingested by a phagocyte, and forms a phagosome. The phagosome fuses with lysosome, the microorganism is digested with lysosomal enzymes, and the destroyed materials are released from the

cell.

Macrophages and neutrophils are the two major types of phagocytes. They are mobile phagocytes that travel throughout the body in search of invading pathogens. Macrophages are located in most tissues. Neutrophils, also called polymorphonuclear leukocytes (PMN), are primarily found in the bloodstream and comprise the majority of circulating leukocytes. During an acute inflammation process, neutrophils move promptly through the endothelium onto the site of infection through the blood vessels and then through interstitial tissue *via* a process called chemotaxis.

Dendritic cells (DC) are phagocytes, found mainly in the skin, and in T cell and B cell areas of lymphoid tissues. DCs recognise antigens from all types of sources, and present mainly to T cells (Chapter 1.3.2). They play an important role in driving the activation of naive T cells and further differentiation into effector T cells (Murphy *et al.,* 2008). A subset of plasmacytoid DCs (pDCs) play a role in the production of IFN upon virus infection.

1.3.2 Adaptive immunity

The relatively non-specific innate immune response is a prerequisite to initiate the primary adaptive immune response. The adaptive immune response is initiated when a pathogen overcomes the innate immune response. The adaptive immune system is capable of recognising particular microorganisms and develops a strong immune response. Unlike the innate immune system, adaptive immunity is specific to particular antigen challenges, which allows for the generation of responses tailored to specific pathogens or pathogen-infected cells. The adaptive immune response usually results in elimination of the pathogen, and recovery of the host from a disease, as well as establishment of an immunological memory in the host, enabling the host to respond more rapidly and effectively to any subsequent infection by that particular pathogen.

The adaptive immune system is mediated by special lymphocytes, T cells and B cells. Adaptive immunity involves the activation and proliferation of antigen-specific T cells and B cells, subsequent cell-mediated immune activity by T cells, and humoral immunity mediated by B cells, which involves the production of antibodies in response to an antigen.

T lymphocytes and T cell-mediated immunity

T cell-mediated immune response is activated in response to an infection when the naive T cell encounters an appropriate antigen. Antigens are internalised by antigen-presenting cells (APCs) by phagocytosis or endocytosis. APCs then display a fragment of the antigen on their membrane in association with major histocompatibility complex (MHC) molecules, and present the specific antigen to T cells (Figure 1.3.2.1). There are two subpopulations of T cells: T helper (TH) cells and cytotoxic T (CTL) cells, which are usually distinguished by differences in membrane glycoprotein expression. When a TH cell is activated following antigen presentation by APCs, it develops into effector T cells by proliferation, and secretes cytokines. As well as producing effector T cells, the proliferation of naive T cells also produce memory T cells, which will establish long-term immunity to the specific antigen, and generate protective activity whenever re-infection takes place. Cytokines produced by TH cells are important in the activation of both the cell-mediated and the humoral immune response (Figure 1.3.2.1).

B lymphocytes and humoral immunity

The humoral immune response triggers cells to proliferate and secrete large amounts of specific antibodies against extracellular microorganisms and prevent the spread of intracellular infections. B cells are activated in many cases when a naive B cell recognises an antigen, and the antigen is displayed by the B cell as peptide:MHC class II complex, which is recognised by and activates TH cells (Figure 1.3.2.1). Activated TH cells trigger the naive B cell to differentiate and proliferate into effector B cells, known as plasma cells, and memory B cells. Activated B cells produce antibody

against the antigen, which would combat such microorganism, and prevent the spread of infection.

1.3.3 Immunological memory

Pathogens which successfully overcome the generic reactivity of the innate immune system will induce the development of the specific adaptive immunity. The initial adaptive immune response to a pathogen takes a longer time to establish. T cells and B cells, in response to the infection, proliferate and differentiate into effector cells to combat the pathogen, which then leads to the clearance of the pathogen. An immunological memory is developed at the same time, which protects the host from reinfection of the same pathogen. The development of the immunological memory to establish protective immunity against reinfection is an important consequence of the adaptive immune response. The production of long-lived memory lymphocytes during this initial response will generate more prompt response to reinfection of the same pathogen.

1.3.4 Vaccines

Vaccines are inactivated or attenuated virus particles or material derived from viruses. Vaccination artificially induces protective immunity, which will respond to and eliminate the previously encountered specific antigen upon subsequent infection with the natural virus. The development and application of vaccines is probably the most remarkable and exceptional achievement in the field of immunology.

Live attenuated virus vaccines

Live attenuated virus vaccines are a mutant strain of a wild-type virus, whose ability to inhibit host immune responses is impaired. The virus can still infect its natural host, but unlike wild-type virus, the virus will not generally cause severe disease. Whole virus immunisation can induce immunity to antigens in their natural state. As mutated viruses replicate in the host, the number of antigens increases, which means less viruses are required for injection to induce protective immunity. Furthermore, they

can induce a wide range of immune responses, which includes memory-based cellular and humoral immune responses (Carter & Saunders, 2007).

Potential virus mutant strains are selected by passage in the laboratory over several generations under conditions that make them less virulent. The passage process is repeated and thus the ability of viruses to cause disease is diminished. Virus strains with reduced virulence are then selected as candidates for vaccine development.

There are shortcomings of using live attenuated virus vaccines. As the attenuated virus is a modified form of the wild-type virus, there is always a possibility the attenuated strain will revert to the wild-type. The vaccine can cause disease in patients who are immunologically compromised. That said, live attenuated vaccines are still thought to be the most effective and safe vaccine type, with a large number of live attenuated vaccines currently licensed and manufactured. Known examples of live attenuated virus vaccine include: mumps and measles vaccine in the MMR (mumps, measles and rubella) vaccine; oral polio vaccine derived from strains that have lost its ability to infect neurones (Carter & Saunders, 2007); and varicella (chicken pox) vaccine.

Inactivated virus vaccines

Inactivated virus vaccines share common features with live attenuated vaccines, and both provide the whole virus in some way. Viruses are killed or inactivated by heat shock or chemicals such as formaldehyde. Inactivated virus will not cause disease, but the strain is still able to trigger an immune response. A typical example of an inactivated vaccine is the original Salk polio vaccine. The vaccine is generated by treating the wild-type virus with formaldehyde, so that the virus is unable to reproduce in the vaccinated individuals. If inactivation is efficient, the advantage of these vaccines is that very low level of infection is generated. Additionally, this vaccine can be used safely on immunocompromised individuals.

The problem with inactivated virus vaccines is that during the inactivation process, the viruses may be over inactivated and consequently become non-immunogenic. In addition, finding the ideal chemical concentration, reaction time (Carter & Saunders, 2007), temperature and other pertinent conditions to inactivate the virus is difficult. Finally, to stimulate sufficient immunity, a large amount of virions are essential, since the virus cannot replicate, and periodic boosters are required to maintain immunity.

Subunit vaccines

Subunit vaccines are derived from viral antigens free of viral nucleic acid, and contain only a small portion of the viral protein, rather than the whole virus. The vaccine protein is the essential part which can stimulate immune response. For example, the influenza virus vaccine contains the surface glycoproteins haemaggultinin (HA) and neuraminidase (NA) (Carter & Saunders, 2007). To generate this subunit vaccine, influenza virus is treated with formaldehyde. Glycoproteins are removed from the virus envelope and purified using sucrose gradient centrifugation. As the isolated vaccine protein contains no viral genomic RNA, there is no chance of causing a disease. The advantages of subunit type vaccines are they are unlikely to trigger an adverse reaction, and subunit vaccines are not infectious, so that they can be applied to immunosuppressed people. The disadvantages of subunit vaccines are that subunit vaccine require multiple doses and adjuvants and may not be able to generate a powerful immune response as well as a whole virus vaccine.

Recombinant vaccines

Recombinant vaccines have the genes for a desired viral antigen, which is immunogenic and crucial to virus function introduced into a vector, with a view to induce a protective immune response. The gene encoding the antigen is cloned into bacteria, yeast, mammalian cells or viruses for expression in large quantities. The purified gene product is then used as the vaccine. To generate a recombinant vaccine, low virulence or harmless vectors can be chosen, antigen which would not induce protective immunity is eliminated, and only a single non-virulence antigen is

expressed. Therefore, it is unlikely for the vaccinated individuals to become ill from the vaccine. However, generation of such recombinant vaccines is costly and time-consuming. One example of licensed recombinant vaccines for human is the Hepatitis B Virus (HBV) vaccine. An alternative method to generate recombinant vaccine is the production of hybrid virus vaccines. A well-known example for this is vaccinia. The idea is to introduce foreign DNA into a plasmid vector containing the vaccinia virus genome. The resulting recombinant vector is then transfected into cells infected with vaccinia viruses, resulting in the formation of a stable recombinant virus. The advantages of hybrid virus vaccines are they induce a wide range of immune responses and are relative easy to be produced. However, virulence tends to increase over times as live vaccines.

1.4 IFN system

As described in Chapter 1.3, the immune system is an amazingly effective system which recognises, kills and disposes of non-self molecules. The development of an effective immune response involves the secretion of cytokines, which bind to specific receptors on cells in response to various stimuli, to trigger the signal transduction pathway between cells (Kindt *et al.*, 2006).

The IFN system (Figure 1.4.1), which has been comprehensively reviewed by Randall & Goodbourn, 2008, provides essential defence against viral infections at an early stage (hours to days) in the immune response. IFNs are a group of cytokines secreted in response to viral infections, which have antiviral and immunomodulatory functions. When a cell recognises it is infected, it produces IFNs, which then exert their effect on neighbouring cells and itself. This leads to the upregulation of hundreds of ISGs, which may have direct or indirect antiviral functions. The result is the establishment of an antiviral state which limits the capability of viruses to replicate effectively.

Three major classes of IFNs exist based on their amino acid sequences: are classified into three major classes: Type I, Type II and Type III IFNs. Type I IFNs (Isaacs &

Lindenmann, 1957) consist of IFN-α, -β, -ω, -ε, -τ, -δ and –κ and are directly induced in virus-infected cells; Type II IFN (IFN-γ), is a pro-inflammatory IFN which produced by activated T cells and natural killer (NK) cells; Type III IFNs comprise IFN-λ1, -λ2 and -λ3, also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively (Platanias, 2005). All IFNs are induced in response to viral infections, but with limited tissue distributions (Meager *et al.*, 2005; Mennechet & Uze, 2006; Zhou *et al.*, 2007).

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1.4.1 Activation of IFN-β promoter for gene transcription

Induction of type I IFN genes (IFN-α and IFN-β) is an important host response to virus infections. PRRs present in host cells detect viral components in the form of viral proteins or viral nucleic acids and triggers activation of the IFN-β promoter (Chapter 1.4.2). IFN-β subsequently signals both the infected cells and neighbouring cells. This leads to the expression of IFN-stimulated genes (ISGs) with antiviral functions to establish an antiviral state.

The IFN-β promoter region serves as a platform to assemble transcription factors and regulate the activation of IFN-β gene in response to viral infections. The IFN-β promoter region is composed of multicomponent transcription factors, which are collectively termed the enhanceosome and initiate transcription in a cooperative way.

As illustrated in Figure 1.4.1.1, the transcription factors that bind the IFN-β promoter are the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), IFN regulatory factor family (IRFs) (IRF-3, IRF-7) and activating transcription factor 2 (ATF-2)/c-Jun complexes, which assemble cooperatively at the enhancer to initiate transcription (Merika & Thanos, 2001). The activation of IFN-β without the binding of NF-κB and ATF-2/c-Jun has also been reported (Goodbourn *et al.*, 1985; Ellis & Goodbourn, 1994; King & Goodbourn, 1994; Peters *et al.*, 2002), while IRF-3 binding appears to be essential (Kawai & Akira, 2006). Each transcription factor has an influence on IFN-β gene induction, although the impact may vary in cell types and

inducers. The assembly requires the essential mediating protein – the high mobility group (HMG) I protein - HMGI(Y) to bind DNA to form the enhanceosome (Thanos & Maniatis, 1995) and assists the binding of NF-κB and ATF-2/c-Jun. HMGI(Y) binding to the promoter bends the DNA, allowing the activators to bind to the DNA for enhanceosome assembly. Cooperation is essential for IFN-β gene activation, as individual activators have limited binding affinity to the promoter, and would not be able to induce optimal IFN-β gene activation. The assembled enhanceosome then recruits the transcriptional coactivator cAMP-responsive-element-binding-protein (CREB)-binding protein (CBP) and p300 (CBP/p300) to assemble the basal transcriptional machinery for the initiation of IFN-β transcription (Merika *et al.*, 1998).

1.4.2 IFN induction

Host PRRs are proteins expressed by cells to distinguish non-self molecules and act as the first line of defence against invading organisms. Certain PRRs such as the membrane-associated Toll-like receptors (TLRs), and the cytoplasmic RNA helicases (retinoic acid inducible gene-I (RIG-I) and/or melanoma differentiation associated gene 5 (mda-5), are crucial factors in the induction of IFN.

1.4.2.1 TLR-dependent pathway

TLRs have been grouped into a super family with the interleukin-1 receptors (IL-1Rs), and are generally termed Toll/IL-1R (TIR) receptors. The TLRs and IL-1Rs share a common structure of around 200 amino acids in their cytoplasmic regions, known as the TIR domain (Akira & Takeda, 2004). There are 13 mammalian TLRs, and they are distributed in a variety of tissue types. TLRs 1, 2, 4, 5, 6 and 11 are located on the cell surface and they recognise lipids, lipoproteins or peptidedoglycans presented by bacteria, fungi or protozoa. TLRs 3, 7, 8, and 9 are found in intracellular membranes `and these are involved in the detection of bacterial and viral nucleic acids (Baccala *et al.*, 2007). Different TLRs induce IFN production by recognising specific ligands – TLR3 recognises dsRNA and a synthetic analog of viral dsRNA -

polyinosinic-polycytidylic acid (polyI:C). TLR7 and 8 detect ssRNA, and TLR9 for unmethylated CpG-containing DNA (Baccala *et al.*, 2007). The CpG motif refers to DNA containing cytosine (C) and a guanosine (G) linked by a phosphodiester bond (p).

TLR3-dependent signalling pathway

TLR3 is an important factor in antiviral defence. Evidence has shown that induction of IFN is reduced in TLR3-deficient mice infected with murine cytomegalovirus (MCMV) (Tabeta *et al.*, 2004). The broad tissue distribution of TLR3 enables the detection of viral nucleic acid presented to the cell surface in fibroblasts, or produced by uncoating or degradation of the internalised viral particles on endosomes (Matsumoto *et al.*, 2003; Johnsen *et al.*, 2006) (Figure 1.4.2.1.A). Upon ligand binding, TLR3 is tyrosine phosphorylated (Sarkar *et al.*, 2004). TLR3 then recruits Toll-interleukin (IL)-1-resistance (TIR)-domain-containing adaptor protein inducing IFN-β (TRIF), which acts as a scaffold to recruit kinases required for either the IRF-3 or the NF-κB pathway.

On the IRF-3 side of IFN induction (Figure 1.4.2.1.A), TRIF activates tumour necrosis factor (TNF) receptor-associated factor 3 (TRAF3); TRAF3 then binds to TRAF family member-associated NF-κB activator (TANK); TANK interacts with TANK-binding kinase 1 (TBK-1)/inhibitor of NF-κB (IκB) kinase (IKK)ε complex. Studies have shown that the NF-κB-activating kinase (NAK)-associated protein 1 (NAP1) is a subunit of TBK-1/IKKε, associated with TRIF. NAP1 interacts with TBK-1/IKKε to assist the activation of IRF-3 (Sasai *et al.*, 2005) for IFN-β gene expression. NAP1, as well as TANK and another protein (less well understood) - similar to NAP1 TBK1 adaptor (SINTBAD) all participate in the activation of IRF-3 (Sasai *et al.*, 2005; Guo & Cheng, 2007; Ryzhakov & Randow, 2007). However, the mechanisms remain unclear. The IRF-3 carboxyl-domain is activated *via* phosphorylation by TBK-1/IKKε upon viral infection, and forms IRF-3 dimers, resulting in exposure of a nuclear-localisation signal (NLS) (Lin *et al.*, 1998, Dragan

et al., 2007; Panne *et al.*, 2007). IRF-3 translocates into the nucleus, and induces IFN-β gene transcription with the cooperation of CBP/p300 (Akira & Takeda, 2004), NF-κB and ATF-2/c-Jun. IRF-3 is utilised to directly induce the activation of primary IFN genes (IFN-β and IFN-α4), which positively feedback onto cells and induce the production of IRF-7, under continuous infection. Production of IRF-7 promotes the enhanced activation of primary IFN genes IFN-β and IFN-α4 at the transcription level, and the transcription of secondary IFN genes (IFN-α genes) (Marie *at al.*, 1998; Sato *et al.*, 1998; Prakash *et al.*, 2005). IFN-β is first produced upon infection, as a primary response, whereas, IFN-α is released in the amplification stage of an IFN response (Marie *et al.*, 1998). However, IFN-α gene induction is less well understood. As a consequence of positive feedback of IFN-β gene activation, IRF-7 is activated by the TBK-1/IKKε complex as in the IRF-3 pathway (TLR3-dependent). IRF-7 translocates into the nucleus upon activation, and binds to the IFN-β promoter site along with other transcription factors (NF-κB, ATF-2/c-Jun) (Chapter 1.4.1) to activate the IFN-β gene transcription cooperatively.

The NF-κB side of the TLR3-dependent pathway (Figure 1.4.2.1.A), through TRIF, essentially involves the activation of TRAF6 and receptor-interacting protein 1 (RIP1). TRAF6 and RIP1 activation gives rise to polyubiquitination of both factors (Chen, 2005). TRAF6 and RIP1, by complexing with transforming growth factor β-activated kinase (TAK1), TAK1-binding protein (TAB)2, and TAB3, activate IKKs. NF-κB, when associated with the NF-κB inhibitor - IκB, is inactive in the cytoplasm. Activated IKKs phosphorylate IκB, allowing NF-κB to be released and transported into the nucleus. The translocated NF-κB binds to the IFN-β promoter at a different site from IRF-3 and induces the IFN-β gene transcription.

TLR7- and TLR9-dependent signalling pathways

TLR7 and TLR9, which recognise various nucleic acid ligands produced by viruses are also key receptors participating in innate immunity to viruses. TLR7 and TLR9 are indispensable in certain cell types, which do not express TLR3. A subset of DCs,

known as pDCs, produce a significant amount of IFN, in response to some viral infections (ssRNA viruses), but they do not express TLR3. pDCs are one of the few cell types that express TLR7 and TLR9. TLR7 and TLR9 share the same myeloid differentiation factor 88 (MyD88)-dependent pathway of IFN induction. MyD88 plays an important role as a scaffold to mediate IRF-7 and NF-κB activation, in response to ssRNA recognition by TLR-7, and CpG DNA recognition by TLR9 (Kawai & Akira, 2006). The MyD88-dependent pathway (Figure 1.4.2.1.B) involves the activation of IL-1-R associated kinases (IRAKs), and TRAF6. Once activated, IRAK1 and TRAF6, in turn, through complexing with TAK1, TAB2, and TAB3, activate IKKs, which leads to the activation of NF-κB to induce IFN.

pDCs also express a high level of IRF-7 (Kerkmann *et al.*, 2003; Prakash *et al.*, 2005; Haller *et al.*, 2006), providing another IFN induction pathway. In the IRF-7 pathway, MyD88 recruits IRAK1 and IRAK4, TRAF6 and RIP1. The MyD88-IRAK1-IRAK4-TRAF6 complex binds IRF-7 (Honda *et al.*, 2004; Kawai *et al.*, 2004; Uematsu *et al.*, 2005). TRAF6 recruits IRF-7 through IRF-7 polyubiquitination (Kawai *et al.*, 2004) with IRF-7 interacting with polyubiquitinated RIP1 (Huye *et al.*, 2007). IRF-7 is then phosphorylated by IRAK1 and translocates to the nucleus, still associating with MyD88, TRAF6 and IRAK1, where it binds to the IFN-β promoter region to activate gene expression. However, this model is different from the positive feedback model of IRF-7 activation under IRF-3 iuduction (Chapter 1.4.2.1 TLR3-dependent signalling pathway). IRF-7 is further upregulated in response to primary IFNs production, mediated by IRF-3 through the TLR-3-dependent pathway. In myeloid-derived dendritic cells (mDCs), instead of IRF-7, IRF-1 is utilised to assist the activation of IFN-β in response to TLR-9 ligand CpG (Negishi *et al.*, 2006, Schmitz *et al.*, 2007) through MyD88 adaptor and IRAKs.

1.4.2.2 RIG-I & mda-5 pathway (TLR-independent pathway)

Receptor-mediated detection of pathogen-derived nucleic acids requires a broad range of receptors to participate in this event. TLRs are mainly involved in nucleic acids

recognition in the endosomal compartments. However, in contrast to TLRs, RIG-I and/or mda-5 RNA helicases act as crucial sensors which detect viral dsRNA in cytoplasm in a replication-dependent manner. RIG-I and mda-5 contain a C-terminal DExD/H-box RNA helicase domain, which binds dsRNA through its ATPase activity, and two N-terminal caspase recruitment domain (CARD) domains (Gitlin *et al.*, 2006; Childs *et al.*, 2007). The N-terminal CARD domains, mediate protein-protein interaction upon dsRNA binding to the helicase domain, causing domain conformational changes (Yoneyama *et al.*, 2004). RIG-I and mda-5 can both recognise RNA viruses and polyI:C in cytosol, and their activities are not redundant. Studies of RIG-I and mda-5 in response to polyI:C show that *in vivo* type I IFN induction can be impaired in mda-5 deficient mice, but rapid induction of type I IFN has been detected in wild-type and RIG-I deficient mice (Kato *et al.*, 2006). In contrast, dsRNA transcribed *in vitro* does not induce significant amounts of IFN-β in RIG-I deficient mouse embryonic fibroblasts (MEFs), but in mda-5 knocked-out MEFs (Kato *et al.*, 2006). RIG-I can also recognise RNA with 5'-triphosphate. During the replication events of many viruses, RNA synthesis generates transient cytosolic viral uncapped unmodified 5'-triphosphate RNA intermediates, which can be detected by RIG-I (Hornung *et al.*, 2006). The C-terminal regulatory domain of RIG-I binds viral 5'-triphosphate RNA, dimerises RIG-I and activates RIG-I ATPase (Cui *et al.*, 2008). RIG-I and mda-5 also play different roles in the recognition of viruses. Studies have shown that, mda-5 is the dominant sensor for picornaviruses (Gitlin *et al.*, 2006; Kato *et al.*, 2006) such as encephalomyocarditis virus (EMCV) (Kato *et al.*, 2008), and also for MeV (Berghall *et al.*, 2006). RIG-I recognises other RNA viruses, such as paramyxovirus and rhabdovirus families (Kato *et al.*, 2008), and Influenza A Virus (FLUAV) (Kato *et al.*, 2006). It is well accepted that the cysteine-rich C-terminus of the paramyxovirus PIV5-V protein targets mda-5, but not RIG-I, neutralising host defence (Andrejeva *et al.*, 2004). This may explain why RIG-I is the primary route for inducing IFN response to the majority of negative stand RNA viruses, including paramyxovirus. Enhanced type I IFN production has been detected in response to NDV, and vesicular stomatitis virus (VSV) following RIG-I overexpression (Gitlin *et*

al., 2006). Some viruses, for example, Dengue virus and reovirus are recognised by both RIG-I and mda-5 (Loo *et al.*, 2008; Kato *et al.*, 2008).

Cytoplasmic viral RNA, generated by viral replication, activates the RIG-I/mda-5 pathway (Figure 1.4.2.1.C) by binding to RIG-I/mda-5 C-terminal RNA helicase domains. Both RIG-I and mda-5 N-terminal CARD domains recruit the cellular protein – IFN-β promoter stimulator (IPS)-1, also known as mitochondrial antiviral signalling protein (MAVS), CARD adaptor inducing IFN-β (CARDIF), or virus-induced signalling adaptor (VISA) (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). IPS-1 is found in the outer mitochondrial membrane (Gitlin *et al.*, 2006), and regulates the activation of IRF-3, IRF-7, NF-κB and ATF-2/c-Jun pathways, which finally leads to the expression of IFN-α/β genes (Figure 1.4.2.1.C). The N-terminus of IPS-1, containing a CARD-like domain, interacts with the CARD domain of RIG-I/mda-5 (Kawai *et al.*, 2005), and activates IRF-3 and IRF7 through TBK1/IKKε. TBK-1/IKKε in turn phosphorylates the transcription factor IRF-3 and IRF-7. The IFN induction cascades through TLR3 and RIG-I/mda-5 share the TBK1/IKKε pathway. Phosphorylated IRF3 and IRF-7 then translocate into the nucleus as homodimers, where they bind to the IFN-α/β promoter region to initiate type I IFN gene transcription with the assistance of other transcription factors. In parallel, downstream of RIG-I/mda-5, IPS-1 triggers the NF-κB pathway *via* the adaptor Fas-associated death domain (FADD) and the kinase RIP1, which in turn, activates IKKs (IKKαβγ) (Kawai *et al.*, 2005). Like NF-κB activation through the TLR-dependent pathway, NF-κB becomes active through the degradation of IκB and translocates to the nucleus for IFN-β and inflammatory cytokine genes activation. Production of IFN-α/β upregulates RIG-I/mda-5 (Kato *et al.*, 2006), which can lead to propagation of the IFN signal in the continued presence of viral nucleic acid.

1.4.3 IFN signalling

The classical IFN signalling pathway has been extensively studied. The signalling

pathway is activated by the binding of IFN-α/β to type I IFN receptors. This leads to the activation of the receptor-associated Janus/just another kinase (JAK) family and the signal transducers and activators of transcription (STATs) pathway, which plays a important role for the induction of ISGs.

The biological activity of IFN-α/β is initiated by binding to cell surface heterodimeric IFN receptors - IFNAR1 and IFNAR2 (Figure 1.4.3.1) at the cell surface. In the absence of IFN, there is no close association between IFNAR1 and IFNAR2. Prior to ligand binding, the intracellular domains of IFNAR1 and IFNAR2 are also physically associated with the cytoplasmic JAKs - cytoplasmic signalling tyrosine kinase 2 (Tyk2) (associated to IFNAR1) and JAK1 (associated to IFNAR2) (Figure 1.4.3.1). STAT2 is associated with IFNAR2 prior to receptor stimulation, and weakly interacts with STAT1 (Stancato *et al.*, 1996; Precious *et al.*, 2005a; Tang *et al.*, 2007). IFN-α/β binding to IFNAR2 results in the association of IFNAR2 and IFNAR1. Tyk2 then phosphorylates IFNAR1 (Tyr 466), thus providing a docking site for STAT2. The STAT2 molecule itself then becomes tyrosine-phosphorylated (Tyr 690), creating a binding site for STAT1. JAK1 tyrosine-phosphorylates STAT1 (Tyr 701), and the phosphorylation allows STAT1 and STAT2 to form a stable heterodimer. Prior to phosphorylation, STAT2 is primarily localised in the cytoplasm. Phosphorylation of STAT2 causes STAT2 nuclear import (Frahm *et al.*, 2006) by creating a novel NLS, so that STAT1/2 dimers are imported into and retained in the nucleus, until their dephosphorylation (Reich & Liu, 2006). The STAT1/2 heterodimer recruits IRF-9, which contains a helix-turn-helix domain at N-terminus for DNA binding (Tang *et al.,* 2007). STAT1/2 and IRF-9 form the IFN-α/β stimulated gene factor 3 (ISGF3) heterotrimer. ISGF3 binds to the IFN-stimulated Response Element (ISRE) site, which locates in the promoter region of most IFN-inducible genes, to induce ISG transcription.

Type III IFN signalling is similar to that of type I IFN and uses IL-10R2 and IFN-λ receptor 1 (IFNLR1) as receptors. Upon binding of type III IFN to the receptors, Tyk2

associated IL-10R2 and JAK1 associated IFNLR1 activate STAT1 and STAT2 by phosphorylation using a similar strategy as described in the type I IFN signalling pathway (Figure 1.4.3.1).

IFN-γ induced type II IFN signalling, triggers STAT activation in a different manner. In this case, only STAT1 protein is involved in this process (Figure 1.4.3.1), by forming a homodimer rather than the heterodimer in the IFN-α/β pathway. IFN-γ receptors IFNGR1 and IFNGR2, associate with each other weakly in an inactive state. The IFN-γ binding to IFNGR1 and IFNGR2, activates the signalling pathway, and causes IFNGR1 and IFNGR2 to dimerise. Stimulation of receptors brings JAK1 (associated with the cytoplasmic domain of IFNGR1) and JAK2 (associated with the cytoplamic domain of IFNGR2), to close proximity, and activates JAK2, which in turn activates JAK1 by trans-phosphorylation. The activated JAKs phosphorylate the IFNGR1 cytoplasmic domain, and provide a pair of binding sites for STAT1. Two STAT1s form a homodimer *via* their Src-homology-2 (SH2) domains (a protein motif mediates/assists the signalling transduction by the interaction with tyrosine-phosphorylated sequences), and are phosphorylated (Tyr 701), which allows for two STAT1 molecules to dissociate from the receptor and migrate to the nucleus. In the IFN-γ pathway, STAT1/1 homodimer binds to the gamma-activation sequence (GAS) element in the nucleus to activate transcription, without the assistance of IRF-9 (Tang *et al.*, 2007).

IFN-induced gene transcription is activated in cooperation with some of the other co-activators. STAT complexes interact with these factors to assist the activation of genes. For example, p300 and CBP (Zhang *et al.*, 1996; Bhattacharya *et al.*, 1996; Platanias, 2005), increase IFN-α or IFN-γ-inducible gene transcription through regulating chromatin remodelling. Tang *et al.* 2007 has revealed that the IFN receptors assist the assembly of the transcription machinery. The transcriptional cofactor CBP is recruited to the IFNAR chain of the receptor in response to IFN-α, which is thought to directly or indirectly regulate acetylation of the factors involved in

the signalling cascade for gene transcription. CBP catalyses IFNAR2 acetylation on lysine (Lys) 399 and STAT2 on Lys 390; IFNAR2 acetylation provides a docking site for IRF-9; IRF-9, STAT1 and STAT2 molecules are also acetylated by CBP. Acetylation of IRF-9 is indispensable for DNA binding, and acetylation of the STAT factors may assist ISGF3 complex assembly.

1.4.4 IFN-induced antiviral state

The binding of type I IFN activates the JAK-STAT signalling cascade, which in turn, switches on more than 400 ISGs. Upregulation of such ISGs establishes an antiviral state (Sadler & Williams, 2008). Not all ISGs are activated by IFN signalling through the JAK-STAT pathway. A portion of ISGs are induced in response to enveloped viruses by IRF-3, but independent of IFNs (Guo *et al.*, 2000).

Important ISGs such as protein kinase R (PKR), the 2',5'-oligoadenylate synthetase (OAS), RNase L, and the myxovirus resistance protein (Mx) guanosine triphosphate (GTP) GTPases have all been intensively investigated, since they play important roles in anti-viral activities.

PKR

PKR is a serine-threonine IFN inducible protein kinase, which is activated by dsRNA or the cellular PKR activating protein (PACT). PKR is constitutively expressed at low levels in the cell, and is induced by type I and type III IFNs. Under normal conditions, PKR stays inactive as a monomer in cells. PKR is activated by binding of ligands, including viral dsRNAs, to dsRNA-binding domain at the N-terminal of PKR, and undergoes a conformational change, forms a homodimer, and induces autophosphorylation to become an active enzyme (Cole, 2007). Activated PKR phosphorylates the α subunit of protein synthesis eukaryotic initiation factor 2 (eIF2α) at serine (Ser) 51 (Samuel, 1979; Pathak *et al.*, 1988), and inhibits protein synthesis at the translation level. eIF2 is an essential factor for protein synthesis, and the substrate which is well characterised for PKR antiviral effect. In addition to its antiviral

activities, PKR also mediates apoptosis and cell growth control (Der *et al.*, 1997; Lee & Estaban, 1994; Chong *et al.*, 1992).

OAS and RNase L

OAS is present in the cytoplasm in its inactive monomeric form. OAS is upregulated by type I IFN signalling and is activated in response to dsRNA. OAS oligomersises ATP resulting in the production 2',5'-oligodenylates, which in turn activates RNase L. RNase L is expressed as an inactive monomer, and activated through 2',5'-oligomer binding to form a homodimer. Activated RNase L targets viral genomic ssRNA to prevent replication (Silverman, 2007). Activated RNase L cleaves viral mRNA (Austin *et al.*, 2005) to inhibit viral protein synthesis and the cleavage of cellular mRNA and rRNA to shut off host protein synthesis (Silverman, 2007).

Mx

Mx proteins are GTPases, which are induced by IFN, have antiviral activities. The Mx GTPase family consists of human MxA, MxB proteins, and mouse Mx1 and Mx2 proteins. Human MxA protein is associated with antiviral activity induced by IFN-α/β, but not IFN-γ (Simon *et al*., 1991; Samuel, 2001), against both cytoplasmic and nuclear viruses, such as orthomyxoviruses, paramyxoviruses, rhabdovirues, togaviruses and bunyaviruses (Sadler & Williams, 2008). The Mx protein contains an N-terminal GTPase domain, a central interacting domain (CID) and a C-terminal leucine zipper (LZ) domain. Both CID and LZ are essential for the recognition of the viral nucleocapsid-like structure, formed close to the smooth endoplasmic reticulum (ER) (Sadler & Williams, 2008). During viral infection, Mx proteins inhibit viral replication by preventing transport and localisation of the nucleocapsid-like viral proteins, and thus restrict the amplification of viruses in the early stages of infection (Kochs & Haller, 1999, Haller *et al.*, 2009). The Mx GTPases prevent the growth of FLUAV, such as H5N1 viruses or the pandemic H1N1 virus strain of 1918 (Haller *et al.*, 2009).

ISG15

ISG15 is one of the most prominent IFN-α/β-inducible genes (Sadler & Williams, 2008). ISG15 is a 15kDa protein recognised as a homologue of ubiquitin (Ub), and functions in parallel with Ub. ISG15 belongs to a subset of ISGs that are transcriptionally induced in parallel with IFN-β as a primary response to viral infections stimulated directly *via* the IRF family, and/or as a secondary cytokine response through the IFN signalling pathway. In contrast to type I IFNs, IFNγ is a poor inducer of ISG15. Like Ub, ISG15 utilises similar strategies to modulate post-translational modification of cellular proteins, termed ISGylation. ISGylation requires an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase enzyme (Sadler & Williams, 2008). ISGylation and ISG15 appear to play a broad spectrum role in inhibition of viral amplification. ISGylation targets substrate proteins which are important component in the type I IFN signalling pathway, including RIG-I, JAK1, STAT1, MxA, PKR, and RNase L (Zhao *et al.*, 2005), and the enzymes involved in the ISGylation process regulated by IFNs. Unlike ubiquitination, ISG15 does not promote substrate degradation (Liu *et al.*, 2003), but instead activates target proteins (Okumura *et al.*, 2006). Evidence has shown that ISG15 enhances IFN-β production by preventing virus-mediated IRF-3 degradation (Lu *et al.*, 2006). Mice deficient in ISG15 are hypersensitive to infection by viruses, such as FLUAV, influenza B viruses (FLUBV), Sindbis virus (SINV) and herpes simplex virus 1 (HSV-1) (Lenschow *et al.*, 2007; Harty *et al.*, 2009). Mice deficient in Ubp43, the protease required to remove ISG15 from the ISGylation state, are reported to be more sensitive to IFN and polyI:C (Malakhova *et al.*, 2003), and are also resistant to lymphocytic choriomeningitis virus (LCMV) and VSV (Ritchie *et al.*, 2004). The above evidence suggests that ISG15-mediated protein modulation plays a role in generating an efficient IFN response.

ISG56

As another prominent member of the ISG family, ISG56 responds strongly to viral infections. In untreated cells, p56 (the protein product of ISG56) is expressed at low

level. However, expression of ISG56 is greatly increased by virus infection, IFN, and dsRNA (Der *et al.*, 1998; Terenzi *et al.*, 2008). Evidence has shown that ISG56 carries out its antiviral activity by interacting with the DNA replication origin-binding protein E1 of human papillomavirus (HPV), and therefore inhibits the DNA helicase activity of E1 and E1 mediated HPV DNA replication (Terenzi *et al.*, 2008).

1.5 Virus inhibition of the IFN response

The IFN response is an incredibly powerful innate immune mechanism, establishing antiviral defences at different stages to prevent and/or limit viral amplification within the host. In order to survive and establish infection in the host, viruses have developed strategies to overcome the IFN response. The IFN response against viruses and the virus inhibition of IFN response act in such ways as to have a negative effect on each other. Many (if not all) viruses express IFN antagonists which may counteract the IFN response at multiple levels. IFN antagonists are usually non-structural viral proteins which are non-essential for virus amplification. These IFN antagonists are often multifunctional proteins which interact with multiple components to inhibit host IFN induction and signalling. To allow more efficient inhibition of IFN response, a virus may interfere with different pathways by expressing more than one antagonist protein, and a single viral protein may be able to target different stages of the IFN response (Figure 1.5.1). Although different viruses have different strategies, they do share some strategies in common, as described below.

1.5.1 IFN antagonism

Although many (if not all) viruses encode factors that antagonise IFN response, there are five common strategies that allow viruses to inhibit IFN functioning. Many viruses (i) inhibit IFN induction; (ii) inhibit IFN signalling pathway; (ii) inhibit the IFN-induced antiviral state; (iv) globally inhibit the transcription or translation for preventing host cell ISGs expression; (v) adopt a replication strategy insensitive to IFN action.

1.5.2 Inhibition of IFN induction

Viruses adopt a number of ways to interfere with host cell IFN production (Figure 1.5.1.A). Many viruses limit the production of PAMPs or prevent their recognition by the IFN induction machinery. Many viruses produce dsRNA binding proteins to prevent dsRNA binding to viral nucleic acid sensors, such as PRRs. The bovine viral diarrhoea virus (BVDV) Erns protein is secreted to the outside of the cell, and degrades dsRNA, thus preventing TLR3 recognition of dsRNA (Iqbal *et al.*, 2004). The N-terminus of NS1 protein of FLUAV sequesters dsRNA through an RNA binding domain, thus preventing the activation of IFN production at an early stage (ligand recognition and binding) (Wang *et al.*, 2002; Ferko *et al.*, 2004).

The TLR and RIG-I/mda-5 signalling cascade is also a potential target for some viruses. The NS3/4A protease of HCV cleaves the essential TLR3 signalling mediator TRIF at cysteine (Cys) 372 *in vitro*, accelerates TRIF degradation *in vivo*, and consequently disrupts TLR3 signalling (Li *et al.*, 2005a). The V proteins of paramyxoviruses bind mda-5, and block the subsequent activation of the IFN-β promoter (Andrejeva *et al.*, 2004). The FLUAV NS1 protein interacts with RIG-I and subsequently inhibits IRF-3 translocation into the nucleus induced by RIG-I and RIG-I mediated IFN-β promoter activation (Mibayashi *et al.*, 2007).

Further downstream of the IFN induction pathway, intracellular proteins which mediate either the TLR-dependent pathway or the RIG-I/mda-5 pathway are all possible intervention sites that IFN antagonists target. As an example of an IFN antagonist which interferes with the IFN pathway at various stages, the HCV NS3/4A protein cleaves the mitochondrial anchor site of IPS-1 at its C-terminus, and as a consequence, the signalling pathway between IPS-I and TBK1/IKKε is abolished (Li *et al.*, 2005b; Meylan *et al.*, 2005; Lin *et al.*, 2006a). The V proteins of certain paramyxoviruses: PIV5, HPIV2, and MuV act as alternative substrates for TBK1/IKKε, therefore preventing the downstream phosphorylational activation of IRF-3 (Lu *et al.*, 2008).

NF-κB is a crucial transcription factor to activate the IFN-β promoter gene, many viruses encode proteins to target NF-κB. The African swine fever virus (ASFV) A238L protein, as an I-κB homologue, inhibits NF-κB activation (Powell *et al.*, 1996; Revilla *et al.*, 1998; Tait *et al.*, 2000).

Viruses have also evolved abilities to interfere with IRF transcription factor family binding to IFN-β promoter region. For example, the HPV type 16 (HPV-16) E6 protein binds to IRF-3 (Ronco *et al.*, 1998; Johnson & Knipe 2009) and inhibits IRF-3 transcriptional activity (Ronco *et al.*, 1998). The N-terminal protease (Npro) of BVDV targets IRF-3 for degradation (Hilton *et al.*, 2006), therefore blocking IFN gene transcription.

1.5.3 Inhibition of IFN signalling

Viruses have developed strategies to bypass the IFN antiviral response by targeting the IFN signalling cascade at different stages (Figure 1.5.1.B).

Some viruses target IFN signalling prior to IFN binding to the receptors. For example, the vaccinia virus (VACV), and most other orthopoxviruses, express proteins that interfere with IFN-α/β binding to its receptors (Colamonici *et al.*, 1995; Symons *et al.*, 1995). The VACV protein B18R is secreted to the outside of the cells, and binds IFN, therefore preventing the antiviral response in both infected and neighbouring cells (Alcami *et al.*, 2000). Poxviruses secrete soluble proteins to sequester IFN-α and IFN-γ (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Upton *et al.*, 1992; Alcami & Smith, 1995; Mossman *et al.*, 1995a; Mossman *et al.*, 1995b), so that IFN binding to their receptors is prohibited and receptor activation is prevented. The K3 and K5 proteins of human herpesvirus 8 (HHV-8) target the IFNGR1 subunit of IFN-γ receptor for ubiquitination, endocytosis and degradation (Li *et al.*, 2007).

Given the fact that STATs play a critical role in the IFN signalling cascade, many viruses express viral antagonists that target STATs. STAT function is inhibited by

paramyxoviruses primarily through a single virus-encoded protein – V protein. Some paramyxoviruses target STATs for protesomal degradation. PIV5 is demonstrated to target both IFN-α/β and IFN-γ by targeting STAT1 for proteasome-mediated degradation *via* the activity of its V protein (Didcock *et al.*, 1999; Precious *et al.*, 2005b; Precious *et al.*, 2007). MuV and SV41 V proteins use similar strategies, targeting STAT1 for degradation. HPIV2 V protein targets STAT2 for degradation, therefore, blocking IFN-α/β signalling pathway. The paramyxovirus V protein-targeted STATs degradation is STAT-dependent. For example, PIV5 target STAT1 for degradation only if cells expressing STAT2, whereas HPIV2 fails to mediate STAT2 degradation in the absence of STAT1 (Parisien *et al.*, 2002; Andrejeva *et al.*, 2002; Stock *et al.*, 2005). STAT proteins are targeted by polyubiquitylation and efficient proteasomal degradation. Studies have also shown that sufficient amounts (*i.e.* high levels) of V protein have to be expressed by some paramyxoviruses, in order to generate prompt degradation of STAT protein. This is supported by infecting both naive and IFN-pretreated cells with UV-inactivated PIV5 (Didcock *et al.*, 1999; Stock *et al.*, 2005). Low level expression of V protein in UV-inactivated PIV5 would target STAT1 for degradation in naive cells, where relatively low levels of STAT1 are present. However, in the IFN-pretreated cell, where STAT1 is upregulated by IFN treatment, the amount of V protein expressed in UV-inactivated virus could not degrade STAT1 completely.

The V proteins of both Nipah virus (NiV) and Hendra virus (HeV) of the Henipavirus genus block both IFN-α/β and IFN-γ singalling by preventing the nuclear translocation of STAT1 and STAT2 (Rodriguez *et al.*, 2003; Stock *et al.*, 2005). The Henipavirus V proteins sequester STATs proteins into high molecular weight complexes independent of degradation. The NiV V protein behaves as a nuclear-cytoplasmic shuttle and alters the distribution of STAT1. The NiV V protein moves freely between cytoplasm and nucleus, targets STAT1, shuttles STAT1 back to the cytoplasm, resulting in cytoplasmic sequestration of STAT1 (Rodriguez *et al.*, 2002; Stock *et al.*, 2005).

Viruses also block IFN signalling through the downregulation of STAT phosphorylation. MeV V protein expression subverts IFN-α/β and IFN-γ signalling. The wild-type MeV V protein interacts with the IFNα/β receptor to block IFN signalling by preventing the phosphorylation of STAT1 and STAT2, whereas the MeV Edmonston vaccine strain (MeVEd) V and C proteins block both IFN-α/β and IFN-γ signalling downstream of STAT phosphorylation (Stock *et al.*, 2005). VACV dephosphorylates and deactivates STAT1 *via* the expression of a viral phosphatase, VH1 (Najarro *et al.*, 2001). HCV core protein inhibits STAT1 phosphorylation to prevent its association with STAT2, and therefore inhibit ISGF3 formation (Lin *et al.*, 2006b). HBV and HCV downregulate STAT1 transcriptional activity *via* upregulation of protein phosphatase 2A (PP2A) (Duong *et al.*, 2004; Christen *et al.*, 2007). As an enzyme, PP2A inhibits protein arginine methyltransferase 1 (PRMT1). PRMT1 is an enzyme that catalyses the methylation of STAT1. Hypomethylated STAT1 increases its association with its inhibitor - protein inhibitor of activated STAT1 (PIAS1), and becomes less active (Christen *et al.*, 2007). PIAS inhibits STAT DNA binding, and thus reduces the transcriptional activation of IFN-stimulated genes (Duong *et al.*, 2004; Duong *et al.*, 2005; Christen *et al.*, 2007).

In addition to V protein, viruses also express antagonists to target STATs. HSV-1 infected cell protein 27 (ICP27) inhibits IFN-induced STAT1 phosphorylation and nuclear accumulation (Johnson *et al.*, 2008; Johnson & Knipe, 2009). The inhibition of STAT1 phosphorylation is at or before JAK-1 activation (Johnson & Knipe, 2009).

Apart from STATs, IRF-9 is also targeted by several viruses, due to its role in the formation of ISGF3 transcription complexes. Adenovirus (AdV) E1A protein decreases IRF-9 expression to inhibit IFN-α signalling (Leonard & Sen, 1996). HPV-16 E7 prevents ISGF3 formation by direct targeting of IRF-9 (Barnard & McMillan, 1999).

1.5.4 Inhibition of IFN-induced antiviral enzymes & global protein synthesis

As discussed above, IFN induces the activation of a variety of antiviral proteins, such as PKR and OAS, in response to dsRNA stimulation. Some viruses, including poxvirus, HSV, influenza virus and reoviruses, express viral dsRNA-binding proteins to sequester dsRNA away from those antiviral proteins, to prevent the activation of such proteins.

PKR plays a pivotal antiproliferative role in the IFN response, as it is responsible for cell growth and proliferation in uninfected cells (Stark *et al.*, 1998). Viruses have introduced diverse mechanisms to inhibit the activation and production of PKR. FLUAV has two strategies to subvert PKR antiviral activity. PKR is regulated through the physical association with its cellular inhibitor $p58^{IPK}$. $p58^{IPK}$ activity is upregulated upon FLUAV infection (Lee *et al.*, 1990; Hale *et al.*, 2010), which results in the dissociation of $p58^{IPK}$ from its natural inhibitor - heat shock protein 40kD (HSP40) (Melville *et al.*, 1997; Melville *et al.*, 1999; Hale *et al.*, 2010). FLUAV NS1 protein also binds directly to the N-terminal 230-amino acid region of PKR (Li *et al.*, 2006) and at positions 123-127 of PKR to prevent PKR activation (Min *et al.*, 2007). AdV virus associated RNA I (VAI RNA) forms a complex with PKR to prevent PKR autophosphorylation and activation (Katze *et al.*, 1987). OAS/RNase L is targeted or inhibited by viral proteins in a dsRNA-independent manner. Human immunodeficiency virus (HIV-1) (Martinand *et al.*, 1999) and EMCV (Martinand *et al.*, 1998) upregulate the cellular RNase L inhibitor to downregulate the activity of RNase L.

In addition to targeting transcription factors, and the inhibition of antiviral enzyme activation, viruses also slow down or impair the host mRNA transcription and protein synthesis (Goodbourn *et al.*, 2000). Foot-and-mouth disease virus (FMDV) L proteinase switches off host protein synthesis. However, increased expression of IFNs is detected in cells infected with the attenuated FMDV strains with mutations in genes enconding L proteinase (Chinsangaram *et al.*, 1999; Goodbourn *et al.*, 2000). Viruses

are also able to mimic or generate host cell protein homologues to reduce the IFN production efficiency (Goodbourn *et al.*, 2000). For example, EBV produces a homologue of IL-10 to minimise the IFN response (Bejarano & Masucci, 1998). One of the biological functions of IL-10 is to inhibit the activation of TH1 cells, as activated TH1 cells produce a number of cytokines, including IFN-γ (Goodbourn *et al.*, 2000). Viruses also express antagonists to modulate host cell gene expression. FLUAV NS1 is one of the examples, which interacts with the two zinc-finger domains of the nuclear 30kDa subunit of cleavage and polyadenylation specificity factor (CPSF30). The binding prevents the CPSF30 modulated 3' ends processing of pre-mRNA into polyadenylated mRNAs (Nemeroff *et al.*, 1998; Hale *et al.*, 2010).

1.6 Aims

My project aims to develop stable cell-lines that can be used to rapidly isolate mutant viruses, which are better inducers of IFN, as live attenuated virus vaccine candidates. Cell-lines are used to screen the viruses by specifically responding to the invasion of viruses. Using wild-type viruses to infect the engineered cell-lines, cells infected by IFN-inducing mutant viruses would activate the IFN-β promoter and a downstream reporter gene under the control of the IFN-β promoter. Those mutant virus-infected cells will then be selected based on their distinctive characteristics, *i.e.* green fluorescent protein (GFP) expression or cell surface expression of V5 epitope tag. We can then isolate mutant viruses from the selected cells using fluorescence-activated cell sorting (FACS) (for GFP) or panning (of the V5 epitope). In essence, these specifically engineered cell-lines are working as a sorter to select mutant viruses. Ideally, this system would be developed as a universal sorter to isolate various types of live attenuated vaccine candidates.

The isolated viruses will be genetically analysed and functionally studied. Whole genome sequencing may be carried out to provide information on the location of mutations which ablate the viruses' ability to express a functional IFN antagonist. Studying the genetic defects will help us better understand the mechanisms these

viruses use to cause diseases in the host.

Finally, the generated stable cell-line (A549/pr(IFN-β).GFP) has been used routinely to screen for compounds that inhibit IFN induction and/or signaling or block the activity of viral IFN antagonists with a view to successful antiviral drug discovery.

Figure 1.2.1 Classification of the *Paramyxoviridae* family according to the Eighth Report of ICTV (2005).

Family ***Paramyxoviridae***

Subfamily ***Paramyxovirinae***

Genus ***Respirovirus***
- Sendai virus (SeV)
- Human parainfluenza virus types 1 & 3 (hPIV1/3)
- Bovine parainfluenza virus type 3 (bPIV3)

Genus ***Rubulavirus***
- Simian virus type 5 (SV5)
- Simian virus type 41 (SV41)
- Mapuera virus (MPRV)
- Mumps virus (MuV)
- Human parainfluenza virus type 2 (hPIV2)
- Human parainfluenza virus types 4a & 4b (hPIV4a/4b)

Genus ***Morbillivirus***
- Measles virus (MeV)
- Cetacean morbillivirus (CeMV)
- Canine distemper virus (CDV)
- Peste-des-petits-ruminants virus (PPRV)
- Phocine distemper virus (PDV)
- Rinderpest virus (RPV)

Genus ***Henipavirus***
- Hendra virus (HeV)
- Nipah virus (NiV)

Genus ***Avulavirus***
- Newcastle disease virus (NDV)
- Avian paramyxovirus 1 to 9 (APMV-1 to -9)

Genus ***"TPMV-like Viruses"***
- Tupaia virus (TPMV)

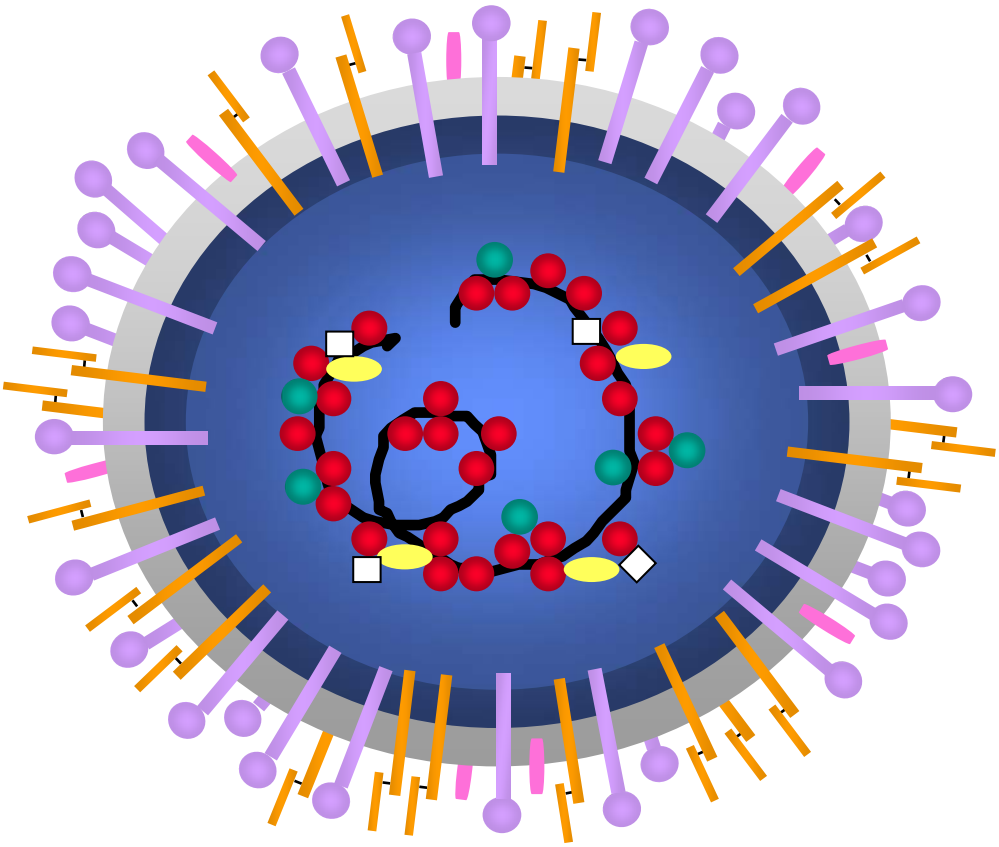
Subfamily ***Pneumovirinae***

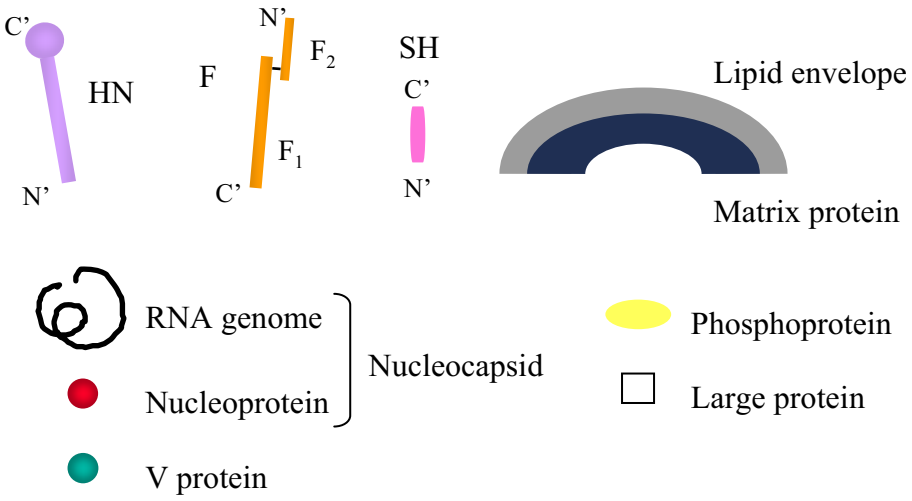
Genus ***Pneumovirus***
- Human respiratory syncytial virus (hRSV)
- Bovine respiratory syncytial virus (bRSV)
- Murine pneumonia virus (MPV)

Genus ***Metapneumovirus***
- Turkey rhinotracheitis virus (TRTV)

adapted from Carlos, 2005

Figure 1.2.2.1



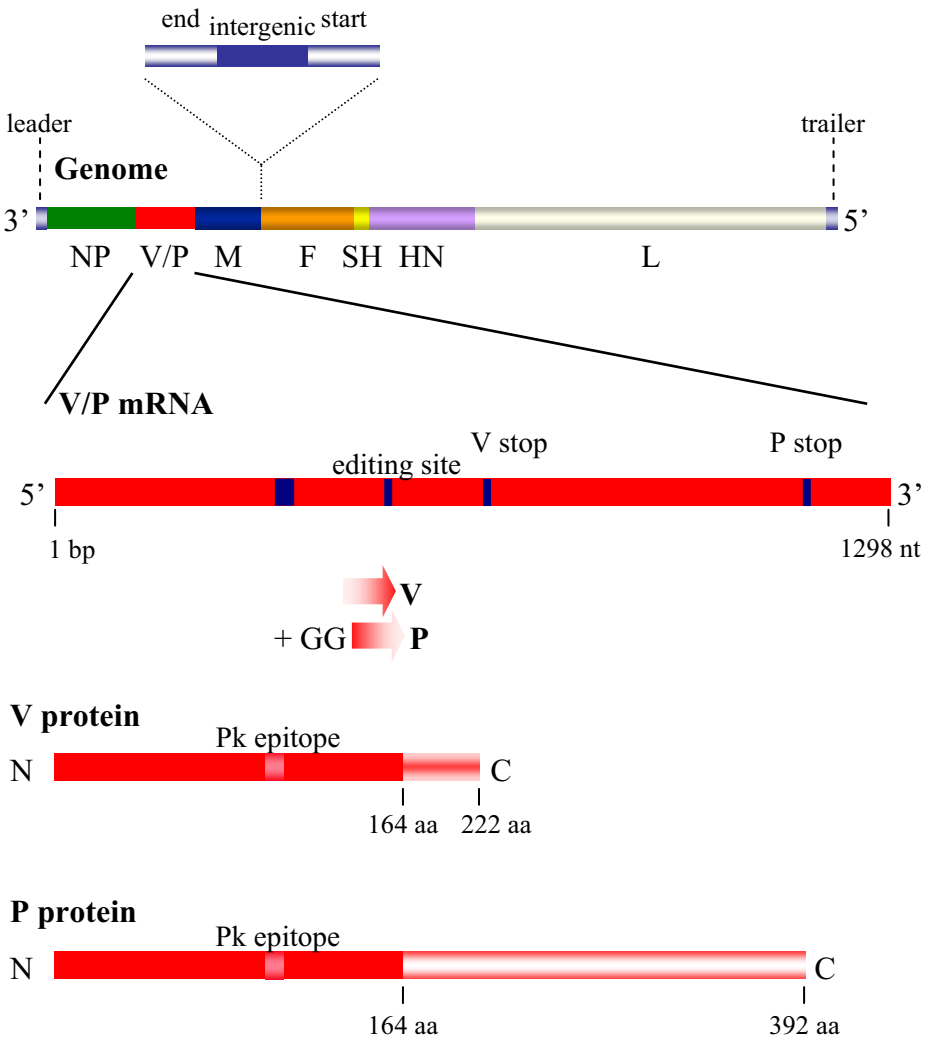


adapted from Carlos, 2005

Figure 1.2.2.1 Schematic diagram of a PIV5, a typical paramyxovirus (adapted from Carlos, 2005).

Virions are decorated with two surface glycoproteins, hemagglutinin-neuraminidase (HN) attachment glycoprotein and the (F) fusion glycoprotein into the lipid bilayer. Underlying the lipid bilayer is the viral matrix (M) protein. The nucleocapsid is composed of genomic RNA that interact with the nucleoprotein (N) and the components of the viral RNA polymerase complex (phosphoprotein P and large (L) protein) The small integral membrane protein, SH, is found only in certain rubulaviruses, including PIV5. The V protein is a structural protein, found in rubulaviruses.

Figure 1.2.3.1

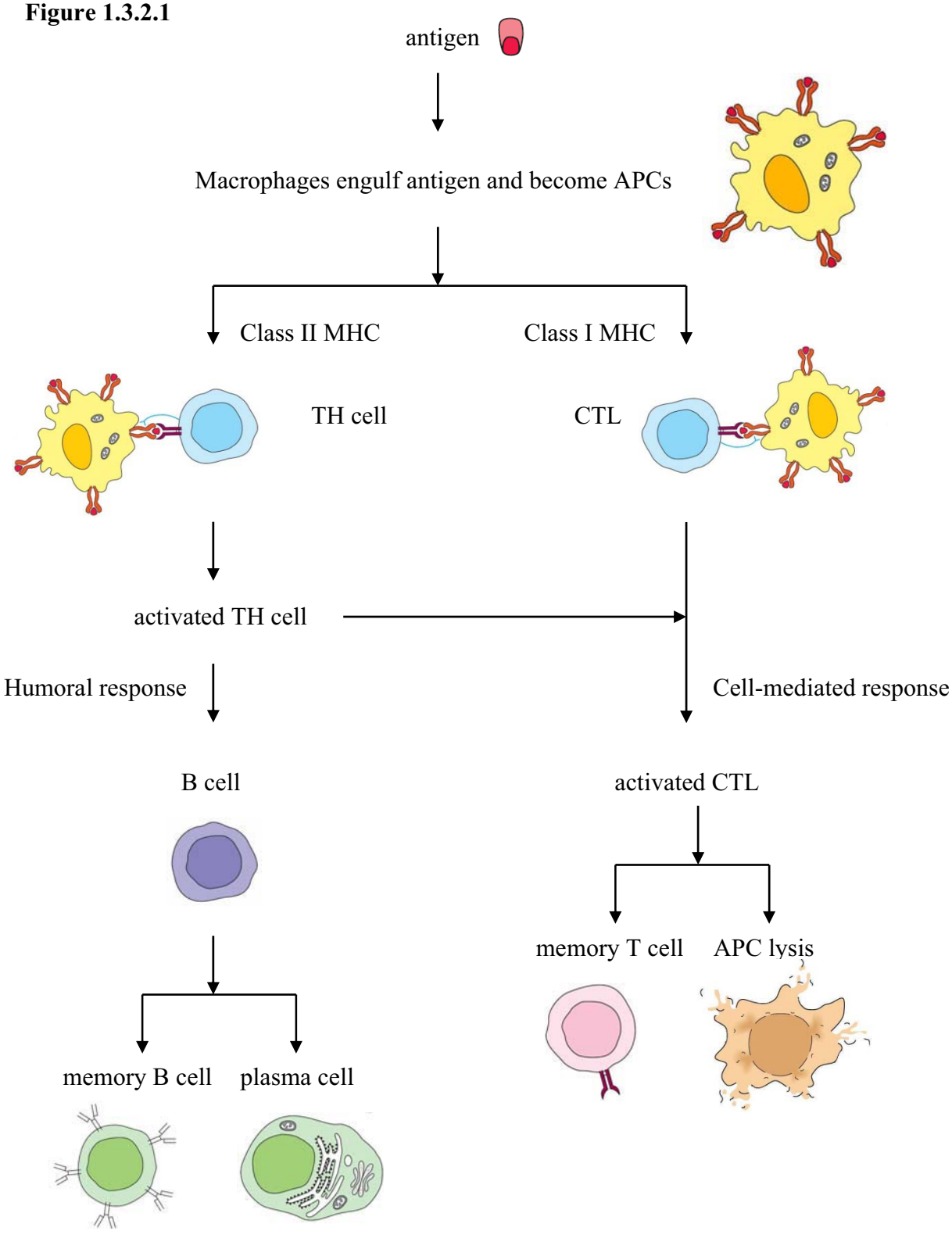


modified from Chatziandreou, 2002

Figure 1.2.3.1 Representation of the PIV5 genome, the structure of the V/P gene and encoded V and P proteins (modified from Charziandreou, 2002).

The PIV5 genome is single-stranded, non-segmented, negative sense RNA and contains seven genes that code for eight proteins (NP, P and V, M, F, SH, HN and L). Extragenic regions contain a 3'leader sequence and the 5' trailer sequence. Each gene contains transcription start/stop control sequences as part of the gene. The V/P gene codes for two proteins, the V and P proteins. The open reading frames of V and P gene overlap, V mRNA is transcribed as a complete copy, whereas the P mRNA is transcribed with an addition of two non-templated G residues. As a consequence, V and P proteins have the N-terminus in common. However, their C-termini are unique for each protein.

Figure 1.3.2.1

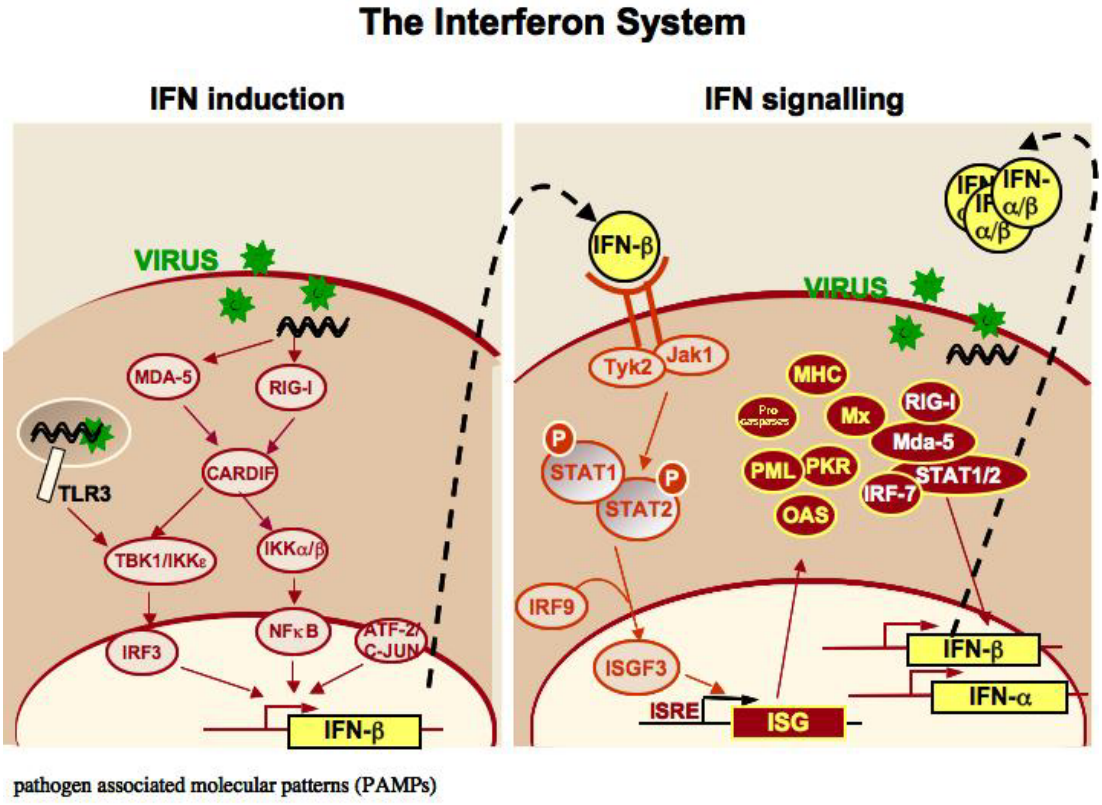


images modified from Kindt *et al.*, 2006

Figure 1.3.2.1 Flowchart for the overview of the humoral and cell-mediated immune response (images modified from Kindt *et al.*, 2006).

The MHC molecules bind antigens presented by APCs. TH cells recognise class II MHC molecules, whereas CTLs recognise antigen with class I MHC molecules. T cell is activated by the binding of MHC-antigen, and activated T cell secretes cytokines, which activates B cells, CTLs and other cells. B cells interacting with antigen differentiate into B memory cells and antibody-secreting plasma cells. Produced antibodies neutralise the antigens for clearance. Activated CTLs either develop into T memory cells, or lyse the APCs.

Figure 1.4.1



modified from Haller *et al.*, 2006

Figure 1.4.1 The IFN system (modified from Haller *et al.*, 2006).

The IFN system is an efficient system which responds to virus infections. Certain host pattern recognition receptors (PRRs) recognise dsRNA, and activate IFN genes through transcription factors IRF-3 and NF-κB. IFN induction upregulates the JAK-STAT pathway to activate IFN-stimulated genes (ISGs) to inhibit viral replication and cell proliferation.

Figure 1.4.1.1

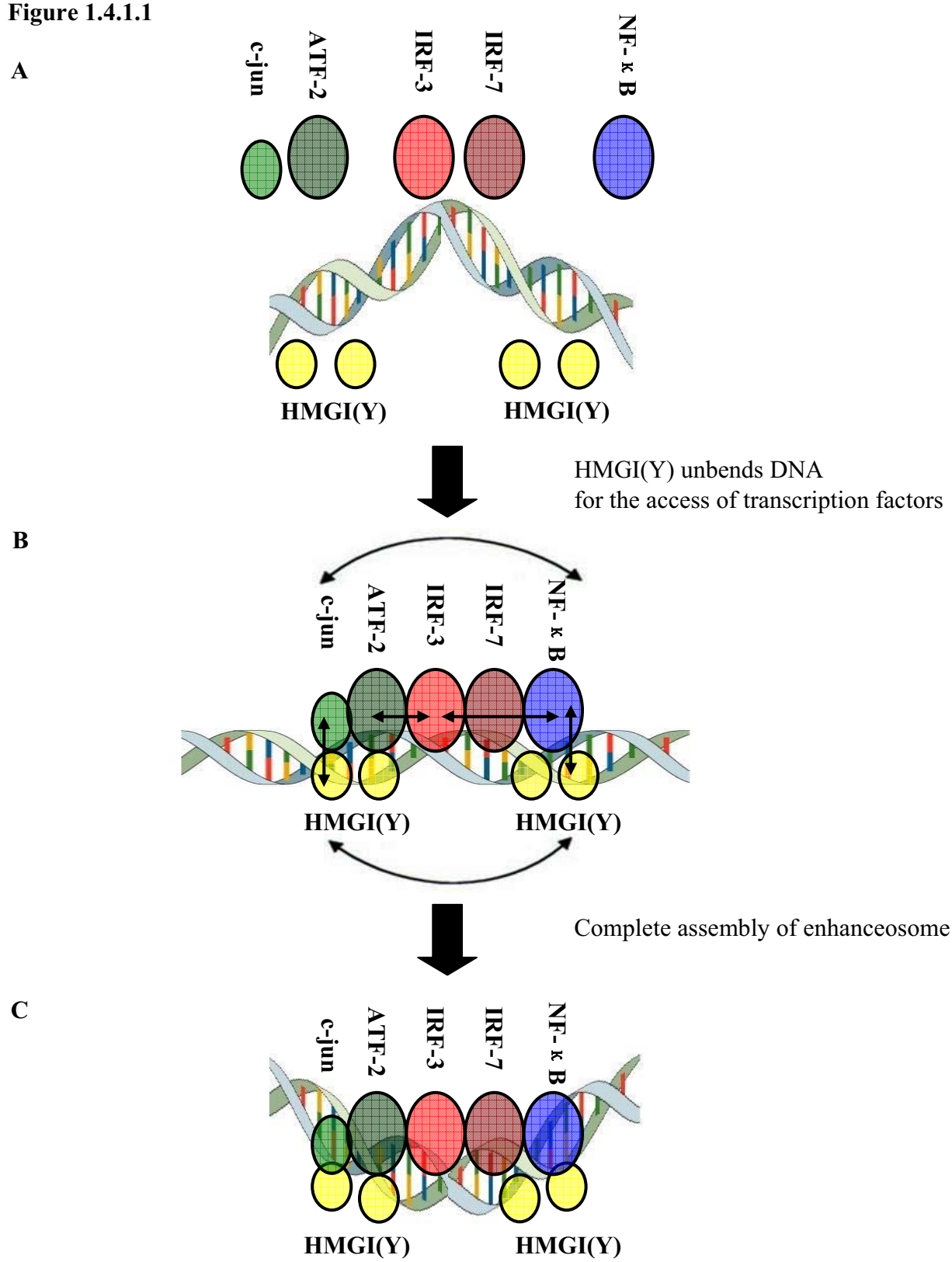
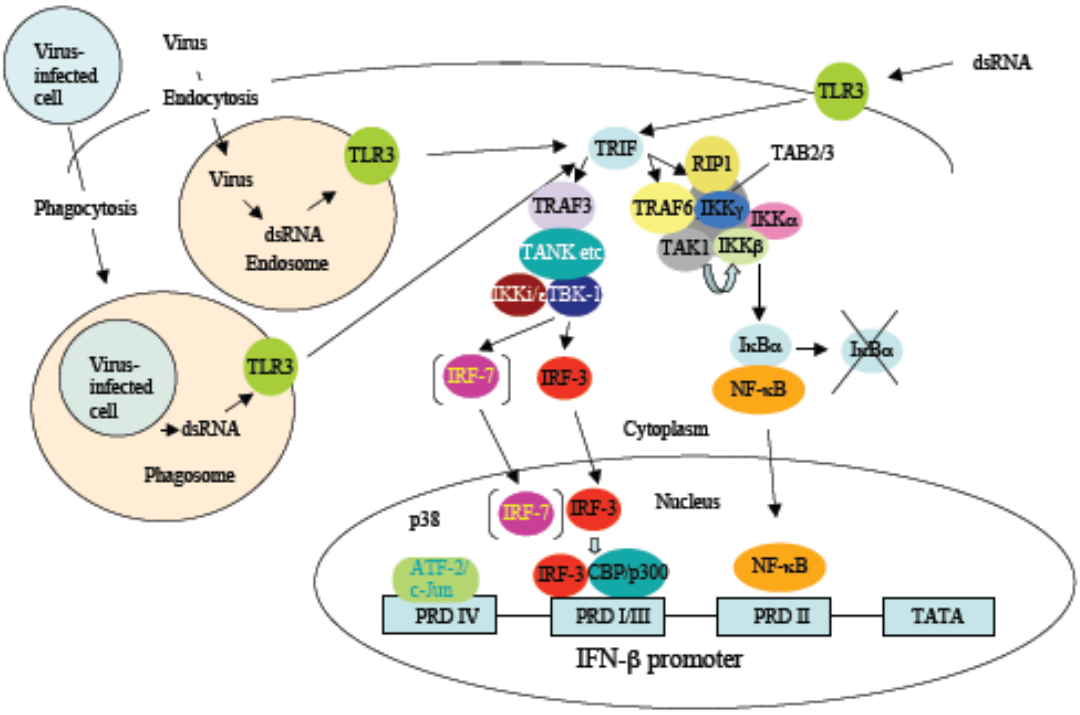

A
c-jun
ATF-2
IRF-3
IRF-7
NF- κ B
HMGI(Y)
HMGI(Y)
HMGI(Y) unbends DNA
for the access of transcription factors
B
c-jun
ATF-2
IRF-3
IRF-7
NF- κ B
HMGI(Y)
HMGI(Y)
Complete assembly of enhanceosome
C
c-jun
ATF-2
IRF-3
IRF-7
NF- κ B
HMGI(Y)
HMGI(Y)

Figure 1.4.1.1 Enhanceosome assembly for activation of the IFN-β promoter.

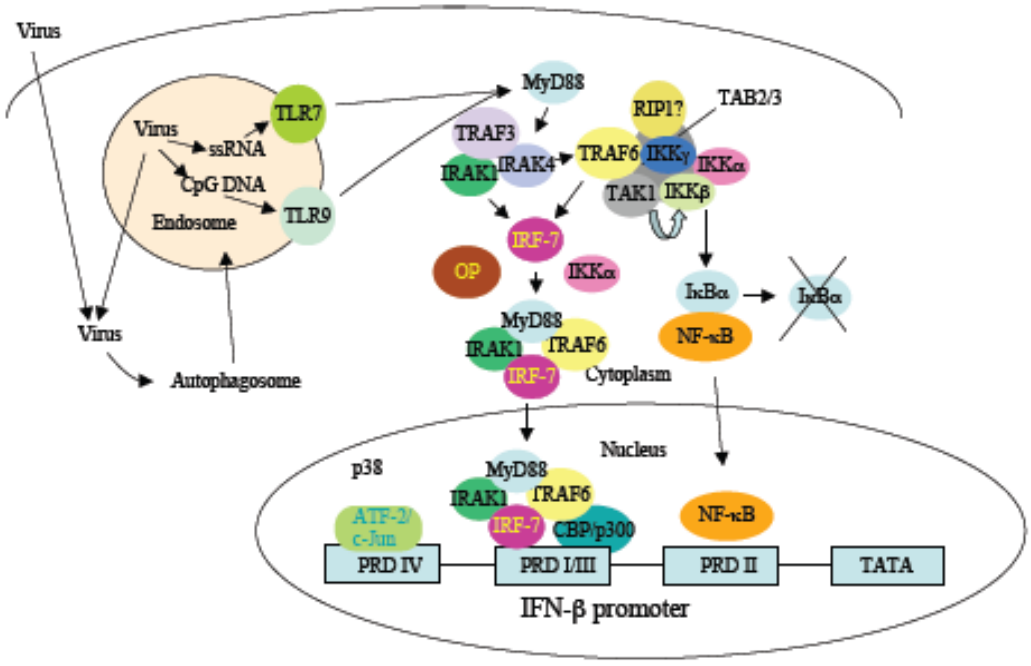
The intrinsic DNA structure (Panel A) of the promoter needs to be flattened, to allow binding of transcription factors (e.g. IRFs, NF-κB, ATF-2, c-Jun) to the promoter (Panel B). HMG I(Y) assists binding by unbending/straightening the DNA, which significantly enhances the transcription factors binding affinity to the enhanceosome. Protein–protein interactions (arrows) between all the components, allow the formation of a stable nucleoprotein structure (Panel C).

Figure 1.4.2.1

A



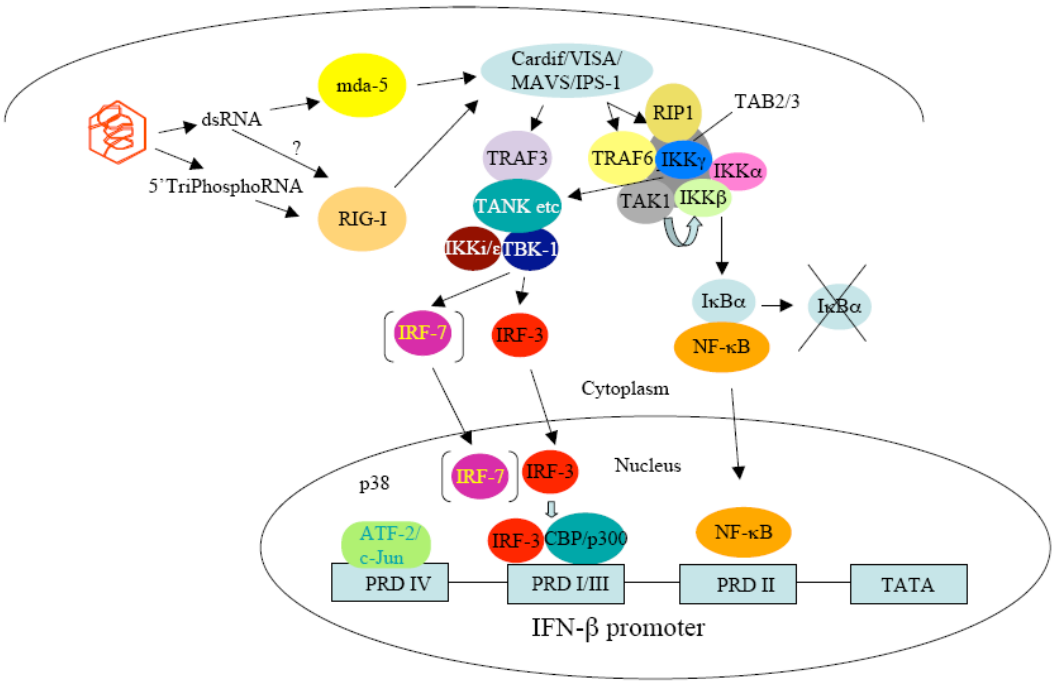
B



adapted from Randall & Goodbourn, 2008

Figure 1.4.2.1

C

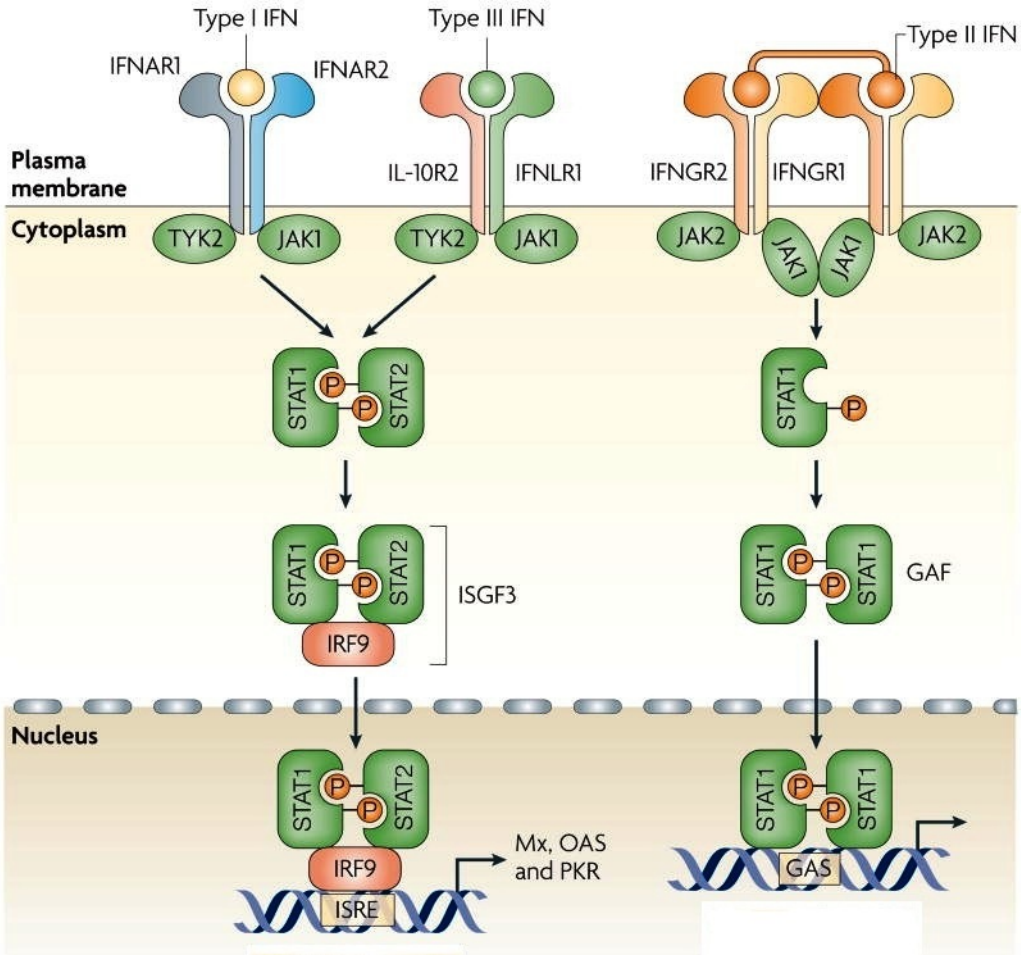


adapted from Randall & Goodbourn, 2008

Figure 1.4.2.1
A. TLR3-dependent IFN induction pathway.
B. TLR7- and TLR9-dependent IFN induction pathway
C. RIG-I and mda-5-dependent IFN induction pathway
(adapted from Randall & Goodbourn, 2008).

In response to the recognition of different viral ligands, these recruit TRIF, Cardif/VISA/MAVS/IPS-1 or adaptor proteins respectively, and activate IRF3 (or IRF7) and NF-κB transcription factors. Activated IRFs and NF-κB then translocate to the nucleus and bind to different sites on the IFN-β promoter and induce the transcription of the IFN-β gene.

Figure 1.4.3.1



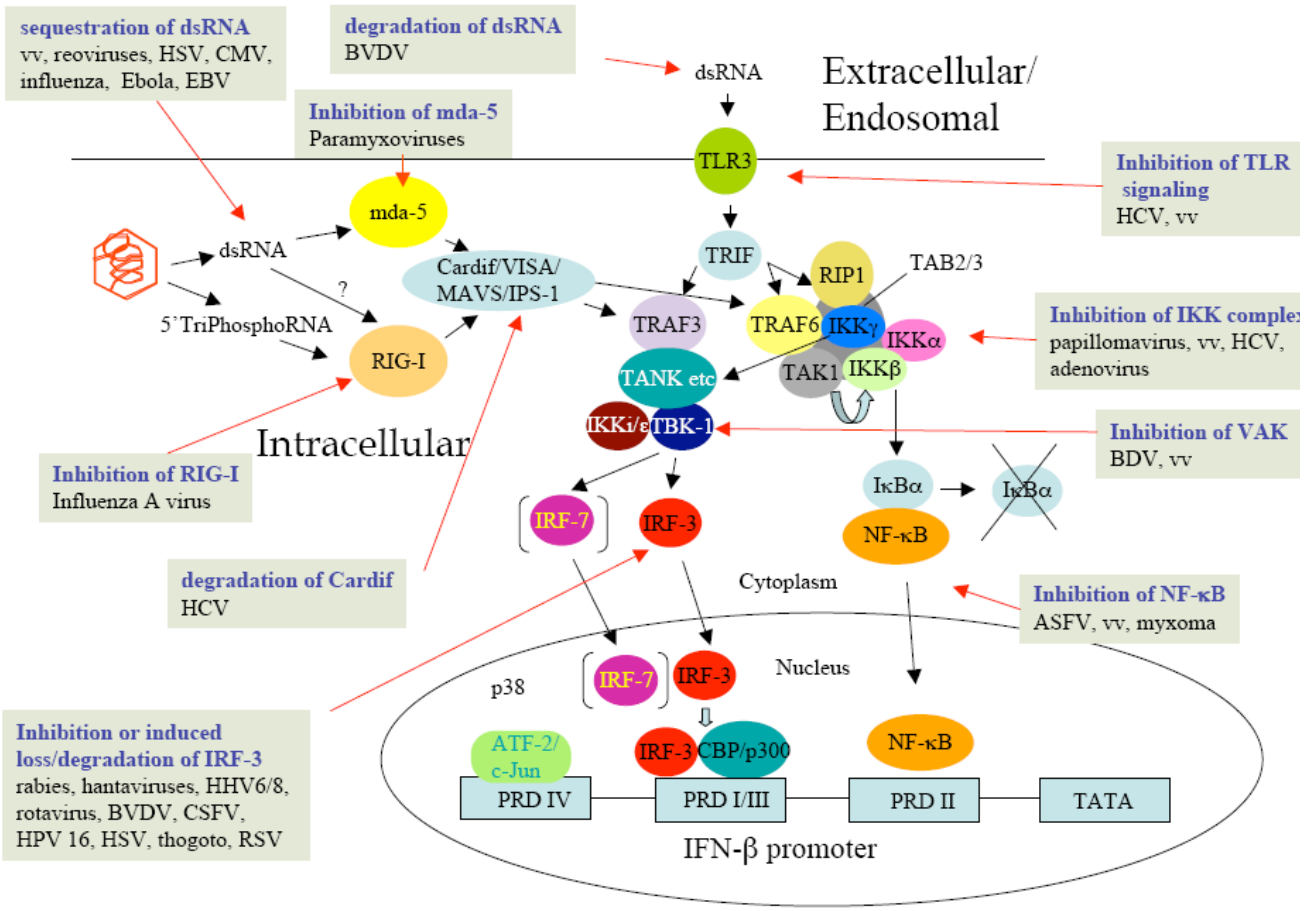
adapted from Sadler & Williams, 2008

Figure 1.4.3.1 IFN signalling pathways (adapted from Sadler & Williams, 2008).

The interferon-induced JAK-STAT signalling pathway is one of the crucial pathways, and utilises three main receptor units to accomplish the process: the heterodimer of IFN-α receptor 1 (IFNAR1) and IFNAR2 for type I IFNs; interleukin-10 receptor 2 (IL-10R2) and IFN-γ receptor 1 (IFNLR1) complex for type III IFNs; and IFNGR1 and IFNGR2 for type II IFN. The binding of IFNs to their recrptors activates receptor-associated tyrosine kinases, which in turn activate STATs molecules by phosphorylation. With the help of other transcription factors, phosphorylated STATs dimers are translocated into the nucleus, bind to their target promoter site, and induces the transcription of a number of ISGs.

Figure 1.5.1

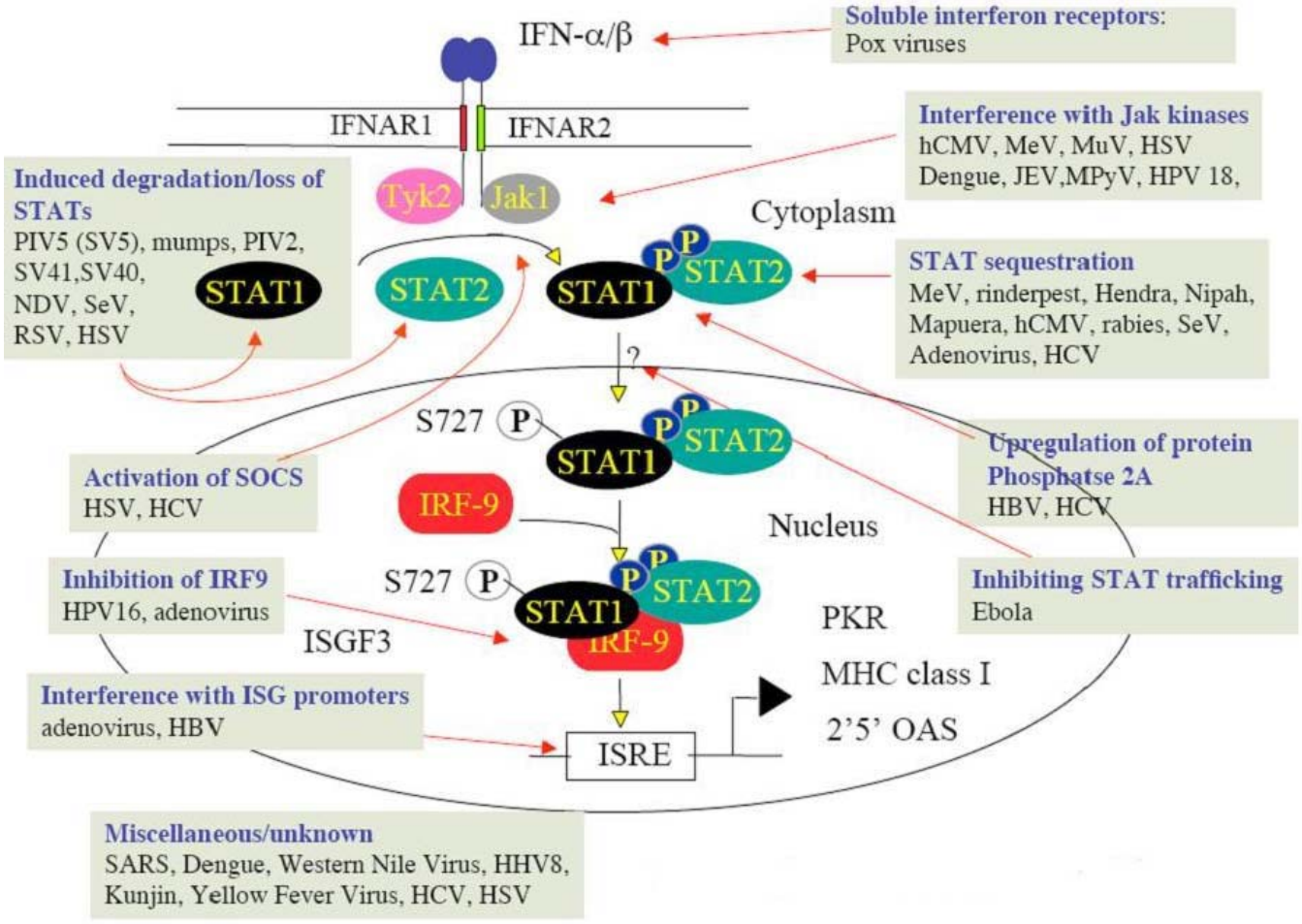
A



adapted from Randall & Goodbourn, 2008

Figure 1.5.1

B



adapted from Randall & Goodbourn, 2008

Figure 1.5.1 Virus inhibition of the IFN response.

A. Virus inhibition of IFN induction.
B. Virus inhibition of IFN signalling.
(adapted from Randall & Goodbourn, 2008).

Viruses have developed methodologies to express IFN antagonists to subvert the host IFN response at multiple levels. Different viruses express different IFN antagonists targeting the same cellular molecule in the IFN system (e.g. BVDV Npro and HPV-16 E6 proteins both target IRF-3 in the IFN induction pathway), or a virus express a single antagonist targeting various cellular molecules (e.g. PIV5 V targets mda-5 in the IFN induction cascade, and STAT1 in the IFN signalling pathway).

2. MATERIALS & METHODS

2.1 Mammalian cells & tissue culture

2.1.1 Cell-lines used in this study

A549

human lung epithelial cells (European Collection of Cell Cultures (ECACC)).

A549/Npro

A549 cells stably expressing the Npro protein of BVDV with an N-terminal V5 tag.

Vero

fibroblast-like cells originating from the kidney of an African green monkey (ICN Pharmaceuticals Ltd., UK).

293T

human embryonic kidney cells (provided by Prof. R. Iggo, University of St Andrews).

MDCK

derived from the kidney of an adult cocker spaniel (ECACC).

MDCK/V

MDCK cell-line constitutively expressing the V protein of PIV5 (Precious *et al.*, 2005)

In addition to the basic cell-lines mentioned above, the following permanent cell-lines were also generated and used as part of this study:

A549/pr(IFN-β).GFP

A549 cell-line stably expressing the green fluorescence (GFP) gene under the control of the IFN-β promoter.

A549/pr(IFN-β).GFP/BVDV-NPro

A549/pr(IFN-β).GFP cell-line stably expressing the Npro protein of BVDV with an N-terminal V5 tag.

A549/pr(IFN-β).GFP/HCV-NS/4A

A549/pr(IFN-β).GFP cell-line stably expressing the NS/4A protein of HCV.

A549/pr(IFN-β).GFP/V

A549/pr(IFN-β).GFP cell-line stably expressing the V protein of PIV5 W3 strain. Blasticidin is used as mammalian selection marker.

A549/pr(IFN-β).GFP/KO.STAT1

A549/pr(IFN-β).GFP cell-line stably expressing shRNA to STAT1. Blasticidin is used as mammalian selection marker.

A549/pr(IFN-β).GFP.V5

A549/pr(IFN-β).GFP cell-line stably expressing the V5 tag on the cell surface under the control of IFN-β promoter.

A549/pr(IFN-β).GFP.V5/V

A549/pr(IFN-β).GFP/V cell-line stably expressing the V5 tag on the cell surface under the control of IFN-β promoter.

MDCK/V/Npro

MDCK/V cell-line stably expressing Npro protein of BVDV.

2.1.2 Cell maintenance

Cell monolayers were cultured in 25cm^2 or 75cm^2 tissue culture flasks (Greiner Bio-One, UK) in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum (FCS, Lonza, Belgium) and incubated at 37°C/5% CO_2. Depending on the growth rate of the cell-line, cells were routinely passaged, trypsinised (Trypsin, ethylenediaminetetraacetic acid (EDTA); Becton Dickinson Ltd., UK) and passed every 3-5 days as appropriate.

2.1.3 Cell stock freezing & resuscitation

Freezing

Adherent cells were trypsinised, resuspended in a small volume of normal growth medium, and pelleted at 1500rpm for 5mins. Cells were then resuspended in freezing medium, DMEM supplemented with 30% FCS and 10% (v/v) dimethyl sulfoxide (DMSO), and aliquoted into cryovials. The cells were kept in a polystyrene box (to slow down temperature decrease) and frozen at -70°C before long-term storage in

liquid nitrogen.

Resuscitation

Cryovials were rapidly thawed at 37°C before centrifugation at 1500rpm. The supernatant was aspirated off, and the pellet of cells resuspended and grown in normal growth medium at 37°C/5% CO_2 overnight. Medium was replaced after 24h in order to remove traces of DMSO.

2.1.4 Treatment of cells

As required, cells were treated with media supplemented with recombinant human IFNαA/D (PBL Biomedical Labs). For stimulation of IFN-responsive promoters, cells were incubated overnight with 10^3units/ml IFN-α. Transfection of cells by plasmids (and synthetic dsRNA; polyI:C) was carried out using the FuGENE® 6 transfection reagent (Roche Diagnostics, UK), according to the manufacturer's instructions.

2.1.5 Subcloning

Subcloning was required to generate homogeous cell-lines. Cells were trypsinised, counted using a haemocytometer, and diluted to around 1 cell/100µl in DMEM (10% FCS), and plated into 96-well microtitre plates (Greiner Bio-One, UK). To compensate for mis-counting, cells were also diluted to 3 cells/100µl, and 1 cell/300µl, and plated into 96-well microtitre plates. Cells were normally cultured, replacing growth medium every 3-7 days, and observed under the microscope to pick single cell colonies growing in single wells. The cells from selected wells were trypsinised, and passed into either a 24-well microtitre plate (Nunc A/S, Denmark) or 25cm^2 tissue culture flask according to the growth rate and cell number. When enough cells were obtained from single cell colonies, each candidate was tested under specific drugs regimes, or infected with viruses, or western blotted to check whether the protein of interest was expressed. Protein expressing colonies were then frozen in DMEM (30% FCS, 10% DMSO) at either -70°C, or in liquid nitrogen for long-term storage.

2.2 Mammalian viruses & infection of cells

2.2.1 Main replicating viruses used in this study.

MuV Enders clone 3/30

The MuV Enders stock was prepared by plaque-purifying and amplifying the virus by passage at low multiplicity of infection (MOI) and the resulting virus being termed MuV Enders clone 3/30 (MuV cl3/30) (Young *et al.*, 2009).

MuV Enders (DI)

The MuV Enders (DI) stock was generated by a high MOI of infection of Vero cell which increases the number of DIs, which induces significant IFN production.

PIV5 W3

Wildtype laboratory strain of PIV5 (Choppin, 1964).

PIV5 VΔC

A strain of PIV5 which has lost the V protein function to target STAT1 (He *et al.*, 2002)

PIV5 VΔC (P2)

The PIV5 VΔC (P2) is generated by infecting confluent Vero cells with the original PIV5 VΔC at a high MOI in order to increase the number of DIs within the virus population.

CPI+

Canine isolate of PIV5 (Evermann *et al.*, 1980; Evermann *et al.*, 1981)

CPI-

Canine isolate of PIV5 from the brain of a gnotobiotic dog infected with CPI+ virus which establishes persistent infections more readily than CPI+ *in vitro* (Southern *et al.*, 1991). This strain is unable to block IFN signalling and is also reported to induce the synthesis of IFN (Poole *et al.*, 2002).

FLUAV rUd wt

Recombinant wildtype FLUAV (A/Udorn/72 H3N2) generated and kindly provided by Dr. David Jackson (Hale *et al*., 2006).

FLUAV rNS1-ran5

FLUAV rNS1-ran5 mutant virus was generated by Dr. David Jackson in our

laboratory using reverse genetics. The segment 8 of the FLUAV Udorn viral genome (encodes NS1 and NS2) flanked by the human RNA polymerase I promoter and terminator sequences was amplified from the pHH-NS rescue plasmid (used to rescue virus) by PCR using the GeneMorph® II random mutagenesis kit (Stratagene, US). The PCR products were generated and included in the rescue system in place of the pHH-NS rescue plasmid to create different viral stocks containing different random mutations. All recovered viruses (rNS1-ran1 – rNS1-ran6) and were then placed onto the A549/pr(IFN-β).GFP/V reporter cells. Only the rNS1-ran5 mutant resulted in GFP-positive cells, *i.e.* induced higher level of IFN.

2.2.2 Virus infection

PIV5

Monolayers of cells were infected with virus suspended in DMEM (2% FCS) at an appropriate MOI. Monolayers were washed prior to infection in phosphate buffered saline (PBS) to remove all traces of serum. After an adsorption period of 1-2h on a rocking platform at 37°C, the virus inoculum (or DMEM only for mock infections) was removed and replaced with DMEM (2% FCS). Cells were incubated at 37°C/5% CO_2 until harvested.

FLUAV

Monolayers of cells were infected with virud suspended in DMEM (serum free) at an appropriate MOI. Monolayers were washed prior to infection in PBS to remove all traces of serum. After an adsorption period of 1-2h on a rocking platform at 37°C, the virus inoculum (or DMEM only for mock infections) was removed and replaced with DMEM (serum free) and 2.5μg/ml N-acetyl trypsin (NAT). For infections where determination of infectious virus was not required, trypsin was not added to the medium post inoculation. Cells were incubated at 37°C/5% CO_2 until harvested.

2.2.3 Preparation of virus stocks

Stocks of the paramyxoviruses - PIV5 W3, PIV5 VΔC, CPI+, and CPI-, were kindly

maintained and provided as required by Mr. Dan Young in our laboratory. For paramyxovirus virus stock preparation and to obtain sufficient virus for further experiments, 90% confluent Vero cells were washed twice in sterile PBS in order to remove traces of serum, and then infected with virus suspended in DMEM (2% FCS) at an MOI of ~ 0.001 plaque-forming unit (PFU)/cell (wildtype viruses) or 10 PFU/cell (viruses with DIs). After 1h rocking at 37°C/5% CO2, cells were incubated for 3-4 days in 25cm^2 tissue culture flasks. The medium was then harvested into a centrifuge tube and spun at 4000rpm for 5mins. The supernatant was then added to 90% confluent Vero cells cultured in roller bottles with 10-15ml DMEM (2% FCS) medium for 1 hour virus absorption, replaced by freshly prepared 20-25ml DMEM (2% FCS), and incubated at 37°C/5% CO_2 for 3-4 days, until a CPE could be observed. The medium was again harvested and spun at 4000rpm for 5mins. Virus supernatant was aliquoted and stored at -70°C. Virus titre was subsequently determined by plaque assay.

2.2.4 Plaque assay

PIV5

Monolayers of Vero cells in 6-well plates (Greiner Bio-One, UK) were cultured until 80-90% confluent. Virus stock was titrated in a series of 10-fold dilutions in DMEM (2% FCS). Cells were infected with 1ml of each virus stock dilution per well. Infected cells were inoculated for 2h at 37°C, in a sealed gas box (5% CO_2) on a rocking platform to allow virus binding and spread onto the cells. 5-10ml of overlay (autoclaved 0.5% (w/v) carboxy methyl cellulose (Methocel MC; Sigma-Aldrich Co. Ltd., UK) and DMEM (2% FCS) was applied onto the cell monolayer in each well, in order to prevent virus diffusion. The cells were left in the incubator under 37°C/5% CO_2 for 10-15 days. When plaques were observed as cell fusions, the medium was aspirated off with the aid of vacuum line. Cell monolayers were fixed with 10% (v/v) formaldehyde in PBS for more than 30mins. Fixative reagent was replaced by 0.05% (w/v) crystal violet. Typically, 10-20mins were required on a rocking platform for sufficient colour development. The stained plates were then washed with water to

remove any fixative or stain solution. Plaques were then observed as holes surrounded by cell fusions. Virus titre (plaque forming unit per virus preparation; PFU/ml) was determined by counting the number of plaques in each well with the corresponding dilution taken into account.

FLUAV

Monolayers of Vero cells in 6-well plates were cultured until 80-90% confluent. Virus stock was titrated in a series of ten-fold dilutions in DMEM (serum free). Cells were infected with 500µl of each virus stock dilution per well. Infected cells were inoculated for 1h at 37°C, in 5% CO_2 on a rocking platform to allow virus binding and spread onto the cells. The virus inoculum was removed, and 2ml of molten agarose overlay (serum-free DMEM supplemented with 0.2% (w/v) bovine serum albumin (BSA), 2µg/ml NAT, and 0.9% (w/v) agarose (NuSeive GTG; Cambrex, UK) was added to each well. The plates were left at room temperature for 15mins for the agarose overlay to solidify, and incubated in 37°C, at 5% CO_2 for 3-4 days until distinct plaques could be visualized. The plates were fixed with 10% formaldehyde in PBS on top of the agarose, and left overnight. Plaques were visualised by immunostaining. Virus titre was determined by counting the number of plaques in each well with the corresponding dilution taken into account.

2.2.5 Visualisation of virus plaques by immunostaining.

To visualise paramyxovirus or FLUAV plaques, an immunostaining technique was used. Fixed monolayer of cells were permeablised (5% (v/v) NP-40, 10% (w/v) sucrose in PBS) for 15mins, and then washed in PBS supplemented with 1% FBS. Areas of virus infection (plaques) were visualised by detecting viral structural proteins. Monolayers were incubated for 1h at room temperature with 500µl/well of appropriately diluted primary antisera (mouse antisera raised against PIV5 (V5 epitope); or rabbit antisera raised against whole X31 (H3N2); diluted in PBS/1% FBS. Cells were washed with PBS/1% FBS, and monolayers incubated for 1h at room temperature with 500µl/well of appropriately diluted secondary anti-mouse (PIV5) or

anti-rabbit (FLUAV) Immunoglobulin G (IgG) alkaline phosphatase (AP)-conjugated antibody (Ab). Monolayers were subsequently washed with PBS/1% FBS, and incubated for ~30mins with 500µl/well of AP substrate (as per manufacturer's instructions; Sigma-Aldrich Co. Ltd., UK), or until sites of virus infection were easily visualised. The reaction was stopped by rinsing with water.

2.3 Lentivirus-mediated generation of stable cell-lines

2.3.1 Lentivirus preparation

The 293T cells, a packaging cell-line for lentivirus, were grown in DMEM (10% FCS) at 37°C/5% CO_2 to obtain 90% confluence. To generate a lentivirus plasmid, a reporter gene of interest is cloned into the lentivirus vector sequence containing long terminal repeats (LTRs) and the Psi-sequence. The LTRs contains the promoter and integration sequences and serves to integrate the reporter gene into the host genome. The Psi-sequence is a signal sequence for virus packaging. Plasmid pCMVR8.91 (pCMV-R) expresses HIV *gag* gene, encoding virus core protein, and HIV *pol* gene, encoding virus replication enzymes (Zufferey *et al.*, 1997). pMD-G (pVSV-G) expresses the vesicular stomatitis virus (VSV) envelope protein (Naldini *et al.*, 1996). LTRs are replaced with a human cytomegalovirus (CMV) promoter and Psi-sequence is deleted from the packaging plasmid. Thus, the packaging sequences will not be incorporated into the virion and the virus produced is not able to replicate. The lentiviral expression plasmids pCMV-R, pVSV-G and the lentivirus plasmid expressing target protein, were co-transfected into 293T cells using FuGENE® 6 overnight (14-16h) at 37°C. Virus was collected after 48 and 72h and filtered through 0.45µm Tuffryn membrane filters (Invitrogen, UK), and replaced with fresh DMEM (10% FCS) each time.

2.3.2 Transduction of target cells

The A549 derived reporter cell-lines were established using the lentivirus system to obtain stably protein expressing cell-lines. Cell numbers were grown to obtain monolayers that were 30-50% confluent on the day of infection. For a 25cm^2 tissue

culture flask, the A549 cells were infected with the lentivirus suspended in DMEM (serum free) and polybrene (Sigma-Aldrich Co. Ltd., UK; 8µg/ml). Virus was incubated for 1h at 37°C/5% CO_2. After inoculation, the virus containing medium was added with an equal volume of fresh DMEM (10% FCS) to allow the cells to recover for 48h. The cells were then placed under selection drugs - puromycin (2µg/ml 2d) or blasticidin (10µg/ml 10d) depending on the mammalian selection marker in the genome.

2.4 Molecular biology

2.4.1 Agarose gel electrophoresis

DNA was analysed by gel electrophoresis in horizontal mini-gels of 1% agarose (Sigma-Aldrich Co. Ltd., UK) in 1X TBE buffer (5X TBE, 45mM Tris-borate, 1mM EDTA). DNA samples were mixed with the appropriate volume of DNA loading buffer (Promega Ltd., UK), prior to electrophoresis. Samples were run at 90V in 1X TBE buffer (containing 1µg/ml ethidium bromide), until bands were clearly resolved. Along with the samples, DNA size markers were also run (100bp ladder and 1kb ladder; Promega Ltd., UK). Resolved DNA bands of interest were excised under UV light, and DNA was recovered using a QIAquick™ Gel Extraction Kit (following manufacturer's instructions; QIAGEN® Ltd., UK).

2.4.2 Restriction enzyme digestion of DNA

Individual backbone vector DNA and purified PCR products were mixed with 2µl 10X enzyme buffer, 0.5µl (5U) each of the desired restriction enzymes (Promega, UK), and 0.2µl 10mg/ml acetylated BSA, in a reaction mixture made up to 20µl with de-ionised H_2O. Reactions were incubated at 37°C (for the majority of enzymes) for 2-4h, or overnight as appropriate.

2.4.3 Ligation of DNA fragments (vector/insert)

Insert and vector DNA were prepared by enzyme digestion/gel purification as described above. Insert, vector, ligase buffer and T4 DNA ligase were mixed with

de-ionised H_2O. Reactions were incubated overnight at 16°C and stored at –20°C until ready for use. Ligation reaction products were used for heat shock transformation of competent *E. coli* DH5α cells as described below.

2.4.4 Heat shock transformation of competent cells

10µl of ligated reaction mix (or ~1µg pre-purified plasmid) was added directly to 100µl of thawed, competent cells. After incubation on ice for 1h, cells were heatshocked in a 42°C water bath for 2mins before being immediately transferred back to ice for a further 2mins. Cells were resuspended in 1ml LB broth and incubated at 37°C for 1h (in order to assist cell recovery). The suspension was then plated out onto solid LB-agar plates supplemented with an appropriate antibiotic (90mm-diameter petri dishes; Scientific Laboratory Supplies Ltd., UK). Plates were inverted and incubated at 37^{o}C overnight. Mini-cultures were prepared from selected colonies.

2.4.5 Preparation of plasmid DNA

For small-scale preparations, bacterial cell cultures of 10ml (in LB broth containing appropriate antibiotic) were grown overnight at 37°C in a shaking incubator. DNA was extracted from cells and purified on silica gel membrane columns using the QIAGEN® DNA mini-prep kit (QIAGEN® Ltd., UK), according to the manufacturer's instructions. For large-scale preparations, bacterial cultures of 200- 250 ml were grown overnight at 37°C in a shaking incubator, and DNA was extracted from cells using the QIAfilter™ Plasmid Maxi Kit (following manufacturer's instructions; QIAGEN® Ltd., UK). The extraction of DNA using these QIAGEN® kits is based on the alkaline lysis of bacterial cells, followed by adsorption of DNA onto silica in the presence of high salt.

2.5 Plasmid DNAs

2.5.1 Plasmids used in this study

Several plasmids were used in this study, either directly, as backbone vectors for

cloning, or to provide DNA sequences for the construction of new plasmids:

pdl'pr(IFN-β).GFP(puro)

lentivirus vector for expressing green fluorescence protein. Expression is controlled by the IFN-β promoter together with a puromycin resistance product for mammalian cell selection.

pdl' BVDV-Npro.V5(N-ter)(puro)

lentivirus vector expressing N-terminally V5-tagged BVDV-Npro with a puromycin resistance product for mammalian cell selection.

pdl'HCV-NS3/4A(puro)

lentivirus vector expressing NS3/4A protein of HCV with a puromycin resistance product for mammalian cell selection.

pdl'PIV5-V(W3)(bla)

lentivirus vector expressing V protein of PIV5 (W3) with a blasticidin resistance product for mammalian cell selection.

pLKO.STAT1(bla)

lentivirus vector expressing shRNA to STAT1 with a blasticidin resistance product for mammalian cell selection (generously provided by Dr. Lena Andrejeva, University of St Andrews).

2.5.2 Plasmids generated in this study

Novel plasmids were generated for transfections, and the isolation of stable cell-lines. The integrity of all new constructs was confirmed by immunofluorescence prior to use:

pDisplay.V5

cDNA coding for the V5 epitope of PIV5 (W3) expressed on the cell surface. The commercial pDisplay™ vector backbone was purchased from Invitrogen, UK.

pdl'pr(IFN-β).V5

lentivirus vector expressing V5 protein of PIV5 (W3) on the cell surface under the control of IFN-β promoter with a puromycin resistance product for mammalian cell selection.

2.6 Protein analysis

2.6.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were prepared in SDS-PAGE disruption buffer (6M Urea, 2M β-mercaptoethanol, 4% (w/v) sodium dodecyl sulfate (SDS), bromophenol blue) and heated at 100°C for 2mins prior to analysis. Polypeptides were separated through 4-12% NuPAGE polyacrylamide gradient gels (Invitrogen, UK) by electrophoresis at 180V until maximum resolution of polypeptide bands was noted.

2.6.2 Antibodies

Antibodies used for western blotting and immunofluoresence.

Primary antibodies

Target protein/Tag	Manufacturer
β-actin	Sigma-Aldrich Co. Ltd.
MuV-NP	Abcam
PIV5-V (polyclonal)	Serum produced by Diagnostics Scotland. Affinity-purified by Dr Bernard Precious.
V5 (reacts with PIV5-V protein, monoclonal)	Serotec
V5 (HPR-conjugated, monoclonal)	Serotec
STAT1α p91 (monoclonal)	Santa Cruz Biotechonology
X31 (H3N2, polyclonal)	Dr. Alan Douglas (National Inititute of Medical Rereach, UK)
IFIT1 (N-16) (goat polyclonal) - ISG56	Santa Cruz Biotechonology

Secondary antibodies

Anti-mouse IgG Texas Red and fluorescein isothiocyanate (FITC) conjugated antibodies were from Oxford Biotechnology Ltd., UK. Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibodies were from

Amersham Bioscience, UK. Goat anti-rabbit IgG AP-conjugated antibody was from Santa Cruz Biotechnology, USA.

2.6.3 Immunoblotting

Polypeptides were separated by SDS-PAGE as described in 2.6.1, and transferred to polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module according to the manufacturer's instructions (Invitrogen, UK). Following transfer, membranes were blocked for 30mins in 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween20 in PBS (blocking buffer), and incubated for 1h with the appropriate primary antibodies diluted (in blocking buffer) as directed by the supplier. After extensive washing with 0.1% Tween20 in PBS, the protein:antibody interactions were detected by incubation for 1h with HRP-conjugated anti-mouse or anti-rabbit IgG (Amersham Bioscience, UK) diluted appropriately in blocking buffer. Following final washing in 0.1% Tween20 in PBS, specific polypeptide bands were visualised by enhanced chemiluminescence (ECL) (Amersham Biosciences Ltd., UK) according to the manufacturer's protocol.

2.6.4 Immunofluorescence

On coverslips

Monolayer of cells (~70-90%) were cultured on 10mm coverslips in 5% formaldehyde, 2% sucrose in PBS, and permeabilised with 0.5% NP-40, 10% sucrose in PBS for 5mins. The permeabilising buffer helps antibody to penetrate the cell membrane for sufficient binding. Cells were washed three times with PBS containing 1% FCS. Cells were incubated with primary antibody for 1h, and washed with PBS to remove unbound antibody. Cells were incubated with Texas Red or FITC-conjugated secondary antibody (Oxford Biotechnology Ltd., UK) was added onto the monolayer (1/100 dilution) for 1h at room temperature. For DNA/nuclei staining, DNA-binding fluorochrome 4',6-diamidino-2-phenylindole (DAPI); 0.5µg/ml) was added with secondary antibody solution. Cells were then washed once in permeabilisation buffer, and three times in PBS (1% FCS, 1% (w/v) sodium azide). For fluorescence, cells

were fixed with fixative (PBS, 5% formaldehyde, 2% sucrose), and washed with PBS (1% FCS, 1% sodium azide). Finally, before microscopy observation, cells were mounted in Citifluor AF-1 mounting solution (Citifluor). Stained features such as GFP, viral antigen, nuclei, could be visualized using specific filters using a Nikon Microphot-FXA microscope.

2.7 Miscellaneous assays

2.7.1 polyI:C induction

4µg of polyI:C, 6µl of FuGENE® 6 and 200µl of OPTI-MEM were mixed and incubated at room temperature for 30mins and then added to a monolayer of cells in a 25cm^2 tissue culture flask with 4ml DMEM (10% FCS).

2.7.2 Transient trasfection

3µl of FuGENE® 6, 2µg of DNA were mixed with serum free DMEM in a sterile eppendorf tube in a total volume of 100µl and incubated at room temperature for 30mins. The transfection mix was added to 4ml of DMEM (10% FCS) before adding to a preformed monolayer of 293T cells in a 25 cm^2 tissue culture flask and incubating for 48h. The cells were then subjected to testing.

2.7.3 CPE reduction bioassay for IFN

The amount of IFN secreted by cells was estimated by using a biological EMCV-inhibition assay in A549/Npro cells. Culture supernatants from infected cells were harvested, centrifuged at 1500xg for 10min to pellet cellular debris, UV-treated to inactivate residual virus. 50% confluent Vero cells in 96-well plates were pre-treated with UV-inactivated 2-fold serial dilutions of culture media containing IFN for 24h prior to infection with EMCV (0.05 PFU/cell) (sensitive to IFN). The development of CPE was monitored 36hpi by crystal violet staining. Units of IFN were estimated as the dilution of media required to reduce the CPE by ~50%.

2.7.4 Flow cytometry

Following desired treatment, adherent A549 cells were trypsinised and agitated to keep them in suspension. GFP was measured using FL1 and FL2 detectors respectively on a FACSCalibur flow cytometer (BD Biosciences, USA). Cells positive for GFP were sorted into single cell, and separated into 96-well microtitre plates (1 cell/well).

2.7.5 Panning

Tissue culture petri dish

Two 60mm tissue culture petri dishes were either pre-incubated with anti-PIV5-V antibody (10μg/sample) or with PBS (control) at 4^{o}C overnight and then washed with ice-cold PBS (control) to wash away any unbound antibodies. Monolayers of 293T cells which had been transfected with pDisplay.V5 construct were cultured in two 75cm^2 tissue culture flask and treated with EDTA (1mM) in PBS at 37^{o}C for 5mins to detach cells from the surface of the tissue culture flask. Cells were pipetted into single cells suspension and centrifuged to remove EDTA/PBS at 1500rpm and then resuspended to single cell suspension with 4ml/sample of ice-cold PBS. Cells were added to either the petri dish pre-coated with anti-PIV5-V antibody or the control plate and incubated at 4^{o}C for 1h to allow cells binding to the antibody. Each plate was washed with PBS to wash away any unbound cells. Any cells expressing V5 epitope on their surface would bind to the antibody attached to the surface of the plate and therefore remained on the plate.

Dynabeads® Protein A

Monolayer of A549/pr(IFNβ).GFP.V5/V cells cultured in 75cm^2 tissue culture flasks were infected with PIV5 W3 with an MOI of 1 PFU/cell for 12h. Infected cells were treated with 5mM EDTA at 37^{o}C for 5mins, and pipetted into single cell suspension, and incubated with UV-inactivated mouse anti-PIV5-V antibody (5μg/ml) for 1h at 4°C with tumbling. The cell suspension was incubated with 10μl pre-washed Dynabeads® Protein A (Invitrogen Ltd., UK) in 1ml 1mM EDTA/PBS buffer for 1h at

4°C with tumbling. Cells expressing V5 epitope tag on their surface bound to the Protein A of the beads and were separated from the unbound cells with a magnet. The beads migrated to towards the magnet and any unbound cells remained in the supernatant were removed by washing with PBS. Selected cells were added onto a preformed monolayer of A549/pr(IFN-β).GFP/V cells in a 25cm^2 tissue culture flask to allow virus propagation for ~3 days when a CPE was observed. Culture medium was collected and virus titre was determined by plaque assay.

3. RESULTS

When infected by viruses, the host immune system generates protective products to eliminate the viruses. The innate immune response is activated upon virus infection, and induces the production of IFN-α/β. The IFN then signals neighbouring cells to activate the JAK-STAT pathway, which in turn, switches on the expression of ISGs to establish an antiviral state. In order to survive, viruses target the host IFN induction and/or signalling cascades to prevent them from functioning properly. However, there are always naturally occurring mutants in the virus population that have lost their ability to circumvent the host immune response. Such mutant viruses would not be able to cause severe disease in the host, and thus may be used as live attenuated virus vaccine candidates.

My project was to develop a cell-line that can be used isolate mutant viruses that stimulate IFN production. In other words, to develop a rapid method for selecting mutant viruses that are good IFN inducers.

3.1 Generation of the A549/pr(IFN-β).GFP reporter cell-line

3.1.1 Generation of a reporter cell-line using a lentivirus vector system

The aim was to develop a methodology using engineered cells to isolate IFN-inducing mutant viruses. To achieve this, we generated cell-lines in which reporter protein expression is driven by the IFN-β promoter. By selecting cells which express reporter proteins, the aim was to use them to identify and isolate the IFN-inducing mutant viruses from wildtype viruses.

A lentivirus vector pdl'pr(IFN-β).GFP (plasmid generated by Dr Mara Rodrigues in our laboratory), which expresses the enhanced green fluorescence protein (eGFP) and puromycin resistant gene (*pac*) under the control of IFN-β promoter (Figure 3.1.1.1), was used to transfect 293T cells. The lentivirus construct map and the generation of the reporter cell-lines are illustrated in Figure 3.1.1.1 and Figure 3.1.1.2, respectively.

Lentiviruses are complex retroviruses whose distinguishing ability is to integrate into non-dividing cells without disassembly of the nuclear membrane. Lentivirus plasmid pdl'pr(IFN-β).GFP and packaging plasmids pCMVR8.91 and pVSV-G were co-transfected into 293T cells to produce the designed lentivirus. The harvested recombinant viruses were then used to infect A549 cells which were then subjected to selections to generate a cell-line, in which the reporter proteins are expressed in the cells when the IFN-β promoter is activated. For this pdl'pr(IFN-β).GFP construct, we used two strategies to activate the IFN-β promoter: (i) IFN induction by dsRNA, (ii) infect cells with a MuV Enders strain preparation enriched with defective interfering (DI) particles – MuV Enders (DI), which has been indentified as a good IFN inducer.

Cells were transfected with synthetic dsRNA - polyI:C, to activate the IFN-β promoter. dsRNA is recognised by intracellular sensor RIG-I and/or mda-5, which activates the IFN signalling cascade to switch on IFN production (Chapter 1.4.2.2). If the lentivirus infection is successful, GFP expression should be detectable by both methods. Puromycin selection process was preformed post polyI:C transfection to remove the non-successfully transduced cells, *i.e.* cells that did not express GFP when the IFN-β promoter was activated.

3.1.2 Puromycin selection of lentivirus transduced cells

The lentivirus-infected A549 cells were transfected with dsRNA to activate the IFN-β promoter. 1µl polyI:C (5µg/µl) and 6µl FuGENE® 6 were mixed with 200µl OPTI-MEM at room temperature for 40 minutes, and then added to a monolayer of the transduced A549 cells growing in a 25cm^2 tissue culture flask with 2ml DMEM (10% FCS). At 6h post polyI:C transfection, puromycin (2µg/ml) was added to the culture medium and maintained for 2 days. When transfected with dsRNA, cells which express the *pac* gene survived puromycin selection, whereas uninfected cells died. Approximately 10% of total starting cells survived the selection. The surviving cells were grown to confluence and passaged for further characterisation and analysis.

3.1.3 FACS sorting and subcloning to generate homogeneous A549/pr(IFN-β).GFP reporter cell-lines

In order to isolate a homogeneous IFN-β promoter tightly regulated, GFP expressing cell-line, FACS separation was carried out after the puromycin selection of the lentivirus-infected A549 cells. FACS is a type of flow cytometry for sorting a heterogeneous mixture of cells based upon the light scattering and fluorescent characteristics of each cell. GFP is one of the measurable parameters of FACS sorting. Monolayers of the lentivirus-infected A549 cells were transfected with polyI:C for 6h, trypsinised and resuspended in DMEM (10% FCS) to obtain a single cell suspension, and sorted by FACS to separate the GFP-positive cells and GFP-negative cells. The pool of the FACS sorted A549 GFP-positive cells were then subjected to subcloning to obtain a homogenous cell population, in which all cells would express GFP in an inducible manner. The pool of FACS sorted cells was subcloned into 96-well microtitre plates. Colonies were selected from 96-well microtitre plates in which cells grew in of roughly 20% of the wells, thereby ensuring that each colony was likely to be derived from a single cell. When a reasonable monolayer of cells from the single cell became established, cells were trypsinised and passed into a 24-well microtitre plate. Cell colonies were subsequently passaged and confirmed microscopically as GFP-negative when not treated with dsRNA or infected with viruses enriched with DIs (*i.e.* when the IFN-β promoter is not activated). Individual selected clones were then subjected to IFN-induced GFP expression. Cells from each colony were separately cultured on coverslips, and infected with MuV Enders (DI) at an MOI of 10 PFU/cell. Cells were fixed and immunostained for viral antigen (MuV-NP) at 6hpi, and examined under a Nikon Microphot-FXA immunofluorescence microscope (data not shown). Cells expressing GFP were selected and named with clone numbers. From those selected cell-lines, the one which expressed GFP the most rapidly and with the most intensity was selected and termed A549/pr(IFN-β).GFP cell-line (referred as the naive GFP reporter cell-line in figures). This resulting cell-line was amplified and frozen in liquid nitrogen.

3.2 Characterisation of the A549/pr(IFN-β).GFP reporter cell-line

As a potential cell-line for isolating IFN-inducing mutant viruses, a series of characterisation experiments were performed on the A549/pr(IFN-β).GFP cell-line to ascertain how the cell-line would respond to virus infection, and to identify potential problems that may arise when isolating IFN-inducing mutant viruses.

3.2.1 GFP induction assay on A549/pr(IFN-β).GFP reporter cell-line

FACS and immunofluoresence analysis were performed to determine how quickly the IFN-β promoter was activated and GFP expressed following virus infection. The A549/pr(IFN-β).GFP cells were cultured on coverslips and in 25cm^2 tissue culture flasks in parallel, and infected with MuV Enders (DI) at an MOI of 10 PFU/cell to guarantee that every cell was infected. Coverslips were were fixed at 2, 4, 6 and 8hpi immunostained for viral antigen (MuV-NP) and GFP expression was visualised microscopically (Figure 3.2.1.1.A). Infected cells cultured in 25cm^2 tissue culture flasks were trypsinised into single cell suspension, fixed at 2, 4, 6 and 8hpi (one flask of cells per time point), and analysed by LYSYS program on a Becton Dickinson FACScan (Figure 3.2.1.1.B). Immunofluorescence result showed that during the time course studied, GFP expression was induced at 6hpi in response to MuV Enders (DI) infection and a small amount of viral protein was synthesised. At 8hpi, there was a significant increase in GFP expression and the amount of MuV-NP viral protein synthesised. DAPI stain for nuclei confirmed approximately >90% of the infected cells expressed GFP. Quantitative FACS (Figure 3.2.1.1.B) which is more precise was in agreement with the immunofluorescence results. By 4hpi, 35% of the total cells were weakly positive for GFP. At this stage, GFP was not detectable by immunofluorescence. At such an early time point, the GFP expression intensity may be too low to be visualised by immunofluorescence. FACS data has also shown that by 6hpi, 80% of the cells were positive for GFP, and by 8hpi, about 90% of the cells were strongly positive for GFP.

In order to investigate how well GFP induction correlates with IFN production,

A549/pr(IFN-β).GFP cells which had been grown on coverslips in 24-well microtitre plates were infected with differing multiplicities of MuV (DI). At 24hpi the number of cells expressing GFP was visualised by fluorescence and culture medium was collected and any residual viruses were inactivated by UV treatment. IFN level from each sample was measured by CPE reduction bioassay for IFN. Results show that the number of cells expressing GFP correlated with the amount of IFN produced (Figure 3.2.1.2 A&B). Also, since GFP is expressed from a transcript which also encodes *pac*, these cells will express *pac* under the control of the IFN-β promoter.

3.2.2 Effect of knocking out key signalling molecules involved in the IFN induction cascade

To further characterise the A549/pr(IFN-β).GFP reporter cells, we constitutively expressed either BVDV-Npro or HCV-NS3/4A in these cells to determine the effects of knocking out crucial signalling molecules on IFN induction and GFP expression. As discussed in Chapter 1.5.2, BVDV-Npro blocks IRF-3 (a transcription factor crucial for IFN promoter activation) from binding to DNA, and targets IRF-3 for protein degradation (Hilton *et al.*, 2006). HCV-NS3/4A cleaves Cardiff/VISA/MAVS/IPS-1, thereby blocking both mda-5 and RIG-I signalling and thus disrupts IRF-3 phosphorylation and NF-κB activation (Foy *et al.*, 2005; Sumpter *et al.*, 2005; Meylan *et al.*, 2005; Kawai & Akira, 2006). HCV-NS3/4A also mediates TRIF cleavage and reduces TRIF abundance, inhibits polyI:C-activated TLR3 signalling, which consequently leads to the blockage of IRF-3 activation (Li *et al*. 2005a).

To generate the cell-lines, we infected the A549/pr(IFN-β).GFP cells with lentiviruses expressing either BVDV-Npro or HCV-NS3/4A protein. Both lentiviruses have *pac* as their selection marker. The pdl'BVDV-Npro and pdl'HCV-NS3/4A lentiviruses were generated by Dr. Yun-Hsiang Chen in our laboratory. Following infections with lentiviruses, the A549/pr(IFN-β).GFP cells were treated with puromycin (2μg/μl) for 2 days. Cells survived the selection were named as A549/pr(IFN-β).GFP/Npro or

A549/pr(IFN-β).GFP/NS3/4A cell-line (abbreviated to BVDV/Npro or NS3/4A cells in Figure 3.2.2.1). Monolayers of A549/pr(IFN-β).GFP, A549/pr(IFN-β).GFP/Npro and A549/pr(IFN-β).GFP/NS3/4A cells cultured on coverslips were infected with MuV Enders (DI) of an MOI of 10 PFU/cell, and fixed at 16hpi, and immunostained for MuV-NP. As shown in Figure 3.2.2.1., GFP expression was significantly reduced in cells expressing BVDV-Npro or HCV-NS3/4A, where key signalling molecules of IFN induction pathway has been knocked out. Further, for A549/pr(IFN-β).GFP reporter cells, GFP expression was up-regulated by the IFN-β promoter in response to MuV Enders (DI) virus infection. As expected, both BVDV-Npro and HCV-NS3/4A were effective in blocking IFN-β promoter activation, leading to inhibition of GFP expression in A549/pr(IFN-β).GFP reporter cells. In addition, BVDV-Npro and HCV-NS3/4A blocking GFP expression also confirmed IRF-3 as a crucial factor which activates the IFN-β promoter.

3.2.3 The importance of DIs in the induction of IFN

The above results confirmed that GFP expression in the A549/pr(IFN-β).GFP reporter cell-line works as a reliable marker for measuring the activation of the IFN-β promoter. We next performed virus infection assays to investigate whether virus preparations containing few DIs activated the IFN-β promoter, compared to virus stocks rich in DIs (Figure 3.2.3.1.A). In addition, culture media collected from the infections were used to carry out a CPE reduction bioassay to measure the amount of IFN produced (Figure 3.2.3.1.B).

The MuV Enders (DI) stock, which we know to be a good IFN inducer and has been used for our previous studies, was generated by a high MOI of infection of Vero cell which increases the number of DIs. The DI-poor MuV Enders stock used in this study was prepared by plaque-purifying and amplifying the virus by passage at low MOI – the resulting virus being termed MuV Enders clone 3/30 (MuV cl3/30) (Young *et al.*, 2009). Monolayers of the A549/pr(IFN-β).GFP reporter cells were infected either with MuV Enders (DI) or MuV Enders cl3/30 at an MOI of 5 PFU/cell, fixed at 16hpi and

immunostained for viral antigen (MuV-NP). Figure 3.2.3.1.A showed that there was only a small number of cells expressing GFP in A549/pr(IFN-β).GFP reporter cells infected with DI-poor MuV Enders cl3/30 virus. This suggested a small percentage of viruses in the MuV Ender cl3/30 virus population were able to induce the production of IFN. This subpopulation of viruses may be the IFN-inducing mutant viruses which occur naturally in the virus population.

Culture medium from those virus infections were harvested at 16hpi and UV-inactivated to perform a CPE reduction bioassay for IFN (Figure 3.2.3.1.B) to measure the amount of IFN produced by the cells post-infection. The amount of IFN produced was used to quantify the viruses' ability to induce IFN. Figure 3.2.3.1.B confirmed that the MuV Enders (DI) stock was better able to induce IFN than the non-defective MuV Enders cl3/30, in agreement with the immunofluorescence results.

3.2.4 Puromycin selection of IFN-inducing mutant viruses using A549/pr(IFN-β).GFP reporter cell-line

As alluded to earlier, naturally occurring IFN-inducing mutant viruses have the potential to be used as live attenuated virus vaccines. To determine whether the A549/pr(IFN-β).GFP cell-line could be used to isolate IFN-inducing mutant viruses from the wildtype virus population using one of the selection markers – *pac*, a model system was designed (Figure 3.2.4.1). This selection marker should lead to either (i) survival of the cell when the cell is infected by an IFN-inducing mutant virus, or (ii) cell death when it is infected by a virus which fails to activate the IFN-β promoter. Briefly, if a cell is infected with an IFN-inducing mutant virus, it should not block the host IFN response, the IFN-β promoter would be activated, the *pac* gene expressed, and the cell would survive puromycin selection. On the other hand, if a cell is infected with a wildtype virus, the functional viral IFN-antagonist(s) would block the activation of the IFN-β promoter, the *pac* gene would not be expressed, and as a conquence, the cell would die in the presence of puromycin.

In order to select IFN-inducing mutant virus, an additional requirement for this system to work is to ensure the cells are not co-infected with a wildtype virus and an IFN-inducing mutant virus. If a cell was infected with both an IFN-inducing mutant and a wildtype virus, the wildtype virus would express IFN antagonist(s), and would block the activation of IFN. Cells then require to be infected at an MOI of approximately 1 PFU/cell, so that the majority of the cells are infected but co-infection is largely avoided.

Monolayers of A549/pr(IFN-β).GFP reporter cells were cultured to confluence and infected either with MuV Enders cl3/30 or MuV Enders (DI) (control) at an MOI of 1 PFU/cell. At 6hpi, puromycin (2μg/ml) was added to the media and the infected cells cultured in the presence of puromycin for 48h. Culture medium containing viruses was harvested at 54hpi. A high percentage (>95%) of dead cells were observed amongst cells infected with MuV Enders cl3/30, whereas only a small percentage (~10%) of cells died in the flask of cells infected with the MuV Enders (DI) (data not shown). The harvested viruses were then cultured on preformed monolayers of the A549/pr(IFN-β).GFP reporter cells to allow virus amplification. The cells were incubated after infection until a CPE was observed and the supernatant was then collected. Viruses released from the A549/pr(IFN-β).GFP reporter cells were titrated, however the virus titre was 10^4-fold lower (data not shown) compared to the original MuV Enders cl3/30 virus stock.

It was important to ascertain whether the selected virus population had been enriched in IFN-inducing mutant viruses, *i.e.* whether the isolated viruses were any better in their ability to induce IFN than the original virus stock. An immunofluorescence assay was performed to examine whether there was an increase in the number of GFP-positive cells in cells infected with the isolated virus population compared to cells infected with the original MuV Enders cl3/30 virus (Figure 3.2.4.2). Monolayers of A549/pr(IFN-β).GFP reporter cells were cultured to confluence and infected with MuV Enders cl3/30, MuV Enders (DI), and the rescued virus of MuV Enders cl3/30 at

an MOI of 5 PFU/cell, fixed at 16hpi and immunostained for MuV-NP.

We show that A549/pr(IFN-β).GFP reporter cells infected by MuV Enders (DI) induced intensive GFP expression, however, surprisingly only a few cells infected with rescued virus expressed GFP. There was also no significant increase in the number of GFP expressing cells, when comparing the isolated virus with the original MuV Enders cl3/30 infected cells. Unfortunately, puromycin selection of IFN-inducing mutant viruses using the A549/pr(IFN-β).GFP cell-line was not successful. One possible reason for this may be that the mutant viruses which were isolated may have been lost during the amplification process using A549/pr(IFN-β).GFP reporter cells post puromycin selection. Further characterisation experiments were carried out to follow this up.

3.2.5 Characterisation of the A549/pr(IFN-β).GFP reporter cell-line for the ability to support virus replication

Careful examination of immunofluoresence studies showed that there was little viral antigen present in cells in which GFP was activated (Figure 3.2.4.2 MuV Enders (DI)). In striking contrast, in cells that were negative for GFP, a lot of viral antigen could be detected (Figure 3.2.4.2 MuV Enders cl3/30). There may be a number of reasons for this. One potential explanation is that the GFP-positive cells have been infected by defective viruses which cannot replicate and therefore cannot be amplified. Or secondly, an antiviral state is induced in the cells in which IFN-β promoter has been activated that inhibits virus replication. To address the question about establishment of an antiviral state, subsequent plaque assays were performed.

Plaque assays were designed to visualise virus spread *vs* production of IFN by infecting A549 cells with PIV5 VΔC, a strain of PIV5 which has lost the V protein function to target mda-5 (He *et al.*, 2002), at an MOI of 0.01 PFU/cell. Monolayers of cells were fixed at 2, 4 and 6 days post-infection (dpi). MxA, an IFN-induced antiviral protein, was used as a monitor of IFN induction. Immunofluorescence (Figure 3.2.5.1)

showed that a significant amount of MxA was produced at 4 and 6dpi in the surrounding cells of an observed virus plaque. In addition, no significant plague development (increase in the number of infected cells) was observed (Figure 3.2.5.1 4 & 6dpi). In conclusion, virus amplification may have been eliminated by the production of IFN by A549 cells. In other words, the establishment of an antiviral state induced *via* IFN pathway prevented virus replication.

3.2.6 Modifications of the A549/pr(IFN-β).GFP reporter cell-line

In the A549/pr(IFN-β).GFP cell-line, expression of GFP does not alter its native ability either to respond to or to produce IFN. As observed previously (Chapter 3.2.4), the IFN-inducing mutant viruses we hoped to isolate may be sensitive to IFN. A549 cells produce a significant amount of IFN following infection with IFN-inducing mutant virus which may establish an antiviral state. Therefore, it may not be possible to use the A549/pr(IFN-β).GFP reporter cell-line to isolate IFN-inducing mutant viruses. Consequently, we modified this cell-line so that it became non-responsive to IFN. Since STAT1 is an essential transcriptional factor needed for IFN signalling, the A549/pr(IFN-β).GFP reporter cell-line was modified to prevent IFN signalling by targeting STAT1. Two approaches were used to knock out STAT1. The first was to generate an analogous cell-line expressing shRNA to knock out STAT1. Secondly, a cell-line which constitutively expresses the V protein of PIV5, which targets STAT1 for proteasome-mediated degradation (Didcock *et al.*, 1999; Precious *et al.*, 2005b; Precious *et al.*, 2007) was isolated.

The A549/pr(IFN-β).GFP cell-line was infected with either lentiviruses expressing shRNA to STAT1 (KO.STAT1) or PIV5-V protein (Figure 3.2.6.1). Both lentivirus vectors had blasticidin as their selection marker. The pLKO.STAT1(bla) lentivirus was generated by Dr Lena Andrejeva and pdl'PIV5-V(W3)(bla) lentivirus has been generated by Dr. Yun-Hsiang Chen in our laboratory. Following infection with lentiviruses, the GFP reporter cells were treated with blasticidin (10μg/μl) for 10 days. Cells surviving blasticidin selection were analysed by immunofluoresence (Figure

3.2.6.2.A) and western blotting (Figure 3.2.6.2.B) for STAT1 to confirm STAT1 expression had been inhibited. The cell-lines were named A549/pr(IFN-β).GFP/KO.(knocked-out)STAT1 cell-line (abbreviated to KO.STAT1 reporter cell-line in figures) and A549/pr(IFN-β).GFP/V cell-line (abbreviated to GFP/V cell-line in figures). Generation of the cell-lines is illustrated in Figure 3.2.6.1.

Immunofluorescence results (Figure 3.2.6.2.A) shows STAT1 expression had been significantly reduced in A549/pr(IFN-β).GFP/KO.STAT1 and A549/pr(IFN-β).GFP/V cell-lines. Encouragingly, western blot data (Figure 3.2.6.2.B) suggested STAT1 could not be detected with the A549/pr(IFN-β).GFP/V cell-line (+IFN & -IFN) as expected. In contrast, while there was a significant reduction of STAT1 with the shRNA expressing cells, a small amount of STAT1 was still present nevertheless. Thus we had successfully modified the cells and found that the V expressing cell-line a better cell-line to knock out IFN signalling, compared to the shRNA expressing cell-line. Therefore, the A549/pr(IFN-β).GFP/V cell-line was used in subsequent studies rather than the A549/pr(IFN-β).GFP/KO.STAT1 cell-line. The modified cells were then infected by MuV Enders (DI) which confirmed that the cells were still able to express GFP in response to virus infection (data not shown).

3.2.7 Investigation of the A549/pr(IFN-β).GFP/V reporter cell-line as a permissive cell-line for virus growth

Once the A549/pr(IFN-β).GFP/V cell-line had been generated, it was important to confirm that it was permissive for the growth of mutant viruses that were sensitive to IFN. The strains used to carry out this study were CPI+ and CPI-. CPI+ is a canine isolate of PIV5 (Evermann *et al.*, 1981). CPI- (Poole *et al.*, 2002) is a mutant of CPI+ which is IFN sensitive, and no longer able to produce a functional V protein that targets STAT1 (Chatziandreou *et al.*, 2002). If plaques of CPI- were able to develop in the A549/pr(IFN-β).GFP/V cell-line, but not in the A549/pr(IFN-β).GFP cell-line, then it would provide evidence that the A549/pr(IFN-β).GFP/V could support the propagation of IFN-sensitive viruses and thus may be used as a potential cell-line for

isolating IFN-inducing mutant viruses.

A549/pr(IFN-β).GFP and A549/pr(IFN-β).GFP/V cells were infected with either CPI+ or CPI- at an MOI of 0.01 PFU/cell. Cells were fixed at 2, 4 and 6dpi and immunostained for PIV5-NP&P. The results (Figure 3.2.7.1) illustrated that the A549/pr(IFN-β).GFP/V cell-line was better able to support the replication of CPI- compared to the A549/pr(IFN-β).GFP reporter cell-line in terms of plaque development, *i.e*. CPI- virus replicated relatively more efficiently in the V protein expressing GFP reporter cells than in the A549/pr(IFN-β).GFP reporter cell. Based on these findings, the A549/pr(IFN-β).GFP/V cell-line, may be considered a potential cell-line for isolating IFN-inducing mutant viruses.

3.3 FACS selection of IFN-inducing mutant viruses

3.3.1 FACS selection of IFN-inducing mutant viruses using A549/pr(IFN-β).GFP/V reporter cell-line

Having generated and characterised the A549/pr(IFN-β).GFP/V reporter cell-line, we next attempted to use the system to isolate IFN-inducing mutants of PIV5 and FLUAV. The FLUAV rNS1-ran5 mutant virus used in this study was generated by Dr. David Jackson in our laboratory using reverse genetics. The segment 8 of the FLUAV Udorn viral genome (encodes NS1 and NS2) flanked by the human RNA polymerase I promoter and terminator sequences was amplified from the pHH-NS rescue plasmid (used to rescue virus) by PCR using the GeneMorph® II random mutagenesis kit. Six PCR products were generated and included in the rescue system in place of the pHH-NS rescue plasmid to create 6 different viral stocks containing different random mutations. All recovered viruses (rNS1-ran1 – rNS1-ran6) and were then placed onto the A549/pr(IFN-β).GFP/V reporter cells. Only the rNS1-ran5 mutant resulted in GFP-positive cells. Therefore, FLUAV rNS1-ran5 mutant virus was used in the FACS sorting experiment as a positive control (Figure 3.3.1.1 and 3.3.1.2.A). In this procedure, on the basis of GFP expression, FACS was used to separate cells in which the IFN-β promoter had been activated from cells in which it had not. The advantage

of FACS is that we should be able to isolate single viruses from single cells. FACS sorting will separate GFP-positive cells either as a bulk population or as single cells into 96-well microtitre plates. The potential advantage of sorting cells into a 96-well microtitre plate is that individual mutant viruses may be isolated, while from the pooled population of GFP-positive cells, it is likely that a mixture of viruses would be isolated. The reason for using the A549/pr(IFN-β).GFP/V cells for rescuing any potential mutant viruses is that not only would the cells not enter an IFN-induced antiviral state once IFN has been released from the GFP-positive cells, but also it should be possible to rapidly determine whether IFN-inducing mutant viruses had indeed been selected. Prior to virus isolation, the GFP-positive cells were either pooled, or were sorted into individual cells in a 96-well microtitre plate. Following FACS isolation of the GFP-positive cells, the selected cells were co-cultured with preformed monolayers of A549/pr(IFN-β).GFP/V cells in order to rescue virus from cells in which the IFN-β promoter had been activated.

FACS sorting experiments for GFP cells were carried out at the University of Edinburgh, UK. Prior to sorting, monolayers of the A549/pr(IFN-β).GFP/V cells were cultured to confluence in 75cm^2 tissue culture flasks for infection. The A549/pr(IFN-β).GFP/V reporter cells were infected with FLUAV rNS1-ran5 mutant viruses or PIV5 W3 at an MOI of 1 PFU/cell. Infected reporter cells were trypsinised, resuspended as single cell suspensions in serum free DMEM, and sorted by FACS at 12hpi (Figure 3.3.1.2). Approximately 13% of the A549/pr(IFN-β).GFP/V reporter cells were sorted as GFP-positive for FLUAV rNS1-ran5 mutant virus and about 0.3% of the A549/pr(IFN-β).GFP/V reporter cells were sorted as GFP-positive for PIV5 W3. GFP-positive cells were collected either pooled and co-cultured with A549/pr(IFN-β).GFP/V cells grown as monolayers in 25cm^2 flasks, or individual GFP-positive cells were sorted into single wells of a 96-well microtitre plate with preformed monolayers of A549/pr(IFN-β).GFP/V cells. These were transported back to St Andrews in a sealed CO_2 gas box, transferred into a tissue culture incubator (37°C, 5% CO_2) for 16h to allow settlement/attachment of the sorted cells. Cells were

then incubated on a platform rocker, the medium was harvested when a CPE was observed and the virus titrated. The A549/pr(IFN-β).GFP/V reporter cells were then infected either with the original viruses (PIV5 W3 or FLUAV rNS1-ran5 mutant virus) or their rescued viruses at 5 PFU/cell to determine whether the selected virus population had been enriched with IFN-inducing mutant viruses. If successful, there would have been a significant increase in the number of GFP-positive cells in monolayers infected with the rescued virus population (Figure 3.3.1.3 (FLUAV data not shown)). Infected cells were fixed at 24hpi and monitored both for GFP expression and for viral antigen (PIV5-P&V) following immunostaining. However, disappointingly, there was no significant increase in the number of GFP expressing cells when infecting the A549/pr(IFN-β).GFP/V reporter cell-line with the isolated virus population. Similarly, no IFN-inducing mutants of FLUAV rNS1-ran5 were isolated in parallel experiments (data not shown).

The FACS-based method to isolate infectious mutant viruses using A549/pr(IFN-β).GFP/V reporter cells was not successful. Mutant viruses which are IFN inducers may have been initially selected for, since we were able to separate GFP-positive cells as a small population from GFP-negative cells. However, if so, the viruses selected were not subsequently amplified during co-cultivation of the GFP-positive cells with the A549/pr(IFN-β).GFP/V cells. Therefore, further characterisation assays were undertaken to confirm if this cell-line had the ability to support the growth of rescued mutant viruses (see below).

3.3.2 Further investigation of the A549/pr(IFN-β).GFP/V reporter cell-line as a permissive cell-line for virus growth

We were able to isolate virus-induced GFP-positive cells using FACS, however the only virus that was isolated was non-defective wildtype virus that failed to activate the IFN response. One possible reason for the failure to isolate mutant viruses from GFP-positive cells, is that, although the IFN-induced antiviral activity would have been prevented, the A549/pr(IFN-β).GFP/V cells responsible for the activation of the

IFN-β promoter may still not be able to support the growth of the mutant viruses. A suggestion that this might be the case came from examining the activation of the IFN-β promoter during plaque development (Figure 3.3.2.1). The A549/pr(IFN-β).GFP/V reporter cells were infected with CPI+ (Chapter 3.2.7) virus at an MOI of 0.01 PFU/cell, fixed at 2dpi and the expression of GFP and the presence of virus antigen (PIV5-NP&P), following immunostaining individual cells were visualised by fluorescence microscopy. The results shown in Figure 3.3.2.1 suggested that within a developing plaque of CPI+ in A549/pr(IFN-β).GFP/V cells, although an antiviral state induced by IFN was prevented, the virus antigen level was still low in cells that were positive for GFP, and the cells strongly positive for viral antigen were negative for GFP. There are two possibilities for this. Firstly, GFP-positive cells may have been infected with DIs, which cannot replicate without the help of a non-defective virus within the same cell. Secondly, since IRF-3 activates a subset of ISGs as well as the IFN-β promoter, GFP-positive cells in which the IFN-β promoter was activated may have been in an IFN-independent antiviral state, and were thus unable to support efficient viral replication.

The A549/pr(IFN-β).GFP/V cells were further characterised to examine whether an antiviral state can be induced in them independently of IFN. The A549/pr(IFN-β).GFP, A549/pr(IFN-β).GFP/V, A549/Npro cells, and Vero cells were used in this study. A549/pr(IFN-β).GFP/V cells constitutively express PIV5-V protein which targets STAT1 for proteasome-mediated degradation (Didcock *et al.*, 1999; Precious *et al.*, 2005b; Precious *et al.*, 2007) and therefore IFN signalling is blocked in these cells. A549/Npro cells constitutively express the Npro protein of BVDV which target IRF-3 for degradation and thus the induction of IFN as well as any antiviral activity induced *via* IRF-3 is prevented. Vero cells are IFN-deficient (Desmyter *et al.*, 1968), they cannot produce any IFN in response to virus infections. Unpublished data generated in our laboratory by Dr Lena Andrejeva has also suggested that Vero cells are IRF-3 deficient. Therefore, any antiviral response activated though IRF-3 is impaired in Vero cells. The virus used in this study was PIV5 VΔC (P2). PIV5 VΔC is a strain of PIV5

W3 which produces a C-terminus truncated V protein of PIV5 (He *et al.*, 2002). The PIV5 VΔC (P2) was generated from the original PIV5 VΔC stock by infecting confluent Vero cells at a high MOI in order to increase the number of DIs within the virus population.

In this study, PIV5 VΔC (P2) viruses were diluted in a series of 10-fold dilutions, starting with an MOI of 10 PFU/cell. Each diluted stock was then used to infect A549/pr(IFN-β).GFP cells or A549/pr(IFN-β).GFP/V cells. In addition, to monitor virus replication, A549/Npro cells or Vero cells were also infected with the same virus stock in parallel. Virus-infected cells were fixed at 16hpi and immunostained for viral antigen (PIV5-NP&P). GFP expression and viral protein synthesis were then visualised using fluorescence microscopy. Figure 3.3.2.2 (10 PFU/cell) showed the majority of A549/pr(IFN-β).GFP and A549/pr(IFN-β).GFP/V cells were strongly positive for GFP when all the cells were infected, however the amounts of viral proteins synthesised were low in both cells which showed intensive GFP expression. The pattern of viral protein synthesis was shown as dots. In contrast, both Vero cells and A549/Npro cells showed high levels expression of viral antigen (Figure 3.3.2.2 10^{-1} and 10^{-2} PFU/cell).The viral proteins were synthesised in an evenly distributed pattern in the cytoplasm. This indicates these cells are better able to support the virus replication compared to A549/pr(IFN-β).GFP/V cells, suggesting that the mutant viruses that activate the IFN response also induce an IFN-independent antiviral state though IRF-3 activation.

As well as being essential for the activation of the IFN-β promoter, IRF-3 can also up-regulate the expression of a subset of ISGs directly, independent of IFN, including ISG56 (Grandvaux *et al.*, 2002). Therefore, we also determined the level of ISG56 expression in order to further investigate the establishment of an IFN-independent antiviral state induced by IRF-3 following the previous studies. Thus, if ISG56 expression can be detected in cells in which IFN signalling is inhibited (A549/V cells) but not in cells where IRF-3 is degraded (A549/Npro cells), this would confirm that

antiviral state is induced directly *via* IRF-3 but independent of IFN. If this was the case then the A549/pr(IFN-β).GFP/V cells may enter in an antiviral state following activation of IRF-3 by mutant viruses that activate the IFN-β promoter. This may also therefore help explain the reason why very low levels of viral antigen were detected in A549/pr(IFN-β).GFP/V cells that were strongly positive for GFP. In this study of ISG56 expression, the virus stocks used were MuV Enders cl3/30 and MuV Enders (DI). The MuV Enders (DI) stock has been characterised as a good IFN inducer in previous studies. A549, A549/V and A549/Npro cells were mock infected or infected with MuV Enders cl3/30 or MuV Enders (DI) at 5 PFU/cell. At 24hpi, expressions of ISG56 and actin were detected in the total cell extracts by immunoblot analysis. Results shown in Figure 3.3.2.3 revealed that cells intact for IFN and IRF-3 (A549s) and cells deficient in IFN (A549/Vs) induced a significant amount of ISG56 in response to DIs, whereas in cells deficient in IRF-3 pathway (A549/Npros) did not, confirming that ISG56 expression could occur independently of IFN pathway through the activation of IRF-3. This also provides evidence for the establishment of an antiviral state in the PIV5-V expressing cells *via* an IFN-independent pathway.

Having undertaken a series of characterisations and modifications of the GFP reporter cells, we conclude that (i) expression of BVDV-Npro in the A549/pr(IFN-β).GFP reporter cells blocks the activation of IRF-3, therefore the reporter cells are no longer able express GFP (Figure 3.2.2.1); (ii) expression of PIV5-V protein in the A549/pr(IFN-β).GFP reporter cells prevents the antiviral activity *via* IFN response, however the antiviral state induced *via* IRF-3 still occurred. As a consequence, we may be able to use the A549/pr(IFN-β).GFP/V cell-line to initially isolate mutant viruses by FACS, but we may need to use other cell-lines deficient in their IFN-independent antiviral response to grow the isolated mutant viruses. Unfortunately, by knocking our IRF-3 we lose the opportunity to monitor the mutant viruses' ability to induce IFN by GFP expression during the amplification process.

3.4 Generation of the A549/pr(IFN-β).GFP.V5 and the A549/pr(IFN-β).GFP.V5/V reporter cell-lines

There are safety issues about sorting virus-infected cells by FACS. Therefore we developed an alternative strategy for isolating cells in which the IFN-β promoter had been activated. The strategy was to express an epitope tag on the cell surface when the IFN-β promoter was activated, thereby facilitate antibody selection of cells expressing the epitope tag. The V5 epitope of PIV5 was chosen because of the availability of the antibody in the laboratory (The V5 epitope tag is derived from a small epitope (Pk) present on the P and V proteins of PIV5). The surface expression of the epitope was achieved by cloning the epitope tag sequence into a commercially available vector pDisplay™ (Invitrogen Ltd., UK) (Figure 3.4.1.1). The expressed epitope is flanked at its N-terminus with Ig κ-chain leader sequence in the pDisplay™ vector, which directs the protein to the secretary pathway, and at its C-terminus with the platelet derived growth factor receptor (PDGFR) transmembrane domain, which anchors the protein to the plasma membrane, displaying the V5 tag on the extracellular side. The gene sequence encoding the epitope tag fused to the transmembrane domain was subcloned into a pdl'pr(IFN-β).GFP lentivirus vector such that its expression was under the control of IFN-β promoter. The generated lentivirus plasmid was then used to make lentiviruses and infect cells to generate a reporter cell-line, in which the epitope tag would be used as a selection marker and expressed on the cell surface driven by the IFN-β promoter.

In more detail, two complementary oligonucleotides (Eurogentec Ltd., UK) encoding the V5 epitope tag were made,

Forward

5'-GATCTGGAAAGCCGATCCCAAACCCTCTATTAGGTCTGGACTCCACCCTGCA-3'

Reverse

3'-ACCTTTCGGCTAGGGTTTGGGAGATAATCCAGACCTGAGGTGGG-5'

These were annealed at 95^{o}C, slowly cooled down to room temperature and then cloned into the pDisplay™ vector between the *Bgl*II and *Pst*I sites. 6μg of the

construct and 9μg of FuGENE® 6 were mixed with 100μl of serum free DMEM, incubated at room temperature for 30 mins and then used to transfect a monolayer of 293T cells growing in a 25cm^2 tissue culture flask. Transfected 293T cells were fixed and immunostained for V5. Figure 3.4.1.2 Step 3a showed 10% of the transfected cells expressed V5 epitope on their surface, confirming the cloning of V5 epitope sequence into the pDisplay™ vector was successful. The successfully cloned construct was named as pDisplay.V5.

In order to test whether cells which express V5 epitope tag on their surface can be separated from the ones do not, using an antibody-dependent selection method, a panning experiment was carried out. Pre-incubation of petri dish with antibody facilitates the binding of antibody to the plastic. Two 60mm dishes were either incubated with PIV5-V antibody (10μg/sample) or with PBS (control) at 4^{o}C overnight. Both petri dishes were then rinsed with ice-cold PBS (control) to wash away any unbound antibodies. Two 75cm^2 tissue culture flasks of 293T cells transfected with pDisplay.V5 construct were treated with EDTA (1mM) in PBS at 37^{o}C for 5 mins to detach the cells from the surface of the tissue culture flask. The cell suspension was centrifuged to remove EDTA/PBS, resuspended to single cell suspension with 4ml/sample of ice-cold PBS, added to either the plate pre-coated with anti-PIV5-V antibody or the control plate and incubated for 1h to allow cells to bind to the antibody. Each plate was rinsed with PBS to wash away any unbound cells. Figure 3.4.1.2 Step 3b showed a significant number of cells bound to the plate pre-coated with antibody, whereas only a few cells bound to control plate. This confirmed that using an antibody based method to separate cells expressing the V5 epitope tag on their surface was possible.

The fragment enconding the V5 tag linked to the transmembrane domain of pDisplay™ vector was digested from the pDisplay™ backbone vector between the *BamH*I and *Not*I sites and ligated into the pdl'pr(IFN-β).GFP lentivirus backbone vector (Figure 3.1.1.1) by replacing eGFP between the *BamH*I and *Not*I sites. The

resulting lentivirus vector (Figure 3.4.1.2 Step 4) contains the cell surface-expressing V5 epitope and *pac* gene under the control of IFN-β promoter. In order to test whether the molecular cloning of the pDisplay.V5 fragment into the pdl'pr(IFN-β).GFP lentivirus backbone vector was successful, the lentivirus plasmid together with packaging plasmids pCMVR8.91 and pVSV-G were co-transfected into 293T cells to produce the lentivirus, which was to be tested. Supernatant containing lentiviruses was harvested at 2 days post transfection and then used to infect A549/pr(IFN-β).GFP or A549/pr(IFN-β).GFP/V cells. Both reporter cell-lines were used as parental cell-lines. As a consequence, the generated cell-line would not only express GFP in the cytoplasm, but also express V5 epitope tag on the cell surface, when the IFN-β promoter was activated. After infection with lentiviruses, a small portion of the cells were separately cultured on coverslips and transfected with dsRNA to activate the IFN-β promoter (refer to Chapter 3.1.2 polyI:C transfection). At 24h post transfection, the cells were fixed, immunostained for V5 and examined microscopically (data not shown) for cell surface V5 epitope expression. The immunofluorescence data confirmed 10% of cells expressed V5 epitope on the cell surface and therefore the molecular cloning of pDisplay.V5 fragment into the pdl'pr(IFN-β).GFP lentivirus backbone vector was successful. The lentivirus plasmid was termed as pdl'pr(IFN-β).V5. Both the A549/pr(IFN-β).GFP and the A549/pr(IFN-β).GFP/V cells infected with pdl'pr(IFN-β).V5 lentivirus were then subjected to subcloning to generate homogenous cell-lines, in which the V5 epitope was expressed on the cells when the IFN-β promoter was activated. Lentivirus-infected cells were directly subcloned into 96-well microtitre plates and the process was the same as for the generation of A549/pr(IFN-β).GFP cell-line (Chapter 3.1.3). One cell colony from both the A549/pr(IFN-β).GFP cell-line and the A549/pr(IFN-β).GFP/V cell-line which also expressed the V5 epitope on cell surface with the highest percentage of cells that was V5-positive was selected. A549/pr(IFN-β).GFP.V5 cell-line (referred to as GFP.V5 cell-line in figures) and a A549/pr(IFN-β).GFP.V5/V cell-line (referred to as GFP.V5/V cell-line in figures) were generated. Subsequent characterisation assays were carried out to further investigate the properties of the cell-lines.

3.5 Characterisation of A549/pr(IFN-β).GFP.V5 reporter cell-line

To initially characterise the A549/pr(IFN-β).GFP.V5 cells, immunofluorescence was performed to determine how quickly the V5 epitope tag was expressed on the cells surface when the IFN-β promoter was activated following virus infection. A549/pr(IFN-β).GFP.V5 cell cultured on coverslips were either mock infected or infected with PIV5 VΔC (P2) at an MOI of 5 PFU/cell and fixed at 4, 8, 16 and 24hpi. GFP-positive cells and cell surface expression of V5 epitope tag, following immunostaining, were visualised by fluorescence microscopy. Immunofluorescence results (Figure 3.5.1) confirmed both the surface V5 expression and GFP expression were induced when the IFN-β promoter was activated. These studies showed an increase in the expression of GFP starting from 4hpi to 8hpi and which remained stable until at least 24hpi. The expression of the surface V5 epitope however was not as stable as GFP. There was an increase in the intensity of the surface V5 epitope signal starting from 8hpi. Although the expression was strong at 16hpi, this was significantly reduced by 24hpi. Given the fact that further mutant virus isolation would be based on the expression of surface V5 epitope, this expression peak should be taken into account.

One explanation for the above results is that following infection with PIV5 VΔC (P2), the IFN-β promoter is activated, but subsequently it gets switched off, and that the stability of the V5 tagged protein is much less than GFP. To compare the stability of GFP and surface V5 expression further, and characterise the expression half-life of the V5 epitope tag, a comparable immunofluorescence experiment was carried out. A549/pr(IFN-β).GFP.V5 cells grown on coverslips were either mock infected or infected with MuV Enders (DI) at an MOI of 5 PFU/cell. Cycloheximide (CHX) (50μg/ml) was added into the culture media at 12hpi to block further protein synthesis. In the presence of CHX, we can compare the stability of GFP with the V5 epitope. Cells were then fixed at 12(0), 16(4), 20(8) and 24(12)hpi (post CHX treatment) and immunostained for V5. Results (Figure 3.5.2) were in accordance with the original characterisation experiment (Figure 3.5.1), and re-confirmed the cell surface

expression of the V5 epitope tag was transient. The expression peaked between 12-16hpi, and decreased from 20hpi. In contrast, cells remained GFP-positive even at 12h post CHX treatment. In conclusion, if the V5 surface expression is to be used as a selection marker for isolating mutant viruses, then the isolation process should be performed within 12-16hpi to separate the V5 positive cells from cells that are negative for V5.

3.6 Panning selection of IFN-inducing mutant viruses using A549/pr(IFN-β).GFP.V5/V reporter cell-line

Having generated and characterised the A549/pr(IFN-β).GFP.V5/V cell-line, we then tried to use A549/pr(IFN-β).GFP.V5/V cell-line to isolate mutant viruses that induce an IFN response. The strategy was to infect the cell-line with PIV5 W3 virus and use antibody selection of V5 expressing cells. Any cell infected with a mutant virus within the wildtype virus population that induces the IFN-β promoter would express V5 epitope tag on the cell surface. To separate those V5 epitope tag expressing cells from the cells that were not, a panning selection was carried out using Dynabeads® Protein A.

Monolayers of A549/pr(IFN-β).GFP.V5/V cells cultured in 75cm^2 tissue culture flasks were infected with PIV5 W3 with an MOI of 1 PFU/cell. At 12hpi, infected cells were treated with EDTA (5mM) in PBS to detach the cells from the surface of the tissue culture flask. The cell suspension was centrifuged to remove EDTA/PBS, resuspended to single cell suspension with 1ml/sample of ice-cold EDTA (1mM) in PBS. Anti-PIV5-V antibody (5µg/ml) was added to the cell suspension and incubated with tumbling for 30 mins. During the incubation, anti-PIV5-V antibody bound to the V5 epitope displayed on the cell surface. The unbound antibody was then washed away with ice-cold PBS by centrifugation. Supernatant was removed and the cell pellet was resuspended into single cell suspension with 1ml fresh ice-cold PBS. Dynabeads® Protein A (10µl/sample) were added to cell suspension and incubate for 30 mins to allow antibody binding to the protein A of the beads. Each sample was then placed

again on a magnet. The beads migrated towards the magnet side, any unbound cells remained in the supernatant and were removed by washing with PBS. Selected cells were added onto a preformed monolayer of A549/pr(IFN-β).GFP/V cells in a 25cm^2 tissue culture flask to allow virus propagation approximately for 3 days until a CPE was observed. Culture medium was collected and virus was titrated. Fresh monolayers of A549/pr(IFN-β).GFP/V were infected with PIV5 W3, rescued PIV5 W3 or PIV5 VΔC (P2) (control) at an MOI of 5 PFU/cell and fixed at 16hpi. GFP expression and viral antigen (PIV5-NP&P), following immunostaining, were visualised using fluorescence microscopy. Results (data not shown) were the same as what we observed from FACS mutant virus isolation. There was no significant increase in the number of GFP-positive cells infected by viruses rescued from the magnetic beads separation. Unfortunately, using a panning method to isolate mutant viruses was not successful. The reason(s) for this may be the same as why we failed to isolate mutant viruses from the FACS sorting experiment and will be considered in the Discussion.

3.7 Applications of the A549/pr(IFN-β).GFP reporter cell-line(s)

3.7.1 Screening for compounds that inhibit IFN induction

Compounds that inhibit IFN production may be developd as anti-inflammatory compounds to treat certain autoimmune diseases where there is over-inflammation caused by over-production of IFN and may be harmful to individuals. These compounds may potentially be used in the laboratory as potential drugs to mimic virus antagonists that inhibit IFN production. Therefore, development of a rapid method for high throughput screening assays to search for compounds that inhibit IFN production may be of pharmaceutical companies' interest in the aspect of drug development. Potentially, the A549/pr(IFN-β).GFP cell-line could be used for high throughput screening assays to screen compounds that inhibit IFN production by monitoring GFP expression. Collaborative studies have been carried out with Medical Research Council Technology (MRCT) at Mill Hill, UK. In an independent project they identified a TBK-1 inhibitor, known as compound X, as a potential anti-cancer agent. TBK-1 is critical for the phosphorylation of IRF-3 (McWhirter *et al.*, 2004;

Oganesyan *et al.*, 2006) and causes its activation and nuclear translocation to induce IFN-β. In order to demonstrate the potential of the A549/pr(IFN-β).GFP cell-line for high throughput screening and also to characterise compound X's ability to inhibit IFN induction (potentially binding to TBK-1), a series of GFP screening assays were performed using the A549/pr(IFN-β).GFP cell-line. Monolayers of the cells grown on coverslips in a 24-well microtitre plate were pretreated with compound X of various concentrations (0-10μM) for 30 mins and then either mock infected or infected with PIV5 VΔC (P2) in the presence of X at an MOI of 10 PFU/cell. If TBK-1 is targeted by X, then GFP expression should be inhibited in the A549/pr(IFN-β).GFP cells when infected with PIV5 VΔC (P2). The infected A549/pr(IFN-β).GFP cells were fixed at 10hpi. GFP expression and viral antigen (PIV5-V) synthesis, following immunostaining, were visualised by fluorescence microscopy (Figure 3.7.1.A). Culture media of the infected cells was collected and inactivated by UV treatment to kill any residual viruses at 10hpi, and IFN level from each sample was measured by CPE reduction bioassay for IFN (Figure 3.7.1.B). Both fluorescence microscopy and CPE reduction bioassay for IFN showed that PIV5 VΔC (P2) induced GFP expression (Figure 3.7.1.A) and IFN production (Figure 3.7.1.B) in the absence of compound X. Compound X inhibited IFN induction (Figure 3.7.1.B) and GFP expression (Figure 3.7.1.A) but also led to cell death at concentrations of 5μM and above (Figure 3.7.1.A). The optimal concentration of compound X to inhibit IFN induction was 2.5μM. Based on these findings, A549/pr(IFN-β).GFP cells are eligible as a methodology to test drugs which target the IFN induction cascade.

Figure 3.1.1.1

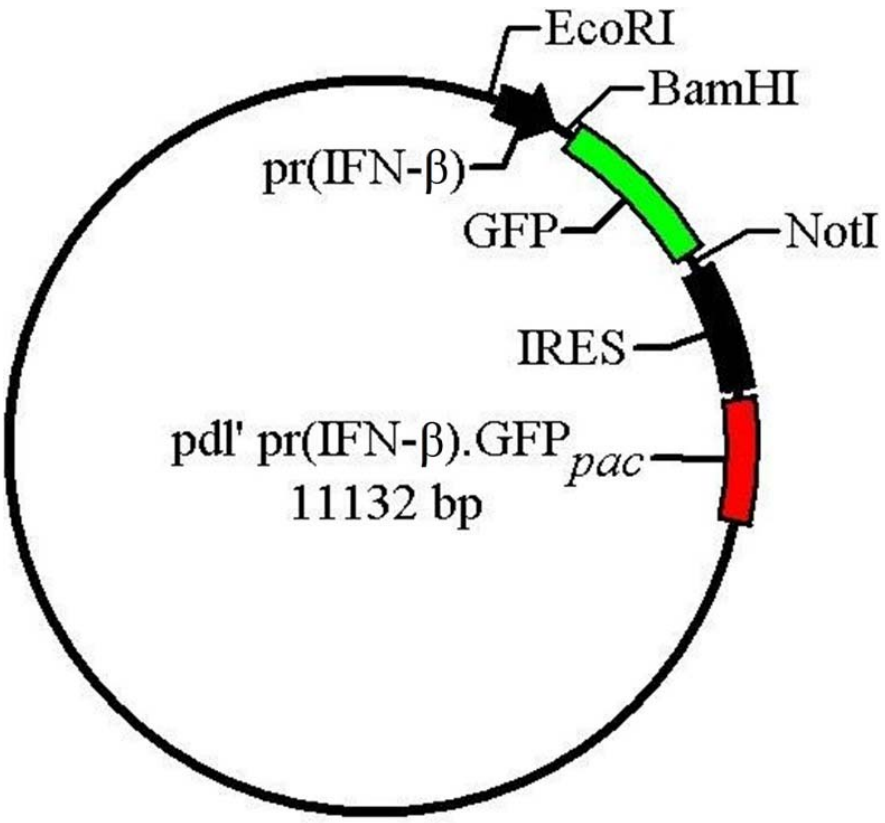


Figure 3.1.1.1 Schematic representation of the pdl'pr(IFN-β).GFP lentivirus vector.

The pdl'pr(IFN-β).GFP lentivirus vector expressing GFP and puromycin resistance gene (*pac*) under the control of the IFN-β promoter. An internal ribosome entry site (IRES) is located downstream of GFP and upstream of *pac*, to allow for translation initiation in the middle of an mRNA sequence for protein synthesis.

Figure 3.1.1.2

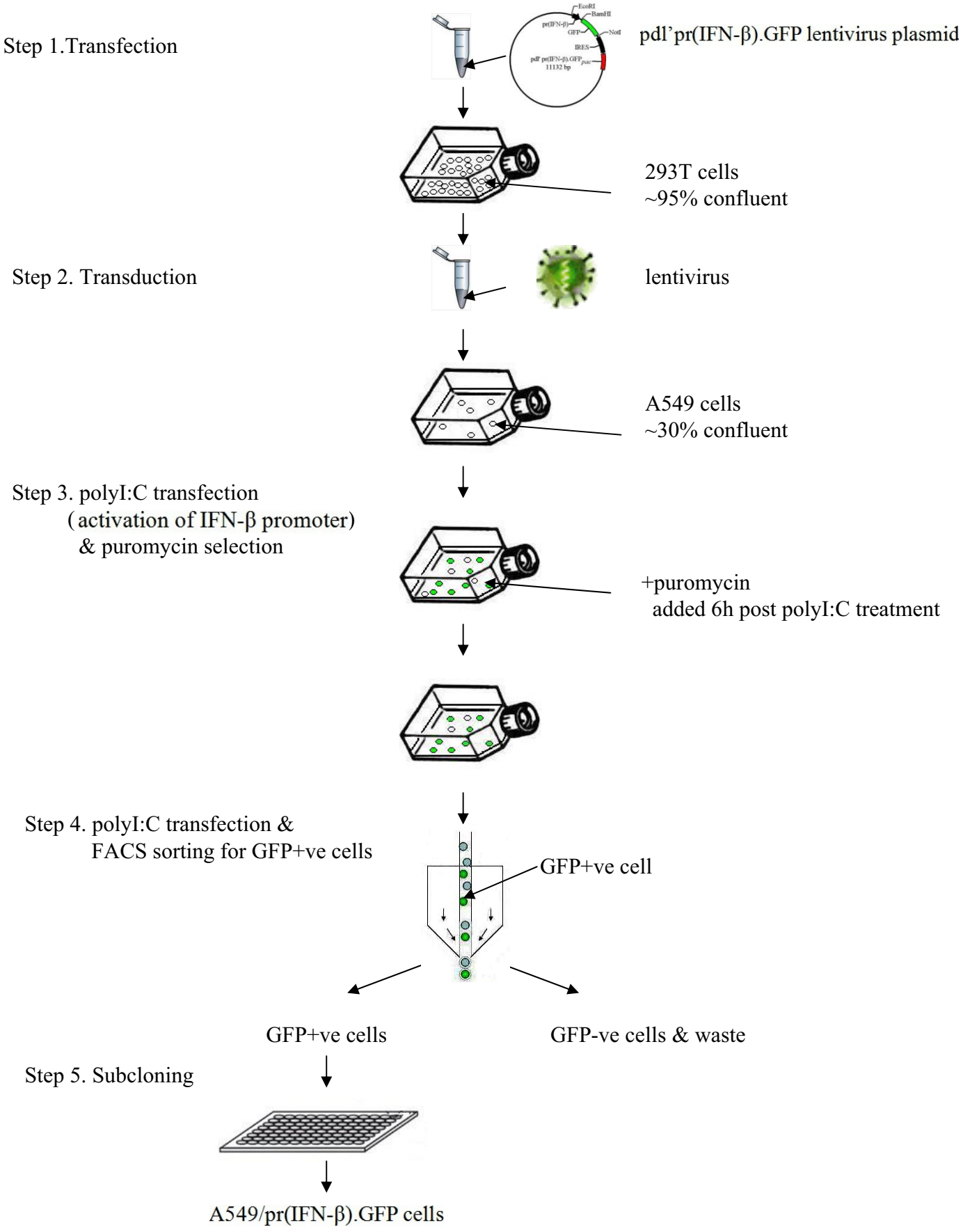

Step 1.Transfection
pdl'pr(IFN-β).GFP lentivirus plasmid
293T cells
~95% confluent
Step 2. Transduction
lentivirus
A549 cells
~30% confluent
Step 3. polyI:C transfection
(activation of IFN-β promoter)
& puromycin selection
+puromycin
added 6h post polyI:C treatment
Step 4. polyI:C transfection &
FACS sorting for GFP+ve cells
GFP+ve cell
GFP+ve cells
GFP-ve cells & waste
Step 5. Subcloning
A549/pr(IFN-β).GFP cells

Figure 3.1.1.2 Generation of the A549/pr(IFN-β).GFP cell-lines.

Step 1. The lentivirus plasmid pdl'pr(IFN-β).GFP was co-transfected into 293T cells with packaging plasmids: pCMVR8.91 and pVSV-G. The lentivirus supernatant was harvest at 48 & 72h post transfection. The resulting supernatant was then centrifuged to remove cell debris.

Step 2. Then the lentivirus supernatant was used to infect naive A549 cells.

Step 3. To activate the IFN-β promoter, lentivirus transduced cells were transfected with polyI:C, and at 6h post polyI:C stimulation, puromycin was added to the culture medium to kill any non-transduced cells.

Step 4. The lentivirus transduced cells were then further sorted by FACS machine for GFP-positive cells when the IFN-β promoter is activated.

Step 5. The FACS sorted cells were subcloned using 96-well microtitre plates to generate homologous cell-lines – the A549/pr(IFN-β).GFP cell-lines.

Figure 3.2.1.1
A

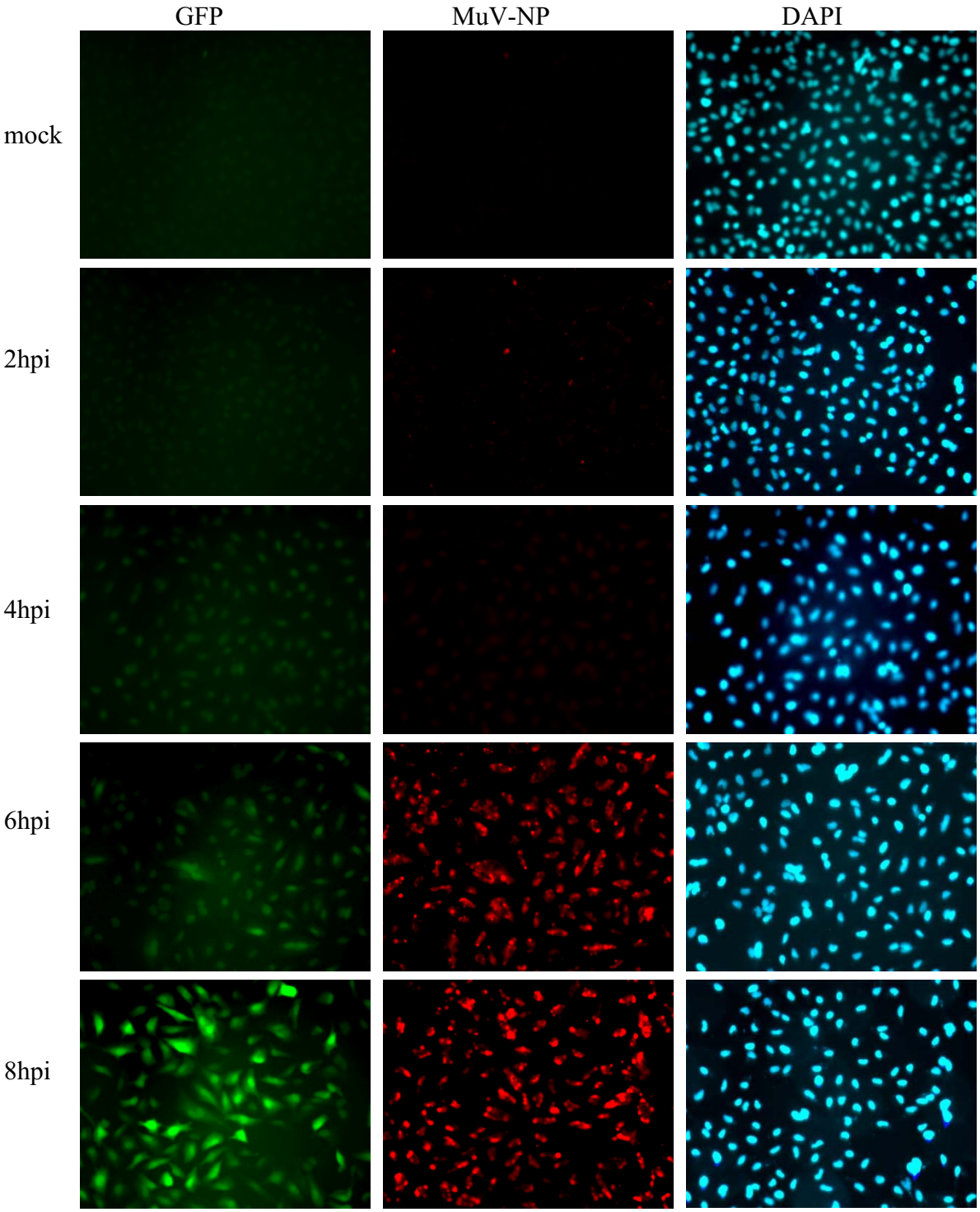


Figure 3.2.1.1
B

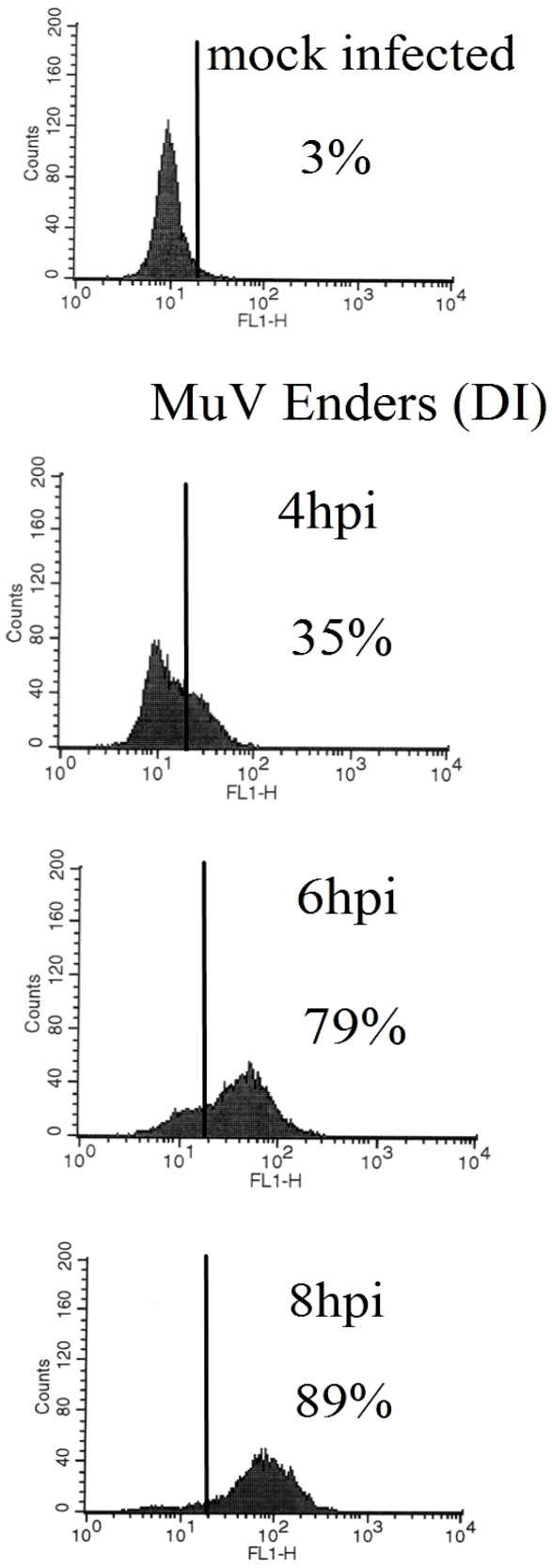


Figure 3.2.1.1 Characterisation of the A549/pr(IFN-β).GFP reporter cell-line in the ability to induce GFP regulated by IFN-β promoter.

A. Immunofluoresence visualisation of GFP expression *vs* viral protein (MuV-NP) synthesis at various time points.
B. FACS analysis of GFP expression intensity *vs* time.

Monolayers of A549/pr(IFN-β).GFP cells were infected with MuV Enders (DI) of an MOI of 10 PFU/cell. Cells were fixed at 2, 4, 6 & 8hpi.

MuV viral protein expression was detected from 6hpi by immunofluorescence and GFP expression was observed from 8hpi. FACS analysis showed 35% of the cells were GFP-positive by 4hpi and significant increases in the number of GFP-positive at 6 & 8hpi.

Figure 3.2.1.2
A

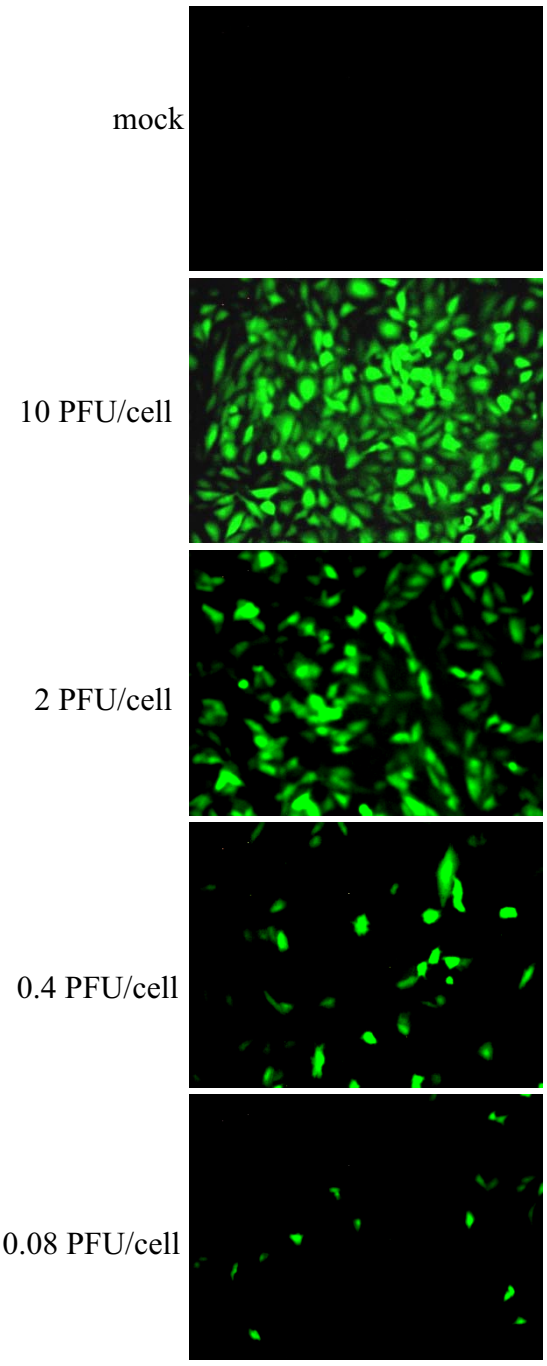


Figure 3.2.1.2
B

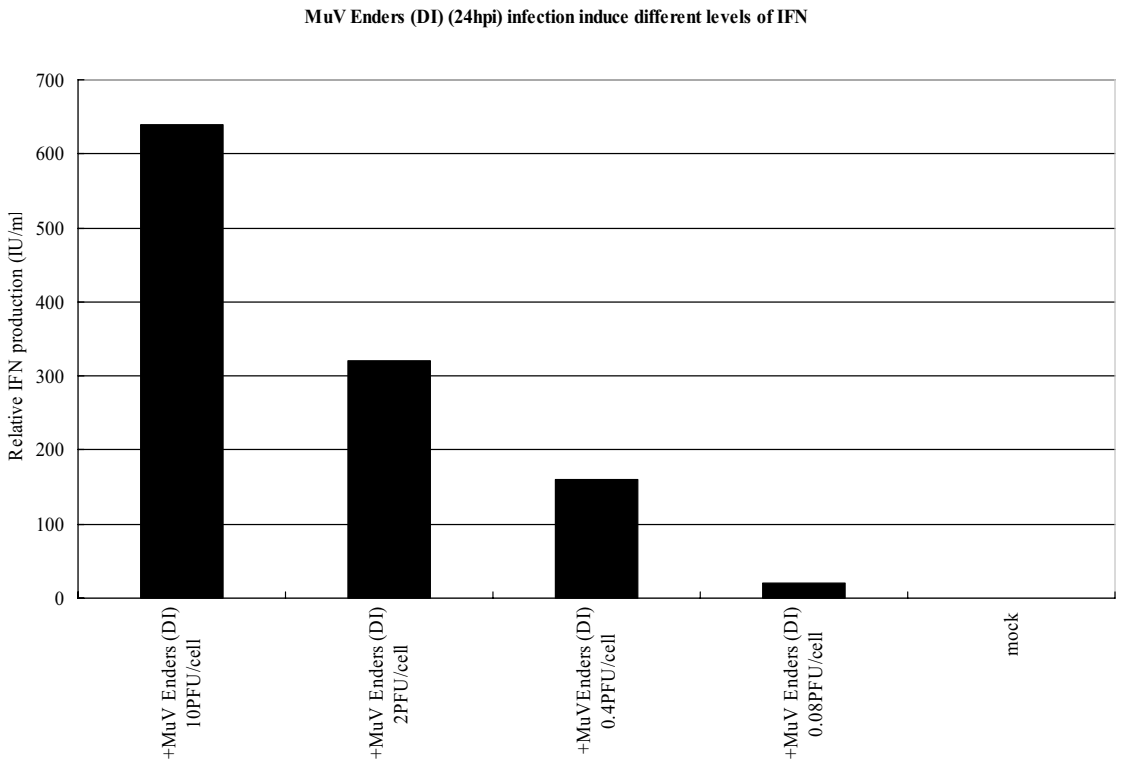


Figure 3.2.1.2 GFP induction assay on A549/pr(IFN-β).GFP reporter cell-line.

A. MuV Enders (DI) virus infection with multiple MOIs on A549/pr(IFN-β).GFP reporter cells.
B. CPE reduction bioassay for IFN for the analysis of GFP and IFN correlation.

Monolayers of A549/pr(IFN-β).GFP reporter cells were cultured on coverslips in 24-well microtitre plates, and infected with MuV Enders (DI) at MOIs of 10, 2, 0.4 or 0.08 PFU/cell. The culture supernatants were harvested, and UV-inactivated to kill any residual viruses at 24hpi. CPE reduction bioassay for IFN was performed to determine the amount of IFN produced by each infection unit. The coverslips were fixed and immunostained for MuV-NP.

Figure 3.2.2.1

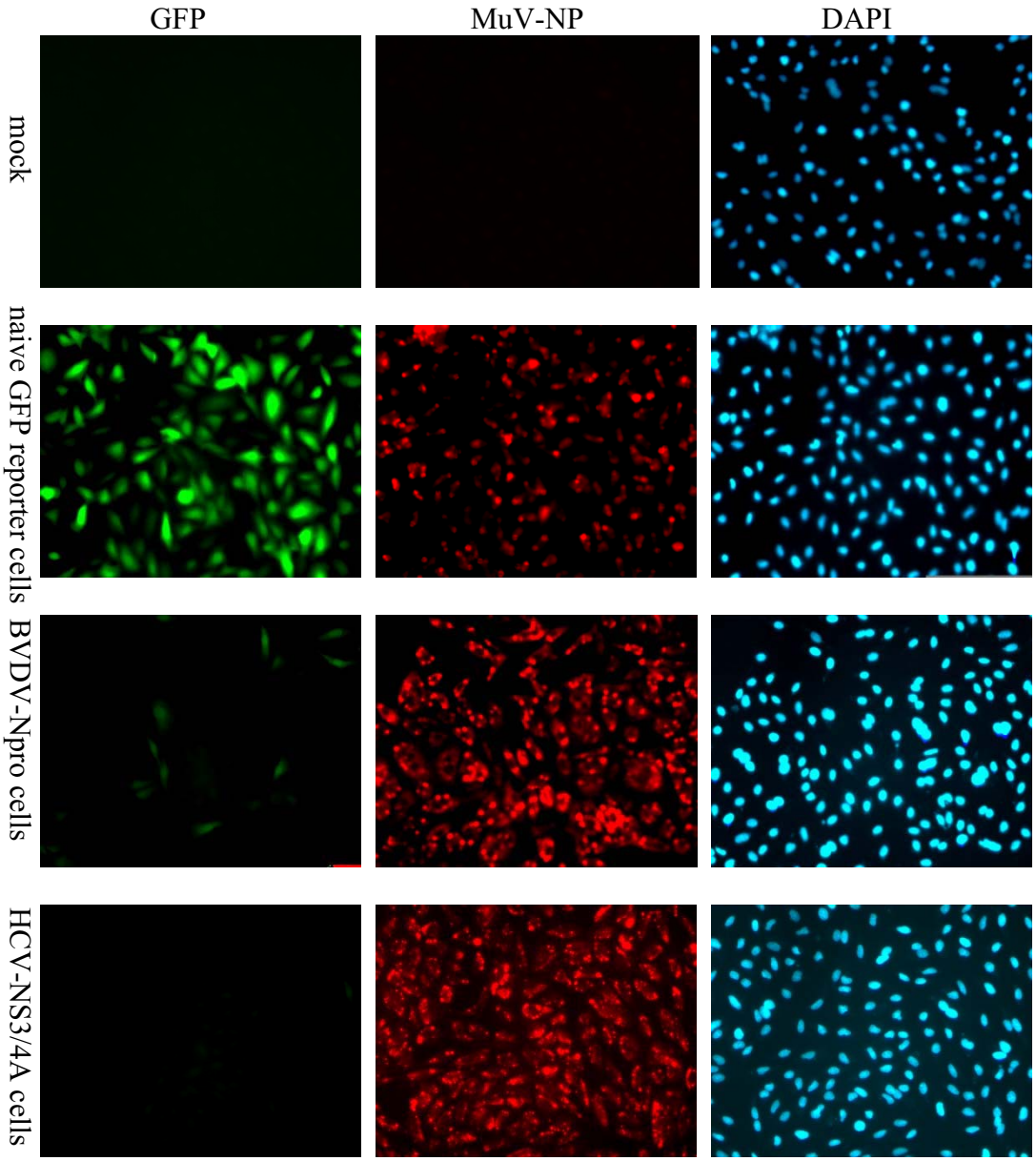

GFP
MuV-NP
DAPI
mock
naive GFP reporter cells
BVDV-Npro cells
HCV-NS3/4A cells

Figure 3.2.2.1 Effect of knocking out key signalling molecules involved in the IFN-induction cascade.

The A549/pr(IFN-β).GFP reporter cells were engineered to constitutively express either BVDV-Npro or HCV-NS3/4A to determine the effect of knocking out crucial signalling molecules on IFN induction and GFP expression. A549/pr(IFN-β).GFP, A549/pr(IFN-β).GFP/BVDV-Npro and A549/pr(IFN-β).GFP/HCV-NS3/4A cells were individually infected by MuV Ender (DI) virus at an MOI of 10 PFU/cell, and fixed at 16hpi and immunostained for MuV-NP.

The assay demonstrated that GFP expression was under the control of IFN-β promoter. Expression of either BVDV-Npro or HCV-NS3/4A prevented induction of GFP expression following infection of the cells with MuV Enders (DI).

Figure 3.2.3.1
A

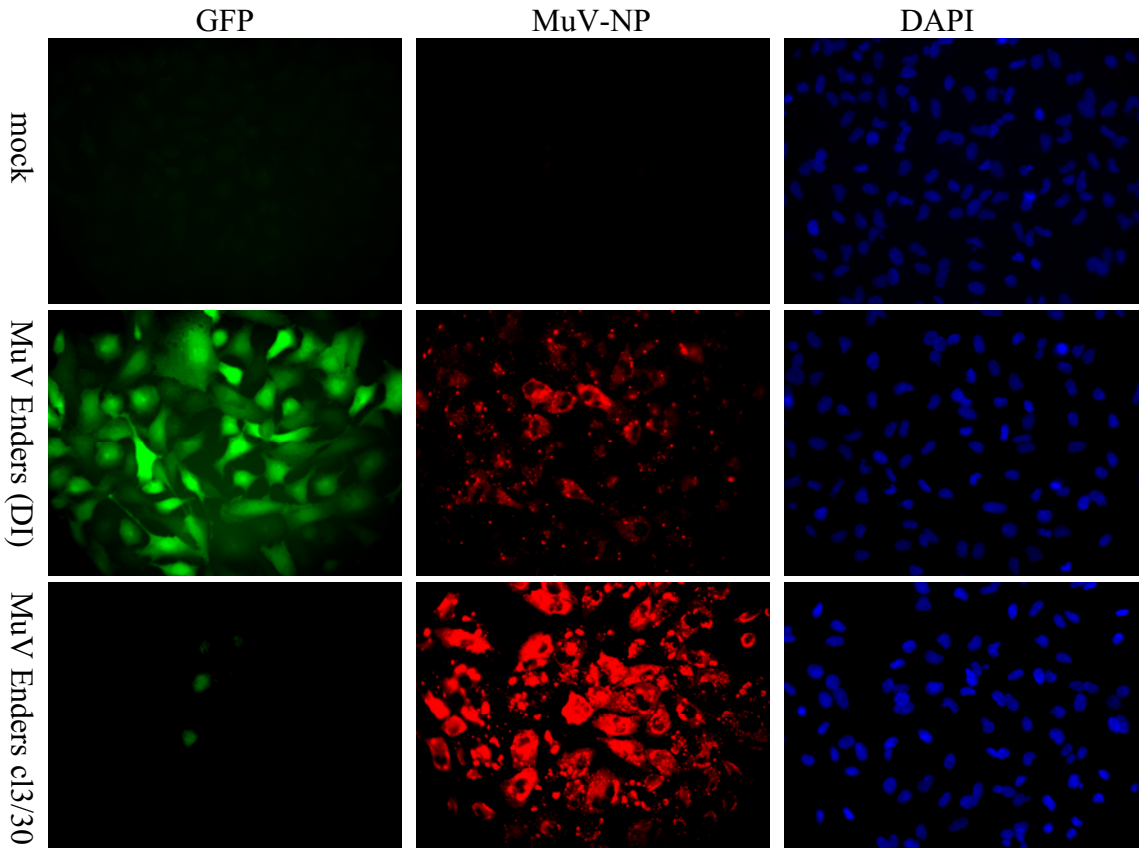


Figure 3.2.3.1
B

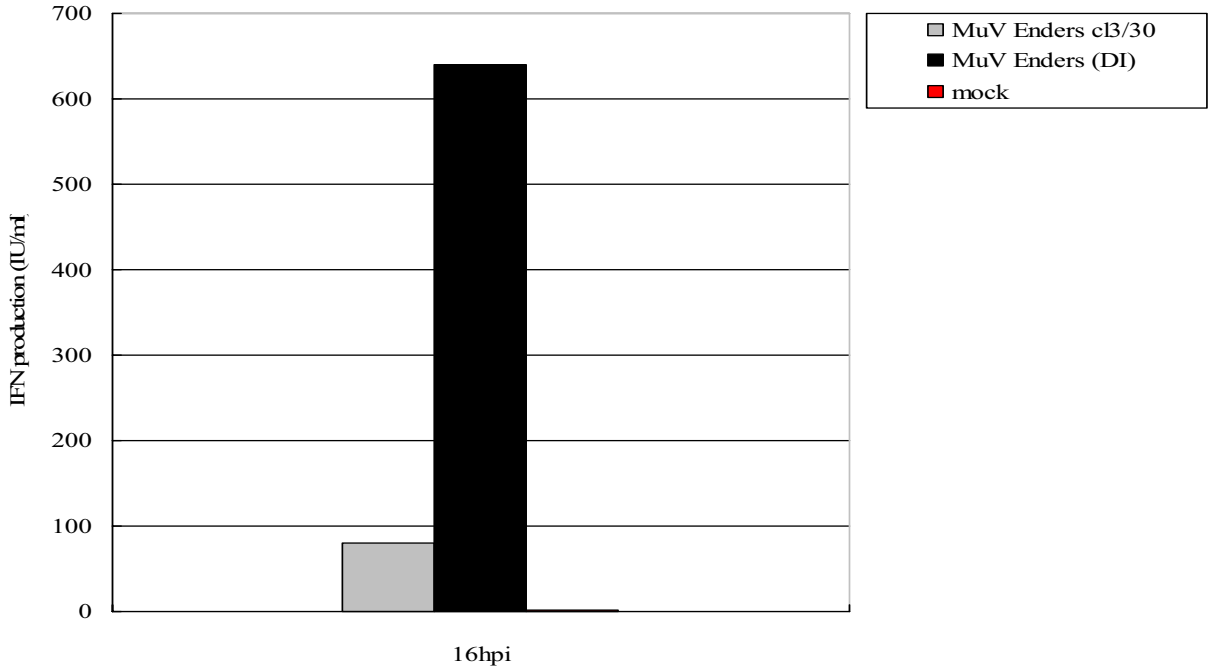


Figure 3.2.3.1 Investigation of DI-rich and DI-poor virus preparations in the ability to activate the IFN-β promoter using A549/pr(IFN-β).GFP reporter cells.

A. GFP expression following infections of A549/pr(IFN-β).GFP reporter cells with MuV Enders cl3/30 (DI-poor) or MuV Enders (DI) (DI-rich).
B. CPE reduction bioassay for IFN to quantify the IFN produced by the A549/pr(IFN-β).GFP reporter cells in response to MuV Enders cl3/30 or MuV Enders (DI) infection.

Monolayers of the A549/pr(IFN-β).GFP reporter cells were infected with MuV Enders cl3/30 or MuV Enders (DI) at an MOI of 5 PFU/cell. Cells were fixed at 16hpi and immunostained for MuV-NP. Supernatants were harvested and UV-inactivated to perform the CPE reduction bioassay to quantify the IFN produced by the cells.

DIs have better ability to induce IFN (>90% green cells) and the amount of IFN produced by MuV Enders (DI) infected cells was 8-fold higher than MuV Enders cl3/30 infected cells. A small portion of viruses within the MuV Enders cl3/30 population were able to induce IFN, but is significantly lower than the DIs.

Figure 3.2.4.1

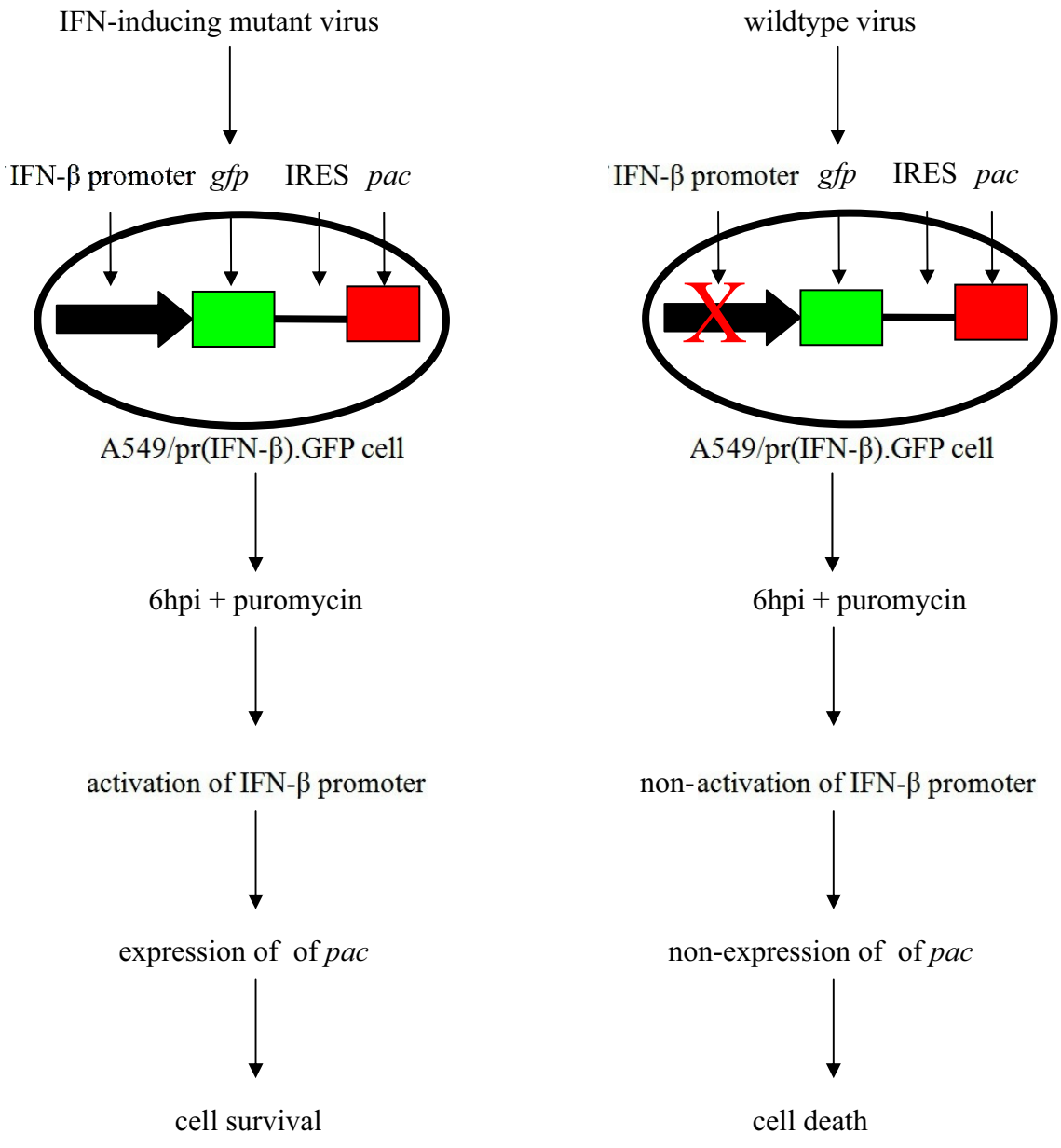

IFN-inducing mutant virus
IFN-β promoter gfp IRES pac
A549/pr(IFN-β).GFP cell
6hpi + puromycin
activation of IFN-β promoter
expression of of pac
cell survival
wildtype virus
IFN-β promoter gfp IRES pac
X
A549/pr(IFN-β).GFP cell
6hpi + puromycin
non-activation of IFN-β promoter
non-expression of of pac
cell death

Figure 3.2.4.1 Schematic representation of post-infection puromycin selection process in the A549/pr(IFN-β).GFP reporter cell-line that should ultimately lead to the selection of IFN-inducing mutant viruses from a wildtype virus population.

Figure 3.2.4.2

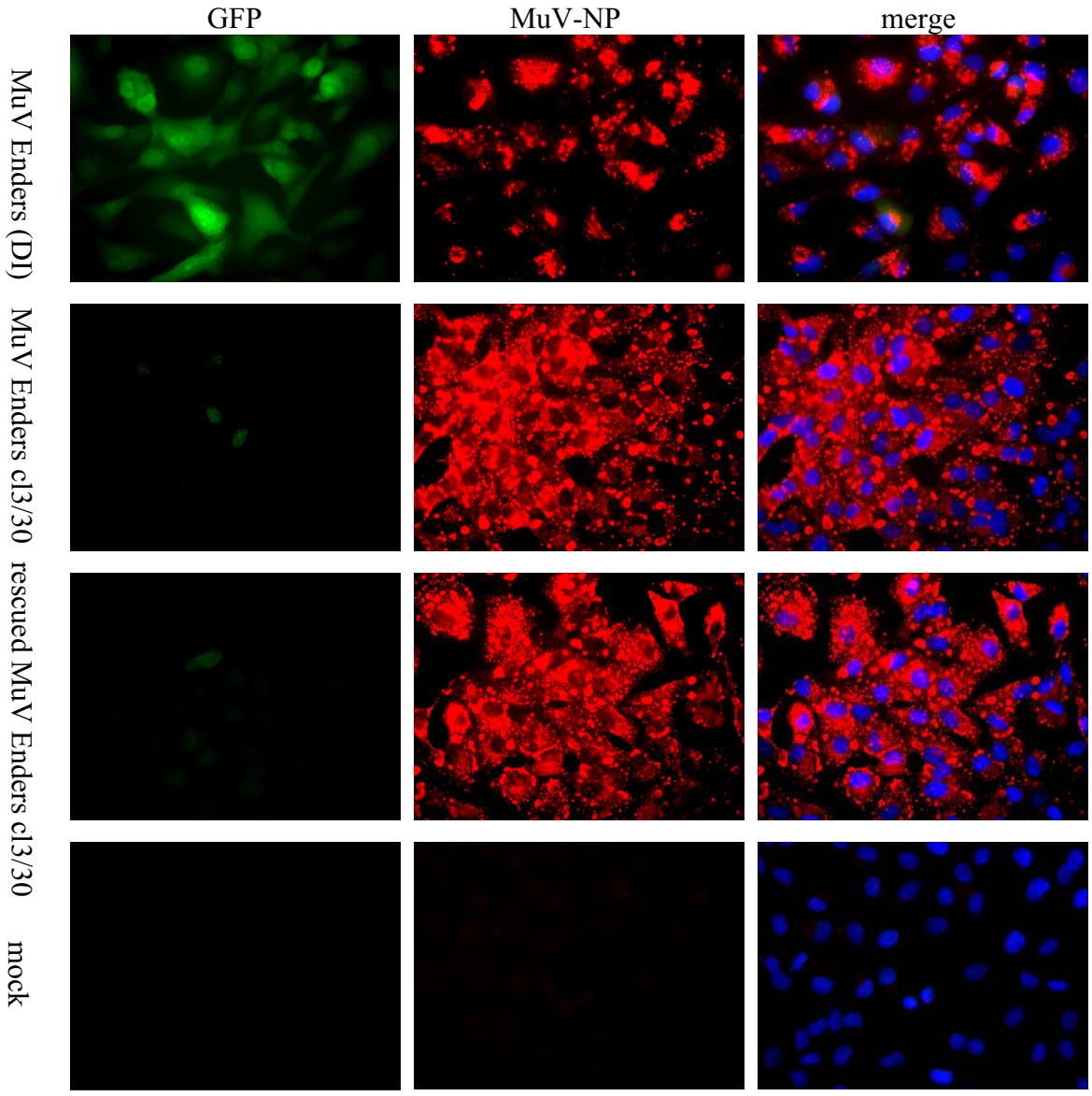

GFP
MuV-NP
merge
MuV Enders (DI)
MuV Enders cl3/30
rescued MuV Enders cl3/30
mock

Figure 3.2.4.2 Investigation of the puromycin selected viruses in the ability to induce IFN.

The A549/pr(IFN-β).GFP reporter cells were infected with MuV Enders (DI), MuV Enders cl3/30 or resuced MuV Enders cl3/30 viruses at an MOI of 5 PFU/cell. Cells were fixed at16hpi and immunostained for MuV-NP.

The rescued MuV Enders cl3/30 viruses were no better at inducing IFN (number of green cells *vs* virus infected cells) than the original MuV Enders cl3/30.

Figure 3.2.5.1

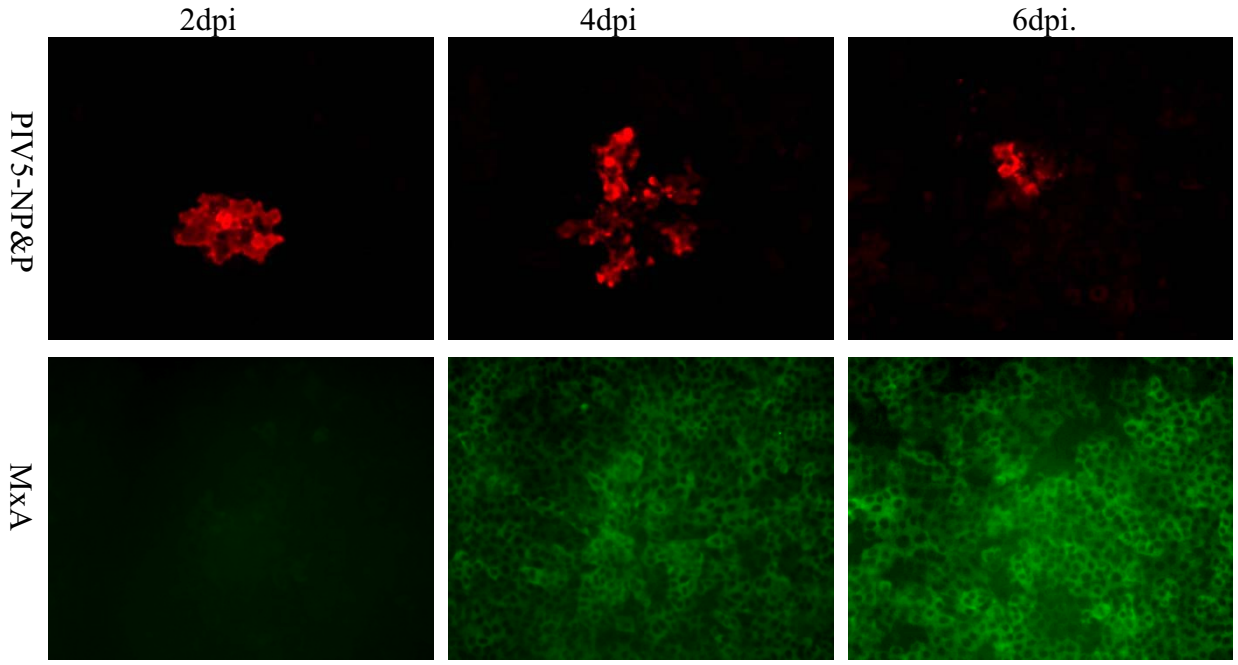


Figure 3.2.5.1 Investigation of IFN production in establishing an antiviral state to inhibit virus growth.

A549 cells were set up in 6-well plates and infected with PIV5 VΔC at an MOI of 0.01 PFU/cell. Monolayers of infected cells were fixed at 2, 4 & 6dpi and immnostained for PIV5-NP&P (Texas red) and MxA (FITC).

No significant virus plaque development was observed. However, an antiviral state (MxA) had been established and spread in cells neighbouring infected cells, confirming the production of IFN by virus-infected and neighbouring cells.

Figure 3.2.6.1

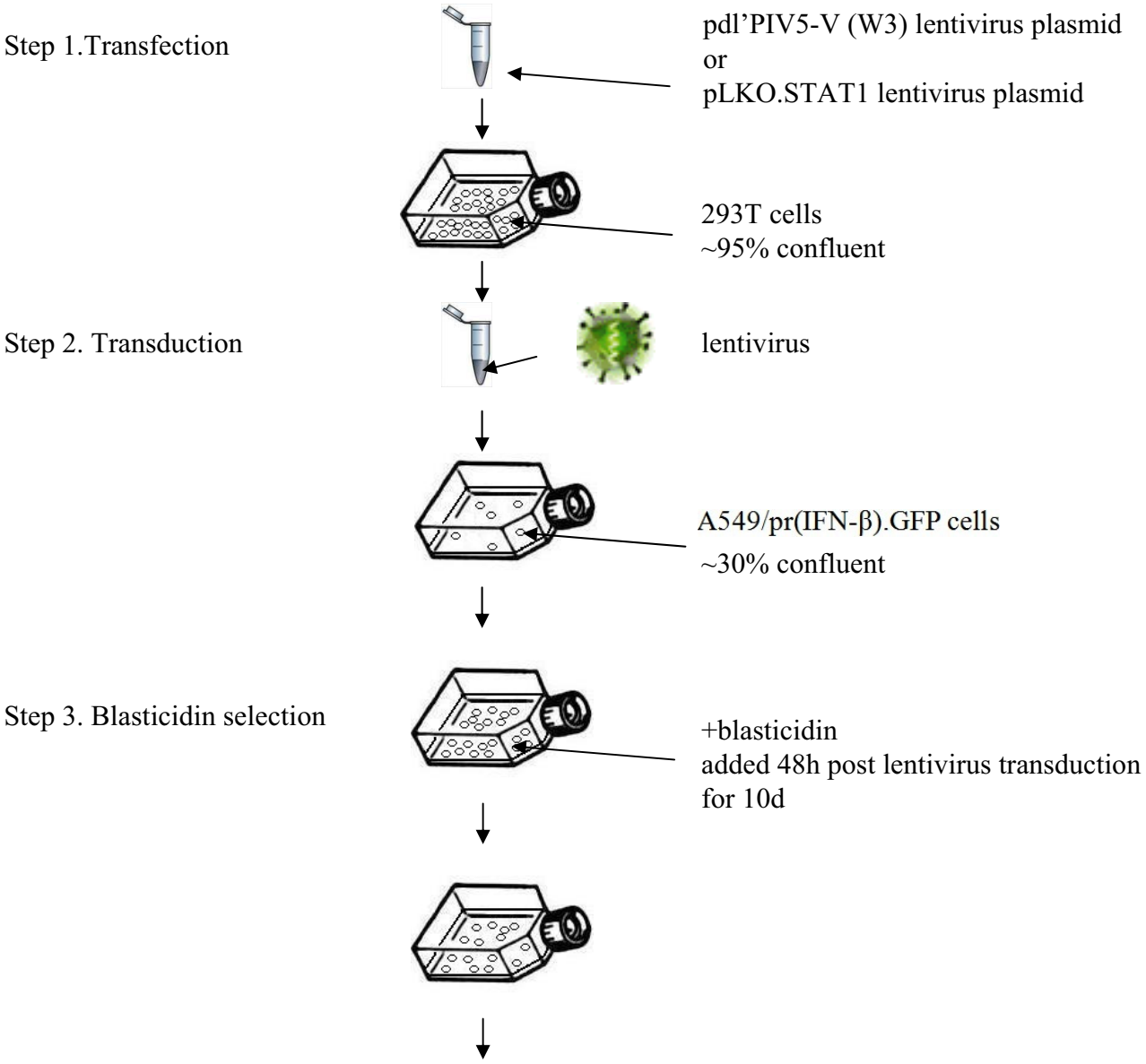

Step 1.Transfection
pdl'PIV5-V (W3) lentivirus plasmid
or
pLKO.STAT1 lentivirus plasmid
293T cells
~95% confluent
Step 2. Transduction
lentivirus
A549/pr(IFN-β).GFP cells
~30% confluent
Step 3. Blasticidin selection
+blasticidin
added 48h post lentivirus transduction
for 10d
Step 4. Characterisation of the cells for inhibition of STAT1 expression

Figure 3.2.6.1 Generation of the A549/pr(IFN-β).GFP/KO.STAT1 and the A549/pr(IFN-β).GFP/V cell-lines.

Step 1. The lentivirus plasmids expressing shRNA to STAT1 or PIV5-V(W3) were co-transfected into 293T cells with packaging plasmids: pCMVR8.91 and pVSV-G. The lentivirus supernatant was harvested at 48 & 72h post transfection. This supernatant was centrifuged to remove cell debris.

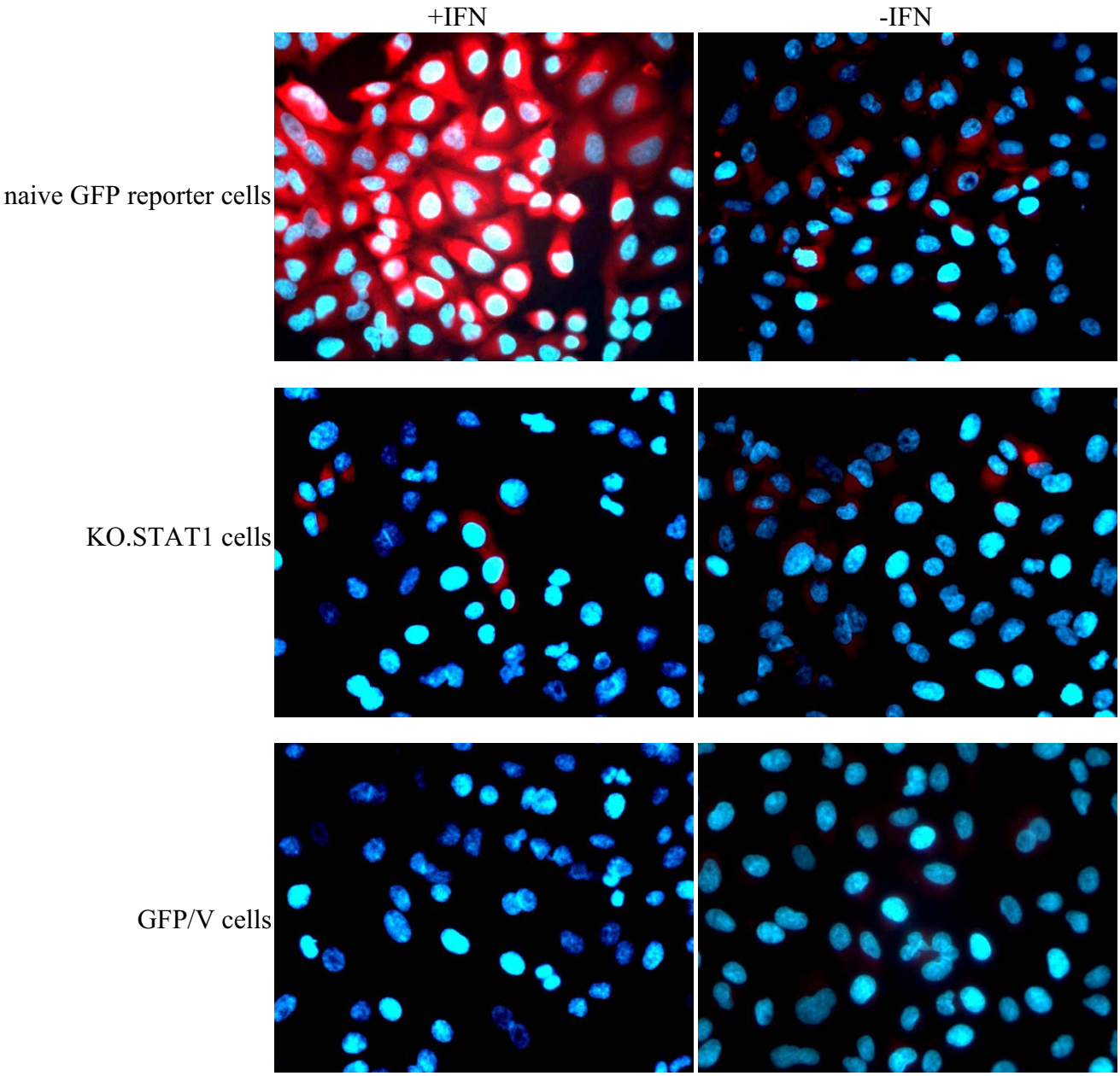
Step 2. The lentivirus supernatant was used to infect the A549/pr(IFN-β).GFP reporter cells.

Step 3. Lentivirus transduced cells were selected using blasticidin. The selection was performed 48h post lentivirus infection.

Step 4. The lentivirus transduced cells were then further characterised for inhibition of STAT1 expression (Figure 3.2.6.2).

Figure 3.2.6.2

A



B

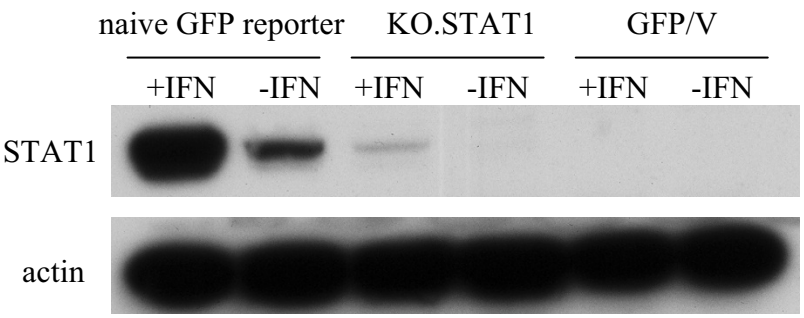


Figure 3.2.6.2 Expression of shRNA to STAT1 or PIV5-V significantly reduced STAT1 expression in A549pr(IFN-β).GFP reporter cells.

Monolayers of A549pr(IFN-β).GFP reporter cells transduced with shRNA to STAT1 or PIV5-V lentiviruses were cultured on coverslips in 24-well microtitre plates and were pre-treated with IFN-α (Roferon A) (10^4IU/ml) overnight.

A. Lentivirus transduced cells cultured on coverslips were fixed and immunostained for STAT1.
B. Lentivirus transduced cells cultured in 24-well microtitre plates were harvested. Lysates were subjected to SDS-PAGE and western blot.

Immunofluoresence and western blot date showed successful expression of shRNA to STAT1 or PIV5-V in A549pr(IFN-β).GFP reporter cells reduced STAT1 expression in A549pr(IFN-β).GFP/KO.STAT1 cells or inhibited it in A549pr(IFN-β).GFP/V cells.

Figure 3.2.7.1

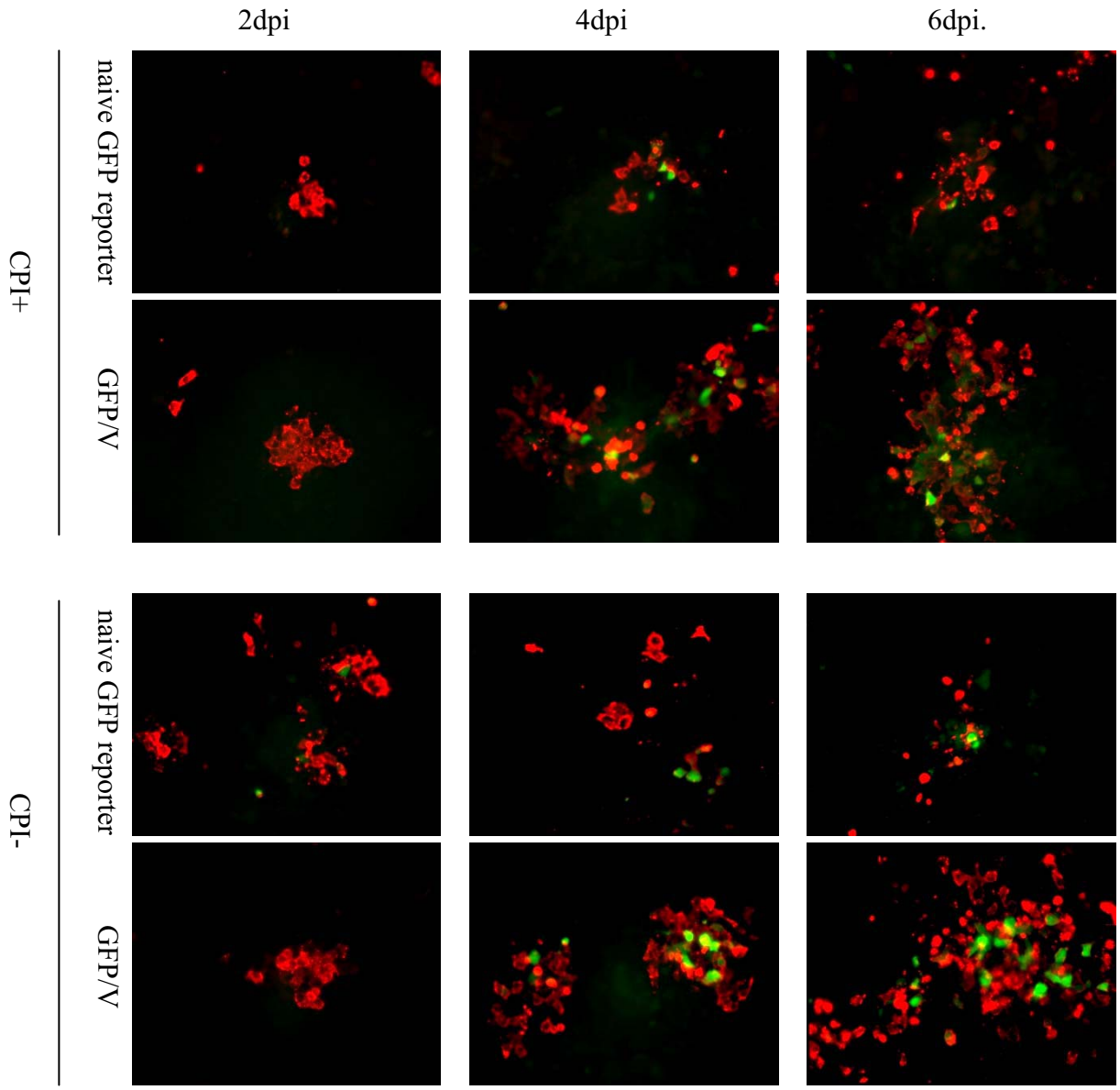

2dpi
4dpi
6dpi.
CPI+
naive GFP reporter
GFP/V
CPI-
naive GFP reporter
GFP/V

Figure 3.2.7.1 Plaque assay comparison of the abilities of A549/pr(IFN-β).GFP and A549/pr(IFN-β).GFP/V cell-lines in the ability to support the growth of viruses.

A549/pr(IFN-β).GFP and A549/pr(IFN-β).GFP/V reporter cells were infected with CPI+ or CPI- at an MOI of 0.01 PFU/cell, fixed at 2, 4 & 6dpi and immunostained for PIV5-NP&P.

The immunofluorescence assay demonstrated that the A549/pr(IFN-β).GFP/V reporter cells was better able to allow CPI- replication, than the A549/pr(IFN-β).GFP reporter cells, as the development of plaques (plaque size) was observed in the A549/pr(IFN-β).GFP/V reporter cells, but not in the A549/pr(IFN-β).GFP reporter cells.

Figure 3.3.1.1

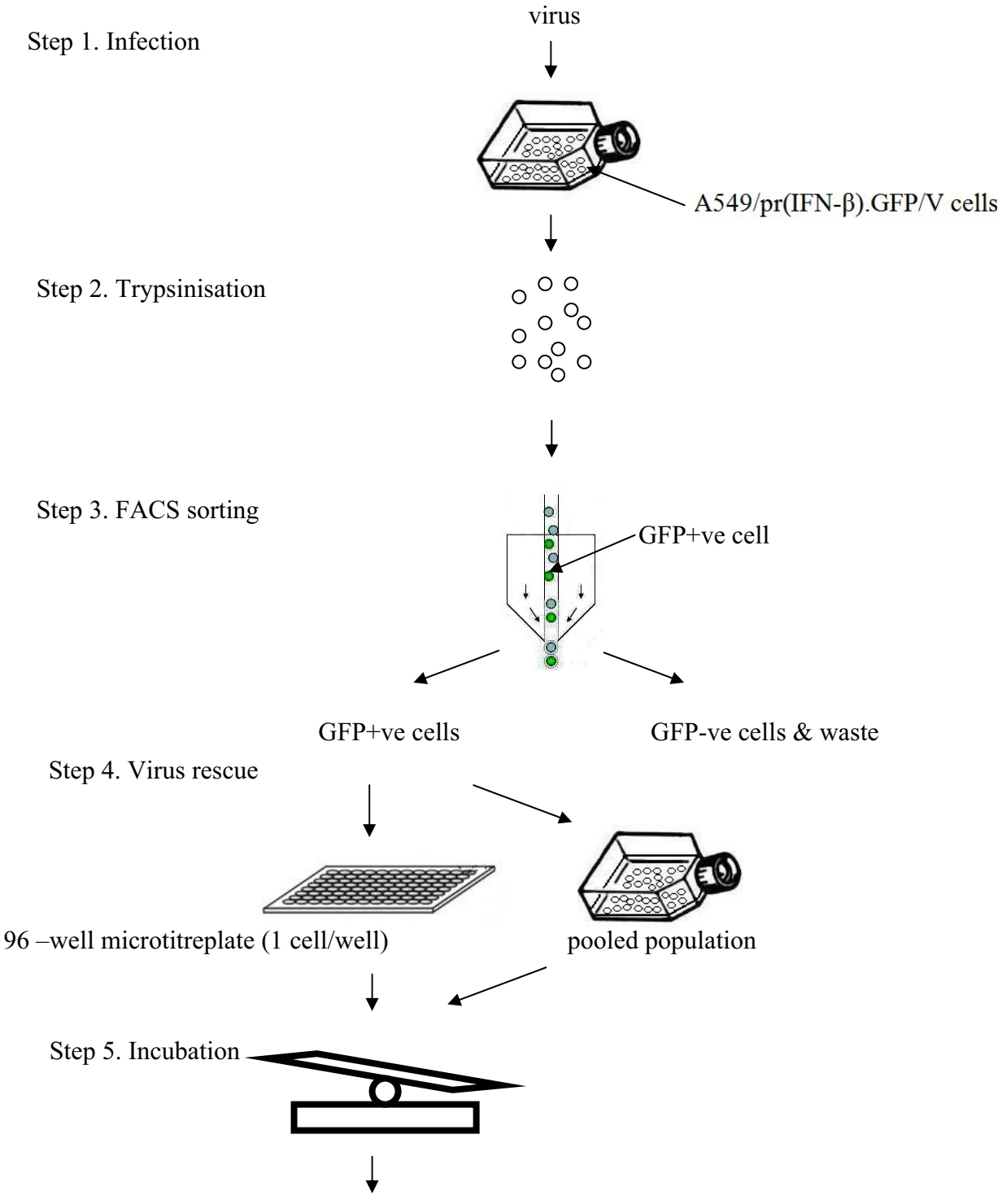

Step 1. Infection
virus
A549/pr(IFN-β).GFP/V cells
Step 2. Trypsinisation
Step 3. FACS sorting
GFP+ve cell
GFP+ve cells
GFP-ve cells & waste
Step 4. Virus rescue
96 –well microtitreplate (1 cell/well)
pooled population
Step 5. Incubation
Step 6. Microscopy screening for GFP+ve colonies

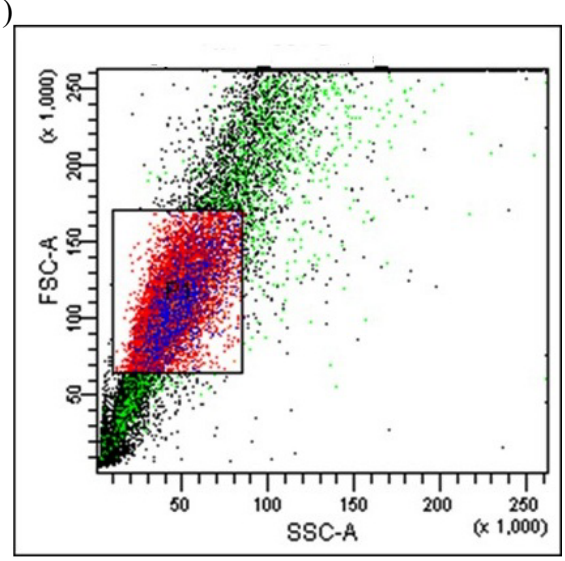
Figure 3.3.1.1 Schematic representation of FACS selection for IFN-inducing mutant virus-induced GFP-positive cells.

Monolayers of A549/pr(IFN-β).GFP.V reporter cells were infected with wildtype viruses at an MOI of 1 PFU/cell, trypsinised, and resuspended into single cell suspension, ready for FACS sorting. GFP-positive cells were separated by FACS into single cells into 96-well microtitre plates, or as a pooled population into 25cm^2 tissue culture flasks with preformed monolayers of the A549/pr(IFN-β).GFP.V reporter cells. Sorted cells were cultured on platform rockers, and visualised microscopically for GFP expression.

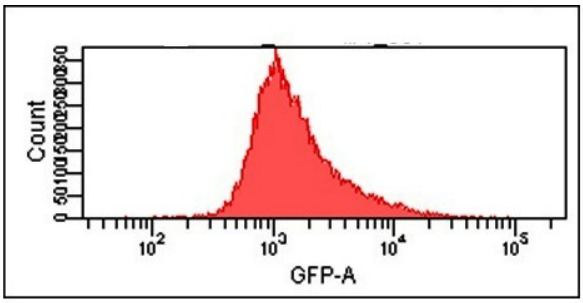
Figure 3.3.1.2

A. FLUAV rNS1-ran5 mutants

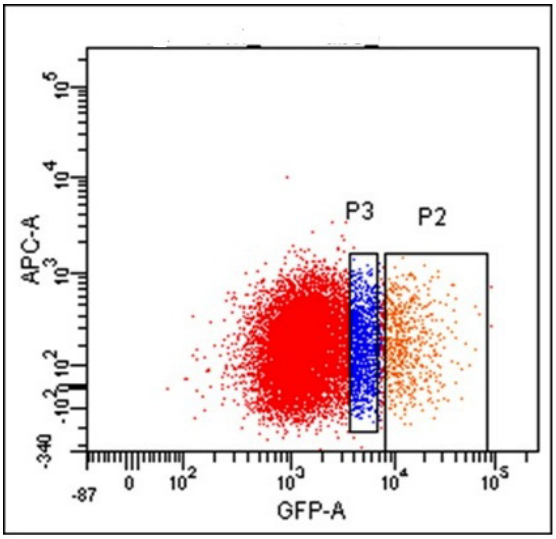
(1)



(2)



(3)



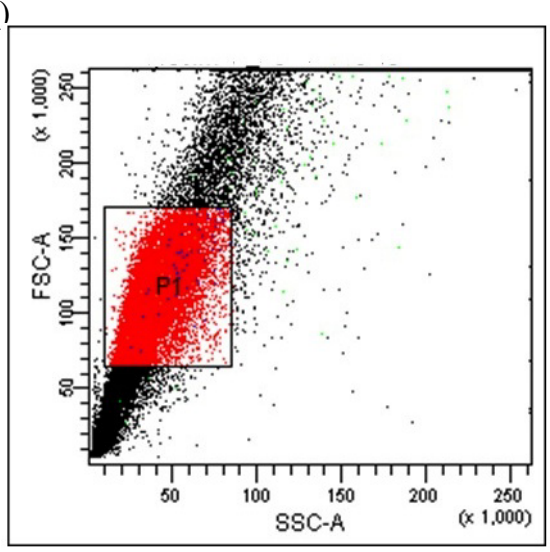
(4)

Population	#Events	%Parent	%Total
All Events	23,165		100.0
P1	13,800	59.6	59.6
P2	601	4.4	2.6
P3	1,178	8.5	5.1

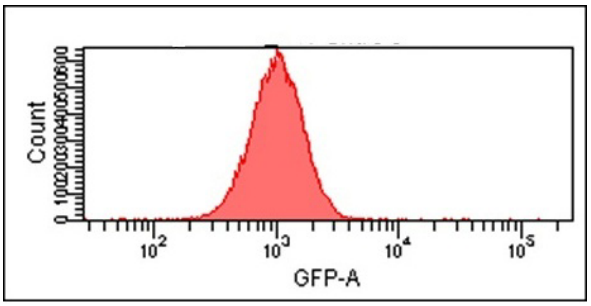
Figure 3.3.1.2

B. PIV5 W3

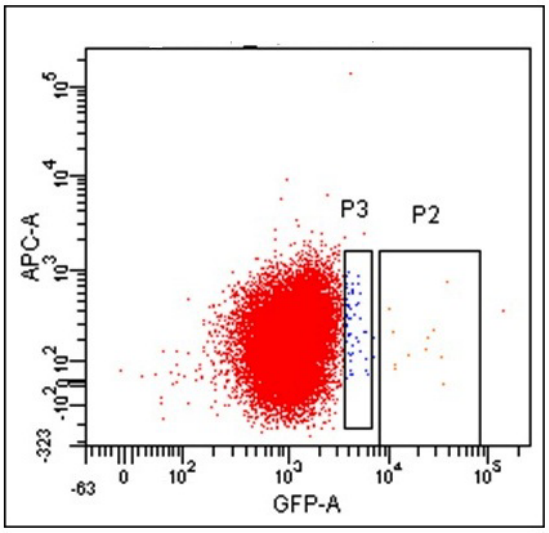
(1)



(2)



(3)



(4)

Population	#Events	%Parent	%Total
All Events	33,631		100.0
P1	20,064	59.7	59.7
P2	12	0.1	0.0
P3	55	0.3	0.2

Figure 3.3.1.2 FACS sorting of GFP-positive cells to isolate IFN-inducing mutant viruses.

75cm^2 tissue culture flasks of A549/pr(IFN-β).GFP/V reporter cells were infected with **(A)** FLUAV rNS1-ran5 random mutants, or **(B)** PIV5 W3 at an MOI of 1 PFU/cell. Samples were then analysed by FACS at 18hpi. Before cells were sorted by fluorescence intensity, all cells were (1) gated by FSC (forward scatter) and SSC (side scatter). This means cells were selected by size and granule intensity to prevent dead and/or aggregated cells being analysed. (2) Selected cells from the gated portions were analysed for GFP intensity. The histogram shows the intensity of GFP with the number of total cells designated on the x-axis. (3) The gated cell population was determined and sorted as GFP-positive or GFP-negative, with x-axis representing the GFP intensity of individual cells. (4) The sample chart: P1 is the total cell population analysed, P2 and P3 both are the cells gated as GFP-positive, P2 is the GFP cell population gated further along to the left on the x-axis which shows stronger GFP expression than P3. Approximately 13% of the total gated cells (P1) were GFP-positive for FLUAV rNS1-ran5 mutant viruses and about 0.3% of the total gated cells were GFP-positive for PIV5 W3. Separated GFP-positive cells (P2&P3) were inoculated on preformed monolayer of A549/pr(IFN-β).GFP/V reporter cells for the rescued virus to amplify.

Figure 3.3.1.3

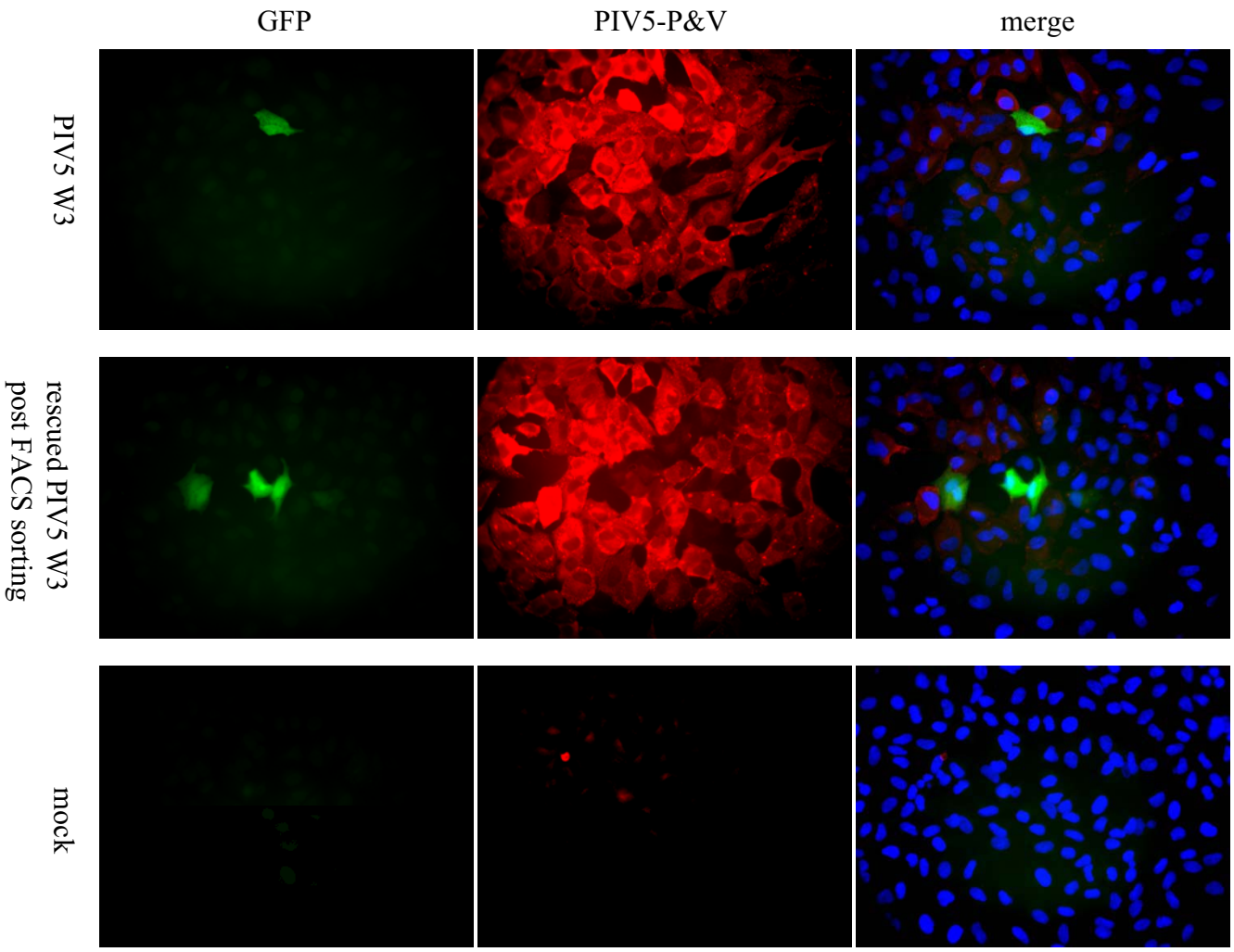

GFP
PIV5-P&V
merge
PIV5 W3
rescued PIV5 W3 post FACS sorting
mock

Figure 3.3.1.3 Characterisation of FACS sorting rescued PIV5 W3 virus in the ability to induce IFN.

A549/pr(IFN-β).GFP/V reporter cells infected with PIV5 W3 or rescued PIV5 W3 viruses of an MOI of 5 PFU/cell. Cells were fixed and immunostained for PIV5-P&V.

No significant increase in the number of GFP expressing cells was observed when we compared FACS rescued viruses to wildtype viruses. FACS selection of IFN-inducing mutant viruses was not successful.

Figure 3.3.2.1

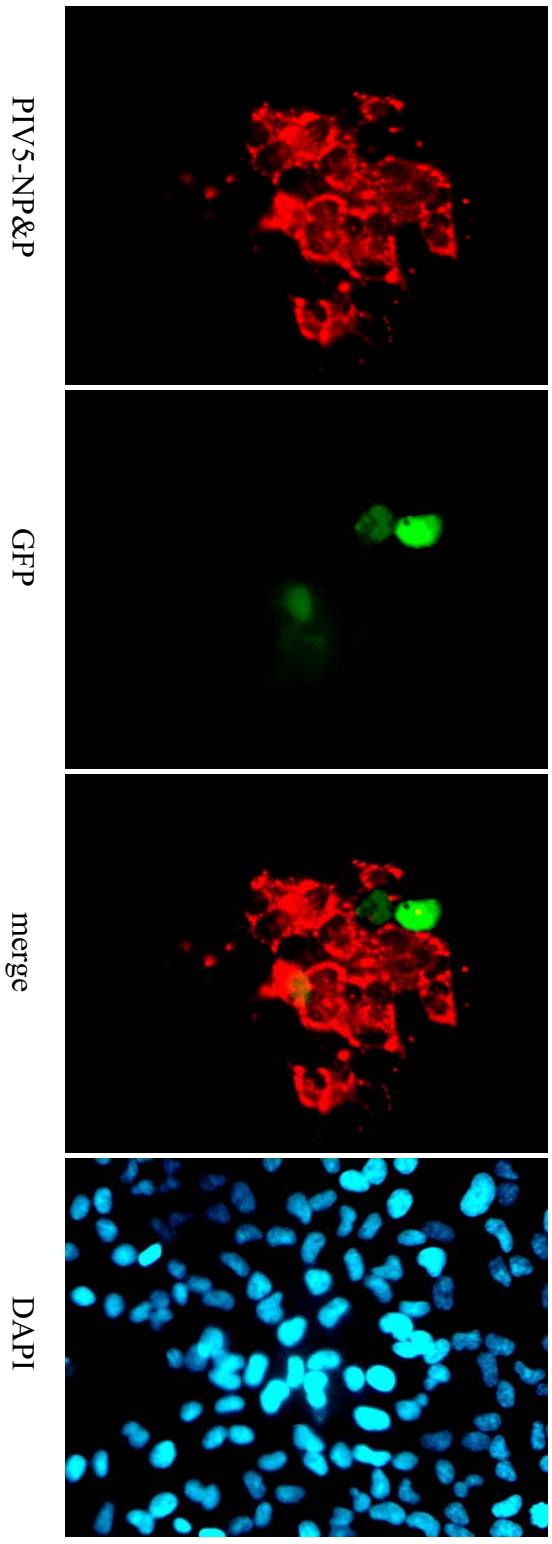


Figure 3.3.2.1 Virus replication is inhibited in A549/pr(IFN-β).GFP/V cells when IFN-β promoter is activated.

A549/pr(IFN-β).GFP/V reporter cells were infected with CPI+ at an MOI of 0.01 PFU/cell, fixed at 2dpi and immunostained for PIV5-NP&P.

Within a developing plaque, very litter viral antigen was detected in cells strongly positive for GFP. In conclusion, virus replication is inhibited in the A549/pr(IFN-β).GFP/V cells where IFN-β promoter is activated.

Figure 3.3.2.2

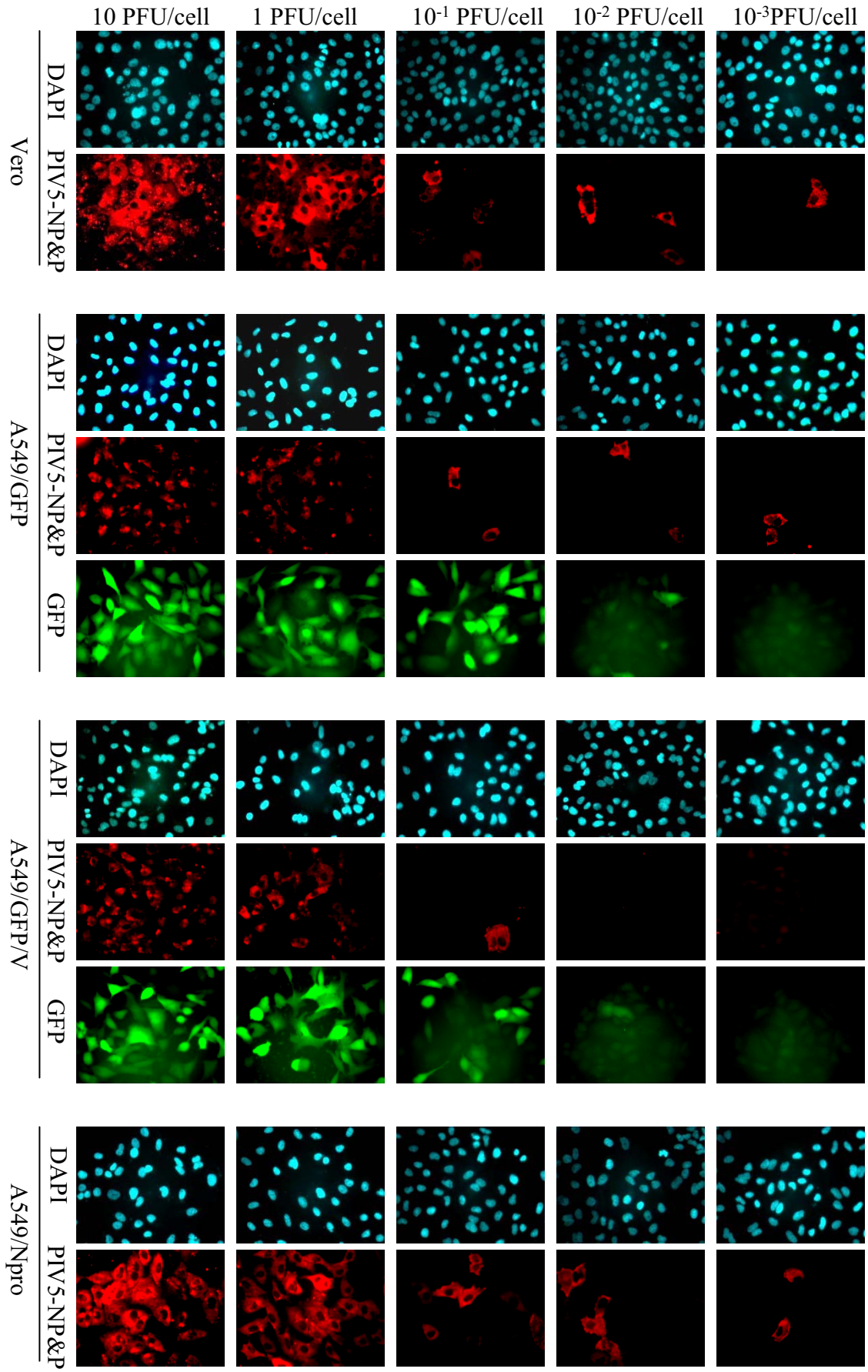

10 PFU/cell
1 PFU/cell
10-1 PFU/cell
10-2 PFU/cell
10-3PFU/cell
Vero
DAPI
PIV5-NP&P
A549/GFP
DAPI
PIV5-NP&P
GFP
A549/GFP/V
DAPI
PIV5-NP&P
GFP
A549/Npro
DAPI
PIV5-NP&P

Figure 3.3.2.2 Investigation of the induction of an IFN-independent antiviral state and virus replication.

PIV5 VΔC (P2) viruses were diluted in a series of 10-fold dilutions, and each diluted stock was used to infect monolayers of the A549/pr(IFN-β).GFP, A549/pr(IFN-β).GFP/V, A549/Npro, or Vero cells at MOIs of 10 PFU/cell – 10^{-3} PFU/cell. Cells were fixed at 16hpi and immunostained for PIV5-NP&P.

A549/Npro and Vero were permissive for virus replication.

Figure 3.3.2.3

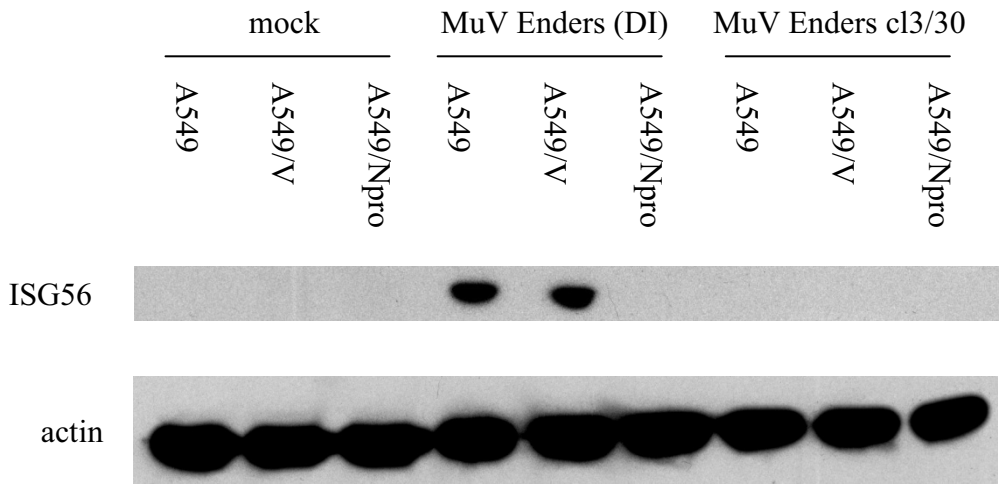

mock
MuV Enders (DI)
MuV Enders cl3/30
A549
A549/V
A549/Npro
A549
A549/V
A549/Npro
A549
A549/V
A549/Npro
ISG56
actin

Figure 3.3.2.3 Determination of ISG56 expression to investigate the establishment of an IFN-independent antiviral state induced by IRF-3.

A549, A549/V, A549/Npro cells were infected with MuV Enders cl3/30 or MuV Enders (DI) and harvested at 24hpi. Lysates were subjected to SDS-PAGE and western blot for ISG56 expression..

Western blot confirmed ISG56 expression can be activated independent of IFN. DIs induced activation of ISG56 in cells intact for IFN and IRF-3 (A549s), and in cells deficient in IFN signalling (A549/Vs), however, not in cells where IRF-3 pathway is targeted (A549/Npros).

Figure 3.4.1.1

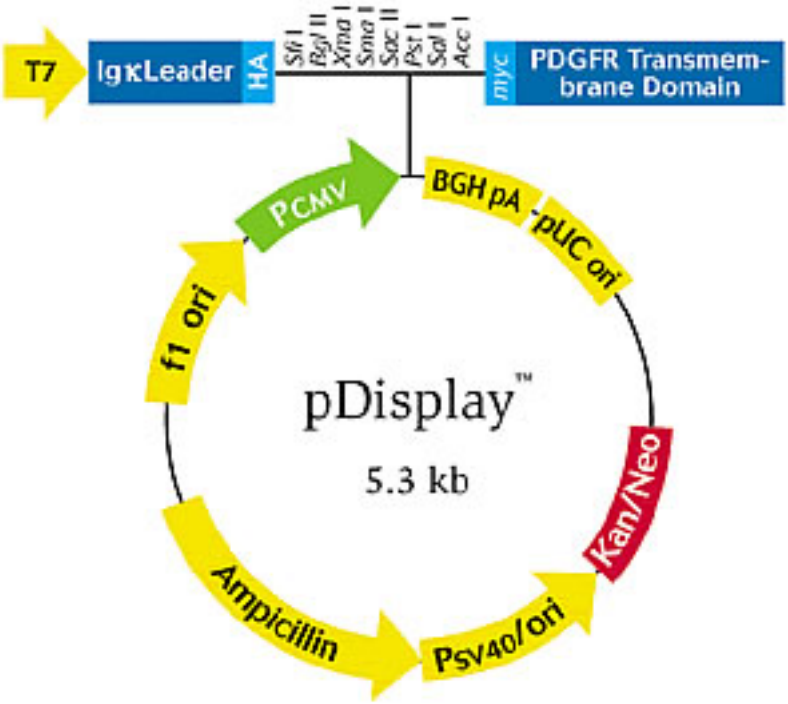

T7
IgκLeader
HA
Sfi I
Bgl II
Xma I
Sma I
Sac II
Pst I
Sal I
Acc I
myc
PDGFR Transmem-
brane Domain
PCMV
BGH pA
pUC ori
f1 ori
pDisplay™
5.3 kb
Kan/Neo
Ampicillin
PSV40/ori

Figure 3.4.1.1 Map of pDisplay^{TM} vector (adapted from Invitrogen catalog no. 660-20 Version C).

pDisplayTM is a commercial vector produced by Invitrogen, Ltd., UK. pDisplay^{TM} is a 5.3kb mammalian expression vector which allows display of proteins on the cell surface. The target protein expressed from pDisplay^{TM} was fused at its N-terminus to the murine Ig κ-chain leader sequence, which targets protein to secretory pathway and at its C-terminus the platelet derived growth factor (PDGFR) transmembrane domain (Gronwald *et al.*, 1988).

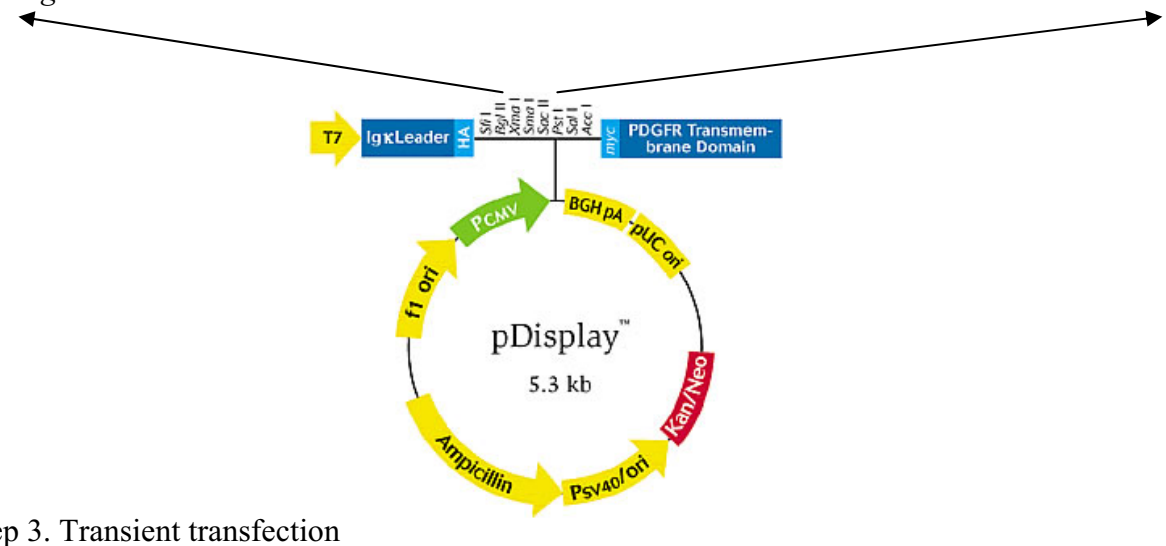
Figure 3.4.1.2

Step 1. Oligos annealing
Forward
5’-GATCTGGAAAGCCGATCCCAAACCCTCTATTAGGTCTGGACTCCACCCTGCA-3’
Reverse

3’-ACCTTTCGGCTAGGGTTTGGGAGATAATCCAGACCTGAGGTGGG-5’

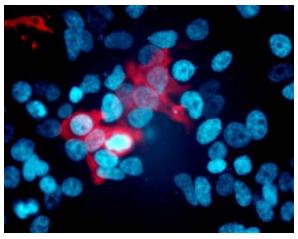
Step 2. Cloning

GATCTGGAAAGCCGATCCCAAACCCTCTATTAGGTCTGGACTCCACCCTGCA
*Bgl*II *Pst*I



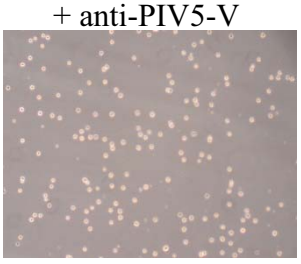
Step 3. Transient transfection

a. Immunofluorescence

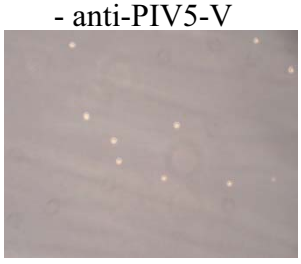


b. Panning

+ anti-PIV5-V



- anti-PIV5-V



Step 4. Generation of thepdl’pr(IFN-β).V5 lentivirus plasmid

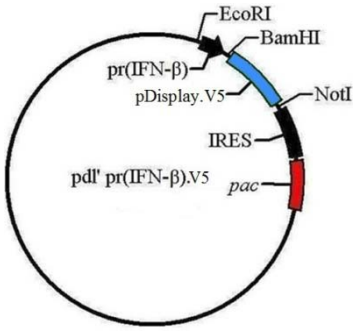


Figure 3.4.1.2 Generation of a lentivirus plasmid expressing the surface V5 epitope under the control of IFN-β promoter.

Step 1. Oligos of complementary strands of V5 epitope were annealed.

Step 2. V5 epitope sequence was cloned into the pDisplay™ vector.

Step 3. Plasmid was transfected into 293T cells.

a. Successful cloning resulted in the surface expression of V5 by immunofluorescence.
b. Panning experiments showed cells expressing the V5 epitope on the surface successfully bound to anti-PIV5-V antibody soaked in tissue culture dishes.

Step 4. The fragment enconding the V5 tag linked to the transmembrane domain of pDisplay™ vector was digested from the pDisplay™ backbone vector between *BamH*I and *Not*I sites and cloned into the pdl'pr(IFN-β).GFP lenvirus vector between *BamH*I and *Not*I sites by replacing eGFP, under the control of IFN-β promoter, generating the pdl'pr(IFN-β).V5 plasmid.

Figure 3.5.1

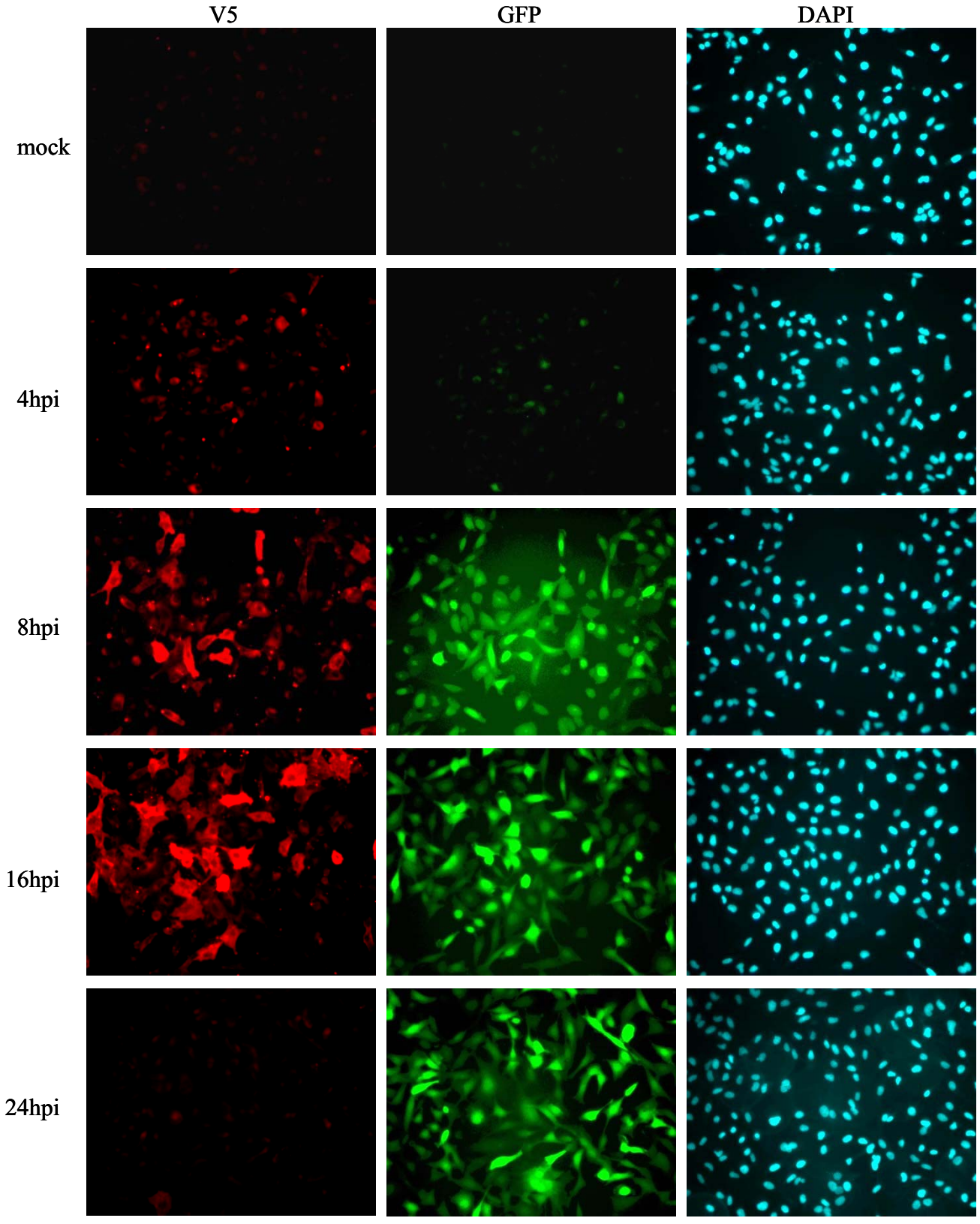


Figure 3.5.1 Characterisation of the extracellular V5 epitope tag expression when the IFN-β promoter is activated.

A549/pr(IFN-β).GFP/V5/V cells were infected with PIV5 VΔC (P2) virus at an MOI of 5 PFU/cell, fixed at 4, 8, 16 & 24hpi and immunostained for extracellular PIV5-V.

The A549/pr(IFN-β).GFP/V5/V reporter cells started to express GFP from 4hpi. The expression increased between 4 to 8hpi and remained stable until at least 24hpi. The V5 epitope tag was inducible in response to DIs. The V5 epitope cell surface expression was transient and expression was strong at 12-16hpi.

Figure 3.5.2

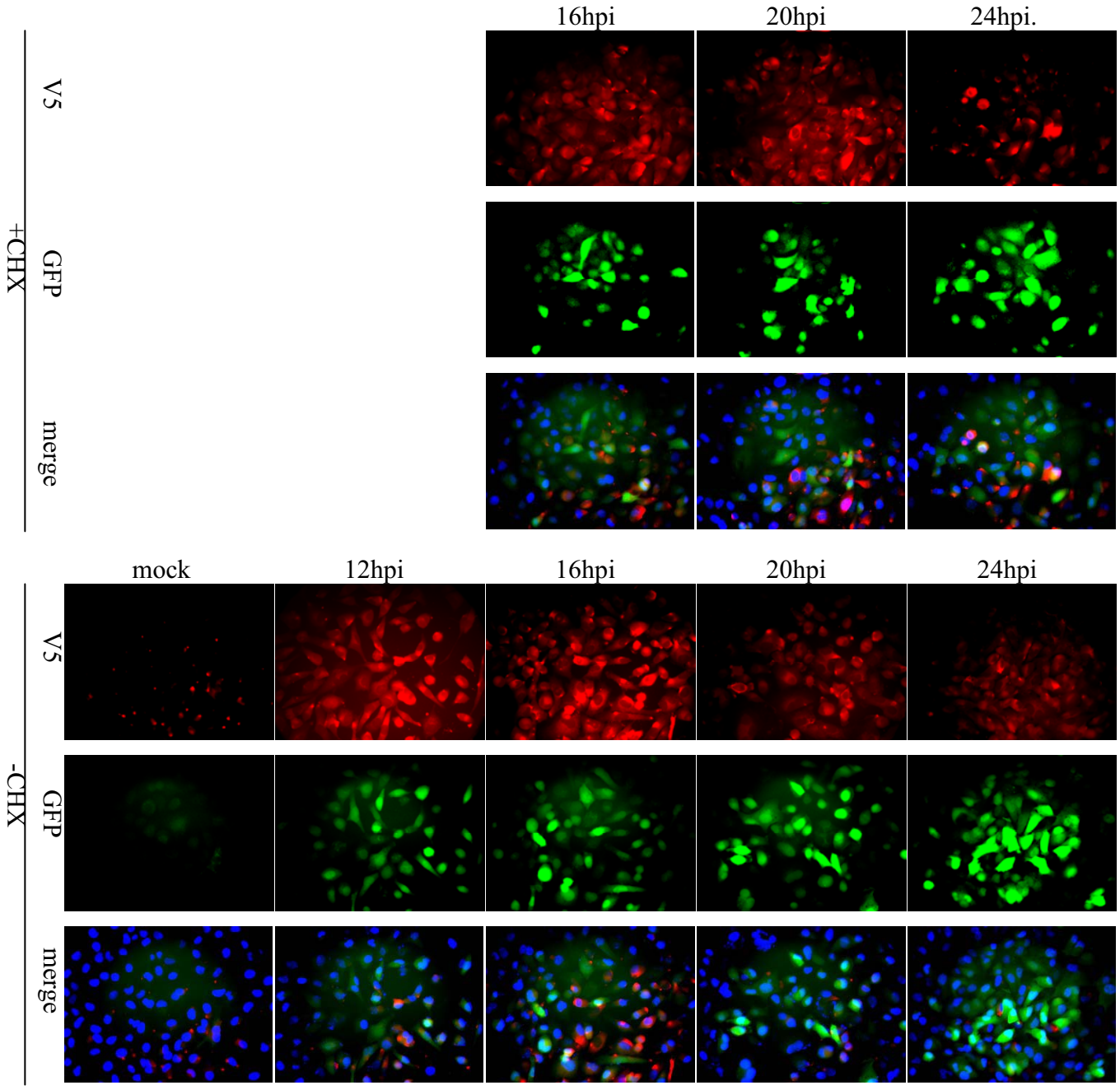

16hpi
20hpi
24hpi.
+CHX
V5
GFP
merge
mock
12hpi
16hpi
20hpi
24hpi
-CHX
V5
GFP
merge

Figure 3.5.2 Comparison of the stability of GFP *vs* V5 expression.

A549/pr(IFN-β).GFP/V5/V cells were infected with MuV Enders (DI) at an MOI of 5 PFU/cell. Cells were treated with CHX at 12hpi to inhibit viral protein synthesis, fixed at 12 (0), 16 (4), 20 (8), 24 (36)hpi (post CHX treatment) and immunostained for extracellular PIV5-V.

Both GFP and V5 epitope expression were under the control of the IFN-β promoter. Intense GFP expression was observed at 12hpi and remained stable. By contrast, V5 epitope expression was detectable at 12hpi, but started to decrease after reaching a peak at 16hpi.

Figure 3.7.1

A

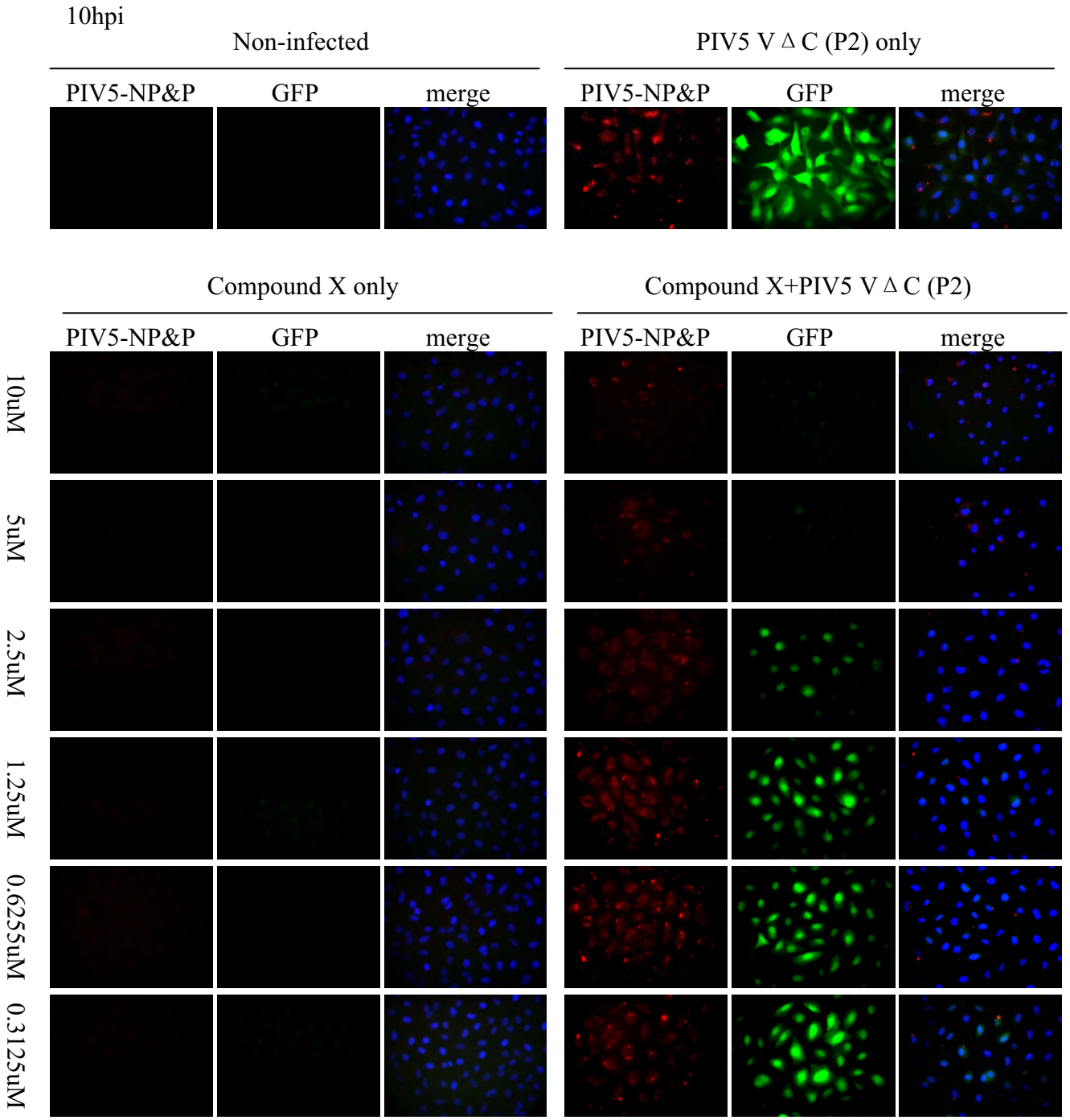

10hpi
Non-infected
PIV5 V Δ C (P2) only
PIV5-NP&P
GFP
merge
PIV5-NP&P
GFP
merge
Compound X only
Compound X+PIV5 V Δ C (P2)
PIV5-NP&P
GFP
merge
PIV5-NP&P
GFP
merge
10uM
5uM
2.5uM
1.25uM
0.6255uM
0.3125uM

Figure 3.7.1

B

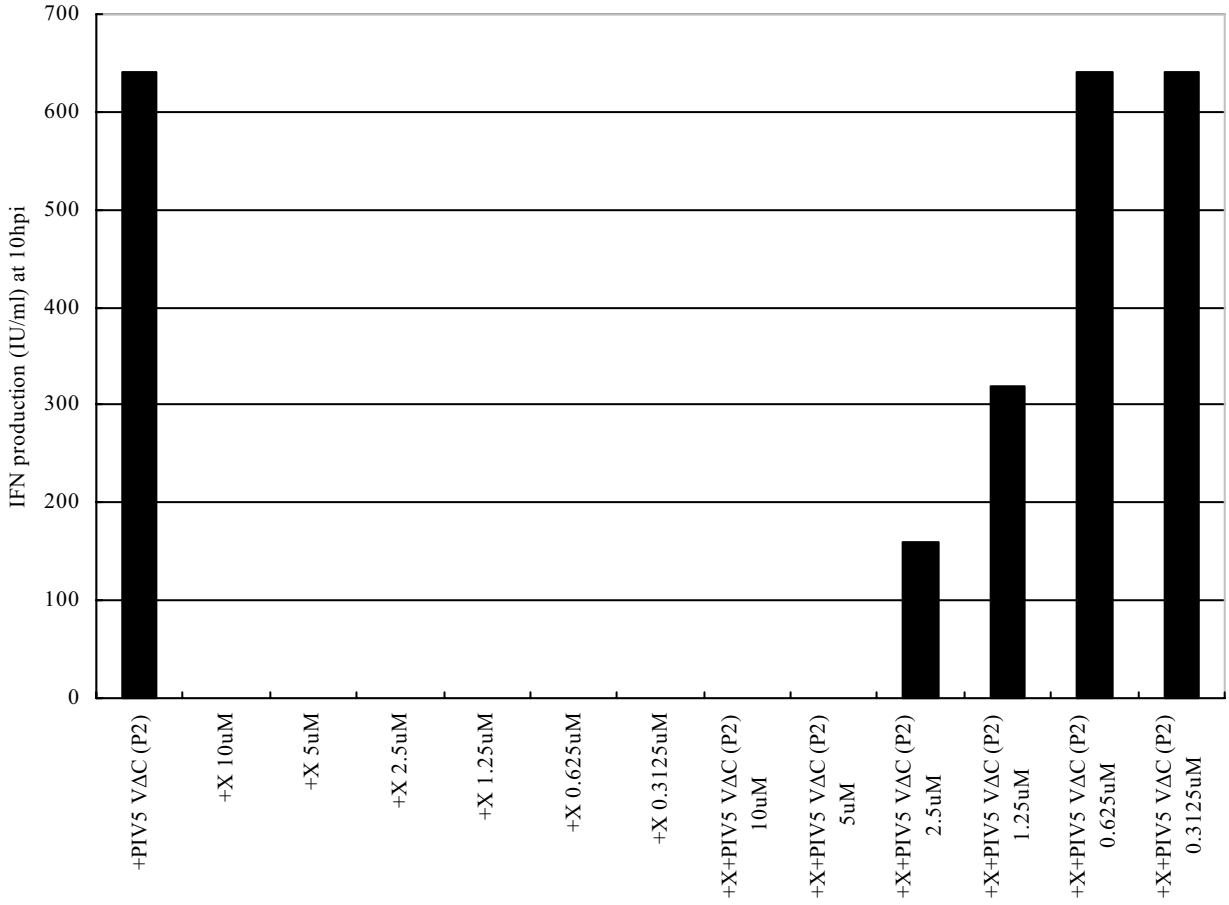

IFN production (IU/ml) at 10hpi
700
600
500
400
300
200
100
0
+PIV5 VΔC (P2)
+X 10uM
+X 5uM
+X 2.5uM
+X 1.25uM
+X 0.625uM
+X 0.3125uM
+X+PIV5 VΔC (P2) 10uM
+X+PIV5 VΔC (P2) 5uM
+X+PIV5 VΔC (P2) 2.5uM
+X+PIV5 VΔC (P2) 1.25uM
+X+PIV5 VΔC (P2) 0.625uM
+X+PIV5 VΔC (P2) 0.3125uM

Figure 3.7.1 Screening for compounds that inhibit IFN induction using A549/pr(IFN-β).GFP reporter cell-line.

A. PIV5 VΔC (P2) vary in ability to induce GFP expression in the presence of various concentrations of TBK-1 inhibitor (compound X) in A549/pr(IFN-β).GFP reporter cells.
B. PIV5 VΔC (P2) vary in ability to induce IFN in the presence of various concentrations of TBK-1 inhibitor.

A549/pr(IFN-β).GFP cells cultured on coverslips in a 24-well microtitre plate were pretreated with various concentrations of compound X for 30 mins and infected with PIV5 VΔC (P2) in the presence of compound X at an MOI of 10 PFU/cell. At 10hpi, cells were fixed. Viral protein (PIV5-NP&P) synthesis, following immunostaning, and GFP expression (Panel A) were visualised using fluorescence microscopy. Culture supernatants were collected and the amount of IFN present was measured in by CPE reduction bioassay for IFN and IFN production is plotted (Panel B).

Compound X inhibition of IFN induction was observed by the reduction of IFN production and the inhibition of GFP expression. The optimal concentration of compound X to inhibit IFN induction was ~2.5μM.

4. DISCUSSION

Characterisation of the A549/pr(IFN-β).GFP and its derivative reporter cell-lines demonstrated that (i) following infection of the A549/pr(IFN-β).GFP reporter cells with a stock of MuV Enders known to be a good inducer of IFN, approximately 90% of the cells became GFP-positive by 6-8hpi, demonstrating the ability of the majority of the A549/pr(IFN-β).GFP cells to respond quickly to virus infection; (ii) CPE reduction bioassay for IFN showed that the amount of IFN produced by the A549/pr(IFN-β).GFP reporter cells correlated with the number of cells that were positive for GFP; (iii) the level of IFN induction correlated with the amount of DIs present in the virus stock; (iv) expression of the IFN antagonists, BVDV-Npro and HCV-NS3/4A, were able to block the induction of IFN-β promoter and thus GFP expression.

4.1 IFN-inducing mutant viruses as live attenuated virus vaccines

Mutant viruses that can induce IFN are considered to be potential candidates for the development of live attenuated vaccines. These are viruses with attenuating mutations or deletions in genes which encode the viral IFN antagonist(s) that blocks the IFN production. In addition, these viruses should still conserve the ability to infect and replicate in host cells. However, because these viruses would be unable to circumvent the IFN response, the virulence would be reduced dramatically. One of the traditional and currently widely used methods of generating live attenuated virus vaccine is reverse genetics that could be used to introduce point mutations or deletions in the viral genome to knock out the viral IFN antagonist(s). Using reverse genetics, the mutation or deletion in the gene would be known and therefore it would be easy to monitor the development, utilisation, manufacture, and application of the vaccine. However, the artificial engineering of viruses may have potential problems, as the process is time consuming and the technology demanding. Also, as IFN antagonists are always multifunctional, a deletion in the genes encoding the viral IFN antagonists may over-attenuate the virus, and therefore end viruses may not be sufficiently

immunogenic. Moreover, reverse genetics are not available for all viruses. An alternative to reverse genetics would be to isolate IFN-inducing mutant viruses from wildtype virus population. The mutant viruses of interest would be the naturally existing viruses in the wildtype virus population which have deletions or mutations in genes that encode viral IFN antagonist(s).

4.1.1 Using A549/pr(IFN-β).GFP(V) reporter cell-line(s) to try to isolate IFN-inducing mutant viruses

A cell based methodology was developed and characterised to try and isolate IFN-inducing mutant viruses. A549 cells were engineered by expressing lentivirus vector in which GFP and *pac* expression were under the control of IFN-β promoter. IFN-inducing mutant virus isolation from wildtype virus population was first attempted by infecting the cells with MuV Enders cl3/30 virus and subsequently selecting cells with puromycin, which would only be present in cells in which the IFN-β promoter had been activated. A small percentage (<5%) of MuV Enders cl3/30 infected cells survived the puromycin selection. The viruses present in these selected cells were grown on A549/pr(IFN-β).GFP reporter cells, so that the viruses' ability to induce IFN could be monitored in terms of GFP expression. However, when comparing the rescued virus with the original MuV Enders cl3/30 in their ability to induce IFN, there was no significant increase in the number of GFP expressing cells in the A549/pr(IFN-β).GFP reporter cells infected with the rescued virus. Given that we were able to select for *pac*-positive cells surviving the puromycin selection following virus infection, it may have been that these cells had been infected with IFN-inducing mutant viruses but that we had subsequently been unable to isolate them by growing them on the A549/pr(IFN-β).GFP cells. To investigate this further, we subsequently decided to monitor the plaque development following infections with PIV5 VΔC in A549 cells and correlated this with MxA (an antiviral protein induced by IFN) induction. The result showed that A549 cells produced a large amount of IFN in response to virus infection and signalled to neighbouring cells, which was supported by the observation that the cells surrounding these plaques were positive for

MxA and were thus in an IFN-induced antiviral state. Therefore, the failure to isolate IFN-inducing mutant viruses using the A549/pr(IFN-β).GFP reporter cell-line showed that by activating the IFN-β promoter, we also induced an IFN-dependent antiviral state which presumably inhibited the isolation of mutant viruses with defects in their IFN antagonist(s).

In an attempt to overcome the problem of an IFN-dependent antiviral activity induced in the A549/pr(IFN-β).GFP reporter cells when trying to isolate IFN-inducing mutant viruses, we constitutively expressed PIV5-V protein in the A549/pr(IFN-β).GFP reporter cells. As PIV5-V targets STAT1 for proteasome-mediated degradation (Didcock *et al.*, 1999; Precious *et al.*, 2005b; Precious *et al.*, 2007) and consequently blocks the IFN signalling pathway, the A549/pr(IFN-β).GFP/V cells were no longer able to respond to IFN.

We next compared the A549/pr(IFN-β).GFP/V cells with the A549/pr(IFN-β).GFP cells in their ability to support replication of mutant viruses with defects in their IFN antagonist(s). Plaque development assay following infections with CPI- (Figure 3.2.7.1) which cannot target STAT1 for degradation showed that the A549/pr(IFN-β).GFP/V reporter cell-line was better able to support CPI- replication compared to the A549/pr(IFN-β).GFP reporter cell-line. However, when we then used FACS sorting to isolate IFN-inducing mutant viruses using the A549/pr(IFN-β).GFP/V reporter cells and FACS technique, we were still unable to select any IFN-inducing mutant viruses. Clearly, although we were able to isolate GFP-positive cells using FACS sorting, the isolated mutant viruses may not be able to replicate in the A549/pr(IFN-β).GFP/V reporter cell-line.

We noted that within developing plaques in A549/pr(IFN-β).GFP/V cells (Figure 3.3.2.1) very little virus antigen was detected in cells in which the IFN-β promoter had been activated (*i.e.* those cells which were positive for GFP). Possible reasons for this could be that an IFN independent antiviral state may have been induced in the

GFP-positive cells that inhibited virus protein synthesis and replication; and/or the GFP-positive cells may have been infected with DIs which because of their defective nature, could not subsequently be isolated (discussed further below). Since little virus antigen was detected in any cell that was positive for GFP expression in the PIV5 V expressing cells, it suggested that induction of an antiviral state did occur. This may have been because the activation of IRF-3 in the GFP-positive cells resulted in the expression of a subset of IRF-3 responsive ISGs independently of the action of IFN. To study this further, firstly, we investigated the establishment of an IRF-3-dependent, IFN-independent antiviral state by looking at virus replication in A549/pr(IFN-β).GFP (naive), A549/pr(IFN-β).GFP/V (these cells express sufficient PIV5 V to block IFN signalling but not enough to block IFN induction) and A549/Npro (IRF-3 deficient) cells following their infection with PIV5 VΔC (P2). Viral antigen was expressed at significantly higher levels in A549/Npro cells than in the A549/pr(IFN-β).GFP and A549/pr(IFN-β).GFP/V cells. Thus, blocking IFN response alone would not completely inhibit antiviral activities of the host cell. Therefore, viruses still have difficulties replicating in IFN deficient cells. However by blocking antiviral activity induced *via* IRF-3 might allow efficient virus replication. To investigate whether viruses that activated the IFN-β promoter could induce an IRF-3-dependent, IFN-independent antiviral response, we studied ISG56 expression, a key ISG that can also be induced directly *via* IRF-3. In other words, we examined whether virus stocks that were good inducers of IFN could, in the absence of an IFN response, also induce the expression of ISG56. In response to MuV Enders (DI) infection, ISG56 expression was clearly detected in A549 and A549/V cells, but not in A549/Npro cells where the IRF-3 pathway is blocked. We therefore conclude that ISG56 can be activated *via* IRF-3, independent of IFN in response to virus infections.

To conclude, constitutive expression of PIV5 V protein in the A549/pr(IFN-β).GFP/V reporter cell-line partially helped prevent an antiviral state induced *via* the IFN pathway. However, IFN-independent antiviral activities induced by IRF-3 have to be prevented to generate an ideal reporter cell-line permissive for the growth of

IFN-inducing mutant viruses.

4.1.2 Modifications of the A549/pr(IFN-β).GFP/V reporter cell-line as a permissive cell-line for the growth of IFN-inducing mutant viruses

We have shown that blocking IRF-3 aids the replication of PIV5 VΔC (P2), by constitutive expression of BVDV-Npro. However, inhibition of IRF-3 activation also leads to inhibition of IFN-β promoter activation and consequently GFP expression. Consequently, it is not possible to knock out IRF-3 activation in the A549/pr(IFN-β).GFP cells and use of them to isolate IFN-inducing mutant viruses. Alternative strategies therefore need to be developed.

There is evidence to show that ISG56 has a powerful antiviral function independent of IFN (Guo *et al.*, 2000), but dependent of IRF-3 (Grandvaux *et al.*, 2002). ISG56 is the major gene (Grandvaux *et al.*, 2002; Dr Lena Andrejeva unpublished observations), if not sole (e.g. ISG54 and ISG60 (Grandvaux *et al.*, 2002)), induced *via* IRF-3, dependent and independent of IFN. Current studies by Dr. Lena Andrejeva in our laboratory also showed that ISG56 is an essential ISG which inhibits PIV5 replication (data not shown). Therefore, we would try to express shRNA to ISG56 in the A549/pr(IFN-β).GFP/V5/V cell-line, which would knock down the expression of p56 (the protein product of ISG56). Consequently, by inhibiting ISG56 activation, we should therefore significantly prevent the antiviral response induced by IRF-3, which may allow better replication of IFN-inducing mutant viruses in the A549/pr(IFN-β).GFP/V5/V reporter cells.

4.1.3 Development of an A549/pr(NF-κB).GFP reporter cell-line to isolate IFN-inducing mutant viruses

Activation of the IFN-β promoter is dependent on the cooperative binding of several transcription factors, of which IRF-3 and NF-κB are crucial. We have confirmed that upon a viral infection, the host cell can establish an antiviral state *via* two major pathways, the IFN pathway and the IRF-3 pathway (IFN-independent). Therefore, in

order to avoid the host cell developing an antiviral activity, we could try to generate a reporter cell-line, which would express a reporter protein in response to IFN-inducing mutant virus infection, while any IFN-dependent or IFN-independent antiviral activities are prevented. One possibility is to isolate mutant viruses that induce IFN *via* the NF-κB pathway so we could express GFP as a reporter protein under the control of NF-κB promoter and knock out IRF-3 in these cells. The idea would be to generate an NF-κB cell-line similar to the A549/pr(IFN-β).GFP reporter cell-line. To achieve this, we could replace the IFN-β promoter with the NF-κB promoter sequence (Figure 4.1.3.1) in the pdl'pr(IFN-β).GFP lentivirus vector, and infect A549 cells with the pdl'pr(NF-κB).GFP lentivirus. Lentivirus-infected cells may be subject to subcloning to obtain homogenous cell-lines and characterisation of the cell-lines performed if necessary. If an A549/pr(NF-κB).GFP reporter cell-line was isolated, we would then engineer the cell-line to block both the IRF-3-dependent and IFN-dependent antiviral activities. As demonstrated previously, constitutive expression of PIV5 V targets STAT1 for degradation and therefore IFN signalling blocked. We have also provided evidence that by expressing BVDV-Npro, the IRF-3 pathway is efficiently blocked. Therefore, by blocking both IFN signalling and IRF-3 pathways to prevent any possible antiviral activities in an A549/pr(NF-κB).GFP reporter cell-line, we may be able to isolate mutant viruses which induce IFN.

4.1.4 The role of DIs in the induction of IFN

As discussed previously (Chapter 4.1.1), possible reasons for the failure to isolate IFN-inducing mutant viruses could be: (i) IFN-inducing mutant viruses may induce an IRF-3-dependent antiviral state within the cell as a direct response to virus infection, and as a consequence, this IFN-independent antiviral activity may inhibit the replication of mutant viruses; (ii) activation of the IFN-β promoter may only occur following infections of cells with defective interfering viruses (DIs). As DIs always have deletions in their genomes, they will not be able to replicate in the absence of non-defective helper viruses, which are able to provide gene functions missing in the DIs. If this is the case, we may not be able to isolate any mutant viruses that activate

the IFN-β promoter. However, at this stage, we are not able to distinguish between the two possibilities of why we failed to isolate IFN-inducing mutant viruses, namely the induction of an antiviral state or the fact that DIs are an absolute requirement for IFN induction. The importance of DIs in the induction of IFN has been previously studied and it is believed that presence of DIs within the virus preparation can significantly induce high levels of IFN (Johnston, 1981; Poole *et al.*, 2002; Strahle *et al.*, 2006; Strahle *et al.*, 2007). The ability of some SeV has long been known to strongly activate IFN-β is associated with DI genomes (Johnston, 1981; Poole *et al.*, 2002). Studies have shown that the ability of SeV DIs to induce IFN is associated to (i) their ability to compete with their helper non-defective viral genomes for their replication substrate and consequently result in low levels viral products (e.g. SeV V and C proteins) which block the host IFN response to dsRNA; (ii) their copyback DI genomes; (iii) the level of DI genome replication (Strahle *et al.*, 2006). Our own experiments suggest that DIs may be extremely important for activating the IFN response. For example, there are significant differences in the number of GFP-positive in the A549/pr(IFN-β).GFP reporter cells when infected with DI-rich (MuV Enders (DI)) and DI-poor (MuV Enders cl3/30) viruses. The majority (~90%) of the A549/pr(IFN-β).GFP reporter cells infected with MuV Enders (DI) were GFP-positive, however, less than 1% of the A549/pr(IFN-β).GFP reporter cells infected with MuV Enders cl3/30 were GFP-positive.

The A549/pr(IFN-β).GFP/(V) reporter cell-lines are currently being used in our laboratory to follow the dynamics of IFN induction by viruses. Using these cell-lines, we have reported on the dynamics of IFN induction by a variety of negative strand RNA viruses, including PIV5, MuV and FLUAV. Infection assays on the A549/pr(IFN-β).GFP reporter cells have suggested that wildtype viruses or DI-poor virus stocks induce a very limited amount of IFN during the infection process. However, viruses enriched with DIs induced significant IFN production. It will be important to characterise the DIs and how they activate the IFN response at the molecular level.

Previous studies have shown that there is heterocellular induction of IFN in response to PAMPs (Apostolou & Thanos, 2008; Enoch *et al.*, 1986; Hu *et al.*, 2007; Senger *et al.*, 2000; Zawatzky *et al.*, 1985). However, the molecular basis of the cellular restriction of induction has not been carefully studied, and it has been generally assumed to be a property of the host cell; for example, it has been suggested that only cells at certain stages of the cell cycle may be responsive for the induction (Zawatzky *et al.*, 1985) and recently it has been suggested that the availability of transcription factors (e.g. IRF2, IRF3 or p65) may interfere with the percentage of cells able to support induction (Apostolou & Thanos, 2008). However our data has clearly shown that the heterocellular induction of IFN is not restricted by either of these assumptions, given the fact that GFP can be induced in at least 90% of the A549/pr(IFN-β).GFP cells. Further observations using the A549/pr(IFN-β).GFP cells have clearly shown that the heterocellular induction of IFN is stimulated by the properties of infecting virus used in the study.

4.2 Other applications

The A549/pr(IFN-β).GFP/(V) reporter cell-lines are used to monitor the IFN induction activity by GFP expression, directly or indirectly induced by viruses, and also as a tool for fundamental studies of how viruses interfere with the host IFN system in our laboratory.

The A549/pr(IFN-β).GFP reporter cell-line is now being used to screen for compounds which inhibit the IFN induction pathway in our laboratory. Compounds that are IFN induction inhibitors could be potentially developed as anti-inflammatory drugs for the treatment of symptoms caused by over-inflammation. Any compound that blocks IFN induction will be characterised and investigated to better understand the mechanism of inhibition. Similarly, if an A549/pr(NF-κB).GFP reporter cell-line becomes available, we will use it to screen for compounds that inhibit IFN induction *via* the NF-κB pathway. Again, such compounds are potential candidates as anti-inflammatory drugs. Furthermore, the reporter cell-lines may be developed as a

method to screen for compounds which have novel antiviral activities. For example, the A549/pr(IFN-β).GFP/(V) reporter cell-line will be pretreated with the compounds of interest then infected with wildtype virus to study the GFP expression. Any compound that is capable of inhibiting the viral IFN antagonists may result in the activation of the IFN-β promoter and then GFP expression in the reporter cells.

4.3 Conclusion

The aim of this project was to develop a cell-line, which could be used to rapidly isolate mutant viruses that induce IFN production. Having generated and characterised the A549/pr(IFN-β).GFP and its derivative reporter cell-lines, we confirmed that those cell-lines were potential reporter cell-lines that could be used to monitor the induction of IFN by either GFP expression or cell surface expression of V5 epitope. However unfortunately, we were not able to use these cell-lines to isolate mutant viruses that activate the IFN-β promoter. One of the major problems may be that IRF-3, which is essential for the activation of the IFN-β promoter, also induces the activation of a subset of ISGs which can establish an IFN-independent antiviral state within the cell as a direct response to virus infection. Consequently, this IFN-independent antiviral activity may inhibit the replication of any mutant virus which has been isolated using the reporter cell-line(s). To try and overcome this problem, we modified the cell-lines to generate cells deficient both in IFN and IRF-3. We found that by targeting IRF-3 (by expression of BVDV-Npro or NCV-NS3/4A) was not feasible, as the GFP expression was also inhibited (Chapter 3.2.2). However, ISG56 is known as one of the major ISGs that induced by IRF-3 (Grandvaux *et al.*, 2002; Dr Lena Andrejeva unpublished observations) and functions as a crucial factor to establish an antiviral state in host cells (Fensterl *et al.*, 2008). Therefore we are currently expressing shRNA to knock out ISG56 in the reporter cell-lines and hopefully this may prevent the antiviral activities induced *via* IRF-3 and therefore make the reporter cell-lines permissive for the growth of IFN-inducing mutant viruses. Any isolated mutant viruses will subsequently be genetically analysed and functionally studied. Genetic studies of the defects or mutations of the viruses may

provide further insights into the interaction of viruses with the IFN system. Of course, it remains possible that IFN is only induced by DIs, and if so it may not be possible to isolate self-replicating IFN-inducing mutant viruses. This is also one of the issues that we are actively investigating in our laboratory. Finally, the A549/pr(IFN-β).GFP and its derivative reporter cell-lines, are being applied as tools to study the fundamental side of virus infection and IFN induction, as well as a method to screen for potential anti-inflammatory and antiviral compounds.

Figure 4.1.3.1

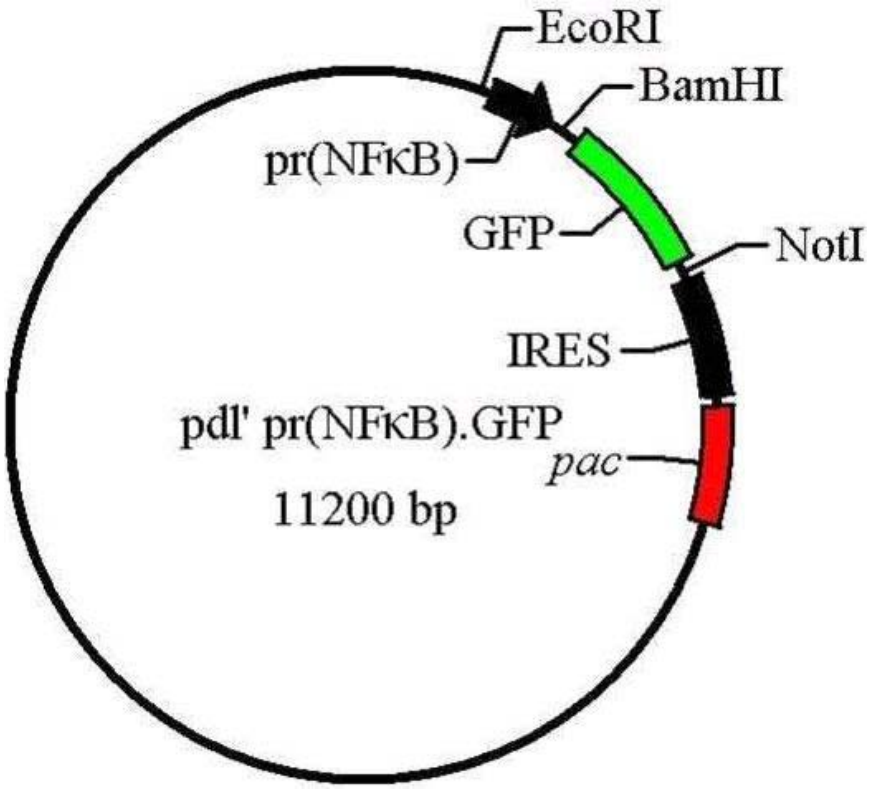


Figure 4.1.3.1 Schematic representation of the pdl'pr(NF-κB).GFP lentivirus vector.

The pdl'pr(NF-κB).GFP lentivirus vector expressing GFP and puromycin resistant gene (*pac*) under the control of the NF-κB promoter. An IRES is located downstream of GFP and upstream of *pac*, to allow for translation initiation in the middle of an mRNA sequence for protein synthesis.

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Chen, S., Short, J. A. L., Young, D. F., Killip, M. J., Schneider, M., Goodbourn, S. & Randall, R. E. (2010). Heterocellular Induction of Interferon by Negative-Sense RNA Viruses. *Virology* **47**, 247-255.