Development and application of a vaccinia virus based system to study viral proteins modulating interferon expression and interferon induced antiviral activities

by

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ABSTRACT

The interferon (IFN) system is integral to antiviral innate immunity in vertebrate hosts. Inside a cell, viral pathogen associated molecular patterns (PAMPs) trigger the IFN response, comprised of IFN induction and an IFN-induced antiviral state. However, viruses have evolved strategies to counteract the IFN system. The E3 protein of vaccinia virus (VV), encoded by the E3L gene, impedes cytokine expression and suppresses the activation and function of antiviral proteins. Deletion of the E3L gene (VVΔE3L) produces an IFN sensitive mutant virus that is replication defective in most human cell lines. Due to the limited human cell lines available to support VVΔE3L replication, the capacity of E3 inhibition of human IFN-induced antiviral activities is not well defined. In this study, VVΔE3L was generated and characterized to facilitate the study of other viral IFN antagonists at modulating human IFN-induced antiviral responses. A human liver carcinoma cell line, Huh7, was found to support VVΔE3L replication. A comprehensive analysis of VVΔE3L IFN sensitivity revealed E3 inhibits all human type I and type II IFN-induced antiviral activities by modulation of the protein kinase R (PKR) pathway.

Influenza non-structural protein 1 (NS1) is well-known to mediate the suppression of IFN induction and IFN action in influenza virus infections. However, the IFN antagonizing potential of influenza NS1 may be virus subtype and/or isolate specific. VVΔE3L was next applied as an expression vector to study influenza NS1 function in modulating IFN-induced antiviral activities and IFN induction in human cells. Recombinant viruses were generated to express influenza NS1 (from avian H5N1 and pandemic viruses 1918 pH1N1, 1968 pH3N2, and 2009 pH1N1) in replacement of E3. It was found that influenza NS1 inhibits human IFN-induced antiviral activity in a subtype and isolate specific manner. Moreover, influenza NS1 differentially regulates human IFN

expression in a virus isolate-dependent manner. Altogether, this work highlights the potential of VVΔE3L as an excellent virus model system to study viral proteins modulating IFN expression and IFN-induced antiviral activities in human cells.

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"The happiest person is the person who thinks the most interesting thoughts." -Buddha

Dedication

This work is dedicated foremost to my parents, Tony and Nila Arsenio:

My most valuable lessons learned in life were not given in any of my many classes, but were taught by you. I could not have done this without your continued love, encouragement, and support. Thank you, Mom and Dad.

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And to my beautiful nieces, Kennedy and Kelsey:

'Anything is possible' when you find what you love to do, work hard to do well, and, above all, have fun with it ☺.

All of you have been my support in everything I have done, and you will always be my foundation in everything I choose to do. 'Home is where the heart is' no matter where this crazy world will take us.

***

Words I choose to live by:

"Be the change you wish to see in the world."
- Mahatma Gandhi

"Do what you love. Love what you do. Take less. Give more. Never quit.
Never follow. Be passionate. Be bold. Be honest. Respect people.
Respect the environment. Always bring out the best in your family and friends.
Change is the only constant. Fear is an illusion. Attitude is everything."
- Sonnie Trotter, Canadian professional rock climber

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"Science is 1% inspiration and 99% perspiration."
- Albert Einstein

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LIST OF ABBREVIATIONS

1918 pH1N1	1918 pandemic H1N1 virus
1968 pH3N2	1968 pandemic H3N2 virus
2009 pH1N1	2009 pandemic H1N1 virus
3pRNA	uncapped 5'-triphosphate RNA
a.a	amino acid
A549	human adenocarcinoma lung epithelial cells
AAF	IFN-α activated factor
AD293, 293T	human embryonic kidney cells
ADAR	adenosine deaminase
AIM2	absent in melanoma 2
AP-1	activated protein 1
AraC	cytosine arabinoside
ATF-2	activated transcription factor-2
ATP	adenosine triphosphate
BHK21	baby hamster kidney cells
bp	base-pair
C-Add	C-terminal tail extension
CARD	caspase recruitment and activation domain
Cardif	CARD containing adaptor protein
CBP	CREB-binding protein
CEFs	chicken embryonic fibroblasts
CID	central interacting domain
Cop	Vaccinia virus Copenhagen strain
CPSF30	cleavage and polyadenylation specificity factor
CRE	c-AMP-responsive element
cDNA	complementary DNA
cRNA	complementary RNA
CTD	C-terminal domain
DAI	DNA-dependent activator of interferon regulator factors
DAK	dihydroacetone kinase
DCs	dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DPI	days post-infection
DRBDs	dsRNA binding domain(s)
dsRBD	double-stranded RNA binding domain
dsRNA	double-stranded RNA
ED	effector domain
EEV	extracellular enveloped virion
EGFP	enhanced green fluorescence protein
EMCV	Encephalomyocarditis virus
ER	endoplasmic reticulum
GAGs	glycosaminoglycans
GAS	Gamma activated sequence

GMP	guanosine monosphosphate
gpt	guanine phosphoribosyltransferase
GTP	guanosine triphosphate
H	hour
HA	hemagglutinin
HDAC	histone deacetylases
HeLa	human cervical carcinoma epithelial cells
HeLa-S3	human cervical carcinoma epithelial spinner-3 cells
HepG2	human hepatocellular carcinoma epithelial cells
HPI	hours post-infection
HPT	hours post-transfection
HSV-1	Herpes simplex virus-1
Huh7	human hepatocellular carcinoma epithelial cells
IFN	Interferon
IFNAR1/2	IFN alpha receptor 1/2
IFNγ	IFN-gamma
IL-1R	IL-1 receptor
IL-1β	interleukin-1β
IL-6	interleukin-6
IMP	inosine monophosphate
IMV	intracellular mature virion
IPS-1	IFN-β promoter stimulator 1
IRAK	IL-1R-associated kinase
IRF	IFN regulatory factor
IRS	insulin related protein
ISG	IFN stimulated gene
ISGF3	IFN stimulated gene factor 3
ISRE(s)	IFN stimulated response element(s)
Jak(s)	Janus kinase(s)
Kb	kilobase
kDa	kiloDalton
LGP2	laboratory and genetics physiology 2
LZ	leucine zipper
M1, M2	matrix protein 1, matrix protein 2
M2	matrix 2 protein
MAVS	mitochondrial antiviral signalling protein
MDA5	melanoma differentiation associated 5
MOI	multiplicity of infection
MRC5	human lung fibroblasts
mRNA	messenger RNA
NA	neuraminidase
NEP/NS2	nuclear export/non-structural protein 2
NF-κB	nuclear factor kappa B
NLR	nucleotide-binding oligomerization domain (NOD)-like receptors
NLS	nuclear localization signals
NoLs	nucleolar localization sequence
NP	nucleoprotein

NS1	Non-structural protein 1
OAS	oligoadenylate synthetase
ORF	open reading frame
PA	polymerase acid
PABP1	poly(A)-binding protein
PAMP	pathogen associated molecular pattern
PB1	polymerase basic 1
PB2	polymerase basic 2
PBS	phosphate buffered saline
pDCs	plasmacytoid dendritic cells
pfu	plaque forming units
PI3K	phosphatidylinositol 3' kinase
PKR	Protein Kinase R
polyIC	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
REV	Revertant control virus
REV	revertant virus control
RIG-I	Retinoic inducible gene I
RK-13	rabbit kidney cells
RLR	RIG-I like receptor
RNaseL	ribonuclease L
RNP	viral ribonucleoprotein complex
RT-PCR	reverse transcription polymerase chain reaction
SH2	Src homology 2
SHP-1	SH2 domain-containing phosphatase-1
siRNA	small interfering RNA
SOCS	suppressor of cytokine signalling protein
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription protein
STING	stimulator of interferon genes
SW13	human adrenal carcinoma cells
TBK	tank binding kinase
TBK-1	TANK binding kinase-1
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
TRAP	translocon-associated protein
TRIM25	tripartite motif 25
TriMut	triple mutant
U/ml	Units/ml
UV	ultraviolet
VISA	virus induced signalling adaptor
vRNA	viral RNA
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
VVΔE3L	VV E3L deletion mutant

WR	Vaccinia virus Western Reserve strain
XGPRT	hypoxanthine-guanine phosphoribosyltransferase

CHAPTER I: INTRODUCTION

I. Innate interferon response to viral infections

The innate immune system is the first line of protection against viral infections. At the cellular level, the rapid recognition of an invading pathogen activates the innate immune response. Innate immune responses include the production of various proinflammatory and antiviral cytokines, for example, type I interferons (IFNs)[212]. The IFN system is a critical component of innate immunity against viral infections. Type I IFNs induce several signalling cascades in an autocrine and paracrine manner to stimulate the expression of multiple IFN stimulated genes (ISGs). Some ISGs are well-known for their functions to inhibit viral replication[222].

I.1 IFN induction

I.1.1. IFN inducing pathogen associated molecular patterns

Pathogen associated molecular patterns (PAMPs) are molecular signatures that are generally unique to pathogens[104]. In virus-infected cells, the presence of viral PAMPs induces IFN gene expression. Viral PAMPs include intracellular viral DNA or RNA species, among which double-stranded RNA (dsRNA) is the major viral PAMP. dsRNA PAMPs can be derived from viral genomic dsRNA, the replication intermediates of ssRNA viral genomes, and annealed complementary mRNAs that are encoded on opposite strands in the viral genomes of DNA viruses[255]. Uncapped 5'triphosphate RNA (3pRNA) of the genomes of most RNA viruses and *in vitro* transcribed transcripts can also induce IFN expression[117,218].

The recognition of viral PAMPs by host pathogen recognition receptors (PRRs) activates PRR signalling. PRR signalling pathways lead to type I IFN (primarily IFN-β) induction (Fig. 1). The transcription of the IFN-β gene can be mediated by three families

of transcription factors, including interferon regulatory factors (IRFs), mainly IRF-3 and IRF-7, which bind to interferon stimulated response elements (ISREs), nuclear factor (NF)-κB , which binds to the *κ*B site, and the heterodimer c-Jun/activated transcription factor-2 (ATF-2), an activated protein (AP-1) which binds to the c-AMP-responsive element (CRE)[222]. Phosphorylation of the transcription factors mediates their translocation to nucleus to induce IFN expression. Constitutively expressed IRF-3 is activated by phosphorylation by two noncanonical IκB kinases: TANK binding kinase-1 (TBK-1) and IKKε[90,225]. In an unstimulated state, the NFκB inhibitor IκB maintains NFκB heterodimers in the cytoplasm. Upon viral infections, IKK phosphorylates IκB which leads to proteosomal degradation of the inhibitor, thereby releasing NFκB[213,231]. Phosphorylation of the AP-1 dimer is mediated by MAP kinases, p38 and c-Jun NH2-terminal kinases (JNK), which activate ATF-2 and c-Jun, respectively[71,108]. The activated transcription factors translocate to the nucleus. IRF-3 homo-dimerizes and associates with transcriptional activators NFκB and AP-1. This transcription complex recruits the transcriptional coactivator, CREB-binding protein (CBP), to induce IFN-β mRNA synthesis[89]. The IFN-β promoter contains several transcription factor binding sites at which the cooperative binding of the transcription factors, also known as the enhanceosome complex, can mediate maximal promoter activation[185,186].

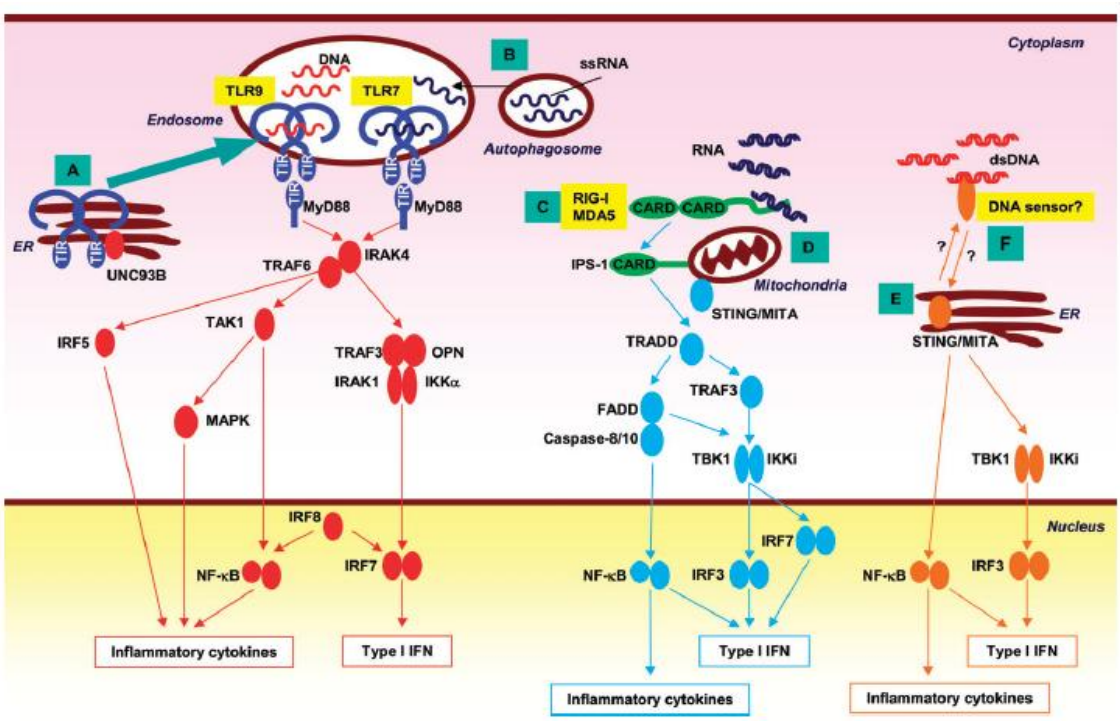


Fig.1 Activation of PRR signalling pathways by viral PAMPs. Recognition of viral PAMPs by host PRRs activates downstream signalling pathways to induce type I IFN and inflammatory cytokine gene expression. (A) Located on the ER in pDCs, TLR7 and TLR9 interact with UNC93B and are trafficked to the endosome to recognize viral ssRNA and DNA, respectively. TLR7/9 recruit MyD88, IRAK4 and TRAF6 to activate TAK1, IRF5 and TRAF3. TAK1 mediates the activation of NF-κB and MAPK, to induce inflammatory cytokine gene expression. TRAF3 activates IRAK1 and IKKα, which phosphorylate IRF7 to induce type I IFN genes. OPN is involved in IRF7 activation. IRF8 facilitates NF-κB and IRF7 activation. (B) Autophagy induction occurs in pDCs, which deliver viral RNA to the endosome or lysosome, where TLR7 is expressed. (C) In the cytoplasm, viral RNA species are recognized by RLRs. RIG-I and MDA5 recruit their shared adapter IPS-1 via CARDs. IPS-1 is localized to mitochondria, recruits TRADD, which then forms a complex with FADD, caspase-8 and caspase-10 to activate NF-κB. TRADD also recruits TRAF3 to activate TBK1–IKKi–IRF3. FADD is involved in IRF3 activation. STING (also known as MITA) localizes to (D) mitochondria or (E) ER; in mitochondria, STING (MITA) interacts with IPS-1 and RIG-I to activate NF-κB and IRF3. (F) Cytoplasmic dsDNA is sensed by host DNA sensors including DAI (ZBP2) or AIM2. In the ER, STING (MITA) is involved in the response to dsDNA. DsDNA activates NF-κB and IRF3 via the IKK complex and TBK1–IKKi, respectively.

I.1.2 Pattern recognition receptors

PRRs can be classified into two main families, membrane-bound Toll-like receptors (TLRs) and cytoplasmic receptors such as retinoic acid-inducible gene-I (RIG-

I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and cytosolic DNA sensors.

TLRs are transmembrane proteins and can be expressed on endosomes or on the cell surface of plasmacytoid dendritic cells (pDCs), eosinophils, and neutrophils[1,100]. TLRs contain a conserved Toll/IL-1 receptor (IL-1R) (TIR) domain responsible for transducing intracellular signalling following the recognition of viral PAMPs. TLR3, TLR7, TLR8, and TLR9 recognize nucleic acid PAMPs and activate the antiviral immune response by stimulating type I IFN and proinflammatory cytokine production. TLR3 recognizes double-stranded RNA (dsRNA)[3], TLR7 and TLR8 detect single-stranded RNA (ssRNA) from RNA viruses such as HIV and influenza viruses and synthetic uridine-rich ssRNA[48,149]. TLR9 recognizes unmethylated CpG DNA of DNA viruses such as herpes simplex viruses[148]. Activation of TLRs by the recognition of specific viral PAMPs results in receptor dimerization and the interaction between the TLR TIR domains and TIR-domain containing adaptor proteins[180]. The TLR adaptor proteins transduce downstream signals to IL-1R-associated kinases (IRAK) and tumor necrosis factor (TNF) receptor-associated factor (TRAF) proteins. IRAK and TRAF proteins then converge at the activation of IκB kinase (IKK) family members, including IKKα, IKKβ, IKKε, and TBK to regulate the activation of IRFs and NFκB.

Cytosolic recognition of viral RNA PAMPs is mediated by RLRs, including retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2)[271,272]. RIG-I, MDA5, and LGP2 are RNA helicases containing an ATP-binding motif in their central DExD/H helicase domain. The helicase domain unwinds dsRNA and the ATP-binding motif which contains lysine 270 (K270) is critical to mediate IFN-β induction[243]. A C-terminal

domain (CTD) in RIG-I, MDA5, and LGP2 binds dsRNA. RIG-I and MDA5, but not LGP2, have two caspase recruitment and activation domains (CARD) at their N-termini, while a repressor domain (RD) (also called the regulatory domain) is present towards the C-termini in RIG-I and LGP2, but not in MDA5[265]. A negative regulatory role of the RD was shown when the overexpression of the RD inhibited virus-induced IFN-β induction[209]. The overexpression of the CARD alone stimulates IFN production and demonstrates the CARD region is required to mediate downstream signalling[209,272]. The CARD region of RIG-I or MDA5 interact with the CARD region of their shared adaptor protein interferon-β promoter stimulator -1 (IPS-1) (also known as MAVS, VISA, and Cardif), which is located on the outer mitochondrial membrane. IPS-1 in turn activates two IKK-related kinases, IKK-ε and TBK1 through TRAF3 to mediate the activation of IRF3 and NFκB[86].

RIG-I and MDA5 recognize specific types of viral RNA PAMPs[270]. For instance, RIG-I detects ssRNA containing the uncapped 5' 3pRNA of influenza A virus, *in vitro* transcribed RNA[94,194], panhandle regions in the genome of influenza viruses[55,201] and homopolymeric ribonucleotide sequences such as the polyuridine tract of the hepatitis C virus ssRNA genome[210]. Short dsRNA species (21-27 nucleotides in length) lacking the 3pRNA can also be selectively recognized by RIG-I[153]. Unlike RIG-I recognition of short dsRNA sequences, MDA5 detects longer dsRNA[109] and higher order RNA structures or RNA webs (mismatched basepairs in dsRNA sequences) that were isolated from encephalomyocarditis (EMCV) or vaccinia virus (VV) infected cells[195]. Both RIG-I and MDA5 signalling can be activated by small cellular RNA species cleaved by a cellular ribonuclease L (RNaseL)[150] and RNA species derived from VV infections[44,170]. Reports have shown LGP2 may function as a positive or negative regulator of RIG-I and

MDA5 signalling, and may be virus-specific. For example, VSV infection, but not EMCV infection, results in increased type I IFN responses in LGP2 deficient mice[258].

Direct interactions with other proteins and/or motifs can positively or negatively regulate RLR signalling. For example, a direct interaction between dihydroacetone kinase (DAK) and MDA5 was shown to negatively regulate MDA5 activity[47]; NLRX1 (also known as NOD9) directly interacts with IPS-1 to negatively regulate IPS-1 signalling[160]; Src homology 2 (SH2) domain-containing phosphatase-1 (SHP-1) activates RIG-I mediated signalling by binding directly to RIG-I[5]. More recently, an ER-associated protein called STING (stimulator of interferon genes; also known as MITA/MPYS/ERIS) has been shown to be an important regulator of IFN production in response to both RNA and DNA species[98]. In response to VSV infection, STING was shown to interact with RIG-I, but not MDA5, to induce type I IFN expression via both NFκB and IRF3 transcriptional activities[99]. STING was shown to interact with SSR2 (also known as TRAPβ), a member of the translocon-associated protein (TRAP) complex required for translocation of proteins across the ER membrane following mRNA translation[99]. STING was also shown to mediate IFN production in response to intracellular DNA species and infection with HSV-1 in DCs[98,99].

Ubiquitination of RLRs can also enhance or suppress RLR signalling. For example, tripartite motif 25 (TRIM25), a RING finger E3 ligase, mediates the Lysine 63-linked ubiquitination of the CARDs of RIG-I required for efficient RIG-I activation[57]. REUL, a RIG-I specific E3 ubiquitin ligase, ubiquitinates lysine 154, 164, and 172 residues of the RIG-I CARD to stimulate RIG-I signalling[58]. Another E3 ubiquitin ligase, RNF125 ubiquitinates RIG-I, MDA5, and IPS-1 to suppress their functions[8].

NLRs recognize a wide range of microbial PAMPs including bacterial peptides and RNA species[113]. NLRs activate cytokine production through NFκB transcriptional activity or via an inflammasome, which causes the proteolytic cleavage of multiple caspases to stimulate the production of inflammatory cytokines, such as interleukin-1β (IL-1β). For example, influenza A virus infection induces IL-1β production through activation of a NLR family member, NLRP3, a component of the inflammasome which may be cell-type specific[4,96].

Cytosolic DNA sensors, such as DNA-dependent activator of interferon regulator factors (DAI) or ZPB2[244], can also activate IFN production through NFκB and IRF3 activities in response DNA species associated with DNA viruses such as herpesviruses (HSV) and VV. Another cytosolic DNA PRR, absent in melanoma 2 (AIM2), plays a role in inflammasome mediated production of IL-1β in response to DNA viruses[199].

I.2 IFN-induced antiviral state

I.2.1 IFN-induced signalling pathways

In humans, the IFN family consists of three main classes: type I IFNs, comprised of IFN-α subtypes, IFN-β, IFN-ω, IFN-ε, and IFN-κ, type II IFNs, which includes a single member IFN-γ, and type III IFNs (IFN-λ1, IFN-λ2, and IFN-λ3). There are 13 IFN-α genes and 4 pseudogenes, in which 12 different IFN-α proteins or subtypes have been identified, including IFN-α1,-α2, -α4, -α5, -α6, -α7, -α8, -α10, -α13, -α14, -α16, -α17, -α21. Since IFN-α1 and IFN-α13 share 98.9% nucleotide sequence identity, identical proteins are produced[32,64,263]. Members of each IFN class bind to their cognate receptors expressed on the cell surface to induce intracellular signalling cascades and transcriptionally regulate the expression of host defence genes, including apoptosis and antiviral immunity associated genes.

Type I IFNs are well-characterized antiviral cytokines. All IFN-α subtypes are structurally similar and share the same receptor with IFN-β, the type I IFN receptor. The type I IFN receptor is composed of two transmembrane subunits, IFNAR1 and IFNAR2[37,192]. Classical IFN-induced signalling is mediated by the Jak/STAT pathway. Type I IFN binding to IFNAR1/2 results in receptor activation by the cross-phosphorylation of two Janus Kinases (Jaks), Tyk2 and Jak1, associated with IFNAR1 and IFNAR2, respectively[93]. Activated Jaks recruit and mediate the phosphorylation of two signal transducers and activators of transcription proteins (STAT1 and STAT2). STATs1/2 form heterodimers and the phosphorylation of critical amino acid residues tyrosine 701 in STAT1 and tyrosine 690 in STAT2 promotes their association with DNA binding protein, IRF9, to form the transcriptional complex, interferon stimulated gene factor 3 (ISGF3)[39,132,196,217]. The phoshorylation of serine 727 in STAT1 is required for its transcriptional activity[264]. ISGF3 translocates to the nucleus and bind to a specific DNA sequence, known as the IFN-stimulated response element (ISRE), which are located approximately 200 bp upstream to the promoter regions of more than 300 ISGs[197] (Fig.2).

Type II IFNs are important immunoregulators of innate and adaptive immunities. Type II IFN-induced signalling involves IFN-γ binding to its IFNGR2/IFNGR1 receptor, followed by the activation of Jak1 and Jak2 and subsequent phosphorylation of STAT1. STAT1 homodimers assemble and translocate into the nucleus to activate gene transcription by binding to gamma activated sequences (GAS) (Fig.2). IFN-γ regulates the pro-inflammatory response and is a key regulator of T cell responses, including promotion of the Th1 T cell response and downregulation of the generation of IL-4 and IL-10 producing Th2 T cells[22].

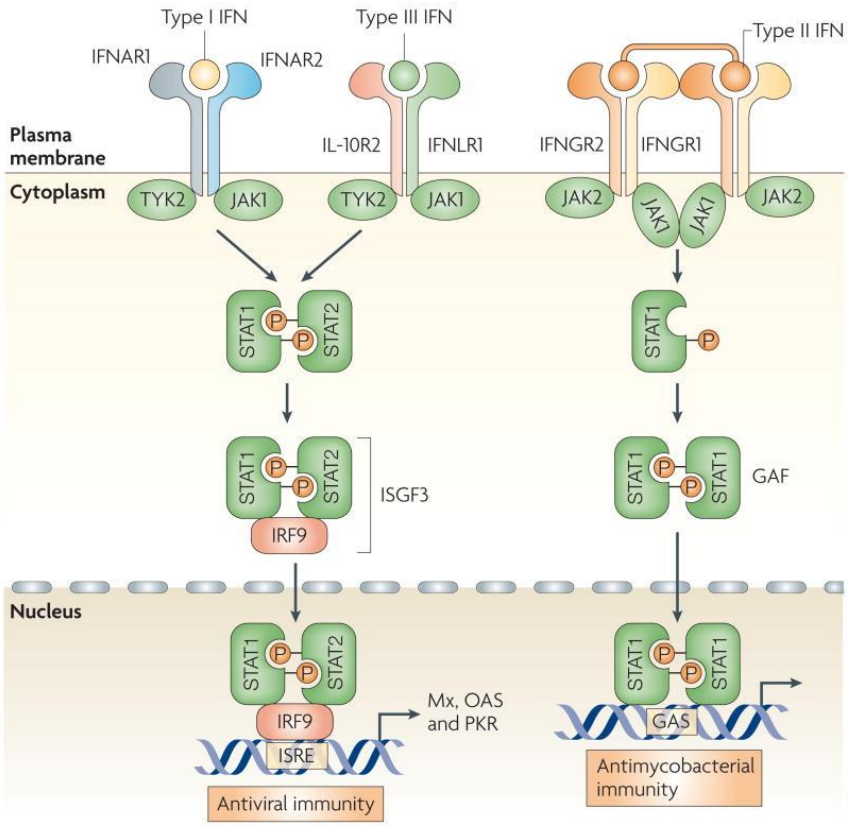


Fig.2 IFN-induced signalling. Type I IFNs bind to their cognate receptors to activate signal transduction events. Phosphorylation of IFNAR1/2 by Jak1 and Tyk2 is followed by the phosphorylation of STATs1/2. Phosphorylated-STATs1/2 proteins associate with IRF-9 and form the transcription complex ISGF3. ISGF3 transcriptionally induces ISGs expression by binding to ISRE promoter regions.

IFN-induced signalling amplifies the antiviral response. In this positive feedback loop, IFN-induced signalling results in the expression of another main IFN transcription factor, IRF-7. Activated IRF-7 associates with activated IRF-3 to bind to ISREs and coordinate the robust expression of IFN-β and IFN-α genes[37,63,132,155,193,222].

Type I IFNs can also induce signalling pathways that are independent of ISGF3 mediated signalling. The activated IFNAR1/2 can interact with other STAT proteins, such as STAT3 and STAT5, to mediate the interaction with cellular proteins such as Crk proteins, insulin related proteins (IRS), and phosphatidylinositol 3' kinase (PI3K)[112,197]. Type I IFN-induced signalling can regulate other cellular pathways such as proliferation, differentiation, and adhesion pathways, or apoptotic pathways[21,241]. Furthermore, IFN-induced signalling is also implicated in the activation of MAP Kinases (MAPKAPK2 and MNK1) in the p38 MAP kinase pathway[136,137], the mTOR/p70 S6 kinase pathway[131], and histone deacetylases (HDAC), which play a role in the regulation of IFN/ISG mRNA translation and ISG transcription[29,178].

I.2.2 ISGs and antiviral activities

Genome-wide microarray studies have revealed that more than 300 genes can be induced by type I and type II IFNs in human and murine cells[42,46]. ISGs encompass a multitude of proteins involved in host defence, inflammatory cytokine signalling, apoptosis, and protein degradation[42]. ISGs have been shown to directly function as antiviral ISGs. Classical examples of antiviral ISGs include protein kinase R (PKR), oligoadenylate synthetase (OAS)/ribonuclease L (RNaseL), and MxA.

PKR, also known as EIF2αK2, is constitutively expressed in all tissues and is a well-characterized ISG[59]. PKR mediates host defence responses including antiviral activity and apoptosis. In addition, it also plays a role in other cellular pathways such as

proliferation and gene transcription[59]. PKR is a member of a family of protein kinases that regulate protein synthesis in response to distinct stress signals. Other kinases in this family include HRI (also known as EIF2αK1), PERK (also known as EIF2αK3), and GCN2 (also known as EIF2α4). All members of this family share in common their substrate, the mammalian translation initiation factor eIF2α. In response to distinct stimuli, each kinase mediates the phosphorylation of serine residue 51 in the α subunit of eIF2. Phosphorylation of eIF2α represses its function in the exchange of GDP to GTP to inhibit mRNA translation initiation[206]. PKR contains a dsRNA binding domain (dsRBD) comprised of two dsRNA binding motifs at its N-terminus and a C-terminal catalytic serine-threonine kinase domain. Under normal conditions, PKR exists as an inactive monomer. Ligands including viral dsRNA, polyanionic compounds such as heparin and the cellular protein activator, PACT, activate PKR[61]. Ligand binding to the N-terminal dsRNA binding domain of PKR induces a conformational change. Activation of PKR by autophosphorylation at residues threonine 446 and threonine 451 via its C-terminal kinase domain causes the protein to dimerize. Activated PKR mediates the phosphorylation of eIF2α to halt global mRNA translation[59,61]. PKR has been shown to mediate antiviral activity against a broad range of viruses, including RNA viruses HCV[176], EMCV[269] and DNA viruses, including HSV-1[2].

Similar to PKR, the oligoadenylate synthetase (OAS) family of genes are constitutively expressed and are well characterized ISGs[200,212]. OAS exists as a monomer in a normal state. In the presence of viral dsRNA, OAS oligomerizes (tetramers) to synthesize 2', 5'-linked phosphodiester bonds to polymerize ATP into oligomers of adenosine, or 2', 5'-

oligodenlylates[95,116,200]. 2', 5'-oligomers polymerize and bind to cellular ribonuclease L (RNaseL) monomers to activate the enzyme. Upon activation, RNaseL dimerizes and mediates RNA degradation[27,35]. The antiviral activity of RNaseL has been characterized against RNA virus families including *Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, Orthomyxoviridae, Flaviviridae and Retroviridae* families[232].

Type I and type III IFNs induce the expression of Mx proteins. Mx proteins are guanine-hydrolyzing proteins or GTPases. They contain an N-terminal GTPase domain, a central interacting domain (CID), and a C-terminal leucine zipper (LZ). In humans, MxA and MxB proteins have been identified, among of which MxA proteins have been shown to exert antiviral activity[76,78,79,212]. Viral nucleocapsid-like structures are recognized by the CID and LZ of MxA proteins in humans[120]. MxA proteins exist as oligomers, are localized on the ER within the cytoplasm and exert antiviral activity by binding to and degrading viral nucleocapsids. A broad range of viruses including *hantaviruses*[6], *measles virus*[252], and *influenza viruses*[79] have been reported to be susceptible to MxA antiviral activity.

I.3 Viral antagonizing mechanisms to counteract the IFN system

Co-evolution of viruses with their hosts has led viruses to develop strategies to evade the IFN system[77]. Viral hosts have sophisticated and highly efficient mechanisms to detect viral dsRNA PAMPs to activate the IFN response. However, many viruses have developed countermeasures to compromise the IFN response. One such countermeasure is viral encoded dsRNA binding proteins, which inhibit dsRNA PAMP activated antiviral innate immune responses. Examples of viral encoded dsRNA binding proteins are reovirus σ3, HSV-1 US11, avian reovirus σA, hepatitis C virus NS5a, Ebola virus VP35,

vaccinia E3 and influenza virus NS1[97,102,126,144,169] (Fig.3). In particular, vaccinia E3 and influenza NS1 share in common the ability to inhibit more than one component of the IFN response and target similar signalling pathways involved in IFN induction and IFN-induced antiviral functions[51,189].

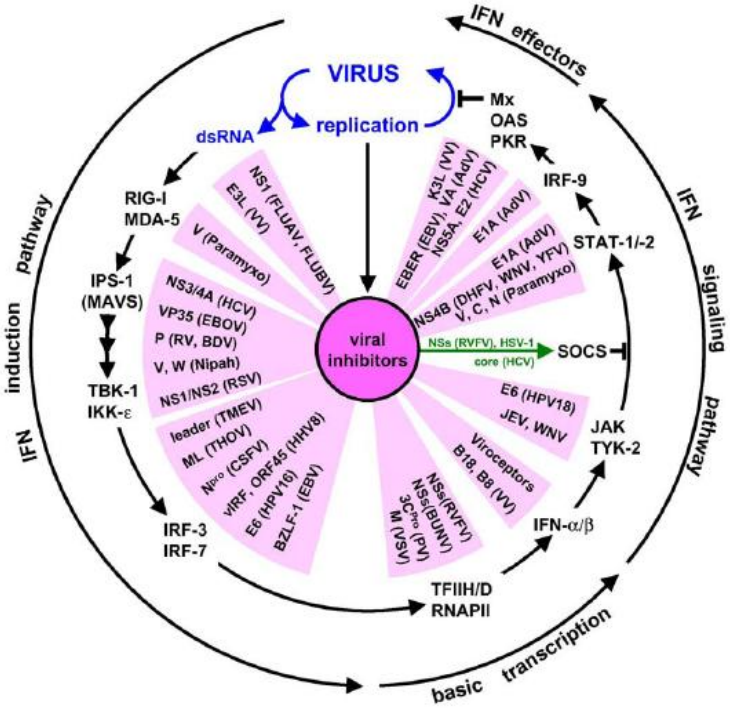


Fig.3 Viral antagonists of the IFN system. Viral proteins target IFN induction, IFN-induced signalling, and IFN-induced antiviral effector functions to evade the cellular IFN response.

I.3.1 Vaccinia E3

A pioneer virus system that was used to study the anti-IFN functions of viral proteins was vaccinia virus (VV)[53,181,182]. VV has a broad vertebrate host range and is the prototypic member of the Orthopoxvirus genus within the subfamily *Chordopoxvirinae* in the family *Poxviridae*. VV contains an intronless, linear double-stranded DNA (dsDNA) genome, approximately 185-200 kb, from which at least 200 proteins are produced. The central region of the genome is highly conserved between different orthopoxviruses,

whereas the ends are hypervariable. VV replication occurs in a cascade of transcription events in virus-factories in the cytoplasm of infected cells. VV contains three classes of genes: early, intermediate, and late genes[267]. Early genes are immediately transcribed under the control of early promoters through the activity of the viral transcriptosome, followed by replication of the viral genome mediated by the viral DNA-dependent DNA polymerase. Following DNA replication, the transcription of intermediate viral genes occurs under the control of intermediate promoters. Intermediate gene expression is then followed by the transcription of late viral genes containing late-stage promoters by the viral RNA polymerase[162].

VV early gene products comprise numerous proteins that function in antagonizing host immune responses[165,189]. In general, poxviral encoded immunomodulators are classified into three main categories: secreted virokines (viral homologues of cellular chemokines that bind cellular receptors, such as complement control protein VCP[121], secreted viroreceptors (viral homologues of cellular receptors which sequester cellular ligands, such as type I IFN inhibitor)[242], and intracellular viral proteins that modulate signalling and effector pathways, such as VV E3[165].

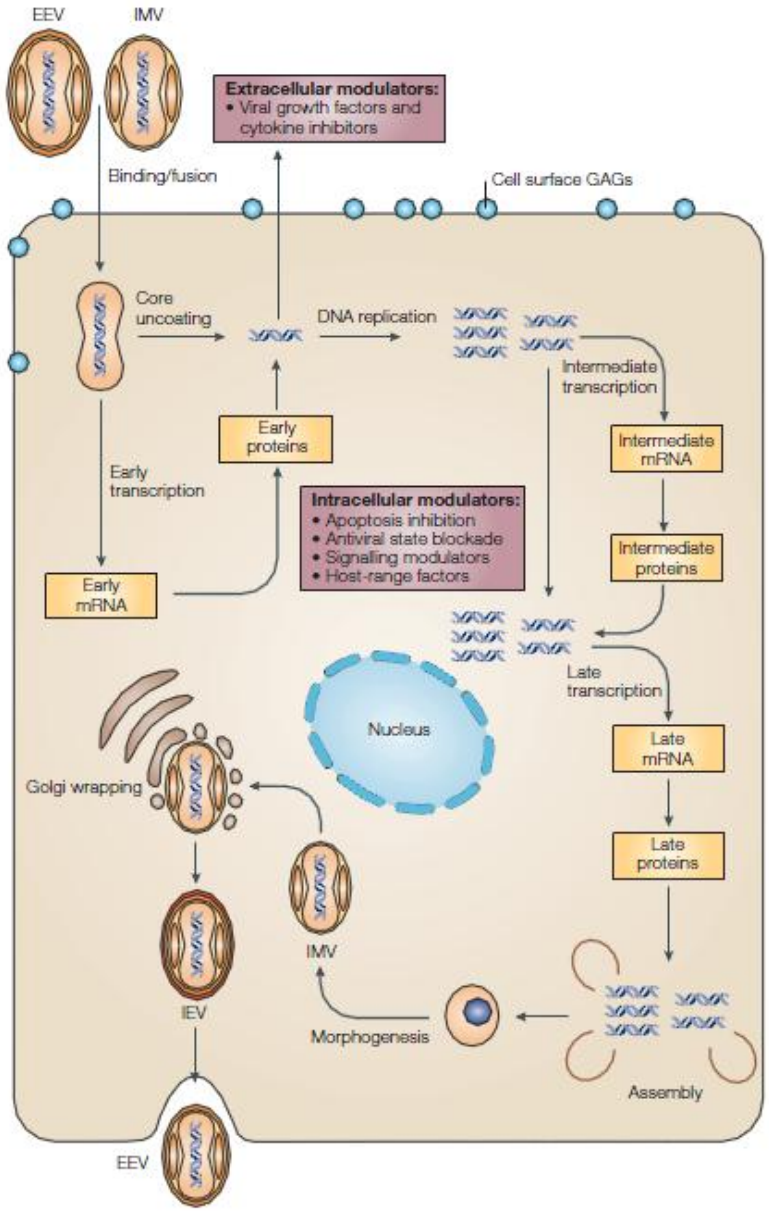


Fig.4 Schematic representation of the VV life cycle. Two infectious forms of poxviruses are the intracellular mature virion (IMV) and the extracellular enveloped virion (EEV)[141]. Virion binding and entry involves several viral proteins, including VV-A28, and the ubiquitously expressed cellular protein, glycosaminoglycans (GAGs)[164]. Following virion fusion with the cellular membrane, the virus core is released into the cytoplasm. Early genes are immediately transcribed by the viral transcriptosome. Uncoating of the viral core releases the viral genomic DNA and template for replication of the virus genome mediated by the viral DNA-dependent DNA polymerase. Transcription of intermediate viral genes follows DNA replication. Intermediate gene expression is then followed by the transcription of late viral genes. Simultaneous to late gene expression, virion morphogenesis occurs, in which the intracellular mature IMV virions assemble and migrate through microtubule trafficking through the Golgi apparatus. IMV encased in a pair of Golgi-derived membranes comprise the intracellular enveloped virion (IEV). Virion release occurs through fusion of the external virus envelope membrane with the cell membrane, producing infectious extracellular enveloped virions (EEV)[162].

The E3 protein, encoded by a VV early gene E3L, is a well-known IFN antagonist. VV E3L is a host range gene required for virus replication in most human cell lines[15] and virulence in mice[23]. The E3 protein is a 25 kiloDalton (kDa) protein with two functional domains, an N-terminal Z-DNA binding domain and a C-terminal dsRBD. The ability of E3 to mediate the inhibition of cytokine expression is largely dependent on the activity of its dsRBD. For example, E3 was shown to suppress IPS-1 dependent signalling[44], and inhibit the phosphorylation and activation of IRF-3 and IRF-7[234,268], thereby impeding IFN-β induction. E3 was also shown to inhibit the expression of proinflammatory cytokines, such as TNFα and IL-6, by inhibiting the PKR dependent activation of NFκB and MAPK p38 pathways[172]. The dsRBD of E3 was also shown to inhibit ssRNA and DNA dependent IFN-β induction through RIG-I and RNA polymerase III-mediated dsDNA sensing pathways[152,257]. E3 inhibits the activation and function of multiple ISGs. For example, the dsRBD of E3 mediates the inhibition of activation of dsRNA-dependent antiviral proteins, 2',5' OAS and PKR, although the N-terminal Z-DNA binding domain has also been shown to directly interact with and inhibit PKR activation[31,40,208]. It was further demonstrated that E3 directly interacts with ISG15 to inhibit protein ubiquitylation[70], and the dsRBD of E3 can suppress the RNA editing activity of the cellular enzyme ADAR1[140]. The IFN antagonizing activity of E3 was shown in E3-transgenic mice that were more sensitive to infection with *Leishmania major* infections[49]. E3 also exhibits antiapoptotic functions. For example, the N-terminal Z-DNA binding domain was shown to inhibit apoptosis in human HeLa cells[125], and E3L expression in NIH3T3 cells confers resistance to dsRNA induced apoptosis[60]. In summary, E3 mediates the inhibition of multiple host defence pathways.

The deletion of the E3L gene produces a mutant virus (VVΔE3L) with a distinct phenotype. First, VVΔE3L exhibits a restricted host range in cell culture and is nonvirulent in mice[23]. For instance, in comparison with the wild-type virus, VVΔE3L is unable to replicate in monkey kidney Vero cells, murine L929 cells, or HeLa cells[15,127,229,230]. It is well known that PKR and RNaseL play major roles in determining the highly restricted host range of a VV E3L deletion mutant (VVΔE3L)[15,127]. Second, VVΔE3L is sensitive to IFN treatment[16]. Previous studies on the IFN sensitivity of a VV E3L deletion mutant (VVΔE3L) were based on HeLa[128] or murine L929 cells[14,15,268]. However, in these cells, the replication of a VVΔE3L is defective, even in the absence of IFN treatment. It remains inconclusive which IFN-inducible antiviral proteins are the major players in mediating the inhibition of VVΔE3L replication in an IFN-induced antiviral state in human cells. Thus, inhibition of IFN-induced antiviral activity by VV E3 is still not well defined.

Complementation of E3 function can be mediated by the expression of other viral dsRNA binding proteins in replacement of E3 (Fig.5)[65]. Previous studies that have used VVΔE3L in this manner include the expression of reovirus σ3 protein[13], porcine C rotavirus NSP3 protein[130], hepatitis C virus NS5A protein[84], avian reovirus σA protein[66], and ORF virus E3 homolog encoded from the OV20.0L gene[259]. Studies of the anti-IFN functions of these viral proteins expressed in VVΔE3L were performed in HeLa, murine L929, rabbit kidney RK13, or chicken embryonic fibroblasts (CEFs) cells. However, it is difficult to investigate which IFN-inducible antiviral proteins are modulated since VVΔE3L cannot replicate in HeLa or murine L929 cells, even without IFN treatment. Thus, HeLa and murine L929 cells are not ideal cell lines to test the IFN antagonizing activity of E3 or other viral dsRNA binding proteins in an IFN-induced antiviral state. In

addition, while VVΔE3L can replicate in RK13 cells and CEFs[15], treatment of these cell lines with rabbit or chicken IFN-α/β may induce different signalling pathways than human IFN treatment due to species-specificities. Thus, RK13 cells and CEFs are not ideal cell systems to study E3 inhibition of human IFN-induced antiviral activities.

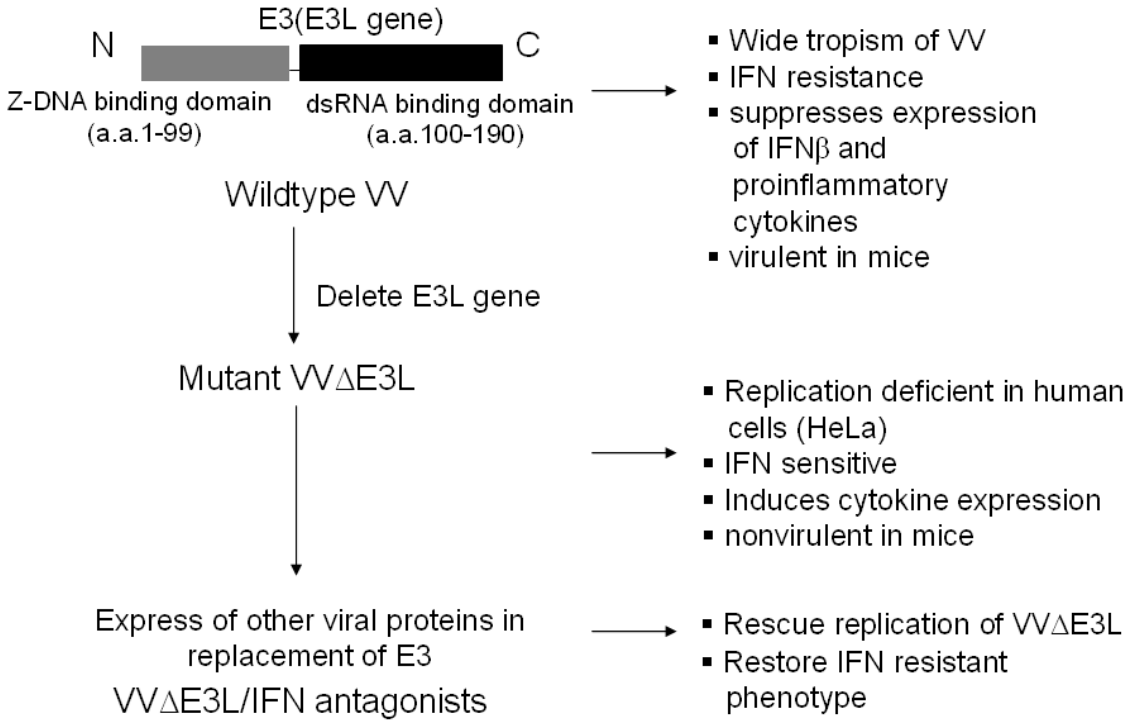


Fig.5 Schematic representation of VV E3 protein and summary of the phenotypes associated with wildtype VV (expressing E3 protein) and a VV E3 deletion mutant, VVΔE3L (devoid of E3 protein). Recombinant viruses expressing viral dsRNA binding proteins in replacement of E3 complements E3 function.

I.3.2 Influenza non-structural protein 1 (NS1)

Influenza A virus belongs to the *Orthomyxoviridae* family, comprised of five genera: influenza viruses A, B, and C, *Thogotovirus* and *Isavirus*[114]. The viral structural proteins include glycoproteins hemagglutinin (HA), neuraminidase (NA), a matrix 2 (M2) protein that project outwards through the lipid membrane, and a second matrix protein (M1) located below the lipid membrane. The virion core contains the viral ribonucleoprotein (RNP) complex, comprised of the viral RNA segmented genome coated with nucleoprotein (NP), viral polymerase proteins: polymerase basic 1 (PB1), polymerase basic 2 (PB2), and polymerase acid (PA). Nuclear export/non-structural protein 2 (NEP/NS2) are located under the M1 layer. The influenza viral genome contains eight negative-sense ssRNA gene segments that encode 11 or 12 proteins depending on the viral strain (Fig.6)[183]. The viral RNA dependent polymerase mediates the transcription and replication of the viral RNA genome in the nucleus. Each negative-sense viral RNA segment (vRNA) is transcribed into mRNA by the RNA-dependent RNA polymerase complex and requires the synthesis of a 5' capped primer of cellular pre-mRNA mediated by cellular RNA polymerase II activity. These capped and polyadenylated viral mRNA species are exported into the cytoplasm and translated into viral proteins. The newly synthesized viral proteins (PB1, PB2, and PA) translocate back into the nucleus to facilitate virus replication. Replication of the viral genome requires the conversion of the negative-sense vRNAs to full-length, positive-sense copies or complementary RNA (cRNA). The cRNA is the template for viral replication and newly synthesized vRNAs and occurs in a cap-independent manner[52,233] (Fig.6). The synthesis of small viral RNAs are speculated to provide the switch between transcription to replication[190,256]. All influenza vRNAs contain a 5' 3pRNA moeity and conserved

noncoding sequences at their 5' and 3' ends. The partially complementary conserved 13 nucleotides at the 5' end can base-pair with the 12 conserved nucleotides at the 3' end to form a panhandle secondary structure. Both the 3pRNA moiety and panhandle structure can act as influenza viral PAMPs to activate the RIG-I pathway[156].

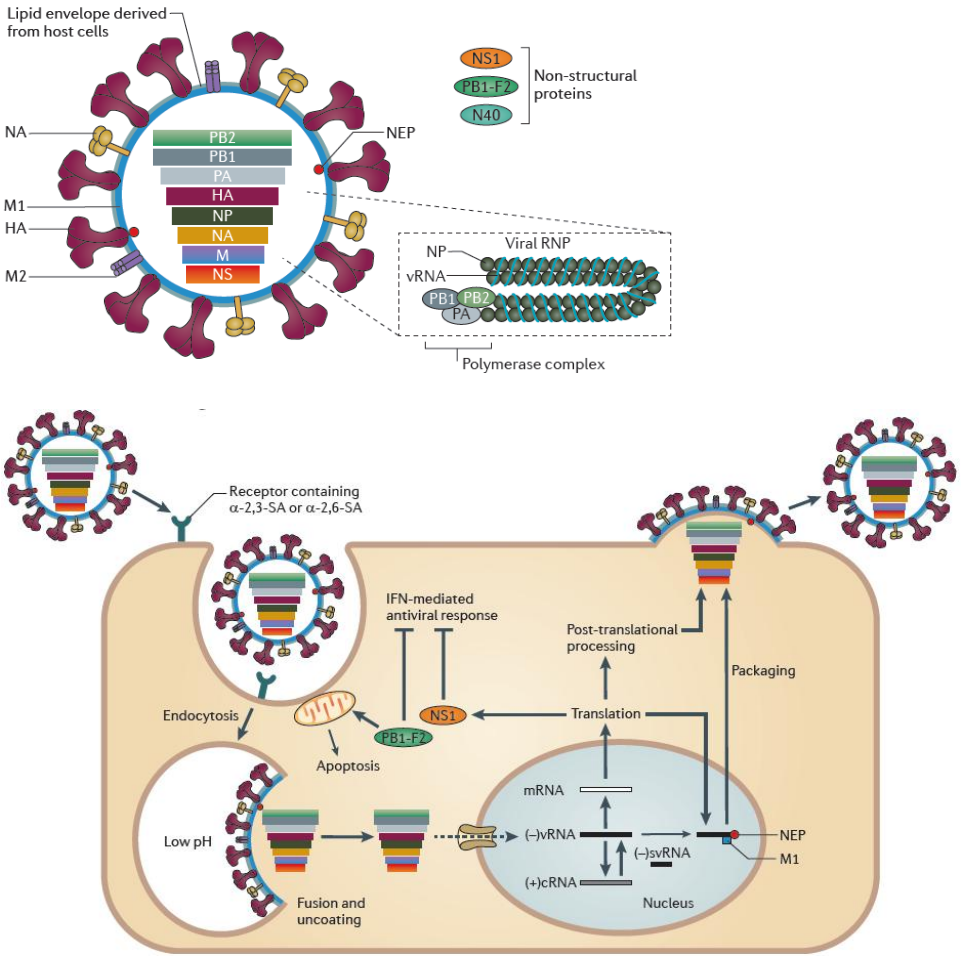


Fig.6 Schematic representation of the influenza virus particle and life cycle.
The influenza viral genome comprises 8 gene segments. Also shown is a recently identified protein N40, encoded on segment 2 (PB1 segment)[266]. The HA of human influenza viruses preferentially binds to N-acetylneuraminic acid attached to a galactose sugar at a α2,6 linkage (sialic acid-α2,6), whereas avian influenza HA preferentially binds to sialic acid with a α2,3 linkage (sialic acid-α2,3). Receptor mediated endocytosis and low pH dependent fusion of the viral and cellular endosomal membranes mediates viral particle entry and uncoating, releasing the viral RNP complex into the cytoplasm. The viral RNP is imported into the nucleus via nuclear localization signals (NLS) present in the NP. The viral RNA dependent viral polymerase mediates the transcription and replication of the viral RNA genome in the nucleus. Each negative-sense viral RNA segment (vRNA) is transcribed into mRNA by the RNA-dependent RNA polymerase complex and requires the synthesis of a 5'capped primer of cellular pre-mRNA mediated by cellular RNA polymerase II activity. Replication of the viral genome requires the conversion of the negative-sense vRNAs to full-length, positive-sense copies or complementary RNA (cRNA). Progeny RNPs are exported mediated by the NEP and M1 proteins for packaging. HA, NA, and M2 are transported to the plasma membrane through the trans-Golgi secretory pathway for viral particle assembly and release from the cells by budding[52,183].

Influenza mRNAs also contain 5' and 3' splice sites that resemble consensus sequences of exon/introns boundaries in cellular mRNA transcripts[166]. Among the eight genome segments, splicing of influenza gene segment 7 (M) and segment 8 (NS) occurs to produce proteins from unspliced and spliced mRNA variants. The NS segment encodes for non-structural protein 1 (NS1), the translation product of the unspliced mRNA, and NEP/NS2, the translation product of the spliced mRNA. NS1 functions in the virus life cycle through interaction with other viral and cellular proteins. For example, NS1 was shown to interact with the vRNP to enhance vRNA replication[151,253]. Additionally, NS1 enhances viral mRNA synthesis through interaction with cellular proteins, translation initiation factor, eIF4GI, and poly(A)-binding protein, PABP1[7,24]. Studies have also suggested NS1 plays a role in the regulation of influenza M1 mRNA splicing[143,204,205]. Thus, NS1 has several regulatory functions in the viral replication cycle.

Like VV E3, NS1 is a dsRNA binding protein and a well-known inhibitor of IFN responses. Structurally, NS1 contains a dsRBD at its N-terminus (a.a. 1-73) a central effector domain (ED) (a.a.74-207). Amino acid residues beyond the ED comprise a disordered C-terminal tail that contains two nuclear localization sequences (NLS1 and NLS2) and a nucleolar localization sequence (NoLS) and is viral-strain dependent[73]. NS1 mediates the evasion of the IFN system in influenza infections through the activities of its dsRBD and/or ED. NS1 has been shown to inhibit IFN induction. For instance, NS1 inhibits dsRNA dependent activation of transcription factors c-Jun/ATF-2[147], NFκB[260], and IRF-3[246] required for IFN gene transcription. NS1 interacts with E3 ubiquitin ligase TRIM25, required for RIG-I CARD ubiquitination, to prevent RIG-I dependent type I IFN induction.[56] At the posttranscriptional level, studies have demonstrated NS1 inhibits

the processing of IFN-β mRNAs through the direct interaction between its ED and the 30 kDa subunit of a cellular protein required for cellular mRNA processing, cleavage and polyadenylation specificity factor (CPSF30)[38,173,175]. NS1 disrupts IFN-induced signalling by upregulating the expression of suppressors of cytokine signalling (SOCS) proteins, SOCS-1 and SOCS-3, inhibitors of the Jak/STAT pathway, to block STAT1 and STAT2 signalling[105]. NS1 also blocks the activation and functions of IFN-induced antiviral proteins. For example, the dsRBD activity of NS1 prevents the activation and function of the OAS/RNaseL system[158,232]. Amino acid residues arginine 38 and lysine 42 in the dsRBD of NS1 have been shown to be critical for its dsRNA binding activity. While the dsRNA binding activity is not the main mechanism by which NS1 inhibits PKR activation in influenza infections, a.a residues 123-127 in the ED of NS1 have been shown to interact with PKR and inhibit its activation by dsRNA and PACT[133,159,248]. Moreover, NS1 mediates the evasion of apoptosis in an Akt-dependent manner through a direct interaction with the p85β subunit of PI3K[74,228,135], or through p53 mediated apoptosis by NS1 directly binding to p53[261]. Furthermore, NS1 proteins may also contain a C-terminal four a.a. residue (ESEV or EPEV) PDZ-ligand binding motif which can bind cellular PDZ proteins to disrupt cell signalling and polarity[80,177]. In all, NS1 exhibits the capacity to suppress multiple cellular pathways in the host response to influenza virus infection (Fig.7).

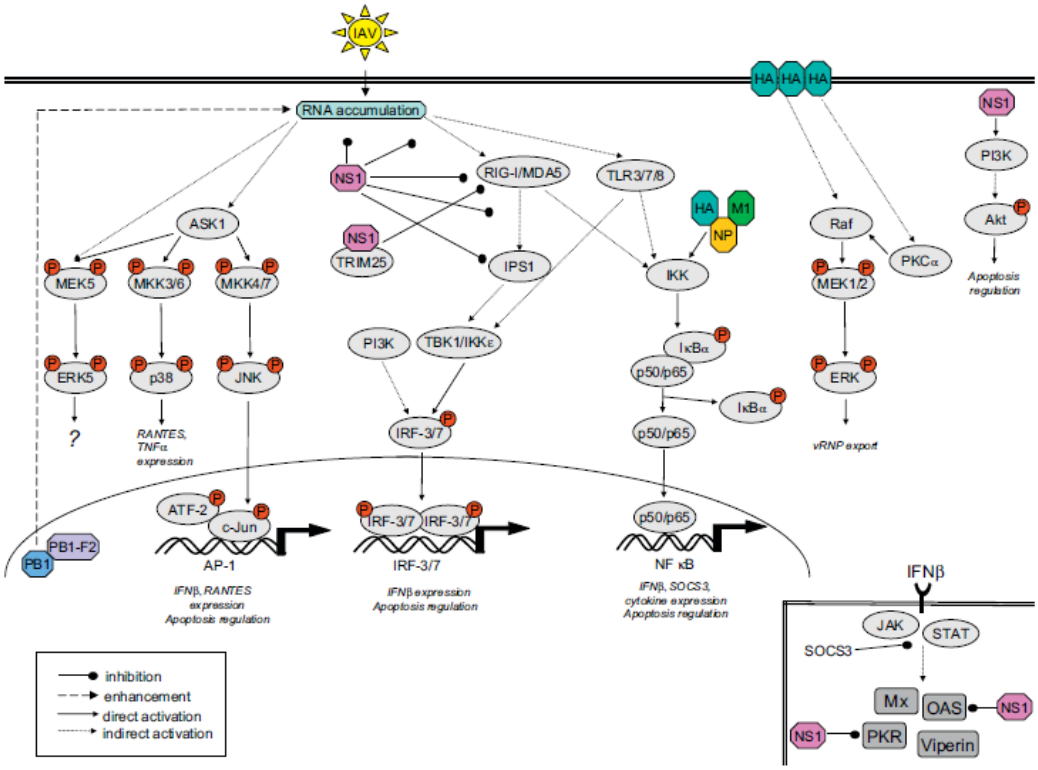


Fig.7 NS1 mediates the evasion of cellular antiviral pathways in influenza infections. NS1 impedes cytokine induction in influenza infections through the inhibition of RIG-I signalling. NS1 inhibits the activation and of IFN-inducible PKR and OAS antiviral effectors. NS1 may also exert anti-apoptotic activity through interaction with the PI3K pathway.

Studies have reported critical a.a. residues in both the dsRBD and ED of NS1 are associated with the ability of NS1 to exert its immunomodulatory functions. For example, critical a.a residues for the dsRNA binding activity include arginine 38 (R38), lysine 41 (K41), arginine 35 (R35), arginine 46 (R46), serine 42 (S42), and threonine 49 (T49)[33]. Residues in the ED of NS1, including tyrosine 89/methionine 93[74], proline 164/proline 167[228] and leucine-141/glutamic acid-142[135] were shown to bind to the p85β subunit of PI3K. A summary of the reported critical a.a. residues in NS1 for its interference with IFN induction and IFN-induced antiviral functions is included in Table 1.

Table 1 Summary of reported critical amino acid residues associated with influenza NS1 mediated regulation of IFN induction and IFN-induced antiviral activity

Influenza A virus strain of NS1 studied	Amino acid or NS1 domain	IFN induction	IFN-induced antiviral activity	Ref.
H3N2 A/Udorn/72	R48, K41		Inhibits OAS/RNaseL	[158]
H3N2 A/Udorn/72	123-127		Inhibits PKR activation	[159]
H5N1 Egret/Hong Kong/02	F103, M106	stabilizes CPSF30-NS1 interaction to inhibit IFN expression	Downregulates MxA expresssion	[215]
H5N1 CK/Hong Kong/97	E92	Induces IFNα		[215]
H5N1 Egret/Hong Kong /02	D92	Inhibits IFN expression		[134]
H5N1 Egret/Hong Kong /02 and CK/Hong Kong/91	A149	No effect in human cells	No effect in human cells	[215]
H5N1 A/Egret/Hong Kong//02	5 a.a. deletion at position 80-84, D92	Inhibits IFN-α expression in CEFs		[215]
H1N1 A/Brevig Marburg/1/18 and H1N1 A/Tx/36/91	F103, M106	Inhibits IFN expression by CPSF30 binding		[119]
H5N1 A/Hong Kong/156/97	E92		Resistance to IFN and TNF in pigs	[134,223]
H5N1/Goose/Guandong/1/96	A149	Inhibits IFN induction in CEFs		[138]
H5N1/Goose/Guandong/1/96	A149V	Induces IFN in CEFs		[138]
H5N1/Goose/Guandong/1/96	A149		IFN resistance in chickens	[138]
H1N1/PR8	G184 – enhances virulence	No effect	No effect	[240]
H1N1 A/PR8/34	E96/E97	Interacts with		[56]

		TRIM25 to inhibit RIG-I dependent IFN induction		
	R38AK41AS42G (Deficient dsRNA binding)	Induces IFN in A549 and MDCK		[50]
	R35, R46, S42, T49	Important for dsRNA binding activity		[33]
H1N1 A/PR/8/34	C-terminal truncations	Induce IFN in MDCK cells		[81]
H1N1 A/TX/91	C-terminal truncations	Induce IFN in differentiated human tracheobronchial epithelial cells and A549 cells		[251]
2009 pH1N1 (A/California/04/09)	R108, E125, G189, but contains F103, M106	Insufficient binding to CPSF30		[75]

It is important to note that the reported NS1 functions associated with specific a.a residues or motifs in NS1 are often virus strain specific. For instance, NS1 blocks gene expression by directly binding to and inhibiting the function of CPSF30[173,175,254]. Phenylalanine 103 (F103) and methionine 106 (M106) which are present in the NS1 proteins of influenza strains H5N1 Egret/Hong Kong/02, H1N1 A/Brevig Marburg/1/18 and H1N1 A/Tx/36/91 were demonstrated to be critical for efficient binding to CPSF30[119,215]. However, F103 and M106 are also present in NS1 of the influenza strain 2009 pH1N1 (A/California/04/09)[75], yet this NS1 protein was unable to block IFN expression efficiently.

Several studies have shown that influenza A viruses differ in their capacity to regulate the IFN system and suggested the NS1 protein is a contributing factor to viral-strain specific differences in virus-induced modulation of IFN responses [82,260]. For example, in comparison to human H1N1 viruses, infection with highly pathogenic avian H5N1 isolates was shown to strongly induce type I IFN and proinflammatory cytokine expression in primary human alveolar and bronchial epithelial cells[28]. H5N1 viral strain-dependent patterns of cytokine expression were observed in CEFs, in which differences in NS1 proteins were speculated to partly contribute to the difference in virus-induced cytokine profiles[215]. In human lung carcinoma, A549 cells, infection with influenza reassortant viruses, including a 1918 pH1N1 reassortant containing NS1 of a seasonal human influenza virus isolate and a seasonal human influenza virus reassortant containing 1918 pH1N1 NS1, showed that the 1918 pH1N1 NS1 mediated upregulated cytokine and chemokine gene expression, yet strongly suppressed the expression of multiple IFN-stimulated genes (ISGs) and host genes involved in lipid metabolism[18]. The 2009 pH1N1 infection induced TNFα and IFNγ expression in human macrophages[211], and IL-6 and type I IFNs in the lungs of infected ferrets[107]. More recently, it was reported that the NS1 protein of 2009 pH1N1 was inefficient in blocking host gene expression[75]. While the transient expression of the 2009 pH1N1 NS1 was shown to limit IFN-β production in 293T cells, it exhibited weaker inhibitory activity on IFN-β production in comparison to NS1 proteins of A/Texas/36/91 and A/Brevig Mission 1/18, both of which are known to potently block IFN-β induction[75]. Thus, the modulation of IFN responses by NS1 is a complex function, which is subtype and likely isolate dependent. Further characterization of isolate dependent functions of NS1 is highly desirable for understanding its role in influenza virus modulation of innate immunity and virus pathogenicity.

I.3.3 Challenges in studying influenza NS1 function in the modulation of the IFN system

The development of methods to genetically manipulate the genome of RNA viruses to generate infectious influenza viruses has advanced the understanding of influenza pathogenesis[62,154]. Reverse genetics techniques allow for the reconstruction of infectious influenza viruses. The generation of chimeric influenza viruses by reverse genetics techniques has permitted the study of strain-specific differences in the functions of individual viral proteins. However, the genetic manipulation of one vRNA gene segment that encodes for two different proteins (such as segment eight which encodes for NEP and NS1) may affect the expression and function of the two protein products and may result in attenuated viruses[62,236].

There are challenges that can limit comparative studies on the role of subtype and/or strain-specific NS1 function in modulating IFN expression and IFN-induced antiviral activities. First, the choice of a suitable cell culture system and animal model for swine, human and avian origin is limited due to differences in tissue tropism and differences in virus replication and virulence have been reported in different systems[179]. Second, the examination of NS1 function in the context of influenza infection often requires the laborious procedure of extensive reverse genetic manipulation of the viral genome. Third, influenza virus gene transcription to produce the NS1 protein requires RNA splicing, thus mutagenesis of NS1 in the influenza viral genome may affect the expression and function of other influenza viral proteins, such as NS2. Fourth, NS1 is a multifunctional protein in the influenza life cycle, thus, in the context of influenza viral

infection, it can be technically challenging to analyze the specific function of NS1 that is exclusive to the modulation of the IFN response.

I.3.4 Recombinant VVs as model systems for studies of viral immunomodulators

There are several advantages in using recombinant VVs as model systems for studies of viral immunomodulators. First, the stability and easy manipulation of the viral DNA genome makes it an ideal virus platform for generating recombinant viruses. Second, VV genes can be deleted or non-VV genes can be inserted into the VV genome using standard, routine homologous recombination techniques[25,161]. Large DNA sequences (over 25 000 bp) can be stably integrated into the VV genome without compromising virus infectivity. Third, VV has a wide vertebrate host range. Fourth, the immunomodulatory functions of several VV early gene products have been identified[72]. Using the homologous recombination technique, other viral genes can be expressed in replacement of the deleted VV ORF to study the immunomodulatory functions of the protein products[25,161]. Thus, recombinant VVs are suitable expression vectors to safely and efficiently study the functions of other viral proteins in modulating the host IFN response in a wide range of cell lines.

I.4 Rationale

VV E3 is a well-known inhibitor of IFN induction and IFN-induced antiviral activity. However, previous studies that have examined the IFN sensitivity of a VV E3L deletion mutant (VVΔE3L) were performed in cell lines, such as human HeLa cells and murine L929 cells, in which VVΔE3L replication is defective, even without IFN treatment. Other studies have examined the IFN sensitivity of VVΔE3L in rabbit kidney RK-13 cells[30] and chicken embryonic fibroblasts (CEFs)[66]. Although VVΔE3L can replicate in rabbit kidney RK-13 cells[30] and CEFs[15], and was shown to be sensitive to treatment with rabbit IFN-α or chicken IFN-α in these cell lines[230], it is likely that there are species-specific differences in the IFN-induced signalling pathways and ISG expression, in comparison to human IFNs. Thus, these cell lines are not suitable to precisely analyze the antagonizing activity of E3 in a human IFN-induced antiviral state. In addition, in humans, there are 12 different IFN-α subtypes known whose antiviral activities are less characterized. Therefore, the characterization of IFN-induced cellular responses (which involves more than 300 genes)[42] in relation to virus replication has not been well defined due to the limited cell lines and reagents available for such analysis.

It is important to fully examine E3 inhibition of IFN-induced antiviral activities for two main reasons. First, E3 is highly conserved among its poxvirus orthologues. Thus, the characterization of E3 inhibition of IFN responses would provide a better understanding of its role in mediating poxvirus pathogenicity and host species specificity. Second, recombinant VVs are valuable tools to study the immunomodulatory potential of viral gene products expressed in the virus backbone. In particular, recombinant VVΔE3L mutants have been used previously to express other viral IFN antagonists in replacement of E3. However, there are gaps in the current understanding of E3 inhibition of IFN-

induced antiviral responses. First, the full potential of E3 inhibition of IFN-induced antiviral responses in human cells is unknown. Second, the mechanistic pathways related to E3 mediated inhibition of human IFN-induced antiviral activities as well as IFN induction signalling pathways are less defined. Thus, a thorough characterization of E3 inhibition of human IFN responses is important for the full application of VVΔE3L as an expression vector to study other viral proteins with potentials to inhibit human IFN responses. Collectively, VVΔE3L would be a useful expression vector to study other viral proteins capable of targeting similar components of the IFN system, including IFN induction and IFN-induced antiviral action, as VV E3.

Similar to VV E3, influenza NS1 is well-known for its functions in modulating IFN induction and IFN-induced antiviral activities. Both E3 and influenza NS1 target similar host pathways to impede IFN expression such as IRF-3, NFκB, and IPS-1 dependent pathways. E3 and NS1 also prevent the activation and functions of the OAS/RNaseL and PKR pathways. However, the activity of NS1 to modulate the IFN system has been shown to be virus subtype and/or strain-specific and cell-type specific[18,119]. Furthermore, it is technically challenging to study NS1 function that is exclusive to its functions in modulating IFN induction and IFN-induced antiviral activities and independent of its functions in the influenza replication life cycle. Thus, VVΔE3L can be used to study the immunomodulatory function of NS1, when expressed in replacement of E3. Specifically, VVΔE3L can be used to study influenza strain specific NS1 functions in the modulation of IFN-induced antiviral activity and IFN induction that are independent of its functions in the influenza replication life cycle.

I.5 Hypothesis

It is known that VV E3 can inhibit IFN-induction and IFN-induced antiviral activity. A complete characterization of E3 IFN antagonizing activity and the signalling pathways involved in its inhibition of IFN-induced antiviral activity and IFN induction would better develop VVΔE3L as an ideal expression vector to study other viral proteins modulating both arms of the IFN response. The overall hypothesis that drives this research is a VV E3L deletion mutant, VVΔE3L, can be used as a vector to investigate other immunomodulatory proteins, such as influenza NS1, for their functions and related cellular signalling pathways in the modulation of IFN induction and IFN-induced antiviral activities.

I.6 Objective

The overall objective of this study is to explore VVΔE3L as a platform to study other viral proteins for their functions in modulating IFN induction and IFN-induced antiviral activities. The first main objective is to characterize the potential of E3 in the inhibition of human IFN-induced antiviral activities. Specifically, comprehensively examine E3 inhibition of all known human type I and type II IFN-induced antiviral activities and the signalling mechanisms involved. The second main objective is to apply VVΔE3L as a vector to study the functions of another viral IFN antagonist in modulating the IFN responses. In this study, VVΔE3L will be applied to investigate the IFN-antagonizing activity of influenza NS1 for three main reasons. First, VV E3 and influenza NS1 share in common the ability to inhibit IFN induction and IFN-induced antiviral activity through similar signalling pathways. Second, the expression of different NS1 proteins in VVΔE3L will allow for a comparative analysis of influenza strain-specific differences in

NS1 antagonizing IFN responses in the same cell line. Third, the expression of NS1 in replacement of E3 will permit the study of NS1 function in modulating IFN-induced antiviral activity and IFN induction independent of its functions in the influenza replication life cycle, but in the context of live virus infection.

Chapter II. Materials and Methods

1. Cell and viruses

Baby hamster Kidney (BHK21), human cervical carcinoma epithelial (HeLa-S3) (HeLa), human adrenal carcinoma (SW13), human lung fibroblasts (MRC5), human hepatocellular carcinoma (Huh7) (HepG2), human adenocarcinoma lung epithelial (A549), human embryonic kidney (AD293) (293T) (ATCC) and human hepatoma (Huh7, used in Results – Chapter I) (provided by Dr. G.Y. Minuk, University of Manitoba) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). The viruses used in this study are vaccinia virus Copenhagen strain (provided by Dr. G. McFadden, University of Florida) and vaccinia virus Western Reserve strain (WR-V119) (ATCC). Influenza A viruses were provided by Dr. Darwyn Kobasa, National Microbiology Laboratory.

2. Reagents

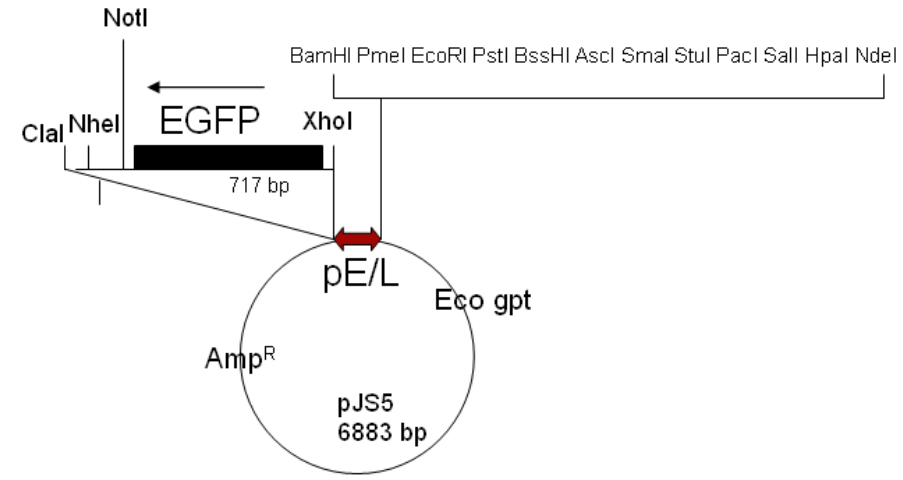
Human interferons: Type I IFNs (12 IFN-α subtypes, IFN-β1a, IFN-ω), leukocyte IFN (a mixture of IFN-α subtypes and IFN-ω) and type II IFN (IFN-γ) were purchased from PBL Biomedical Laboratories. Cytosine arabinoside (AraC) was purchased from Sigma. Antibodies include VV E3L, D12, G8 (Genscript), HA (Sigma-Aldrich), β-actin (Sigma-Aldrich), STAT1 (Upstate), phosphorylated-STAT1 (Tyr701) (Upstate), phosphorylated-STAT1 (Ser727) (Cell Signalling Technology), PKR (Cell Signalling Technology), phosphorylated-PKR (Thr446) (Epitomics), eIF2α and phosphorylated-eIF2α (Ser51) (Invitrogen), RNaseL (Cell Signalling Technology), MDA5 (Bethyl Laboratories), RIG-I (was a generous gift from Dr. Rongtuan Lin, University of McGill), IPS-1 (Bethyl Laboratories), HRP-conjugated mouse and rabbit secondary antibodies (Sigma-Aldrich), IRdye-labelled Mouse Secondary antibody (Licor-Odyssey), IRdye-

labelled Rabbit Secondary antibory (Licor-Odyssey), human IFN-response array (SABiosciences-Qiagen).

3. Construction of VV Copenhagen E3L deletion (pΔE3L/EGFP) recombinant vectors

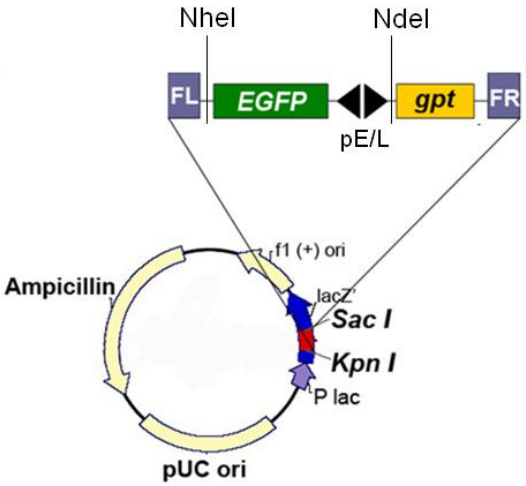
For homologous recombination, the left and right flanking regions of the *E3L* gene were PCR amplified using VV Copenhagen strain genomic DNA as the template. BHK21 cells were infected with VV Copenhagen strain at a MOI of 1 for 24 H. VV Copenhagen strain genomic DNA was purified from infected BHK21 cells by phenol:chloroform extraction. The left flanking region was synthesized with primer pair of E3L_FLF (ATTA***CTCGAG***TGATGTTCATATATTGGTTCATACAT) and E3L_FLR (***TTGTCCCGGGTTGAAGCTTACTACATATGAGAATGCTAGCTAGAT***TCTGATTCT AGTTATCAATAACA), while the right flanking region was synthesized with primer pair of E3L_FRF (***ATCTAGCTAGCATTCTCATATGTAGTAAGCTTCAACCCGGGACAA***CCAGCAAT AAAACTGAACCTACT) and E3L_FRR (TGGC***GAGCTC***TCAAGAATATAGGTAATACAAAATCTAA). The complementary sequences representing multiple cloning sites are shown in bold and italics. The left and right flanking sequences were ligated by a second round PCR with primers FLF and FRR and were inserted into cloning vector pBS-KS+ (Stratagene) between the XhoI and SacI sites, resulting in plasmid pΔE3L. Sequence fidelity was confirmed by DNA sequencing. The *gpt* gene driven by an early and late promoter, p7.5, was subcloned into pΔE3L at the SmaI site. A cassette containing *EGFP* gene, driven by an early and late promoter, was digested from pJS5-EGFP (Fig.8a) and cloned into pΔE3L between NheI and NdeI sites, yielding VV Copenhagen *E3L* deletion vector, pΔE3L/EGFP (Fig.8b).

A dominant selectable marker that has been used to generate recombinant VVs is the *Escherichia coli* (*E.coli*) guanine phosphoribosyltransferase (gpt) gene. The gpt gene is cloned into the expression vector under the control of the poxviral p7.5 early and late promoter[54]. The gpt gene encodes for the enzyme hypoxanthine-guanine phosphoribosyltransferase (XGPRT). XGPRT mediates the conversion of hypoxanthine and guanine to inosine monophosphate (IMP) and guanosine monosphosphate (GMP), respectively. The function of this enzyme permits the selective growth of recombinant VVs under culture media containing hypoxanthine/aminopterin/thymidine[157,167,168].



EGFP gene (717 bp) was cloned into pJS5 at NotI and XhoI

(a)



(b)

Fig.8 Schematic representation of expression vectors a) pJS5-EGFP and b) pΔE3L/EGFP. A cassette containing EGFP and early/late promoter was cloned into pΔE3L-p7.5gpt in between NheI and NdeI restriction enzyme sites. pΔE3L-p7.5gpt contains the flanking left and right sequences to the E3L ORF of VV Copenhagen strain.

To construct the revertant control vector, the *E3L* gene and its authentic promoter region was PCR amplified from VV Copenhagen strain genomic DNA with primer pairs E3L_N-XhoI 5′-ATTACTCGAGATTTCGCAATCTTAATGTTACAACG-3′ and E3L_C-SmaI 5′-ATAACCCGGGTCAGAATCTAATGATGACGTAACCAA-3′. The *E3L* gene was cloned into expression vector pJS5 in between XhoI and SmaI. A cassette containing EGFP and *E3L* was cloned into pΔE3L/EGFP in between NheI and NdeI, resulting in plasmid pΔE3L-Rev. The sequence fidelity of pΔE3L/EGFP and pΔE3L-Rev was confirmed by DNA sequencing.

4. Construction of VV Western Reserve E3L deletion (pΔE3L/EGFP) recombinant vector

The E3L deletion mutant recombinant expression vector was also generated based on the Western Reserve strain of VV . WR viral genomic DNA was purified from BHK21 cells infected with wildtype WR by phenol:chloroform extraction. The left and right flanking regions of the WR *E3L* gene were PCR amplified from WR genomic DNA with primer pair 5′-TTGGTACCTCTCTTATGAATCGTATATCATCAT-3′and 5′-AGATCTACTACATATGAGTCGACGAATGCTAGCTATTCGATAAGGCAGATGGAAAATCTA-3′ and primer pair 5′-GCTAGCATTCGTCGACTCATATGTAGTAGATCTAATCTCTGCGTTAGAACGCTCGTCGA-3′ and 5′-TAGAGCTCAATAAACCGTCTATTGCCACAAATT-3′.
The left and right flanking sequences were assembled together by recombinant PCR and inserted into cloning vector pBS-KS+ (Stratagene) between the KpnI and SacI sites, resulting in plasmid pWRΔE3L. The *gpt* gene driven by an early and late promoter, p7.5, was subcloned into pWRΔE3L at the BglII site. A cassette containing the enhanced GFP (EGFP) gene, driven by an early and a late promoter, was cloned between the NheI and

NdeI sites, resulting in pΔE3L/EGFP (Fig.9). Sequence fidelity of pWRΔE3L/EGFP was confirmed by DNA sequencing.

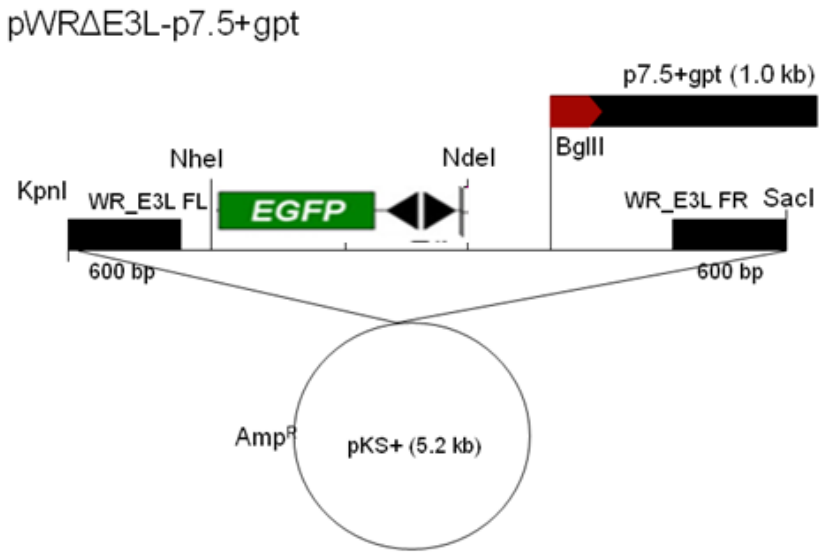


Fig.9 Schematic representation of pWRΔE3L/EGFP expression vector

5. Construction of recombinant vectors to express influenza A virus NS1 proteins

NS1 genes that encode for full-length NS1 proteins are listed in Table 2. Mutant NS1 proteins were constructed (based on the 2009 pH1N1 NS1/I123 sequence, Accession number FJ971074), including NS1 truncation mutants: NS1ΔN: a.a. residues 80-219; NS1ΔC: a.a. residues 1-79; NS1 ED mutants: NS1/I123V: isoleucine to valine mutation at a.a. residue 123; NS1TriMut: triple a.a substitutions arginine to lysine at residue 108 (R108K), glutamic acid to aspartic acid at residue 125 (E125D), and glycine to aspartic acid at residue 189 (G189D)[75]; NS1 TriMutC-Add: triple a.a. substitutions (R108K, E125D, G189D) with extended C-terminal tail at residues 220 to 230, RKMERTIEPEV (based on Accession number AF115289 (H5N1 NS1)); and NS1C-Add: C-terminal tail extension of residues 220 to 230, RKMERTIEPEV (based on Accession number AF115289 (H5N1 NS1) (Table 3). A hemagglutinin (HA) tag, 5'-ATGTACCCATACGATGTTCCAGATTACGCT-3', was added to the amino-terminus of each NS1 gene for detection of the NS1 protein. The HA-tagged NS1 genes were individually cloned into pUC57 (Genscript) and subcloned into pJS5-EGFP in between BamHI or EcoRI (at the N-terminus of the HA-tagged NS1) and SmaI (at the C-terminus of NS1) restriction sites (Fig.10a). A cassette containing EGFP and each HA-tagged NS1 sequence (from positive clones of pJS5-EGFP/NS1) was cloned into pWRΔE3L in between the Nde and NheI restriction sites to produce expression plasmids for homologous recombination (Fig.10b).

Table 2 Summary of influenza NS1 genes encoding full-length NS1 proteins

Full-length NS1	**Influenza A virus subtype**	**Virus strain**	**Accession number**	**Species isolate**	**Restriction enzyme sites added to N- and C- termini of *NS1*)**
H5N1-duck	H5N1	A/Chicken/Yunnan/493/05(H5N1)	DQ095705	duck	EcoRI/ SmaI
H5N1-human	H5N1	A/Hong Kong/486/97(H5N1)	AF256181	human	BamHI/ SmaI
Swine-origin 2009 pH1N1	H1N1	A/California/06/2009 (H1N1) *NS1 gene contains isoleucine 123 residue	FJ971074		BamHI/ SmaI
1918 pH1N1	H1N1	A/Brevig_Mission/1/18(H1N1)	AF333238		EcoRI/ SmaI
1968 pH3N2	H3N2	A/Beijing/1/68(H3N2)	CY008160		EcoRI/ SmaI

Table 3 Summary of influenza NS1 mutant proteins based on the 2009 pH1N1 NS1 sequence

Mutant NS1	**Mutation(s)**	**Restriction enzyme sites added to N- and C- termini of *NS1*)**
NS1ΔN	a.a. residues 80-219	EcoRI/ SmaI
NS1ΔC	a.a. residues 1-79	EcoRI/ SmaI
NS1/V123	isoleucine to valine at residue 123	EcoRI/ SmaI
NS1TriMut	R108K, E125D, G189D[75]	EcoRI/ SmaI
NS1C-Add	11 a.a C-terminal extension at residues 220 to 230, RKMERTIEPEV (based on Accession number AF115289 (H5N1 NS1)	EcoRI/ SmaI
NS1TriMutC-Add	R108K, E125D, G189D[75] with 11 a.a C-terminal extension at residues 220 to 230, RKMERTIEPEV	EcoRI/ SmaI

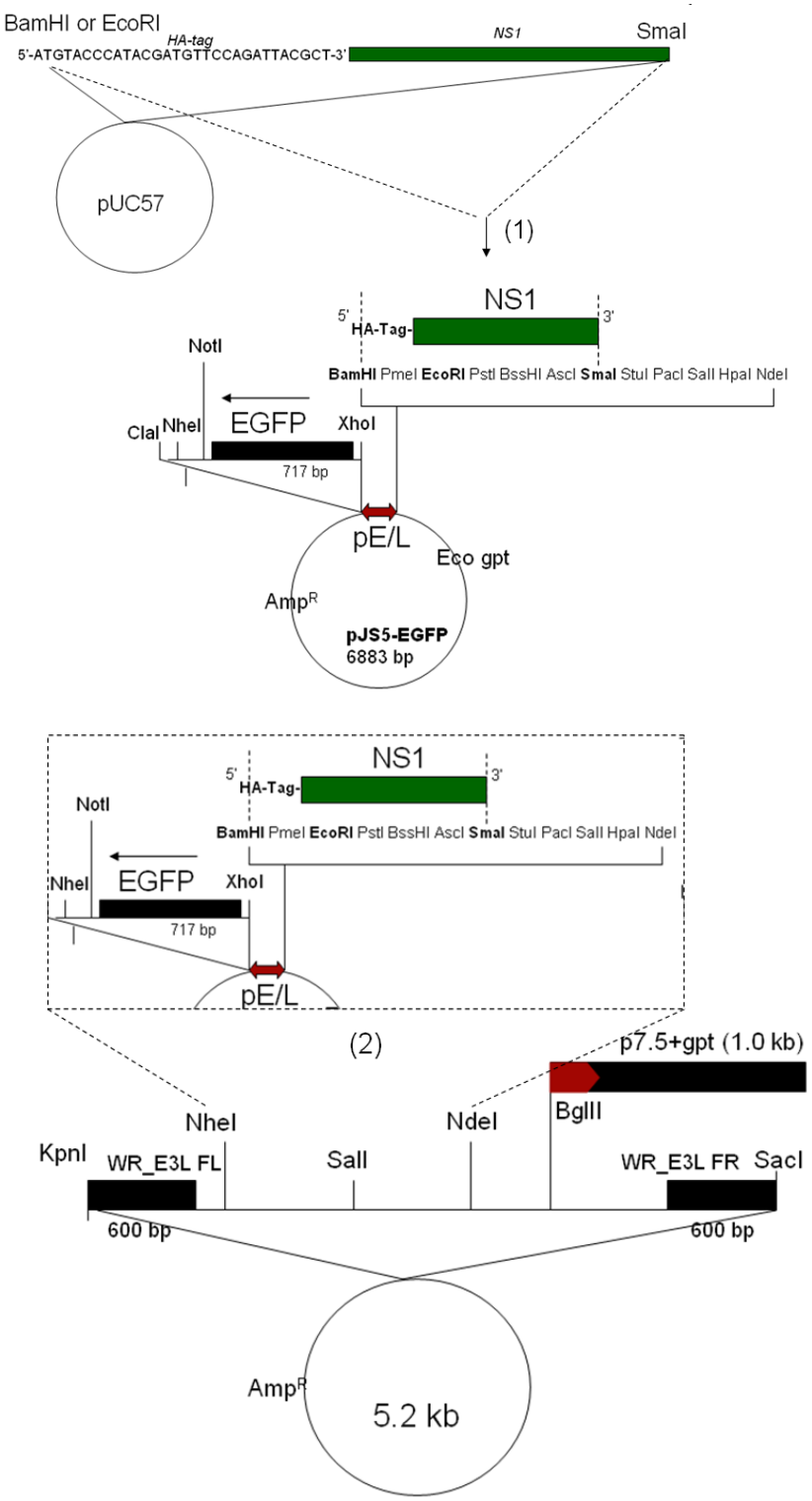


Fig.10a Construction of WR E3L deletion recombinant expression vectors containing NS1 genes (see Tables 1, 2 for NS1 genes). HA-tagged NS1 genes were synthesized and inserted into pUC57 (Genscript). (1) Each HA-tagged NS1 gene was cloned into pJS5-EGFP vector at restriction sites BamHI (or EcoRI) and SmaI. (2)

Cassettes containing EGFP, early/late promoter, and HA-tagged NS1 were next cloned into pWRΔE3L-p7.5+gpt vector in between NheI and NdeI restriction sites.

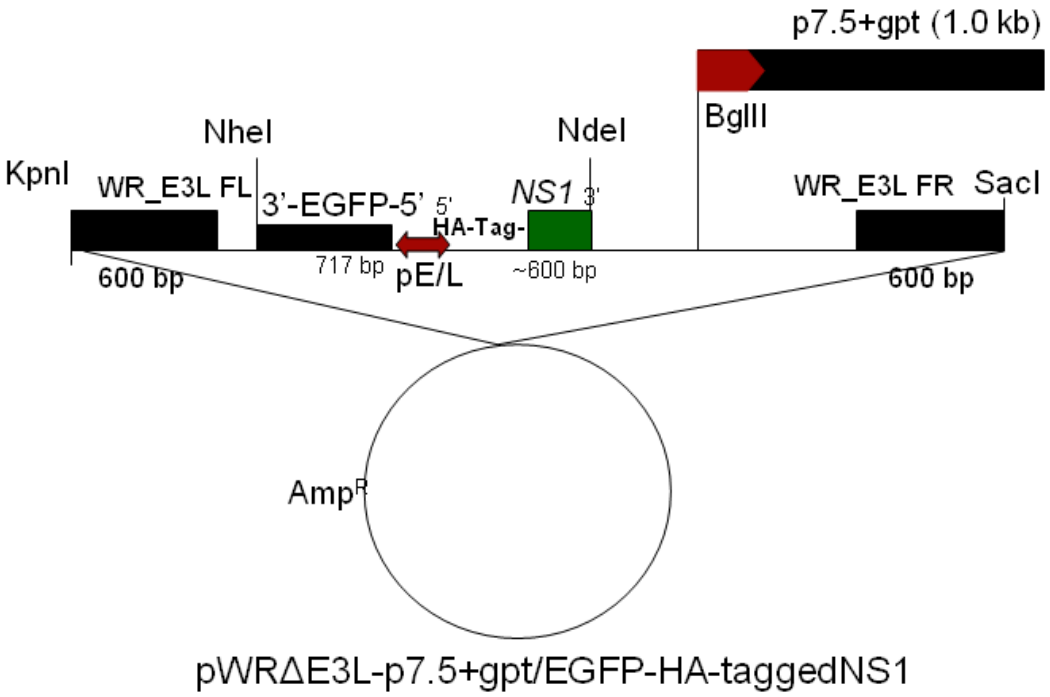


Fig.10b Schematic representation of resultant NS1 recombinant expression vectors. See Table 3 for list of recombinant expression vectors and resultant recombinant viruses expressing NS1.

The resultant recombinant expression vectors containing the NS1 genes are listed in Table 4. The sequence fidelity of all recombinant vectors was verified by DNA sequencing.

6. Generation of VV Copenhagen E3L deletion (Cop-ΔE3L) and revertant control (Cop-ΔE3L-REV) recombinant viruses

5 μg of plasmid DNA pΔE3L/EGFP (for generation of the E3L deletion mutant, Cop-ΔE3L) was transfected into BHK21 cells infected with Copenhagen strain wild-type using Effectene Transfection Reagents according to the manufacturer's instructions (Qiagen). Recombinant poxvirus was grown in selective media containing mycophenolic acid[54], xanthine, and hypothaxine, and purified after 4 rounds of plaque purification. For generation of the revertant control virus, 5 μg of plasmid DNA pΔE3L-Rev was transfected into BHK21 cells infected with the E3L deletion mutant virus. Purification of the revertant virus, which expresses the E3 protein, was performed in HeLa cells. The expression of E3 in Cop-ΔE3L rescues Cop-ΔE3L replication in HeLa cells. The purity of the Cop-ΔE3L mutant and revertant control Cop-ΔE3L-Rev viruses was confirmed by PCR with primer pair flanking the E3L ORF, forward primer 5′-TGATAAAGTAGGTTCAGTTTTATTGCTGGTTGT-3′ and reverse primer 5′-TGTTATTGATAACTAGAATCAGAATCTA-3′. Adjacent ORFs were amplified by PCR with the following primer pairs: E2L forward primer 5′-TGGATTCTGTCCAATGATGATGAAACG-3′ and reverse primer 5′-TCTTCCCTCTATCATGTTCACTACTGG-3′; E4L forward primer 5′-ATACATTAGTAGTTACTCATCCAA-3′ and reverse primer 5′-ATCATCCTCTGGTGGTTCGTCGTT-3′.

7. Generation of VV WR E3L deletion mutant (VVΔE3L) and revertant control (VVΔE3L-REV)

Generation of recombinant viruses (VVΔE3L and VVΔE3L-REV) were performed as described above with the exception of the wildtype VV strain used. The presence and absence of the WR E3L gene in VVΔE3L-REV and VVΔE3L viruses, respectively, were confirmed by PCR using pairs WR E3L locus forward primer 5'-ACGACGAACCACCAGAGGATGATGAA-3' and *E3L* locus reverse primer 5'-AGAGAATATACTAGTCGCGTTAATAGTA-3'.

8. Generation of recombinant VV expressing NS1 proteins

5 μg of each recombinant expression vector containing NS1 genes (Table 4) was transfected into BHK21 cells infected with WR strain wild-type using Attractene Transfection Reagents according to the manufacturer's instructions (Qiagen). Recombinant poxviruses expressing NS1 proteins were passaged four rounds in selective media containing mycophenolic acid[54], and purified after eight rounds of plaque purification in BHK21 cells. Western blotting using antibodies against HA-tag (to detect NS1) and E3 were performed at each round of passage and plaque purification to verify the expression of NS1 proteins and deletion of E3 in the recombinant VVs expressing NS1. The resultant recombinant viruses expressing NS1 proteins are listed in Table 4.

Table 4 Summary of NS1 recombinant expression vector constructs and the resultant recombinant WR viruses expressing NS1

NS1	Recombinant expression vector	Recombinant virus
H5N1 NS1-duck	pWRΔE3L-EGFP/H5N1 NS1-duck	VVΔE3L/H5N1 NS1-duck
H5N1 NS1-human	pWRΔE3L-EGFP/H5N1 NS1-human	VVΔE3L/H5N1 NS1-human
2009 pH1N1 NS1 (labelled NS1/I123 in Results – Section III)	pWRΔE3L-EGFP/2009 pH1N1 NS1	VVΔE3L/2009 pH1N1 NS1 (labelled VVΔE3L/NS1/I123 in Results – Section III)
1918 pH1N1 NS1	pWRΔE3L-EGFP/1918 pH1N1 NS1	VVΔE3L/1918 pH1N1 NS1
1968 pH3N2 NS1	pWRΔE3L-EGFP/1968 pH3N2 NS1	VVΔE3L/1968 pH3N2 NS1
NS1ΔN	pWRΔE3L-EGFP/NS1ΔN	VVΔE3L/NS1ΔN
NS1ΔC	pWRΔE3L-EGFP/NS1ΔC	VVΔE3L/NS1ΔC
NS1/V123	pWRΔE3L-EGFP/NS1/V123	VVΔE3L/NS1/V123
NS1TriMut	pWRΔE3L-EGFP/ NS1TriMut	VVΔE3L/ NS1TriMut
NS1C-Add	pWRΔE3L-EGFP/NS1C-Add	VVΔE3L/NS1C-Add
NS1TriMutC-Add	pWRΔE3L-EGFP/NS1TriMutC-Add	VVΔE3L/NS1TriMutC-Add

9. Virus replication assays

The replication capacities of Cop-ΔE3L and Copenhagen wildtype (Cop) viruses were analyzed in BHK21, and human cell lines HeLa-S3, HepG2, SW13, MRC5, AD293, and Huh7 cells and WR recombinant viruses in BHK21 and Huh7 cells. Confluent cell monolayers were infected at a MOI of 1 with Cop-ΔE3L or Cop and WR recombinant viruses and collected 24 hours post-infection (hpi) for virus titrations. Virus replication assays were performed in triplicate.

10. Interferon sensitivity assays

Huh7 cell monolayers were grown in 12-well culture plates to 80% confluency and subsequently treated overnight at 37°C with varying concentrations of human IFNs (12 Human IFN-α subtypes, IFNs-β, ω, and γ) at 0, 2, 20, 200, and 2000 U/ml or IFN-β at 0, 1, 10, 100, and 1000 U/ml. Cell monolayers were infected at a multiplicity of infection (MOI) of 1 with Cop-ΔE3L-Rev, Cop-ΔE3L, WR based VVΔE3L, VVΔE3L-REV, and VV recombinant viruses expressing NS1 proteins and incubated at 37°C. 1 HPI, total virus and media containing IFN were aspirated, and replaced with fresh media containing the same concentration of IFN, and incubated for an additional 24 H for Copenhagen based recombinants or 48 H for WR based recombinants at 37 °C. Control wells contained growth media without IFN.

11. Plaque assays

Virus infections with and without IFN treatments were collected at the time points indicated and serial dilutions of each were used to infect 100% confluent BHK21 cell monolayers. To calculate virus titre (pfu/ml), plaques were enumerated 24 hpi under UV. Titrations were performed in triplicate.

12. Western blot

Cells were collected in 1 ml phosphate buffered saline (PBS) and centrifuged at 6000 rpm for 5 minutes. Cell pellets were lysed in 300 µl of 1X Laemmli buffer. Protein samples were separated by electrophoresis on 4-12% polyacrylamide gels (Bio-Rad) at 150 V for 1 h, transferred onto H-bond nitrocellulose membranes (Fisher-Scientific) at 150 mA for 1 h, and incubated in 5% skim milk in TBST (1X Tris-borate sulphate and 0.2% Tween20) with the antibodies as indicated. For the detection of phosphorylated proteins, to inhibit cellular phosphatase activity, cells were treated with 5 µl of Phosphatase Inhibitor Cocktail III (Calbiochem) for 10 minutes at 37ºC prior to lysis. Protein expression was detected using chemiluminescence-based reagents Western Lightning Reagent (Perkin Elmer LAS, Inc, Millipore). Protein quantification by was performed using infrared imaging (Licor-Odyssey).

To examine the profile of signalling proteins in Huh7 cells primed with type I and type II IFNs, Huh7 cells were treated overnight at 37°C with 1000 U/ml of either leukocyte IFN, IFNs-β, ω, and γ, and infected at a MOI of 5 with all recombinant viruses for 6 H. To inhibit cellular phosphatase activity, Phosphatase Inhibitor Cocktail I (Sigma-Aldrich) was added to all wells for 10 minutes at 37ºC prior to cell lysis in PBS containing SDS.

13. RNA gel for RNaseL activity

Huh7 cells were treated with 1000 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ, and infected at a MOI of 5 with either Cop-ΔE3L-Rev or Cop-ΔE3L. Total RNA was extracted 6 and 12 HPI using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Samples were prepared according to the NorthernMax Protocol

(Ambion, Inc.) and loaded onto 1% agarose-LE (Ambion, Inc.), electrophoresed at 100 V for 1 h, and visualized under UV light.

14. RNA interference by small interfering RNA (siRNA) transfections

For each siRNA experiment, two or three negative control or non-targetting siRNAs were included to address the sensitivity and to rule out false-positives of off-target siRNA effects. Negative control siRNAs included Silencer Cy3-Labeled Negative Control #1 (Catalog #4621, Ambion, Inc.), scrambled siRNA (Dharmacon RNA Technologies), and a control siRNA sequence identical to the MDA5 siRNA sequence with a two base-pair mismatch highlighted in bold: 5′-CAA***CC***AGGCCCUACAAAUU-3′ (Dharmacon RNA Technologies). For each gene targeting siRNA, the knockdown efficiency of each gene specific siRNA was first tested among four different siRNA sequences per gene (Dharmacon RNA Technologies). The siRNA sequence that achieved the best knockdown efficiency of each gene was used for subsequent experiments.

PKR and MxA RNA silencing in Huh7 cells

Huh7 cells were seeded at 2.5×10^5 cells per well in 6-well culture plates and transfected with 100 nM of small interfering RNA (siRNA) specific to human PKR 5′-P-ACUUUGUCUAGUUUCUCGCUU, and MxA 5′-P-UUCGUCUUCCGGUAUGUCGUU (Dharmacon RNA Technologies) and Silencer Cy3-Labeled Negative Control #1 (Catalog #4621, Ambion, Inc.) using Hiperfect Transfection Reagent (Qiagen) or siPORT Amine transfection reagent (Ambion) in DMEM and incubated at 37 °C. 48 h later, cells were treated with 100 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ overnight, then infected at a MOI of 1 with viruses as indicated. Virus titres were determined by plaque assays. PKR silencing was confirmed by Western blot and the suppression of MxA was confirmed by RT-PCR.

RIG-I, MDA5, IPS-1, PKR, and STING siRNA transfections in HeLa cells

HeLa cells seeded at 1.5 x 10^5 cells/well were mock transfected or transfected with 100 nM of siRNA for 48 H using Hiperfect transfection reagent (Qiagen). Scrambled siRNA (Dharmacon) was used as the negative control siRNA. The siRNA target sequences (Dharmacon) include RIG-I siRNA 5′-CCACAACACUAGUAAACAA-3′, MDA5 siRNA 5′-CAAUGAGGCCCUACAAAUU-3′, PKR siRNA 5′-GCGAGAAACUAGACAAAGU-3′, IPS-1 siRNA 5′-CAUCCAAAGUGCCUACUAG-3′, and STING siRNA 5′-GCAUCAAGGAUCGGGUUUA-3′. Cells were infected 48 HPI with recombinant viruses at a MOI of 5 and collected 9 HPI for qRT-PCR analysis. Gene expression knockdowns were confirmed by Western blot or RT-PCR.

15. Reverse transcription polymerase chain reaction (RT-PCR)

1 μg of RNA samples were used for the sythesis of complementary DNA (cDNA) sequences by reverse transcription using Advantage RT-for-PCR Kit (Clontech) and Quantitect Reverse Transcription kit (Qiagen). All RNA samples were treated with DNaseI (Qiagen) to remove residual genomic DNA. Negative cDNA controls were also prepared under identical reaction conditions without reverse transcriptase. PCR reactions were performed as indicated in Results Sections. Primers include MxA forward primer 5′-AAGGTCAGTTACCAGGACTACGAGA-3′ and reverse primer 5′-ACAATCATGTAACCCTTCTTCAGGT-3′; human RNaseL forward primer 5′-TATGGCTTCACAGCCTTCATGGAA-3′ and reverse primer 5′-ACAATCTGTACTGGCTCCACGTTT-3′; human GAPDH forward primer 5′-AAGGTGAAGGTCGGAGTCAACGGA-3′ and reverse primer 5′-TTACTCCTTGGAGGCCATGT-3′; vvD12L forward primer 5′-ATGGATGAAATTGTAAAAAATATCCGGGA-3′ and reverse primer 5′-

TCACAGCAGTAGTTTAACTAGTCT-3′; vvG8R forward primer 5′-AATGTAGACTCGACGGATGAGTTA-3′ and reverse primer 5′-TCGTCATTATCCATTACGATTCTAGTT-3′; human IFNα2 forward primer 5'-TCCTGGTGCTCAGCTGCAAGTCAA-3'; and reverse primer 5'-TCATGATTTCTGCTCTGACAACCT-3'; human IFNα4 forward primer 5'-TCAGCTACAAATCCATCTGTTCTC-3' and reverse primer 5'-TCATGATTTCTGCTCTGACAACCT-3'; human IFNα5 forward primer 5'-TGCTCAACTGCAAGTCAATCTGTTCT-3' and reverse primer 5'-TCATGATTTCTGCTCTGACAACCT-3'; human IFNα8 forward primer 5'-GTGGCCCTAGTGGT GCTCAG-3'; human IFNα8 reverse primer 5'-GGAGACTCTATCAC CCCCACTTC-3'; human RIG-I forward primer 5'-AAAGCTAGTGAGGCACAGC-3' and reverse primer 5'-TGCACCTGCCATCATCCC-3'; human STING forward primer 5'-AGGAGAGCCACCAGAGCACACTCT-3' and reverse primer 5'-TGTTGCTGTAAACCCGATCCTTGA-3'.

16. Quantitative Real-time PCR and Reverse-transcription PCR (RT-PCR)

HeLa cells were seeded at $5.5x10^5$ cells per well in 6-well culture plates and infected with the recombinant viruses at a MOI of 5. Total RNA was extracted at 9 or 12 HPI (Qiagen) and residual genomic DNA contamination was removed using RNase-free DNase Kit (Qiagen). Mock infections were used as the control. qRT-PCR analysis was performed using Taqman Gene Expression Assays (Applied Biosystems). qRT-PCR was performed with human IFN-β and β-actin primers. The IFN-β forward primer sequence was 5′-TGGCTGGAATGAGACTATTGTTGAG-3′, the reverse primer sequence was 5′-CAGGACTGTCTTCAGATGGTTTATCT-3′ and the probe sequence was 5′-CCTCCTGGCTAATGTC-3′. The β-actin forward primer sequence was 5′-

CACACTGTGCCCATCTACGA-3′, the reverse primer sequence was 5′-GCCAGCCAGGTCCAGAC-3′ and the probe sequence was 5′-CCCATGCCATCCTGC-3′. Results represent the relative expression of IFN-β in virus infected cells, normalized to actin expression, in comparison to mock infected cells.

17. Influenza A virus infections

Infections with influenza A viruses, including pandemic 2009 H1N1 with NS1 containing I123, A/Canada/RV1535-NS/2009, or V123, A/Canada/RV1532-AB/2009 and A/Canada/RV1527-ON/2009, pandemic 1918 H1N1 A/South Carolina/1/18, and a seasonal H1N1 viral strain A/Canada/RV733/2007 were performed in biosafety containment level 4 (CL4). HeLa cell monolayers were washed 1x with virus culture medium (MEM/0.1% BSA + 1x Penicillin/Streptomycin). Virus infections were performed at a MOI of 3 in duplicate for the 3 and 6 H time-points, and incubated at 37ºC, 5% CO_2 in a humidified incubator. At 3 and 6 HPI, the supernatants were removed and 600 µl of lysis buffer RLT (with 10µl β-mercaptoethanol per ml RLT) (Qiagen) was added and incubated for 5 min at room temp. Lysates were collected into a 2 ml tube each. Samples were removed from CL4 according to SOP and stored overnight at 4ºC.

18. Immunoprecipitations

293T cells were transfected with 1 µg of pCAGGS vector/FLAG-tagged human CPSF30 for 24 H. Cells were mock infected or infected with VVΔE3L recombinant viruses expressing NS1/I123, NS1/V123, NS1TriMutC-Add, NS1TriMut, and NS1C-Add at a MOI of 5 and collected 8 HPI. Cells were lysed in 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10% glycerol, 1% Triton-X-100, and 0.5 M EDTA, supplemented with protease inhibitor cocktail (Roche). Cell lysates were incubated with 25µl of anti-FLAG

resin (Sigma) for 2 h at 4°C. Precipitated proteins were washed 5 times in lysis buffer, removed from the resin according to the manufacturer's instructions (Sigma) and analyzed by Western blotting. DsRNA binding assays were performed as described previously[171].

19. Quantitative real-time PCR (qRT^2-PCR) array analysis

RNA was isolated from HeLa cells that were mock infected or infected with REV, VVΔE3L, VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1/I123 viruses at a MOI of 5 for 12 H. RNA samples were converted to cDNA using RT^{2}First Strand Kit (SABiosciences). Gene expression profiles induced by virus infection were analyzed using the human IFN-α/β response PCR array (SABiosciences, Catalogue Number PAHS-016). Cytokines detected with a threshold cycle value of greater or equal to 35 were not considered significantly expressed and excluded from the analysis. qRT^2-PCR arrays were performed in triplicate for each virus infection. Data analysis was performed using the integrated web-based software designed specifically for all RT^2 Profiler PCR Arrays, PCR Array Data Analysis Web Portal (SABiosciences).

20. Construction of H5N1 NS1 expression plasmids, pcDNA3.0/H5N1 NS1-duck, and pcDNA3.0/H5N1 NS1-human

The complete NS1 gene sequences of H5N1 NS1-duck and H5N1 NS1-human (Table 2) were blunt end cloned into the eukaryotic expression plasmid, pcDNA3.0, digested with SmaI. Positive clones were confirmed by Western blot detection of each NS1 protein following transient transfections with each expression plasmid in BHK21 cells.

CHAPTER III: RESULTS

I: ***Antagonizing activity of VV E3 on human type I and type II IFN-induced antiviral activities (Virology 2008. 377(1):124-132)***

II: ***Comparative analysis of NS1 proteins from influenza viruses of different species-origins for their interference of IFN-induced antiviral activities***

III: ***The effector domain of NS1 of 2009 pH1N1 enhances human IFN-β gene expression in an isolate-dependent manner through RIG-I, MDA5, STING, and PKR signalling pathways***

IV: ***NS1 proteins of different avian H5N1 isolates induce an isolate-specific pattern of human IFN-α expression***

RESULTS

I: *Antagonizing activity of VV E3 on human type I and type II IFN-induced antiviral activities*

Introduction. Previous studies that have demonstrated the IFN antagonizing activity of E3 were based in cell lines that cannot support the replication of a VVΔE3L [15,16,127,268]. *VVΔE3L can replicate in rabbit kidney, RK-13 cells*[30]*, and was shown to be sensitive to treatment with rabbit IFN-α in this cell line*[230]*. However, the full potential of E3 inhibiting human IFN-induced antiviral activity in relation to virus replication remained unclear due to the limited human cell lines available for such analysis. Here, a comprehensive characterization of the inhibition potential of E3 on human IFN-induced activities was performed in a human cell line that was found to support VVΔE3L replication.*

The specific objectives included 1) comparatively examine human cell lines that may support the replication of VVΔE3L, 2) analyze the inhibition potential of VV E3 on all human type I and type II IFN-induced antiviral activities in an IFN responsive human cell line that is permissive to VVΔE3L, and 3) examine the role of IFN-induced classical signalling pathways in mediating antiviral activities.

I.1. The VV E3L deletion mutant can replicate efficiently in the human hepatoma cell line Huh7

The E3L deletion mutant (Cop-ΔE3L) and its revertant (Cop-ΔE3L-REV) were constructed based on the Copenhagen strain as described in Materials and Methods. The only difference between the E3L deletion mutant and revertant viruses is the absence and presence of the E3L ORF, respectively (Fig.11a). The difference between the revertant virus and the wild-type Copenhagen strain is the inclusion of gpt and EGFP for easy

plaque identification and orientation of the E3L ORF. The inclusion of gpt and EGFP has no effect on virus replication and IFN sensitivity (data not shown). As shown in Fig.11a, the mRNA expression of two adjacent ORFs, E2L and E4L, was not affected by deletion of E3L.

The replication capability of Cop-ΔE3L was tested in six human cell lines and compared to BHK21. The human hepatoma cell line Huh7, was the only cell line to efficiently support replication of Cop-ΔE3L, while it completely failed to replicate in human lung fibroblasts (MRC5), human embryonic kidney (AD293), and human cervical carcinoma epithelial (HeLa) cells (Fig.11b).

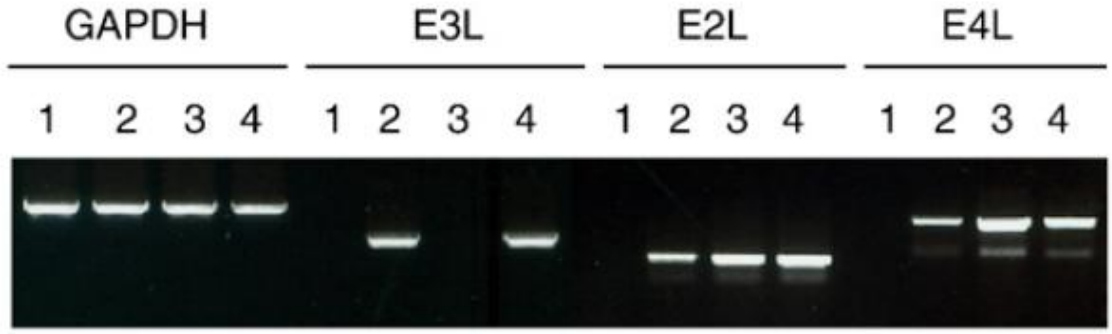


Fig.11a Confirmation of the deletion of E3L in the E3L deletion mutant (Cop-ΔE3L) and the expression of E3L in the revertant control viruses. (a) Total RNA was extracted from BHK21 cells infected with Cop-ΔE3L, Cop-ΔE3L-REV, and wild-type VV Cop. RT-PCR analysis confirm E3L mRNA expression is absent in the BHK21 cell control (lane 1), and in the E3L deletion mutant (Cop-VVΔE3L) (lane 3), but is present in wild-type VV Cop (lane 2) and in the revertant control (Cop-ΔE3L-REV) (lane 4). Deletion of E3L did not affect the mRNA expression of adjacent ORFs, E2L and E4L.

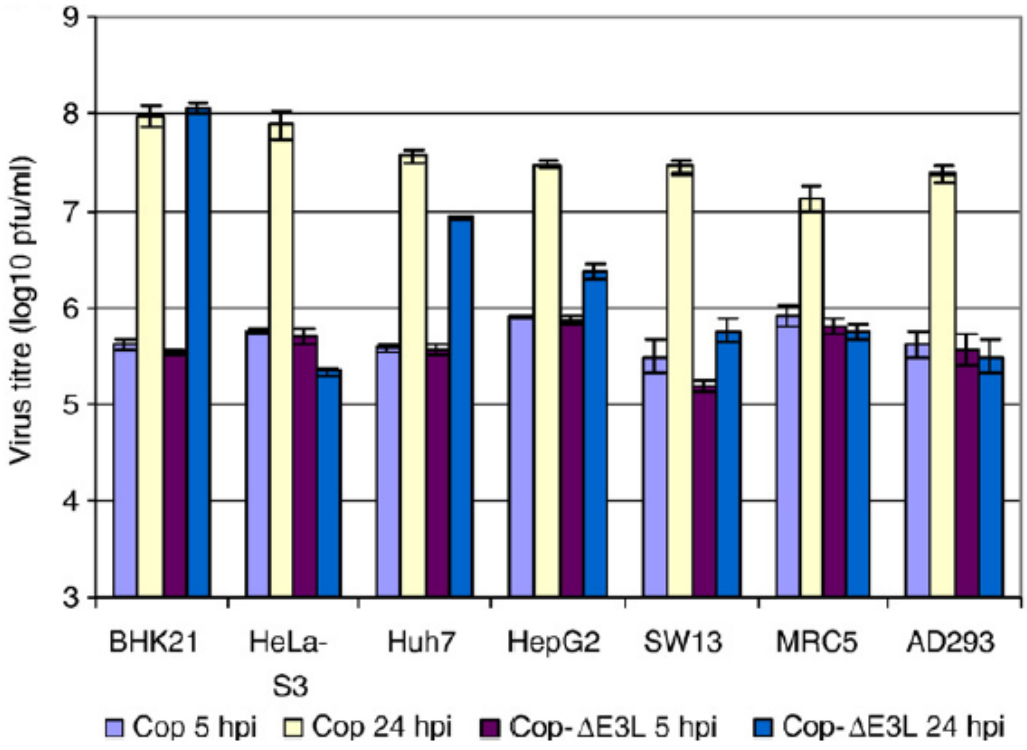


Fig. 11b Human hepatocellular carcinoma, Huh7 cells, efficiently support Cop-ΔE3L replication. Confluent cell monolayers were infected at a MOI of 1 with wild-type VV Copenhagen strain (Cop) and Cop-ΔE3L and harvested 5 and 24 hpi. Virus titres (log_{10} pfu/ml) were measured in triplicate. Error bars indicate standard error of the mean. Data shown are representative of three independent experiments.

I.2. VV E3 is a potent inhibitor of all human type I and type II interferons in Huh7 cells

Huh7 cells have been reported to be responsive to human type I IFNs[19]. The Huh7 cell line used in this study is responsive to both type I and type II IFNs, as confirmed by the activation of STAT1 by phosphorylation at two residues, tyrosine 701 and serine727 (Fig. 12). To analyze the inhibition activity of the E3 protein on human IFNs in Huh7 cells, Cop-ΔE3L and its revertant were comparatively analyzed for their sensitivity to both type I and type II IFNs (including the 12 IFN-α subtypes, IFN-β, IFN-ω, and IFN-γ). As shown in Fig. 13a, Cop-ΔE3L-REV is resistant to treatment of all type I and type II IFNs tested, depicted by the consistent pattern of replication of the revertant virus in the presence of all IFN species. Generally, a slight decrease in Cop-ΔE3L-REV replication is seen with an IFN dose of 200 to 2000U/ml of type I and type II IFN species. In contrast, deletion of the E3L gene results in a dramatic increased sensitivity to all human IFN species: IFN-β, IFN-ω, IFN-γ, and the IFN-α subtypes (Fig.13b). Cop-ΔE3L replication is inhibited in the presence of type I and type II IFNs in a dose-dependent manner. Even at a low dose of 20 U/ml, IFN species demonstrated potent inhibition, decreasing virus replication by 100-fold, seen in IFN-β, IFN-ω, and IFN-γ treated Huh7 cells. Among all IFNs tested, the most potent IFN species was IFN-α14, which inhibited replication of Cop-ΔE3L by 1000-fold at its highest dose of 2000 U/ml. Following IFN-α14, were type I IFNs: IFN-α2a, IFN-β, and type II IFN-γ, each of which inhibited replication of Cop-ΔE3L between 100 to 1000-fold. In contrast, the least effective at inhibiting viral replication was IFN-α7, followed by IFN-α5.

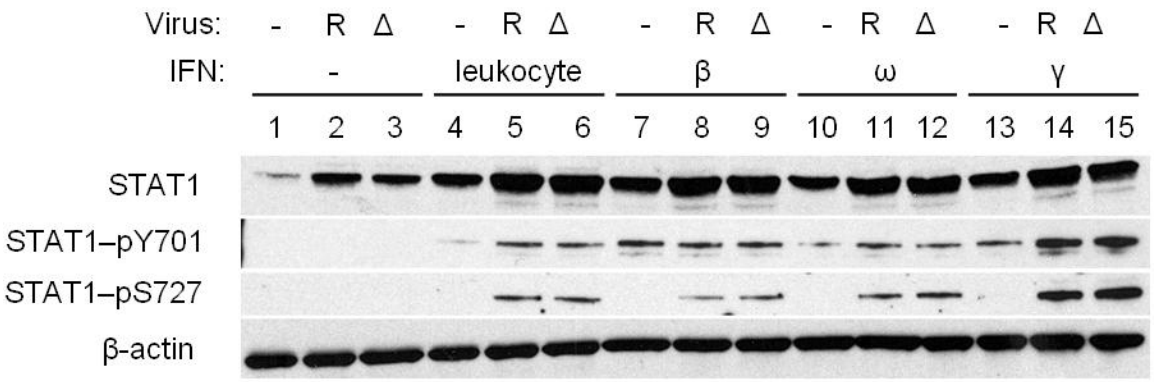


Fig.12 Huh7 cells are responsive to type I and type II IFN treatment. Activation of STAT1 by phosphorylation at residues tyrosine 701 (Y701) and serine 727 (S727) was monitored by Western blotting in Huh7 cells treated with 1000 U/ml of type I IFN (IFN-β) and type II IFN (IFN-γ) for 24 h at 37°C and infected with the revertant control virus (R) or the E3L deletion mutant virus (Δ). Data shown are representative of three independent experiments.

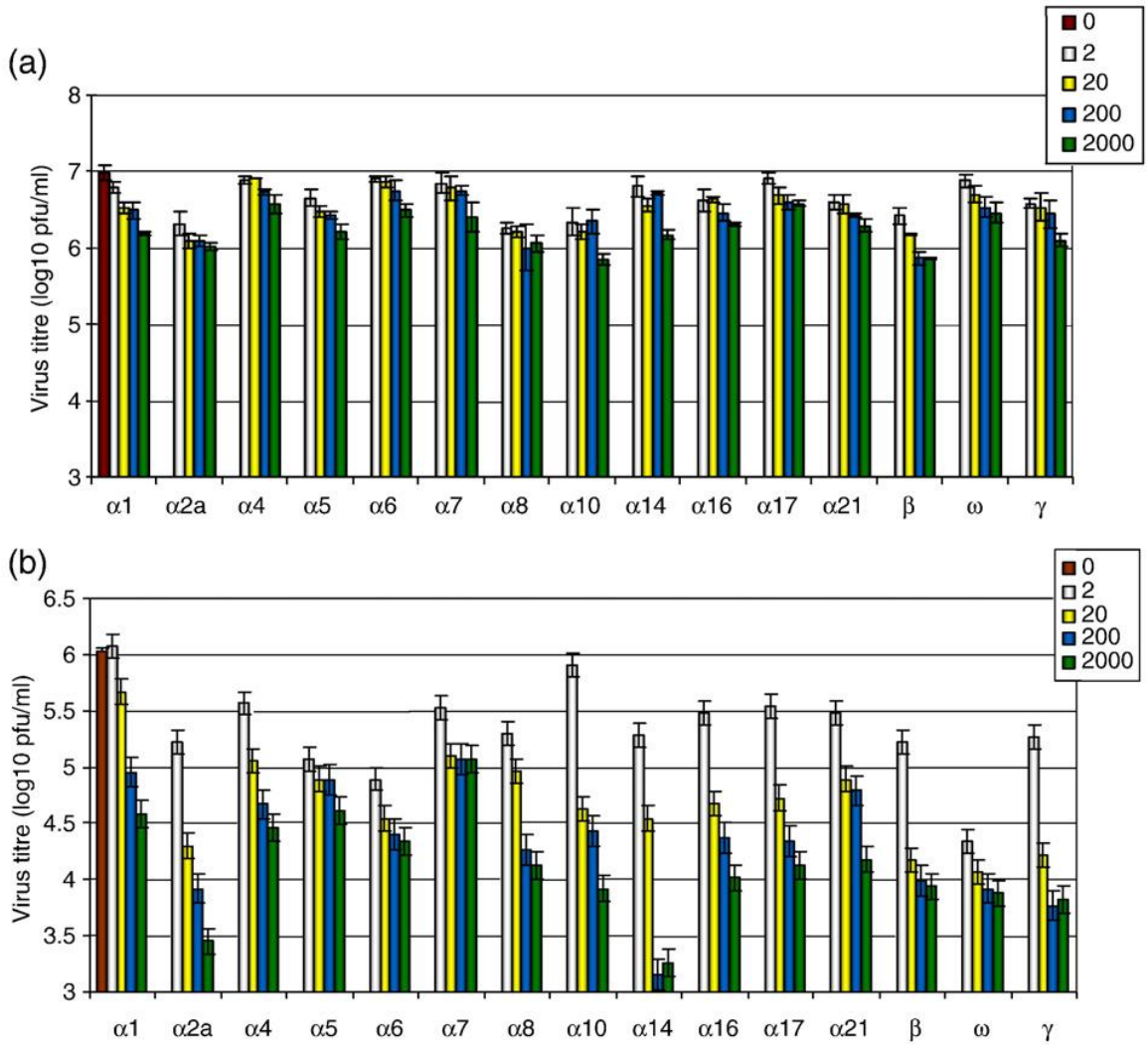


Fig.13 Comparative analysis of the sensitivity of Cop-ΔE3L-REV and Cop-ΔE3L to type I and type II human IFNs. (a) Replication of Cop-ΔE3L-REV is unaffected by IFN treatment. (b) Replication of Cop-ΔE3L is inhibited by type I and type II human IFNs in a dose-dependent manner. Huh7 cell monolayers (5×10^5 cells/ml) were pretreated with human IFNs (12 IFN-α subtypes, IFN-β, ω, and γ) at different concentrations of 0, 2, 20, 200, or 2000 U/ml for 24 h at 37 °C. The IFN-treated cells were infected at a MOI of 1, with Cop-ΔE3L-REV or Cop-ΔE3L and harvested 24 h later. Titrations were performed in triplicate. Error bars indicate standard error of the mean. Data shown are representative of three independent experiments.

I.3. Cop-ΔE3L IFN sensitivity is mediated by PKR

To further characterize the mechanism of IFN resistance of Cop-ΔE3L-REV and sensitivity of Cop-ΔE3L mutant viruses (Fig.13), the roles of each of the three main IFN-induced classical antiviral pathways: PKR, 2′–5′ OAS/RNaseL, and MxA pathways were examined. Since IFNs transcriptionally induce PKR, to investigate the PKR pathway, any change in endogenous PKR protein levels in type I (leukocyte, -β,-ω) and type II (γ) IFN-treated Huh7 cells in the absence and presence of virus infection with Cop-ΔE3L-REV and Cop-ΔE3L were first monitored. As shown in Fig.14, the level of PKR in Cop-ΔE3L-REV infected Huh7 cells is comparable to the level of PKR in the cell control in the absence of IFN treatment. However, the level of total PKR appears to be reduced in Huh7 cells infected with Cop-ΔE3L in the absence of IFN treatment. Treatment with type I (leukocyte, -β,-ω) and type II (γ) IFNs slightly enhanced the level of endogenous PKR in the absence of virus infection. Even in the presence of both type I and type II IFNs, total PKR seems to be slightly reduced in all Cop-ΔE3L infected cells, whereas in all Cop-ΔE3L-REV infected cells, there is no difference in PKR in the absence or presence of IFN treatment. It is important to note that the antibody used in this study recognizes endogeneous PKR protein and was synthesized against residues adjacent to a critical phosphorylation residue, threonine 451. Thus, because of this antibody's specificity at detecting endogeneous PKR, the absolute total PKR protein level may not be fully detected as PKR dimerizes and autophosphorylates following virus infection.

The activation/phosphorylation of PKR and corresponding phosphorylation changes in its substrate, eIF2α was examined next. Phosphorylated PKR is readily detected following infection with Cop-ΔE3L and is enhanced after IFN treatment

(Fig.14). A minimal level of phosphorylated PKR is detected in Huh7 cells infected with Cop-ΔE3L-REV in the absence and presence of IFN treatment. Total eIF2α levels slightly increased post viral infection, regardless of IFN treatment. The level of phosphorylated eIF2α increases after viral infection and IFN treatment. The only exception is observed in IFN-β treated Huh7 cells, in which total eIF2α and phosphorylated eIF2α is slightly reduced in the absence of virus infection (Fig.14). Although infection with both Cop-ΔE3L-REV and Cop-ΔE3L induced eIF2α phosphorylation, a significantly greater level of phosphorylated eIF2α is detected in Huh7 cells infected with Cop-ΔE3L than Cop-ΔE3L-REV.

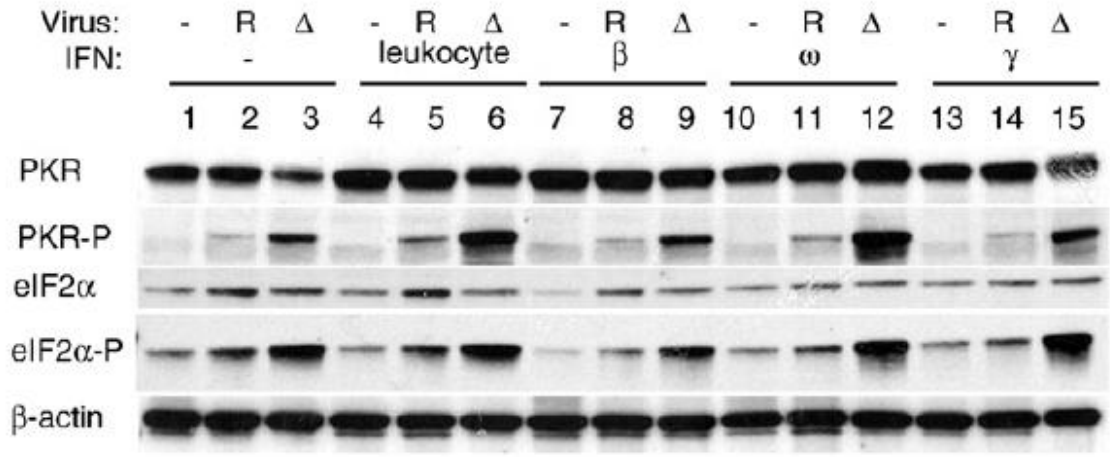
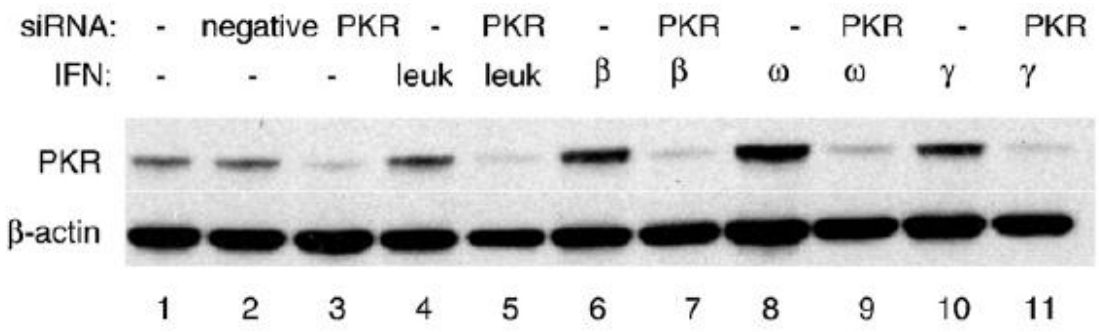


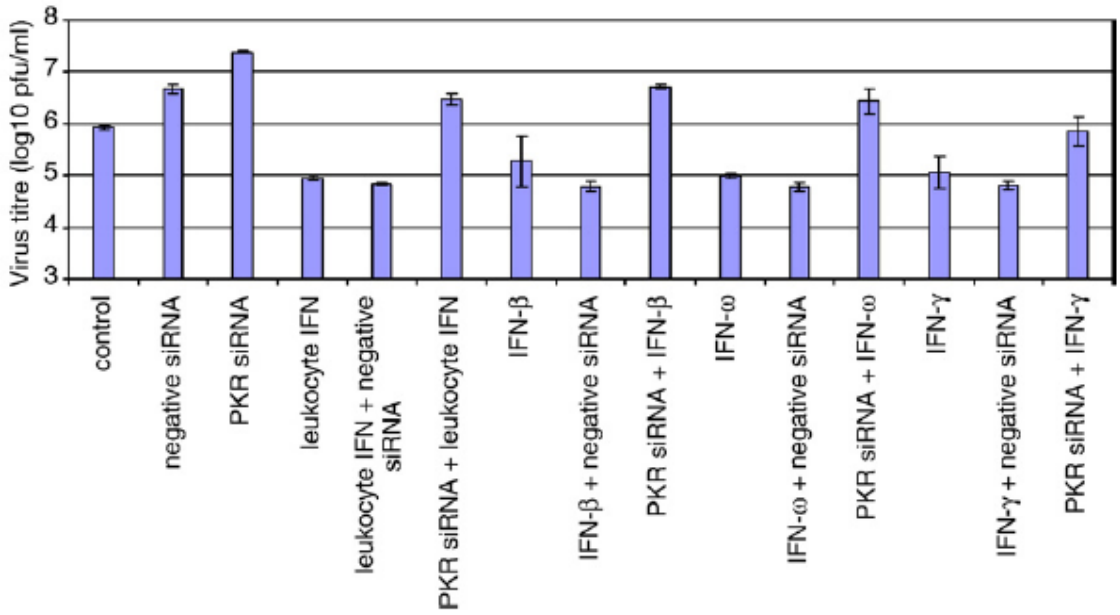
Fig.14 E3 mediates evasion of the PKR/eIF2α pathway in Huh7 cells. Western blot analysis of endogenous and phosphorylated levels of PKR and eIF2α in Huh7 cells with no IFN (lanes 1, 2, and 3) or treated with leukocyte IFN (lanes 4, 5, and 6), IFN-β (lanes 7, 8, and 9), IFN-ω (lanes 10, 11, and 12), IFN-γ (lanes 13, 14, and 15), and infected with Cop-ΔE3L-REV (R) or Cop-ΔE3L (Δ). Data shown are representative of three independent experiments.

To further characterize the role of the PKR pathway in the IFN sensitivity of Cop-ΔE3L, siRNA was used to specifically suppress the expression of PKR in Huh7 cells. Huh7 cells transfected with PKR siRNA were treated with 100 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ and infected with Cop-ΔE3L for 24h. In all cells transfected with PKR siRNA, the expression of PKR was significantly reduced (Fig.15a). Suppression of PKR in Huh7 cells abolished the effect of type I and type II IFN antiviral activity, in that when transfected with PKR siRNA, the replication of Cop-ΔE3L in the presence of type I and type II IFNs was at the same level as or higher than the control without IFNs (Fig.15b). In addition, in comparison with the control cells not transfected or transfected with the negative siRNA, the knockdown of PKR expression significantly enhanced the replication of Cop-ΔE3L in Huh7 cells not treated with IFN. It is intriguing that transfection with the negative siRNA also moderately enhanced Cop-ΔE3L replication.

To verify that transfection of Huh7 cells with this negative siRNA does not affect the antiviral activity of type I and type II IFNs, Huh7 cells were also transfected with the negative control siRNA then subsequently treated with 100 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ and infected with Cop-ΔE3L. The E3L deletion mutant remains sensitive to IFN treatment, indicating that the increase in virus titre in all negative control siRNA transfected cells in the absence of IFN treatment is an effect specific to this particular control siRNA, regardless of Cop-ΔE3L IFN sensitivity to type I and type II IFN (Fig.15b).



(a)



(b)

Fig.15 PKR mediates the IFN sensitivity of Cop-ΔE3L. (a) Western blot confirmation of the knockdown of PKR protein in Huh7 cells transfected with PKR siRNA in the absence (lane 3) and presence of leukocyte IFN (lane 5), IFN-β (lane 7), IFN-ω (lane 9), and IFN-γ (lane 11). Controls include Huh7 cells only (lane 1), and negative siRNA transfection (lane 2). (b) Huh7 cells were untreated (control) and transfected with negative siRNA (negative), PKR siRNA, and treated with leukocyte IFN, IFN-β, IFN-ω, and IFN-γ. PKR and negative siRNA transfected cells and IFN treatment are as indicated. Titrations were performed in triplicate. Error bars indicate standard error of the mean. Data shown are representative of three independent experiments.

I.4. RNaseL is not expressed in the Huh7 cells

To examine the role of the 2′–5′ OAS/RNaseL pathway in the cellular response to Cop-ΔE3L infection, RNaseL activity was monitored by measuring 28S and 18S rRNA degradation in IFN-treated and VV infected Huh7 cells. As depicted in Fig.16a, RNaseL activity was not detected in Huh7 cells observed at 6 and 12 HPI. Given the limited RNaseL activity in this cell system, the endogeneous RNaseL protein was monitored in Huh7 cells and in a positive control, HeLa-S3 cells, by Western blot. Total RNaseL protein was undetectable in Huh7 cells even in the presence of IFN treatment and virus infection, yet present in HeLa-S3 cells (Fig.16b). To further examine the endogenous expression of RNaseL mRNA, RT-PCR analysis of RNA extracted from Huh7 cells treated with type I and type II IFNs and infected with both Cop-ΔE3L and Cop-ΔE3L-REV was performed. As shown in Fig.16c, RNaseL mRNA is detected in HeLa-S3 cells, while it is not detected in Huh7 cells even in the presence of IFN treatment and virus infection. Since we were unable to detect the basal level of RNaseL in Huh7 cells by Western blot and RT-PCR, RNaseL does not contribute to the IFN sensitivity of Cop-ΔE3L in Huh7 cells.

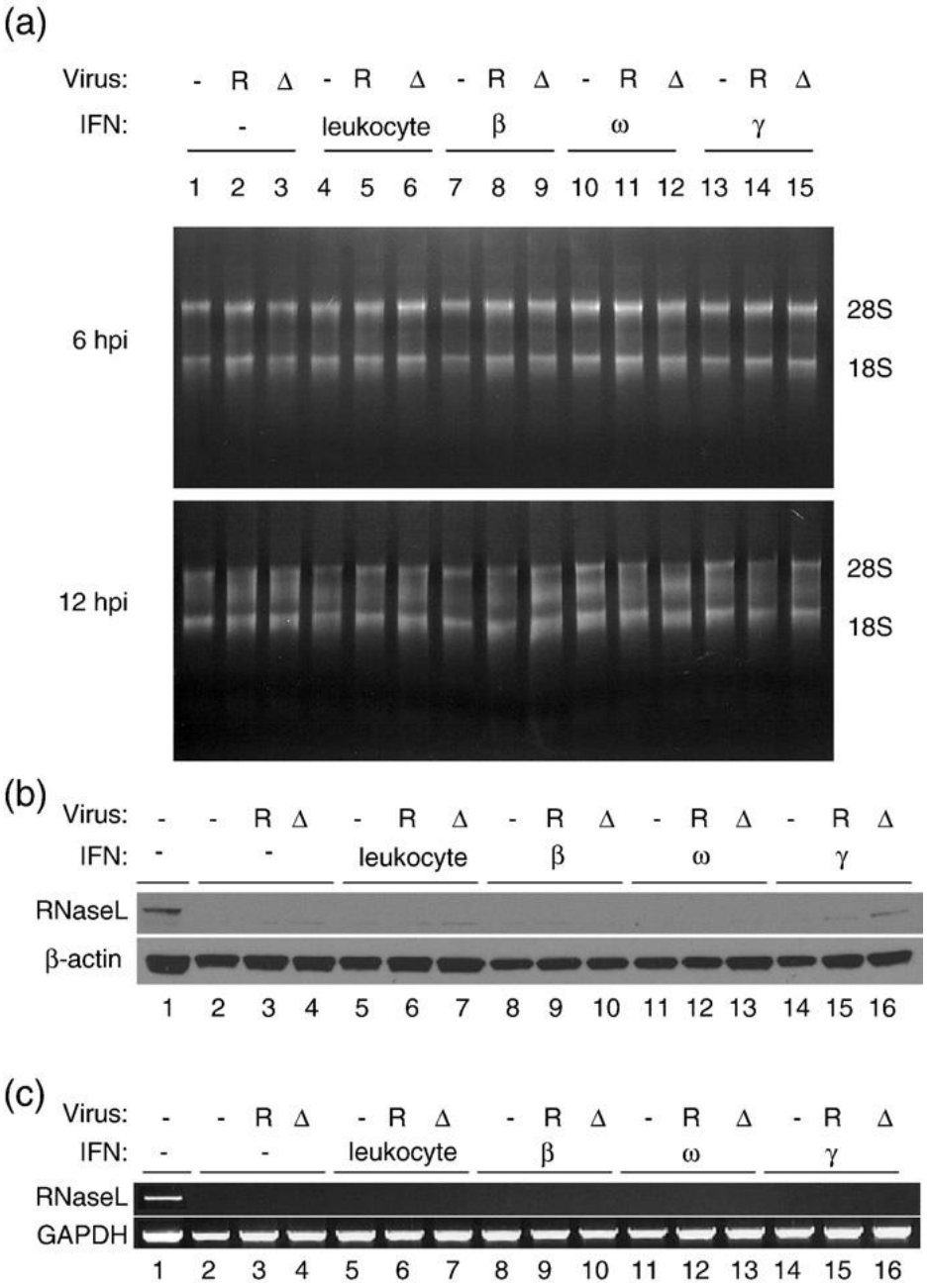


Fig.16 RNaseL activity is not detected in Huh7 cells. (a) Huh7 cells treated with type I and II IFNs and infected with Cop-ΔE3L-REV (R) and Cop-ΔE3L (Δ). 18S and 28S ribosomal subunits remain intact 6 and 12 HPI as indicated. Data shown are representative of three independent experiments. (b) RNaseL protein is detected in HeLa-S3 cells (lane 1) and is absent in Huh7 cells in the absence of IFN treatment and virus infection (lane 2) and in the presence of leukocyte IFN (lanes 5, 6, and 7), IFN-β (lanes 8, 9, 10), IFN-ω (lanes 11, 12, 13), and IFN-γ (lanes 14, 15, and 16) and virus infection with Cop-ΔE3L-REV (R) and Cop-VVΔE3L (Δ). (c) RNaseL mRNA expression is present in HeLa-S3 cells (lane 1), but is undetectable in Huh7 cells untreated or treated with type I and type II IFNs and infected with Cop-ΔE3L-REV (R) and Cop-ΔE3L (Δ) as indicated.

I.5. IFN-induced MxA does not inhibit Cop-VVΔE3L replication

IFN-induced MxA expression has been linked to the inhibition of virus gene transcription, viral mRNA translation, and virus nucleocapsids transportation of several RNA viruses[85]. Currently, no evidence has been reported of the role of MxA in the cellular immune response to poxviruses. To examine the inhibitory potential of MxA activity with regards to Cop-ΔE3L replication, MxA specific siRNA was used to knockdown the expression of MxA and its effect observed on virus replication. The knockdown of MxA expression in Huh7 cells transfected with MxA siRNA and treated with leukocyte IFN, IFN-β, IFN-ω, and IFN-γ was confirmed by RT-PCR (Fig.17a). Type I IFNs (leukocyte IFN, IFN-β, and IFN-ω) induced stronger MxA expression than IFN-γ. MxA expression was not detected in cells in the absence of IFN. Knockdown of MxA in Huh7 by siRNA did not significantly affect the ability of the E3L deletion mutant to replicate in the absence or presence of leukocyte IFN, IFN-β, IFN-ω, or IFN-γ (Fig.17b). Cop-ΔE3L remained sensitive to type I and type II IFN treatment in Huh7 cells transfected with MxA siRNA.

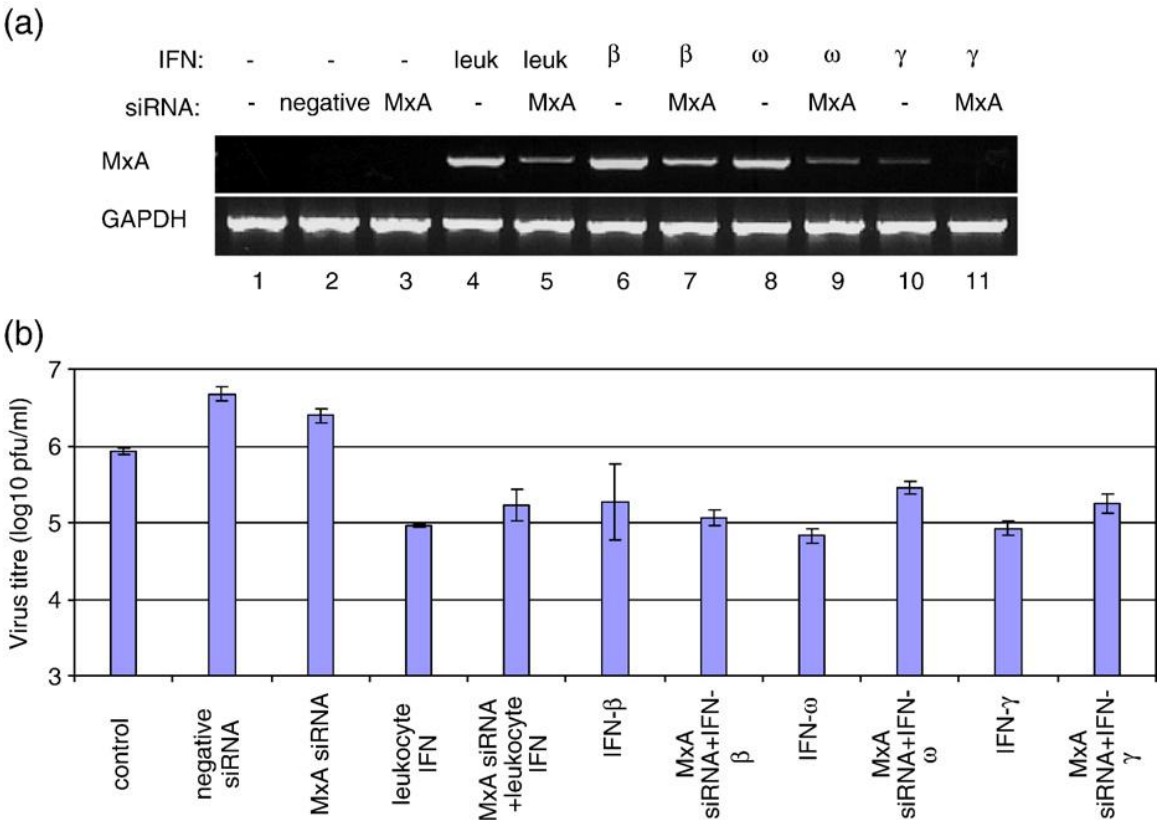


Fig.17 Effect of MxA knockdown by siRNA on the replication of Cop-ΔE3L in Huh7 cells in the absence and presence of type I and type II IFNs. (a) Knockdown of type I and type II IFN induced MxA expression in Huh7 cells by siRNA, confirmed by RT-PCR. (b) Effect of MxA activity on Cop-ΔE3L replication. Huh7 cells transfected with MxA siRNA and treated with type I and type II IFNs are as indicated. Virus control in Huh7 cells only (control) and negative siRNA transfection control (negative siRNA) are included. Titrations were performed in triplicate. Error bars indicate standard error of the mean. Data shown are representative of three independent experiments.

I.6. IFNs inhibit translation of intermediate genes in Cop-ΔE3L infections

Since a higher level of eIF2α phosphorylation in Cop-ΔE3L than in Cop-ΔE3L-REV infected Huh7 cells was observed (Fig.14), the association of such eIF2α phosphorylation with virus protein synthesis was investigated. The effect of IFN treatment on the expression of two VV genes, an early gene, D12L, which encodes for a capping enzyme[87], and an intermediate gene, G8R, whose protein product has been identified as a late transcription factor[115] was measured. RT-PCR analysis demonstrate the mRNA expression of D12L and G8R in Huh7 cells infected with both the revertant control and E3L deletion mutant viruses was not impaired in either the absence or presence of type I and type II IFNs (Fig.18a). However, at the level of protein expression, translation of the early gene, D12L, was largely unaffected in either the absence or presence of IFN or virus infection. In contrast, type I IFN treatment completely blocked translation of G8R, while type II IFN treatment also significantly inhibited G8R protein synthesis. It should be noted that G8R protein translation was compromised in Cop-ΔE3L infections in comparison with Cop-ΔE3L-REV infections even in the absence of IFN treatment (Fig. 18b). This partial block of G8R may account for the difference in the replication capacity between Cop-ΔE3L and Cop-ΔE3L-REV in Huh7 cells (Fig. 11b).

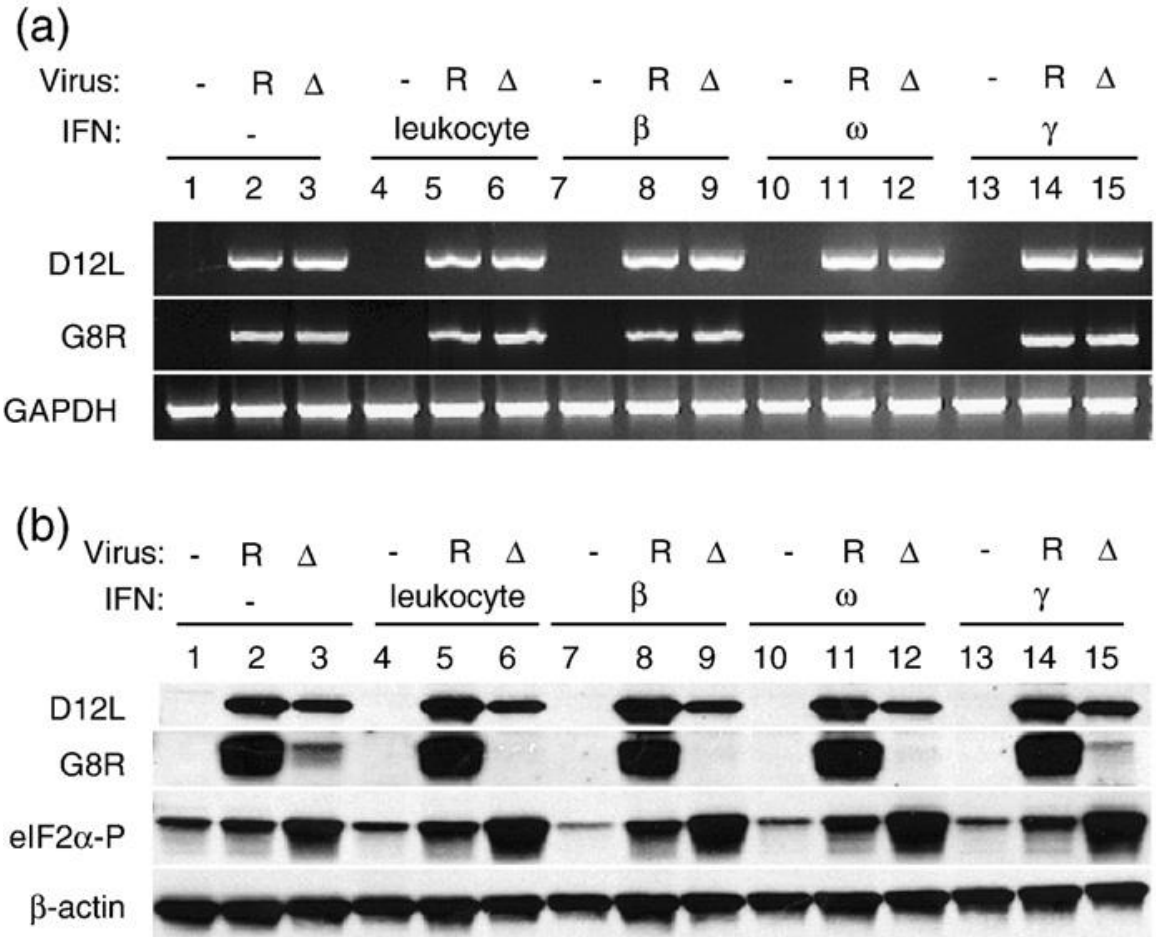


Fig.18 IFN treatment inhibits VV intermediate protein synthesis. (a) Total RNA was extracted from Huh7 cells treated with 1000 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ for 24 h at 37 °C and infected with Cop-ΔE3L-REV (R) and Cop-ΔE3L (Δ) at a MOI of 5 for 6 h. D12L, G8R and human GAPDH as a control were amplified by RT-PCR. (b) Western blot analysis of Huh7 cells treated with type I and type II IFNs (1000 U/ml) and infected with Cop-ΔE3L-REV (R) and Cop-ΔE3L (Δ) at a MOI of 5 for 6 h, using polyclonal antibodies against D12L and G8R. Data shown are representative of three independent experiments.

I.7. Discussion

IFNs can regulate the expression of over 300 genes[42]. PKR and RNaseL pathways are the two best characterized innate antiviral immune responses regulated by IFNs. It is well known that VV E3, a dsRNA binding protein, plays a critical role in determining the virus tropism by inhibiting activation of PKR and RNaseL[15,128,268]. IFN-induced antiviral activities, including activation of PKR and RNaseL, against VV were examined in HeLa[129] or L929[16] cells using wild-type VV and its E3L deletion mutant. However, the replication of the E3L deletion mutant virus is defective in these cell lines even without IFN treatment. By co-expression of E3, IFN-induced antiviral activities can be compromised against VSV replication[230]. A rabbit kidney cell line, RK13, can efficiently support Cop-ΔE3L replication and this replication could be blocked by rabbit IFN-α[30]. However, due to the limited availability of reagents, E3 mediated IFN resistance particularly against human IFNs was not comprehensively examined.

In this study, a human hepatoma cell line, Huh7, which is IFN responsive, was identified to efficiently support the replication of a vaccinia E3L deletion mutant. The capacity of E3 suppressing both human type I (12 IFN-α subtypes, IFN-β, and IFN-ω) and type II (IFN-γ) IFN-induced antiviral activities against VV was investigated using this cell line. It was found that E3 plays the major role in mediating antagonistic activity against all human type I and type II IFNs in a human liver carcinoma cell line, Huh7. This is the first comprehensive analysis of the inhibitory activity of E3 against all human type I and type II IFNs in a human cell line.

In humans, several type I IFN species, including 12 IFN-α subtypes, IFN-β, IFN-ω, and one type II IFN-γ have been identified. IRF-3 mediates the first wave of IFN-β production, and is followed by the expression of the latent transcription factor, IRF-7,

which mediates the second wave of IFN production, inducing expression of IFN-α2, -α5, -α6, and -α8[43,132,192,216]. The difference in induction times and variable biological functions associated with the 12 IFN-α subtypes make it possible for variable degrees of antiviral strength. In Fig.13b, different IFN-α subtypes exhibited different levels of inhibition activity against the E3L deletion mutant. The specific mechanisms underlying this variation in inhibition activity among IFN-α subtypes and type II IFN remain to be investigated.

The E3L deletion mutant induced a greater degree of eIF2α phosphorylation than infection with the revertant virus in both the absence and presence of IFN treatment (Fig.14). The increased level of eIF2α phosphorylation associated with Cop-ΔE3L infection may play a critical role in IFN-induced inhibition of the virus replication, since it has been shown that a 10–20% difference of eIF2α phosphorylation could have significant impact on the inhibition of protein translation[88].

In Cop-ΔE3L-REV infected Huh7 cells, phosphorylated eIF2α was present although a low level of phosphorylated PKR was detected (Fig.14). In addition to PKR, three other cellular kinases have been shown to phosphorylate eIF2α in regulating global translation in response to distinct stimuli. These kinases include GCN2, triggered by amino acid starvation; PERK, activated by cellular stress and protein misfolding at the ER membrane; and HRI, which is responsive to iron deficiency[83]. It is possible that these additional kinases may contribute to eIF2α phosphorylation in Cop-ΔE3L-REV infected cells, although the actual kinase involved in this case is unknown. In the system described in this study, the level of phosphorylated PKR correlates with the enhanced level of eIF2α phosphorylation in Cop-ΔE3L infected Huh7 cells (Fig.14). This is in agreement with several previous studies, which have shown that an increased level of

eIF2α phosphorylation induced by Cop-ΔE3L infection is associated with an increased level of PKR/phosphorylated PKR[31,221,226]. This association in the context of IFN treatment and virus infection has not been reported prior to this study. In addition, the specific suppression of PKR by siRNA in Huh7 cells rescues Cop-ΔE3L replication upon type I and type II IFN treatment. Apart from its dsRNA binding capability and the subsequent inhibition of PKR activation, the E3 protein has also been shown to directly bind to both regulatory and substrate binding regions of PKR and inhibit PKR function[226]. Thus, this data indicates that the E3 protein exerts its antagonizing activity against IFN by blocking PKR function to activate antiviral signals, such as eIF2α phosphorylation, in the cell system described here. In addition to its classical role in phosphorylating eIF2α, PKR is also implicated in the regulation of other signalling pathways such as proinflammatory cytokines, growth factors, oxidative stress and apoptosis[59] and such activities may also contribute to the IFN-induced inhibition of Cop-ΔE3L replication in Huh7 cells described in this study and pathogenesis *in vivo*.

It was shown that PKR is the key factor in determining the replication defective phenotype of the VV E3L deletion mutant in HeLa cells[273]. It requires at least 12 h of incubation (data not shown) for IFNs to mount effective antiviral signalling, while replication of the VV E3L deletion mutant is aborted at early times of infection[31,146]. Therefore, it is unlikely that PKR mediated antiviral activity, which is antagonized by the E3 protein, in HeLa cells is regulated by IFN. The data presented here is strong evidence showing that the E3 protein antagonizes antiviral signalling pathways induced by IFN, in which PKR is the main player. Since IFNs can regulate the expression level of greater than 300 genes simultaneously[42], it is very likely that the mechanism of IFN-induced and PKR mediated antiviral signalling observed in Huh7 cells is different to the recent report

on the role of PKR in restricting the replication of the VV E3L deletion mutant in HeLa cells[273].

Inhibition of the 2′–5′ OAS pathway by E3 was shown in various cell systems that include PKR knockout murine fibroblasts and immortalized MEFs whose constitutive RNaseL expression suppressed VVΔE3L replication, but had no effect on wild-type VV[203,268]. Moreover, the E3 protein of modified vaccinia virus (MVA) was shown to inhibit the 2′–5′ OAS/RNaseL pathway in HeLa cells[145]. Although the 2′–5′ OAS/RNAseL pathway is induced by both type I and type II IFNs, the induction of antiviral pathways may be cell type-specific[239]. In this study, RNaseL activity was not detectable in Huh7 cells treated with type I and type II IFNs during VV infection. It was further demonstrated by the lack of RNaseL mRNA and protein expression in this cell line (Fig.16). Therefore, the role of E3 inhibiting RNaseL mediated antiviral activity cannot be evaluated using Huh7 cells as described in this study. However, it is plausible to further investigate the role of E3 at inhibiting RNaseL activity using this human cell line. To elucidate the function of RNaseL in inhibiting replication of the E3L deletion mutant, constitutively expressing Huh7-RNaseL cell lines can be generated. This would allow for an expanded characterization of VV E3 and its inhibition of RNaseL activity in human cells.

MxA is induced primarily by type I IFNs or type III IFNs[79] and has been reported to inhibit both RNA and DNA viruses[11,68,239]. The mechanism by which MxA inhibits viral replication remains unknown and the role of this IFN-induced protein in suppressing VV replication has not been elucidated. In this study, MxA has no detectable activity in suppressing Cop-ΔE3L replication, in that the knockdown of MxA gene expression by siRNA does not significantly abolish the antiviral activity of either type I or type II IFNs

against Cop-VVΔE3L (Fig.17). It is also possible that MxA mediated antiviral signalling is defective in Huh7 cells, as this antiviral activity has been shown to be cell type specific[219].

VV replication was shown to be sensitive to pretreatment with murine IFNs-α/β in Balb/c mice and that this inhibition is associated with the blockage of early viral gene expression[207]. In the human cell line, Huh7, both type I and type II IFNs inhibit intermediate ORFs translation of a VV mutant devoid of the E3L ORF, while early and intermediate gene transcription is unaffected (Fig.18). IFNs exert a different spectrum of antiviral activity through synchronizing with other cellular factors such as TNF-α *in vivo* in comparison with the *in vitro* tissue culture system[276]. Since G8R is a late gene transcriptional activator, it is predictable that late gene expression is blocked. Moreover, in the presence of IFN treatment, in the E3L deletion mutant infections, the G8R protein is absent. Apart from antagonizing PKR induced eIF2α phosphorylation, it is also possible that E3 can directly regulate the expression of other VV genes involved in viral transcription and/or translation. In fact, a study that describes the association of VV transcription and translation with cytoplasmic DNA factories illustrates the relocation of E3 to these DNA factories at times post-infection at which intermediate and late viral gene expression would occur[110].

In this first results section, there are three contributions to characterizing the IFN antagonizing properties of VV E3. First, a human cell line, Huh7, was identified to efficiently support the replication of the VV E3L deletion mutant and is suitable to examine the antiviral activity of a wide range of human IFNs and/or viral IFN antagonists expressed in replacement of VV E3. Second, in this Huh7 cell system, it was conclusively demonstrated that VV E3 is a universal antagonist against both type I and

type II IFNs. Third, in this specific virus/cell system, mechanistically, PKR is the major IFN regulated signal suppressed by VV E3. Collectively, this study highlights an excellent human cell line system in which to study the inhibition activity of a strong IFN antagonist, VV E3, against all human type I and type II IFN species.

RESULTS

II: *Comparative analysis of NS1 proteins from influenza viruses of different species-origins for their interference of IFN-induced antiviral activities*

Introduction. IFNs play crucial roles in mediating cellular defences against viral infections. Many viruses produce proteins to counteract IFN-induced antiviral activities. The NS1 protein of influenza viruses has been shown to mediate the inhibition of IFN-induced antiviral activities. However, due to different cell tropisms and a lack of a common cell culture system for influenza viruses of different species origins, the activity of NS1 proteins of influenza viruses originated from different animal hosts in modulating IFN-induced antiviral responses is not well characterized.

Here, VVΔE3L was applied as an expression vector to perform studies on the inhibition activity of NS1 proteins from different species of origin on human IFN-induced antiviral responses. The NS1 proteins in this analysis include NS1 of two highly pathogenic avian H5N1 isolates, avian (duck) and human isolates, pandemic human viruses 1918 pH1N1 and 1968 pH3N2, and swine-origin 2009 pH1N1. It was hypothesized that NS1 proteins of animal origins, for example, swine or avian origins, have the capacity to inhibit human IFN-induced antiviral activities.

The specific objectives of this study included: 1) examine the replication capacities of recombinant viruses expressing NS1 proteins from the influenza viruses of swine and avian origins in Huh7 cells 2) to comparatively examine the antagonizing activities by various NS1 proteins against both human type I and type II IFN-induced antiviral activities; and 3) to investigate the role of the PKR/eIF2α pathway in mediating antiviral activity against recombinant viruses expressing NS1 proteins.

II.1. Generation of recombinant VVΔE3L viruses expressing NS1 proteins

An alignment of the NS1 amino acid (a.a.) sequences of two avian H5N1 NS1 proteins (duck isolate and human isolate), swine-origin 2009 pH1N1 NS1, human 1918 pH1N1 NS1, and human 1968 pH3N2 NS1 is shown in Fig.19. Distinct differences in the NS1 protein sequences, as shown in red, include a five a.a. deletion (a.a.80-84) in NS1 of avian H5N1-duck, the shortened C-terminal length of 2009 pH1N1 NS1 (219 a.a.), and the extended C-terminal tail of 1968 H3N2 NS1 (237 a.a.). All recombinant viruses were generated based on the WR strain of VV. Expression of VV E3 of the revertant virus control (VVΔE3L-REV) and NS1 in replacement of E3, in the recombinant viruses expressing NS1, were confirmed by Western blot (Fig.20). The resultant recombinant viruses expressing NS1 proteins included VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, VVΔE3L/2009 pH1N1 NS1, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/1968 pH3N2 NS1.

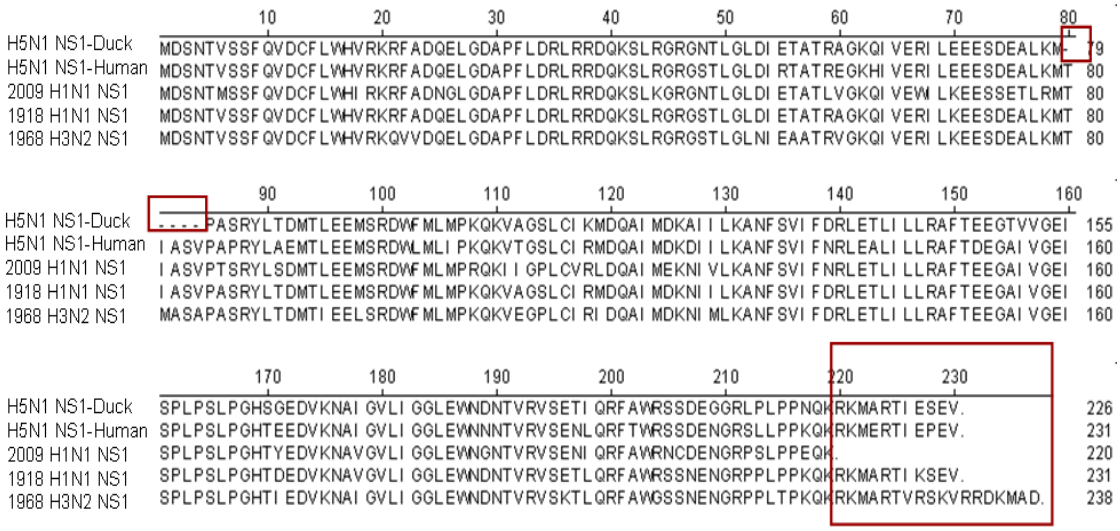


Fig.19 Amino acid (a.a) sequence alignment of influenza NS1 proteins. The NS1 proteins were from two avian H5N1 viral strains of different species isolates, duck isolate (H5N1 NS1-duck) and human isolate (H5N1 NS1-human), pandemic 2009 H1N1 swine-origin (2009 pH1N1 NS1), pandemic 1918 H1N1 (1918 pH1N1 NS1), and pandemic 1968 H3N2 (1968 pH3N2 NS1). Highlighted in red include a five a.a. deletion in H5N1 NS1-duck (a.a. 80-84), PDZ binding motif in H5N1 NS1-duck and H5N1 NS1-human (a.a.226 to 230), truncated C-terminal end sequence of 2009 pH1N1 NS1, and extended C-terminal end sequence of 1968 pH3N2 NS1.

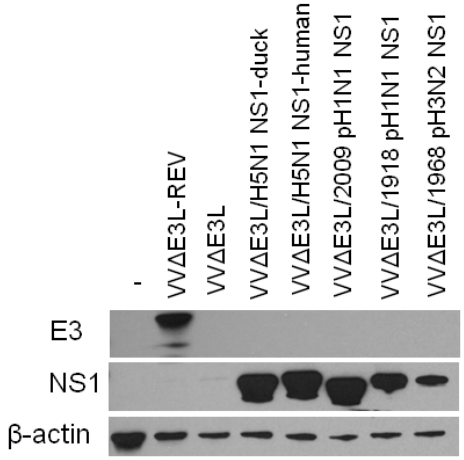
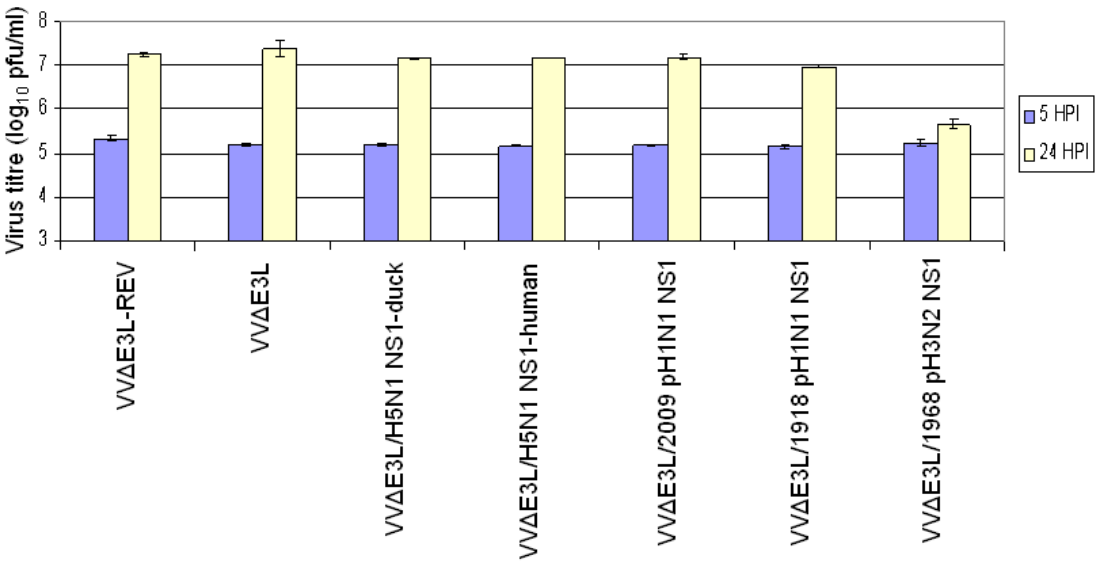


Fig.20 Expression of NS1 proteins. Western blot confirmation of NS1 expression (HA-tag antibody) in replacement of VV E3 in all NS1 recombinant viruses, E3 expression in the revertant control virus (VVΔE3L-REV) and absence of E3 expression in the E3L deletion mutant viruses (VVΔE3L).

II.2. Recombinant viruses expressing NS1 proteins replicate efficiently in Huh7 cells

To investigate whether Huh7 cells support the replication of the recombinant viruses expressing NS1 proteins, Huh7 cell monolayers were infected at a MOI of 1 with all recombinant viruses and harvested at 5 and 24 HPI for virus titrations (Fig.21). BHK21 cells, which are equally permissive to both wild-type VV and the E3L deletion mutant, were included as a control. It is interesting to note that the replication of VVΔE3L/1968 pH3N2 NS1 was compromised in BHK21 cells, in comparison to VVΔE3L (Fig.21a). While all recombinant viruses expressing NS1 were able to replicate in Huh7 cells, VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 replicated to viral titres comparable to or slightly better than VVΔE3L (Fig.21b). In contrast, the viral titres of VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 consistently remained 5 fold less than that of VVΔE3L.

(a)



(b)

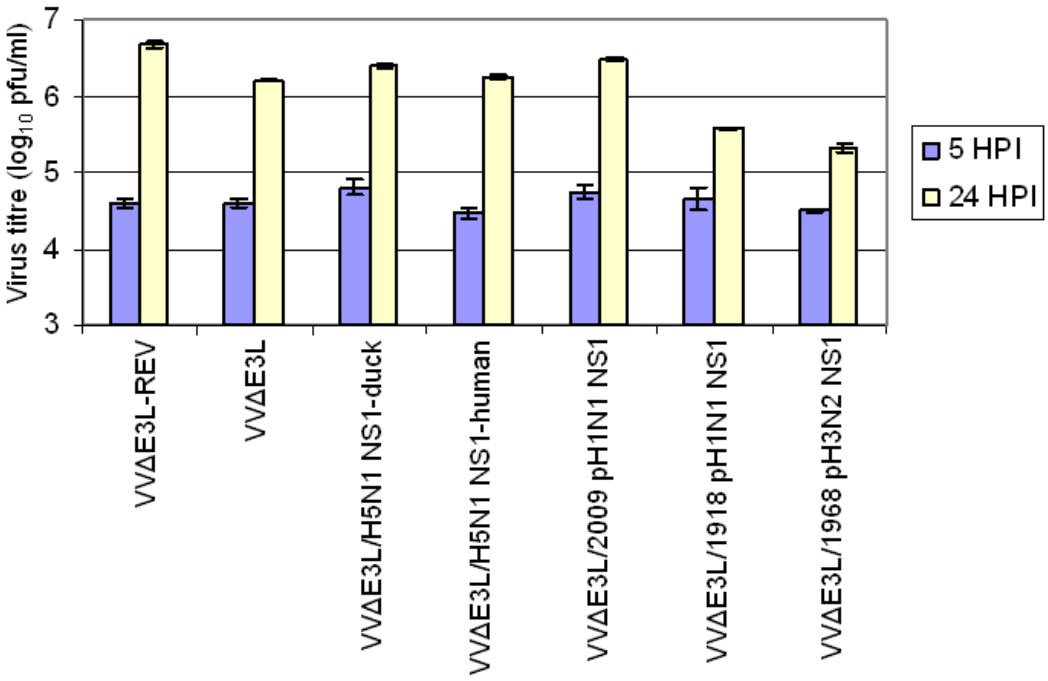


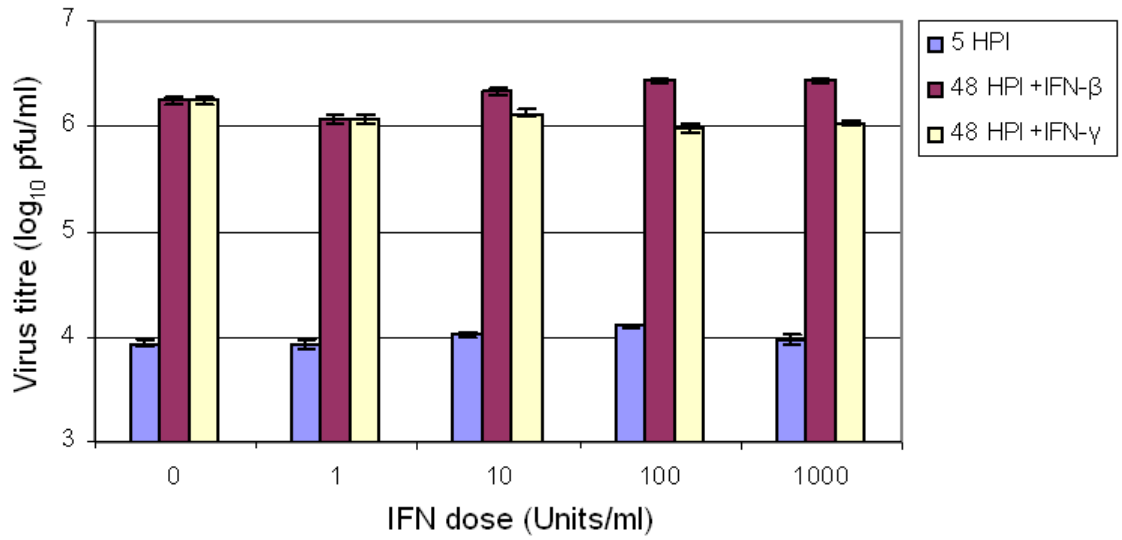
Fig.21 Replication capacities of recombinant viruses expressing NS1 in (a) BHK21 cells and (b) human Huh7 cells. Confluent cell monolayers were infected at a MOI of 1 with all viruses as indicated and harvested 5 and 24 HPI for virus titrations. Virus titrations were performed in triplicate. Error bars indicate standard error of the mean. Data shown are representative of three independent experiments.

II.3. NS1 proteins of H5N1-duck, H5N1-human, and 2009 pH1N1 mediate stronger resistance to human type I and type II IFN-induced antiviral activities than 1918 pH1N1 NS1 and 1968 pH3N2 NS1

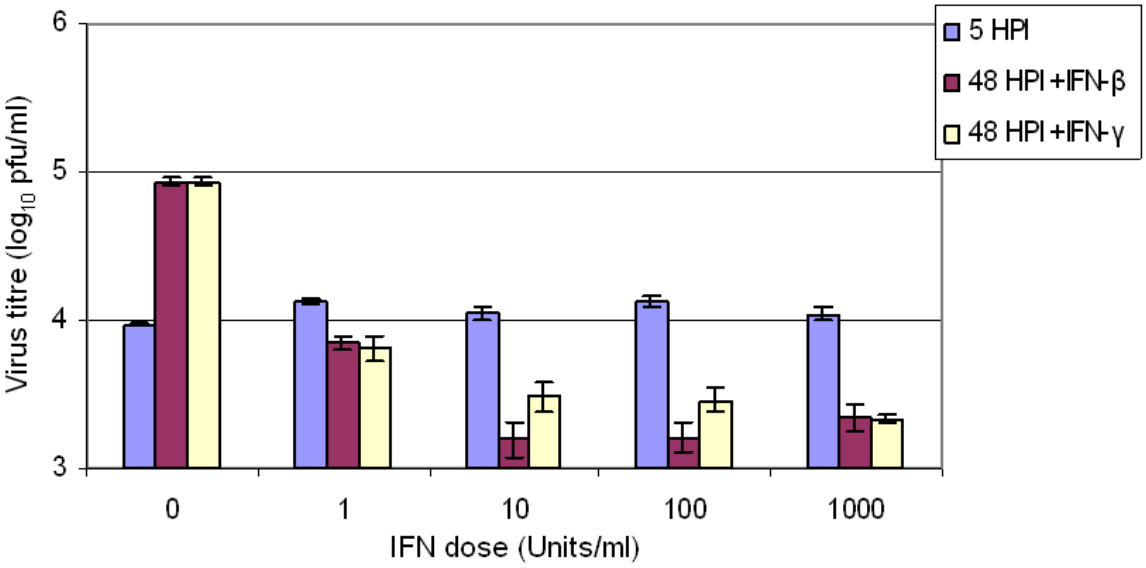
To investigate the antagonizing activity of the NS1 proteins against human IFN-induced antiviral activity, the sensitivity of the recombinant viruses expressing NS1 to human type I (IFN-β) and type II (IFN-γ) IFNs was analyzed in Huh7 cells. As shown in Fig.22a, replication of the revertant control virus (VVΔE3L-REV) was unaffected by type I or type II IFN treatment even at the highest IFN dose (1000 U/ml). In contrast, VVΔE3L is highly sensitive to type I and type II IFN treatment (Fig.22b), in that the lowest IFN dose (1 U/ml) was sufficient to suppress VVΔE3L replication by 10 fold. Recombinant viruses expressing NS1 proteins of the H5N1-duck isolate (Fig.22c) and H5N1-human isolate (Fig.22d) were highly resistant to type I and type II IFN treatment, in that VVΔE3L/H5N1 NS1-duck and VVΔE3L/H5N1 NS1-human recombinant viruses replicated to comparable titres (10^6 pfu/ml) even at the highest dose of 1000 U/ml of both type I and type II IFNs in comparison to the non-IFN controls. Similar to VVΔE3L/H5N1 NS1-duck and VVΔE3L/H5N1 NS1-human, the expression of 2009 pH1N1 NS1 in this virus system also rendered the virus highly resistant to both type I and type II IFN treatment, in that VVΔE3L/2009 pH1N1 NS1 replicated to 10^6 pfu/ml virus titres even at the highest IFN dose of 1000 U/ml (Fig.22e). In contrast, recombinant viruses expressing NS1 of 1918 pH1N1 and 1968 pH3N2 were partially resistant to type I and type II IFN treatment overall (Fig.22f, 22g). Both VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 exhibited greater resistance to type II IFN treatment than type I IFN treatment. Thus, NS1 proteins of avian H5N1 and swine 2009 pH1N1

mediated stronger resistance to both type I and type II human IFN-induced antiviral activities than NS1 proteins of human 1918 pH1N1 and 1968 pH3N2.

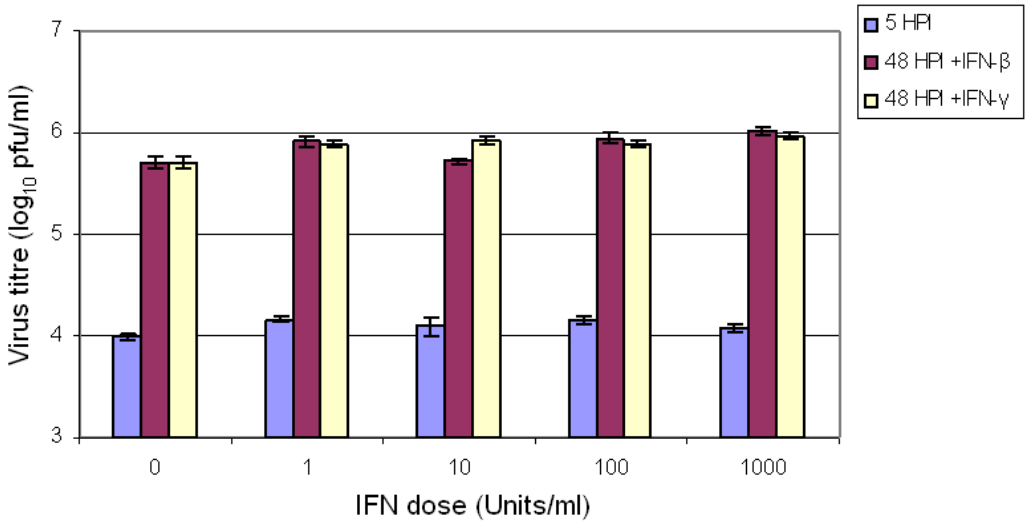
(a)



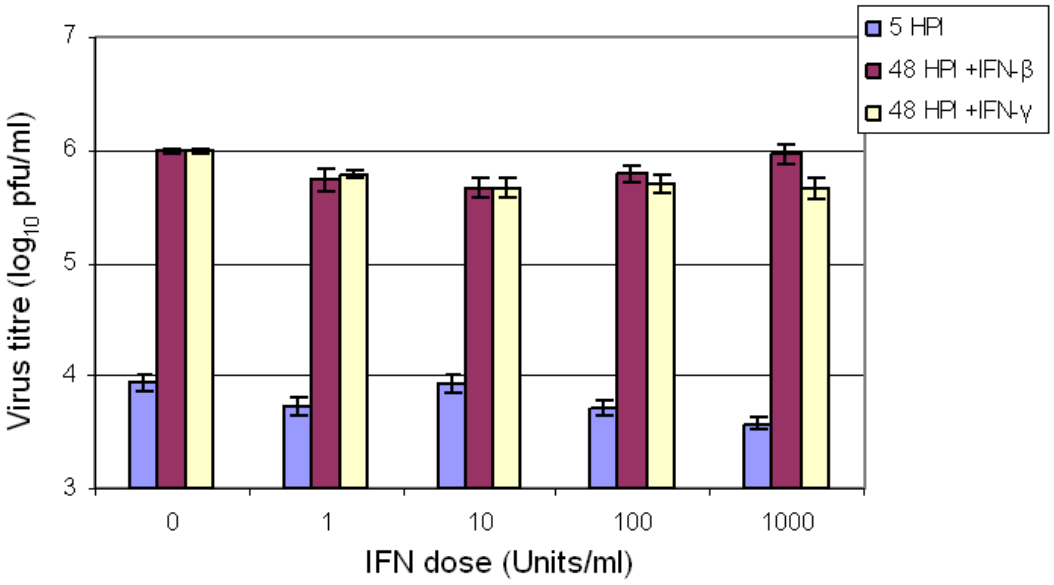
(b)



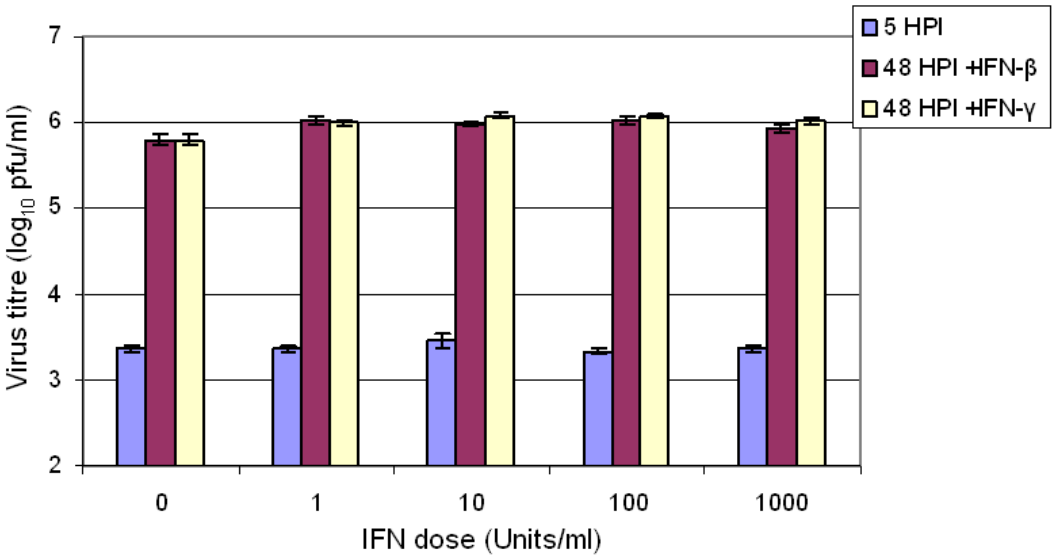
(c)


5 HPI
48 HPI +IFN-β
48 HPI +IFN-γ
Virus titre (log10 pfu/ml)
7
6
5
4
3
0
1
10
100
1000
IFN dose (Units/ml)

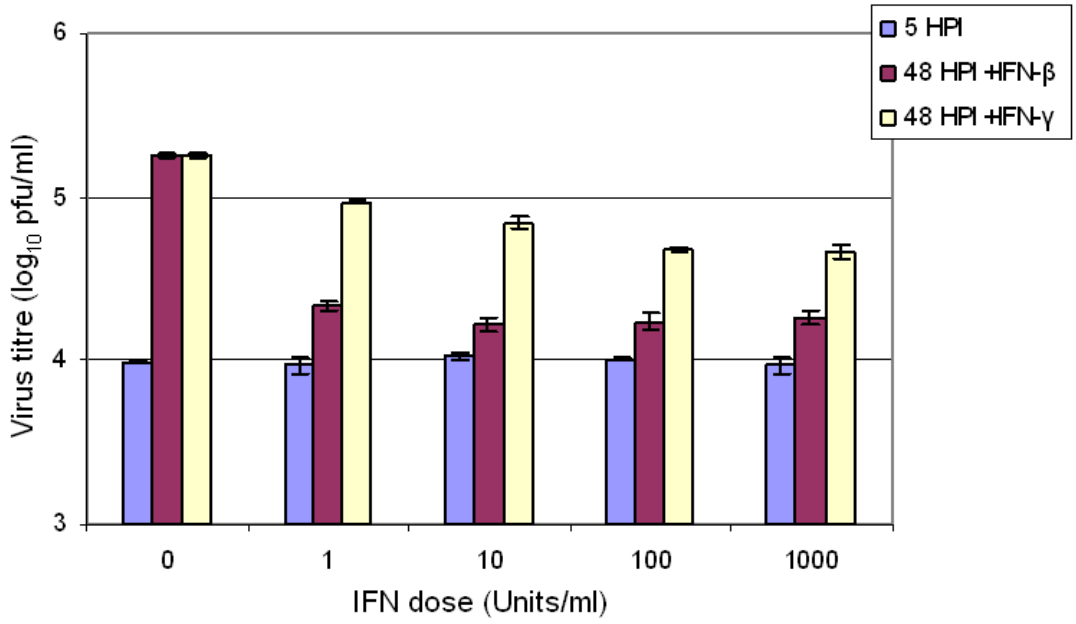
(d)


5 HPI
48 HPI +IFN-β
48 HPI +IFN-γ
Virus titre (log10 pfu/ml)
7
6
5
4
3
0
1
10
100
1000
IFN dose (Units/ml)

(e)


5 HPI
48 HPI +IFN-β
48 HPI +IFN-γ
Virus titre (log10 pfu/ml)
7
6
5
4
3
2
0
1
10
100
1000
IFN dose (Units/ml)

(f)


5 HPI
48 HPI +IFN-β
48 HPI +IFN-γ
Virus titre (log10 pfu/ml)
6
5
4
3
0
1
10
100
1000
IFN dose (Units/ml)

(g)

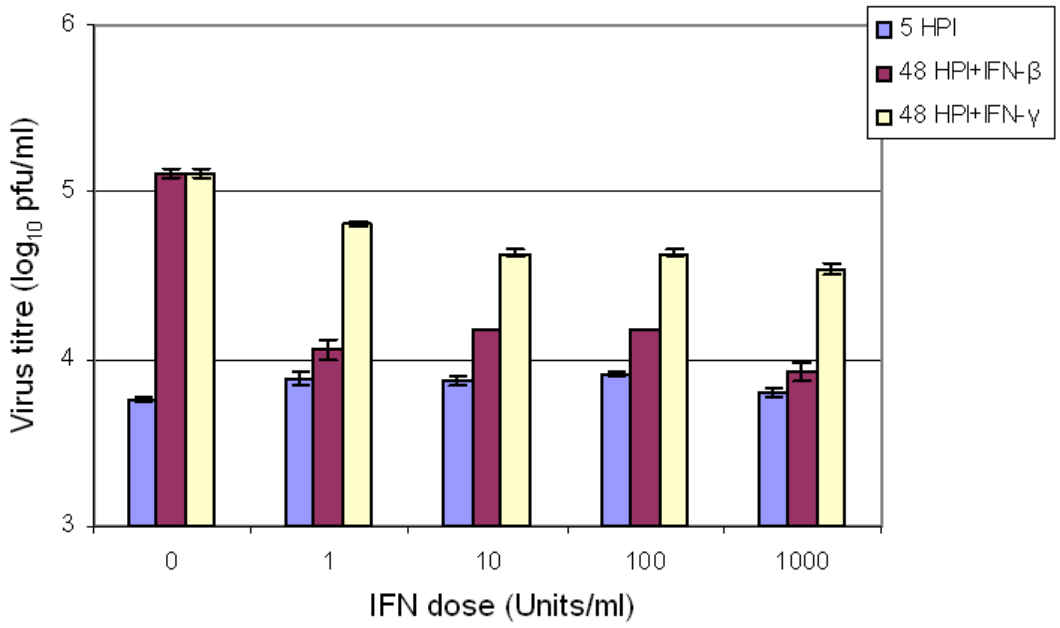


Fig.22 Comparative analysis of the IFN sensitivities of recombinant viruses expressing NS1 to human type I and type II IFN treatment in Huh7 cells. Data illustrates the effect of IFN treatment on the replication of control viruses (a) VVΔE3L-REV and (b) VVΔE3L and recombinant viruses expressing NS1 proteins (c) VVΔE3L/H5N1 NS1-duck, (d) VVΔE3L/H5N1 NS1-human, (e) VVΔE3L/2009 pH1N1 NS1, (f) VVΔE3L/1918 pH1N1 NS1, and (g) VVΔE3L/1968 pH3N2 NS1. IFN sensitivity assays were performed in triplicate. Titrations were performed in triplicate. Error bars represent standard error of the mean. Data shown are representative of three independent experiments.

II.4. NS1 proteins of different influenza viruses differentially inhibit PKR/eIF2α phosphorylation

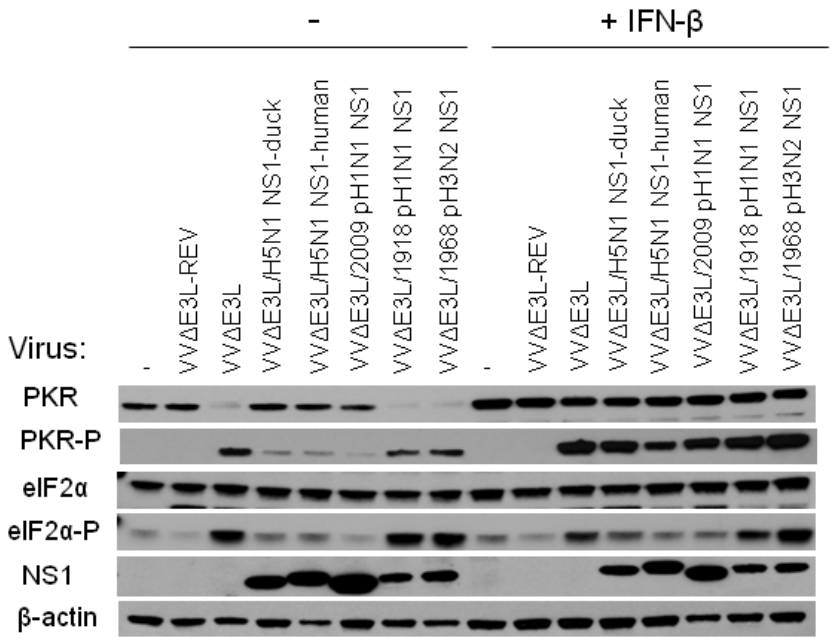
In IFN-treated Huh7 cells, VVΔE3L sensitivity was mainly mediated by PKR, but not RNaseL or MxA (Results - I, Fig.15-Fig.17). While VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 were more sensitive to type I IFN than type II IFN treatment, there was no major difference in the IFN resistant phenotypes of VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 to type I IFN or type II IFN treatment. To investigate the role of the PKR/eIF2α pathway in the differential IFN resistance patterns mediated by the different NS1 proteins, the activation/phosphorylation of PKR and its substrate eIF2α was examined by Western blotting. Huh7 cells were untreated or treated overnight with 1000 U/ml of IFN-β and infected at a MOI of 5 for 6 H with VVΔE3L-REV, VVΔE3L, VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, VVΔE3L/2009 pH1N1 NS1, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/1968 pH3N2 NS1 recombinant viruses.

In the absence of IFN treatment, phosphorylated-PKR was readily detected in the VVΔE3L/1918 pH1N1 NS1, VVΔE3L/1968 pH3N2 NS1, and VVΔE3L infections (Fig.23a). In contrast, VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 recombinants were stronger at inhibiting PKR/eIF2α phosphorylation without IFN treatment. In the presence of IFN treatment, infection with VVΔE3L and all recombinants expressing NS1 induced PKR phosphorylation. While the VVΔE3L/2009 pH1N1 NS1 exhibited the greatest inhibition of PKR phosphorylation in the presence of IFN, in comparison to the other recombinants expressing NS1 proteins, it was not able to fully inhibit PKR phosphorylation comparable to the VV E3 protein in the VVΔE3L-REV infection. Quantitation of phoshorylated-PKR protein in the absence and

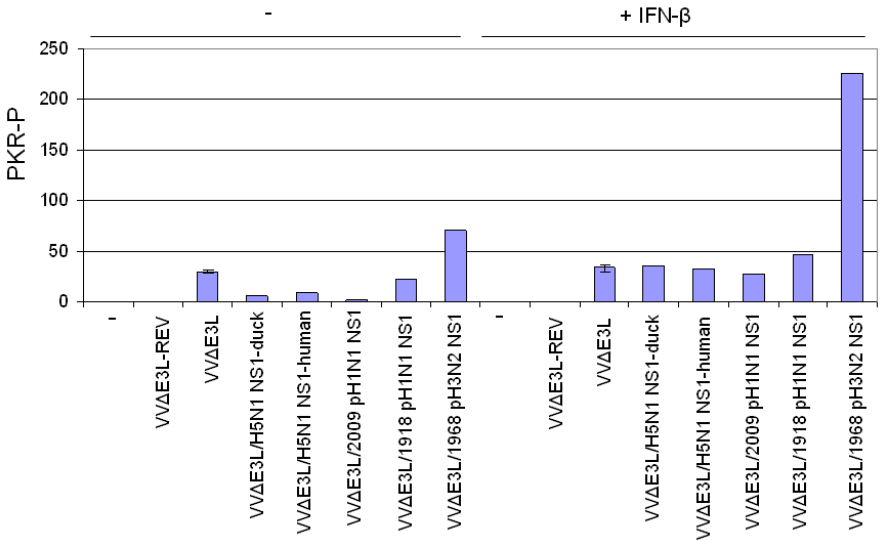
presence of IFN following virus infections was also performed (Fig.23b). The NS1 proteins of 1918 pH1N1 and 1968 pH3N2 NS1 did not mediate the inhibition of PKR phosphorylation (even without IFN treatment). In contrast, the NS1 proteins of avian H5N1 and 2009 pH1N1 induced significantly less PKR phosphorylation than VVΔE3L, 1918 pH1N1 NS1 and 1968 pH3N2 NS1 (with and without IFN treatment). However, while the NS1 proteins of avian H5N1 and 2009 pH1N1 mediated comparable resistance to IFN-induced antiviral activity as the VV E3 protein (Fig.22c, 22d, 22e), none of these IFN resistant NS1 proteins inhibited PKR phosphorylation comparable to E3. In all, NS1 proteins of different influenza viruses differentially inhibit PKR phosphorylation.

Infection with VVΔE3L, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/1968 pH3N2 NS1 resulted in a significant increase in eIF2α phosphorylation in the absence and presence of IFN treatment (Fig.23a, 23c). VVΔE3L/1968 pH3N2 NS1 infection induced the greatest eIF2α phosphorylation with and without IFN. In the presence of IFN, a slight increase in eIF2α phosphorylation was observed following infection with VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 viruses, but not VVΔE3L-REV. It is interesting to note that infections with the IFN resistant recombinants VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 resulted in a considerable increase in PKR phosphorylation but only a slight increase in eIF2α phosphorylation in the presence of IFN. Thus, while the IFN resistant recombinants are more effective at inhibiting PKR/eIF2α phosphorylation than the IFN sensitive recombinants, they can also induce PKR and eIF2α phosphorylation in IFN-treated Huh7 cells.

(a)


-
+ IFN-β
Virus:
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/H5N1 NS1-duck
VVΔE3L/H5N1 NS1-human
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/1918 pH1N1 NS1
VVΔE3L/1968 pH3N2 NS1
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/H5N1 NS1-duck
VVΔE3L/H5N1 NS1-human
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/1918 pH1N1 NS1
VVΔE3L/1968 pH3N2 NS1
PKR
PKR-P
eIF2α
eIF2α-P
NS1
β-actin

(b)


-
+ IFN-β
PKR-P
250
200
150
100
50
0
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/H5N1 NS1-duck
VVΔE3L/H5N1 NS1-human
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/1918 pH1N1 NS1
VVΔE3L/1968 pH3N2 NS1
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/H5N1 NS1-duck
VVΔE3L/H5N1 NS1-human
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/1918 pH1N1 NS1
VVΔE3L/1968 pH3N2 NS1

(c)

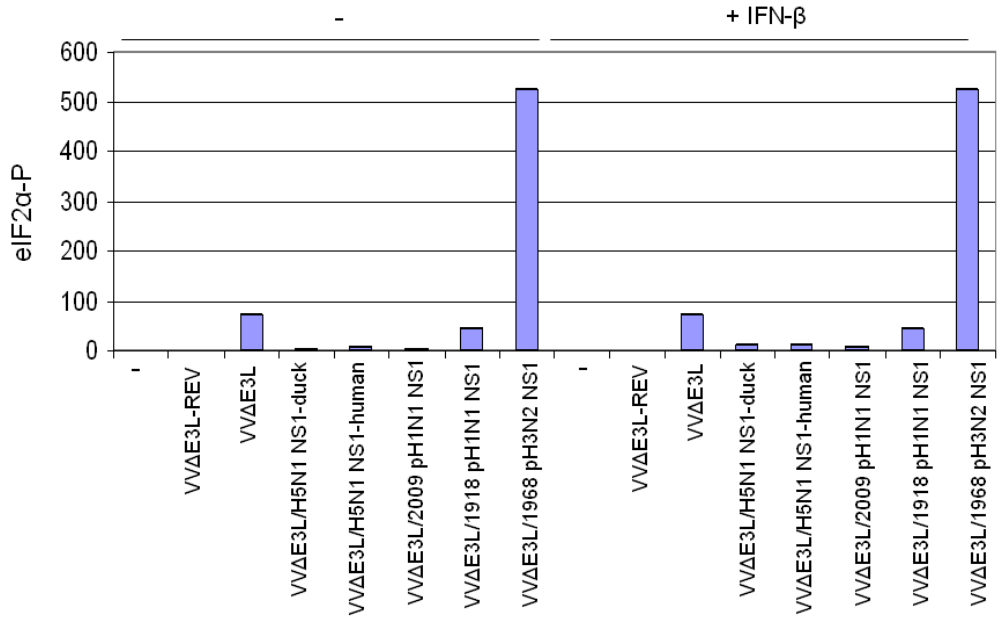


Fig.23 PKR/eIF2α phosphorylation in untreated and IFN-treated Huh7 cells following infection with recombinant viruses expressing NS1. Confluent Huh7 cell monolayers with and without IFN-β (1000 U/ml) treatment were infected at a MOI of 5 with the indicated viruses and harvested 6 HPI for Western blotting. (a) Western blot analysis of signal changes in PKR/eIF2α induced by virus infection. (b) Quantification of phosphorylated-PKR and (c) eIF2α proteins. Data shown are representative of three independent experiments. Error bars represent one standard deviation.

II.5. NS1 proteins of different influenza viruses differentially bind dsRNA.

Recombinant viruses expressing the NS1 proteins of H5N1-duck isolate (Fig.22c), H5N1-human isolate (Fig.22d), and 2009 pH1N1 isolate (Fig.22e) were resistant to type I and type II IFN-induced antiviral activity. To investigate whether the resistance to IFN treatment by VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 is associated with their capacities to bind dsRNA, dsRNA binding assays were performed. Immunoprecipations with polyIC-coated agarose beads demonstrate the IFN resistant NS1 proteins (H5N1 NS1-duck, H5N1 NS1-human, and 2009 pH1N1 NS1) bind dsRNA stronger than the NS1 proteins of the IFN sensitive viruses, VVΔE3L/1918 pH1N1 NS1 and 1968 pH3N21918 pH1N1 NS1 and 1968 H3N2 NS1 (Fig.24). Additionally, the dsRNA binding capacity is comparable among the NS1 resistant proteins. Thus, NS1 proteins of different influenza viruses differentially bind dsRNA, which may account for their differences in mediating resistance to IFN-induced antiviral activity.

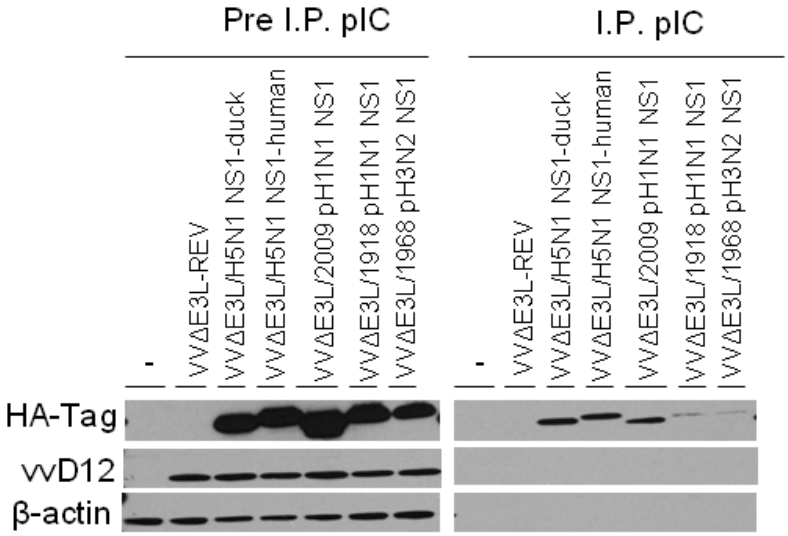


Fig.24 NS1 proteins of different influenza viruses differentially bind dsRNA. IFN resistant NS1 proteins (H5N1 NS1-duck, H5N1 NS1-human, and 2009 pH1N1 NS1) strongly bind dsRNA, in comparison to IFN sensitive NS1 proteins (1918 pH1N1 NS1 and 1968 pH3N2 NS1). BHK21 cells were infected at a MOI of 5 with the recombinant viruses as indicated and cell lysates were collected 8 HPI. Expression of NS1 proteins and control VV early protein D12 was confirmed in lysates collected prior to immunoprecipitation (Pre I.P.). Pull-downs show the dsRNA binding activity of IFN resistant proteins is comparable, and is minimal in 1918 pH1N1 NS1 and 1968 pH3N2 NS1 infections. The revertant control was included as a positive control for D12 expression. It should be noted that the dsRNA binding activity of VV E3 was confirmed previously[171], but is not shown here. Immunoprecipitations (I.P.) were performed in triplicate.

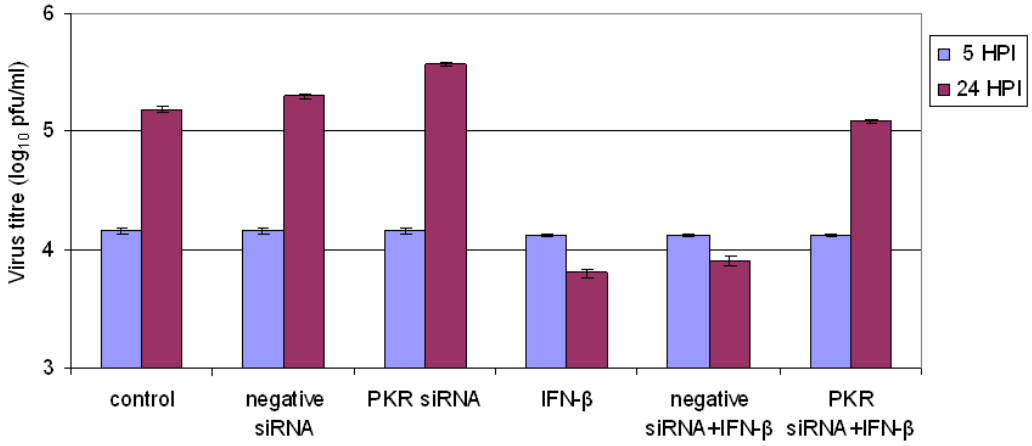
II.6. PKR contributes to the IFN sensitivities of VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1

VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 H3N2 NS1 exhibited sensitivities to type I IFN treatment (Fig.22f, 22g). Infection with VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 viruses resulted in phosphorylated-PKR and phosphorylated-eIF2α profiles similar to VVΔE3L with and without IFN treatment (Fig.23). In the presence of IFN, infection with the IFN resistant recombinants also stimulated PKR phosphorylation. To further investigate the role of PKR in mediating the IFN sensitivity of VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1, siRNAs were used to observe the effect of PKR knockdown on the IFN sensitivity of VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 viruses Huh7 cells.

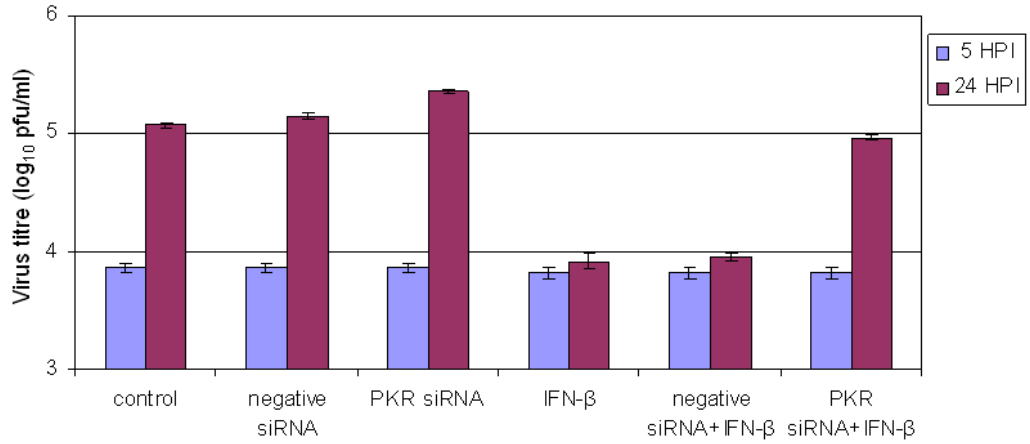
Huh7 cells were transfected with PKR siRNA or a non-targetting negative control siRNA at 100 nM for 48 H, then treated with IFN-β (100 U/ml) overnight at 37ºC. Untreated or IFN-treated Huh7 cells with and without siRNA treatment were infected at a MOI of 1 with VVΔE3L, VVΔE3L/1918 pH1N1 NS1, VVΔE3L/1968 pH3N2 NS1 and VVΔE3L/2009 pH1N1 NS1 viruses, and harvested at 5 and 24 HPI for virus titrations. VVΔE3L was included as a positive control, since it was shown that PKR mediates VVΔE3L sensitivity to IFN (Results – I, Fig.15).

As shown in the previous results section (Fig.15), PKR knockdown rescued VVΔE3L replication in the presence of IFN treatment (Fig.25a). Similarly, PKR knockdown enhanced the replication of VVΔE3L/1918 pH1N1 NS1 (Fig.25b) and VVΔE3L/1968 pH3N2 NS1 (Fig.25c) in the presence of IFN treatment. Thus, PKR mediates the IFN sensitivities of VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1.

(a)


6
5
4
3
Virus titre (log10 pfu/ml)
5 HPI
24 HPI
control
negative siRNA
PKR siRNA
IFN-β
negative siRNA+IFN-β
PKR siRNA+IFN-β

(b)


6
5
4
3
Virus titre (log10 pfu/ml)
5 HPI
24 HPI
control
negative siRNA
PKR siRNA
IFN-β
negative siRNA+ IFN-β
PKR siRNA+ IFN-β

(c)

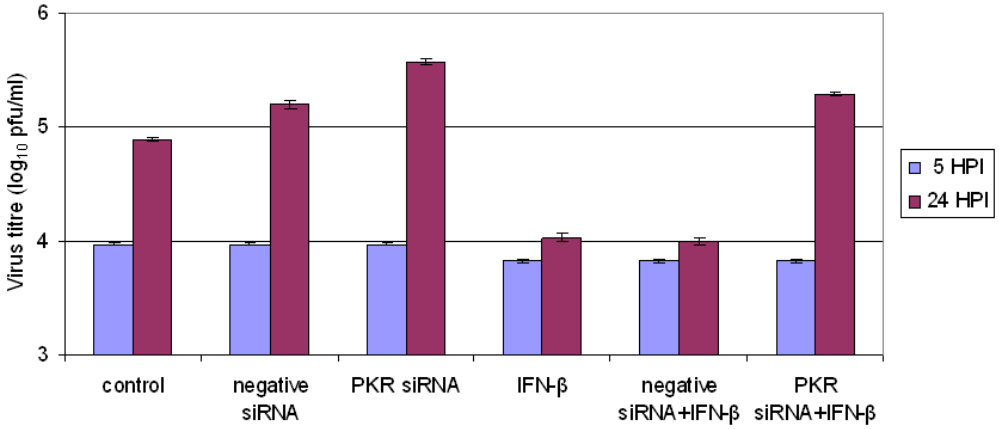


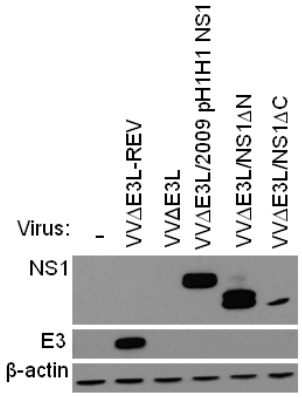
Fig.25. PKR mediates the IFN sensitivities of VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1. PKR knockdown rescues replication of (a) VVΔE3L, (b) VVΔE3L/1918 pH1N1 NS1 and (c) VVΔE3L/1968 pH3N2 NS1 in the presence of type I IFN. Huh7 cells ($2.5x10^5$ cells/well) were transfected with 100 nM of PKR siRNA or a non-targetting negative control siRNA for 48 H. Cells with and without siRNA treatment were treated overnight at 37°C with 100 U/ml IFN-β prior to infection with the indicated viruses. Cells were harvested at 5 and 24 HPI for virus titrations. Titrations were performed in triplicate. Error bars represent standard error of the mean. Data shown are representative of three independent experiments.

II.7. The dsRBD of NS1 mediates the resistance to IFN-induced antiviral activity

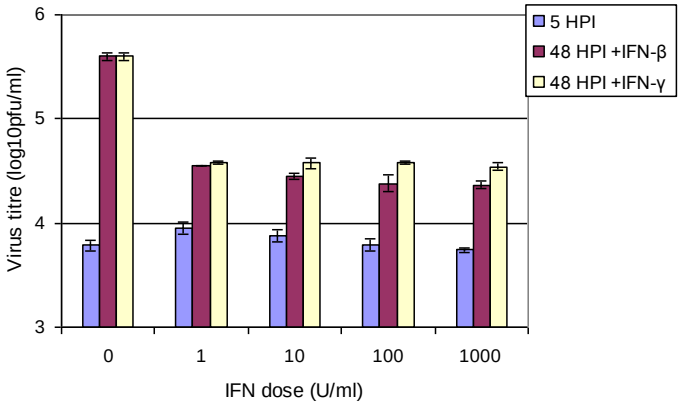
NS1 contains two functional domains, an N-terminal dsRNA binding domain (dsRBD) (residues 1-73) and an effector domain (ED) towards the C-terminus (residues 74-209)[122]. The generation of VVΔE3L/NS1ΔN and VVΔE3L/NS1ΔC recombinant viruses were based on the 2009 pH1N1 NS1, as a representative of the IFN resistant NS1 proteins in this study. To investigate the mechanism by which NS1 mediates resistance to IFN-induced antiviral activity, recombinant viruses expressing truncated mutants proteins of NS1 were generated. Truncated mutant viruses expressed either the ED of NS1 (VVΔE3L/NS1ΔN) or the dsRBD of NS1 (VVΔE3L/NS1ΔC). The expression of the NS1 truncated proteins of purified VVΔE3L/NS1ΔN and VVΔE3L/NS1ΔC recombinant viruses were confirmed by Western blot (Fig.26a).

As shown in Fig.26b, VVΔE3L/NS1ΔN was partially sensitive to IFN treatment, in that type I and type II IFNs inhibited VVΔE3L/NS1ΔN replication by approximately 10 fold, in comparison to the control. However, VVΔE3L/NS1ΔN was not as IFN sensitive as VVΔE3L (Fig.22b), in that VVΔE3L/NS1ΔN was still able to replicate even at the highest IFN dose (1000 U/ml). In contrast, VVΔE3L/NS1ΔC replication was unaffected by type I IFN treatment (Fig.26c). In comparison to the control without IFN, type II IFN treatment slightly inhibited VVΔE3L/NS1ΔC replication (Fig.26c). Overall, VVΔE3L/NS1ΔC exhibited full resistance to type I and type II IFN treatment. Therefore, the dsRBD of NS1 plays a primary role in suppressing IFN-β induced antiviral activity.

(a)



(b)



(c)

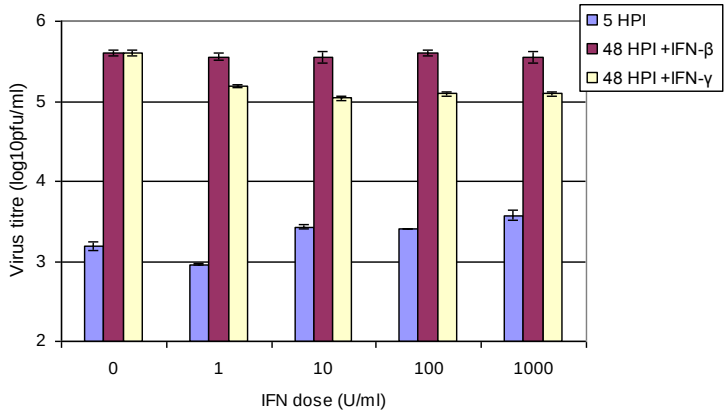
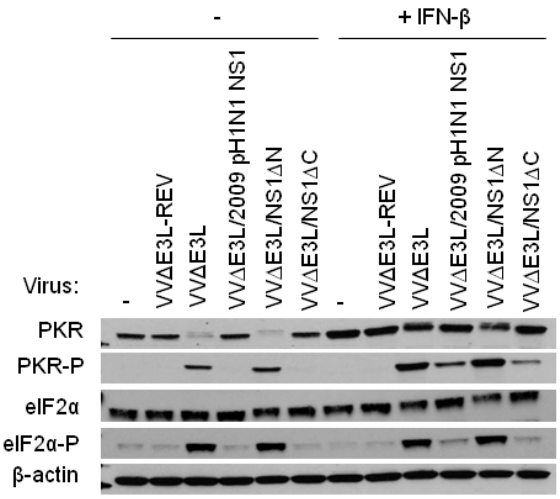


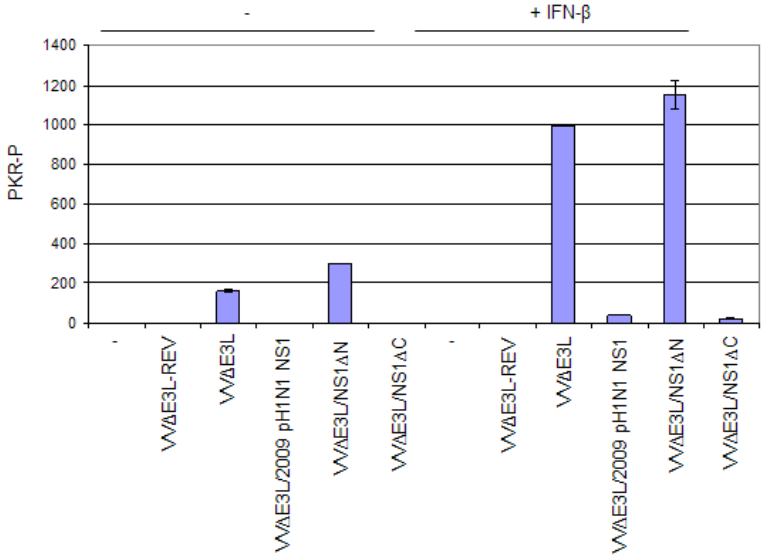
Fig.26 The dsRBD of NS1 primarily mediates the resistance to type I and type II IFN treatment. (a) Western blot confirmation of NS1 mutant proteins in recombinant viruses expressing the ED of NS1 (NS1ΔN) or the dsRBD of NS1 (NS1ΔC). (b) NS1ΔN exhibits partial sensitivity to type I and type II IFN treatment, while (c) NS1ΔC is resistant to type I and type II IFN treatment. IFN sensitivity assays and virus titrations were performed in triplicate. Error bars represent standard error of the mean. Data shown are representative of three independent experiments.

Next, the truncated NS1 (dsRBD and ED) mutants were examined for their effects on the phosphorylation of PKR and eIF2α. VVΔE3L-REV infections inhibited PKR and eIF2α phosphorylation in the absence and presence of IFN (Fig.27). Similar to VVΔE3L-REV, in the absence of IFN, infection with VVΔE3L/2009 pH1N1 NS1 and VVΔE3L/NS1ΔC inhibited PKR/eIF2α phosphorylation. In Huh7 cells untreated and treated with IFN, infection with VVΔE3L/NS1ΔN induced the phosphorylation of both PKR and eIF2α, similar to infection with VVΔE3L. Only in the presence of IFN, were phosphorylated-PKR and phosphorylated-eIF2α detected following infection with VVΔE3L/2009 pH1N1 NS1 and VVΔE3L/NS1ΔC viruses. However, infection with VVΔE3L/NS1ΔC inhibited PKR and eIF2α phosphorylation slightly greater than infection with VVΔE3L/2009 pH1N1 NS1. It should be noted that while the dsRBD of NS1 mediated the full resistance to IFN treatment, it does not fully inhibit PKR phosphorylation.

(a)


-
+ IFN-β
Virus:
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/NS1ΔN
VVΔE3L/NS1ΔC
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/NS1ΔN
VVΔE3L/NS1ΔC
PKR
PKR-P
eIF2α
eIF2α-P
β-actin

(b)


-
+ IFN-β
PKR-P
1400
1200
1000
800
600
400
200
0
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/NS1ΔN
VVΔE3L/NS1ΔC
-
VVΔE3L-REV
VVΔE3L
VVΔE3L/2009 pH1N1 NS1
VVΔE3L/NS1ΔN
VVΔE3L/NS1ΔC

(c)

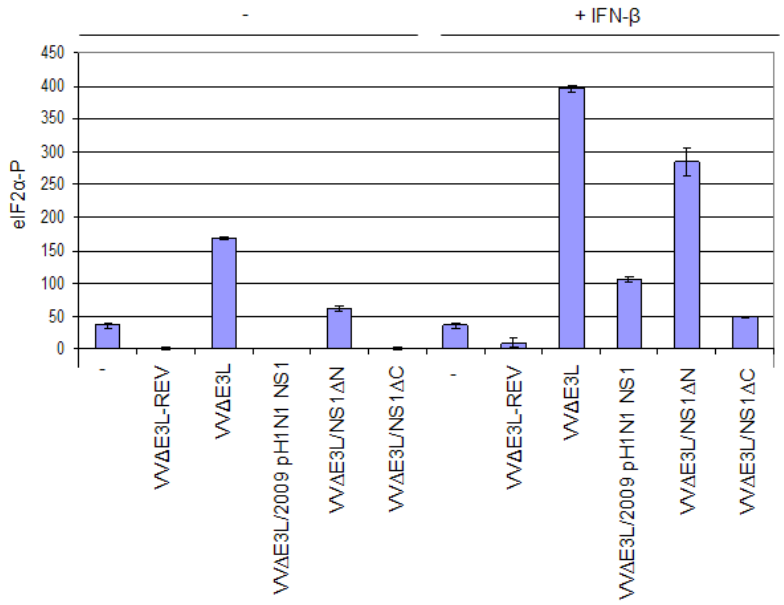


Fig.27 PKR/eIF2α phosphorylation in Huh7 cells following infection with VVΔE3L/NS1ΔN and VVΔE3L/NS1ΔC. Huh7 cells were untreated or treated with IFN-β (1000 U/ml) overnight at 37°C. Virus infections were performed at a MOI of 5 and collected 6 HPI. Phosphorylation of PKR and eIF2α were detected by (a) Western blotting. Quantification of (b) PKR phosphorylation and (c) eIF2α phosphorylation by Licor-Odyssey. Data shown are representative of three independent experiments. Error bars represent one standard deviation.

II.8. Mutations in the ED of 2009 pH1N1 NS1 do not affect its resistance to IFN-induced antiviral activity but compromise its ability to inhibit PKR phosphorylation

In the subsequent Results Section III, it was found that mutations in the ED of 2009 pH1N1 NS1 affect its ability to mediate IFN-β expression. Here, it was investigated whether mutations in the ED of the 2009 pH1N1 NS1 affect its resistance to type I IFN – induced antiviral activity. Among the H5N1 NS1 and 2009 pH1N1 NS1 proteins, there are three distinct differences in their EDs. First, the NS1 protein sequence length is different (H5N1 NS1 proteins are 230 a.a versus 219 a.a. of 2009 pH1N1 NS1). Second, the H5N1 NS1 proteins, but not 2009 pH1N1 NS1, contain a PDZ binding motif at a.a.226 to 230. Third, there are three amino acids in the 2009 pH1N1 NS1 protein, including arginine 108 (R108), glutamic acid 125 (E125), and glycine 189 (G189) that are different amino acids at the same positions in the H5N1 NS1 proteins. In the H5N1 NS1 proteins, a.a residue 108 is lysine (K108), and a.a. residues 125 and 189 are both aspartic acid (D125, D189). It was recently shown that the 2009 pH1N1 NS1 was inefficient in blocking host gene expression due to its inefficient binding to CPSF30[75]. However, this function could be restored with triple a.a. substitutions at R108, E125, and G189 (present in the 2009 pH1N1 NS1 protein) to K108, D125, and D189 (present in the H5N1 NS1 proteins)[75]. Furthermore, among the NS1 proteins of 2009 pH1N1 isolates, a natural mutation occurred in NS1 at position 123 from isoleucine (NS1/I123), predominantly present in virus isolates collected pre-epidemic, to valine (NS1/V123), predominantly present in virus isolates collected during the middle and late phases of the pandemic[184]. The 2009 pH1N1 NS1 protein in the previous experiments contains I123.

The VVΔE3L recombinants expressing NS1 ED mutations included V123 (VVΔE3L/NS1/V123), an 11 a.a. extension to the C-terminal tail of 2009 pH1N1 NS1 that contains a PDZ motif (based on a human isolate of H5N1 NS1) (VVΔE3L/NS1C-Add). In addition, the triple a.a. mutation (R108K, E125D, and G189D as previously reported[75] (VVΔE3L/NS1TriMut), and a combined mutation of the triple a.a. mutation with the 11 a.a. addition to the C-tail (VVΔE3L/NS1TriMutC-Add) were also constructed (Fig.28). To distinguish the 2009 pH1N1 NS1 ED mutants, the recombinant virus VVΔE3L/2009 pH1N1 NS1 (which contains I123) will be denoted VVΔE3L/NS1/I123. The expression of NS1/I123 ED mutant proteins were confirmed by Western blot (Fig.29).

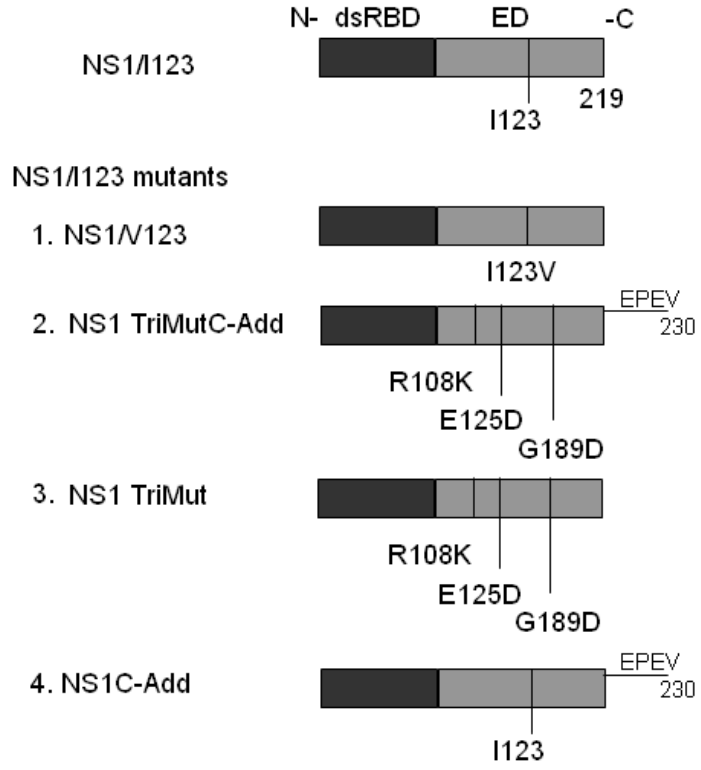


Fig.28 Schematic representation of a.a. mutations introduced in the ED of 2009 pH1N1 NS1, which contains I123. The 2009 pH1N1 NS1 is NS1/I123.

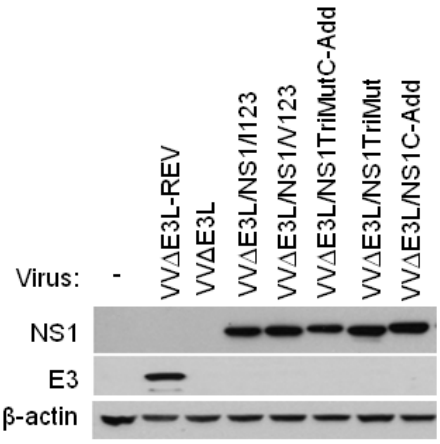
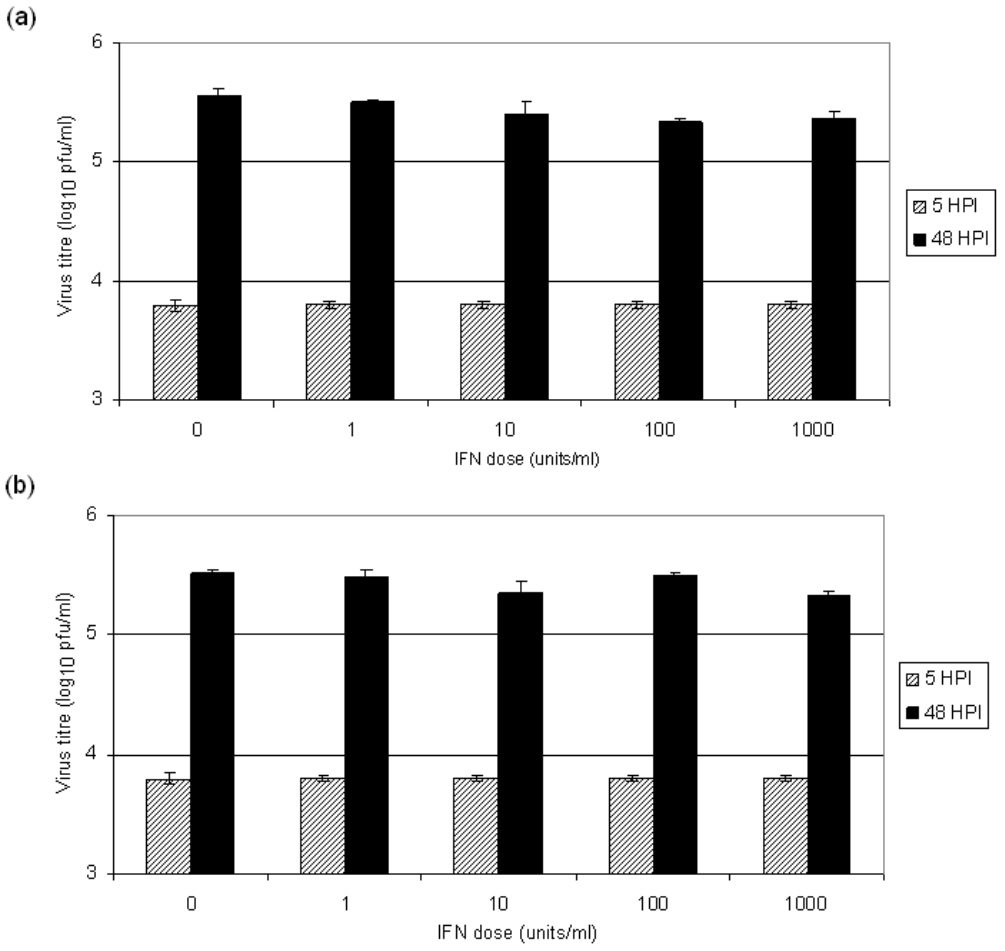


Fig. 29 Western blot confirmation of NS1/I123 mutant proteins expressed in replacement of VV E3. Recombinant viruses expressing NS1/I123 mutant proteins include VVΔE3L/NS1/V123, VVΔE3L/NS1TriMutC-Add (R108K, E125D, and G189D a.a. substitutions with extended C-tail to 230 a.a.), VVΔE3L/NS1TriMut (R108K, E125D, and G189D a.a. substitutions), and VVΔE3L/NS1C-Add (extended C-tail to 230 a.a.).

The NS1 ED mutants were next compared in their sensitivities to IFN-β treatment. All VVΔE3L recombinants expressing the 2009 pH1N1 NS1/I123 ED mutants exhibited resistance to IFN-β treatment (Fig.30a-30d), comparable to VVΔE3L/NS1/I123 (Fig.22e). Thus, mutations in the ED of NS1/I123 have no effect on suppressing IFN-β induced antiviral activity.



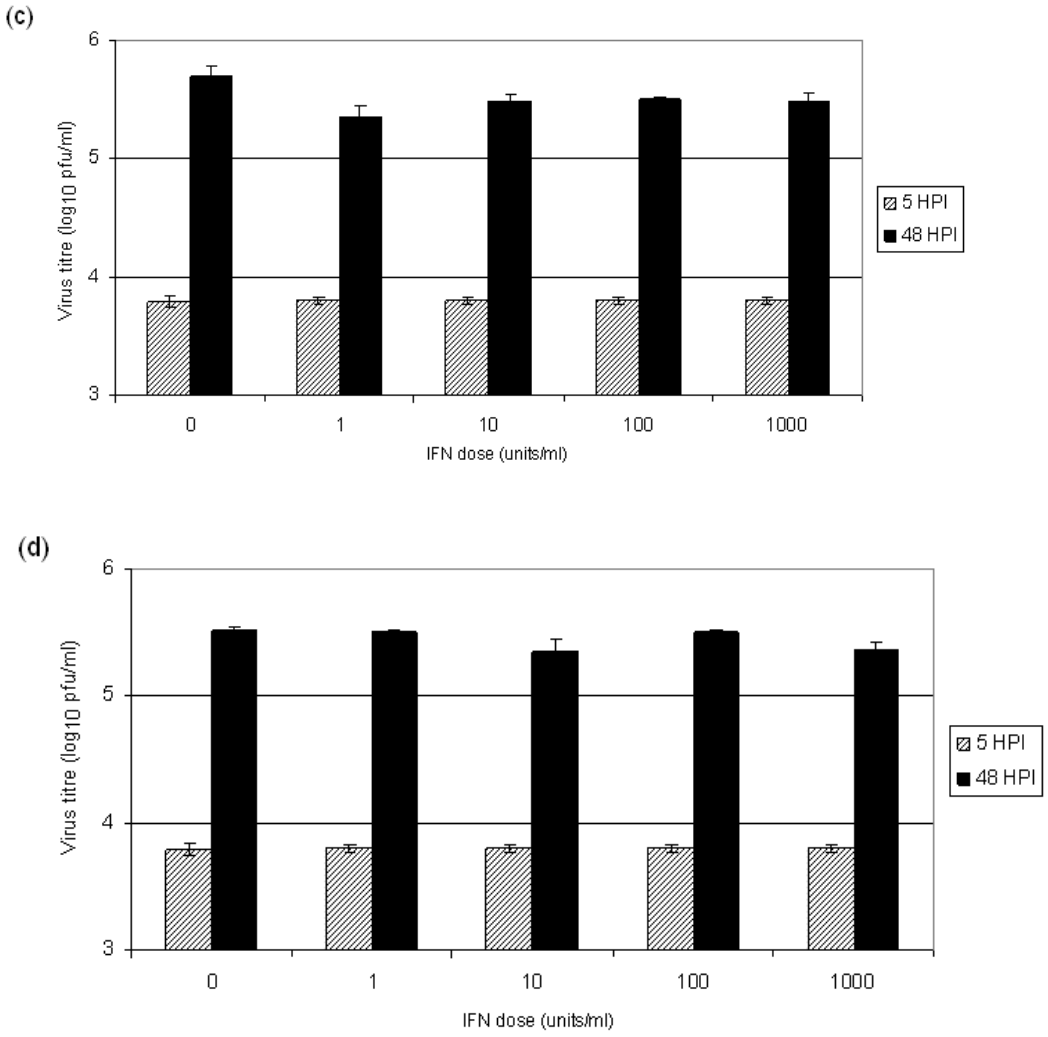
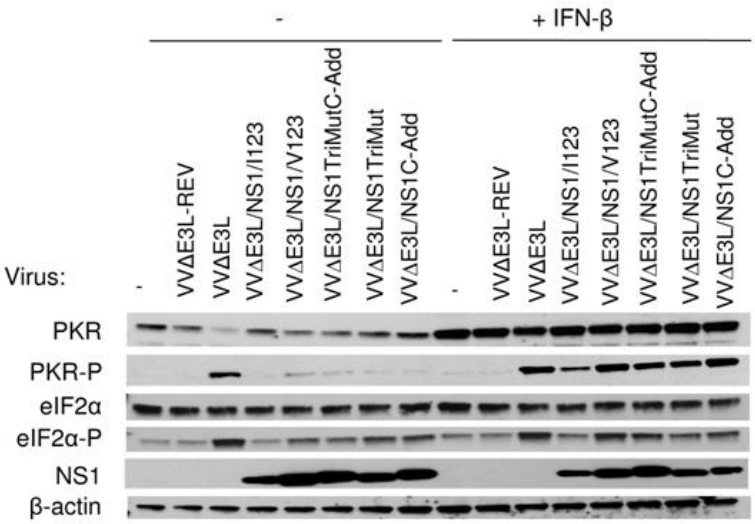


Fig.30 Mutations in the ED of NS1/I123 do not affect its resistance to IFN-induced antiviral activity. Huh7 cells were treated with IFN-β (1000 U/ml) overnight then infected with recombinants expressing (a) NS1/V123, (b) NS1TriMutC-Add, (c) NS1TriMut, and (d) NS1C-Add. Virus-infected cells were collected at 5 and 48 HPI for plaque assays. Titrations were performed in triplicate. Data shown are representative of three independent experiments.

Next, effects of the various mutations in the ED of the NS1 on PKR phosphorylation were examined. In the absence of IFN treatment, NS1/V123, NS1TriMutCAdd, NS1TriMut, and NS1C-Add inhibited the phosphorylation of PKR and eIF2α following virus infection (Fig.31a). Following IFN-β treatment, a significant amount of PKR and eIF2α was phosphorylated in cells infected with the viruses

expressing NS1/V123, NS1TriMutC-Add, NS1TriMut, and NS1C-Add. In contrast, VVΔE3L-REV infections did not induce phosphorylation of PKR or eIF2α in the presence of IFN. In comparison with the wild-type NS1/I123, the ED mutants showed reduced inhibition of PKR/eIF2α phosphorylation. The changes of PKR and eIF2α phosphorylation following virus infections with and without IFN were also quantified and confirmed by Licor-Odyssey infrared imaging (Fig.31b). Thus, the mutations in the ED of the 2009 pH1N1 NS1/I123 do not affect NS1 suppression of IFN-β induced antiviral activity, although these mutations compromised the activity of NS1/I123 to inhibit PKR and eIF2α phosphorylation.

(a)



(b)

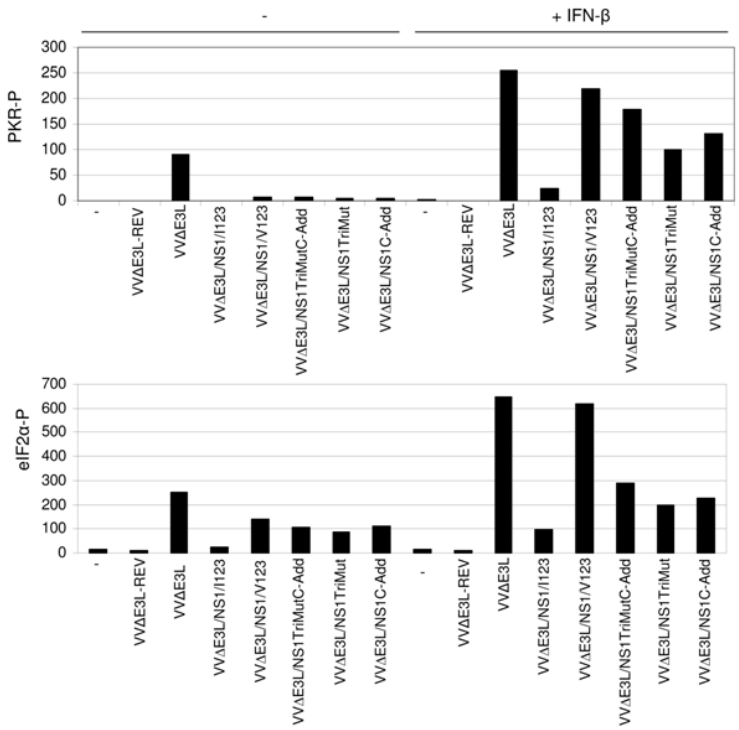


Fig.31 PKR/eIF2α phosphorylation by the NS1/I123 and its mutants following IFN-β treatment and virus infection. Huh7 cells were untreated or treated with human IFN-β (1000 U/ml) overnight at 37°C, then infected with the viruses at a MOI of 5 for 6 H. (a) Phosphorylation of PKR and eIF2α following infection with: VVΔE3L-REV, VVΔE3L, VVΔE3L/NS1/I123, VVΔE3L/NS1/V123, VVΔE3L/NS1TriMutC-Add, VVΔE3L/NS1TriMut, and VVΔE3L/NS1C-Add. (b) Quantification of phosphorylated-PKR and phosphorylated-eIF2α proteins by Licor-Odyssey. Data shown are representative of three independent experiments. Error bars represent one standard deviation.

II.9. Discussion

VVΔE3L was used as an expression vector to comparatively study a panel of NS1 proteins, when individually expressed in replacement of E3, at modulating IFN-induced antiviral activity in human cells. This is the first comparative study of NS1 proteins from influenza viruses of different species-origins, including highly pathogenic avian H5N1 isolated from two different species, an avian isolate (H5N1 NS1-duck) and a fatal human isolate (H5N1 NS1-human), swine-origin 2009 pH1N1, and the human 1918 pH1N1 and human 1968 pH3N2 pandemic viruses, at inhibiting human IFN-induced antiviral activities. The capacity of NS1 to inhibit IFN induction is subtype and/or strain specific[82,119]. However, less is known about subtype and/or strain-dependent differences in NS1 mediated modulation of IFN-induced antiviral activities.

In this section, there are two major findings that contribute to the characterization of influenza NS1 in modulating IFN-induced antiviral activity in human cells. First, NS1 proteins of different species of origin exhibited different antagonizing activities against human type I and type II IFNs. Specifically, NS1 proteins of avian H5N1 (from both the duck and human isolates) and swine-origin 2009 pH1N1 exhibited greater antagonizing activity against human type I and type II IFNs than the NS1 proteins of classic human 1918 pH1N1 and 1968 pH3N2. Mechanistically, the dsRNA binding domain (dsRBD) of NS1 plays a primary role in mediating the IFN resistant phenotype. Second, NS1 proteins differentially inhibit PKR phosphorylation. The NS1 proteins which mediated resistance to IFN-induced antiviral activity (H5N1 NS1-duck, H5N1 NS1-human, and 2009 pH1N1 NS1) were more effective at inhibiting PKR phosphorylation than the NS1 proteins which mediated sensitivity to type I IFNs (1918 pH1N1 NS1 and 1968 pH3N2 NS1) (Table 5).

Table 5 Summary of NS1 functions in modulating IFN-induced antiviral activity (+/- denote yes/no, respectively)

Recombinant virus	IFN resistant or sensitive	Protein dsRNA binding	Inhibition of PKR phosphorylation
VVΔE3L-REV	resistant	+	+
VVΔE3L	sensitive	N/A	-
VVΔE3L/H5N1 NS1-duck	resistant	+	Stronger than 1918 pH1N1 NS1 and 1968 pH3N2 NS1
VVΔE3L/H5N1 NS1-human	resistant	+	Stronger than 1918 pH1N1 NS1 and 1968 pH3N2 NS1
VVΔE3L/2009 pH1N1 NS1	resistant	+	Stronger than 1918 pH1N1 NS1 and 1968 pH3N2 NS1
VVΔE3L/1918 pH1N1 NS1	Sensitive to type I IFN	Minimal	-
VVΔE3L/1968 pH3N2 NS1	Sensitive to type I IFN	Minimal	-

NS1 has been shown to play a major role in influenza pathogenesis by mediating the inhibition of IFN pathways[34,73,275]. It was hypothesized that NS1 proteins of different influenza subtypes/isolates may exhibit different capacities at antagonizing IFN responses. Indeed, differences in the capacities of NS1 to antagonize human IFN-induced functions were found among the NS1 proteins analyzed in this study. The majority of studies on NS1 mediated suppression of IFN responses is limited to type I IFNs[82,119], therefore type II IFNs were included in this analysis. While the NS1 proteins of the highly pathogenic avian H5N1 and swine-origin NS1 mediated resistance to type I and type II IFNs, the NS1 proteins of the classic human 1918 pH1N1 and 1968 pH3N2 viruses exhibited greater sensitivity to type I IFN than type II IFN treatment. Overall, recombinants expressing 1918 pH1N1 and 1968 pH3N2 NS1 proteins were sensitive to IFN-induced antiviral activities. Other viral factors of 1918 pH1N1 and 1968 H3N2 may

have played a more dominant role in virus virulence than NS1. For instance, the 1918 pH1N1 HA, NA, and PB1 genes were shown to mediate efficient virus replication in human bronchial epithelial cells and virulence in mice[36,118,187].

Virus infection in HeLa cells with influenza virus strain A/H1N1/PR/8/34 virus strain inhibited PKR phosphorylation[17,111]. In this study, it was found that the recombinant viruses expressing different NS1 proteins differentially inhibited PKR phosphorylation. Specifically, VVΔE3L/H5N1 NS1-Duck, VVΔE3L/H5N1 NS1-Human, and VVΔE3L/2009 pH1N1 NS1 were more effective at inhibiting both PKR and eIF2α phosphorylation than VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 (Fig.23). PKR has been shown to restrict the host range of certain viruses, including poliovirus, Sindbis virus, West Nile Virus[45,220] and VVΔE3L[273]. NS1 deletion mutants based on A/H1N1/PR/8/34 were shown to be replication defective in a PKR-dependent manner in human fibroblasts, W138 cells, and in mice[17]. Collectively, these findings suggest that ability of NS1 to mediate the inhibition of PKR phosphorylation may be virus-subtype and strain specific.

In the context of influenza infections, it is possible that the activity of other influenza viral proteins of A/H1N1/PR/8/34 may have contributed to the inhibition of PKR phosphorylation, in addition to NS1. It was recently shown that the nucleoprotein (NP) interacts with heat shock protein 40 (Hsp40) to release $P58^{IPK}$, which mediates the inhibition of PKR phosphorylation and activity to downregulate eIF2α phosphorylation[224]. In naïve cells, $P58^{IPK}$ is inactive and exists in a complex with Hsp40. In influenza infections, $P58^{IPK}$ is released from Hsp40 and has been shown to promote influenza replication and inhibit PKR activity[67,198,247].

VVΔE3L is highly sensitive to IFN-induced PKR mediated antiviral activity[9].

The NS1 dsRBD was found to play the major role in mediating IFN resistance (Fig.26c). However, while the expression of H5N1 NS1-duck, H5N1 NS1-human, and 2009 pH1N1 NS1 proteins in VVΔE3L restored the IFN resistant phenotype of VV E3, none of the NS1 proteins were able to fully inhibit PKR phosphorylation comparable to VV E3 (Fig. 23, Fig.27). Therefore, it is reasonable to speculate that the mechanism(s) of IFN resistance exerted by H5N1-Duck, H5N1-Human, and 2009 pH1N1 NS1 proteins may involve additional ISGs. NS1 has been shown to disrupt IFN-induced PI3K/Akt and STAT-mediated signalling[105,135]. However, it was found that the PI3K/Akt signalling pathway was not activated following IFN treatment and virus infections, nor was there a reduction in phosphorylated STAT1 (Tyr701) following infection with the recombinant viruses expressing NS1 observed by Western blot analysis (data not shown).

Mutations in the ED of 2009 pH1N1 NS1 containing I123 (NS1/I123) did not affect its resistance to IFN-induced antiviral activity (Fig.30). Although 2009 pH1N1 NS1/I123 and its dsRBD mediated strong inhibition of PKR phosphorylation, all the ED mutants did not inhibit PKR phosphorylation efficiently. Taken together, these results suggest two things. First, multiple residues and/or motifs of the 2009 pH1N1 NS1/I123 protein may contribute to its ability to inhibit PKR phosphorylation. Second, the antagonizing activity of 2009 pH1N1 NS1/I123 against human type I and type II IFN-induced antiviral activities involves the modulation of ISGs other than PKR.

In this section, the antagonizing activity of a panel of influenza virus NS1 proteins against an IFN-induced antiviral state was examined using VVΔE3L as a platform. It was found that the IFN antagonizing activity of NS1 against human IFNs is subtype-specific. Mechanistically, the dsRBD of 2009 pH1N1 NS1 primarily mediated the resistance to

IFN-induced antiviral activity. Additionally, it was found that different NS1 proteins differentially inhibit PKR phosphorylation. Collectively, the work presented here provides further insight into the characterization of virus subtype and isolate dependent activities of NS1 mediated modulation of human IFN-induced antiviral activities.

RESULTS

III: *The effector domain of NS1 of pandemic 2009 H1N1 enhances human IFN-β gene expression through RIG-I, MDA5, STING, and PKR-dependent signalling*

Introduction. The capacity of NS1-mediated regulation of IFN induction can be virus subtype and/or strain specific and may contribute to the variability of virus virulence. In Results - II, the NS1 proteins of different species origins differentially inhibited IFN-induced antiviral activity. Using VVΔE3L, it was shown that VV E3 suppresses the expression of human IFN-β through modulation of RIG-I, MDA5, and IPS-1 signalling pathways in HeLa cells, and that the major PAMP associated with VVΔE3L infection is likely to be dsRNA species[172]*. In influenza infected cells, the 5' 3pRNA on viral RNAs and the vRNA panhandle secondary structure activate RIG-I dependent signalling to induce IFN*[194]*. Therefore, VVΔE3L and influenza share very similar signalling pathways for IFN induction. Thus, VVΔE3L can be further applied to analyze NS1 function at mediating IFN-β expression.*

Since it was found that different NS1 proteins, when expressed in VVΔE3L, mediate different inhibition activities against human IFN-induced antiviral activity (Results – II), it was hypothesized that the NS1 proteins differentially modulate VVΔE3L-induced IFN-β expression. Here, the specific objectives included: 1) comparatively analyze the IFN-β expression profiles induced by the different NS1 recombinants; 2) analyze the viral mechanism of NS1-mediated modulation of IFN-β expression; 3) investigate the potential cellular signalling mechanisms involved.

III.1. NS1 proteins differentially regulate IFN-β expression

The effect of NS1 on VVΔE3L-induced cytokine expression was first analyzed by real time PCR arrays (APPENDIX). The relative expression of 84 cytokines, including IFNs, ILs, and TNFα were determined in virus-infected HeLa cells, in comparison to mock infections. Infection with VVΔE3L/2009 pH1N1 NS1 resulted in a dramatic increase in IFN-β expression, in comparison to VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, and VVΔE3L infections. To further investigate whether the NS1 proteins of the different influenza A virus subtypes and/or isolates differentially modulate IFN-β induction, IFN-β expression was examined following infection with VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, VVΔE3L/2009 pH1N1 NS1, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/1968 pH3N2 NS1. VVΔE3L-REV and VVΔE3L infections were included as controls.

Previously, it has been reported that VVΔE3L induces type I IFN, such as IFN-β, in several cell lines, including HeLa cells[44,172]. HeLa cells were infected at a MOI of 5 with the recombinant viruses and collected 12 HPI for qRT-PCR analysis of IFN-β expression (Fig.32). Infection with VVΔE3L/2009 pH1N1 NS1 resulted in the greatest expression of IFN-β, in comparison to VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, VVΔE3L/1918 H1N1 NS1, VVΔE3L/1968 H3N2 NS1, and VVΔE3L alone (Fig.32). In comparison to VVΔE3L, infection with VVΔE3L/H5N1 NS1-human and VVΔE3L/1918 H1N1 NS1 suppressed IFN-β expression, whereas VVΔE3L/H5N1 NS1-duck and VVΔE3L/1968 pH3N2 NS1 induced IFN-β expression comparable to VVΔE3L (Fig.32). Thus, NS1 proteins differentially regulate VVΔE3L-induced IFN-β expression.

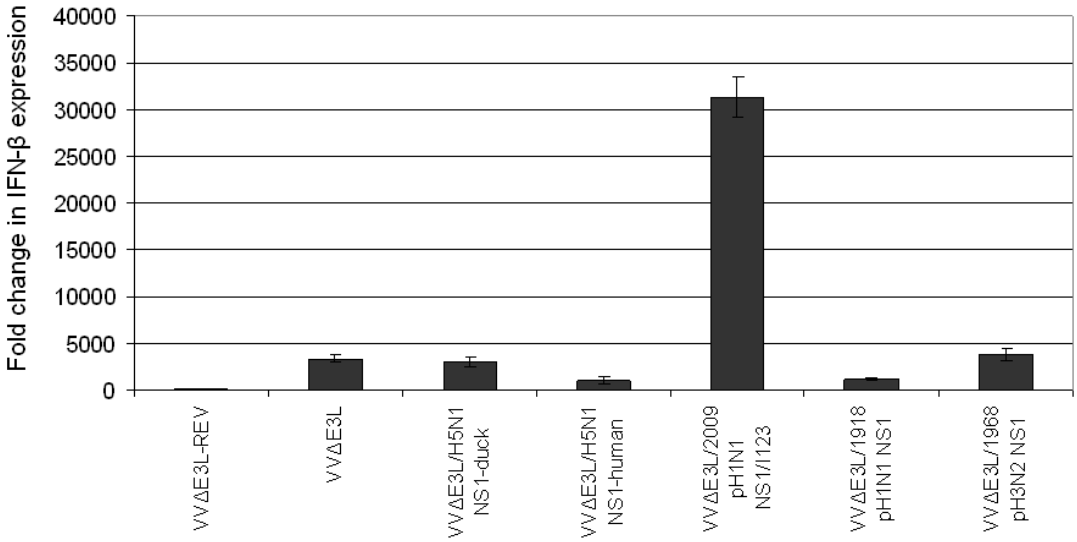


Fig.32 Comparative analysis of IFN-β expression mediated by infection with recombinant viruses expressing NS1. HeLa cells were mock infected or infected with the indicated viruses at a MOI of 5 and collected 12 HPI for qRT-PCR analysis of IFN-β gene expression, performed in triplicate. Data represents the fold change in IFN-β gene expression relative to mock infections. Error bars indicate standard deviation. Data shown are representative of three independent experiments.

III.2. 2009 pH1N1 NS1 containing I123 of the pre- and most of the early epidemic isolates mediates enhanced IFN-β gene expression in human cells

In Results – II, it was found that the 2009 pH1N1 NS1/I123 and the H5N1 NS1-human inhibited IFN-induced antiviral activity, while the 1918 pH1N1 NS1 protein was IFN sensitive. Interestingly, 2009 pH1N1 NS1/I123 strongly increased IFN-β expression, while the H5N1 NS1-human and 1918 pH1N1 NS1 proteins suppressed IFN-β expression, in comparison to the control VVΔE3L (Fig.32). Since influenza virus primarily infects lung epithelial tissues, it was next investigated whether 2009 pH1N1 NS1/I123, H5N1 NS1, and 1918 pH1N1 NS1 mediate the same pattern of IFN-β expression in A549 cells.

A549 cells were infected with VVΔE3L-REV, VVΔE3L, VVΔE3L/2009 pH1N1 NS1/I123, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/H5N1 NS1-human at a MOI of 5 and collected 12 HPI. Similar to the pattern observed in HeLa cells (Fig.32), infection with VVΔE3L/2009 pH1N1 NS1 resulted in the greatest increase in IFN-β expression, relative to mock infections (Fig.33). In addition, VVΔE3L infection induced greater IFN-β expression, in comparison to VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/H5N1 NS1-human recombinants in A549 cells (Fig.33).

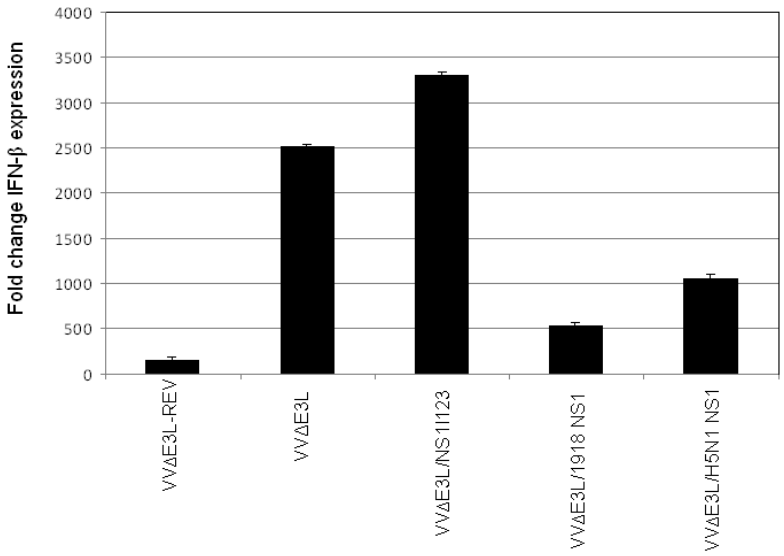
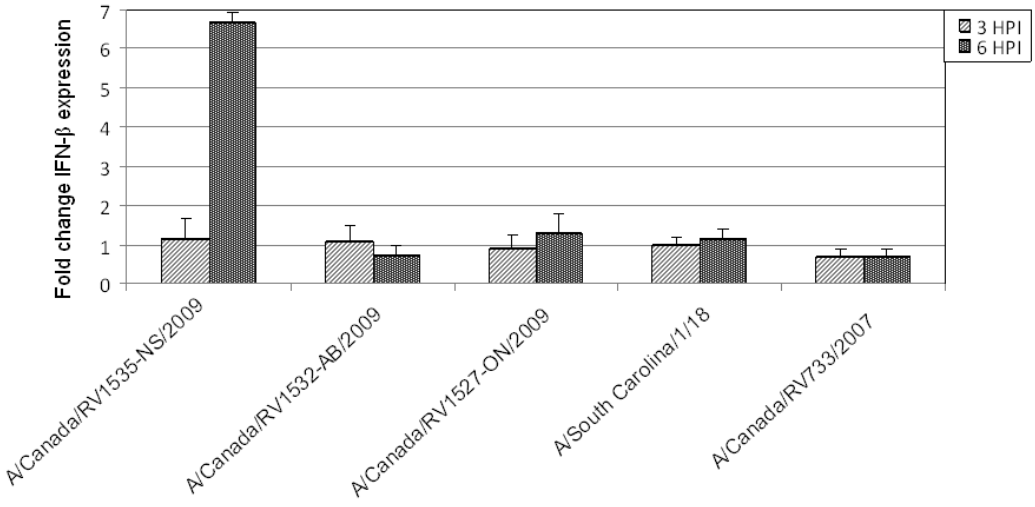


Fig.33 VVΔE3L/2009 pH1N1 NS1/I123 infection mediates increased IFN-β expression in A549 cells. A549 cells were mock infected or infected with viruses at a MOI of 5 and collected 12 HPI. qRT-PCR analysis of IFN-β gene expression was performed in triplicate. Data represents the fold change in IFN-β gene expression relative to mock infections. Error bars indicate standard deviation. Data shown are representative of three independent experiments.

Since NS1 proteins from different subtypes/isolates expressed in the backbone of VVΔE3L mediated differential effects on the expression level of IFN-β, IFN-β expression was next examined in both HeLa and A549 cells infected with a panel of influenza viruses. Among 2009 pH1N1 isolates, a natural mutation occurred in NS1 at position 123 from isoleucine (NS1/I123), predominantly present in virus isolates collected pre-epidemic, to valine (NS1/V123), predominantly present in virus isolates collected during the middle and late phases of the pandemic[184]. Included were three isolates of 2009 pH1N1 viruses, A/Canada/RV1535-NS/2009 (with NS1/I123), A/Canada/RV1532-AB/2009 and A/Canada/RV1527-ON/2009 (with NS1/V123), 1918 pH1N1 virus A/South Carolina/1/18, and a seasonal H1N1 viral strain A/Canada/RV733/2007. It should be noted that between A/Canada/RV1535-NS/2009 and A/Canada/RV1532-AB/2009 or A/Canada/RV1527-ON/2009, there is a single a.a. change in the neuraminidase (NA), nucleoprotein (NP), and PB1 proteins and two a.a. differences in the haemagglutinin (HA) protein, in addition to the I123V change in NS1. Consistent with the observations with VVΔE3L recombinants expressing different NS1 proteins (Fig.32, Fig.33), infection with the A/Canada/RV1535-NS/2009 virus, which contains NS1/I123, induced higher IFN-β expression than other influenza viruses examined in both HeLa (Fig.34a) and A549 cells (Fig.34b). Therefore, NS1 of pre- and early 2009 pH1N1 isolates, in which I123 is present (NS1/I123), enhances IFN-β expression in human cells.

(a)



(b)

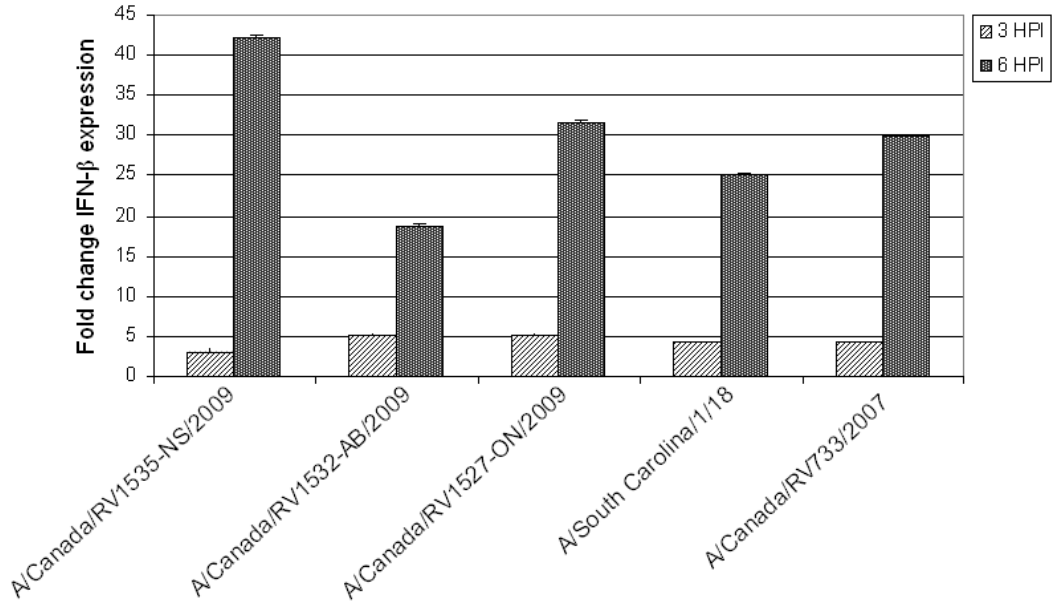


Fig.34 Pandemic 2009 pH1N1 influenza A virus strain with NS1/I123 (A/Canada/RV1535-NS/2009) induces IFN-gene expression in human cells. Fold change in IFN-β gene expression following virus infections with influenza A viruses as indicated in (a) HeLa and (b) A549 cells. Virus infections were performed at a MOI of 5 and collected at the times points as indicated. Error bars indicate one standard deviation. Data shown are representative of three independent experiments.

III.3. NS1/I123 does not directly induce IFN-β expression

Previously, it was reported that RNA species derived from intermediate and/or late stages of VV infection potently induce IFN-β expression[172]. When the transcription of VV intermediate and late genes is blocked with a DNA replication inhibitor, such as araC, the VVΔE3L virus no longer induces IFN-β expression[44,172]. Thus, it was next investigated whether NS1/I123 can induce IFN-β expression in the absence of VV intermediate and late transcription or if NS1/I123 enhances IFN-β expression mediated by VV intermediate and late gene transcripts. HeLa cells were untreated or treated with araC and then were infected with VVΔE3L or VVΔE3L/NS1/I123. Total RNA and cell lysates were collected 9 HPI for qRT-PCR and Western blot analysis. In the absence of araC, relative to the mock infection, VVΔE3L infection increased IFN-β expression by approximately 1600 fold, whereas VVΔE3L/NS1/I123 infection increased IFN-β expression by more than 2000 fold (Fig.35a). However, in the presence of araC, IFN-β expression following VVΔE3L or VVΔE3L/NS1/I123 infection was blocked. This suggests that expression of NS1/I123 in VVΔE3L enhances IFN-β expression, induced by VV intermediate and late gene products. AraC treatment did not affect NS1 protein expression (Fig.35b).

(a) (b)

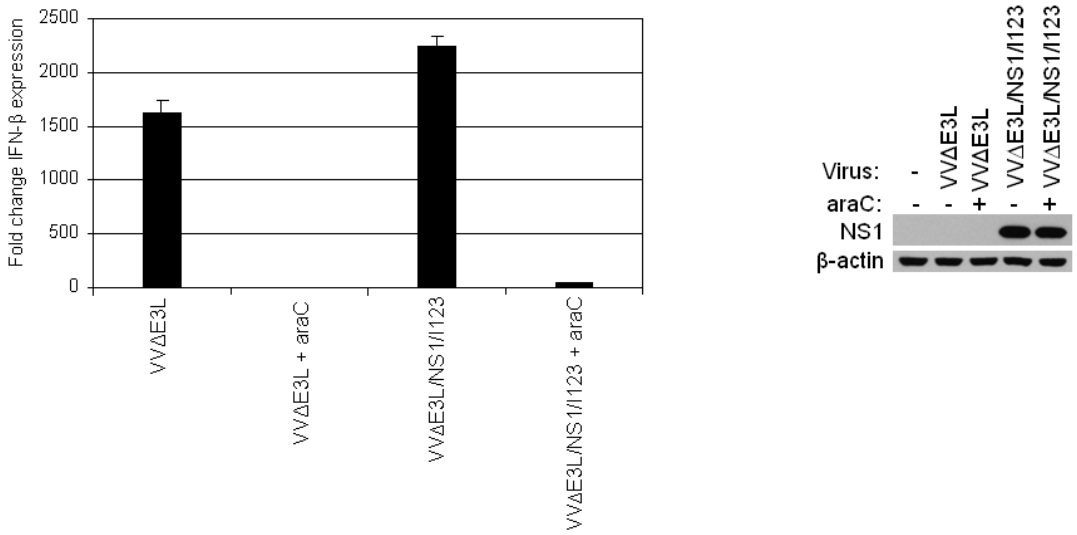


Fig.35 NS1/I123 enhances IFN-β gene expression induced by VV intermediate and late gene products. (a) qRT-PCR analysis of fold change in IFN-β gene expression in HeLa cells infected with VVΔE3L and VVΔE3L/NS1I123 in the absence and presence of cytosine arabinoside (AraC). Confluent HeLa cells were untreated or pretreated with 50 μg/ml of AraC and infected with VVΔE3L and VVΔE3L/NS1I123 at a MOI of 5 and collected at 8 HPI for total RNA and cell lysates. (b) Western blot confirmation of VVΔE3L/NS1I123 protein expression in the absence and presence of AraC following virus infections. Error bars indicate one standard deviation. Data shown are representative of three independent experiments.

III.4. The effector domain of NS1/I123 plays a primary role in mediating enhanced IFN-β expression

To further delineate which functional domain, the ED or dsRBD, of NS1 plays the major role in VVΔE3L/NS1/I123-mediated enhancement of IFN-β expression, IFN-β expression was next examined in HeLa cells infected with the VVΔE3L/NS1 truncation mutant recombinant viruses, VVΔE3L/NS1ΔN (which expresses only the ED (a.a. 80-219)) and VVΔE3L/NS1ΔC (which expresses only the dsRBD (a.a.1-79)). At 9 HPI, VVΔE3L/NS1ΔN infection resulted in the greatest IFN-β expression, in comparison to VVΔE3L, VVΔE3L/NS1/I123, and VVΔE3L/NS1ΔC infections (Fig.36). It is interesting

that lower IFN-β expression was observed following VVΔE3L/NS1ΔC infection than VVΔE3L infection alone (Fig.36). Thus, the ED of NS1/I123 is responsible for NS1-mediated enhancement of IFN-β expression, while the dsRBD of NS1 can inhibit IFN-β expression.

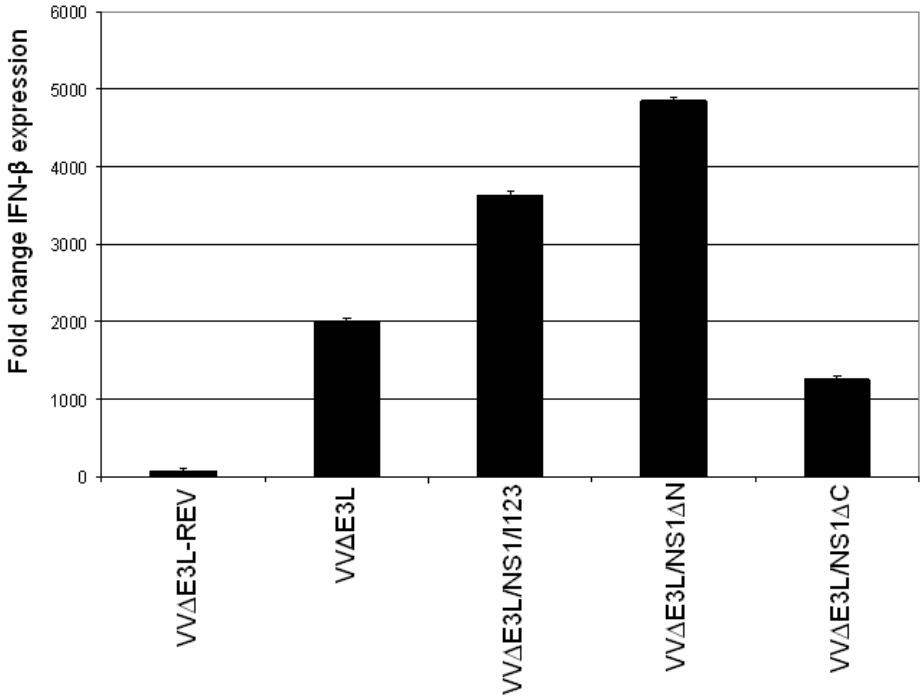


Fig.36 The effector domain (ED) of NS1/I123 is responsible for the enhancement of IFN-β gene expression. qRT-PCR analysis of fold change in IFN-β gene expression following virus infections with VVΔE3L-REV, VVΔE3L, VVΔE3L/NS1I123, VVΔE3L/NS1ΔN, and VVΔE3L/NS1ΔC in HeLa cells. Virus infections were performed at a MOI of 5 and collected at 9 HPI. Error bars indicate standard deviation. Data shown are representative of three independent experiments.

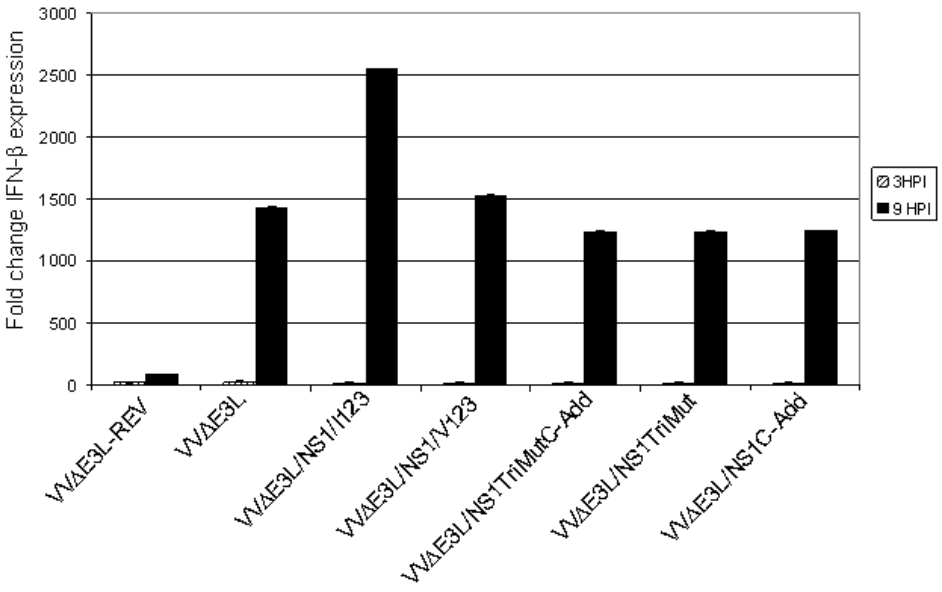
III.5. Isoleucine 123 and the truncated C-tail of NS1/I123 are critical for the enhancement of IFN-β gene expression

Recently, it has been reported that R108, E125 and G189 were important for the NS1/I123 mediated modulation of host cell gene expression, including IFN-β[75]. In this study, the role of I123 and the truncated C-terminal tail in mediating the enhanced IFN-β expression were analyzed. The effects of the mutations in the ED of NS1/I123 were next analyzed in their capacities to modulate NS1/I123 enhancement of IFN-β expression.

HeLa cells were mock infected or infected with VVΔE3L-REV, VVΔE3L, VVΔE3L/NS1/I123, and recombinant viruses expressing the NS1/I123 ED mutants, including VVΔE3L/NS1/V123, VVΔE3L/NS1TriMut, VVΔE3L/NS1C-Add, and VVΔE3L/NS1TriMutC-Add and collected at 3, 9, and 12 HPI. Total RNA was collected for qRT-PCR analysis of IFN-β expression. At 9 HPI, VVΔE3L/NS1/I123 infection resulted in greatest IFN-β expression in comparison with the other recombinant viruses (>2500 fold) (Fig.37a). In contrast, infection with recombinant viruses VVΔE3L/NS1/V123, VVΔE3L/NS1TriMutC-Add, VVΔE3L/NS1TriMut, and VVΔE3L/NS1C-Add resulted in IFN-β expression slightly less than or comparable to VVΔE3L (Fig.37b). The increase in IFN-β expression following VVΔE3L/NS1/I123 infection was more prominent at 12 HPI, in which the increase was greater than 40000 fold (Fig.37b). From 9 to 12 HPI, although the expression of IFN-β increased in VVΔE3L, VVΔE3L/NS1/I123V, and VVΔE3L/NS1C-Add infections, VVΔE3L/NS1TriMut and VVΔE3L/NS1TriMutC-Add infections inhibited IFN-β expression, in comparison to VVΔE3L (Fig.37b). Thus, I123 and the truncated C-tail are

critical for the enhancement of IFN-β gene expression, while the three a.a. substitutions (R108K, E125D, and G189D) are required for the inhibitory function.

(a)



(b)

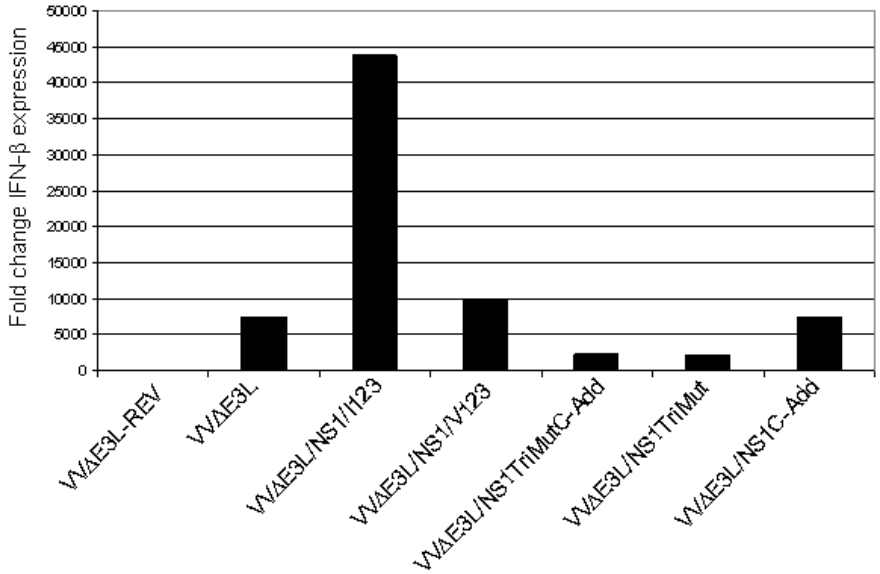


Fig.37 Isoleucine 123 and the shortened C-tail of NS1I123 mediate robust IFN-β gene expression. qRT-PCR analysis of fold change in IFN-β gene expression following virus infections in HeLa cells. Virus infections were performed at a MOI of 5 and collected at (a) 3, 9 and (b) 12 HPI. Error bars indicate one standard deviation. Data shown are representative of three independent experiments.

III.6. Valine 123 and the C-terminal 11 a.a. mutations in NS1/I123 weaken its interaction with CPSF30

CPSF30 is required for the 3′ end processing of cellular pre-mRNAs[10]. NS1-mediated inhibition of IFN-β expression and the suppression of host gene expression are associated with the activity of NS1 to bind to and inhibit the activity of host cellular protein CPSF30[173]. The 2009 pH1N1 NS1 was shown to inefficiently bind CPSF30[75], while three a.a. substitutions in NS1/I123 (R108K, E125D, and G189D) enhanced the direct interaction between NS1/I123 and CPSF30[75]. Therefore, immunoprecipitation experiments were performed, using the previously reported assay system expressing a FLAG-tagged CPSF30[37] to assess the CPSF30 binding activity of 2009 pH1N1 NS1/I123 and its mutant proteins expressed in the VVΔE3L backbone. Expression of CPSF30 and NS1 mutant proteins following viral infection and transfection with the pCAGGs vector were confirmed by Western blot (Fig.38a). In agreement with the observations of Hale and others[75], the NS1 mutant proteins with triple a.a. substitutions (NS1TriMutC-Add and NS1TriMut) had the highest affinity for CPSF30, while NS1/I123 interacted with CPSF30 with less affinity in the FLAG pull-downs (Fig.38b). However, valine 123 and the 11 a.a. C-terminal addition (without R108K, E125D, and G189D) significantly weakened the binding of NS1/I123 to CPSF30 (Fig.38b).

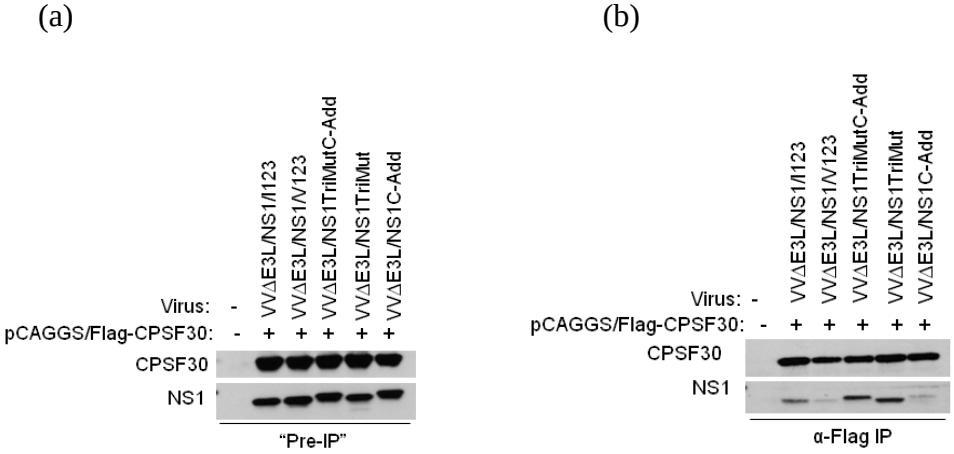


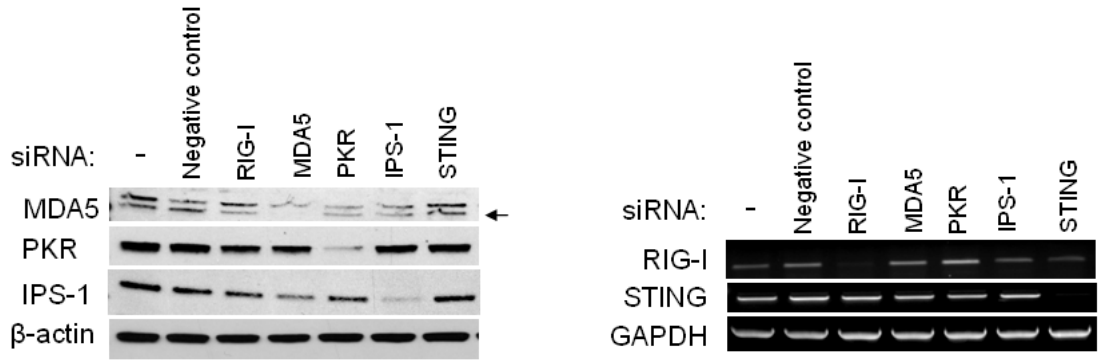
Fig.38 Interaction of NS1/I123 and its ED mutants with CPSF30. FLAG-tagged CPSF30 was transiently expressed in 293T cells prior to infection with VVΔE3L/NS1/I123, VVΔE3L/NS1/V123, VVΔE3L/NS1TriMutC-Add, VVΔE3L/NS1TriMut, and VVΔE3L/NS1C-Add viruses and immunoprecipitated (IP) with anti-FLAG resin. Mock infected 293T cell lysates without FLAG-tagged CPSF30 was the negative control. a) Western blot confirmation of CPSF30 and VVΔE3L/NS1 protein expression as indicated prior to immunoprecipitations ('Pre-IP'). b) Precipitated CPSF30 and VVΔE3L/NS1 proteins were detected by Western blot. Data shown are representative of five independent experiments.

III.7. NS1/I123 enhances IFN-β expression through RIG-I, MDA5, STING, and PKR dependent pathways

Previously, it was reported that VVΔE3L induces IFN-β expression through MDA5 and RIG-I, while PKR also plays a role[172]. Recently, STING (stimulator of interferon genes; alternatively known as MITA/MPYS/ERIS) has also been shown to mediate IFN-α/β expression in response to viral infections[98,99,106,274]. In this study, the role of RIG-I, MDA5, PKR, IPS-1, and STING in mediating IFN-β expression induced by virus infection with VVΔE3L/NS1/I123 was investigated using siRNA knockdown experiments.

HeLa cells were transfected with siRNAs specific to the genes of interest. The knockdown efficiency for each siRNA transfection was confirmed by Western blot or RT-PCR depending on the availability of reagents (Fig.39a). HeLa cells treated with siRNAs were then mock infected or infected with either VVΔE3L or VVΔE3L/NS1/I123 and collected 9 HPI. qRT-PCR analysis of IFN-β expression following virus infections with VVΔE3L (Fig.39b) and VVΔE3L/NS1/I123 (Fig.39c) was performed. VVΔE3L infection without siRNA or with negative control siRNA treatment induced IFN-β expression by 1400 fold, relative to mock infection (Fig.39b). In comparison to VVΔE3L control infections, MDA5 knockdown almost completely abolished VVΔE3L-induced IFN-β expression. IPS-1 and STING knockdown also dramatically reduced IFN-β induction (Fig.39b). The suppression of RIG-I exhibited a slight effect on VVΔE3L-induced IFN-β expression, in comparison to MDA5, IPS-1, and STING knockdowns (Fig.39b). Similar to what was previously reported[172], PKR knockdown also affected VVΔE3L-induced IFN-β expression (Fig.39b).

(a)


siRNA:
-
Negative control
RIG-I
MDA5
PKR
IPS-1
STING
MDA5
PKR
IPS-1
β-actin
siRNA:
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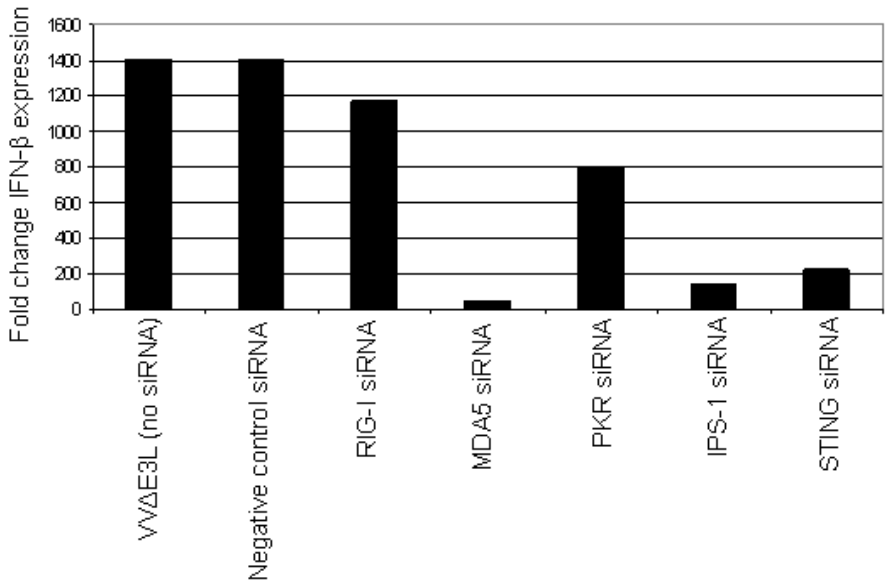
Negative control
RIG-I
MDA5
PKR
IPS-1
STING
RIG-I
STING
GAPDH

(b)


Fold change IFN-β expression
1600
1400
1200
1000
800
600
400
200
0
VVΔE3L (no siRNA)
Negative control siRNA
RIG-I siRNA
MDA5 siRNA
PKR siRNA
IPS-1 siRNA
STING siRNA

(c)

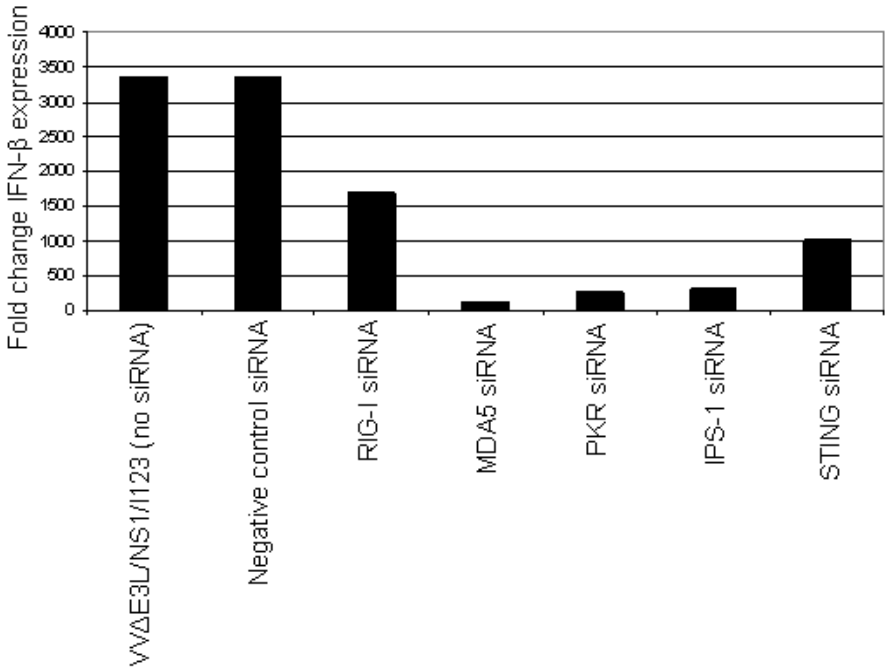


Fig.39 NS1/I123 enhances VVΔE3L-induced IFN-β gene expression through MDA5, IPS-1, STING, and PKR-dependent signalling. 1.5 x 10^5 HeLa cells were transfected with siRNAs as indicated at 100 nM for 48 h then infected with VVΔE3L and VVΔE3L/NS1I123 viruses at a MOI of 5 for 9 H. a) Confirmation of RIG-I, MDA5, PKR, IPS-1, and STING expression knockdowns by Western blot or RT-PCR. qRT-PCR analysis of fold change in IFN-β gene expression in b) VVΔE3L infections and c) VVΔE3L/NS1I123 infections, in comparison to mock infections. 1.5 x 10^5 HeLa cells were transfected with RIG-I, MDA5, and PKR specific siRNAs at 100 nM for 48 h and infected with VVΔE3L/NS1ΔN at a MOI of 5 for 9 H. Error bars indicate standard deviation. Data shown are representative of three independent experiments.

Similarly, IFN-β expression induced by VVΔE3L/NS1/I123 was significantly reduced by MDA5, IPS-1 and STING gene knockdowns (Fig.39c). It is interesting to note that the knockdown of PKR and RIG-I had a more dramatic effect on the IFN-β expression following VVΔE3L/NS1/I123 infection (Fig.39c), in comparison to VVΔE3L infection (Fig.39b).

III.8. Discussion

VVΔE3L was used as an expression vector to examine NS1 function in the regulation of IFN-β induction. This is the first study in which VVΔE3L was applied to study the function of a viral protein, when expressed in replacement of E3, at modulating IFN-β induction. In this section, there are five novel findings that contribute to the characterization of NS1 function in modulating IFN-β expression in human cells. First, NS1 proteins of different influenza subtypes and strains differentially modulate IFN-β expression, in comparison to VVΔE3L. Second, the NS1 proteins of different 2009 pH1N1 isolates can mediate isolate-dependent enhancement of IFN-β expression. Third, the ED of 2009 pH1N1 NS1/I123 mediates the enhanced IFN-β expression, while the dsRBD exhibits an inhibitory effect. Fourth, the truncated C-terminal tail and the residue isoleucine 123 in the ED of 2009 pH1N1 NS1 (NS1/I123) are critical for the enhancement of IFN-β expression. Fifth, mutational analysis of the ED of NS1/I123 suggest the natural occurring mutation of isoleucine to valine at residue 123 may play a role in the 2009 pH1N1 virus adaptation to human hosts.

It is intriguing that the NS1/I123 of the pre- and majority of early 2009 pH1N1 isolates mediated enhanced IFN-β expression, in comparison to the NS1 proteins of the majority of the late 2009 pH1N1 isolates (NS1/V123), the 1918 pH1N1 and avian H5N1 (Fig.32, Fig.37). The majority of the variation between the a.a. sequences of the NS1 proteins examined in this study lies in the ED (Fig.19). Indeed, the ED of the 2009 pH1N1 NS1/I123 was responsible for enhancing IFN-β expression (Fig.36), while the dsRBD inhibited IFN-β expression. In support of this observation, recombinant influenza viruses expressing RNA binding defective NS1 mutants were shown to induce a higher

level of IFN-β than the virus expressing NS1 with a functional dsRBD[50]. It is worth pointing out that the VVΔE3L expressing the ED (VVΔE3L/NS1ΔN) induced a higher level of IFN-β expression than the virus expressing the full length NS1/I123, whilst VVΔE3L expressing the dsRBD (VVΔE3L/NS1ΔC) mediated less IFN-β expression than both VVΔE3L and VVΔE3L/NS1/I123 (Fig.36). This observation clearly indicates that the ED and the dsRBD of the full length NS1 protein mediate opposite effects on IFN-β expression.

The NS1 protein of the 2009 pH1N1 is 219 a.a. in length and lacks 11 a.a. residues at the C-terminal tail in comparison with the 1918 pH1N1 and the avian influenza NS1 proteins (Fig.19). Previous studies have shown that this 11 a.a. sequence may contain a PDZ binding motif (ESEV or EPEV) and is a virulence determinant of avian H5N1 isolates and 1918 pH1N1[101,275]. In this study, it was found that extension of the C-terminal tail of 2009 pH1N1 NS1 (NS1/I123) to contain this extra 11 a.a. sequence (NS1TriMutC-Add, NS1C-Add) abolished the ability of NS1/I123 to enhance IFN-β expression (Fig.37a, 37b). In support of this observation, C-terminal truncations in NS1 have been shown to result in increased IFN expression following infections with a highly virulent variant of A/PR/8/34 (H1N1) in MDCK cells, avian A/turkey/Oregon/71-SEPRL (TK/OR/71-SEPRL) (H7N3) in primary chicken embryo cells, and of the human isolate A/TX/91 in primary differentiated human tracheobronchial epithelial cells and A549 cells[26,81,119,237,251]. Thus, the C-terminal tail containing the PDZ binding motif of NS1 proteins is one determinant of the modulation of IFN-β expression by influenza A viruses.

Mutation trend analysis of 2009 pH1N1 isolates collected during different periods of the pandemic revealed a naturally occurring mutation at a.a. residue 123 in NS1 from isoleucine, present in all pre-epidemic isolates to valine, present in 29% of isolates

collected during the early stage, in 40% of isolates collected during the middle, and in 78% of isolates collected during the late periods of the pandemic[184]. Here, it was shown that this naturally occurring mutation is functionally significant, in that isoleucine 123 is required for the NS1 protein of the pre- and majority of early isolates of the 2009 pH1N1 viruses to mediate enhanced IFN-β expression (Fig.37a, 37b). The mutation from isoleucine to valine in the NS1 protein of most 2009 pH1N1 isolates from the late period of the pandemic renders the NS1 protein unable to mediate enhanced IFN-β expression, indicating that this mutation represents an adaptation determinant of the 2009 pH1N1 isolates to human hosts. It is interesting to note that the isoleucine 123 to valine mutation was not observed in the previous pandemics of 1918, 1957, and 1968[184]. It is reasonable to speculate that isoleucine 123, together with other unique sequences of the 2009 pH1N1 NS1, for example, the truncated C-terminal tail, form a specific structure important for mediating the enhanced IFN-β expression. During the early stages of the emergence of 2009 pH1N1, the major histopathological features among 100 human autopsy specimens that were confirmed to be associated with 2009 pH1N1 infection included diffused alveolar damage, inflammation and edema[227]. It is interesting to note that as the pandemic progressed, the clinical features of the majority of 2009 pH1N1 infections in humans were reported as mild and resembled seasonal influenza symptoms[103,249]. While underlying conditions such as obesity, cardiovascular disease and asthma may have contributed to the severe or fatal outcomes from which the specimens were collected during the early phase of the 2009 pandemic, the precise viral mechanisms that contributed to the pathogenesis in these fatality cases remained uncharacterized[227]. Thus, it can be speculated that NS1/I123 may have been a critical viral factor in mediating virus pathogenesis and the pathology observed in early fatal cases of 2009 pH1N1.

Correspondingly, it can be reasoned that the natural mutation of I123V in NS1 (which occurred with the progression of the pandemic) supports the 2009 pH1N1 virus adaptation to human hosts, in which milder disease symptoms were commonly observed in human infection cases. It would be interesting to further study potential 2009 pH1N1 virus isolate-dependent histopathological features to better define the precise viral mechanisms implicated in virus-induced pathologies.

It is known that the ED of NS1 proteins mediate the suppression of host gene expression by binding to and inactivating the function of CPSF30, thus inhibiting post-transcriptional 3' end processing of cellular pre-mRNA[173]. In a recent study, it was shown that R108, E125, and G189 are critical for a reduced interaction with CPSF30 and the inability to efficiently suppress IFN-β expression [75]. That observation was confirmed in this present analysis, using VVΔE3L expressing those mutant NS1 proteins (Fig.38). In addition, it was found that mutations of I123V or the addition of the 11 a.a. sequence containing the PDZ binding motif at the C-terminus further weakened the binding between 2009 pH1N1 NS1/I123 and CPSF30. However, such mutations did not result in further enhancement of IFN-β expression. On the contrary, they abolished the enhancement of IFN-β expression (Fig.37). Residues phenylalanine 103 (F103) and methionine 106 (M106) outside the NS1 binding pocket for CPSF30 have been shown to stabilize CPSF30-NS1 interaction[38]. A.a. substitutions in NS1 of highly pathogenic H5N1 A/HK/483/1997, L103F and I106M, increased the affinity of NS1 to CPSF30, in comparison to the weak CPSF30 binding capacity of the wildtype NS1[38,124,238]. It is interesting to note that F103 and M106 are present in 2009 pH1N1 NS1, yet it binds CPSF30 inefficiently (Fig.38). Taken together, these results suggest that multiple

residues and/or motifs of the NS1 protein may contribute to the regulation of host gene expression through modulation of multiple cellular targets, in addition to CPSF30.

The 2009 pH1N1 NS1/I123 protein enhances IFN-β expression induced by VVΔE3L intermediate and late gene transcripts (Fig.35), mainly dsRNA species[172]. Since it has been well characterized that VVΔE3L-induced IFN-β expression is dependent on RIG-I/MDA5/IPS-1 pathway[44,170,172] (Fig.39), it is likely that the NS1 protein acts to enhance the signalling of this pathway. In addition, STING was found to be a critical component in VVΔE3L-induced IFN-β expression. It would be interesting to further investigate whether E3 can directly inhibit STING mediated IFN-β expression or if E3 inhibits the expression of other unidentified cytokines through STING.

It is interesting to note that PKR played a more significant role in the enhancement of IFN-β expression in VVΔE3L/NS1/I123 infected HeLa cells than in VVΔE3L infected cells (Fig.39a, 39b). Thus, it is likely that the NS1/I123 interacts with PKR or PKR-regulated signalling molecules to mediate the enhancement of IFN-β expression. PKR has been shown to regulate transcription through IκB/NF-κB, IRF1, and STAT pathways[59] and was also shown to regulate IFN-α/β mRNA stability through activation of the p38 pathway in response to viral infections with encephalomyocarditis virus, Theiler's murine encephalomyelitis, and Semliki Forest virus[45,220]. It would be interesting to further investigate the mechanism by which NS1/I123 interacts with the RIG-I, MDA5, IPS-1 pathway to mediate the enhancement of IFN-β expression.

In summary, the function of NS1 proteins from several different influenza viruses (including two isolates of 2009 pH1N1, the classic human 1918 pH1N1 and a human

isolate of avian H5N1) in the regulation of IFN-β expression was examined in human cell culture using VVΔE3L as an expression vector. The previously reported novel features of the 2009 pH1N1 NS1 protein, ie. the role of the three a.a. residues (R108, E125, and G189[75]) in controlling IFN-β expression was confirmed with this system. Novel properties of the 2009 pH1N1 NS1/I123 protein, such as the distinct roles of the ED and the dsRBD in the modulation of IFN induction were identified. In addition, the specific a.a. residues/motifs in the ED of NS1/I123 were delineated to be critical for enhancing IFN-β expression. It is intriguing that the NS1 protein may have evolved to mediate the enhancement of IFN-β expression (which would be considered to be detrimental to virus replication), yet retain the ability to completely suppress IFN-induced antiviral activity through function of its dsRBD (Results – II). Since NS1 of 2009 pH1N1 is of the swine influenza lineage of viruses[214], it would be interesting to study cytokine expression profiles in porcine cells infected with VVΔE3L/NS1/I123 and VVΔE3L/NS1/V123 to further characterize the functional significance of the I123V mutation in NS1 in different host species. It is possible that 2009 pHIN1 NS1/I123 may have played a dominant role in inducing cytokine expression that would promote inflammation, alveolar damage, and necrosis observed in the lung pathology of deceased patients infected with 2009 pH1N1[191], while aiding virus spread. It is interesting that these similar lung pathologies are seen with the highly virulent 1918 pH1N1 and H5N1[69]. In all, VVΔE3L was applied to study NS1 function in modulating IFN-β induction, in which several novel properties of the 2009 pH1N1 NS1/I123 protein were identified.

RESULTS

IV: NS1 proteins of different avian H5N1 isolates induce an isolate-specific pattern of human IFN-α expression

Introduction. In the previous section (Results- III, Fig.32), recombinant viruses expressing the NS1 proteins from different influenza viruses differentially induced IFN-β expression. In particular, the recombinant virus expressing the 2009 pH1N1 NS1/I123 mediated enhanced IFN-β expression. In this section, it was investigated whether or not the expression of IFN-α subtypes induced by the recombinant viruses expressing the various NS1 proteins is also different. Previous studies have shown that highly pathogenic H5N1 influenza viruses deregulated cytokine expression and caused cytokine storms in fatal human cases. Therefore, further investigation of the role of NS1 in the modulation of human antiviral cytokine expression may provide a better understanding of avian H5N1 pathogenesis in humans. The specific objective of this investigation is to examine the effects of NS1 proteins from influenza viruses of different isolate origins on the expression of IFN-α subtypes and to explore the related mechanisms.

IV.1. NS1 protein of the avian H5N1-Duck isolate upregulates the expression of several IFN-α subtypes in human cells

Real-time PCR array analysis demonstrated VVΔE3L/H5N1 NS1-duck infection resulted in a considerable increase in IFN-α2, IFN-α4, IFN-α5, and IFN-α8 expression in HeLa cells, in comparison to VVΔE3L/H5N1 NS1-human, VVΔE3L/2009 pH1N1 NS1/I123 and VVΔE3L infections (APPENDIX). It was further investigated whether the NS1 proteins from different subtype and isolate origins (including 1918 pH1N1 NS1 and 1968 pH3N2 NS1) mediate the expression of IFN-α subtypes in human cells. HeLa cells were mock infected or infected at a MOI of 5 with VVΔE3L/H5N1 NS1-duck, VVΔE3L/H5N1 NS1-human, VVΔE3L/2009 pH1N1 NS1/I123, VVΔE3L/1918 pH1N1 NS1, VVΔE3L/1968 pH3N2 NS1, and the controls VVΔE3L-REV and VVΔE3L. Total RNA was extracted at 12 HPI for the analysis of several IFN-α subtypes expression. The mRNA expression level of IFN-α subtypes, including IFN–α2, IFN–α4, IFN–α5, and IFN–α8, and IFN-β as a control were monitored by RT-PCR.

An abundance of endogeneous IFN-α2 and IFN–α8 expression were observed in the mock infection, and downregulated upon infection with VVΔE3L-REV and VVΔE3L (Fig.40). The expression of IFN-α2 and IFN–α8 was also suppressed following infection with VVΔE3L/H5N1 NS1-human and VVΔE3L/2009 pH1N1 NS1. In contrast, VVΔE3L/H5N1 NS1-duck, VVΔE3L/1918 pH1N1 NS1 and VVΔE3L/1968 pH3N2 NS1 infections did not suppress the expression of IFN-α2 and IFN–α8. In addition, IFN–α4 and IFN–α5 expression was also upregulated in VVΔE3L/H5N1 NS1-duck, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/1968 pH3N2 NS1 infections. As seen previously (Fig.33), IFN-β expression was strongly enhanced in the VVΔE3L/2009

pH1N1 NS1 infection. Overall, NS1 proteins of the different influenza subtypes and isolates mediate different patterns of IFN-α expression.

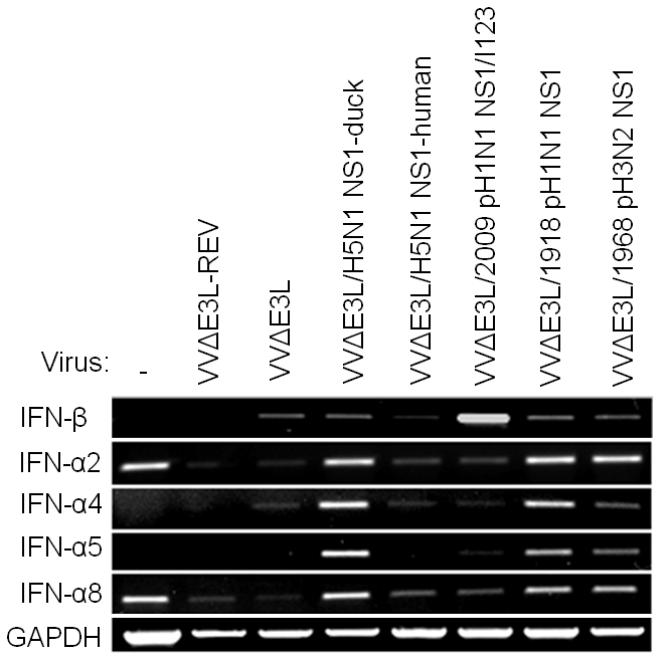


Fig.40 Recombinant viruses expressing NS1 proteins differentially regulate the expression of IFN-α subtypes. HeLa cells ($5x10^5$ cells/well) were infected at a MOI of 5 with recombinant viruses as indicated and collected 12 HPI for total RNA and RT-PCR analysis. Data shown are representative of three independent experiments.

IV.2. VV early gene products downregulate IFN-α2 and IFN-α8 expression

It is interesting to note that the endogeneous expression of IFN-α2 and IFN-α8 was downregulated following infection with VVΔE3L-REV, VVΔE3L, VVΔE3L/H5N1 NS1-human and VVΔE3L/2009 pH1N1 NS1, but was almost or largely unaffected in VVΔE3L/H5N1 NS1-duck, VVΔE3L/1918 pH1N1 NS1, and VVΔE3L/1968 pH3N2 NS1 infections (Fig.40).

Since VVΔE3L cannot replicate in HeLa cells, only VV early genes are expressed. In contrast, in VVΔE3L-REV infected HeLa cells, VV early, intermediate, and late genes

are expressed. Therefore, the downregulation of IFN-α2 and IFN-α8 expression may be due to VV early gene expression. Next, it was tested if inactivated VV could inhibit the expression of IFN-α2 and IFN-α8. The VVΔE3L-REV virus was inactivated by γ-irradiation according to the standard safety procedures. To verify the complete inactivation of VVΔE3L-REV, HeLa cells were infected with VVΔE3L-REV or with γ-irradiated VVΔE3L-REV at a MOI of 1 for 24 H and collected for Western blot detection of VV early proteins (E3 and D12), intermediate protein (G8), and late proteins (A17 and H3). The expression of VV early, intermediate, and late proteins are present in the VVΔE3L-REV infected and absent in γ-irradiated VVΔE3L-REV treated cells (Fig.41a). Additionally, the mRNA level of VV early genes (E3L and D12), intermediate gene (G8), and late gene (A17) were confirmed by RT-PCR (Fig.41b). The mRNAs of early, intermediate, and late genes were only detected in the VVΔE3L-REV infected, but not the γ-irradiated VVΔE3L-REV infected cells. Therefore, the γ-irradiation treatment inactivated the expression of viral genes.

HeLa cells were also infected at a MOI of 5 with VVΔE3L-REV, VVΔE3L, and the γ-irradiated VVΔE3L-REV and collected at 12 HPI for RT-PCR analysis. In contrast to the live VVΔE3L-REV, the endogeneous expression level of IFN-α2 and IFN-α8 is unaffected in HeLa cells treated with γ-irradiated VVΔE3L-REV at 24 HPI (Fig.41c) or 12 HPI (Fig.41d). Thus, it can be speculated that VV early gene products mediated the shutoff of IFN-α2 and IFN-α8 mRNA expression in HeLa cells.

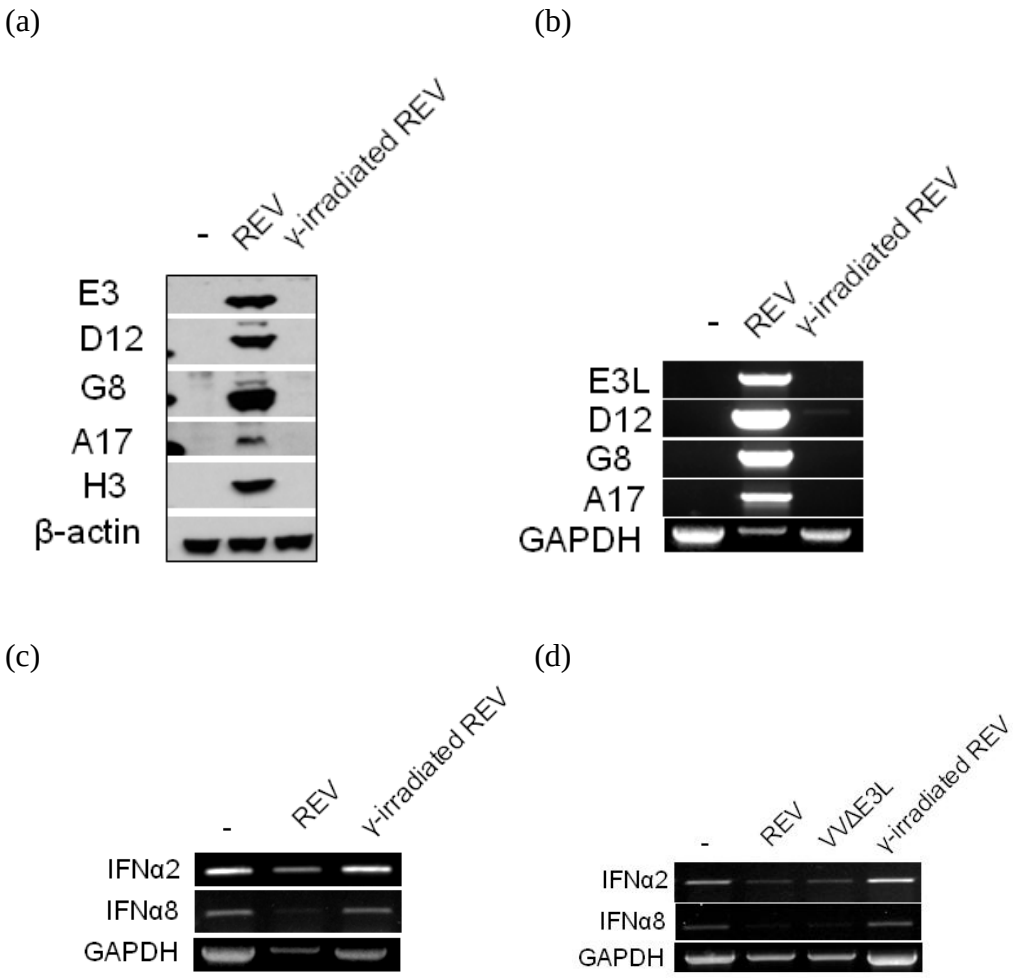


Fig.41 VV early gene products downregulate IFN-α2 and IFN-α8 gene expression in HeLa cells. VVΔE3L-REV virus (REV) was inactivated by γ-irradiation at 8 MRads. (a) Western blot confirmation of VV early proteins E3, D12, intermediate protein G8, and late proteins A17 and H3 in VVΔE3L-REV infection only. (b) RT-PCR confirmation of VV early genes E3L, D12, intermediate gene G8, and late gene A17 in VVΔE3L-REV infection only. (c) Endogeneous IFNα2 and IFNα8 gene expression is unaffected in HeLa cells treated with γ-irradiated VVΔE3L-REV at 24 HPI and (d) 12 HPI. Data shown are representative of three independent experiments.

IV.3. NS1 protein of avian H5N1 duck isolate directly induces the expression of the IFN-α subtypes in human cells

It is interesting that infection with VVΔE3L/H5N1 NS1-duck mediated an enhanced expression of IFN-α2, IFN-α4, IFN-α5, and IFN-α8 expression, in comparison to the VVΔE3L/H5N1 NS1-human infection (APPENDIX). To examine whether the NS1 of the avian H5N1 duck isolate can directly induce the expression of IFN-α subtypes independent of virus infection, expression plasmids pcDNA3.0/H5N1 NS1-duck and pcDNA3.0/H5N1 NS1-human were constructed. HeLa cells were transfected with 500 ng of empty vector control pcDNA3.0, pcDNA3.0/H5N1 NS1-duck and pcDNA3.0/H5N1 NS1-human constructs for 24 H. The expression of NS1 proteins was confirmed by Western blot (Fig.42a). RT-PCR analysis demonstrated that pcDNA3.0/H5N1 NS1-duck, but not pcDNA3.0/H5N1 NS1-human, induces the expression of IFN-α2, IFN-α4, IFN-α5, and IFN-α8 (Fig.42b). Interestingly, IFN-β expression is not detected in pcDNA3.0/H5N1 NS1-duck or pcDNA3.0/H5N1 NS1-human transfected cells (Fig.42b), but was induced following infection with VVΔE3L/H5N1 NS1-duck , VVΔE3L/H5N1 NS1-human and VVΔE3L (Fig.40). Therefore, the NS1 protein of H5N1-duck isolate can directly induce IFN-α2, IFN-α4, IFN-α5, and IFN-α8 expression.

(a) (b)

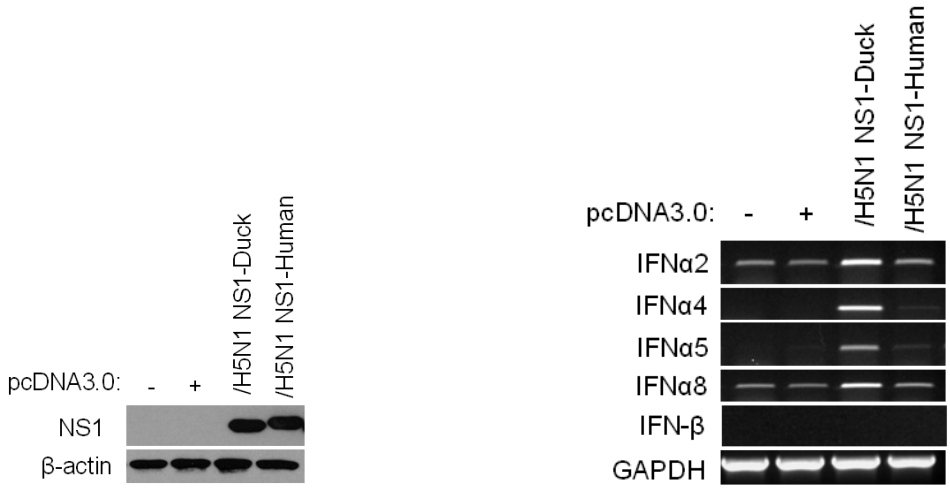
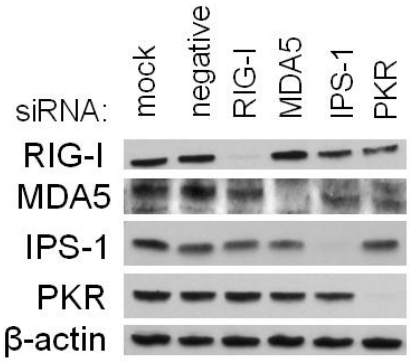


Fig.42 Avian H5N1 NS1-duck protein induces IFN-α gene expression independent of virus infection. HeLa cells (3.0 x10^5 cells/well) were mock transfected or transfected with 500 ng each of empty vector control (pcDNA3.0), and NS1 constructs (pcDNA3.0/H5N1 NS1-duck and pcDNA3.0/H5N1 NS1-human) for 24 H at 37°C. (a) Western blot confirmation of NS1 protein expression 24 HPT. (b) RT-PCR analysis of type I IFN expression following DNA transfections as indicated. Data shown are representative of three independent experiments.

IV.4. VVΔE3L/H5N1 NS1-duck induced expression of IFN-α species requires RIG-I and IPS-1

It was shown that VVΔE3L induces IFN-β expression through the RIG-I/MDA5/IPS-1 pathway in HeLa cells[170]. To investigate the cellular pathways involved in the induction of IFN-α subtypes following infection with VVΔE3L/H5N1 NS1-duck, HeLa cells were transfected with RIG-I, MDA5, IPS-1, PKR, and a negative control siRNA for 48 H, then infected at a MOI of 5 with VVΔE3L/H5N1 NS1-duck and VVΔE3L for 12 hours. The knockdown efficiency were confirmed by Western blot (Fig.43a). In Fig.43b, the knockdown of RIG-I and IPS-1 reduced the expression of all IFN-α subtypes following virus infection with VVΔE3L/H5N1 NS1-duck. MDA5 knockdown had a slightly greater effect on IFN-α2 expression than IFN-α4, IFN-α5, and IFN-α8 expression. Interestingly, PKR knockdown enhanced IFN-α2, IFN-α4, IFN-α5, and IFN-α8 expression in the VVΔE3L/H5N1 NS1-duck infected cells. As expected, VVΔE3L did not induce the expression of the IFN-α subtypes.

(a)



(b)

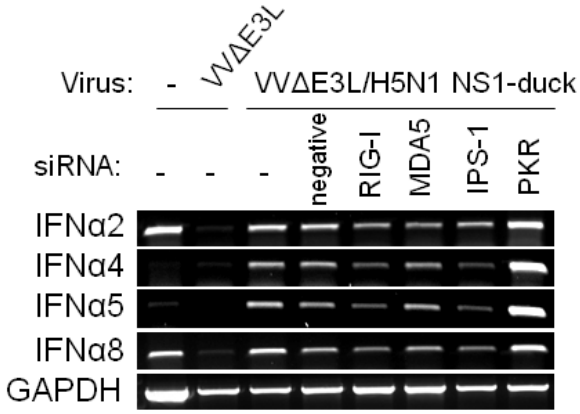


Fig.43 Avian H5N1 NS1-duck, expressed in VVΔE3L, mediates RIG-I and IPS-1 dependent signalling pathways to induce IFN-α expression in HeLa cells. (a) Western blot confirmation of gene expression knockdowns. (b) RT-PCR analysis of IFN-α expression induced by VVΔE3L/H5N1 NS1-duck with or without siRNA treatment. VVΔE3L infection was included as a control. Data shown are representative of three independent experiments.

IV.5. Discussion

The pathogenesis of highly pathogenic avian H5N1 infection is not fully understood. Since the 1997 outbreak, avian H5N1 continues to cause disease in humans with a high mortality rate (60%) through zoonotic transmissions[188]. Possible mechanisms of avian H5N1 pathogenesis include virus spread beyond the respiratory tract, sustained viral replication, higher viral loads, deregulated inflammatory cytokine and chemokine responses[235]. H5N1 infection in human monocyte-derived macrophages and respiratory epithelial cells induces proinflammatory cytokine and chemokine expression[28,34,41,188]. The deregulation of cytokine and chemokine responses in highly pathogenic avian H5N1 infection is proposed to be the major mechanism of pathogenesis in fatal human cases. Avian H5N1 NS1 was shown to inhibit interferon (IFN) induction[138]. However, NS1-mediated modulation of IFN responses can be strain specific.

In this study, it was found that NS1 of different influenza isolates mediate different patterns of IFN-α expression. Specifically, it was found that the NS1 proteins of an avian isolate of H5N1, 1918 pH1N1 and 1968 pH3N2 upregulated the expression of IFN-α4, and IFN-α5, whereas NS1 of 2009 pH1N1 or of a human isolate of H5N1 did not (Fig.40). These results indicate the activity of influenza virus NS1 proteins to modulate human IFN-α subtype expression is subtype and/or isolate dependent.

The downregulation of IFN-α2 and IFN-α8 expression following infection with VVΔE3L-REV, VVΔE3L, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1 excludes the possibility that this effect is due to the activity of E3, since E3 is not expressed in VVΔE3L, VVΔE3L/H5N1 NS1-human, and VVΔE3L/2009 pH1N1 NS1. It can also be excluded that this observation is due to intermediate and late gene products, since only early genes are expressed during VVΔE3L infections in HeLa cells. Thus, it

can be speculated that VV early gene products mediate the shutoff of IFN-α2 and IFN-α8 expression. It is interesting that the expression of H5N1 NS1-duck, 1918 pH1N1 NS1, and 1968 pH3N2 NS1 in VVΔE3L do not mediate the shutoff of IFN-α2 and IFN-α8 expression. It can be speculated that NS1 proteins of H5N1-duck, 1918 pH1N1 and 1968 pH3N2 may interfere with the undefined VV early gene products, thus rendering those early gene products unable to downregulate IFN-α2 and IFN-α8 expression. Alternatively, it is possible that the NS1 proteins of H5N1-duck, 1918 pH1N1 and 1968 pH3N2 may be mediating other signalling pathways to enhance IFN-α subtypes expression, in comparison to the NS1 proteins of H5N1-human and 2009 pH1N1. IRF-7 is essential for efficient IFN-α gene transcription[92]. Modification of IRF-7 by small ubiquitin-related modifiers (SUMOs) has been shown to negatively regulate IRF-7 mediated IFN transcription. An IRF-7 specific SUMO E3 ligase, tripartite motif-containing protein 28 (TRIM28) was recently identified to directly interact with and increase SUMOylation of IRF-7 to negatively regulate its activity[139]. It is possible that the NS1 proteins of H5N1-duck, 1918 pH1N1 and 1968 pH3N2 may be enhancing IRF-7 mediated transcriptional activity by directly or indirectly inhibiting the functions of IRF-7 repressors, such as TRIM28.

Variability in the ED of NS1 is associated with virus virulence[82,119]. Among the H5N1 NS1-duck and H5N1 NS1-human proteins, the major difference is a five a.a. deletion between residues 80-84 in H5N1 NS1-Duck. While this five a.a. deletion in H5N1 NS1 has been shown to confer resistance of the virus to TNFα treatment in avian cells[134], it is less known what effect the a.a 80-84 deletion has on mediating cytokine expression. It is possible that this a.a 80-84 deletion may be critical for the upregulation of IFN-α expression exhibited by H5N1 NS1-Duck, or vice versa, a.a 80-84 are important

for the suppression of IFN-α expression by H5N1 NS1-Human. A PDZ binding motif (ESEV or EPEV at the C-termini of NS1, residues 226-230) is a virulence determinant of H5N1[275]. In this study, the PDZ binding motif can be excluded as a major contributor to the difference in IFN-α expression patterns induced by H5N1 NS1-duck and H5N1 NS1-human proteins, as both proteins contain the PDZ binding motif at their C-termini.

It is interesting that the NS1 of the avian H5N1 duck isolate alone was sufficient to induce IFN-α, but not IFN-β, expression (Fig.43). Thus, it is likely that the signalling pathways involving the induction of IFN-α and IFN-β could be different, although both require RIG-I. The direct interaction between the CARD domains of RIG-I and IPS-1 activates subsequent signals to induce IFN expression. The mechanism by which NS1 of the H5N1 duck isolate directly activates expression of the IFN-α subtypes may involve the direct interaction between the NS1 protein and RIG-I and/or RIG-I dependent downstream signal molecules.

PKR was shown to mediate VVΔE3L induced inhibin beta A (INHBA) and TNFα expression[171] and contributed to IFN-β expression induced by VVΔE3L infection (Fig.39b) and the enhanced IFN-β expression by VVΔE3L/2009 pH1N1 NS1/I123 infection (Fig.39c). It is worth pointing out that PKR knockdown enhanced the IFN-α expression induced by VVΔE3L/H5N1 NS1-Duck infection. Thus, PKR may play an inhibitory role in the induction of these IFN-α subtypes. Further studies, such as investigating the role of PKR in the induction of IFN-α expression by the recombinant viruses expressing other NS1 proteins, will help to delineate the signalling pathways involved in H5N1 NS1-Duck induced IFN-α expression. Nonetheless, the data described in this section suggest that the modulation of antiviral cytokine expression by influenza

virus H5N1 NS1 proteins can be subtype/isolate specific, which may play a role in the virus pathogenesis.

CHAPTER IV: GENERAL DISCUSSION

The IFN system is critical to control viral infections in vertebrate hosts. The two arms of the IFN system include IFN induction and an IFN-induced antiviral state. In virus-infected cells, host PRRs rapidly detect viral PAMPs to induce the expression and production of type I IFNs (mainly IFN-α/β). In humans, type I IFNs (IFN-α/β) can transcriptionally regulate the expression of more than 300 interferon stimulated genes (ISGs), some of which function to induce an antiviral state. Classical IFN-induced antiviral proteins such as PKR, OAS/RNaseL, and MxA, have been identified to exert antiviral activities against a broad spectrum of viruses[1,42,142,212,245].

The outcome of a viral infection is the counterbalance between host defence responses and virus evasion strategies. In the tug-of-war between viruses and IFN responses, viruses have evolved countermeasures to target and suppress all levels of the IFN system. Viral encoded proteins have been shown to inhibit PRR signalling to suppress IFN induction, interfere with IFN-induced signalling, and block IFN-induced antiviral activities[77,262]. DsRNA binding proteins such as VV E3, influenza NS1, and Ebola VP35 prevent the activation of dsRNA-dependent pathways to inhibit IFN induction[12,91]. Influenza NS1 and HCV core proteins upregulate the expression of cellular SOCS proteins to negatively regulate STAT mediated signalling[20,105]. HCV E2 inhibits PKR activation in HCV infections[250]. Although several viral IFN antagonists have been identifed, the mechanistic pathways by which these viral proteins obstruct IFN induction and IFN-induced antiviral pathways are not fully understood[77]. Studies of evasion strategies that are virus-specific is valuable to better understand the mechanisms of pathogenesis and host factors involved in the immune response to a particular

pathogen. Such knowledge is important for the design of better intervention strategies to control viral diseases.

There are challenges that can limit studies of viral IFN modulators in evading IFN responses. First, a suitable cell culture system is often limited for the detailed characterization of the mechanisms involved in the inhibition of the IFN responses, particularly of viruses with different tissue tropisms, such as influenza A viruses[123]. Second, it can be technically challenging and laborious to use reverse genetics techniques to manipulate the viral genomes of an RNA virus, such as influenza, because the manipulation of one viral gene segment that encodes for more than one protein may alter the resulting gene products[202]. Third, it is difficult to exclusively examine the IFN-antagonistic function of a pleiotropic viral protein when it is expressed in its viral backbone.

VV is well known for its ability to infect a broad range of host cell cultures and animals[163]. The VV genome can be easily manipulated to delete nonessential viral genes or insert exogeneous genes to produce stable recombinant viruses by homologous recombination[25,65,161]. VV encodes a plethora of immunomodulators[165,189]. In particular, the E3 protein, encoded by the early gene E3L, is a well-known IFN antagonist. E3, a dsRNA binding protein and VV host range determinant, interferes with both arms of the IFN response, IFN induction and IFN-induced antiviral activities. The activity of its C-terminal dsRNA binding inhibits the activation of dsRNA-dependent IFN induction pathways to thwart IFN and proinflammatory cytokine expression[189,234]. E3 also inhibits the activation of IFN-inducible antiviral and dsRNA binding proteins, PKR and OAS. Deletion of the E3L gene produces a mutant (VVΔE3L) that is sensitive to IFN treatment

and is replication defective in most human cell lines[15,16]. Recombinant viruses have been generated based on VVΔE3L, to express other viral dsRNA binding proteins in replacement of E3. Restoration of E3 function in antagonizing IFN-induced antiviral activity has been shown in this manner[13]. Thus, it is a plausible approach to use VVΔE3L as a vector to investigate other immunomodulatory proteins for their functions and related cellular signalling pathways in the modulation of IFN induction and IFN-induced antiviral activities.

However, the precise mechanistic pathways which are modulated by E3 to inhibit human IFN-induced antiviral activity and inhibit cytokine expression have been less defined. Since VVΔE3L replication is restricted in most human cell lines, the cell lines available for the proper examination of E3 function in the inhibition of human IFN-induced antiviral responses were very limited. Thus, the full potential of E3 mediating the inhibition of human IFN-induced antiviral activities and the related antiviral ISGs were not well defined. Therefore, in order to apply VVΔE3L to examine other viral IFN antagonists modulating human IFN responses, it is essential to characterize the antagonizing activity of E3 on IFN-induced antiviral activity and the related signalling pathways involved in human cells.

To address this gap, in this study, a human cell line, hepatocellular carcinoma (Huh7), was first identified to efficiently support VVΔE3L replication and is responsive to both human type I and type II IFN treatment (Results - I). These two characteristics make Huh7 cells a suitable cell system to test the activity of VV E3 in antagonizing human IFN-induced antiviral activities. It was found that VV E3 is a potent inhibitor of both human type I and type II IFN-induced antiviral activities. Mechanistically, it was

found that PKR mediated the sensitivity of VVΔE3L to type I and type II IFN treatment. Therefore, the VVΔE3L system can be used to study other viral proteins modulating IFN-induced antiviral activities in human cells. In particular, VVΔE3L is a useful model system to study the IFN antagonistic activities of other viral proteins that modulate the PKR pathway.

To this end, VVΔE3L was applied to perform studies of influenza NS1 function in the modulation of human IFN-induced antiviral activities. Similar to E3, NS1 is known to inhibit the activation and functions of IFN-induced antiviral proteins, such as PKR and OAS/RNaseL. In the context of influenza infection, NS1 is a multifunctional protein and promotes viral protein synthesis and viral replication. NS1 functions can be subtype and/or isolate dependent. While current influenza virus reverse genetics technologies have helped to elucidate NS1-mediated modulation of innate immune responses, it remains difficult to delineate the particular functional role of NS1 in suppressing IFN responses due to its additional role in influenza virus replication.

In this study, it was proposed that VVΔE3L can be used as an alternative virus model system to examine the function of NS1 in the modulation of IFN responses in human cells. There are two main advantages to using VVΔE3L to perform such studies on NS1 function. First, VVΔE3L was characterized in the modulation of IFN-induced antiviral activity in human cells, in that VVΔE3L is highly sensitive to type I and type II IFN-induced PKR mediated antiviral activity[9] (Results – I). Second, the VVΔE3L platform can facilitate studies of NS1 independently of other influenza proteins which may influence NS1 function in modulating IFN mediated responses. Of importance,

VVΔE3L can be used to comparatively study the functions of different NS1 proteins from different influenza strains in modulating IFN mediated pathways in the same human cells.

Using VVΔE3L as the expression vector system, NS1 proteins of highly pathogenic H5N1 isolates, and pandemic influenza isolates, including 2009 pH1N1, 1918 pH1N1, and 1968 pH3N2 were compared in their abilities to inhibit IFN-induced antiviral activity in Huh7 cells (Results – II). It was found that different NS1 proteins differentially mediated human IFN-induced antiviral activities. Specifically, NS1 of avian H5N1 virus isolates and the swine-origin 2009 pH1N1, but not NS1 of the classic pandemics 1918 pH1N1 and 1968 pH3N2 isolates, were able to fully inhibit IFN-induced antiviral activity. In addition, it was found that PKR mediated the IFN sensitivities of recombinant viruses expressing 1918 pH1N1 NS1 and 1968 pH3N2 NS1. While NS1 of the avian H5N1 isolates and 2009 pH1N1 were able to fully inhibit IFN-induced antiviral activity, unlike VV E3, none of the NS1 proteins fully inhibited PKR phosphorylation. The precise mechanisms underlying NS1-mediated IFN resistance in IFN-treated Huh7 cells remain undefined, although, antiviral factors such as RNaseL and MxA can be excluded. RNaseL was not expressed in this Huh7 cell system (Fig.16), and MxA did not play a major role in mediating VVΔE3L sensitivity to type I or type II IFN treatment (Fig.17). This finding suggests NS1 may also modulate alternative ISGs, in addition to PKR, in this Huh7 cell system.

VVΔE3L was further applied to investigate the viral mechanism in relation to which functional domain of NS1, the dsRBD or the ED, was responsible for NS1-mediated resistance to IFN-induced antiviral activity. In the context of reverse genetics techniques to construct influenza viruses, alterations in either the dsRBD or ED of the NS1 gene may result in attenuated virus replication in culture and an increase in cytokine

expression, such as IFN production[81,174]. Therefore, it is technically difficult to investigate the role of each NS1 functional domain in inhibiting IFN-induced antiviral activity. The application of VVΔE3L can overcome this limitation, in that recombinant viruses were generated to express truncated mutants of NS1, including VVΔE3L/NS1ΔC (to express only the dsRBD) and VVΔE3L/NS1ΔC (to express only the ED). It was found that the predominant viral determinant of IFN resistance is the N-terminal dsRBD of NS1, while the effector domain (ED) may also play a minor role in NS1-mediated resistance to IFN-induced antiviral activity (Fig.26).

It is interesting that the NS1 proteins differentially inhibited PKR phosphorylation (Fig.23). While the dsRBD of NS1 primarily mediated IFN resistance, it is important to note that the dsRBD (a.a.1-73) is highly conserved among all NS1 proteins included in this analysis (Fig.19). In particular, specific a.a residues that have been reported to be critical for the dsRNA binding activity of influenza NS1 include arginine 38 (R38), lysine 41 (K41), arginine 35 (R35), arginine (R46), serine 42 (S42), and threonine 49 (T49)[33], which are conserved in all NS1 proteins used in this study (Fig.19). It is interesting that while all the NS1 proteins contain the residues that are reported to be critical for dsRNA binding, H5N1 and 2009 pH1N1 NS1 proteins were able to bind dsRNA more efficiently (Fig.24) and suppress PKR phosphorylation to a greater degree than 1918 pH1N1 and 1968 pH3N2 NS1 proteins (Fig.23). In the context of influenza infections, the dsRNA binding activity of NS1 is not the main mechanism by which NS1 mediates the suppression of PKR activity[159]. Rather, it is an interaction between a.a residues 123-127 in NS1 with PKR that suppresses PKR autophosphorylation activity[159]. It is important to note that there are no major differences between in a.a.123-127 among the IFN resistant NS1 proteins (H5N1-duck, H5N1-human, 2009 pH1N1) versus the IFN sensitive NS1

proteins (1918 pH1N1 and 1968 pH3N2). Specifically, all NS1 proteins have I123, M124, and K126 (see Fig.19). There are only differences in a.a 125 and a.a 127. However, these differences are not conserved among the IFN resistant or IFN sensitive NS1 proteins (2009 pH1N1 NS1 has E125, whereas NS1 of H5N1-duck, H5N1-human, 1918 pH1N1, and 1968 pH3N2 have D125; H5N1 NS1-duck has A127, H5N1 NS1-human has D127, whereas 2009 pH1N1 NS1, 1918 pH1N1 NS1, and 1968 pH3N2 NS1 all have N127). Moreover, there is variability in the C-terminal tail length of the NS1 proteins (see Fig.19). Collectively, these findings suggest that there is no definitive residue in NS1 that accounts for its ability to suppress PKR phosphorylation, in that more than one moiety may contribute to this effect. It is possible that structural differences in the ED of NS1 may contribute to differentially inhibiting PKR phosphorylation. In support of this, mutations in the ED of the 2009 pH1N1 NS1/I123 protein affected its ability to inhibit PKR phosphorylation (Fig.31). In all, it was identified that NS1-mediated inhibition of PKR phosphorylation is influenza isolate-dependent and may involve several moieties in the NS1 protein.

E3 is known to suppress IFN induction[189,234]. However, the mechanistic pathways of E3 inhibition of IFN-β expression were less defined. In our lab, using VVΔE3L, it was delineated that E3 suppresses IFN-β expression through RIG-I and IPS-1 dependent-signalling and that dsRNA species are the major PAMPs associated with VVΔE3L induced IFN-β expression[172]. It was further demonstrated that RNA species associated with VV infection activate RIG-I and MDA5-dependent pathways[170]. Therefore, VVΔE3L is also a well-characterized platform to study the functions of other viral proteins to impede IFN expression through RIG-I, MDA5, and IPS-1 pathways.

To this end, VVΔE3L was applied to study the function of influenza NS1 in the modulation of IFN-β expression (Results – III). A well-known function of NS1 is its capacity to inhibit IFN expression in influenza infections, which can also be subtype and/or strain specific[82]. Additionally, in influenza virus infections, the 3pRNA and panhandle structures of the viral RNA are the major IFN inducers through RIG-I signalling[55,201]. Thus, VVΔE3L and influenza share in common similar signalling pathway to induce IFN expression.

In this study, it was found that NS1 proteins from different virus subtypes (H5N1, 2009 pH1N1, 1918 pH1N1, 1968 pH3N2) exhibited different potencies at inhibiting IFN-β expression (Fig.32). Interestingly, it was found that the 2009 pH1N1 NS1 protein mediated the enhancement of IFN-β expression in HeLa and A549 cells, in comparison to VVΔE3L infection (Fig.32-Fig.34). Further analysis into the mechanisms exerted by 2009 pH1N1 NS1 in mediating the enhancement of IFN-β expression was performed. First, it was found that the ED of 2009 pH1N1 NS1 was responsible for mediating the enhancement of IFN-β expression, while the dsRBD exhibited an inhibitory role (Fig.36). Second, mutation analysis in the ED of 2009 pH1N1 NS1 led to the identification that 2009 pH1N1 NS1 enhances IFN-β expression in an isolate-dependent manner. Specifically, a natural mutation occurred in NS1 at position 123 from isoleucine (NS1/I123), predominantly present in virus isolates collected pre-epidemic, to valine (NS1/V123), predominantly present in virus isolates collected during the middle and late phases of the pandemic[184]. Here, it was found that isoleucine 123 in NS1 of pre-epidemic isolates, as well as the C-terminal truncation of 2009 pH1N1 NS1 are the critical viral determinants of the enhanced IFN-β expression. Third, the related cellular signalling pathways were delineated, in that 2009 pH1N1 NS1/123 requires RIG-I, MDA5, IPS-1,

STING, and PKR dependent pathways to mediate the enhanced IFN-β expression. The ability of NS1 to regulate host pre-mRNA processing is largely dependent on the interaction between its ED with CPSF30[175]. It is interesting to note that the residues reported in NS1 to be critical for stabilizing CPSF30 interactions, including phenylalanine 103 (F103) and methionine (106), are present in NS1/I123. However, in agreement with the previous report of Hale and others[75], NS1/I123 did not bind to CPSF30 efficiently in this analysis (Fig.38). In addition, in this study, it was identified that valine 123 and an 11 a.a. extension to the C-terminal end of NS1/I123 weakened its affinity for CPSF30 and yet abolished its ability to enhance IFN-β expression (Fig.37). Collectively, several novel properties unique to the function of 2009 pH1N1 NS1 in regulating human IFN-β expression were delineated using VVΔE3L as the expression vector.

It was interesting that the NS1 proteins of different influenza viruses mediated different patterns of IFN-β expression (Results – III). VVΔE3L recombinant viruses expressing different NS1 proteins were investigated whether NS1 of different influenza viruses regulate different patterns of type I IFN-α expression (Results – IV). Indeed, it was found that NS1 mediates different patterns of type I IFN-α expression in an isolate-dependent manner (Fig.40). Interestingly, it was found that NS1 proteins of the same subtype (H5N1), but isolated from different species (avian versus human isolates) induced different IFN-α expression profiles (Fig.40, Fig.42). Specifically, it was found that H5N1 NS1 isolated from an avian species (duck) directly induces the expression of IFN-α2, IFN-α4, IFN-α5 and IFN-α8 in human HeLa cells (Fig.42). Taken together, it was revealed that avian H5N1 NS1 induces type I IFN-α expression in a species-isolate dependent manner.

In conclusion, VVΔE3L was generated and characterized as a platform to facilitate studies of viral modulators of IFN induction and IFN-induced antiviral activities in human cells. The work presented here illustrates a novel approach to apply VVΔE3L to study the functions of influenza NS1 in the modulation of both arms of the IFN system, IFN induction and IFN-induced antiviral activities. The application of VVΔE3L as an expression vector system to study the IFN-antagonizing function of influenza NS1 in these studies revealed novel insights into influenza subtype and isolate-dependent differences in NS1-mediated modulation of human IFN-induced antiviral activities and IFN induction (Table 6). This knowledge contributes to the current understanding of NS1-mediated mechanisms of pathogenesis in human hosts. Taken together, VVΔE3L is an excellent system to study virus evasion mechanisms of IFN induction and IFN-induced antiviral activities.

Table 6 Summary of different influenza NS1 activities in the modulation of the IFN system in human cells. The effect of NS1 expression in VVΔE3L on IFN induction and IFN-induced antiviral activity is summarized.

Influenza A virus strain of NS1 studied	IFN induction	IFN-induced antiviral activity	Amino acid or NS1 domain	Reference
H5N1 – Duck isolate A/Chicken/Yunnan/493/05		*Resistant to type I and type II IFN treatment in Huh7 cells*		*Results - II*
H5N1 – Human isolate A/Hong Kong/486/97		*Resistant to type I and type II IFN treatment in Huh7 cells*		*Results - II*
2009 pH1N1 (A/California/04/09)		*Resistant to type I and type II IFN treatment in Huh7 cells*	*dsRBD*	*Results - II*
1918 pH1N1 A/Brevig_Mission/1/18(H1N1)		*Sensitive to type I and type II IFN treatment and PKR activity in Huh7 cells*		*Results - II*
1968 pH3N2 A/Beijing/1/68(H3N2)		*Sensitive to type I and type II IFN treatment and PKR activity in Huh7 cells*		*Results - II*
2009 pH1N1 (A/California/04/09)	*Enhances IFN-β expression in HeLa and A549 cells*		*C-terminal truncation at 219 a.a. and I123*	*Results - III*
2009 pH1N1 (A/California/04/09)	*Weakens binding to CPSF30 without enhancing IFN expression*		*V123 and 11 a.a. extension to C-terminus*	*Results - III*
H5N1 – Duck isolate A/Chicken/Yunnan/493/05	*Enhanced IFN-α2, IFN-α8 and induces IFN-α4, IFN-α5 expression in HeLa cells*			*Results - IV*
H5N1 – Human isolate A/Hong Kong/486/97	*Downregulated IFN-α2, IFN-α8 expression in HeLa cells*			*Results - IV*
2009 pH1N1 (A/California/04/09)	*Downregulated IFN-α2, IFN-α8 expression in HeLa cells*			*Results - IV*

1918 pH1N1 A/Brevig_Mission/1/18(H1N1)	*Upregulated IFN-α4, IFN-α5 expression in HeLa cells*			*Results - IV*

Future Directions

In all, several novel viral and cellular determinants of influenza NS1 mediated modulation of the IFN system were delineated using VVΔE3L as a platform in this project. In continuation of these studies, other studies can be further developed. First, different IFN-α species mediated different potencies at inhibiting VVΔE3L replication (Results - I). It would be interesting to further characterize the inhibition efficacy of different IFN-α subtypes on virus replication and further define the signalling pathways that are activated by different IFN-α species. Second, investigation into the mechanisms by which 2009 pH1N1 NS1/I123 mediates enhanced IFN-β expression should be undertaken, such as addressing which stage of the RIG-I, MDA5, IPS-1, STING, and PKR pathways NS1/I123 interacts with to enhance IFN-β signalling. Third, further work is required to identify the precise cellular signalling mechanisms involved in the induction of IFN-α4 and IFN-α5 expression directly induced by the H5N1-duck NS1 protein and whether there are cell-type specificities of this phenotype. Lastly, VVΔE3L demonstrates the potential to study different viral proteins with the potential to modulate cellular signalling pathways related to either arm of the IFN system, IFN induction or IFN-induced-antiviral activity, and/or both arms. Thus, it is feasible to apply VVΔE3L to comparatively study isolate dependent differences in the functions of other viral IFN antagonists in modulating the IFN system.

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APPENDIX

Cytokine	Fold change in expression of HeLa cells infected with virus:				
	VVΔE3L-REV	**VVΔE3L**	**VVΔE3L/H5N1 NS1-duck**	**VVΔE3L/H5N1 NS1-human**	**VVΔE3L/2009 pH1N1 NS1**
IFNA1	5.6	21.7	30.1	8.6	8.4
IFNA2	33.6	121.6	523.0	123.5	38.3
IFNA4	41.3	24.7	426.8	19.7	16.6
IFNA5	8.7	168.8	1127.6	145.5	347.5
IFNA8	5.3	3.6	41.3	9.6	15.0
IFNB1	**80.3**	**7900.8**	**6400.4**	**5617.7**	**30729.6**
IFNG	8.7	21.2	24.2	11.3	16.6
IFNK	6.5	6.8	1.1	3.9	34.5
IL10	16.5	19.6	20.6	9.9	141.1
IL11	32.8	61.3	16.6	42.9	117.2
IL12A	56.8	19.3	17.8	20.8	62.2
IL12B	6.3	7.9	17.8	8.4	52.8
IL13	20.2	10.5	56.2	5.6	271.2
IL15	3.5	8.0	7.1	4.9	8.4
IL16	2.7	6.5	14.8	32.9	89.3
IL17A	8.7	21.2	24.2	11.3	16.6
IL17B	1.1	0.9	10.6	7.3	16.6
IL17C	14.0	115.7	129.4	18.4	20.9
IL25	8.7	21.2	24.2	11.3	16.6
IL18	2.4	4.3	5.2	3.3	5.2
IL19	6.0	57.0	72.3	25.2	20.9
IL1A	5.6	20.7	27.1	14.1	59.4
IL1B	5.7	53.2	71.3	56.3	3.2
IL1F10	8.7	21.2	24.2	11.3	16.6
IL1F5	8.7	21.2	24.2	11.3	16.6
IL1F6	8.7	21.2	24.2	28.0	16.6
IL1F7	9.5	8.3	29.7	14.2	14.4
IL1F8	12.0	21.2	24.2	11.3	28.4
IL1F9	10.7	21.2	27.7	16.8	27.8
IL2	43.3	21.2	43.2	11.3	48.6
IL20	25.7	86.6	221.4	59.5	142.5
IL21	8.7	28.4	24.2	11.3	53.3
IL22	8.7	21.2	24.2	11.3	16.6
IL24	5.5	37.2	41.1	14.7	39.4
IL3	4.9	11.8	38.0	6.3	16.6
IL4	8.7	21.2	38.4	31.7	16.6
IL5	24.3	31.3	20.5	9.6	25.5
IL6	70.2	1479.0	1197.2	920.9	3911.3
INHBA	4.5	119.5	77.1	33.0	1105.6
TNF	11.2	149.3	193.2	128.6	5159.9
GAPDH	1.1	0.7	0.9	1.1	1.1