

**Comparison of host innate immune response
of human and porcine airway epithelial cells
and anti-influenza exploitation**

By

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To my parents,

Without their unconditional love and endless support, I would have
never reached this far to fulfil my dream and become who I am
today.

Abstract

Influenza A virus (IAV) infection is a major global disease that continues to threaten wildlife, livestock production and human health. In humans, IAV infection can be severe leading to the complication of host hyperacute inflammatory response, also known as 'cytokine storm', with ensuing high mortality rate. Pigs infected with IAVs, however, often display no or only mild symptoms. Viral RNA is principally recognised by pattern-recognition receptors retinoic inducible gene-1 (RIG-I) and Toll-like receptor 7/8 (TLR 7/8), each of which signals to activate two key transcription factors, nuclear factor kappa B (NF- κ B) and interferon regulatory factor 3 (IRF3), for the induction of pro-inflammatory cytokines and type I interferons (IFNs), respectively. To examine possible host-species differences in NF- κ B function, we compared the antiviral role of NF- κ B between pig and human primary and immortalised respiratory epithelial cells infected with IAV. Activation of NF- κ B in pig cells significantly inhibited IAV replication and induced strong type I IFN associated gene response. In human cells, however, activation of NF- κ B had limited effects on IFN associated response and IAV replication. Microarray RNA profiling comparison indicated that basal transcriptional activity of NF- κ B associated genes in primary normal human bronchial (NHBE) cells was more active than corresponding genes in primary pig tracheal epithelial cells (PTECs). In particular, *TNFRSF1A*, a key upstream gene of NF- κ B signalling, was found to be highly expressed in NHBE cells. Knockdown of *TNFRSF1A* in NHBE cells blocked IAV replication, but in PTECs had no effect on virus output. Overexpression of *TNFRSF1A* in PTECs increased IAV progeny production, but in NHBE cells had limited effect on virus output.

Aspirin, also known as acetylsalicylic acid (ASA), has been extensively used as an antiviral drug against influenza virus infection during the 1918 H1N1 virus pandemic. The antiviral mechanism of ASA is believed to be through the inhibition of NF- κ B activity in several human cell lines. We, however, found that ASA in PTECs increased NF- κ B activity to block the transcription of IAV; whereas ASA in NHBE cells reduced virus-induced apoptosis to block IAV replication at a post-translational level. Thus, ASA inhibits IAV replication by different mechanisms in PTECs and NHBE cells. Taken together, host species basal activity of NF- κ B associated genes appears to be a key determinant of antiviral response to infection, such that the relatively low basal activity of NF- κ B associated genes (as seen in pig cells) confers a strong antiviral response to NF- κ B activation, whereas high basal NF- κ B associated gene activity (as seen in human cells) is much less responsive to activated NF- κ B.

The management of IAV infection with antivirals is limited by efficacy and development of virus resistance. In this study, ledene, a naturally occurring sesquiterpene from the Australian tea tree, was found to block IAV replication efficiently. Ledene inhibited IAV replication without affecting viral mRNA and protein expression, indicating that the inhibition occurs post-translationally. The selective index (SI), an *in vitro* indicator of efficacy and safety, of ledene was 791, which is higher than that of oseltamivir (SI=10), suggesting that ledene could potentially be more effective than oseltamivir as an antiviral.

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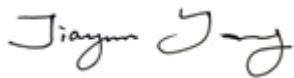
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Author's declaration

I declare that the work in this thesis was carried out in accordance with the Regulations of the University of Nottingham. The work is original, except where indicated by reference in the text, and no part of the thesis has been submitted for any other academic award. Any views expressed in the thesis are those of the author.

SIGNED: 

DATE: 27/09/2021

List of abbreviations

%	=	Percentage
°C	=	Degree Celsius
aa	=	Amino acid
AECs	=	Alveolar epithelial cells
ARD	=	Ankyrin repeat domain
ASA	=	Acetylsalicylic acid
BCP	=	Beta-caryophyllene
bp	=	Base pair
BSA	=	Bovine serum albumin
CAE	=	Cryptoric acid E
CAP	=	7-methylguanosine (m7G)
CC ₅₀	=	Median cytotoxic concentration
cFLAR	=	Caspase-8 and FADD like apoptosis regulator
cIAP1	=	Cellular inhibitor of apoptosis protein 1
cIAP2	=	Cellular inhibitor of apoptosis protein 2
COX	=	Cyclo-oxygenases
cRNA	=	Complementary RNA
DAB	=	3,3'-diaminobenzidine
DBD	=	DNA binding domain
DD	=	Death domain
DEPC	=	Diethylpyrocarbonate
DISC	=	Death-inducing signalling complex
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxyribonucleotide triphosphates
ds-RN	=	Double stranded-RNA

DTT	=	Dithiothreitol
EC	=	Extracellular domain
EID ₅₀	=	Median egg infectious dose
ER	=	Endoplasmic reticulum
FADD	=	Fas-associated death domain
FDR	=	False discovery rate
Ffu	=	Focus forming units
h	=	Hour
HA	=	Haemagglutinin
HAPIV	=	Highly pathogenic avian influenza virus
HCV	=	Hepatitis C virus
HEK 293	=	Human embryonic kidney 293
hpi	=	Hour post infection
HRV	=	Human rhinoviruses
HSV	=	Herpes simplex virus
HSV-1	=	Herpes simplex virus type 1
IAV	=	Influenza A virus
IC	=	Intracellular part
IC ₅₀	=	Median infective dose
ICTV	=	International Committee on Taxonomy of Viruses
IFN	=	Interferon
IgG	=	Immunoglobulin G
IKK	=	I κ B kinase
IKK ϵ	=	Inhibitor of κ B kinase ϵ
IL	=	Interleukin
iNOS	=	Nitric oxide synthases
IPA	=	Ingenuity pathway analysis
IRF3	=	Interferon regulatory factor 3
I κ Bs	=	Inhibitors of NF- κ B

kb	=	Kilobase
kDa	=	Kilodalton
LDS	=	Lithium dodecyl sulfate
LUBAC	=	Linear ubiquitin chain assembly complex
M	=	Matrix protein
mA	=	Milliamps
MAPK	=	Mitogen-activated protein kinase
MDCK	=	Madin-Darby canine kidney
MEF	=	Mouse embryonic fibroblast
mg	=	Milligram
min	=	Minute
mL	=	Millilitre
mm	=	Millimetre
mM	=	Millimolar
MOI	=	Multiplicity of infection
mRNA	=	Messenger RNA
NA	=	Neuraminidase
NEMO	=	NF-kappa-B essential modulator
NEP	=	Nuclear export protein
NeuAc	=	N-Acetylneuraminic acid
NF-κB	=	Nuclear factor kappa B
ng	=	Nanogram
NLS	=	Nuclear localization signals
nm	=	Nanometre
nM	=	Nanomolar
NP	=	Nucleoprotein
NS1	=	Non-structural protein 1
NS2	=	Non-structural protein 2
NSAID	=	Non-steroidal anti-inflammatory drug

P/S	=	Penicillin and streptomycin
PA	=	Polymerase acidic protein
PA	=	Patchouli alcohol
PAMP	=	Pathogen-associated molecular patterns
PB1	=	Basic polymerase protein 1
PB2	=	Basic polymerase protein 2
PBS	=	Phosphate buffered saline
PCA	=	Principle component analysis
PCR	=	Polymerase chain reaction
PG	=	Prostaglandin
PMSF	=	Phenylmethylsulfonyl fluoride
PVDF	=	Polyvinylidene fluoride
qPCR	=	Quantitative PCR
RIG-I	=	Retinoic acid-inducible gene I
RIN	=	RNA integrity number
RIP1	=	Receptor-interacting protein 1
RIPA	=	Radio-immunoprecipitation
RIPK1	=	Receptor-interacting serine/threonine-protein kinase 1
RLR	=	RIG-I-like receptor
RNA	=	Ribonucleic acid
RSV	=	Respiratory syncytial virus
RvD1	=	Resolvin D1
SI	=	Selective index
siRNA	=	Small interfering ribonucleic acid
SL	=	Sesquiterpene lactone
TAD	=	Transactivation domains
TBK1	=	Tank binding kinase 1
TBS	=	Tris buffered saline

TBST	=	Tris buffered saline and Tween 20
TCID ₅₀	=	Median tissue culture infectious dose
TLR	=	Toll-like receptor
TM	=	Transmembrane domain
TMV	=	Tobacco mosaic virus
TNF- α	=	Tumour necrosis factor- α
TP53	=	Tumour protein 53
TPCK	=	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
TRADD	=	TNF-associated death domain protein
TRAF2	=	TNF-receptor associated factor 2
TRAIL	=	Tumour necrosis factor-related apoptosis-inducing ligand
v	=	Volts
vRNA	=	Viral RNA
vRNP	=	Viral ribonucleoprotein
VZV	=	Varicella zoster virus
μ g	=	Microgram
μ l	=	Microlitre
μ M	=	Micromolar

1. Introduction

1.1 Overview of Influenza A virus

Influenza virus is a negative-sense, single stranded RNA virus; it is a member of the family *Orthomyxoviridae*. The International Committee on Taxonomy of Viruses (ICTV) has classified *Orthomyxoviridae* into 7 genera, which are Influenza A virus (IAV), Influenza B virus, Influenza C virus, Influenza D virus, Isavirus, Quaranja virus and Thogoto virus (ICTV, 2020). Based on the antigenic properties of nucleoprotein (NP) and matrix protein (M), influenza viruses are further classified into the A, B, C and D type.

Besides humans, IAV can readily infect a wide range of avian and other mammalian hosts, including poultry, pigs, dogs, cats, horses and sea mammals. As a major disease around the world, IAV poses a threat to human health, poultry and livestock industry. Human beings have encountered several IAV pandemics in our history. In 1918, an H1N1 virus pandemic, known also as 'Spanish flu', which was thought to have originated from a military camp in the US and later brought to Europe during World War I caused approximately 500 million infected people and 50 million deaths (Johnson and Mueller 2002, Akarsu, Burmeister et al. 2003). In 1957, an H2N2 virus pandemic caused more than 1 million deaths globally, and in 1968, an H3N2 virus pandemic originated from Hong Kong, cost another 1 million lives around the world (Lipatov, Govorkova et al. 2004). More recently, in 2009, a swine-derived H1N1 virus caused

the first influenza pandemic of the 21st century (Novel Swine-Origin Influenza, Dawood et al. 2009). Highly pathogenic avian influenza H5N1 (HPAI H5N1) virus became a major concern when the first case of avian to human transmission was reported in Hong Kong in 1997. In that outbreak, a total of 18 human infection cases were reported of which 6 were fatal (Claas, Osterhaus et al. 1998, Subbarao, Klimov et al. 1998). Globally between 2003 and February 2020, however, there were only 861 reported human cases of HPAI H5N1 virus infection of which 455 patients died according to the World Health Organisation (WHO) report (WHO, 2020).

1.2 Influenza A virus structure

The genome size of IAV is approximately 13.6kb, its morphology tends to be variable; cylindrical and spherical shapes are most commonly found each with a diameter of 80 to 120 nm. Its morphology can be filamentous in the early budding phase of the virions (Ryu 2017a). IAV is composed of 8 segments; segment 1 to 8 are viral PB2, PB1, PA, HA, NP, NA, M and NS genes, respectively. The structure of influenza A virus is shown in Figure 1 (Dou, Revol et al. 2018). At the 5' end, all vRNAs are capped with a triphosphate group with an identical 13bp sequence 3' – GGACAAAGAUGAppp – 5'; at the 3' end, all vRNAs contain a hydroxyl group and a 12bp conservative sequence 3' – OH – UCGU(C)UUUCGUCC- 5' (Hoffmann,

Stech et al. 2001). The partially complementary sequences at the 5' and 3' ends form a secondary panhandle structure. The panhandle structure is essential for binding of the IAV RNA polymerase complex, which is crucial for virus replication and transcription (Nagata, Kawaguchi et al. 2008). There is a conservative poly(U) region at the 5' end of each vRNA, which generates poly(A) as a terminate signal during viral mRNA synthesis. This poly(u) region is absent in cRNA, which therefore can be a marker to distinguish viral mRNA and cRNA (Bouvier and Palese 2008).

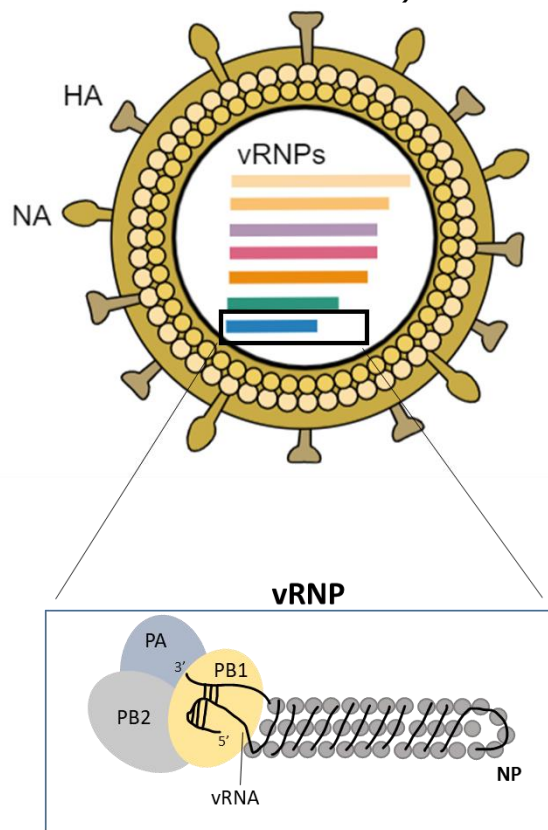


Figure 1-1. Structure of Influenza A virus (Dou, Revol et al. 2018). The envelope of Influenza A virus contains three major transmembrane proteins HA, NA and M2, eight vRNP segments located in the core of the virus and connected to M1 protein which is in contact with the outer bi-lipid layer.

1.3 Protein functions

Segment 1 encodes the basic polymerase protein 2 (PB2); segment 2 encodes the basic polymerase protein 1 (PB1) and an accessory protein, PB1-F2, whose apparent function is to interfere with the host innate immune response (Kamal, Alymova et al. 2017). Segment 3 encodes the basic polymerase acidic protein (PA) and a newly discovered frame-shifting protein PA-X (Jagger, Wise et al. 2012). Segments 4 and 6 encode the surface proteins haemagglutinin (HA) and neuraminidase (NA). Segment 5 encodes NP; segment 7 encodes M1 and the ion channel protein M2; segment 8 encodes the non-structural protein (NS1) and the non-structural protein 2 (NS2) also known as nuclear export protein (NEP).

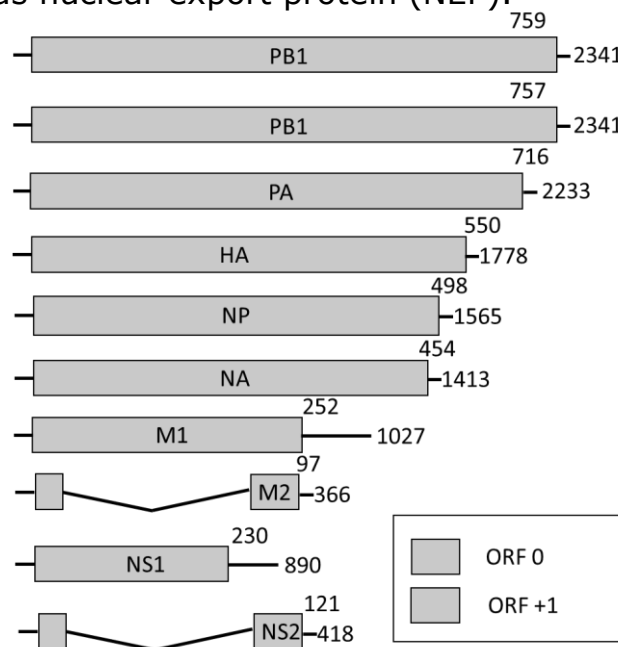


Figure 1-2. The Influenza A virus proteins that are encoded by the 8 segments (Ryu 2017a). Eight viral RNA segments encode 12 proteins, of which 10 non-frameshift-derived proteins are listed in the box. The 2 frameshift proteins, PA-X and PB1-F2, are not listed.

1.3.1 Polymerase complex: PB2, PB1 and PA

The polymerase complex of IAV is derived from three of the largest vRNA segments PB2, PB1 and PA. PB2 and PB1 are basic proteins, and PA is acidic. The negative-sense viral RNA (vRNA) of PB2, PB1 and PA along with NP form viral ribonucleoprotein (vRNP) complex, which plays a crucial role in virus replication and virulence (Eisfeld, Neumann et al. 2015).

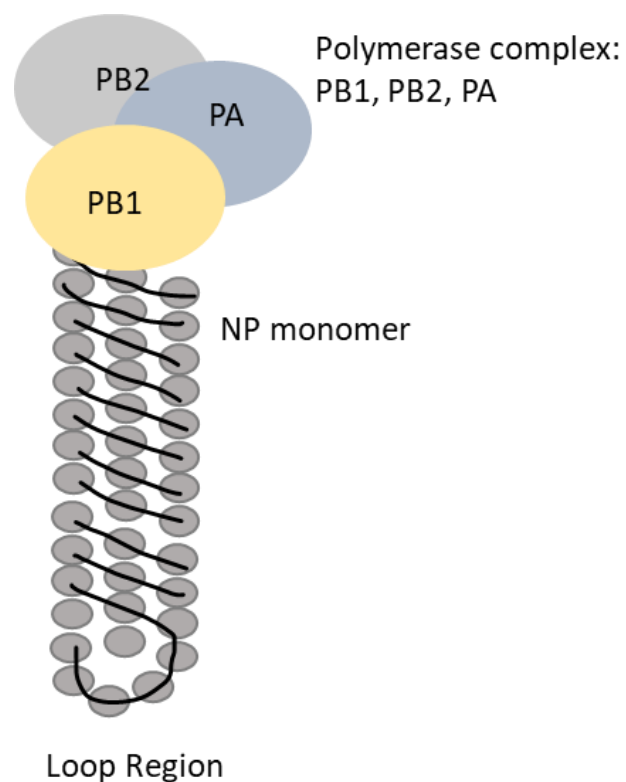


Figure 1-3. The structure of viral ribonucleoprotein (vRNP) complex (Eisfeld, Neumann et al. 2015). The viral vRNP complex is consisted of NP monomers, polymerase complex (PB2, PB1 and PA) and each distinct viral genomic RNA.

The main function of PB2 is the transcription of viral mRNA. PB2 was discovered to have a cap-binding domain at its carboxyl terminus (Blaas, Patzelt et al. 1982, de la Luna, Martinez et al. 1989). PB2 is involved in the initiation of cap-snatching process, in which the host cell pre-mRNA is snatched by PB2 and the snatched residues then serve as 5' cap for subsequent virus replication (Cheung and Poon 2007). Toyoda et al., (1996) reported that the cap-binding site is located near the carboxyl terminus of PB2, and the amino terminus of PB2 directly interacts with PB1. Furthermore, two other domains of PB2 interact with NP, which serve a key role in the formation of vRNP complex. PB2 was also shown to have endonuclease activity (Toyoda, Adyshev et al. 1996). PB2 is also considered as a key determinant for the pathogenicity of IAV infection, position 627 of PB2 being particularly important (Chin, Li et al. 2014).

PB1 is associated with initiation of transcription and chain elongation (Ulmanen, Broni et al. 1981). It is an RNA polymerase, which contains four conserved motifs of RNA-dependent RNA polymerases. Polymerase activity is absent when mutations occur in the conserved motifs (Poch, Sauvaget et al. 1989, Biswas and Nayak 1994). PB1 is the core of the virus RNA polymerase complex and it directly interacts with PB2 and PA through different manners. It was suggested that the N-terminal 140 amino acid of PB1 binds to the C-terminal of PA, and the 158 amino-acid C-terminal of PB1 binds to

the N-terminal of PB2, with no interaction between PB2 and PA (Toyoda, Adyshev et al. 1996).

PA is the smallest protein among the polymerase complex proteins. It is essential for viral RNA transcription and replication, it facilitates PB1 to enter the nucleus, thus, like PB2 and PB1, PA also contains a nuclear localisation signal. Zurcher et al., (1996) found that the PA N-terminal was not necessary for binding to PB1. But following internal or C-terminal deletion of PA, the interaction between PB1 and PA was abolished, suggesting that the C-terminal of PA was essential for PB1-PA interaction (Zurcher, de la Luna et al. 1996). PA was also found to have inhibitory effects on other viral proteins. Over-expression of PA reduced the accumulation of co-expressed viral PB2 and NP, and also reduced the activity of the reconstituted RNA polymerase (Sanz-Ezquerro, de la Luna et al. 1995).

1.3.2 Surface proteins

Based on the antigenic properties of HA and NA, influenza A virus is classified into 18 HA subtypes and 11 NA subtypes (Tong, Li et al. 2012, Tong, Zhu et al. 2013). Both HA and NA are glycoproteins embedded in the viral envelope as spike proteins on the viral surface. The quantity of HA protein is four more times higher than NA protein (Louten 2016).

HA is a type I transmembrane protein with an N-terminal signal peptide outside cell membrane and a C-terminal signal peptide inside cell membrane. The primary roles of HA are to bind sialic acid-containing receptors, and to facilitate the fusion of cell and viral membranes (Cheung and Poon 2007). HA protein is translated by polysomes that are located in the cytoplasm, assembled in the rough endoplasmic reticulum (ER), and then transported through the Golgi apparatus on its way to the cell membrane (Veit and Thaa 2011). In the process of transport, HA undergoes several co- and post-translational modifications (Klenk and Rott 1988). HA₀, the synthesized precursor, is cleaved by host cell proteases post-translationally into two subunits, HA₁ and HA₂, which is a prerequisite for viral infectivity (Chen, Lee et al. 1998, Cheung and Poon 2007). Typically, HA₀ is cleaved by extracellular proteases, however, when multiple basic amino acids appear at the cleavage site of HA₀, it can be cleaved by intracellular proteases, such as furin, which is highly expressed in most organs (Horimoto, Nakayama et al. 1994, Cheung and Poon 2007). IAV subtypes with multiple basic amino acids within the cleavage site of HA₀, like H5 and H7 avian viruses, are considered as highly pathogenic viruses. HA₁ and HA₂ have distinct roles; HA₁ helps the binding of virus to host cells, and HA₂ initiates the fusion with the endosomal membrane (Louten 2016).

NA surface protein is encoded by segment 6. It is a type II transmembrane protein which forms a homotetramer (Veit and Thaa 2011). NA has four domains, which include a globular head, a narrow stalk, a transmembrane domain and a cytoplasmic domain (Varghese and Colman 1991). NA is processed in the same routine as HA. It is first assembled in the ER, then transported through the Golgi apparatus to the plasma membrane. NA serves as 'scissors' to facilitate progeny virion release. When NA is inhibited, progeny viruses cannot be released from infected cells and consequently accumulate intracellularly (Palese, Schulman et al. 1974, Palese and Compans 1976, Cheung and Poon 2007).

1.3.3 Nucleoprotein

NP is encoded by segment 5; it is the second most abundant protein after M1. NP is a highly conserved structural protein that serves as the key determinant to distinguish type A, B and C influenza viruses. The primary function of NP is transcription and replication, together with the polymerase complex (PB2, PB1 and PA) and each vRNA segment form the stable RNP complex (Huang, Palese et al. 1990). In the process of virus replication, NP is the 'checkpoint' factor in controlling RNA polymerase from mRNA synthesis to cRNA and vRNA synthesis, the underlying mechanism of switching however remains unknown (Shapiro and Krug 1988). The RNA binding region of NP is between amino acid residues 91 and 188 as deletion or mutation of

these amino acid residues abolished the binding ability of NP to RNA (Kobayashi, Toyoda et al. 1994).

1.3.4 M1 and M2 proteins

Segment 7 encodes M1 and M2 proteins. M1 protein is encoded by the main co-linear transcript of segment 7; M2 protein is generated by spliced mRNA of segment 7. Phylogenetic analysis revealed that both M1 and M2 had evolved slowly in comparison with PB2, PB1, PA and NP. M1 and M2 show different evolutionary rates in different hosts, with M2 protein exhibiting significant evolution in human and swine lineages but not in avian lineage (Ito, Gorman et al. 1991).

M1 does not have a transmembrane domain and is the most abundant protein that comprises 40% of total viral protein. It is the key viral protein for viral morphogenesis, sited at the inner membrane of viral lipid envelope to separate vRNPs from the viral membrane (Ruigrok, Barge et al. 2000). The key function of M1 is the assembly of viral particles; it is shown that M1 directly binds to RNA without the presence of viral NP, and this binding property is specific for single-stranded RNA (Wakefield and Brownlee 1989). Several studies have indicated that M1 is able to downregulate the transcription of IAV and this inhibition is caused by M1 binding to the RNA (Elster, Fourest et al. 1994, Ruigrok, Barge et al. 2000). The

inhibition of transcription by M1, mediated by amino acid residues 91 and 111, is catalysed by the vRNP complex (Watanabe, Handa et al. 1996). M1 protein is able to oligomerise, acting as a focussed site for the accumulation of viral components during viral assembly and budding (Zhao, Ekstrom et al. 1998, Veit and Thaa 2011).

M2 is one of the three transmembrane proteins of IAV, besides HA and NA. It comprises three segments: a 23 amino-acid extracellular N-terminal domain, a 19 amino-acid transmembrane segment, and a 54 amino acid residues C-terminal cytoplasmic domain (Lamb 1989). M2 is a tetrameric integral membrane protein that has ion channel activity regulated by pH (Pinto, Holsinger et al. 1992). It is highly conserved, in particular in the first 10 amino acids of the extracellular N-terminal, thus serving as a potential target site for vaccine development (Neirynck, Deroo et al. 1999, Fiers, De Filette et al. 2004, Memoli, Han et al. 2020).

1.3.5 NS1 and NEP proteins

The non-structural proteins NS1 and NEP are encoded by segment 8, which is the smallest of the 8 segments. NS1 protein is encoded by the main co-linear transcript of NS gene, with NEP protein translated via alternative splicing of the same parent mRNA of segment 8. NS1 and NEP have an over-lapping 70 amino acid region.

NS1 is synthesized in the early phase of viral infection, whereas NEP is synthesized later (Klenk and Rott 1988).

NS1 protein mainly exists in the nucleus as oligomers (Nemeroff, Qian et al. 1995, Cheung and Poon 2007). It can bind to most types of RNAs, such as vRNA, vRNP, double-stranded RNA (dsRNA), small nuclear RNA (snRNA) and poly(A)-containing cellular RNA (Hatada and Fukuda 1992, Hatada, Takizawa et al. 1992, Qiu and Krug 1994, Qiu, Nemeroff et al. 1995, Marion, Zurcher et al. 1997, Cheung and Poon 2007). Influenza virus lacking NS1 protein produced by reverse genetics technique is still able to replicate efficiently in Vero cells and Madin-Darby canine kidney (MDCK) cells, suggesting that NS1 is not essential in virus replication cycle (Egorov, Brandt et al. 1998).

NS1 is the key viral protein that antagonises host immune response. One of the most extensively studied mechanisms is its inhibition of interferon (IFN) regulatory factor 3 (IRF3), which leads to type I IFN transcription inhibition. NS1 can also block the post-translational process of host mRNAs to the benefit of virus replication (Kochs, Garcia-Sastre et al. 2007). NS1-deficient H1N1 virus is less virulent in comparison with-wild type (wt) H1N1 virus, and NS1-deficient H1N1 virus stimulated a stronger IFN response in human embryonic kidney 293 (HEK 293) cells than the wt H1N1 virus (Kochs, Garcia-Sastre et al. 2007). Several studies have shown that the deletion of NS1 impaired the virulence of H5N1 virus; IFN response was

significantly higher in NS1-deleted H5N1 virus infection than in wild type virus infection (Twu, Kuo et al. 2007, Wang, Zeng et al. 2018). All the above collectively indicate that NS1 inhibits host IFN production and results in increased virulence.

Nuclear export protein (NEP) was previously referred to as NS2. It is a 121 amino acid protein encoded by a spliced variant of segment 8 mRNA. NEP protein can be divided into a 53 amino acids protease-sensitive N-terminal domain and a 68 amino acids protease-resistant C-terminal domain (Akarsu, Burmeister et al. 2003, Paterson and Fodor 2012). The primary function of NEP is in mediating the export of RNP complex from the nucleus (Paterson and Fodor 2012). Several studies have also shown that NEP protein plays a role during virus replication and transcription in addition to RNP export. NEP was found to facilitate the polymerase activity of avian H5N1 virus to achieve efficient virus replication in human cells (Manz, Brunotte et al. 2012). Mutations of NEP lead to defective viral particles and suppression of PA gene cRNA synthesis, suggesting that NEP protein has a role in promoting virus replication (Odagiri and Tobita 1990, Odagiri, Tominaga et al. 1994).

1.3.6 PA-X and PB1-F2

PA-X is a protein encoded by segment 3 that has a 191 amino acid N terminus and a 41 or 61 amino acid C terminus (Jagger, Wise et al. 2012). PA-X is a fusion protein that shares its N-terminal 191 amino acids with PA, and the C terminus is produced by +1 ribosomal frameshift (Jagger, Wise et al. 2012, Shi, Jagger et al. 2012, Vasin, Temkina et al. 2014, Hayashi, MacDonald et al. 2015). PA-X plays a key role in regulating host immune response. PA-X has 'host shutoff' ability that allows IAV to escape from host immune response resulting in increase in progeny virus production (Hayashi, MacDonald et al. 2015). It was also shown that PA-X increased virus replication in mice at the early stage of infection through the accumulation of RNP complexes (Ma, Li et al. 2019).

PB1-F2 is encoded by +1 ORF in segment 2 that is possibly initiated by the Kozak sequence at the start code of PB1 (Chen, Calvo et al. 2001). Chen et al., (2001) found that PB1-F2 is a 87 amino acid protein that induced apoptosis in host cells through mitochondrial targeting. PB1-F2 was also discovered to have functional connection with PB1 to regulate the activity of IAV polymerases (Mazur, Anhlán et al. 2008).

1.4 Life cycle of influenza A virus

The life cycle of IAV is schematically shown in Figure 1-4 (Ryu 2017a). The infection of IAV starts with the binding of HA with cellular receptors that contain sialic acids. After binding to the cellular receptors at the plasma membrane, endocytosis occurs followed by fusion of virion and endosomal membranes. Subsequently, vRNPs with associated polymerase complexes are released into the cytoplasm. Cellular proteins and vRNP then form complexes and enter the nucleus, where the viral transcription occurs. Newly synthesized viral proteins and other viral particles are assembled and released as progeny viruses.

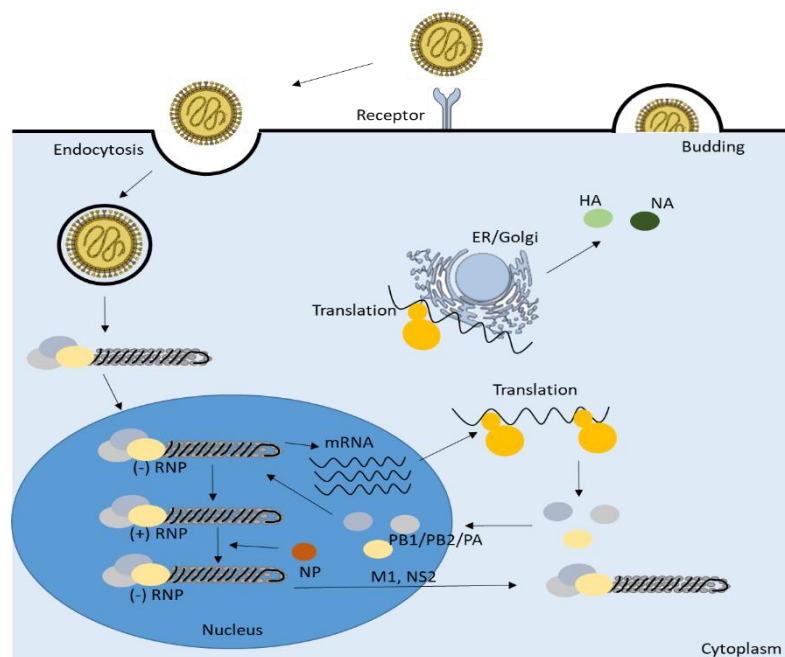


Figure 1-4. Life cycle of influenza A virus (Ryu 2017a). The HA of IAV binds to cellular receptor, and IAV enters into host cell through endocytosis. vRNPs are then released into cytoplasm and transported to nucleus to undergo transcription to synthesize particles of progeny virus. The particles are then assembled and released for infection of neighbouring cells.

1.4.1 Virus entry and uncoating

Influenza A virus primarily binds to the epithelial cells in the respiratory tract along the nasopharynx, trachea, bronchi and bronchioles (Louten 2016). Specifically, IAV recognizes the N-acetylneuraminic (sialic) acid on the cell membrane through HA (Skehel and Wiley 2000, Bouvier and Palese 2008). Based on the binding properties of the sialic acids, there are two major binding linkages, α -2,3- or α -2,6-linkages (Skehel and Wiley 2000, Bouvier and Palese 2008). HA shows preferential binding affinity in different host species; the HA of human or mammalian IAV has high affinity in binding to α -2,6-linkage, and the HA of avian IAV prefers to bind to α -2,3-linkage (Ryu 2017a). The α -2,6-sialic acid is widely distributed in human respiratory tract, hence the replication of avian IAV is in the main restricted in humans (Gagneux, Cheriyan et al. 2003).

Upon the attachment of HA to the sialic acid receptors on the host cell membrane, IAV particles are internalized through endocytosis (Louten 2016), and gain entry into the host cell in endosomes. Endosomes have a pH around 5—6 which triggers the uncoating of influenza virus through (a) the fusion of viral and endosomal membranes and (b) hydrogen ions entry (influx) into the virions through the M2 channel (Bouvier and Palese 2008, Samji 2009, Louten 2016, Ryu 2017a). The low pH causes the conformational

change of HA, so that its fusion peptide becomes stretched and activated, and connects with the cellular membrane to form a fusion pore (Figure 1.5) that releases vRNA into the cytoplasm (Szewczyk, Bienkowska-Szewczyk et al. 2014, Ryu 2017a).

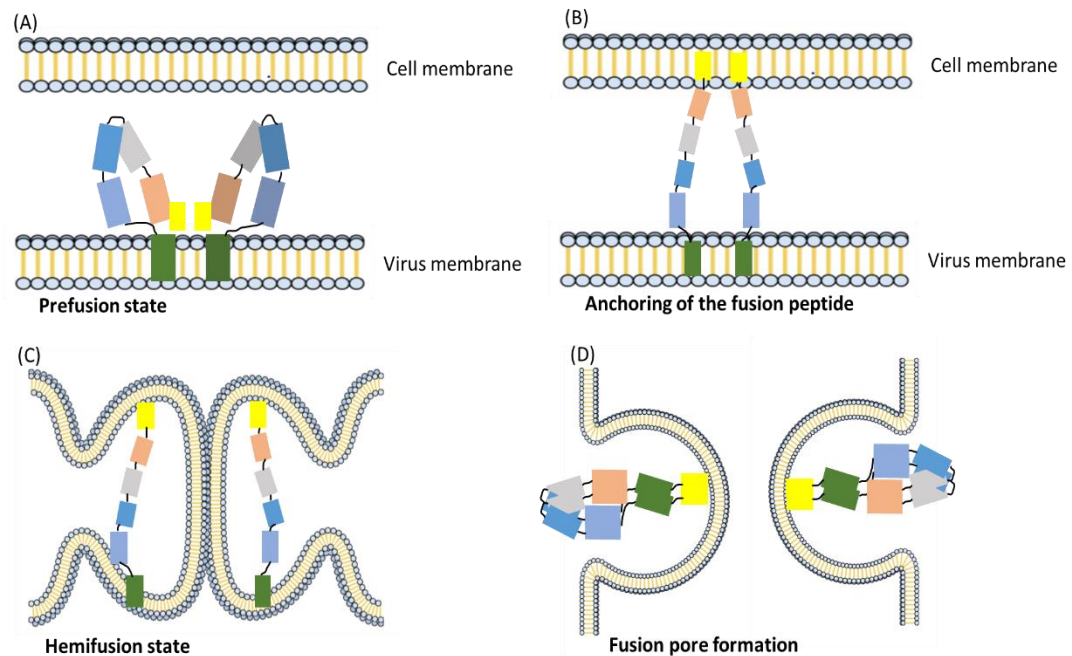


Figure 1-5. The fusion of cellular membrane and viral membrane (Ryu 2017b). HA is folded before the fusion of virus membrane and cell membrane (A). HA is then anchored with cell membrane at low pH (B). A hemifusion state is formed between cell membrane and virus membrane (C) and eventually leads to fusion pore formation (D).

1.4.2 Translocation of vRNP into the nucleus

After their release into the cytoplasm, the vRNPs must be transferred to the nucleus to undergo transcription. The viral proteins in the vRNP, PB2, PB1, PA and NP possess nuclear localization signals

(NLSs) for direction into the nucleus by host cellular proteins (Cros and Palese 2003). Wu et al., (2007) found at least two NLSs in influenza viral NP, one located at the N terminus, and the other one is sited in the middle of the protein. Further data indicated both NLSs are important for the import of vRNP into the nucleus, but the specific mechanism remains unknown (Wu, Sun et al. 2007).

1.4.3 The synthesis of viral RNA

vRNPs are imported to the nucleus, where transcription occurs. Since IAV is a negative-sense RNA virus, the viral genome has to be converted to positive sense as a template for viral RNA synthesis. Using vRNA as template, two sets of positive sense RNAs are synthesized: the viral messenger RNA (mRNA) and viral complementary RNA (cRNA) (Bouvier and Palese 2008). The cRNA is the full-length complementary copy of the corresponding vRNA, but the mRNA is the incomplete complementary copy of the vRNA that lacks the 5' methylated cap and poly(A) tail (Samji 2009). IAV hijacks the transcription machinery of host cells to initiate transcription of the viral mRNA (Figure 1.6). For transcription to commence, the 5' caps of host mRNA transcripts are hijacked by IAV polymerase, and used as primers linked to the 5'-end of viral mRNA to initiate transcription (Ryu 2017a). This is the main reason that the replication of IAV must occur in the nucleus. The 'cap snatching' process requires the involvement of viral polymerases: PB2, PB1 and

PA (Bouloy, Morgan et al. 1979, Bouloy, Plotch et al. 1980). PB2 binds to the 5' caps of host cell mRNAs, PA has endonuclease activity that cleaves the 5' caps of the cellular mRNAs, PB1 then adds nucleotides by using vRNA as template, the transcription continues until it reaches the end of the vRNA (Louten 2016). vRNA contains five to seven uracil residues that are then transcribed to form a poly(A) tail and included in the newly synthesized viral mRNA. The transcription is ended followed by polyadenylation at the 3' end (Luo, Luytjes et al. 1991, Li and Palese 1994).

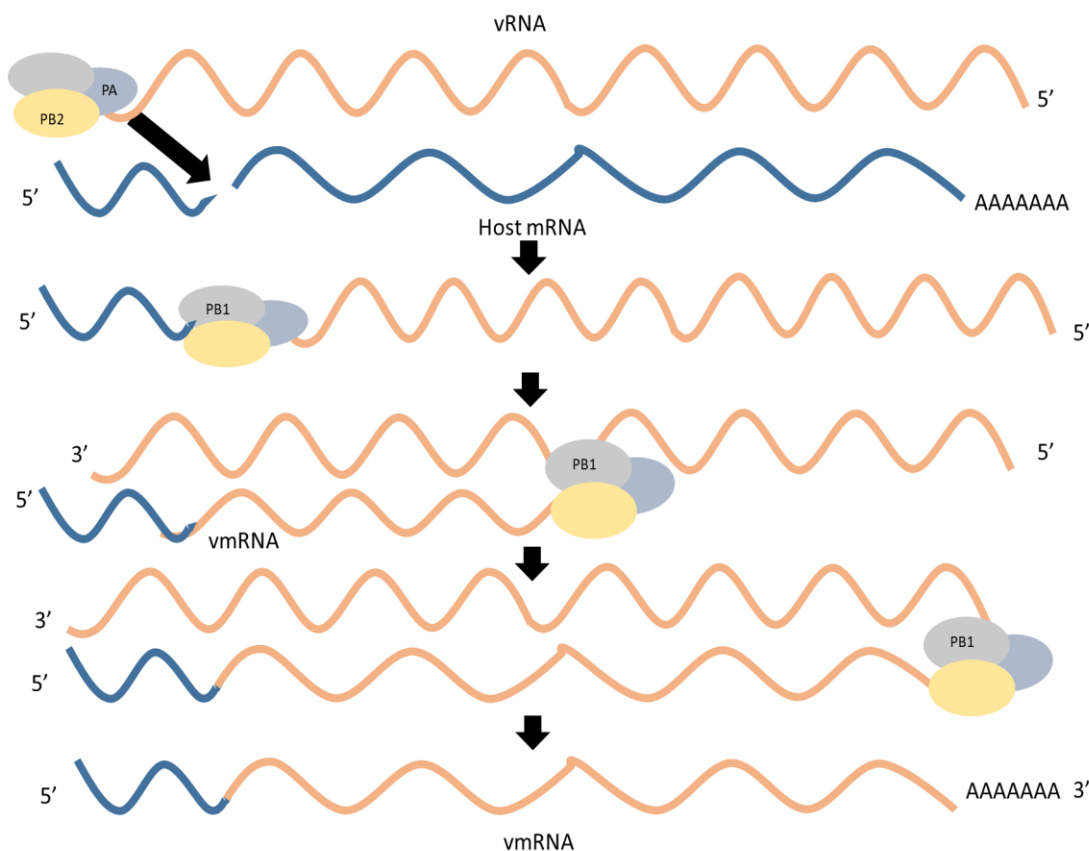


Figure 1-6. IAV transcription via cap-snatching mechanism (Louten 2016).

The 5' end of host cell mRNA is taken by RNP complex. The transcription starts with the binding of PB2 with 5' end of host cell mRNA; PA has endonuclease activity that cuts off the 5' end; PB1 helps to continue the transcription and stops near the end of vRNA. Then the poly(A) tail is added to form a complete viral mRNA.

During replication of IAV, cRNAs become as template to produce full-length copies of negative sense vRNA (Bouvier and Palese 2008). The mechanism that regulates the synthesis of viral mRNA and cRNA is not fully understood. Some suggest that there is no 'switch' regulating the synthesis of viral mRNA and cRNA (Vreede and Brownlee 2007). But others suggest that the amount of NP is the key factor that distinguishes the transcription of vRNAs to mRNAs and cRNAs (Figure 1.7) . At the early stage of IAV replication, with low level of NP, mRNA is the dominant product of transcription; when the accumulation of NP grows, the synthesis of mRNA is reduced and more cRNAs are produced (Ryu 2017b).

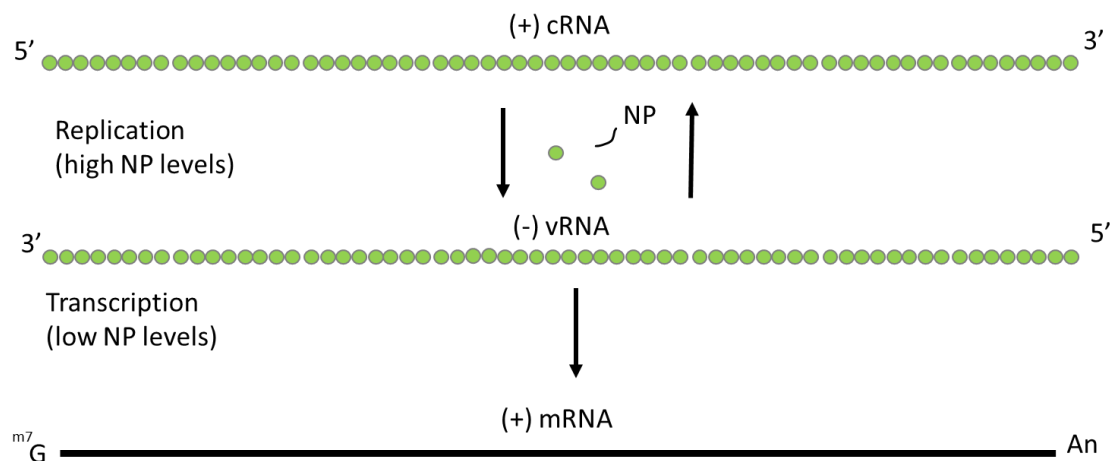


Figure 1-7. The accumulation of NP determines the tendency of vRNA transcribing to cRNA or mRNA (Ryu 2017b). When the NP level is low, (-)vRNA can synthesize more mRNA than cRNA; when the NP level is high, more (-)vRNA are transcribed into cRNA.

1.4.4 The synthesis of viral proteins

After the formation of mature viral mRNAs, they are then transported from the nucleus into the cytoplasm and translated by ribosomes. The viral surface proteins (HA and NA) and the ion channel protein M2 are synthesized at membrane-bound ribosomes and further undergo post-translational modifications in the rough endoplasmic reticulum and the Golgi apparatus (Szewczyk, Bienkowska-Szewczyk et al. 2014). In the ER, HA and NA are folded and glycosylated (Louten 2016). HA, NA and M2 proteins are then transported to the lipid rafts of the host cells. HA is cleaved by protease into HA1 and HA2 with a covalent disulfide bond connecting the two parts. (Figure 1.8).

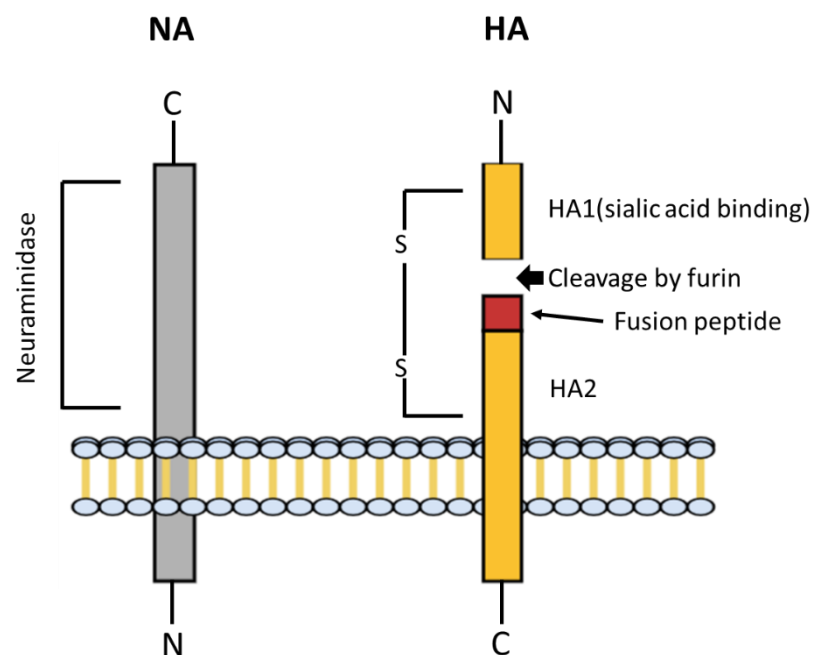


Figure 1-8. Viral HA and NA structure on the lipid rafts (Ryu 2017b). Viral HA is cleaved by furin into HA1 subunit and HA2 subunit, the two subunits are connected by a disulfide bond. NA has neuraminidase activity to help with the release of progeny virus from host cell.

Other viral proteins, including PB2, PB1, PA and NP, are synthesized in the cytoplasmic ribosomes and then transported into the nucleus (Louten 2016). Translated viral proteins in the nucleus together with the synthesized vRNA form RNP complexes (Eisfeld, Neumann et al. 2015). M1 is believed to have a key role in the formation of RNP complexes, and its translation occurs later than other proteins to avoid early assembly and export of RNP complexes to the cytoplasm (Louten 2016). NS2 interacts with M1 to facilitate the export of newly synthesized RNP complexes from the nucleus to the cytoplasm (Bouvier and Palese 2008).

1.4.5 Virus assembly and release

Upon the transport of vRNAs from the nucleus to the plasma membrane, the vRNA segments are assembled at the plasma membrane. IAV must assemble all eight vRNAs to become infectious; however, the assembly mechanism of eight vRNAs is not fully understood. Bancroft et al., (2002) suggested the packaging of vRNAs could be a random and nonspecific process, as the tagged NP, NA and NS were competing for packaging detected by flow cytometry and quantitative analysis of vRNAs in 293T cells (Bancroft and Parslow 2002). An engineered IAV that contains nine RNA segments was generated by RNP transfection method, and was found to be able to replicate *in vivo* (Enami, Sharma et al. 1991). It shows that the assembly of vRNA does not necessarily need eight segments,

indicating the assembly process is random and nonspecific. However, Fujii et al., (2003) showed that the assembly of vRNA segments must incorporate a NA vRNA signal to produce infectious virus. It was suggested that NA provide a signal that guides vRNA segments for assembly (Fujii, Goto et al. 2003).

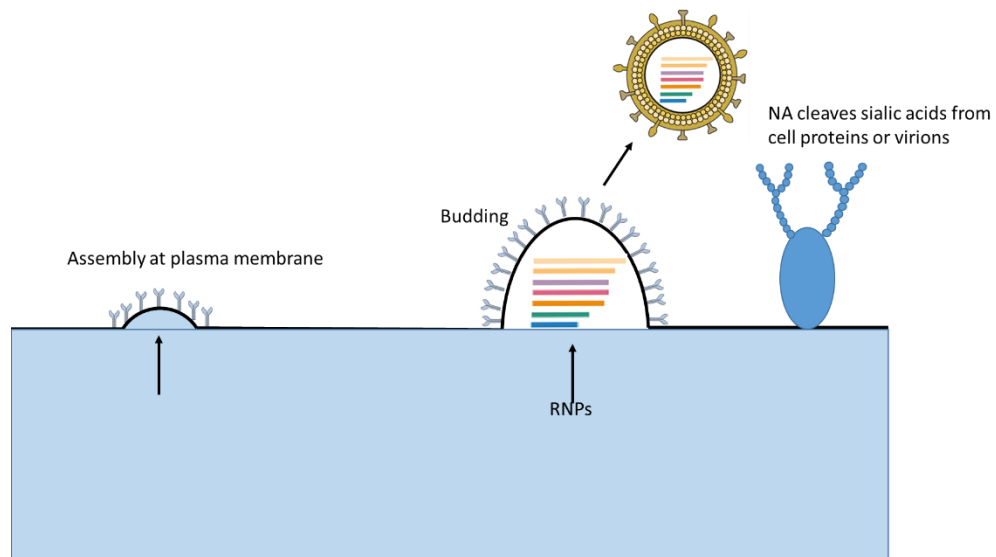


Figure 1-9. The assembly, budding and release of IAV (Louten 2016). The assembly of IAV occurs at the plasma membrane, followed by budding, and eventually progeny virus is released by NA cleavage.

The vRNA segments together with plasma membrane localized HA, NA and M2, and M1-RNP complex assemble into virus particles at the plasma membrane ready for budding. The accumulation of HA and NA at the plasma membrane initiate the budding process (Rossman and Lamb 2011). M1 proteins in the cytoplasm may possibly have a role in initiation of the budding process (Bouvier and Palese 2008). Progeny virions are then released from host cells, but are still attached with the host sialic acids through HA binding. NA has

neuraminidase activity to sever the connection between nascent virus and infected cell surface (Ryu 2017a). Released viruses move on to further infect neighbouring host cells.

1.5 Antigenic drift and shift of influenza A virus

Besides humans, IAVs can readily infect a wide range of avian and mammalian hosts, including poultry, pigs, dogs, cats, horses and sea mammals (Ives, Carr et al. 2002, Wang, Wu et al. 2013, To, Tsang et al. 2014, Zhao, Zhou et al. 2015, Capuano, Miller et al. 2017, Song, Qi et al. 2017, Hemida, Perera et al. 2019, Shin, Siebert et al. 2019, Yang, Wang et al. 2019, Zhou, Sun et al. 2019). IAVs overcome the species barrier through antigenic shift, specifically within HA and NA, which is a process when two or more strains of IAVs combine to become a new subtype. The species barrier can also be overcome by antigenic drift, caused by mutations in the virus genome during viral replication. In other words, antigenic shift refers to the dramatic change of antigenicity after virus reassortment, leading to a new influenza A subtype; antigenic drift refers to progressive and incremental mutations, leading to a new influenza A strain (Jiao, Tian et al. 2008, Earhart, Elsayed et al. 2009, Wang, Lee et al. 2016, Ryu 2017a). The 2009 H1N1 pandemic virus is a triple reassortant through a long course of antigenic shift along with antigenic drift (Garten, Davis et al. 2009). In 2013, a novel H7N9

virus was detected in southern China; it was reassorted from duck-origin H7N3, wild bird-origin H7N9 and Brambling-origin H9N2 viruses (Gao, Cao et al. 2013). This novel H7N9 virus caused 37 deaths out of 132 confirmed deaths in China, which raised major concerns about its threat to public health since 2013 (Zhang, Shi et al. 2013).

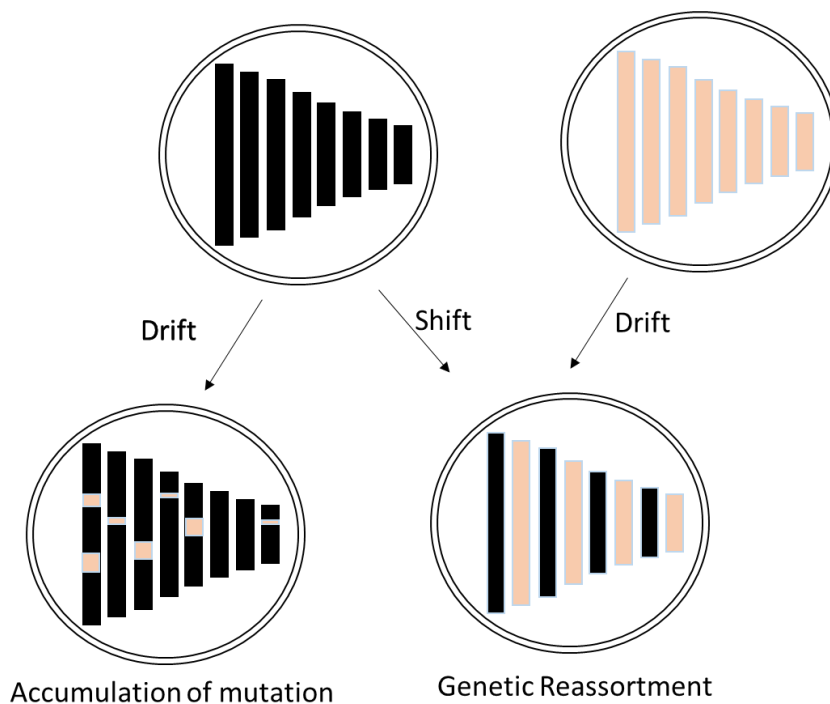


Figure 1-10. Antigenic drift and shift (Ryu 2017a).

Transmission and replication of influenza virus across the species barrier requires host adaptation. The first step of IAV infection is virus entry which is the binding of HA protein to the glycan receptors on host cell surface. Avian influenza virus shows a binding preference for NeuAc α 2,3 sialic acid receptor and human influenza virus prefers to bind to the NeuAc α 2,6 sialic acid receptor. Recently, an avian

H5N5 virus was found to show strong binding to both NeuAc α 2,3 and NeuAc α 2,6 sialic acid receptors, and experimentally, was capable of horizontal transmission between guinea pigs (Linster, van Boheemen et al. 2014, Li, Wang et al. 2015, Gu, Li et al. 2017). The widely circulating avian H9N2 subtype in China has recently acquired the ability to bind both avian and mammalian receptors and is able to transmit between experimental ferrets (Li, Shi et al. 2014). The virus polymerase complex also plays an important role in host adaptation. Position 627 of PB2 is particularly important. A PB2 E627K mutation in avian H9N2 virus enhanced polymerase activity and increase virulence in mice (Subbarao, London et al. 1993, Hatta, Gao et al. 2001, Chen, Bright et al. 2007, Richard, van den Brand et al. 2020). Similarly, a PB2 D701N mutation of avian H5N1 virus also increases pathogenicity and replication in mice (Li, Chen et al. 2005). Serial passages of avian H5N2 virus in mice led to enhanced virulence and horizontal virus transmission within this species which was attributed to a PA T97I mutation (Song, Pascua et al. 2009). Host adaptation can also take the form of combined viral mutational effects. Combined PB2 E627K and PA T97I mutations enhanced replication efficiency in mice (Cheng, Yu et al. 2014). Moreover, the combined mutations in HA and PB2 of an engineered H5N1 virus, acquired during serial passage in ferrets, are sufficient to confer airborne transmissibility (Herfst, Schrauwen et al. 2012). The virulence of H3N2 virus was enhanced by mutations in HA and PB2

(Ping, Dankar et al. 2010). Serial passages of pandemic 2009/H1N1virus in pigs found mutations on PB1, PA, NA and NS1 that can variably enhance replication efficiency in pigs (Gabriel, Herwig et al. 2008, Wei, Sun et al. 2014, Feng, Wang et al. 2016).

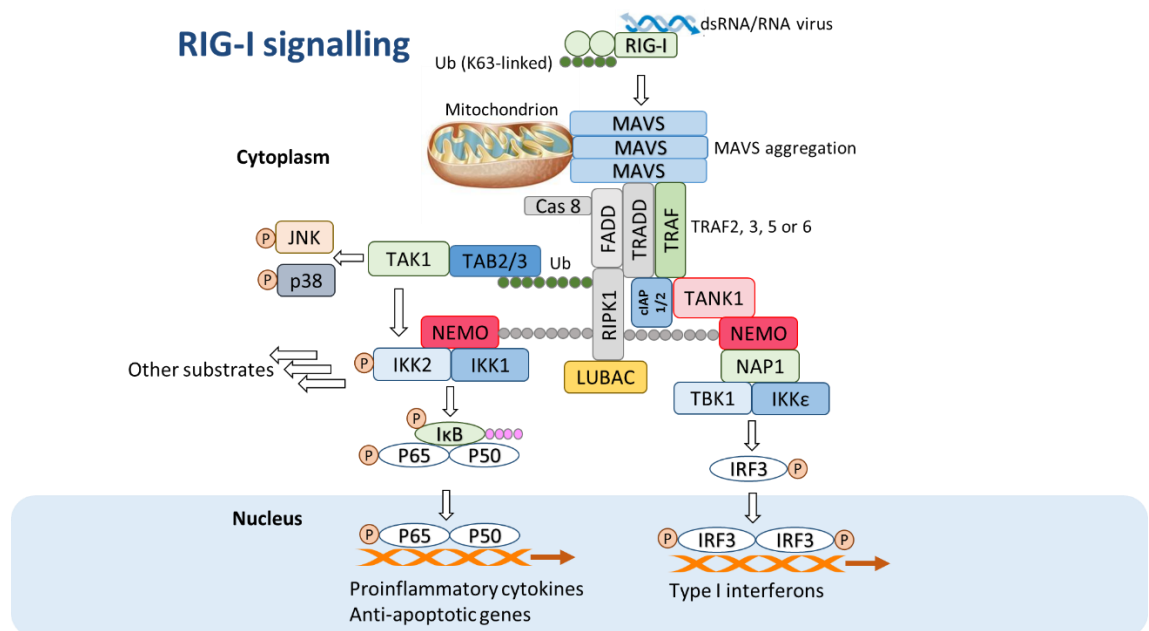
Table 1-1. Effects of influenza A virus mutations (H3 numbering)

Protein	Amino acid	Phenotypic impacts
HA	I155T (Li, Shi et al. 2014)	Increased affinity to human-type receptor
	T160A (Gu, Li et al. 2017)	
	Q226L (Song, Qi et al. 2017)	
	G228S (Song, Qi et al. 2017)	
NA	H274Y (Ives, Carr et al. 2002)	Reduced oseltamivir susceptibility
	N294S (Earhart, Elsayed et al. 2009)	
PB2	Q591K (Wang, Lee et al. 2016)	Enhanced replication efficiency and virulence in mice
	E627K (Subbarao, London et al. 1993)	Mammalian host adaption
	D701N (Li, Chen et al. 2005)	Increased virulence in mice
PB1	H99Y (Linster, van Boheemen et al. 2014)	Increased transmissibility in ferrets
	I368V (Richard, van den Brand et al. 2020)	
	G622D (Feng, Wang et al. 2016)	Increased virulence in mice
PA	T97I (Song, Pascua et al. 2009)	Enhanced polymerase activity and virulence
NP	N319K (Gabriel, Herwig et al. 2008)	Increased replication efficiency
M1	N30D (To, Tsang et al. 2014)	Increased virulence in mice
	T215A (To, Tsang et al. 2014)	
M2	S31N (Wang, Wu et al. 2013)	Reduced susceptibility to amantadine and rimantadine
NS1	P42S (Jiao, Tian et al. 2008)	Increased virulence in mice

1.6 Innate immunity against influenza A virus infection

Innate immunity serves as early defence against pathogen invasion, within hours of infection. It starts with the detection of pathogen-associated molecular patterns (PAMP) by pathogen recognition receptors (PRRs) localised on cell and organelle membranes, and in the cytosol. Viral RNAs are recognised by at least two classes of PRRs

including the membrane bound Toll-like receptors (TLRs); during IAV infection, the most important TLRs are TLR3 and TLR7/8, cytosolic RIG-I-like receptors (RLRs) that include retinoic acid-inducible gene I (*RIG-I* or *DDX58*) and MDA5. Cytosolic RLRs play a more prominent role in the innate immune response to RNA viruses. Viral RNAs bound to *RIG-I* or *MDA5* are recruited to the mitochondrial antiviral signalling protein (MAVS) (also known as VISA, IPS-1 or Cardif) which serves as a docking platform for the assembly of multiple proteins in the downstream activation of transcription factors NF- κ B and IRF3 (Reikine, Nguyen et al. 2014).



1.6.1 IRF signalling pathway

Following IAV infection of host cells, virus induced interferon (IFN) production requires involvement of the IRF family. The first IRF (IRF1) was identified in 1988, it was shown that IRF1 specifically interacted with regulatory region of IFN- β and mediated its transcription induced by Newcastle disease virus (NDV) in mice (Miyamoto, Fujita et al. 1988). Eight other IRFs were identified since the first discovery of IRF1. IRFs have important roles in regulatory response in host innate and adaptive immune response. The primary roles of IRF1 and IRF2 include the regulation of type I IFNs, cell growth, antioncogenic and oncogenic activities (Harada, Takahashi et al. 1994). IRF1 and IRF2 are essential for NK cell development, and IRF1 and IRF2 is essential for CD8⁺ T cells and CD4⁺ differentiation, respectively (Matsuyama, Kimura et al. 1993, Blanco, Contursi et al. 2000). IRF4 and IRF5 are both involved in myeloid cell development to regulate host cell inflammatory response (Jefferies 2019). IRF6 was shown to be constitutively expressed in mouse skin, and was shown to have a key role in keratinocyte differentiation (Ingraham, Kinoshita et al. 2006, Richardson, Dixon et al. 2006). IRF7 was found to be constitutive in B cells and monocytes, and the binding with *MyD88* increased the expression of type I IFNs (Hoshino, Sugiyama et al. 2006). IRF8 is important for B cell development and mainly distributed in nucleus and partially in cytoplasm (Zhao, Kong et al.

2006). IRF9 mediates type I IFN response by regulating interferon-stimulated genes (ISGs) (Jefferies 2019).

IRF3 is constitutively expressed in various tissues and exists in cytoplasm (Lin, Heylbroeck et al. 1998, Grandvaux, Servant et al. 2002). IRF3 gene encodes a 53kDa protein, with the N-terminal containing a DNA binding domain (DBD), and the C-terminal a translational domain. The actions of IKK-related kinases include Tank binding kinase 1 (TBK1) and inhibitor of κ B kinase ϵ (IKK ϵ) result in the activation of IRF3; IKK ϵ activation requires the phosphorylation of its C-terminal serine residue (Tarassishin, Bauman et al. 2013). Activated IRF3 then translocates from the cytoplasm to the nucleus and stimulates DNA binding and transcriptional activity, which leads to the transcriptional induction and eventual release of type I IFNs.

1.6.2 NF- κ B signalling pathway

Ranjan Sen and David Baltimore in 1986 first discovered NF- κ B (nuclear factor kappa B) as a key transcription factor for activation of kappa light genes in B cells (Sen and Baltimore 1986). NF- κ B family has five members. According to their transcriptional activity, RelA (P65), c-Rel and RelB possess transactivation domains (TAD) at their C-terminus and are thereby transcriptionally active; NF- κ B1 (p105) and NF- κ B2 (p100) do not contain the TAD, and serve as precursor proteins to dimerize with other family members which

contain the TAD. NF- κ B1 (p105) and NF- κ B2 (p100) are processed to mature p50 and p52, respectively. Each member of NF- κ B has an approximately 300 amino acid (aa) Rel homology domain (RHD) which is involved in binding to DNA and inhibitors of NF- κ B (I κ Bs) and dimerization (Oeckinghaus and Ghosh 2009).

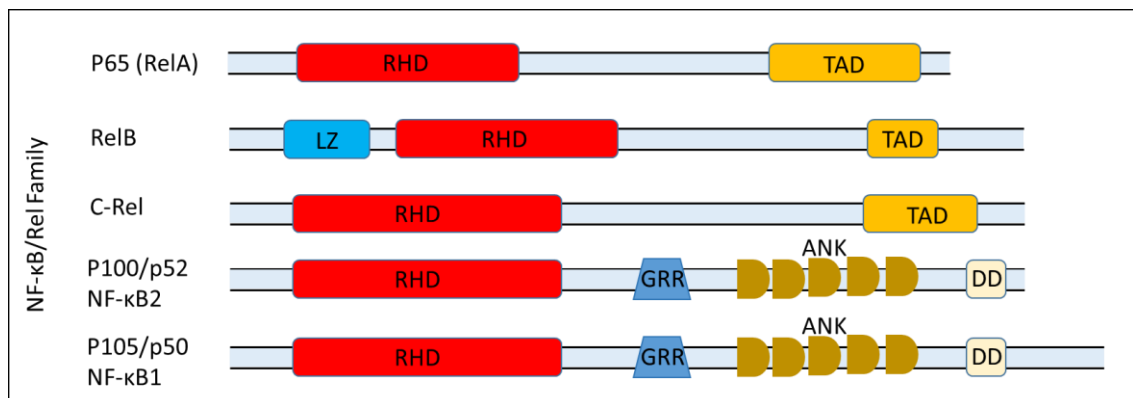


Figure 1-12. Key members of NF- κ B family (Oeckinghaus and Ghosh 2009). Key members from the Rel Family, RelA, RelB and c-Rel are transcriptional ready because they all contain the transactivation domains (TAD).

Degradation of I κ B is an indication of NF- κ B activation. The I κ B family includes I κ B α , I κ B β , I κ B γ , I κ B ϵ , I κ B ζ , Bcl-3, p100 and p105 (Oeckinghaus and Ghosh 2009). The common feature of I κ B is the possession of an ankyrin repeat domain (ARD) (Sun 2011). In an inactive state, ARD of I κ B binds to RHD of NF- κ B, which blocks the nuclear localization signal (NLS) of NF- κ B. I κ B kinase (IKK) complex

is the core molecule of the NF- κ B signalling pathway. When cells are invaded by pathogens, IKK is activated, which stimulates the phosphorylation and subsequent degradation of I κ B (Israel 2010). Three major constituent members of the IKK complex are IKK α , IKK β and IKK γ (NEMO).

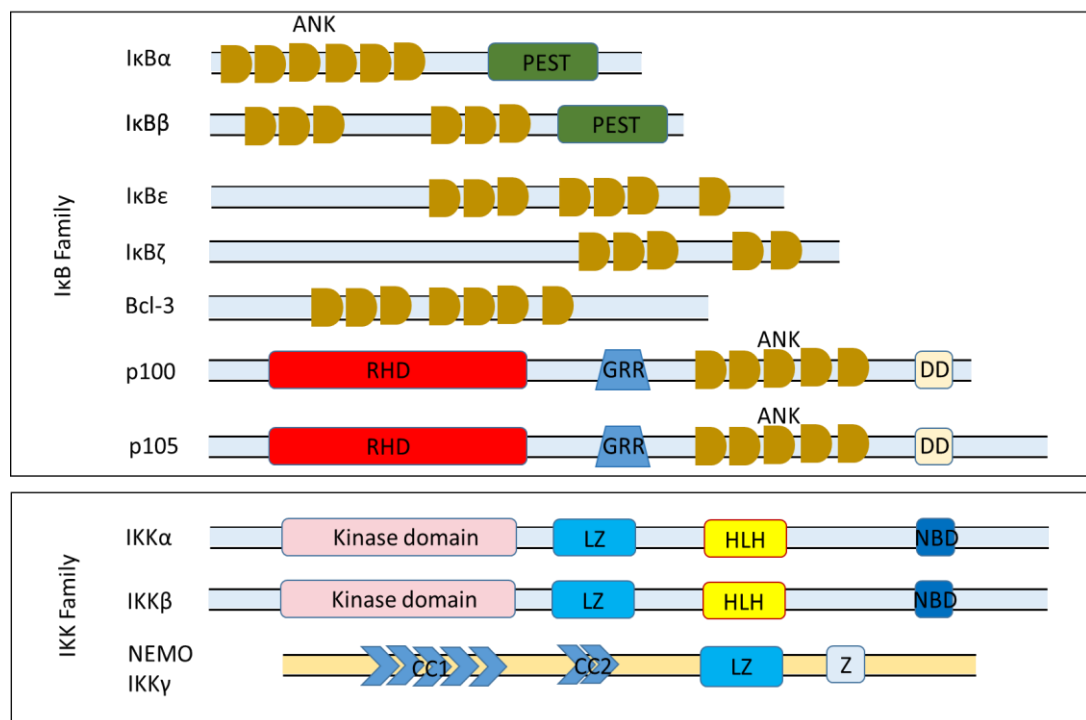


Figure 1-13. Key members of I κ B and IKK complex (Oeckinghaus and Ghosh 2009). Seven members are included in the I κ B family, the activation of I κ B indicates the activation of NF- κ B signalling pathway. IKK complex is consisted of three members: IKK α (IKK1), IKK β (IKK2) and IKK γ .

1.7 Adaptive immunity against influenza A virus infection

Innate immunity is essential to inhibit influenza virus replication in early phase of infection, and adaptive immunity is required for virus clearance and host recovery from virus infection. The initiation of adaptive immunity against IAV infection requires the maturation of antigen-presenting cells (APCs) such as dendritic cells (DCs), the matured DCs then migrate to draining lymphoid tissues to activate naïve T cells and then differentiate into effector cells (Molledo, Lopez et al. 2009). The adaptive immune response serves as the second defence line against influenza virus infection and involves in humoral immune response and cellular immune response.

In humoral immune system, influenza virus infection mainly induces specific antibodies against HA and NA (Gerhard 2001). The binding of HA-specific antibody with the trimeric globular head of viral HA protein blocks the virus attachment to host cells to neutralise virus (Kreijtz, Fouchier et al. 2011). In addition, HA-specific antibody can promote the phagocytosis of infected cells expressing Fc receptor (de Jong, Beyer et al. 2000). NA-specific antibody binds with NA to reduce the enzymatic activity of NA to further limit virus spread (Bahadoran, Lee et al. 2016). Both HA-specific and NA-specific antibodies were found to regulate antibody-dependent cell-mediated cytotoxicity (ADCC) (Kreijtz, Fouchier et al. 2011). Mucosal surface

is the primary entrance for influenza virus infection, and three immunoglobulin classes (IgA, IgM and IgG) are the key antibody isotypes in humoral immune response during IAV infection (Kreijtz, Fouchier et al. 2011). Secretory IgA is able to neutralise the viral surface proteins (HA and NA) to prevent virus from binding with host cells (Mazanec, Coudret et al. 1995). When host cells are infected, IgM is produced during primary response in lymphoid tissues which is crucial to maintain long-term memory to virus infection (Fernandez Gonzalez, Jayasekera et al. 2008). IgG is produced during second response which may occurs in periphery (van de Sandt, Kreijtz et al. 2012).

T cells (CD4⁺ and CD8⁺) and B cells are produced upon infection with influenza virus. CD4⁺ T cells are activated through recognition of virus-derived MHC class II-associated peptides on APCs and induce the expression of co-stimulatory molecules (Tamura and Kurata 2004, Kreijtz, Fouchier et al. 2011, Bahadoran, Lee et al. 2016). The co-stimulatory molecules are important to B cell activation and antibody production (Swain, McKinstry et al. 2012). CD8⁺ T cells can differentiate into cytotoxic T lymphocytes (CTL) upon activation to produce cytokines, and then to eliminate progeny virus production (Chen, Liu et al. 2018). It was shown that the activation of CD4⁺ and CD8⁺ cells was initiated by migration of matured DCs from lungs to draining lymph nodes (Ingulli, Mondino et al. 1997, Chen, Liu et al.

2018). The immune response of B cells is crucial to reduction of mortality from acute respiratory tract infections, promotion of the recovery rate and providing long-lasting protection from viral infection (Lam and Baumgarth 2019). B cells act as APCs to CD4⁺ and CD8⁺ cells, and also induce cytokines to regulate immune response (Shen and Fillatreau 2015). In addition, the antibodies generated by B cells help the elimination of virus particles and the increase of memory CD8⁺ T cells (Rangel-Moreno, Carragher et al. 2008).

1.8 Influenza A virus in pigs

Pigs have an important role in the ecology of influenza virus, especially the reassortment of influenza virus in pigs poses great risk to human health. Pigs are known as the 'mixing vessel' of influenza A virus because a) pigs are susceptible to infection with avian and mammalian influenza virus infection; b) reassortment of avian and human influenza viruses occurs in pigs; c) pigs can transmit reassorted influenza virus to human (Ma, Lager et al. 2009, Crisci, Mussa et al. 2013). Avian influenza virus has the affinity to bind with sialic acid α -2,3 and human influenza virus preferentially binds to sialic acid α -2,6 (Skehel and Wiley 2000). However, pigs can be infected by both avian and human influenza A viruses, because sialic

acids of both linkages are widely distributed in the pig respiratory tract (Ito, Couceiro et al. 1998). The resulting reassortants can subsequently transmit from pigs to humans, which could lead to the beginning of a pandemic virus.

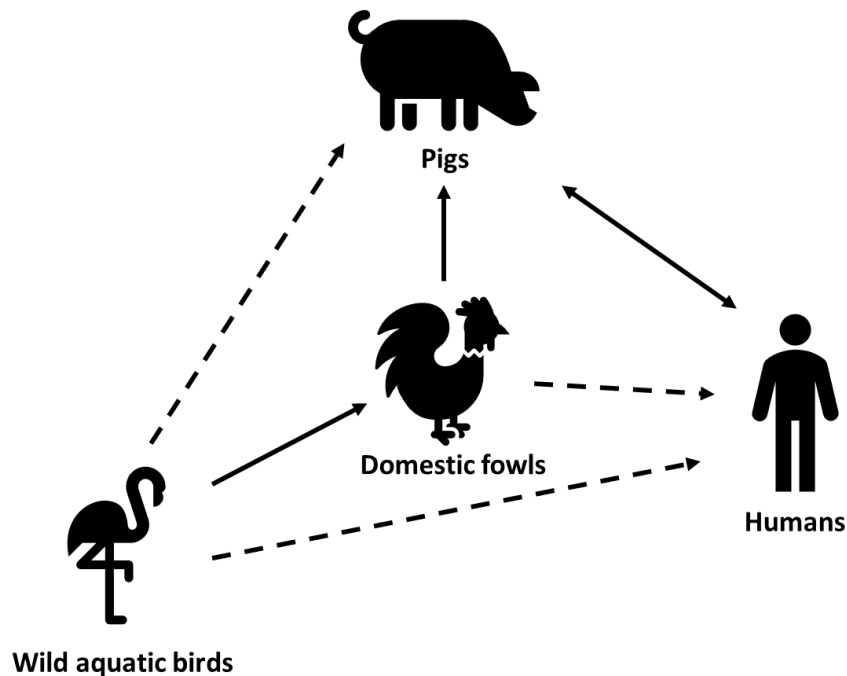


Figure 1-14. Pigs are the 'mixing vessels' of influenza A virus (Ma, Kahn et al. 2008). Wild birds are considered as the natural reservoir of influenza viruses that can infect domestic avian species. Pigs being susceptible to both avian and human influenza viruses can reassort viruses from different species.

1.9 Host response to IAV infection between human and pigs

Pigs typically display no or mild clinical signs to IAV infection and low ensuing mortality. A human-origin H5N1 virus and three avian-origin H5N1 viruses used to infect pigs at a high dose of 10^6 EID₅₀, resulted

in only low virus titres (0.33 to 0.75 EID₅₀) in the tonsil, trachea, lung and liver after 6 days of inoculation (Choi, Nguyen et al. 2005). Two avian H5N1 isolates from Japan were inoculated into chickens, quails, budgerigars, mice and pigs at 10⁸ EID₅₀; all avian species and mice died within 7 days, but all pigs survived with no clinical signs (Isoda, Sakoda et al. 2006). In another study, pigs inoculated with a human H5N1 isolate, and a chicken H5N1 isolate also exhibited mild clinical signs and modest viral titres (Shortridge, Zhou et al. 1998). All above suggest that pigs are relatively resistant to influenza A virus infection, in particular to highly pathogenic avian influenza A (HPAI) virus infection.

In contrast to pigs, humans infected with HPAI virus show severe symptoms. This is particularly true with HPAI H5N1 virus infection, where mortality rate is around 50% (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). A feature of severe influenza virus infection in humans is the development of aggressive hyperacute inflammatory response, described as 'cytokine storm' or hypercytokinemia. In a previous study, human respiratory epithelial cells and macrophages infected with HPAI H5N1 virus were found to show highly elevated cytokine and chemokine gene expression; in contrast, similarly infected pig cells displayed much weaker pro-inflammatory response (Nelli, Dunham et al. 2012). Therefore, *in*

vivo and *in vitro* evidence indicate that pigs are more resistant to IAV infection than humans.

1.10 Development of anti-influenza drugs

Current approved anti-influenza drugs broadly use virus-based antiviral strategy by targeting at different stages of influenza virus replication cycle. Antiviral drugs are used as inhibitors to block the binding of viral particles to host cell receptors, fusion of viral and host cell membranes, viral protein synthesis, viral progeny assembly and the release of newly synthesized virions (L. Eyer 2013).

In order to block the binding of viral HA with host cell receptors that contain sialic acids, several specific molecules are shown to be efficient to block the entry and the endocytosis of influenza virus. The efficient binding between chemoenzymatically synthetic Sialyllactose glycopolymers (SGP) and host cell receptors, α -2,3- and α -2,6-sialic acid, significantly reduced the replications of H5N1 and H3N2 viruses (Narla and Sun 2012). Trivalent chemicals attached with sialyllactose inhibited 85% H1N1 virus progeny production in MDCK cells by binding with host cell sialic acid receptors (Feng, Miura et al. 2010). Polyacrylamides containing α -Sialoside Groups (SA) were shown to reduce the binding affinity of influenza virus A-X31 to further reduce virus replication (Lees, Spaltenstein et al. 1994).

Viral NA surface protein facilitates the release of progeny virions. The active sites of NA between influenza A and B viruses are highly conserved, and the conserved residues tend to develop less resistance against neuraminidase inhibitors (Yen, Hoffmann et al. 2006). Therefore, targeting viral NA has been established as a key strategy for anti-influenza drug development. Several anti-influenza drugs targeting NA have been widely used for patient treatment. Oseltamivir, also known as Tamiflu, is an oral agent for influenza A and B, as well as prophylaxis treatment. Zanamivir with the product name Releza, is a intranasally administrated anti-influenza drug for influenza A and B treatment. Zanamivir was shown to have stronger antiviral effect in comparison to Oseltamivir (Cass, Efthymiopoulos et al. 1999). Some influenza strains have become oseltamivir- and zanamivir- resistant, therefore, peramivir and laninamivir have been developed for treatment of these resistant strains (Bantia, Arnold et al. 2006, Kubo, Tomozawa et al. 2010).

The highly conserved viral M2 protein is another ideal target for anti-influenza drug development. The binding of specific molecules with viral M2 protein prevents viral uncoating and blocks the transport of vRNAs into cytoplasm (Lampejo 2020). Adamantadine, the first synthetic anti-influenza drug targeting M2 discovered in 1964, was shown to have long-lasting antiviral effect up to 72h

(Davies, Grunert et al. 1964). Another known M2 inhibitor is rimantadine, and its antiviral effect was up to 90% (Barik 2012).

Viral RNA polymerase plays a crucial role in virus genome transcription and replication. Favipiravir was specifically designed to inhibit viral polymerase synthesis to further reduce influenza virus replication (Tisdale, Ellis et al. 1995). Another polymerase inhibitor is ribavirin, it was effective to provide 50% protection to mice against H1N1 virus infection (Sidwell, Bailey et al. 2005).

Drug resistance is the major limitation for anti-influenza drug development, as the use of antiviral drugs gives selection pressure for viral target proteins to cause subsequent mutations. The use of NA inhibitor, oseltamivir, caused the emergence of oseltamivir-resistant H1N1 virus since 2007 – 2009, more than 90% of the seasonal H1N1 virus strains were found to be oseltamivir-resistant in USA, Canada, UK and Australia (Dharan, Gubareva et al. 2009, Lampejo 2020). A synthetic HA inhibitor, arbidol, was shown to inhibit the early membrane fusion in H2N2 and H7N7 influenza A viruses, but the resistance to arbidol was observed with HA2 subunit single amino acid mutation (Leneva, Russell et al. 2009). It was shown that the use of M2 inhibitor adamantanes could give up to 90% protection against H1N1 virus in the 1980s, however, about 45% of influenza A viruses developed resistance to adamantanes with several amino acid mutations (L26F, V27A,

A30T/V, S31N, G34E, and L38F) (Hussain, Galvin et al. 2017, Lampejo 2020). IAV infection remains a threat to global public health, with the increasing emergence of drug-resistance influenza virus strains, it is essential to continue our research on the development for novel anti-influenza drug, so we can be prepared for the next influenza outbreak.

1.11 Overarching aims and objectives

There are two overall aims of this thesis: The first aim was to understand host innate immune response to influenza A virus infection between human and pig airway epithelial cells; The second aim was to identify potential anti-influenza drugs and to elucidate their antiviral mechanisms. The specific objectives are:

1. To understand the differences in innate immune response to NF- κ B activation between human and pig airway epithelial cells to virus infection.
2. To characterise the basal transcriptional levels of innate immune genes and to identify the key molecular factors that could play crucial roles in host innate immune response to influenza A virus infection between human and pig airway epithelial cells.

3. To exam the antiviral effect of acetylsalicylic acid and to elucidate its antiviral mechanisms between human and pig airway epithelial cells.
4. To identify a novel anti-influenza drug from screening, and to elucidate its antiviral mechanism in primary human epithelial cells.

2. NF- κ B activation in pig airway epithelial cells, but not in corresponding human cells, blocks influenza virus production

Abstract

Influenza A virus (IAV) infection poses a major threat to global health. Viral RNA is principally recognised by the pattern-recognition receptors retinoic inducible gene-1 (RIG-I) and Toll-like receptor 7/8 (TLR 7/8), each of which signals to activate two key transcription factors, nuclear factor kappa B (NF- κ B) and interferon regulatory factor 3 (IRF3) for the induction of pro-inflammatory cytokines and type I interferons, respectively. The reported roles of NF- κ B during IAV infection appear contradictory; both antiviral and pro-viral effects of NF- κ B have been described. On the one hand, NF- κ B induces production of pro-inflammatory and antiviral mediators (cytokines, chemokines and type I interferons) by the host cell to resist IAV infection. On the other hand, increased NF- κ B activity has been found to enhance IAV replication. Relative basal expression (before infection) of innate immune genes and their regulators could be a key determinant of host cell resistance or susceptibility to IAV infection. We propose that basal activity of NF- κ B or associated proteins could be an underlying factor in host species resistance to IAV infection. To this end, we compared the antiviral role of NF- κ B between pig and human primary and immortalised respiratory epithelial cells infected with IAV. Activation of NF- κ B in porcine respiratory epithelial cells conferred greater resistance to IAV infection, and induced higher phosphorylation of IRF3 and type I IFN-associated gene response. Overexpression of NF- κ B in human respiratory epithelial cells, by contrast, had limited effects on IAV infection, and IRF3 and type I IFN-associated response. This host-species difference between pig and human epithelial cells in virus resistance from NF- κ B activation is accompanied by lower overall basal transcriptional expression of NF- κ B associated genes in pig cells than in corresponding human cells.

2.1 Introduction

2.1.1 Functional contradiction of NF- κ B in influenza virus infection

NF- κ B appears to play contradictory roles during influenza virus infection: induction of an antiviral response and promotion of viral replication. In the main, NF- κ B activates antiviral factors including: IFNs, pro-inflammatory cytokines, chemokines and immuno-receptors (Wang, Basagoudanavar et al. 2010). In A549 cells, double-stranded RNA virus induced a high-level expression of IFN β and pro-inflammatory cytokines due to the activation of NF- κ B (Iordanov, Wong et al. 2001). The activation of NF- κ B, c-JUN and IRF3 co-stimulates the expression of IFN β , a principal antiviral cytokine (Honda, Takaoka et al. 2006). However, NF- κ B has been reported to also be necessary for influenza virus replication. Use of NF- κ B inhibitors BAY11-7085 and BAY11-7082 resulted in significant reduction in influenza virus output from A549 cells and U1752 cells (Nimmerjahn, Dudziak et al. 2004). Wurzer et al. (2004) showed that constitutively active IKK2 conferred an increase in virus output. The constitutive IKK2 activity was associated with NF- κ B-dependent viral induced pro-apoptotic factors, with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and FasL, acting in an autocrine and paracrine manner, respectively (Wurzer, Ehrhardt et al. 2004). Furthermore, a number of studies have shown that the

NF- κ B inhibitors Bay11-7082 or Bay11-7085 impaired influenza virus replication significantly (Kumar, Xin et al. 2008, Pauli, Schmolke et al. 2008, Pinto, Herold et al. 2011). When the IKK2 inhibitor SC75741 was applied to MDCK and A549 cells that had been infected with influenza A (H5N1 or H7N7) or influenza B viruses, the replication of both influenza A virus and influenza B virus was inhibited (Ehrhardt, Ruckle et al. 2013). SC75741 inhibited p65 subunit binding to the promoters of *IL-6* and other NF- κ B responsive genes (but not p65 phosphorylation or its nuclear translocation). This resulted in reduced expression of cytokines, chemokines and pro-apoptotic factors, such as *TRAIL*, and reduced viral activation of caspases, thus impeding caspase-mediated nuclear export of vRNP (Ehrhardt, Ruckle et al. 2013). Viral titres of H1N1 virus decreased after human A549 cells and murine primary alveolar epithelial cells (AECs) were treated with Bay11-7082, indicating that blocking NF- κ B impaired replication of IAV, and it was also demonstrated that both in primary and immortalised cells, viral titres and cytokine expression levels are impaired when NF- κ B is inhibited (Pinto, Herold et al. 2011).

To elucidate how NF- κ B regulates and promotes influenza virus propagation, Kumar et al. (2008) infected A549 cells with H1N1 virus and added the NF- κ B inhibitor PDTC. It was found that influenza virus genomic RNA was significantly reduced in the inhibitor treated

infected cells, but cRNA and mRNA were not affected, indicating that NF- κ B is involved in vRNA synthesis. In addition, siRNA knockdown of p65 resulted in a decreased replication level of influenza virus, which showed that p65 is a key molecule in the NF- κ B signalling pathway that regulates influenza vRNA synthesis (Lamb 1989, Kumar, Xin et al. 2008). Thus, from these experiments, it appears that NF- κ B activity promotes influenza virus gene expression and thus viral replication.

Host innate immune response are an important early defence against influenza virus infection. In addition to viral NS1, PA-X and PB1-F2, the NF- κ B signalling pathway also appears to counteract innate immune defences. NS1 is one of the more well-studied viral proteins. NS1 of certain influenza virus strains inhibits IRF3 activation. It is found that NS1 binds to the E3 ubiquitin ligase TRIM25 to prevent *RIG-I* signalling activation and type I IFN induction (Gack, Albrecht et al. 2009). Pauli et al., (2008) showed that type I IFN signalling is suppressed by SOCS-3 protein, which is dependent on the activation of NF- κ B. The NF- κ B signalling pathway might be a compensatory pathway when both *TLR7* and *MAVS* are absent. When *TLR7*^{-/-} *MAVS*^{-/-} knockout mice were challenged with a sublethal dose of H1N1 virus, virus replication was lower than in wild-type mice; this implies that *TLR7* and *MAVS* are essential for efficient IAV replication. However, when *TLR7*^{-/-} *MAVS*^{-/-} knockout mice were challenged with a lethal

dose of H1N1 virus, more virus replication were detected in *TLR7*^{-/-} *MAVS*^{-/-} knockout mice than wild-type mice (Pang, Pillai et al. 2013). Because the NF-κB signalling pathway supported IAV replication by producing proapoptotic genes (TNF-α and Fas-L), suppression of type I IFNs by NF-κB induced-SOCS3 and promoted influenza virus vRNA synthesis (Ludwig 2009). Therefore, the NF-κB signalling pathway may be a compensatory pathway that facilitates the replication of influenza virus. In high (lethal) dose of virus infection of mice, it was found that the *TLR7* and *RIG-I* mediated response suppressed virus replication (even though infected mice eventually died). However, with a low (sub-lethal) infection dose, *TLR7* and *RIG-I* mediated the promotion of virus replication (Pang, Pillai et al. 2013). Double knockout *TLR7*^{-/-} and *MAVS*^{-/-} mice had reduced virus titres and reduced *TNF-α*, *IL12* and *IFNβ* expression, compared with infected wild type (WT) mice. Mice deficient in *TLR7* or *MAVS* alone supported WT levels of virus titres, indicating that combined deficiencies are required to confer host resistance. Overall, the gradation of *TLR7* and *RIG-I* activation appears critical to the outcome of influenza virus infection: a pro-viral outcome with low (sub-lethal) infection dose, and antiviral outcome with high (lethal) infection dose. It remains to be determined if any of the mechanisms suggested for NF-κB signalling could also be responsible for *TLR7*- and *MAVS*-dependent promotion or inhibition of virus replication.

Taken together, following influenza infection of a host cell, the NF- κ B signalling pathway appears to confer both antiviral and pro-viral functions. On the one hand, the activation of NF- κ B induces pro-inflammatory mediators (cytokines, chemokines and type I IFNs) to clear virus; on the other hand, the inhibition of NF- κ B activity impairs the vRNA synthesis of influenza virus and reduces its infectivity. These findings indicate the complexity of the role of the NF- κ B signalling pathway during influenza infection. The relative basal expression of innate immune genes in pigs and humans, regulated by NF- κ B, prior to infection, could be a key innate determinant of host resistance or susceptibility to IAV infection. Thus, an overarching hypothesis is that the relative basal transcriptional activity of NF- κ B associated genes is a major underlying innate immune factor in dictating the outcome of IAV infection in humans and pigs.

2.1.2 Aims and objectives

The main aim of this thesis is to understand the differences in innate immune response to NF- κ B activation between human and pig cells with IAV infection. The ensuing objectives are:

1. To examine the virus output outcomes between human and pig airway epithelial cells in response to NF- κ B activation.

Primary normal human bronchial epithelial cells (NHBE) and corresponding human cell line (A549 cells), primary porcine tracheal epithelial cells and corresponding porcine cell line (NPTr cells) were used to study the host cell innate immune response to IAV infection after NF- κ B activation. Virus progeny output was analysed by focus forming unit (FFU) assay and mean tissue culture infectious dose (TCID₅₀) assay, and host gene response and host protein response were investigated in the different cell types.

2. To determine basal transcriptional levels of innate immune genes between human and pig primary cells.

The host cell innate immune response to influenza virus infection is likely to be affected by basal levels of gene expression prior to infection. To this end, a microarray transcriptomic approach was used to establish basal gene expression profiles of the two species.

2.2 Materials and Methods

2.2.1 Viruses and cells

Two human isolated H1N1 strains: A/California/2009 (H1N1) and A/USSR/1977 (H1N1) were used in this study. Viruses were propagated in 10-day-old embryonated hen's eggs, and viruses were harvested 72 h after inoculation. Harvested viruses were then aliquoted and stored at -70°C before use. Adenocarcinomic human alveolar basal epithelial (A549) cells, newborn pig tracheal (NPTr) cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum and 1 unit penicillin/streptomycin. Primary normal human bronchial epithelial (NHBE) cells were purchased from Promocell. Three NHBE cells donors were all never smokers, and named as donor 1, donor 2 and donor 3, respectively. Donor 1, 2 and 3 remain the same numbering throughout the thesis. Primary pig tracheal epithelial cells (PTECs) were collected from stripped tracheobronchial mucosae from 3- to 4- month-old pigs. NHBE cells and PTECs were grown in airway epithelial cell medium (PromoCell). Cells were incubated at 37°C with 5% CO₂ in air.

2.2.2 Transfection and infection

Four different plasmids were purchased from Addgene. IKK2 CA (constitutively active I κ B kinase 2) was used to increase the expression of NF- κ B activity; IKK2 inactive was used to reduce the activity of IKK2 to further reduce NF- κ B; RelA-GFP was used to increase NF- κ B activity and determine transfection efficiency, and kc2587 was used as a control vector (Table 2-1). A549, NPTr, NHBE cells and PTECs were seeded into 12-well plates. Cells were transfected with 1 μ g of each plasmid using 2 μ L Lipofectamin 2000 (Thermo Fisher Scientific) transfection reagent for 6 h. The transfected cells were then washed with PBS for three times. The cell lines (A549 and NPTr cells) were maintained with DMEM and primary cells (NHBE cells and PTECs) were maintained in airway epithelial cell medium (PromoCell) for 48 h. Transfected cells were then infected with USSR H1N1 virus and pandemic (PDM) H1N1 virus diluted in Ultraculture medium (Lonza, UK) or airway epithelial cell medium (PromoCell) supplemented with 500 ng/mL L-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma) for 2 h, cells were washed with PBS three times after 2 h. Cell lines were maintained in Ultraculture (Lonza) medium containing 500 ng/mL TPCK and primary cells were maintained in airway epithelial cell medium (PromoCell) containing 250 ng/mL TPCK for 24 h. Spun

down supernatants were collected for progeny virus titration and cells were harvested for total RNA or protein extraction.

Table 2-1. Plasmids used in this study.

Plasmids	description	purpose
kc2587	control plasmid	negative control
IKK2 CA	constitutively active I κ B kinase 2	activate NF- κ B pathway
IKK2 inactive	loss of kinase activity and with effect on binding to NIK	inactivate NF- κ B pathway
RelA-GFP	GFP tagged plasmid	activate NF- κ B pathway

2.2.3 Virus titration by immunocytochemistry assay

MDCK cells were seeded into flat bottom 96-well plates and allowed to grow until 100% confluent. MDCK cells were washed with PBS three times to remove DMEM medium and then infected with collected virus supernatant (diluted with Ultraculture medium at desired dilution) for 2 h. Cells were then washed with PBS and maintained with Ultraculture medium for 4 h and fixed with acetone-methanol (1:1 ratio) for 10 mins. Immunocytochemistry assay was performed by detecting viral NP protein expression. The fixed MDCK cells were washed with Tris buffered saline (TBS) and non-specific binding blocked with 2.5% normal goat serum (Vectorlabs) for 20 mins. Cells were washed once with TBS and incubated with 1:6000 primary mouse monoclonal antibody to influenza virus nucleoprotein

(Abcam) for 1 h at room temperature. Cells were washed three times with TBS and incubated with a polyclonal anti-rat IgG (Vectorlabs) as secondary antibody for another 1 h. After this, cells were rinsed with TBS for three times and incubated with 3,3'-diaminobenzidine (DAB) substrate-chromogen reagent for 5 mins, cells were then washed with TBS for three times and maintained with TBS for microscope imaging. Cells which expressed viral NP (cells with green nuclei) were counted under microscope with 100 times magnification and calculated focus forming units (ffu) of virus per microlitre. $\text{Ffu}/\mu\text{l} = (30A)/x$, A=number of positive cells per field, and x=volume of supernatant used.

2.2.4 Virus titration by the Mean Tissue Culture Infectious Dose (TCID₅₀) assay

MDCK cells were seed into 96-well plates until 70% confluent, cells were then infected by 10-fold serial diluted spun supernatants. Each dilution had 5 replicates. The cultured media were harvested after 48 h and tested for haemagglutination (HA) activity with 0.5% chicken blood cells. The HA activity is shown as log₁₀ (TCID₅₀/mL) and the computation was based on Reed and Muench.

2.2.5 Multiplicity of infection (MOI) determination

The multiplicity of infection (MOI) for stock H1N1 viruses was determined in MDCK cells. MDCK cells were seeded into flat-bottom 96-well plates prior to infection until fully confluent. MDCK cells were then washed with PBS and infected with serial diluted stock viruses harvested from embryonated hen's eggs for 2 h. Cells were washed with PBS for three times and cultured in infection media for 4 h. MDCK cells were then washed and proceeded for immunocytochemistry assay as mentioned above. If 95% to 100% cells were positive for viral NP protein, the amount of virus used in dilution was then considered as 1.0 MOI.

2.2.6 RNA extraction

Cells were lysed by Buffer RLT (QIAGEN) with supplement of beta-mercaptoethanol. Cell lysates were then homogenized by QIAshredder (QIAGEN) for a 1 min 13,000 rpm in benchtop centrifuge at room temperature. Total RNA was extracted by RNeasy Plus Mini Kit (QIAGEN) followed the instructions. Extracted total RNA was then reverse transcribed to cDNA by using SuperScript III first-strand cDNA synthesis kit (Invitrogen). Total of 500 ng RNA was transcribed following the steps below:

1. Add diethylpyrocarbonate (DEPC) H₂O to 500 ng RNA to a final volume of 13.5 µL;
2. Add 1 µL of random hexamers and 1 µL of 10 mM dNTPs;
3. 65°C for 5 mins and then ice incubation for 1 min;
4. Add 4 µL of RT buffer, 1 µL of dithiothreitol (DTT), 1 µL of RNase OUT and 0.5 µL of SuperScript III reverse transcriptase;
5. Incubate the mixture at room temperature for 15 mins;
6. 50°C for 1 h;
7. 70°C for 5 mins to stop the reaction.

cDNA was then diluted by DEPC H₂O 50 times and stored at -20°C freezer for real-time PCR detection.

2.2.7 Real-time PCR amplification

Real-time PCR (qPCR) was performed by using QuantiNova SYBR Green PCR Kit (QIAGEN) followed by the manuscript instruction. Total of 20 µL, including 5 µL of 1:50 diluted cDNA, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 3 µL of DEPC H₂O and 10 µL of SYBR green were mixed and loaded into 96-well plate for host gene expression detection. The qPCR was performed by using a LightCycler-96 instrument (Roche). Each cDNA sample for each gene expression had four replicates, and the gene expression was normalised by the *18S ribosomal RNA* for comparison. The primers

were synthesized by Sigma-Aldrich, and the sequences are as described in Table 2-2.

Table 2-2. Primers used in this study.

Gene	Human		Pig	
	Forward primer (5' to 3')	Reverse primer (5' to 3')	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>18s rRNA</i>	ACGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	Universal primer (same with human)	Reverse primer (5' to 3')
<i>A20</i>	TGGGCTTAAGGGTGTCTGAG	CTTTCTAAGGCCACCTGGGA	CTTCAGCACCCCTCAAGGAGA	AGTCCAATTCCGGGTGTCAT
<i>HES1</i>	GCTCGGGCATTCCAA	AGGTGCTTCACTGTCAATTC	GCTCGAGGCATTCCAAGCT	CGGAGGTGCTTCACTGTGTCAT
<i>HOXB9</i>	TCCCTTCCCCCTGCAA	CGTCCAGAAATGGACATTCTC	TGTCCATTTCTGGGACGCTTA	GCGTCTCACTCTCGTGACTT
<i>IKBKAP</i>	TACCCTGCACCAGTTACCAG	TGCTCTCATAGCATCGAGA	TCACCTGGGTTCAAGGAGAC	TGCAGTGTCTACTGACTTGA
<i>IKKB</i>	CGGCTTCCAACAGCTTACC	GCTTCAGCCACAGTTCTTCA	CTGCCCCGACAGTTTACC	GCTTCAGCCACGAGGTCTTC
<i>IL6</i>	CCAGGAGCCAGCTATGAAC	CCAGGGAGAAGGCAACTG	CGAGCCTTGAGGATTTCC	CAGGTGCCCCAGCTACATTATC
<i>MAVS</i>	GTCAGCCTCCAGGGATCTAC	CCTAGGGCAGTGGAGTGAAA	CTTACCTGCTCGCTCACA	TTCGTACGAGATCAGACA
<i>MCP1</i>	GCTCAGCCAGATGCAATCAA	ACAGATCTCTTGGCCACAA	GAAGAGTCACCAGCAGCAAG	GTCCAGGTGGCTTATGGAGT
<i>NFKBIA</i>	AAGACGAGGAGTACGAGCAG	ACTTCCATGGTCAAGTGCCTT	GGAGTACGAGCAGATGGTGA	TTCATGGTCAAGTGCCTTCT
<i>NGFR</i>	CCTTGCAACACACAGACACA	CCAGTCTCAGCCCAAGAGAA	GGCTACTACCAGGACGAGAC	GCACACGGGTGTTCTGCTTAT
<i>OAS1</i>	TCCAAGCTCAGTCAGCAGAA	CAGGAGAGAGAGTGGCAACA	GAGCTGCAGCGAGACTTCT	GGCGGATGAGGCTCTTCA
<i>PDCD4</i>	CTGGATGTCCACATTACTCT	TTCAGCCTGAAAACATTCTCT	TCCACATTTCATCTCCATGCT	GGAAATTTATCCGGCCTGAAA
<i>RIG-I</i>	GAAGGCATTGACATTGCACAGT	TGGTTGGATCATTTTGATGACA	CCCTGGTTAGGGACGATGAG	AACAGGAACTGGAGAAAAGTGA
<i>RNF19B</i>	CAGCCAGTCTGGTGCAATTAG	TGGAACCGCCATAGCA	Universal primer (same with human)	
<i>SLC7A11</i>	TCCATGAACGGTGGTGTTT	ACCTCTCGAGACGCAACAT	CTCCATGAATGGTGGTGTTT	CCCTCCCGAGATGCAACAT
<i>TDP2</i>	GCATGTGAACGTGCAGGAA	ACTCTGGAGCCTCTTGCAAT	GTGTGTGCATGTGAGTGTGT	ACTCTGGAGCCTCTTGCAAT
<i>TNFRSF1A</i>	ATCCCCAACCCCTTCAG	TGTCTAGGCTCTGTGGCTTG	CCAGCCACATCTGAAACTCG	GCCAGGAAAGACCAAGAA
<i>TNFRSF1B</i>	CAGTCTTGCTGCTGCTG	TCAACTTACCGACCTTGCT	CTCGGTTTCTGCGTGCTTA	GGAGTGCAGGCTTGAGTTTC
<i>TP53</i>	CTGGCATTTGCACCTACCTC	TAACCAGCTGCCCAACTGTA	ATGAGCGCAGCTCTGACTAT	CCATGCAGAGCTGTTACAC
<i>USP13</i>	GTCTGAAGAACCTGGGCAACA	TGCTGAAGATGGCCTGCAT	CCTCAAGAACCTGGGCAACA	TGCTGAAGATGGCCTGCAT
<i>Viral M gene</i>	AGATGAGCCTTCTAACCGAGGTG	TGCAAAAACATCTTCAAGTCTCTG	Universal primer (same with human)	

2.2.8 Protein extraction and protein quantification

Cells were seeded into 12-well plates, and total protein was extracted using radio-immunoprecipitation (RIPA) Lysis Buffer System (Santa Cruz) following the product instructions. In brief, cells in 12-well plates were washed with chilled PBS for 3 times, and then 56 μ L of lysis buffer (includes 1% Phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor cocktail and 1% 100 mM sodium orthovanadate) added to the cells for 10 mins at 4°C. Cell lysates were then transferred to 1.5 mL tubes and centrifuged at 13000 rpm for 15 min. Proteins in RIPA buffer were then aliquoted and stored in -80°C. Protein quantification was performed by using Coomassie Plus (Bradford) Protein Assay Kit (Thermo Scientific Pierce). Protein samples were diluted 100-fold in distilled H₂O. Bovine serum albumin (BSA) at 2 mg/mL was used as protein standard and diluted into serial dilutions. The diluted samples and protein standards were then mixed with Coomassie Plus reagent (1:1 ratio), total of 280 μ L of each sample or protein standard was loaded in triplicate into 96-well plates, and the absorbance was measured at 595 nm by spectrophotometer Fluostar Optima (BMG Labtech).

2.2.9 Western blotting

Total of 5 µg of each sample was mixed with 1.5 µL of Sample reducing buffer (Invitrogen), 5 µL of NuPAGE lithium dodecyl sulfate (LDS) buffer (Invitrogen) and H₂O in a total volume of 15 µL. The mixed cocktail was heated at 70°C for 10 mins and loaded into 1.0 mm, Bis-Tris, 4-12% 12-well NuPAGE Gel (Invitrogen). Each protein sample was loaded twice, and 5 µL of iBright Prestained Protein Ladder (Thermo Fisher) was loaded for the protein size determination. Gel was run at 200 volts (V) and 115 miliamps (mA) in 1:20 diluted Nupage Running buffer (Invitrogen) for 50 mins. Semi-dry method was used for gel transfer. Polyvinylidene fluoride (PVDF) transfer membrane (Thermo Fisher), layers of filter papers and gel were assembled, Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) was set at 10 V and 79 mA for the 1-hour transfer. After 1 h, proteins were transferred to PVDF membrane, the membrane was then washed by Tris-Buffered Saline and Tween 20 (TBST) and blocked by 5% BSA (Sigma) for 1 h. PVDF membrane was washed by TBST for three times (5 mins each) and incubated with primary antibodies diluted at desired concentrations overnight at 4°C. After primary antibody incubation, the membrane was washed three times with TBST and incubated with secondary antibodies at desired dilutions for an hour. The membrane was washed three times with TBST again and added with 1 mL of ECL Select western Blotting

Detection Reagent (Amersham) for a five-minute reaction. The reagent was then removed from the membrane, and the membrane was wrapped with cling film. ChemiDoc Gel Imaging System was used for the final imaging of the membrane. Antibodies used in this study are listed in table 2-3.

Table 2-3. Antibody details.

	Antibody	Molecular Weight (kDa)	Dilution	Supplier
Primary antibody	Phospho-NF- κ B p65(Ser536) (93H1) Rabbit mAb	65	1:1000	Cell Signaling
	NF- κ B p65 (D14E12) Rabbit mAb	65	1:1000	Cell Signaling
	Phospho-IRF-3 p65(Ser396) (D6O1M) Rabbit mAb	45-55	1:1000	Cell Signaling
	IRF-3 p65(D83B9) Rabbit mAb	45-55	1:1000	Cell Signaling
	β -Tubulin (9F3) Rabbit mAb (HRP Conjugate)	55	1:1000	Cell Signaling
	Influenza A NP Rabbit Polyclonal Antibody	56	1:3000	ThermoFisher
	Influenza A M1 Goat Polyclonal Antibody	27	1:2000	Abcam
Secondary antibody	Anti- β -Actin Mouse mAb	42	1:10000	Sigma
	Anti-rabbit IgG (HRP-linked)	-	1:2000	Cell Signaling
	Anti-mouse IgG (HRP-linked)	-	1:2000	Cell Signaling
	Anti-goat IgG (HRP-linked)	-	1:10000	Santa Cruz

2.2.10 Microarray analysis

Two microarray studies were performed: (i) basal transcriptional comparison between NHBE cells and PTECs; (ii) transcriptional changes by overexpression of IKK2 CA in NPTr cells. For the basal transcription comparison study, four biological replicates of NHBE cells and PTECs were seeded into 6-well plates, the cells were then cultured with airway epithelial medium until fully confluent for RNA extraction. For the IKK2 CA overexpression study in NPTr cells, the cells were seeded into 12-well plate and transfected with 1.0 μ g

control vector or IKK2 CA vector for 6 h. Cells were then washed with PBS and cultured in DMEM media for 2 days for RNA extraction. Each transfection had three replicates, in a total of 6 samples. RNA samples were then processed and analysed using Human Gene 2.1 Array Strips (#902114; Affymetrix) and the microarray system, GeneAtlas from Affymetrix. Partek Genomic Suite 6.6 software was used for data analysis and figure generation. The RNA integrity number (RIN) was measured using Agilent 2100 Bioanalyzer. All RNA preps showed $RIN \geq 9.8$, where >7 is the accepted value.

2.2.11 Generation, fragmentation and hybridization of the Sense cDNA on the Array strips

All steps of sense cDNA synthesis, fragmentation and hybridization were performed according to the manufacturer's protocol (GeneAtlas WT Expression Kit User Manual). The resulting fragmented sense cDNA was assessed using the Agilent 2100 Bioanalyzer before hybridisation to the Array Strips. Hybridisation, washing, and scanning were carried out automatically by the GeneAtlas microarray system.

2.2.12 Microarray data analysis

Gene Expression Profile data was generated as CEL files and subjected to analysis by the Partek Genomics Suite 6.6 software. The list of genes of interest comprised genes upregulated or downregulated by at least 2 fold with FDR (false discovery rate)-adjusted p value <0.05.

2.2.13 Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) software (QIAGEN) was used for microarray shortlisted gene analysis. For the basal comparison study between NHBE cells and PTECs, genes of interest that were upregulated or downregulated by at least 2-fold were further filtered. Genes that were upregulated in NHBE cells by at least 4-fold in NHBE cells or upregulated in PTECs by at least 2.5-fold with FDR (false discovery rate), and adjusted p-value<0.05 for selection of genes of interest. In the overexpression of IKK2 CA in NPTr cells study, genes that were upregulated or downregulated by at least 2-fold with FDR by overexpression of IKK2 CA were used for IPA analysis. Shortlisted gene names along with the gene IDs, corresponding fold changes and p-values were imported to IPA for core analysis. Subsequent analysis was carried out by IPA (canonical pathways, upstream regulators, diseases and functions and other functions).

2.2.14 Statistical analysis

All data were analysed by GraphPad Prism 7.0 (GraphPad Software, USA). Significance was determined by one-way ANOVA and two-way ANOVA. Levels of significance were shown in figures, $0.01 < P < 0.05$ was given one asterisk, $0.001 < P < 0.01$, $0.0001 < P < 0.001$ and $P < 0.0001$ were given two, three and four asterisks, respectively. $P > 0.05$ was considered not significant.

2.3 Results

2.3.1 IKK2 CA overexpression comparably activates NF- κ B in human and pig epithelial cells

Expression of p65 and phospho-p65 proteins in human (primary normal bronchial epithelial [NHBE] and human alveolar adenocarcinoma A549) and pig (primary pig tracheal epithelial [PTEC] and immortalised neonatal pig tracheal [NPTr]) cells over-expressing IKK2 CA was assessed by western blotting. The expression profiles of total p65 and phospho-p65 proteins in human and pig cells over-expressing IKK2 CA were similar (Figure 2-1 and Figure 2-2). IKK2 CA overexpression had no consistent effect on the levels of p65 and phospho-p65 in cells of both host species during infection. However, IKK2 CA overexpression consistently increased the basal level of phospho-p65 in uninfected human and pig cells without concomitant increase in total p65 protein which indicates that IKK2 CA overexpression comparably activated NF- κ B in uninfected human and pig cells (Figure 2-1 and Figure 2-2).

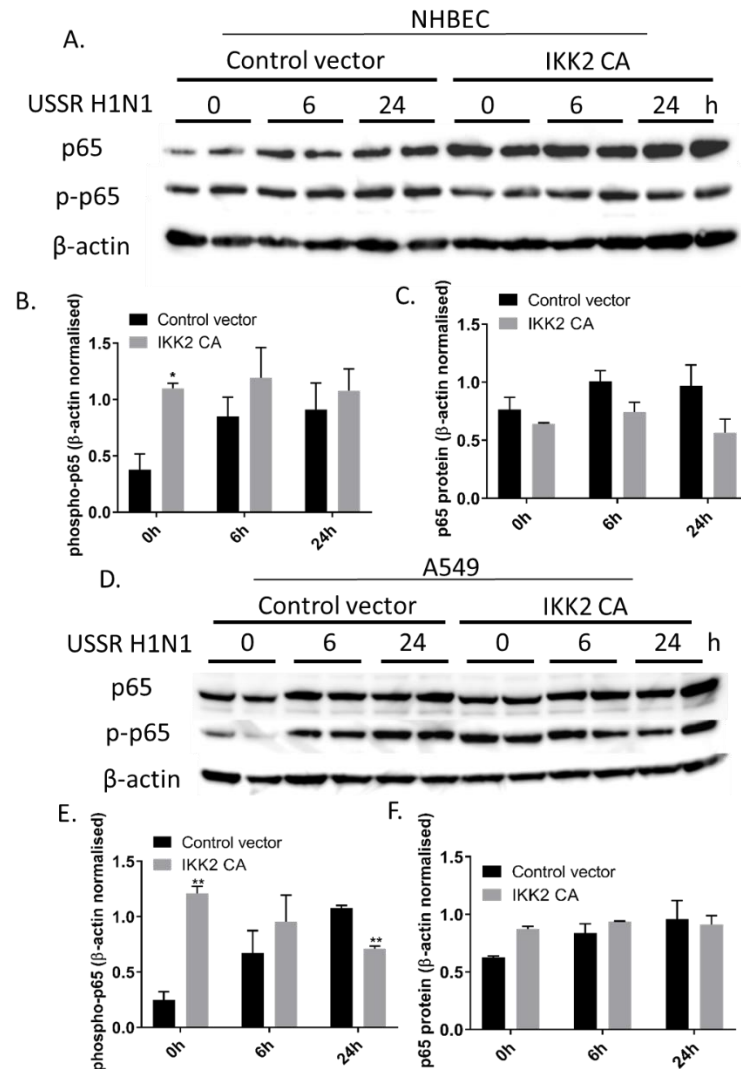


Figure 2-1. Overexpression of IKK2 CA increased NF-κB activity in uninfected human (NHBE and A549) cells. Cells were separately transfected with IKK2 CA or control vector; two days post-transfection, cells were harvested for protein lysates (0 hpi) or infected with 1.0 MOI USSR H1N1 virus and protein lysates were harvested at 6 and 24 hpi. Each protein sample was loaded in duplicate at 5 μg/well. Significance is relative to control vector at each corresponding time point (2-way ANOVA with Sidak's multiple comparisons). (A) p65, phospho-p65 and β-actin protein detection in NHBE cells. (B) phospho-p65 protein expression normalised to β-actin in NHBE cells. (C) Total p65 protein expression normalised to β-actin in NHBE cells. (D) p65, phospho-p65 and β-actin protein expression in A549 cells. (E) phospho-p65 protein expression normalised to β-actin in A549 cells. (F) Total p65 protein expression normalised to β-actin in A549 cells. Results shown representative of three independent experiments. Levels of significance are shown in figures, 0.01 < P < 0.05 was given one asterisk, 0.001 < P < 0.01 was given two asterisks.

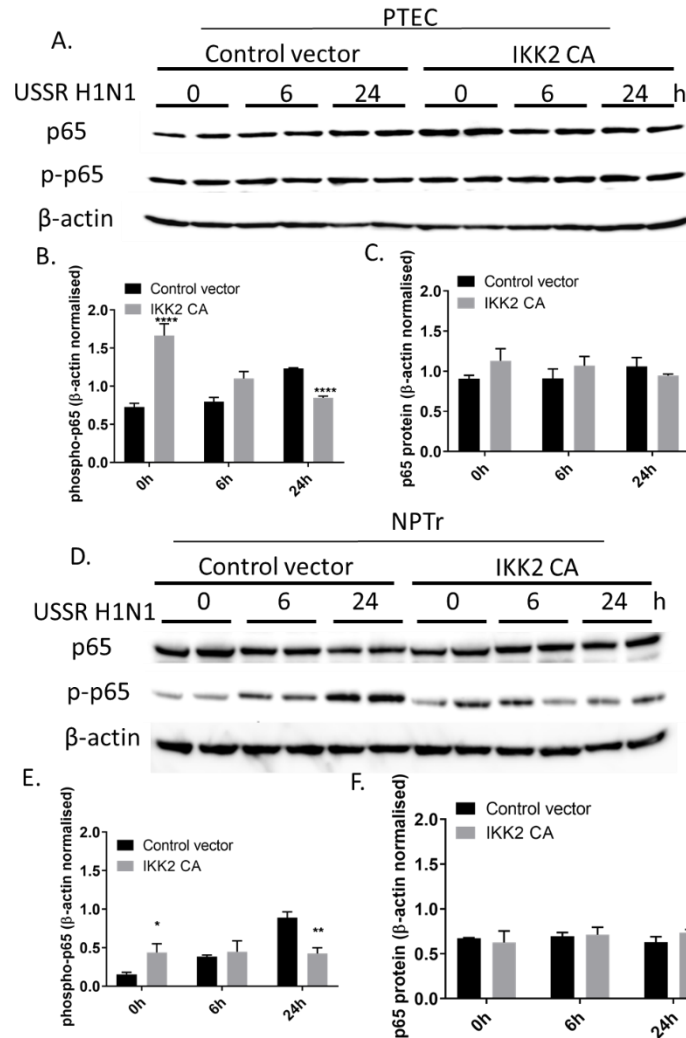


Figure 2-2. Overexpression of IKK2 CA increased NF-κB activity in uninfected pig (PTECs and NPTTr) cells. Cells were transfected with IKK2 CA or control vector; 2 days post-transfection, cells were harvested for protein lysates (0 hpi), or infected with 1.0 MOI USSR H1N1 virus and protein lysates were harvested at 6 and 24 hpi. Each protein sample was loaded in duplicate at 5 μg/well. Significance is relative to control vector at each corresponding time point (2-way ANOVA with Sidak's multiple comparisons). (A) p65, phospho-p65 and β-actin protein in PTECs. (B) phospho-p65 protein expression normalised to β-actin in PTECs. (C) Total p65 protein expression normalised to β-actin in PTECs. (D) p65, phospho-p65 and β-actin protein expression in NPTTr cells. (E) phospho-p65 protein expression normalised to β-actin in NPTTr cells. (F) Total p65 expression normalised to β-actin in NPTTr cells. Results shown representative of three independent experiments. Levels of significance are shown in figures, 0.01 < P < 0.05 was given one asterisk, 0.001 < P < 0.01 and P < 0.0001 were given two and four asterisks, respectively.

2.3.2 IKK2 CA induces dissimilar NF- κ B-inducible gene response to infection between human and pig epithelial cells

NF- κ B responsive genes *IL6* and *MCP1* were used as exemplars to assess host gene response to IKK2 CA overexpression. *IL6* expression is directly linked with NF- κ B pathway as the activation of NF- κ B family induced robust *IL6* expression (Nan, Wang et al. 2018). *MCP1* is monocyte chemoattractant protein that leads to proinflammatory response. It was shown that activation of NF- κ B protein can lead to *MCP1* activation in human peripheral blood monocytes, as well as primary normal human epidermal keratinocytes (NHEKs) and human HaCaT cells (Tang, Liao et al. 2017, Lin, Ye et al. 2018). In the absence of infection, overexpression of IKK2 CA significantly increased basal *IL6* expression in human and pig epithelial cells (Figure 2-3A and C) relative to corresponding uninfected vector controls. Basal *MCP1* expression was also increased by IKK2 CA overexpression in all four uninfected cell types (Figure 2-3B and D).

USSR virus infection of human and pig cells, overexpressing IKK2 CA or vector control, consistently increased *IL6* and *MCP1* expression relative to corresponding uninfected controls (Figure 2-3A and B); with the exception of *IL-6* expression in uninfected-control vector A549 cells relative to infected-control vector A549 cells where no difference was found (Figure 2-3). Although PTECs and NPTr cells

overexpressing IKK2 CA and infected with USSR H1N1 virus also showed increase in *IL6* and *MCP1* expression relative to corresponding uninfected controls (Figure 2-3C and D), unlike human cells, *IL6* and *MCP1* expression in infected-vector control PTECs and NPTr cells was consistently higher than corresponding infected-IKK2 CA cells (Figure 2-3C and D). Thus, in pig cells (PTECs and NPTr cells), transcriptional up-regulation of NF- κ B genes, *IL6* and *MCP1*, in response to USSR virus infection was attenuated by IKK2 CA overexpression, which suggests that pig cells mount a less vigorous downstream gene response to NF- κ B activation than that of human cells.

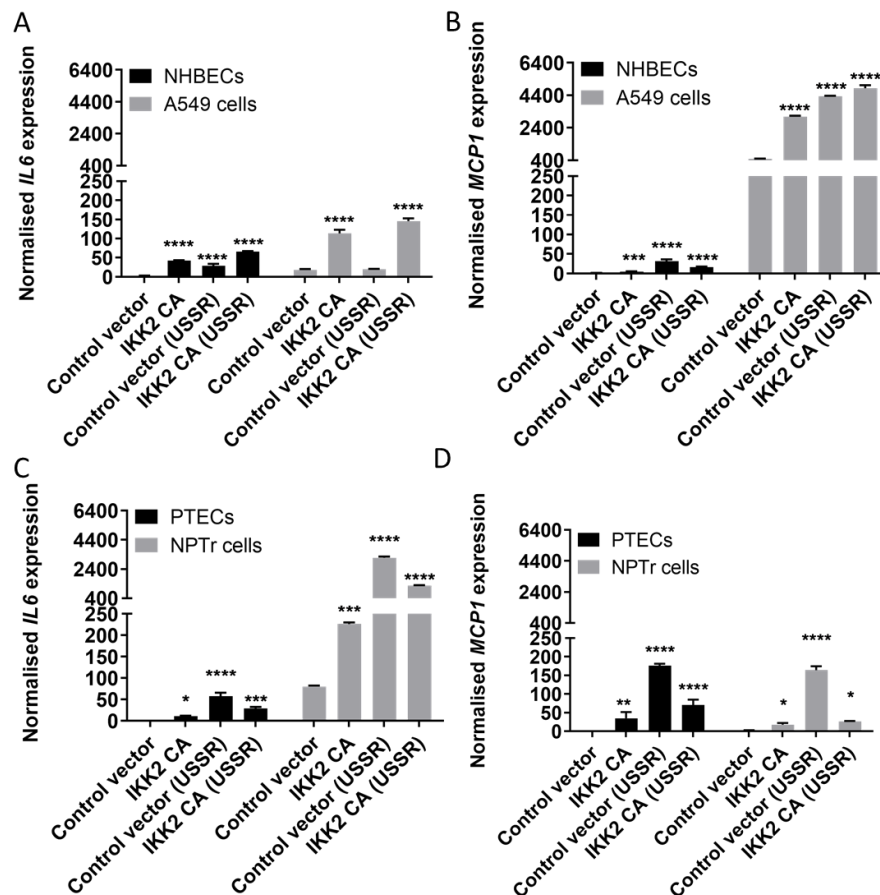


Figure 2-3. IKK2 CA induced transcription of *IL6* and *MCP1* genes in uninfected human and pig cells but during infection IKK2 CA consistently attenuated the up-regulation of NF- κ B responsive genes in pig cells. Cells were transfected with IKK2 CA or vector control; 48 h post-transfection cells were kept uninfected or infected with 1.0 MOI USSR H1N1 virus for 24 h. Cell RNA lysates were harvested for gene expression analysis, normalised to *18S rRNA*. Significance is relative to corresponding uninfected vector control (2-way ANOVA with Dunnett's multiple comparisons). (A) *IL6* and (B) *MCP1* expression in NHBE cells and A549 cells. (C) *IL6* and (D) *MCP1* expression in PTECs and NPTr cells. Based on the use of relative Ct computation, comparisons between human and pig cells can be made. Results shown representative of three independent experiments. Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, $0.001 < P < 0.01$, $0.0001 < P < 0.001$ and $P < 0.0001$ were given two, three and four asterisks respectively.

2.3.3 NF- κ B activation in pig cells blocks influenza virus production, but has no consistent viral effect in human cells

The antiviral effects of NF- κ B activation on IAV were examined in pig and human respiratory epithelial cells. NHBE, A549, PTEC and NPTr cells were separately transfected with different expression plasmids (IKK2 CA, IKK2 inactive and RelA-GFP), and after 48 h were infected with USSR H1N1 virus or PDM H1N1 virus, each at 1.0 MOI for 24 h. Supernatants were then collected and titrated on MDCK cells based on 6 h focus forming assays (FFAs). In primary and immortalised pig epithelial cells, NF- κ B activation from separate over-expression of IKK2 CA and RelA-GFP blocked USSR H1N1 and PDM H1N1 progeny virus output (Figure 2-4A and B). In the two human cell types, the antiviral effects of IKK2 CA and RelA-GFP over-expression were inconsistent compared with pig cells; there was some reduction in USSR H1N1 virus production but to a lesser extent than pig cells, but PDM H1N1 virus output was either unaffected (in A549 cells) or increased (in NHBE cells) from individual IKK2 CA and RelA-GFP overexpression (Figure 2-4C and D). Likewise, the effects of overexpression of IKK2 inactive on progeny virus production were inconsistent and variable in human cell types. Whereas, separate over-expression of IKK2 CA and RelA-GFP was consistently antiviral in both pig epithelial cell types but not in human cells.

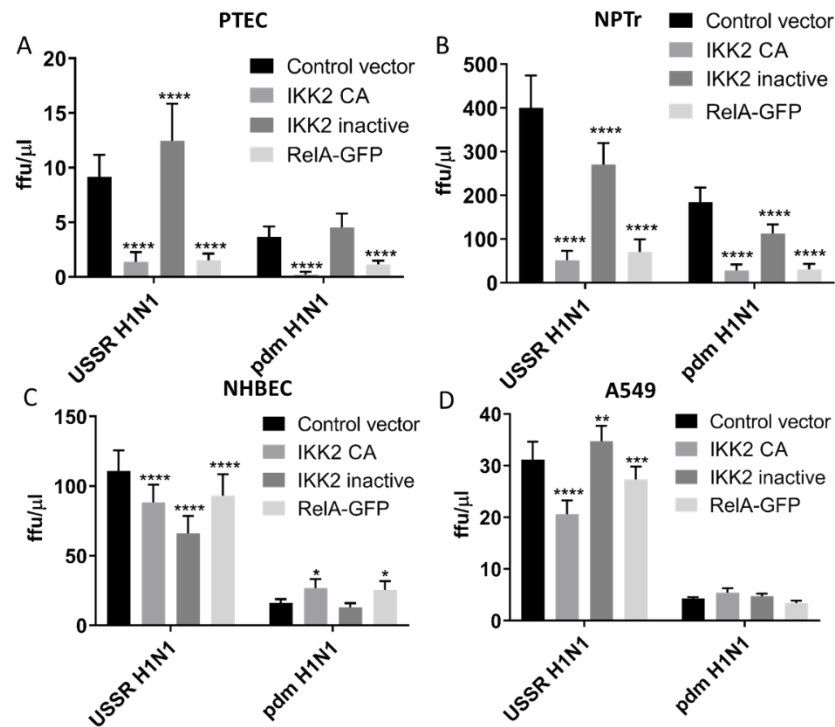


Figure 2-4. Activation of NF- κ B, from IKK2 CA and RelA-GFP over-expression in (A) PTEC and (B) NPTr cells consistently conferred strong reduction in progeny output of human USSR N1N1 virus and PDM H1N1 virus, but in NHBE (C) and A549 (D) cells activated NF- κ B had variable effects on virus replication. Cells were transfected with indicated plasmids for 2 days and subsequently infected with USSR or PDM virus, each at 1.0 MOI (based on 6 h FFAs) for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in FFAs. Results shown are representative of three independent experiments. Significance is relative to corresponding control vector within USSR H1N1 or pdm H1N1 group (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, $0.0001 < P < 0.001$ and $P < 0.0001$ were given three and four asterisks, respectively.

To further demonstrate the differential effects of activated NF- κ B on pig and human epithelial cells, time-course virus output was performed, based on median tissue culture infectious dose (TCID₅₀) assays, on harvested media from the four cell types (NHBE, A549,

PTEC and NPTr cells) which were transiently transfected with IKK2 CA or control vector and after 2 days, the cells were infected with 1.0 MOI of USSR H1N1 virus. Supernatants collected at 3, 6, 12 and 24 h post infection (hpi) were subjected to TCID₅₀ assays using haemagglutination as a read-out for virus presence in the media of reference MDCK cells infected for 48 h with serially diluted supernatants. Consistent with earlier FFA results, overexpression of IKK2 CA significantly reduced progeny virus output in PTECs at 3, 6, 12 and 24 hpi (Figure 2-5A); in NPTr cells, virus output was significantly lower at 12 and 24 hpi, notice that virus output is shown in log change, which indicates that ~100-fold reduction of virus output by IKK2 CA overexpression in NPTr cells at 24 hpi (Figure 2-5B). Overexpression of IKK2 CA in NHBE and A549 cells, however, had no significant effect on virus replication over the 24 h time course (Figure 2-4C and 4D). In summary, although IKK2 CA overexpression activated NF- κ B in human and pig cells, NF- κ B activation by itself did not necessarily indicate virus inhibition; the associated antiviral response was clearly demonstrable in pig cells but not human cells.

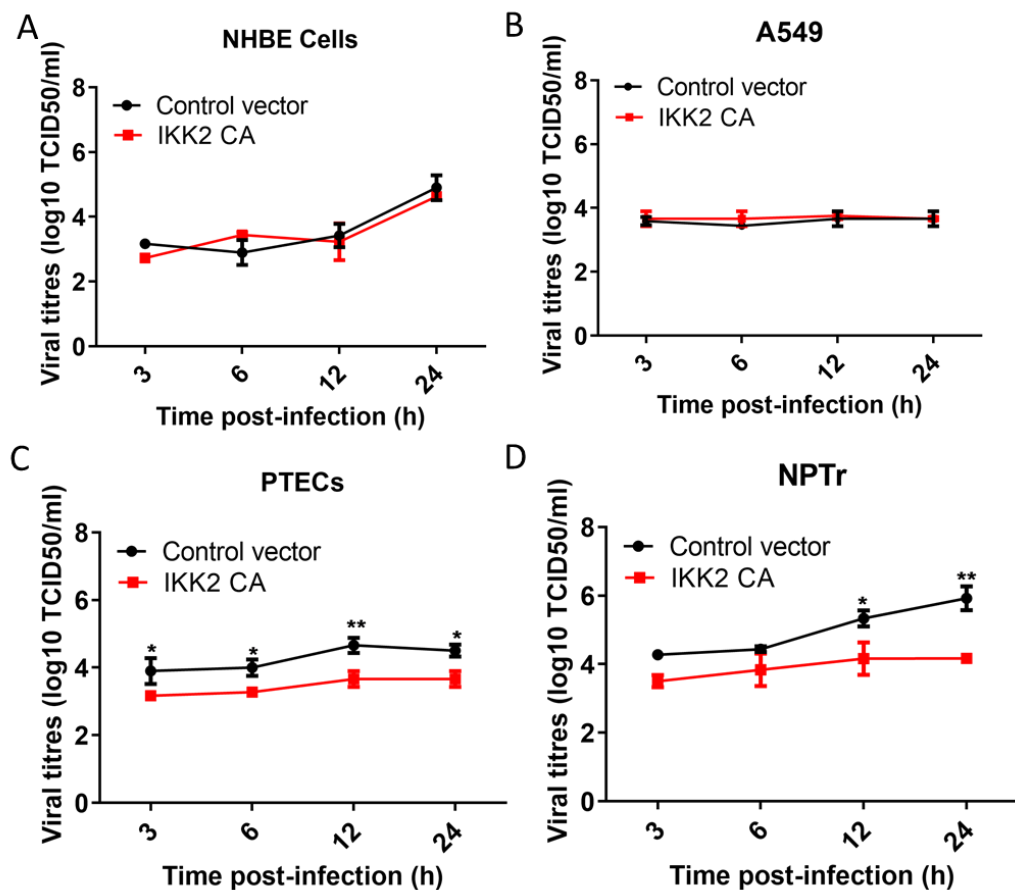


Figure 2-5. Activation of NF- κ B, via IKK2 CA overexpression, reduced virus output from (A) PTECs and (B) NPTr cells, but had little effect on virus replication in human cells (C and D). Cells were transfected with IKK2 CA or control vector; after 2 days they were infected with 1.0 MOI of USSR H1N1 virus for 2 h followed by PBS washes and replacement with fresh infection medium. Supernatants were collected at 3, 6, 12 and 24 hpi for virus titration on MDCK cells by TCID₅₀ assays. Significance is relative to corresponding control vector at each time points (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, 0.01 < P < 0.05 was given one asterisk, 0.001 < P < 0.01 was given two asterisks.

2.3.4 Activation of NF- κ B in pig cells reduces virus transcription

To understand how IKK2 CA overexpression reduced virus replication in pig cells, NPTr cells and PTECs were transfected with IKK2 CA, IKK2 inactive or control vector plasmid, and two days later separately infected with USSR H1N1 and PDM H1N1 virus for 24 h after which total cellular RNA was isolated for quantitative gene expression analysis by qPCR. IKK2 CA overexpression reduced viral *M*-gene expression in both pig cell types (Figure 2-6A and B); conversely, IKK2 inactive overexpression increased *M*-gene expression. Thus, activated NF- κ B inhibited viral *M*-gene transcription in pig cells.

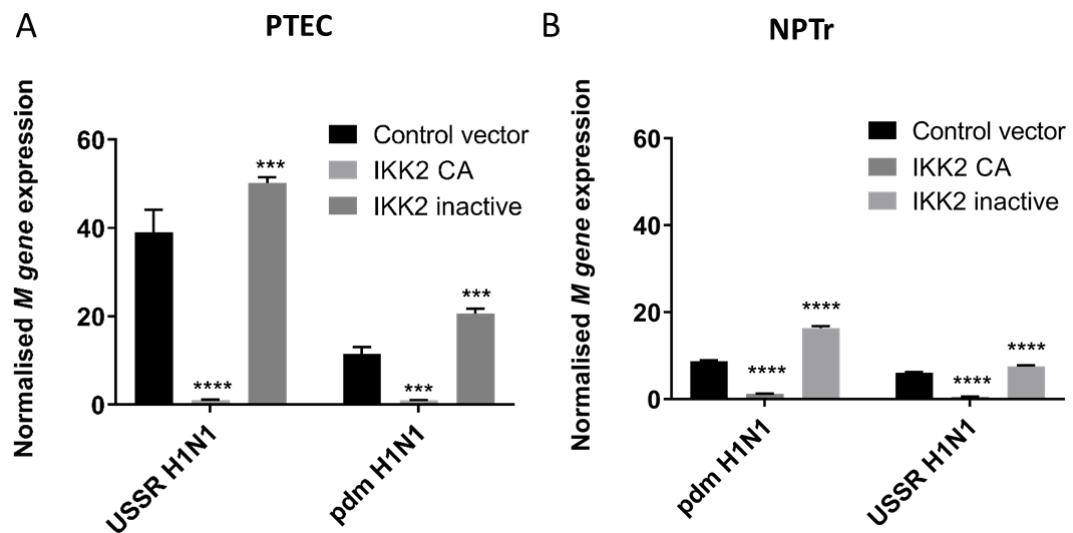


Figure 2-6. Activated NF- κ B, via IKK2 CA overexpression, reduced viral *M-gene* transcription. PTECs (A) and NPTTr cells (B) were transfected with IKK2 CA or control vector; after 2 days cells were infected with 1.0 MOI of USSR H1N1 virus or 2.0 MOI of PDM H1N1 virus for 2 h, then washed with PBS and cultured in fresh infection medium. Cell lysates were collected 24 hpi for RNA extraction and cDNA conversion. Viral *M-gene* expression was normalised to *18S rRNA*. Significance (2-way ANOVA with Dunnett's multiple comparisons) is relative to corresponding control vector for each virus. Levels of significance are shown in figures, $0.0001 < P < 0.001$ and $P < 0.0001$ were given three and four asterisks, respectively. Results shown representative of three independent experiments.

2.3.5 IKK2 CA facilitates type I IFN-associated response in pig cells but not in human cells

RIG-I and *OAS1* were used as exemplars of type I IFN responsive genes to assess the effect of NF- κ B activation on their expression. *RIG-I* sits at the top of the *RIG-I* signalling pathway that recognizes RNA virus infection, then leads to downstream *MAVS* and *IRF3* signalling to produce type I interferon response (Gack, Albrecht et al. 2009). *OAS1* is an interferon-stimulated gene, and it was shown

as a downstream effector of *RIG-I* signalling to inhibit influenza virus replication (Allen, Moore et al. 2011). In uninfected NHBE and A549 cells, IKK2 CA overexpression had no or only modest (1.5-fold increase) effect, on respectively *RIG-I* expression (Figure 2-7A); IKK2 CA increased *OAS1* expression (< 2-fold) in both uninfected human cell types (Figure 2-7B). In uninfected pig (PTEC and NPTr) cells, however, IKK2 CA overexpression strongly up-regulated *RIG-I* and *OAS1* expression (5- to 10- fold increase) (Figure 2-7C and D).

In infected NHBE cells and A549 cells, IKK2 CA overexpression had modest positive or negative effect on *RIG-I* and *OAS1* expression relative to corresponding infected control vectors (Figure 2-7A and B). In infected PTECs and NPTr cells, IKK2 CA overexpression reduced transcriptional activation of *RIG-I* expression; and in infected PTECs, but not NPTr cells, IKK2 CA overexpression also lowered the induction of *OAS1* expression (all relative to corresponding infected controls) (Fig. 2-7C and D). The attenuation in *RIG-I* and *OAS1* expression in infected-IKK2 CA pig cells (relative to corresponding uninfected-IKK2 CA or infected-control pig cells) might well be a regulatory feedback effect of active type I IFN signalling triggered in IKK2 CA-transfected pig cells before infection.

Next, expression of IRF3 and phospho-IRF3 proteins in human (NHBE and A549) and pig (PTEC and NPTr) cells in response to IKK2 CA overexpression were assessed by western blotting. IKK2-CA

overexpression had no effect on IRF3 phosphorylation (IRF3 activation) in both human cell types regardless of infection (Figure 2-8). IKK2 CA transfected PTECs, at 6 and 24 hpi, showed significantly more phospho-IRF3 than corresponding infected controls; IKK2 CA transfected NPTr cells also significantly had more phospho-IRF3 than corresponding control regardless of infection at each time point (Figure 2-9). There was no change in IRF3 protein from IKK2 CA overexpression in both pig cell types (Figure 2-9). Taken together, IKK2 CA overexpression in pig cells facilitates basal activation of type I IFN associated genes (Figure 2-7C and D; Figure 2-9E) which primes cells ahead of infection for enhanced IRF3 activation during infection (Figure 2-9B and E).

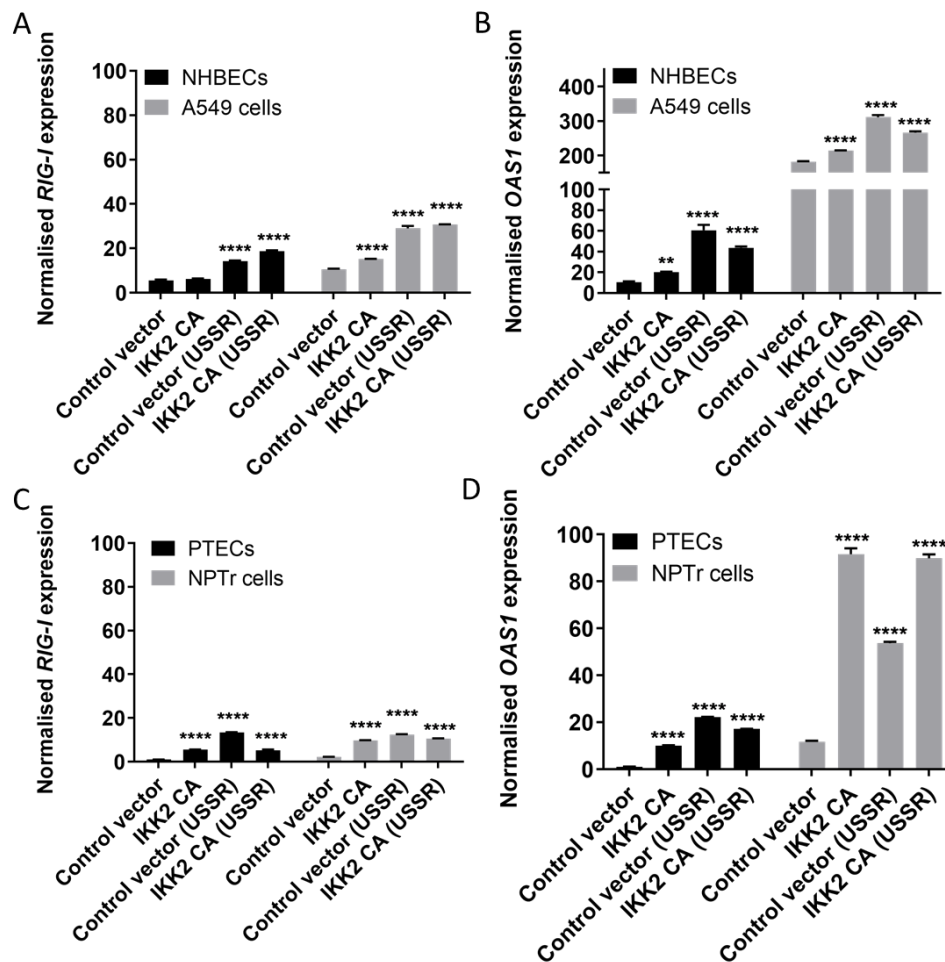


Figure 2-7. IKK2 CA overexpression induced robust type I IFN associated gene response in pig cells. Cells were transfected with control vector or IKK2 CA; two days post-transfection, cells were maintained in fresh media (0 dpi) infected with 1.0 MOI USSR H1N1 virus for 24 h. Cell RNA lysates were harvested for quantitative PCR analysis normalised to *18S rRNA*. Significance (2-way ANOVA with Dunnett's multiple comparisons test) is relative to corresponding uninfected control vector. (A) *RIG-I* and (B) *OAS1* expression in human cells. (C) *RIG-I* and (D) *OAS1* expression in pig cells. Based on the use of relative Ct computation, comparisons between human and pig cells can be made. Levels of significance are shown in figures, $0.001 < P < 0.01$ and $P < 0.0001$ were given two and four asterisks, respectively. Results presented representative of three independent experiments.

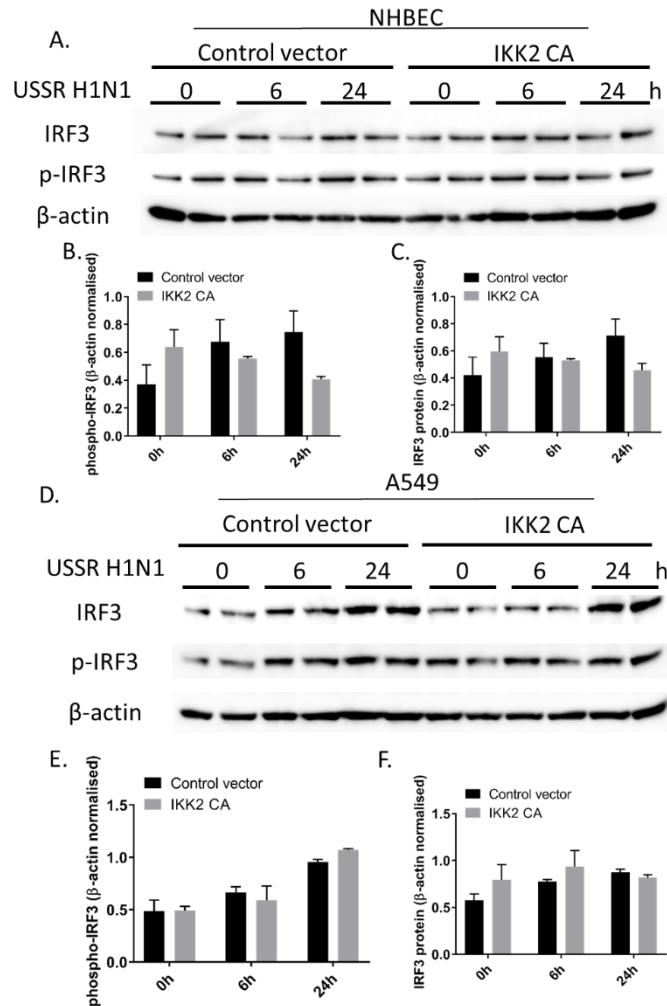


Figure 2-8. Overexpression of IKK2 CA had no detectable effect on IRF3 phosphorylation in human cells. NHBE and A549 cells were transfected with IKK2 CA or control vector; two days post-infection, cells were harvested for protein lysates (0 hpi) or infected with 1.0 MOI USSR H1N1 virus for 6 and 24 h for cell lysate extractions. Each protein sample was loaded in duplicate at 5 μ g/well for Western blotting. Significance (2-way ANOVA with Sidak's multiple comparisons) is relative to corresponding control vector at each time point. (A) IRF3, phospho-IRF3 and β -actin protein expression in NHBE cells. (B) phospho-IRF3 protein expression normalised to β -actin expression in NHBE cells. (C) Total IRF3 protein expression normalised to β -actin in NHBE cells. (D) IRF3, phospho-IRF3 and β -actin protein expression in A549 cells. (E) phospho-IRF3 protein expression normalised to β -actin in A549 cells. (F) Total IRF3 protein expression normalised to β -actin in A549 cells. Results shown representative of three independent experiments.

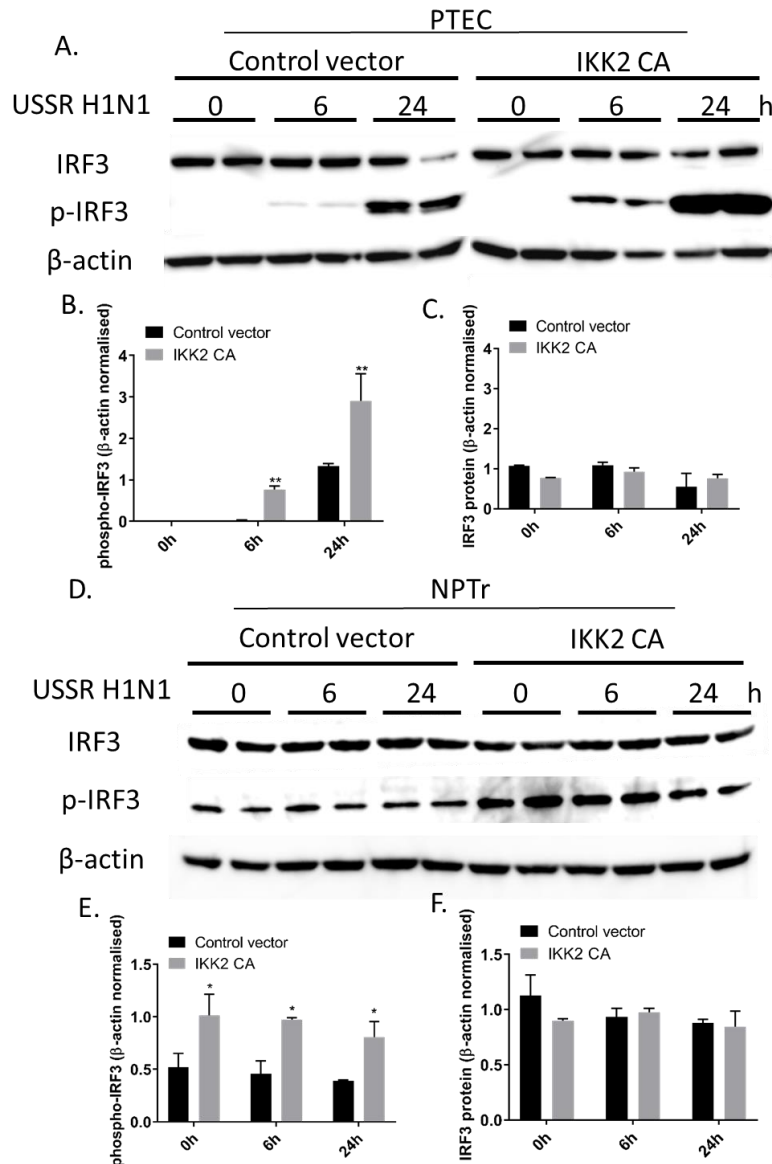


Figure 2-9. Overexpression of IKK2 CA increased IRF3 phosphorylation in pig cells. PTECs and NPTr cells were transfected with IKK2 CA or control vector; two days post-transfection cells were harvested (0 hpi) or infected with 1.0 MOI USSR H1N1 virus for 6 and 24 h for protein lysate extractions. Each protein sample was loaded in duplicate at 5µg/well for Western blotting. Significance (2-way ANOVA with Sidak's multiple comparisons) is relative to corresponding control vector. (A) IRF3, phospho-IRF3 and β-actin protein expression in PTECs. (B) phospho-IRF3 protein expression normalised to β-actin in PTECs. (C) Total IRF3 protein expression normalised to β-actin in PTECs. (D) IRF3, phospho-IRF3 and β-actin protein expression in NPTr cells. (E) phospho-IRF3 protein expression normalised to β-actin in NPTr cells. (F) Total IRF3 protein expression normalised to β-actin in NPTr cells. Levels of significance are shown in figures, 0.01<P<0.05 was given one asterisk, and 0.001<P<0.01 was given two asterisks. Results shown representative of three independent experiments.

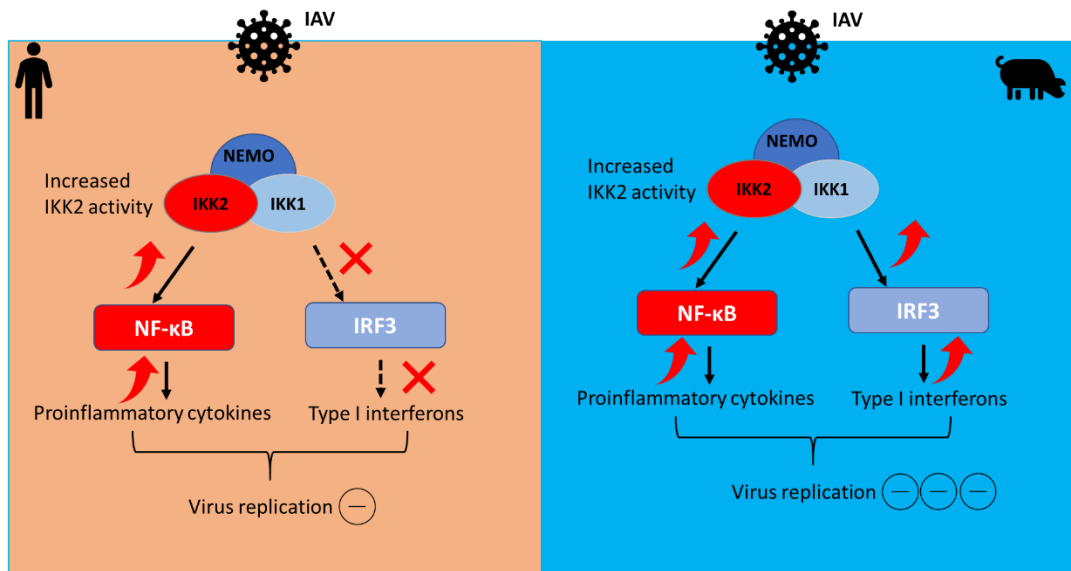


Figure 2-10. Schematic contrast of host species response to IKK2 activation. In human (NHBE and A549) cells, overexpression of IKK2 CA activates NF- κ B to induce production of proinflammatory cytokines, but has little effect on IRF3 activation. In pig cells (PTECs and NPTr cells), overexpression of IKK2 CA leads to NF- κ B as well as IRF3 activation to induce production of proinflammatory cytokines, and type I IFNs to strongly inhibit virus replication. Solid line indicates direct interaction, dashed line indicates weak or no interaction.

2.3.6 Profiling the basal gene expression of NHBE cells and PTECs by microarray RNA analysis

Based on the earlier stated hypothesis that the relative basal expression of innate immune genes, prior to infection, could be a key determinant of host resistance or susceptibility to IAV infection, we performed RNA microarray analysis (on human Affymetrix Gene 2.1 ST Arrays), using four biological replicates of NHBE cells and PTECs, to identify basal (uninfected) differential expression of genes between the two host species (Figure 2-11A). Differential gene expression analysis, using Genespring X, set at ≥ 2 -fold (upregulated) and ≤ -2 -fold (downregulated) at p-values < 0.05 with false discovery rate, identified 2318 genes that were more highly expressed in PTECs, and 4261 genes were more highly expressed in NHBE cells (Figure 2-11B). To take into account possible reduced specificity in heterologous pig-human hybridisation on the chip, genes more highly expressed in pig cells were filtered at ≥ 4 -fold, and genes more highly expressed in human cells were filtered at ≥ 2.5 -fold, resulting in a reduced total gene list of 2868 genes of which 857 genes were more highly expressed in PTECs and 2011 genes more highly expressed in NHBE cells. A selection of differentially expressed genes was successfully subjected to qPCR validation (Figure 2-12).

The filtered gene list of 2868 genes was subjected to predictive functional IPA human analysis. Upstream activated gene analysis strongly suggested that *TNF- α* and NF- κ B were more activated in NHBE cells, with two of the top three highest z-scores (z-score ≥ 2 being statistically significant towards activation) of 8.183 and 6.586 respectively (Figure 2-13A). Two hundred and ninety genes were found to be directly or indirectly connected to *TNF- α* activity; significantly the direction of fold-change in 195 out of the 290 genes was consistent with *TNF- α* activation. Of the 105 genes found to be connected to NF- κ B function, the direction of fold-change in 69 out of the 105 genes was consistent with NF- κ B activation. As there is considerable overlap of genes between *TNF- α* and NF- κ B signalling, and NF- κ B being sited downstream of *TNF- α* , the overall pattern of expression suggests that NF- κ B-related genes, at the basal level before infection, are more highly primed transcriptionally in NHBE cells than PTECs.

Moreover, based on the 2868 differentially expressed genes, IPA diseases or functions analysis predicted increased viral infection in NHBE cells; out of the 364 genes involved in viral infection, the direction of fold-change in 204 genes was consistent with increased viral infection (z-score of 6.525) (Figure 2-13B). Further analysis using the 204 genes identified a subset of 48 genes whose direction of differential expression was consistent with higher NF- κ B-related activity (z-score of 4.8) (Figure 2-13C).

IPA disease or functions analysis also predicted increased cell survival in NHBE cells relative to PTECs; out of the 369 genes involved in cell survival, the direction of fold-change in 202 genes was consistent with increased cell survival (z-score of 5.983) (Fig. 2-13B). Activated NF- κ B has been shown to promote cell survival by suppressing apoptosis (Beg, Sha et al. 1995, Luo, Kamata et al. 2005).

Twenty-eight genes are common in TNF- α signalling, NF- κ B activation, increased viral infection and improved cell survival are found (Figure 2-14A), of which 25 were more highly expressed in NHBE cells, and the remaining 3 (*BBC3*, *CXCR4* and *TIMP2*) were more highly expressed in PTECs (Figure 2-14B). The predicted effects of each of the 28 genes, based on direction of fold-change, are summarised in Figure 3-4C. Downregulated expression of *BBC3*, *TIMP2* and *CXCR4* in NHBE cells was predicted to inhibit TNF α activity. The remaining 25 upregulated genes were predicted to activate TNF activity, of which interleukins (*IL18*, *IL1A*, and *IL1B*) and TNF- α related genes (*TNFRSF10B*, *TNFRSF1A* and *TNFSF10*) were predicted to be activated in NF- κ B signalling. Finally, in analysing the functional connections of the 28 genes, *TNFRSF1A* and *TNFRSF10B* were found to occupy central nodal positions in the predicted network (Figure 2-15). *TNFRSF1A* is a crucial receptor in the mediation of NF- κ B activation and apoptosis; suggesting that high

TNFRSF1A expression could be a determinant of host resistance or susceptibility to IAV infection.

In summary, we found that activation of NF- κ B blocked influenza virus replication in pig airway epithelial cells, but had limited antiviral effect in human airway epithelial cells. The much stronger associated type I interferon response in pig cells, but not in human cells, from NF- κ B activation, would account at least in part for the inhibition of influenza virus in this host species. Conversely, the relatively high basal transcriptional levels of NF- κ B associated genes in human airway epithelial cells could actually impair response to NF- κ B activation, which resulted in a less effective antiviral outcome in human cells.

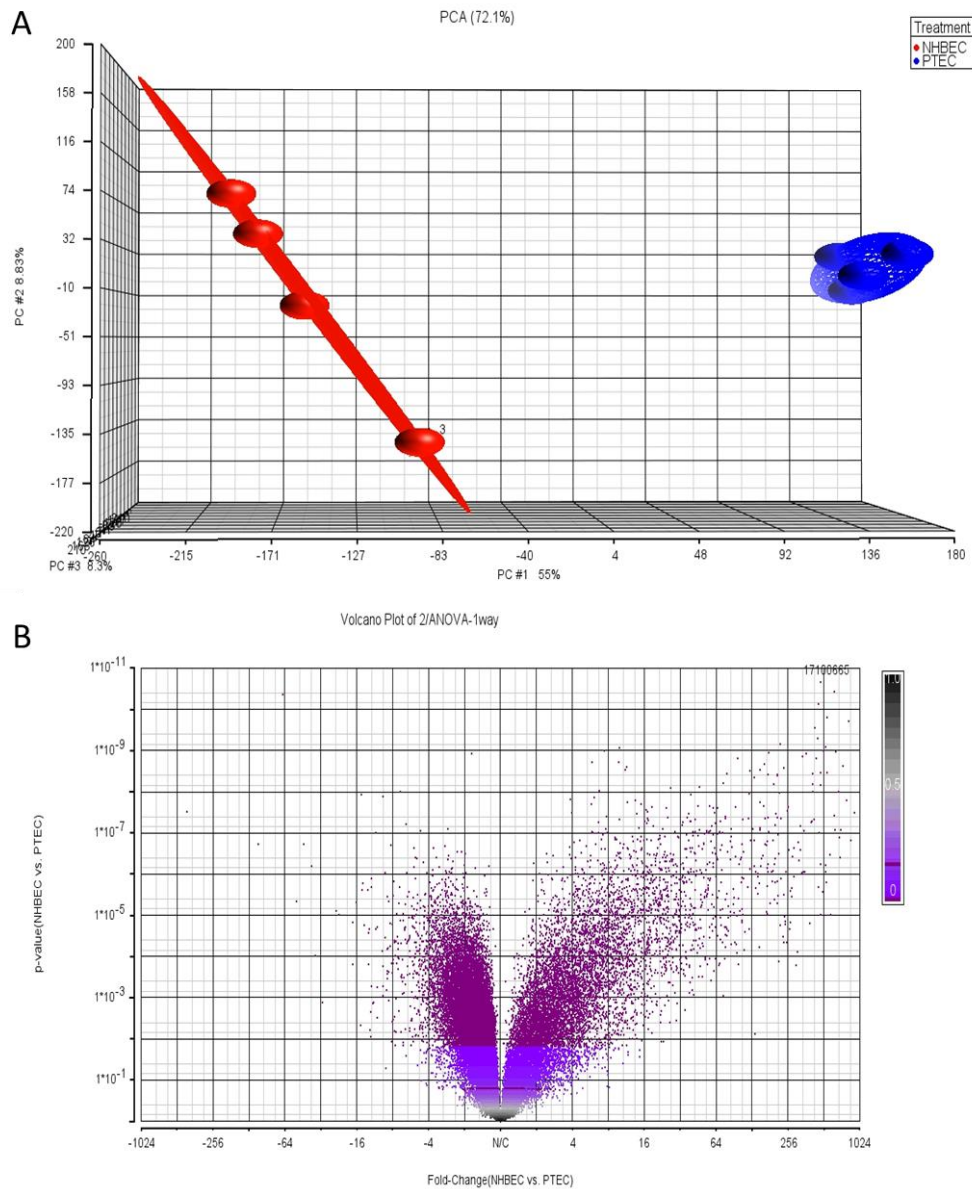


Figure 2-11. Overview microarray analysis of basal transcription levels between NHBE cells and PTECs. (A) PCA plot of four biological replicates of NHBE cells (red), and PTECs (blue) were used for microarray profiling using a human Affymetrix Gene 2.1 ST platform. (B) Volcano plot of differential gene expression. X-axis left of N/C indicates genes more highly expressed in PTECs; x-axis right of N/C more highly expressed in NHBE cells. Y-axis indicates p-values.

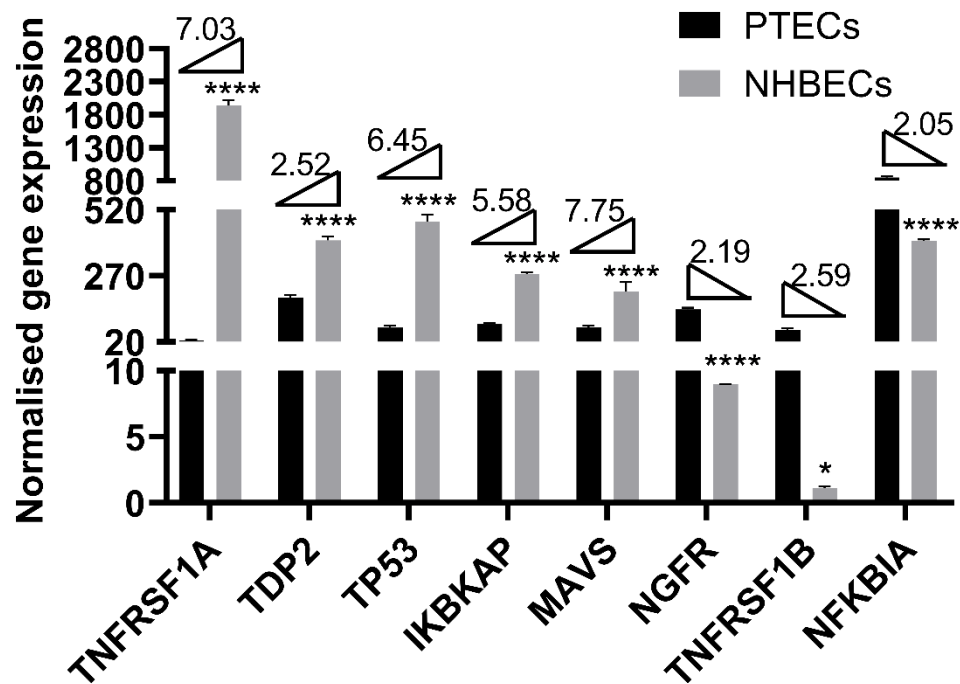


Figure 2-12. qPCR validation of differentially expressed genes between NHBE cells and PTECs identified by RNA microarray analysis. Based on the shortlist of differentially expressed genes, 8 were selected for qPCR, each using human-specific or pig-specific primers; all expression was normalised to *18S rRNA*. Fold-change determined by microarray of each gene is indicated by the direction of corresponding triangle. Significance is between host species of each gene (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, $P < 0.0001$ was given four asterisks.

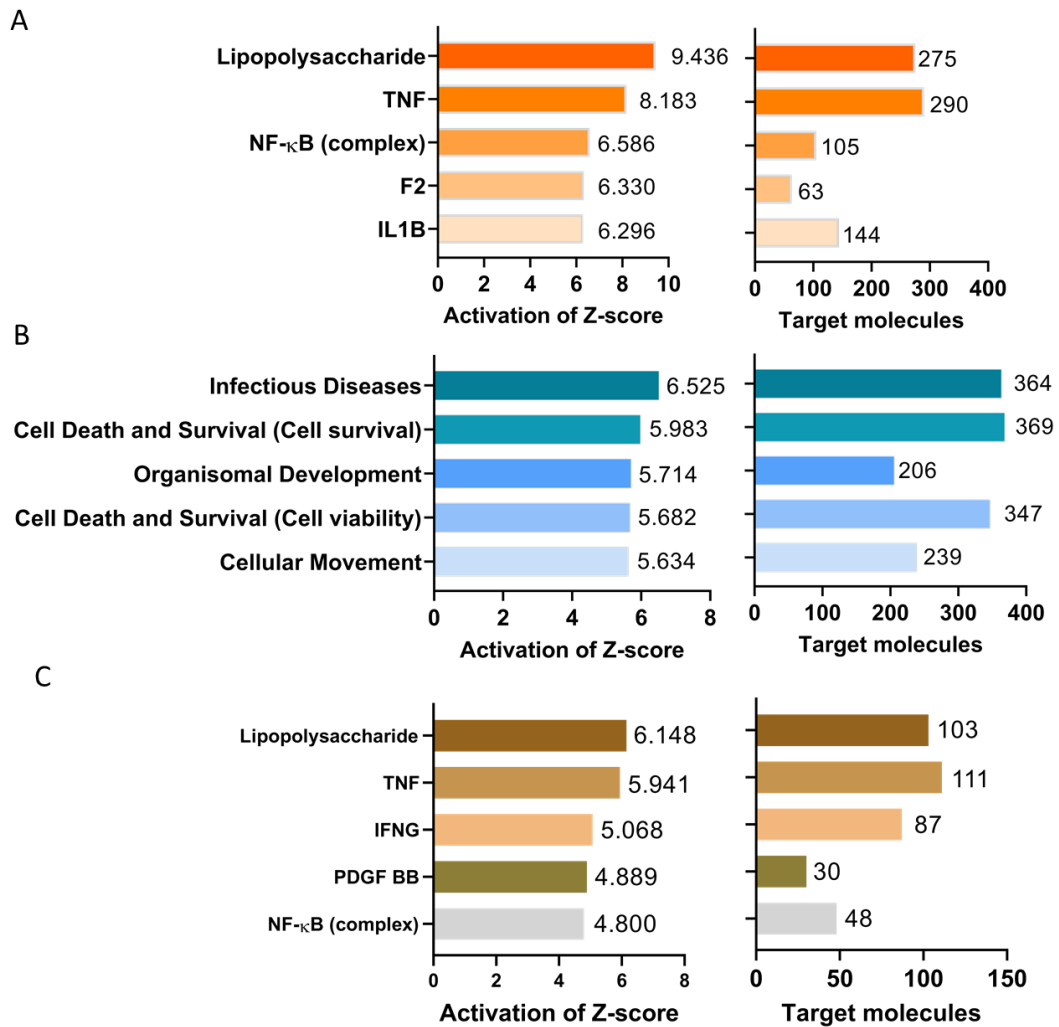


Figure 2-13. IPA predictive analysis for upstream regulators, diseases and functions. Based on the 2868 differentially expressed genes, IPA identified (A) 105 genes that are associated with NF-κB (complex) of which 69 genes have direction of fold-change consistent with activation of NF-κB; (B) 364 genes that are associated with viral infection of which 204 genes have expression direction consistent with promotion of viral infection. IPA also identified 369 genes that are associated with cell survival of which 202 have expression direction consistent with increased cell survival. (C) Of the 204 genes involved in increased viral infection, 48 have direction of fold-change consistent with activation of NF-κB (complex).

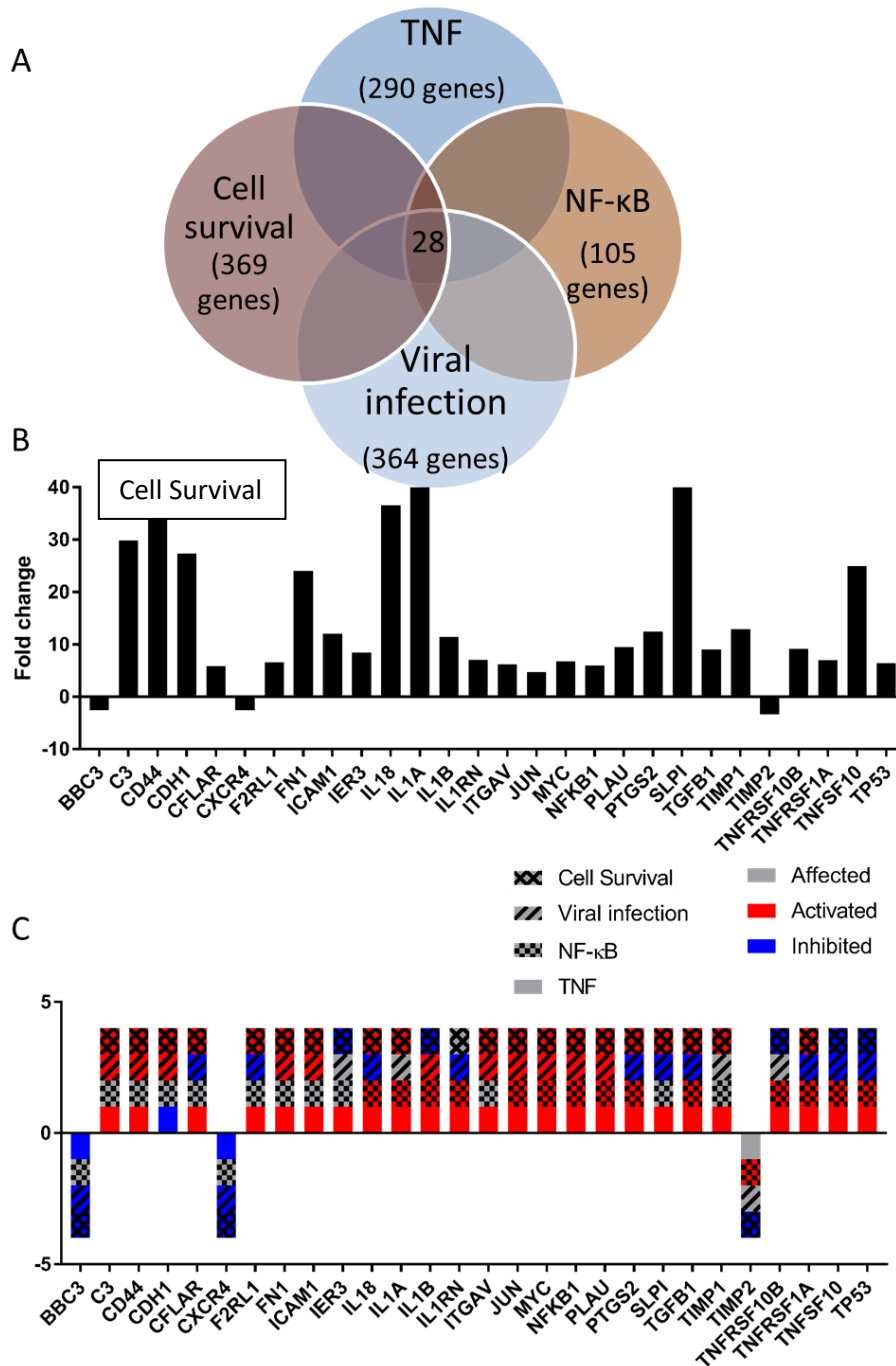


Figure 2-14. Twenty-eight differentially expressed genes were identified that were common to the functional groups of cell survival, TNF- α status, viral infection and NF- κ B status. (A) Venn diagram shows 28 genes common to the 4 functional groups of differentially expressed genes. (B) Fold-change in expression of the 28 genes of which 25 were more highly expressed in NHBE cells, and 3 were more highly expressed in PTECs. (C) Based on direction of fold-change, the predicted effect (indicated as affected, activated or inhibited) of each gene for each of the 4 functions is depicted.

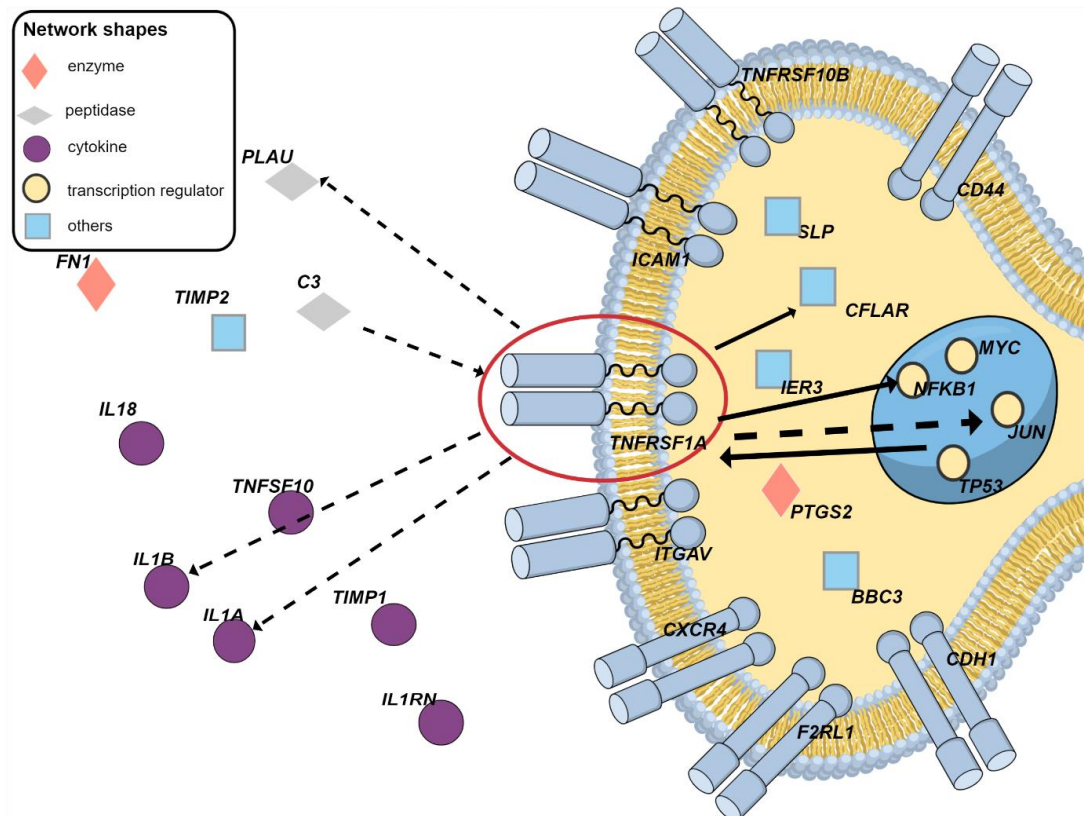


Figure 2-15. IPA predicted connecting pathway based on the 28 selected genes common to the four functional groups of cell survival, *TNF-α* status, viral infection and NF-κB status. *TNFRSF1A* occupies a central nodal position in the predicted network and highlighted in red circle. Solid arrow indicates genes that are directly regulated by *TNFRSF1A*; dash arrow indicates genes that are indirectly regulated by *TNFRSF1A*.

2.3.7 Supplementary section

2.3.7.1 RNA profiling of host genes in NPTr cells overexpressing IKK2 CA

The potent antiviral effect of IKK2 CA overexpression in pig cells appears to be a host species-specific response to NF- κ B activation. To more fully assess the transcriptional changes in NPTr cells subjected to IKK2 CA overexpression, microarray RNA expression assay was performed, using RNA derived from cells transfected with IKK2 CA or vector control without subsequent virus infection. We performed RNA microarray analysis (on human Affymetrix Gene 2.1 ST Arrays), using three biological replicates of each treatment group (6 samples in total).

The resulting PCA plot shows no overlap between the two treatment groups, indicating that IKK2 CA overexpressed group had significantly regulated transcripts relative to control group (Figure S1). The gene selection criteria applied were fold change (up and down) of at least 1.5 and with false discovery rate applied, adjusted p-value of <0.1 . In summary, 18 mapped transcripts are found as differentially expressed between uninfected vector control cells and uninfected IKK2 CA cells (Fig. S2 and Table S1). However, use of pig RNA on a human chip precludes the detection of porcine chemokine and cytokine RNAs owing to low sequence homology between the

host species. Nonetheless, the shortlisted transcripts could still be valuable in detecting changes in basal gene expression in response to IKK2 CA overexpression.

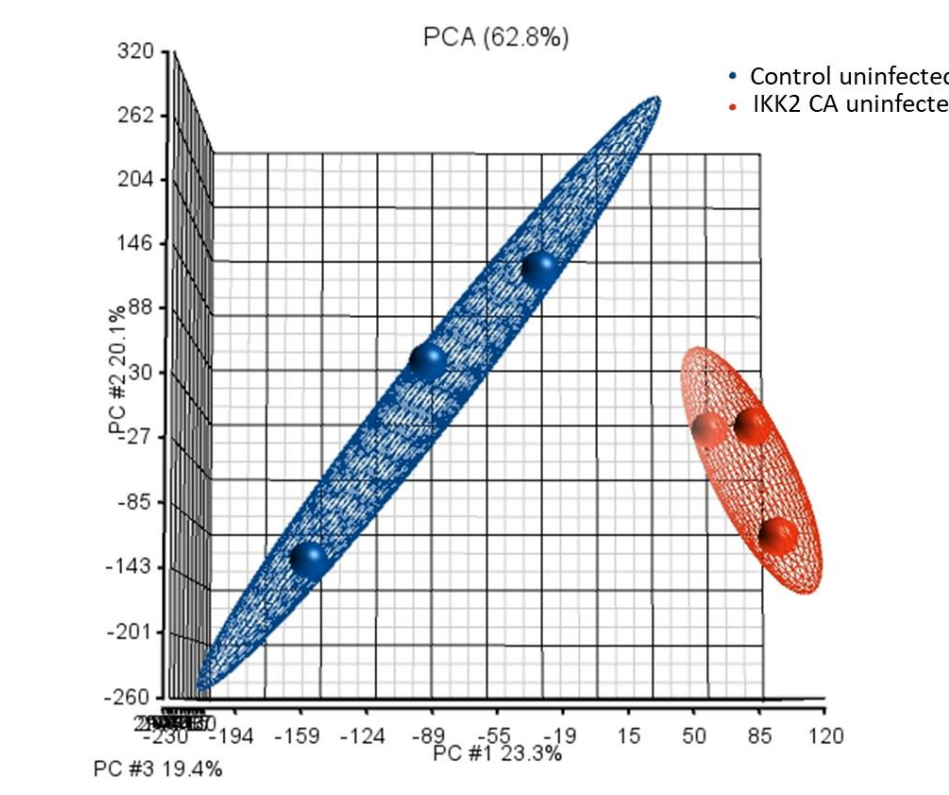


Figure S1. PCA plots of microarray RNA analysis of NPTr cells. Each treatment has three biological replicates and highlighted in different colours, each individual sphere represents a sample. NPTr cells were transiently transfected with IKK2 CA or control vector for 6 h, washed with PBS and maintained in fresh DMEM media supplemented with 10% foetal bovine serum for 2 days. Cells were harvested for total RNA.

2.3.7.2 IKK2 CA overexpression predicted to reduce apoptosis activity in NPTr cells

Seven of the 18 differentially expressed genes from IKK2 CA overexpression were randomly selected for successful qPCR validation (Figure S3). Using the predictive tools of IPA, 8 out of 18 genes were found to be related with apoptosis (Figure S4) of which the directional changes of increased expression of *IKBKB*, *SCL7A11*, *ARF6* and *CLK3*, and reduced expression of *PDCD4* and *TRPM7* (all relative to controls) are predicted to have an inhibitory effect on apoptosis (Figure S4).

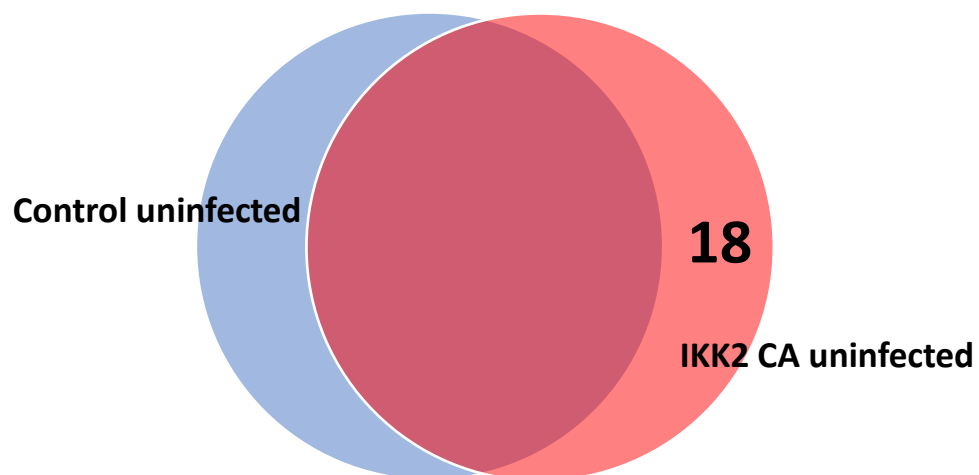


Figure S2. Venn diagram of uninfected IKK2 CA vs uninfected control NPTr cells. Total of 18 genes are significantly differentially regulated by IKK2 CA in uninfected NPTr cells. Genes were selected with a minimum fold change at 1.5 of up regulation or down regulation, applied with false discovery rate and adjusted p-value of <0.1.

Table S1. Top gene list from uninfected control vs uninfected IKK2 CA NPTr cells

Symbol	Entrez Gene Name	Expr p-value	Expr Fold Change
ARF6	ADP ribosylation factor 6	0.0000248	1.659
ARRDC3	arrestin domain containing 3	0.00000336	1.973
CLK3	CDC like kinase 3	0.0000545	1.54
FZD3	frizzled class receptor 3	0.0000515	-1.554
GABRP	gamma-aminobutyric acid type A receptor pi subunit	0.00000325	2.413
GNPTAB	N-acetylglucosamine-1-phosphate transferase subunits alpha and beta	0.00000974	-1.728
HES1	hes family bHLH transcription factor 1	0.00000022	1.569
HOXB9	homeobox B9	0.0000635	1.598
IKBKB	inhibitor of nuclear factor kappa B kinase subunit beta	0.000000243	7.183
MCM6	minichromosome maintenance complex component 6	0.00000283	-1.559
NFIX	nuclear factor I X	0.0000043	1.632
PDCD4	programmed cell death 4	0.0000244	-1.585
PFDN2	prefoldin subunit 2	0.00000124	1.596
PTBP2	polypyrimidine tract binding protein 2	0.0000428	-1.582
RNF19B	ring finger protein 19B	0.0000482	1.547
SLC7A11	solute carrier family 7 member 11	0.0000291	1.985
TRPM7	transient receptor potential cation channel subfamily M member 7	0.0000032	-1.852
USP13	ubiquitin specific peptidase 13	0.00000524	-1.671

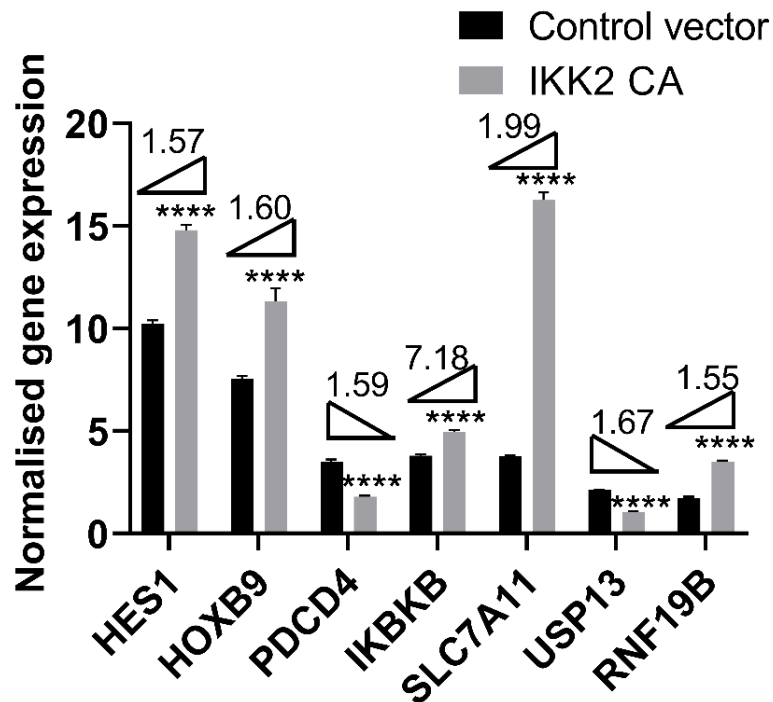


Figure S3. qPCR validation of differential gene expression between and IKK2 CA-transfected uninfected, and control vector-transfected uninfected NPTr cells. Based on shortlisted genes from microarray, all seven selected genes showed differential expression consistent with microarray results. Fold change of microarray analysis of each gene is indicated by direction of corresponding triangle. Gene expression was normalised to *18S rRNA*. Significance is relative to corresponding control vector expression (2-way ANOVA with Sidak's multiple comparisons). Levels of significance were shown in figures, $P < 0.0001$ was given four asterisks.

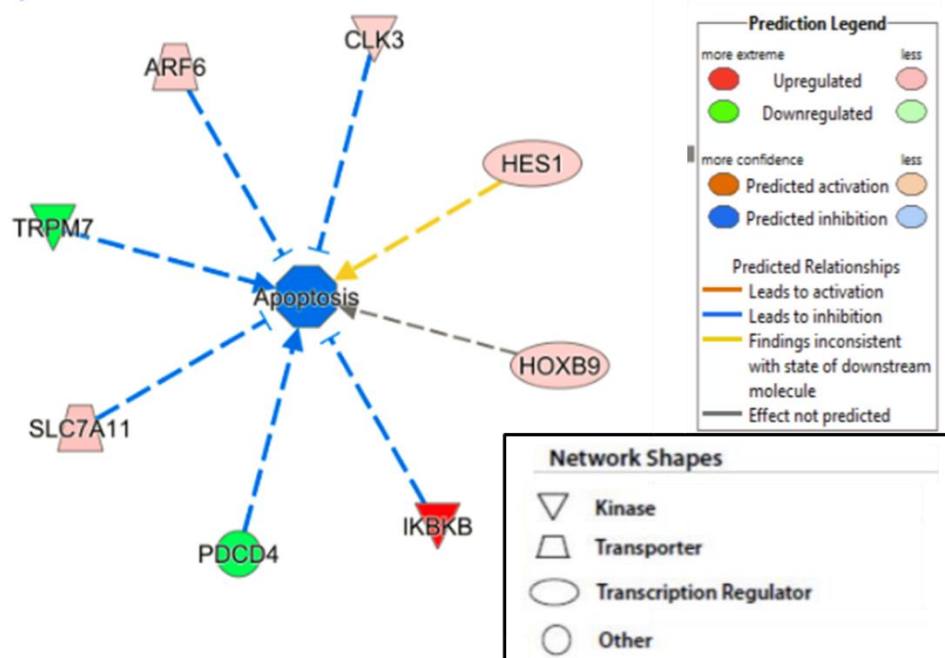


Figure S4. Apoptosis was predicted by IPA to be inhibited in NPTr cells overexpressing IKK2. Eight (shown) out of the 18 genes found to be associated with apoptosis, and the direction of fold-change in expression of 6 out of the 8 genes (*ARF6*, *CLK3*, *IKKB*, *PDCD4*, *SLC7A11* and *TRPM7*) was consistent with IPA-predicted inhibition of apoptosis.

Based on previous IPA analysis, apoptosis was found to be inhibited by IKK2 CA overexpression. Eight genes were identified to be associated with apoptosis, these 8 genes were further filtered based on gene function that is related to NF- κ B or IKK2. Studies have shown that *SLC7A11*, *PDCD4*, *HOXB9* and *HES1* have direct or indirect connections with IKK2 and NF- κ B activity. IKK2 depletion reduced notch target gene *HES1*; *TNF- α* stimulation resulted phosphorylation histone H3 at the Hes-1 promoter, but the signal was lost with IKK2 depletion (Maniati, Bossard et al. 2011). Overexpression of *HOXB9* in SGC7901 cells promoted apoptosis, knockdown of *HOXB9* resulted in reduced phosphorylation of Akt and

NF- κ B dependent Snail expression(Zhang, Wu et al. 2019). Expression of *PDCD4* attenuated phosphorylation of IKK β in renal cancer cells, resulting in inhibition of phosphorylation of its substrate I κ B α , suggesting that *PDCD4* inhibits IKK β activity (Bera, Das et al. 2014). *SLC7A11* inhibition by erastin, sulfasalazine, or shRNA interference sensitizes BAY-induced (I κ B α inhibitor) cell death. Overexpression of *SLC7A11* attenuated BAY-inhibited cell viability (Chang, Chiang et al. 2018). The expressions of *HES1*, *HOXB9*, *PDCD4* and *SLC7A11* were examined on NPTr cells as well as A549 cells for comparison (Figure S5). In uninfected NPTr and A549 cells, IKK2 overexpression increased *HES1*, *HOXB9* and *SLC7A11* expressions, whereas attenuated the expressions of corresponding gene expressions in A549 cells. During infection, *HES1* and *HOXB9* expressions were decreased by IKK2 in NPTr cells in comparison to control infected NPTr cells, but the corresponding gene expression in A549 was not affected by IKK2 overexpression in A549 cells. IKK2 overexpression increased *SLC7A11* expression in NPTr and A549 cells during infection. IKK2 overexpression consistently reduced *PDCD4* expression in NPTr and A549 cells in comparison to corresponding uninfected or infected control vector.

Based on previous result, transfection of IKK2 CA reduced virus output in NPTr cells but had little effect in A549 cells. This indicates that the cellular response prior to infection between NPTr cells and A549 cells could be different, such as NPTr cells are more susceptible

to IKK2 overexpression than A549 cells. IKK2 had different directional change to *HES1*, *HOXB9* and *SLC7A11* expressions in NPTr and A549 cells, it not only shows the different transcriptional changes to IKK2 activation between the two cell types, it also implies the differential response could potentially lead the different virus output outcomes between NPTr and A549 cells.

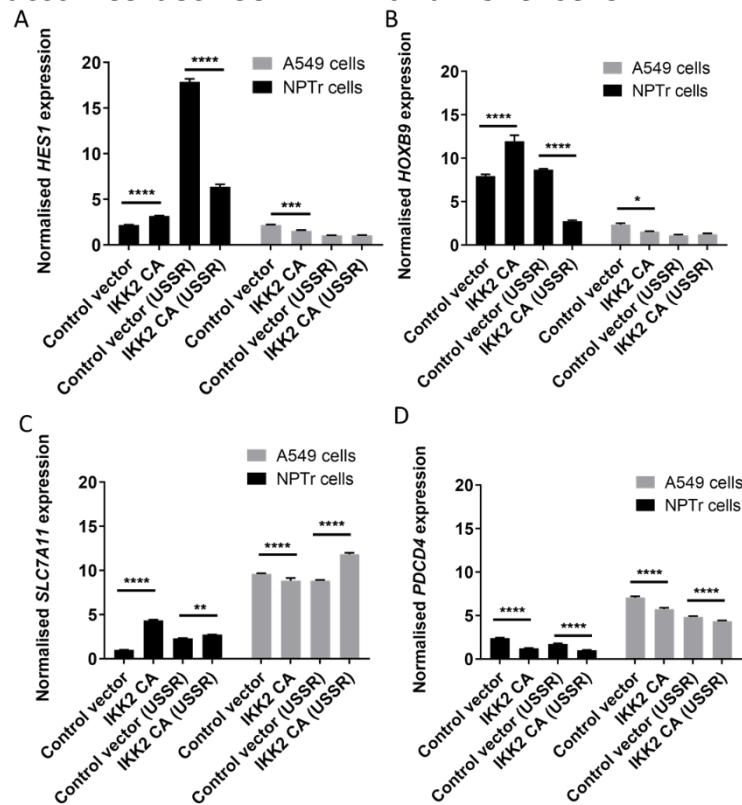


Figure S5. IKK2 activation had opposite effects on *HES1*, *HOXB9* and *SLC7A11* gene expression between NPTr and A549 cells. Four genes, (A) *HES1*, (B) *HOXB9*, (C) *SLC7A11* and (D) *PDCD4*, were chosen based on the 8-gene list of inhibition of apoptosis. The selected four genes are directly or indirectly connected with NF- κ B and IKK2. NPTr and A549 cells were transfected with control vector or IKK2 CA for 2 days, cells were then maintained in fresh media or infected with 1.0 MOI USSR for 24 h. Cell lysates were harvested for RNA extraction and cDNA conversion. Gene expression was normalised to *18S rRNA*. Significance is relative to corresponding control vector transfected cells within uninfected or infected group (2-way ANOVA with Tukey's multiple comparisons). Levels of significance are shown in figures, 0.01 < P < 0.05 was given one asterisk, 0.0001 < P < 0.001 and P < 0.0001 were given three and four asterisks, respectively.

2.4 Discussion

Despite conflicting roles of NF- κ B as an antiviral or pro-viral factor, we showed here that over-expression of IKK2 CA and RelA consistently blocked USSR H1N1 and pdm H1N1 virus production in pig NPTr cells and PTECs. This finding clearly indicates that activation of NF- κ B is antiviral in pig cells. However, there was no clear effect of IKK2 and RelA overexpression on virus progeny output in human cells (A549 cells and NHBE cells).

NF- κ B is important in the induction of *IFN- β* and its associated genes. Wang et al. (2010) showed that *RelA*^{-/-} mouse embryonic fibroblasts (MEF) enhanced Newcastle disease virus (NDV) and Sendai virus (SEV) replication as RelA is necessary for the induction of *IFN- β* expression in early stage of infection. However, NF- κ B was also found to be dispensable in inducing type I IFN response. MEFs that were lacking key components of NF- κ B factors (*RelA*^{-/-} and *p50*^{-/-} *RelA*^{-/-}) showed only mild reduction in *IFN- β* expression in comparison with WT MEFs in NDV and SEV infections (Wang, Hussain et al. 2007). In *p50*^{-/-} *RelA*^{-/-} cells with overexpression of dominant-negative I κ B α to further inhibit the activity of NF- κ B, it was also found that SEV and NDV were able to induce robust *IFN- β* expression (Wang, Hussain et al. 2007). All the above suggest that NF- κ B is able to facilitate but not indispensable to the induction of type I IFN. In the early phase of SEV and NDV infection, NF- κ B is required to

facilitate ATF-2/c-Jun and IRF3 to recruit the transcription co-activator CBP/p300 to the *IFN- β* enhancer. Later in infection, when the expression of IRF3/7 is sufficiently elevated to induce *IFN- β* , the primary role of NF- κ B shifts to induce pro-inflammatory and anti-apoptotic genes (Wang, Hussain et al. 2007, Basagoudanavar, Thapa et al. 2011). In our study, activated IKK2 induced similar NF- κ B response in both pig and human cells, but pig cells mounted much stronger type I IFN associated gene response (*RIG-I* and *OAS1*) and phospho-IRF3 protein response in comparison with human cells. This indicates host-species difference in the ability of NF- κ B to induce type I IFN; NF- κ B activity of pig cells is more able to induce type I IFNs than human cells. Thus, the relatively strong IFN response of pig cells to NF- κ B stimulation could be a key factor that mediates the antiviral effect of NF- κ B activation in pig cells. To understand transcriptional changes in pig cells in response to NF- κ B activation, microarray RNA expression profiling was undertaken by comparing the over-expression of IKK2 CA with control vector in NPTr cells. Although using cellular RNA of NPTr cells on human microarrays failed to detect porcine chemokines and cytokines due to low conservation of sequence homology between pig and human, groups of differentially expressed genes were identified, such that *HES1*, *HOXB9* and *SLC7A11* expression were all clearly elevated in NPTr cells from IKK2 CA overexpression, relative to human A549 cells. This shortlist of differentially expressed genes could still be

candidates responsible for the differential viral effects of NF- κ B activation between pig and human cells.

Studies have indicated that pig and human cells show significantly different innate immune response to influenza virus infection. Cytokine (*TNF- α* and *IL-6*) and chemokine (*CXCL9*, *CXCL10* and *CXCL11*) responses of primary human bronchial epithelial cells and human macrophages to HPAI H5N1 virus infection were strong; however, in primary porcine respiratory epithelial cells and pig macrophages, induction of corresponding cytokines and chemokines was relatively weak (Nelli, Dunham et al. 2012). In another study, type I IFN response was compared between primary human and pig bronchial epithelial cells. Hauser et al. (2013) found that primary NHBE cells mounted higher expression of IFN (*IFN β* and *IFN λ*) and IFN stimulated genes (*ISG*) (*ISG15*, *Mx1* and *OAS1*) than primary normal swine bronchial epithelial (NSBE) cells when infected with H5N3, H5N2 and H1N1 viruses. Furthermore, *RIG-I* activation in NHBE cells from IAV infection was higher than NSBE cells. Thus, the antiviral response of NSBE cells appears less responsive than NHBE cells to virus infection. Taken together, human cells are fundamentally different from pig cells in that human cells mount relatively more vigorous *IFN*, *ISG*, cytokine and chemokine responses than corresponding pig cells in response to IAV infection. Since NF- κ B plays key roles in regulating proinflammatory cytokines

and type I IFNs, it is possible that the relative basal setting of innate immune genes regulated by NF- κ B (in uninfected cells) is a critical determinant of host resistance or susceptibility to IAV infection. Basagoudanavar et al. (2011) showed that the RelA subunit of NF- κ B is crucial for the maintenance of *IFN- β* and *ISG(s)* levels in MEFs before virus infection, and that the role of RelA in inducing IFNs during infection was less important in comparison to its role prior to infection (Basagoudanavar, Thapa et al. 2011). This indicates that the constitutive expression of RelA is important in type I IFN regulation. Based on predictive functional human IPA analysis on the shortlist of 2868 differentially expressed genes, major basal transcriptional differences between NHBE cells and PTECs were clearly evident. *TNF- α* and NF- κ B associated transcriptional activities were stronger in NHBE cells than PTECs.

A major discrepancy of this study is in the microarray analysis in IKK2 CA overexpressed NPTr cells. The primary objective of the microarray assay was to determine the transcriptional changes led by IKK2 CA overexpression in NPTr cells. The use of a human chip in the basal transcriptional comparison between PTECs and NHBE cells was limited by low sequence homology in a range of genes between the two species. Notably, the human chip failed to detect cytokines and chemokine response in NPTr cells. qPCR and western blotting clearly showed a strong type I IFN response by IKK2 CA overexpression in NPTr cells, but the microarray assay failed to

recognise such a response. Therefore, it is necessary in the future to repeat a similar study using pig microarrays.

In conclusion, pig epithelial cells exhibit relatively lower basal transcriptional levels of NF- κ B associated genes than corresponding human cells which could render pig cells more responsive to NF- κ B activation to facilitate stronger type I IFN response to inhibit influenza virus replication. The relatively higher basal transcriptional levels of NF- κ B associated genes in human cells, on the other hand, could predispose the host to the development of acute cytokine dysregulation (cytokine storm).

**3. Different regulatory outcomes of
TNFRSF1A between primary human and pig
airway epithelial cells in influenza virus
infection**

Abstract

Tumour necrosis factor receptor 1 (TNFR1) signalling, triggered by binding of TNF- α (ligand) to TNFR1 (receptor), leads to NF- κ B activation followed by the induction of pro-inflammatory cytokines and chemokines that can either promote cell survival or cell death (apoptosis and necrosis). Through comparison of global basal transcription between primary human bronchial epithelial cells (NHBE cells) and primary porcine epithelial cells (PTECs), we found that the basal expression of *TNFRSF1A* in NHBE cells was strikingly higher than that in PTECs. As *TNFRSF1A* is a key regulator of TNFR1 signalling, relative resting (basal) activity of *TNFRSF1A* could have a bearing on host susceptibility to influenza virus infection. To this end, we assessed the effects of *TNFRSF1A* on influenza virus replication in NHBE cells and PTECs. Knockdown of *TNFRSF1A* in NHBE cells reduced IAV replication but the same knockdown in PTECs had little effect. Virus reduction in NHBE cells from *TNFRSF1A* knockdown was accompanied by attenuation of apoptosis and necrosis activities. Over-expression of *TNFRSF1A* did not affect the progeny virus production in NHBE cells but it increased virus production in PTECs, possibly involving apoptosis-independent necrosis.

3.1 Introduction

3.1.1 Overview of TNFR1 and TNFR2

TNF- α signals through two TNF receptors: TNFR1 (p55) and TNFR2 (p75) (Vandenabeele, Declercq et al. 1995). TNFR1 is a 55 kDa protein encoded by *TNFRSF1A*, and TNFR2 is a 75 kDa protein encoded by *TNFRSF1B* (Okada, Kato et al. 2001). Both TNFR1 and TNFR2 are transmembrane proteins, each comprising an extracellular domain (EC), a transmembrane domain (TM), and an intracellular part (IC) (Vandenabeele, Declercq et al. 1995). A key structural difference between TNFR1 and TNFR2 is the presence of death domain (DD). TNFR1 has an ~80-amino acid DD located in the C-terminal region, but the DD is absent in TNFR2 (Wallach, Varfolomeev et al. 1999). TNF- α binds to TNFR1 and TNFR2 to activate downstream signalling pathways. Activated TNFR1 leads to activation of NF- κ B; activated TNFR2, without a DD, can only modulate NF- κ B activity (Okada, Kato et al. 2001, Xie 2013). The interaction between TNFR1 and TNFR2 post-TNF- α ligation could together induce extrinsic apoptosis in various myeloma cell lines (Rauert, Stuhmer et al. 2011).

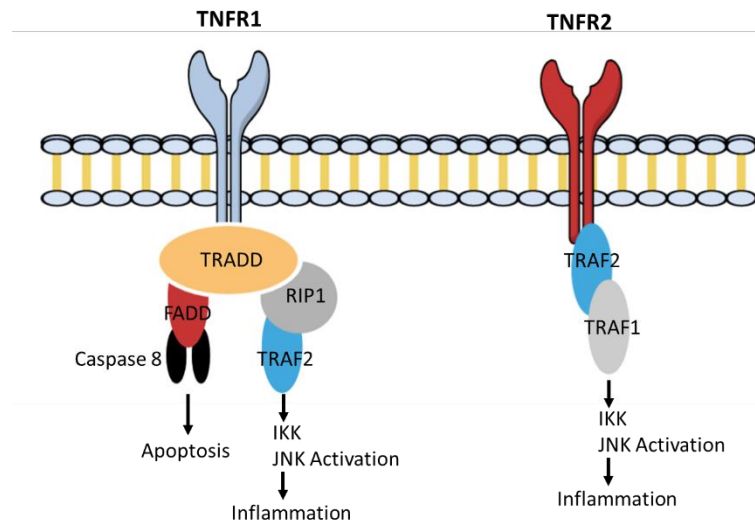


Figure 3-1. Structure of the two TNF receptors and their signalling pathways. TNFR1 forms complex with TRADD, the complex recruits RIP1 and TRAF2 to activate NF- κ B activity, and the apoptotic signals through the binding of FADD and Caspase 8 with TRADD. The lack of death domain can only enable TNFR2 to recruit TRAF2 and TRAF1 to modulate NF- κ B activity (Lopez-Urrutia, Campos-Parra et al. 2017).

3.1.2 TNFR1 signalling pathway

The ligation of TNF- α with TNFR1 initiates the recruitment of the adaptor protein, TNF-associated death domain protein (TRADD). TRADD then serves as a platform for the attachment with receptor-interacting protein 1 (RIP1) and TNF-receptor associated factor 2 (TRAF2) (Baud and Karin 2001). RIP1 and TRADD both contain death domain in the C-terminus that allows interaction with TNFR1 through DD-DD connection (Wajant and Siegmund 2019). TRAF2 is recruited by the TRAF-binding domain in N-terminus of TRADD (Wajant and Scheurich 2011). TNFR1, TRADD, TRAF2, RIP1 and cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2) together form a plasma membrane bound complex, complex I, to induce NF- κ B activation that subsequently leads to production of pro-inflammatory cytokines and chemokines to promote NF- κ B activity (Micheau and

Tschopp 2003). Genes induced by NF- κ B activation were shown to be anti-apoptotic cytokines and chemokines to inhibit apoptosis (Qin, Tao et al. 2007), and activation of NF- κ B promoted cell survival in various normal or cancer cells (Piva, Belardo et al. 2006). As NF- κ B plays a key role in promoting cell survival, TNFR1 activation could lead to NF- κ B activation to improve cell survival. At a later stage of TNFR1 ligation, as complex I is internalised, TRADD, TRAF2 and RIP1 are dissociated from TNFR1, and Fas-associated death domain protein (FADD) and caspase 8/10 are recruited to the DD of TRADD to form complex II (Micheau and Tschopp 2003, Fritsch, Zingler et al. 2017). The formation of complex II can induce apoptosis with the recruitment of caspase 8/10. The complexity of TNFR1 signalling lies with its ability to induce cell survival and apoptosis.

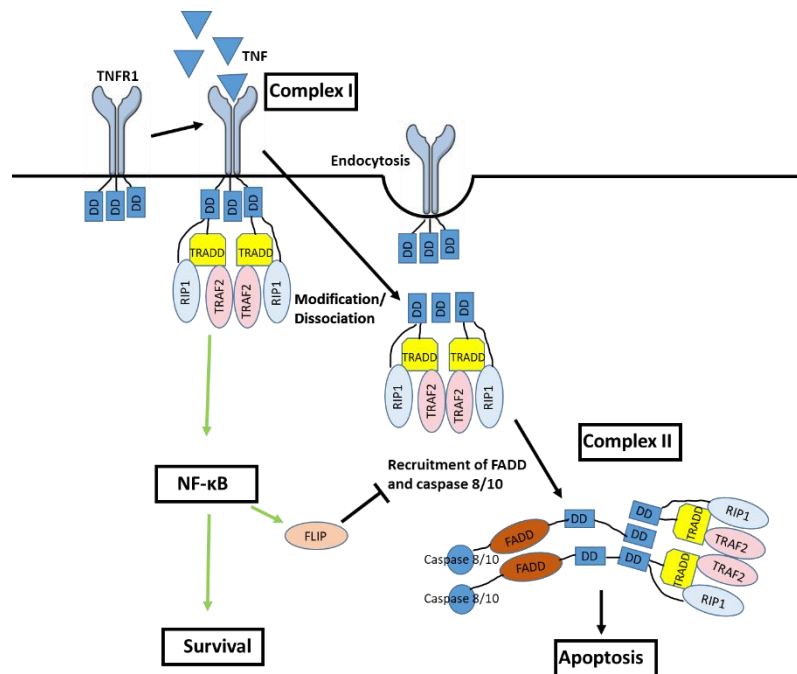


Figure 3-2. Schematic representation of TNFR1 signalling pathway in activation of NF- κ B and apoptosis. TNFR1 is activated upon ligation with TNF- α , then TRADD, TRAF2 and RIP1 (RIPK1) form complex I to activate NF- κ B pathway. Activation of TNFR1 can also lead to the cytoplasmic formation of complex II followed by the dissociation of TRADD, RIP1 and TRAF2 with TNFR1 and their internalisation, FADD and caspase 8/10 are then recruited to form complex II and induce apoptosis. FLIP, also refers to cFLIP, takes part in inhibition of apoptosis (Micheau and Tschopp 2003).

3.1.3 The role of RIP1 in regulating TNFR1 pathway

The receptor-interacting serine/threonine-protein kinase 1 (RIPK1), also known as RIP1, is a key protein that is believed to be an important checkpoint in regulating NF- κ B activity and apoptosis in cells. In Jurkat cells, RIP1 was shown to be a key modulator in the activation of NF- κ B through TNFR1 (Ting, Pimentel-Muinos et al. 1996). Overexpression of RIP1 in Hela and HEK293 cells induced NF- κ B and apoptosis (Hsu, Huang et al. 1996). The ability of RIP1 in inducing NF- κ B and apoptosis could also be time dependent (Ting and Bertrand 2016). In the early phase of TNFR1 ligation, RIP1 is ubiquitinated by TRAF2-cIAP1/2 complex and linear ubiquitin chain assembly complex (LUBAC) E3 ligases. Ubiquitinated RIP1 serves as a platform to recruit NEMO, IKK2 and IKK1 to activate NF- κ B pathway; however, in the later phase of TNFR1 activation, when NF- κ B activity is disrupted, RIP1 then serves as the platform to recruit FADD/Caspase8 and induce apoptosis (Ting and Bertrand 2016).

3.1.4 The role of cIAP1 and cIAP2 in regulating TNFR1 pathway

The cellular inhibitor of apoptosis protein 1 (cIAP1) and 2 (cIAP2) are encoded by *BIRC2* and *BIRC3*, they are known for binding to caspases to inhibit apoptosis (Liston, Roy et al. 1996). When TNFR1 is activated, cIAPs are recruited to TNFR1 through the TRAF2-TRADD

complex. RIP1 attaches to cIAPs and is ubiquitinated by cIAP1/2 to transduce signalling to activate NF- κ B (Zhang, Webster et al. 2019). Thus, cIAP1/2 function as E3 ubiquitin ligase on RIP1. MEF cells that were deficient of cIAP1 and cIAP2 failed to ubiquitinate RIP1 and failed to induce NF- κ B activity. Therefore cIAP1 or cIAP2 are essential for the ubiquitination of RIP1 and NF- κ B activation (Mahoney, Cheung et al. 2008). Deletion of cIAP1 and cIAP2 was shown to be lethal in mice with rapid weight loss and inflammation; however, knockout of caspase-8 in *cIap1*^{-/-}*cIap2*^{-/-} mice could prevent death, which indicates that cIAP1/2 are key modulators that inhibit caspase-8-dependent cell death (Zhang, Webster et al. 2019). Several studies have shown that cIAP1 and cIAP2 have redundant roles. Knockout of cIAP1 and knockout of cIAP2 showed no difference in regulating TNFR1 pathway in mice (Silke and Brink 2010). In murine C2C12 cells and MEF cells, knockout of cIAP1 or knockout of cIAP2 could lead to efficient NF- κ B activation, but knockout of both cIAP1 and cIAP2 attenuated NF- κ B activity significantly (Mahoney, Cheung et al. 2008). Taken together, cIAP1 and cIAP2 share redundant functions and are essential to the ubiquitination of RIP1 and subsequent activation of NF- κ B.

3.1.5 TNFR1 pathway in inducing necroptosis

Apart from regulating NF- κ B and apoptosis, TNFR1 pathway was also shown to be able to induce necrosis, which was defined as a kinase-

regulated process, necroptosis (Vercammen, Beyaert et al. 1998, Degterev, Huang et al. 2005, Degterev, Hitomi et al. 2008). After the internalisation of complex I, FADD, TRADD, caspase-8 and deubiquitylated RIP1 then form complex II, which is also known as cytosolic death-inducing signalling complex (DISC) (Nikoletopoulou, Markaki et al. 2013). When caspase-8 is sufficiently activated, it inhibits RIP1 and RIP3 to initiate caspase dependent apoptosis; when caspase-8 is blocked or degraded, apoptosis fails to proceed and results in necroptosis by the association of RIP1 and RIP3 (Vandenabeele, Galluzzi et al. 2010, Nikoletopoulou, Markaki et al. 2013, Ting and Bertrand 2016). Therefore, necroptosis caused by TNFR1 could be independent of apoptosis.

3.1.6 Aims and objectives

Based on microarray-derived transcriptional profiling, basal transcription of NF- κ B level was to be more found highly expressed in primary human bronchial epithelial cells (NHBE cells) than primary porcine bronchial epithelial cells (PTECs). TNFRSF1A encodes TNFR1, which is a key gene that regulates the TNFR1 pathway and leads to NF- κ B activation. We found TNFRSF1A is highly expressed in NHBE cells. Therefore, it is possible that basal expression of TNFRSF1A between NHBE cells and PTECs could dictate the outcomes of virus replication and host cell response to influenza virus infection. Functional analysis of candidate genes could provide fundamental

insights into the differences in host response to infection between pig and human.

1. To determine influenza virus replication outcomes to TNFRSF1A between NHBE cells and PTECs.

Overexpression and knockdown of TNFRSF1A studies was performed in NHBE cells and PTECs. To compare the effect of TNFRSF1A on influenza virus infection in primary human and pig airway epithelial cells.

2. To study host cell innate immune response to TNFRSF1A with influenza virus infection in NHBE cells and PTECs.

For this objective, real-time PCR, apoptosis assay and necrosis assay were performed to characterise host cell immune response to TNFRSF1A before and during virus infection.

3.2 Materials and Methods

3.2.1 Transfection and infection

TNFRSF1A and control plasmids were purchased from Sino Biological. NHBE cells and PTECs were seeded into 12-well plates, and cells were transfected with 1 µg of each plasmid and 2 µL Lipofectamine 2000 (Thermo Fisher Scientific) transfection reagent for 6 h. Transfected cells were washed with PBS for three times and were maintained with airway epithelial cell medium (PromoCell) for 48 h. Transfected cells were then infected with 1.0 MOI USSR H1N1 virus diluted in airway epithelial cell medium (PromoCell) supplemented with 250 ng/mL L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma) for 2 h, cells were washed with PBS three times after 2 h. Cells were maintained in airway epithelial cell medium (PromoCell) containing 250 ng/mL TPCK for 24 h. Spun down supernatants were collected for progeny virus titration and cells were harvested for total RNA or protein extraction.

3.2.2 siRNA transfection

Human *TNFRSF1A* siRNA, three sets of specifically designed pig *TNFRSF1A* siRNAs and control siRNA were purchased from Thermo Fisher. NHBE cells and PTECs were seeded into 12-well plates until 70% confluent. Cells were then transfected with 1 µL of 10 nM

TNFRSF1A siRNA or control siRNA and 3 µL of Lipofectamine RNAiMAX for 6 h, the transfected cells were washed with PBS and cultured in airway epithelial medium (PromoCell) for another 2 days. Cells were then harvested for protein or RNA extraction, or infected with 1.0 MOI USSR H1N1 virus for 24 h, spun-down supernatants were collected for virus output titration, infected cells were harvested for protein or RNA extraction.

3.2.3 Real-time PCR amplification

Real-time PCR (qPCR) was performed by using QuantiNova SYBR Green PCR Kit (QIAGEN), and qPCR was performed in LightCycler-96 (Roche) as described in 2.2.7. cDNA samples for each gene expression had four replicates, and the gene expression was normalised by the *18S ribosomal RNA* for comparison. The primers were synthesized by Sigma-Aldrich, and the sequences for the primers are shown in the Table 3-1.

Table 3-1. Primers used in this study.

Gene	Human		Pig	
	Forward primer (5' to 3')	Reverse primer (5' to 3')	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>18s rRNA</i>	ACGGCTACCATCCAAAGGA	CCAAATTACAGGGCTCGAAA	Universal primer (same with human)	
<i>A20</i>	TGGGCTTAAGGGTGTCTGAG	CTTTCTAAGGCCACCTGGGA	CTTCAGCACCCCTCAAGGAGA	AGTCCAATTCCGGGGTGTGCAT
<i>BIRC3</i>	ACAAGCAAGAGAACTGATTG	GATCTGAAACATCTTCTGTGG	CAAAACACAGATACCTTTACAAGCAAGA	GATGTTGGCAGCACTATTTCCT
<i>CFLAR</i>	AGGGACAAGTTACAGGAATG	GAGCCTGAAAGTTATTTGAAGG	TAACCTAAGGCTCCAGAAATG	T
<i>IL6</i>	CCAGGAGCCCAAGCTATGAAC	CCAGGGGAGAAGGCAACTG	CGCAGCCTTGAGGATTTC	TTGAATGGATGTCTTCACTG
<i>JAK1</i>	AGCCACCTCAAGAAGCAGAT	GAAACTCAGCTGGCTCATGG	GAAGAGTCAACCAGCAGCAAG	CAGGTGCCCAGCTACATTATC
<i>MCP1</i>	GCTCAGCCAGATGCAATCAA	ACAGATCTCTTGGCCACAA	GAGCTGCAGCGAGACTTCCT	GTCCAGGTGGCTTATGGAGT
<i>OAS1</i>	TCCAAGCTCAGTCAGCAGAA	CAGGAGAGAGAGTGGCAACA	GAAGAGTCAACCAGCAGCAAG	GGCGGATGAGGCTCTTCA
<i>RIG-I</i>	GAAGGCATTGACATTGCACAGT	TGGTTTGGATCATTTTGATGACA	CCCTGGTTTAGGGACGATGAG	AACAGGAACTGGAGAAAAAGTG
<i>STAT1</i>	CTGGCACCAAGAACGAATGAG	GAGTCTGCTCGAGGTCAAT		A
<i>TNFA</i>	GGAGAAGGGTGACCGACTCA	TGCCCAGACTCGGCAAG	CCCAGCTATCTGGACTTTTGTCT	CCAGCCCTCATTTCTCTTTCT
<i>TNFRSF1A</i>	ATCCCCAACCCCTTCAG	TGTCTAGGCTCTGTGGCTTGTG	CCAGCCACATCTGAAACTCG	GCCAGGCAAAAGACCAAGAA
<i>Viral M gene</i>	AGATGAGCCTTCTAACCGAGGTCTG	TGCAAAAACATCTTCAAGTCTCTG	Universal primer (same with human)	

3.2.4 Caspase 3/7 assay

NHBE cells were seeded into 96-well plates and grown until 90% confluent, cells were then rinsed with PBS for 3 times and transfected with control siRNA or *TNFRSF1A* siRNA. After 2 days, cells were infected with USSR H1N1 virus or uninfected. Cells were treated with 100 µl Caspase-Glo 3/7 reagent (Promega) at 6, 12, and 24 hours post infection (hpi) and reacted at room temperature for 30 mins. Lysates were then transferred to white bottom 96-well plate and luminescence was recorded by spectrophotometer Fluostar Optima (BMG Labtech) .

3.2.5 Real-time annexin V apoptosis and necrosis assay

NHBE cells and PTECs were seeded into 96-well plates until 70% confluent. NHBE cells were transfected with control siRNA or *TNFRSF1A* siRNA and Lipofectamine RNAiMAX (ThermoFisher) for 2 days. PTECs were transfected with control vector or *TNFRSF1A* plasmid and FuGENE HD Transfection Reagent (Promega) for 2 days. After 2 days, NHBE cells and PTECs were infected with 1.0 MOI USSR H1N1 virus containing Annexin V apoptosis and Necrosis assay (Promega) reagent. Luminescence and fluorescence activities were

measured by spectrophotometer Fluostar Optima (BMG Labtech) 0, 3, 6, 12 and 24 hpi.

3.3 Results

3.3.1 Knockdown of *TNFRSF1A* reduced virus output in NHBE cells but had no effect in PTECs

NHBE cells and PTECs were transfected with *TNFRSF1A* siRNA or control siRNA; 2 days post-transfection, cells were infected with USSR H1N1 for 24 h. Knockdown of *TNFRSF1A* in NHBE cells from all three biological replicates showed significant reduction in virus output (Figure 3-3A and B). In PTECs, the use of 3 different *TNFRSF1A* siRNAs had no effect on virus production (Figure 3-3C and D). However, knockdown of *TNFRSF1A* RNA in NHBE cells only had marginal effect on viral M1 and NP protein production, and even improved *M-gene* RNA production, suggesting that knockdown of *TNFRSF1A* in NHBE cells inhibited virus production indirectly possibly at post-translation (Figure 3-4A to D).

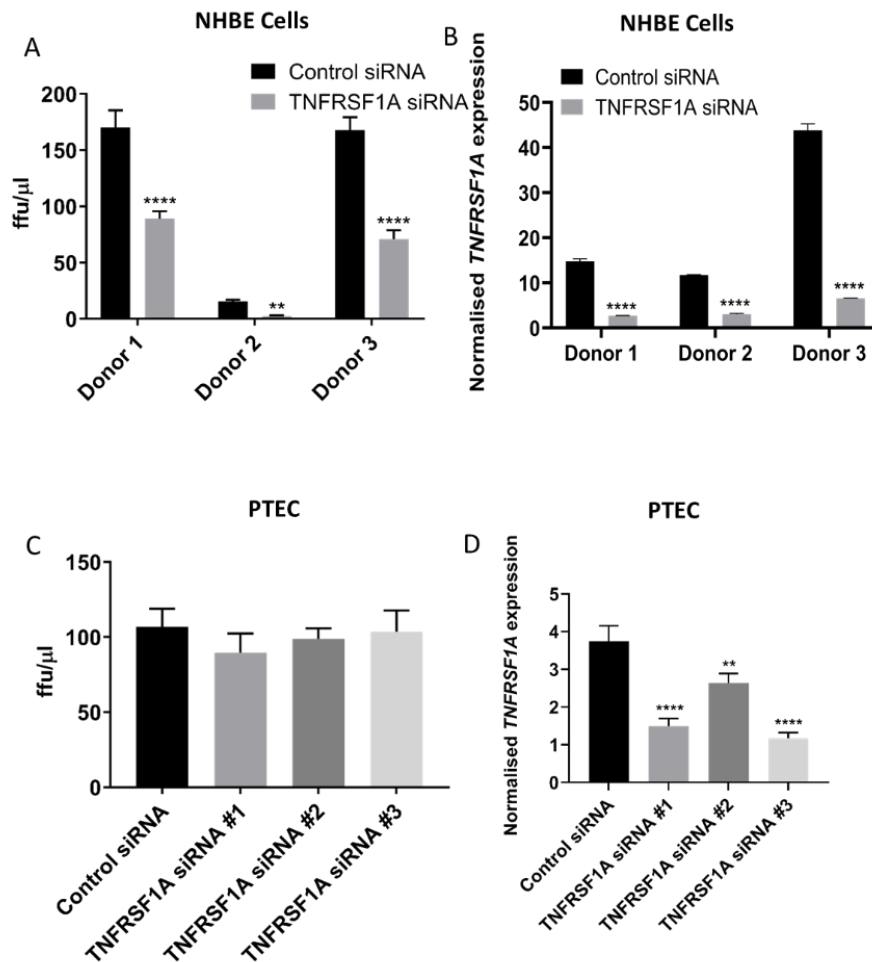


Figure 3-3. Knockdown of *TNFRSF1A* reduced virus output in NHBE cells but had no effect in PTECs. (A) *TNFRSF1A* siRNA reduced virus output in NHBE cells. Significance is relative to the control siRNA of each donor (2-way ANOVA with Sidak's multiple comparisons); (B) reduction in *TNFRSF1A* RNA in *TNFRSF1A* siRNA-transfected NHBE cells. Significance is relative to corresponding control siRNA of each donor (2-way ANOVA with Tukey's multiple comparisons) (C) *TNFRSF1A* siRNAs in PTECs had no effect on virus production, significance is relative to control siRNA (ordinary one-way ANOVA with Dunnett's multiple comparisons). (D) Reduction in *TNFRSF1A* RNA in *TNFRSF1A* siRNA-transfected PTECs, significance is relative to control siRNA (ordinary one-way ANOVA with Dunnett's multiple comparisons). Three biological replicates of NHBE cells were transfected with *TNFRSF1A* siRNA or control siRNA; 2 days post-transfection, cells were maintained in fresh media or infected with 1.0 MOI USSR virus for 24 h. Supernatants of infected cells were collected for MDCK cell titration by focus forming assays. Cell lysates were harvested for RNA extraction, cDNA conversion and qPCR for *TNFRSF1A*. Gene expression was normalised to *18S rRNA*. Levels of significance are shown in figures, 0.001<P<0.01 and P<0.0001 were given two and four asterisks, respectively.

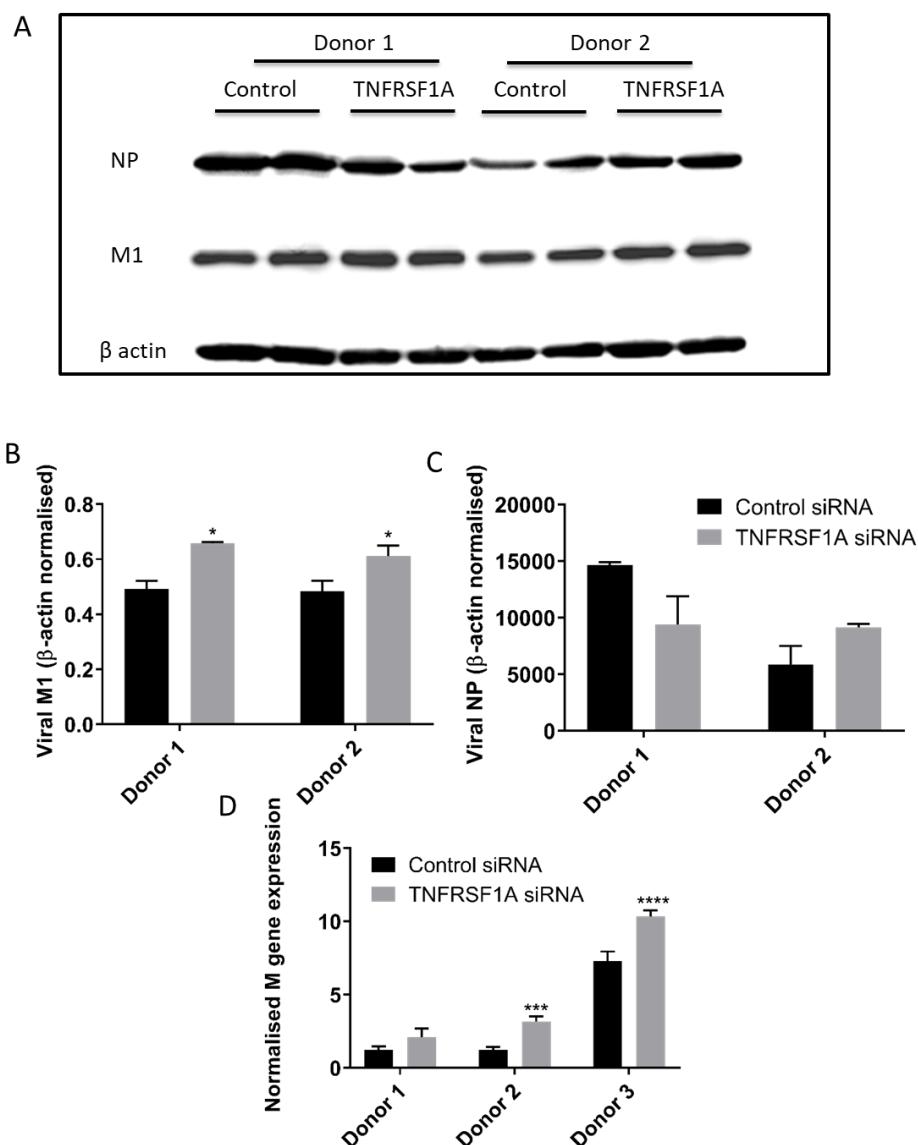


Figure 3-4. Knockdown of *TNFRSF1A* in NHBE cells had marginal effect on viral M1 and NP protein production but improved *M-gene* RNA production.

(A) Viral NP, M1 and β-actin protein detection in NHBE cells; (B) corresponding viral M1 protein expression, and (C) viral NP protein expression normalised to β-actin expression, significance is relative to corresponding control of each donor (2-way ANOVA with Sidak's multiple comparisons). (D) Viral *M-gene* RNA expression in NHBE cells from 2 of 3 donors was promoted by *TNFRSF1A* siRNA transfection. Two days post-transfection, cells were infected with 1.0 MOI USSR H1N1 virus for 24 h. Protein lysates or cell RNA lysates were then collected for western blotting detection or qPCR. For qPCR detection, M gene expression was normalised to *18S rRNA*. Indicated significance is relative to corresponding control (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, $0.0001 < P < 0.001$ and $P < 0.0001$ were given three and four asterisks, respectively.

3.3.2 Knockdown of TNFRSF1A in NHBE cells has no consistent effect on NF- κ B responsive and type-I IFN associated genes

TNFRSF1A contains a cytoplasmic death domain required for signalling pathways associated with apoptosis and NF- κ B activation. Four NF- κ B responsive genes (*TNF- α* , *IL-6*, *A20* and *MCP1*) showed no consistent effect at transcription after *TNFRSF1A* knockdown (Figure 3-5). *TNFRSF1A* siRNA transfected cells showed similar expression before infection in comparison to control siRNA; after infection, there was no consistent effect on gene expression in NHBE cells from three donors (Figure 3-5). Next, four IFN associated genes (*RIG-I*, *OAS1*, *JAK1* and *STAT1*) were also found to show no consistent transcriptional changes knockdown of *TNFRSF1A* (Figure 3-6).

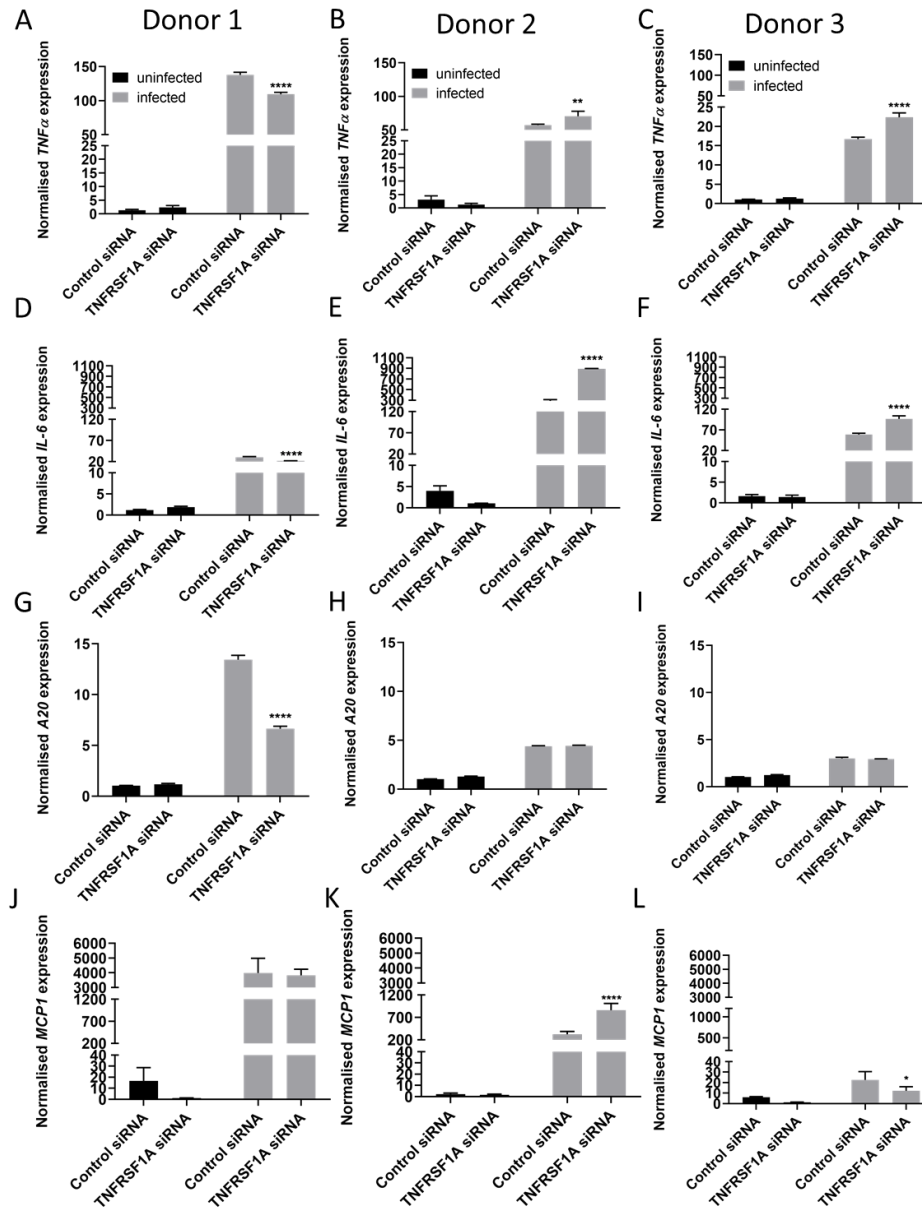


Figure 3-5. Knockdown of *TNFRSF1A* had no consistent effect on NF-κB responsive genes. Four NF-κB responsive genes (*TNF-α*, *IL6*, *A20* and *MCP1*) were selected for analysis. Three biological replicates of NHBE cells were transfected with control siRNA or *TNFRSF1A* siRNA for 2 days, cells were then maintained in fresh media or infected with 1.0 MOI USSR for 24 hours. Cell lysates were harvested for RNA extraction and cDNA conversion. Gene expression was normalised to *18S rRNA*. Significance is relative to corresponding control siRNA transfected cells within uninfected or infected group (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, 0.01<P<0.05 was given one asterisk, 0.001<P<0.01 and P<0.0001 were given two and four asterisks, respectively.

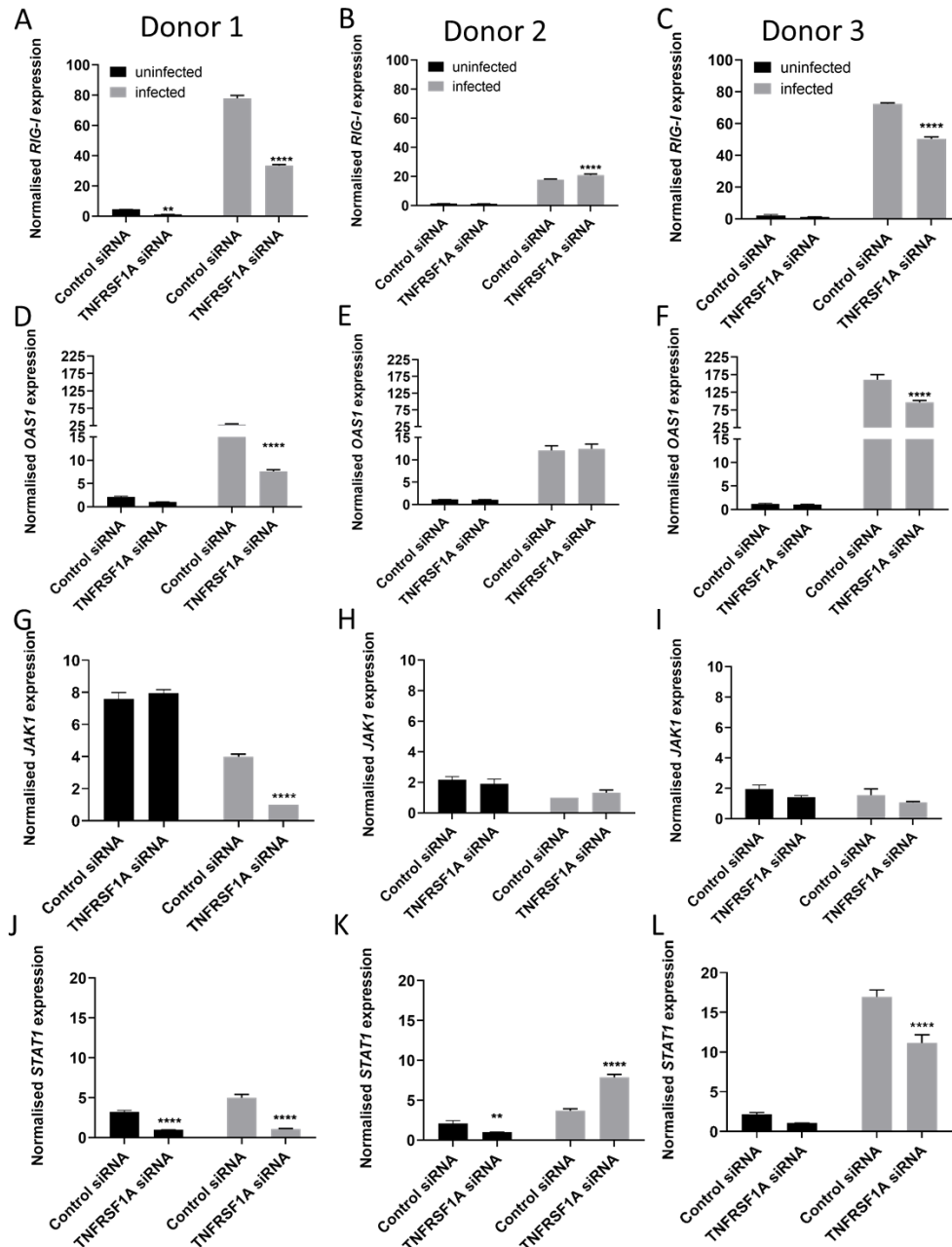


Figure 3-6. Knockdown of *TNFRSF1A* in NHBE cells had no consistent transcriptional effect on type I IFN associated genes. Four type I IFN associated genes (*RIG-I*, *OAS1*, *JAK1* and *STAT1*) were selected in the analysis. Three biological replicates of NHBE cells were transfected with control siRNA or *TNFRSF1A* siRNA for 2 days, cells were then maintained in fresh media or infected with 1.0 MOI USSR for 24 hours. Cell lysates were harvested for RNA extraction and cDNA conversion. Gene expression was normalised to *18S rRNA*. Significance is relative to corresponding control siRNA transfected cells within uninfected or infected group (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures. 0.001 < P < 0.01 and P < 0.0001 were given two and four asterisks, respectively.

3.3.3 Knockdown of *TNFRSF1A* in NHBE cells attenuates apoptosis and necrosis activity

TNFRSF1A is the key molecule that regulates the activity of apoptosis and necrosis (Gaeta, Johnson et al. 2000). The expression of key checkpoint apoptosis gene *BIRC3* (Liu, Hsu et al. 1996, Varfolomeev, Goncharov et al. 2008), and key checkpoint necrosis gene *CFLAR* (Safa 2013) were examined in *TNFRSF1A* knockdown cells; *CFLAR* and *BIRC3* expression in all three biological replicates were reduced post-infection (Figure 3-7). Moreover, *BIRC3* expression was significantly reduced by *TNFRSF1A* siRNA in uninfected cells (Figure 3-7).

Next, end-point caspase 3/7 and real-time necrosis assays were performed to assess apoptotic activity and necrosis activity during *TNFRSF1A* knockdown in NHBE cells. By 24 hpi, *TNFRSF1A* knockdown cells showed significant attenuation in caspase 3/7 activity (Figure 3-8A). *TNFRSF1A* siRNA transfection also attenuated necrosis activity at 24 and 36 hpi (Figure 3-8B). Attenuation of apoptotic activity and necrosis activity in infected NHBE cells could contribute to the reduction of virus output.

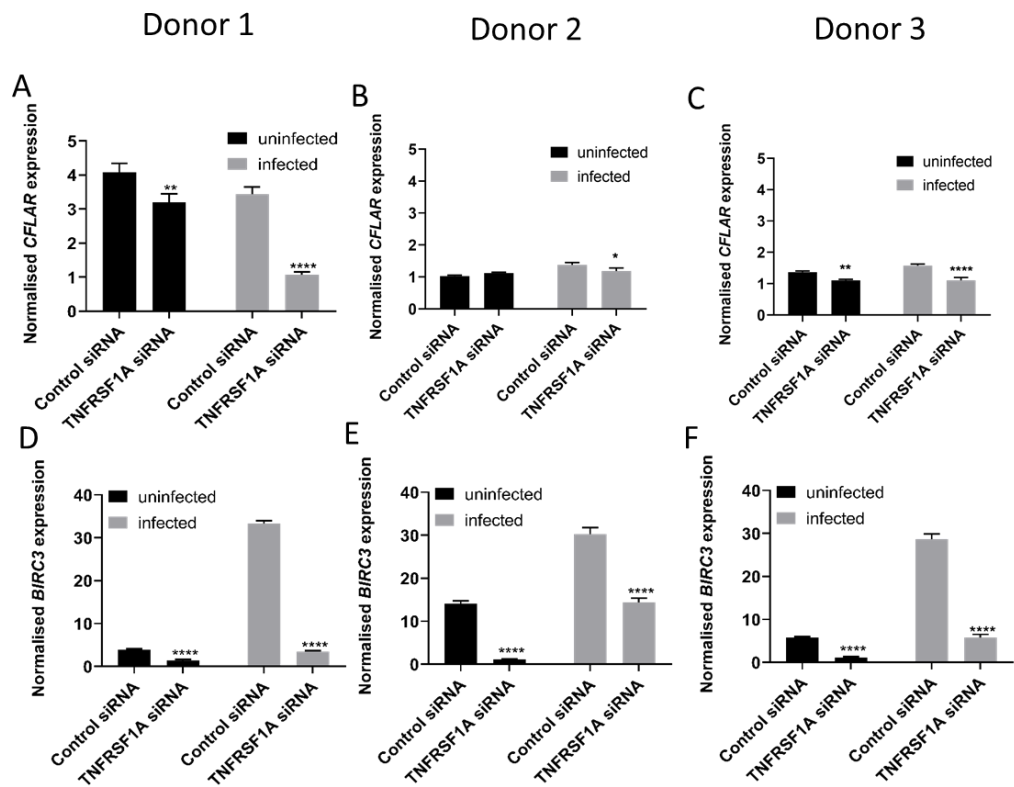


Figure 3-7. Knockdown of *TNFRSF1A* consistently reduced *CFLAR* expression post-infection, and *BIRC3* expression pre- and post-infection.

Three biological replicates of NHBE cells were transfected with *TNFRSF1A* siRNA or control siRNA; 2 days post-transfection, cells were maintained in fresh media or infected with 1.0 MOI USSR for 24 hours. Cell lysates were harvested for RNA extraction and cDNA conversion. Gene expression was normalised to *18S rRNA*. Significance is relative to corresponding control siRNA transfected cells within uninfected or infected group (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, $0.001 < P < 0.01$ and $P < 0.0001$ were given two and four asterisks, respectively.

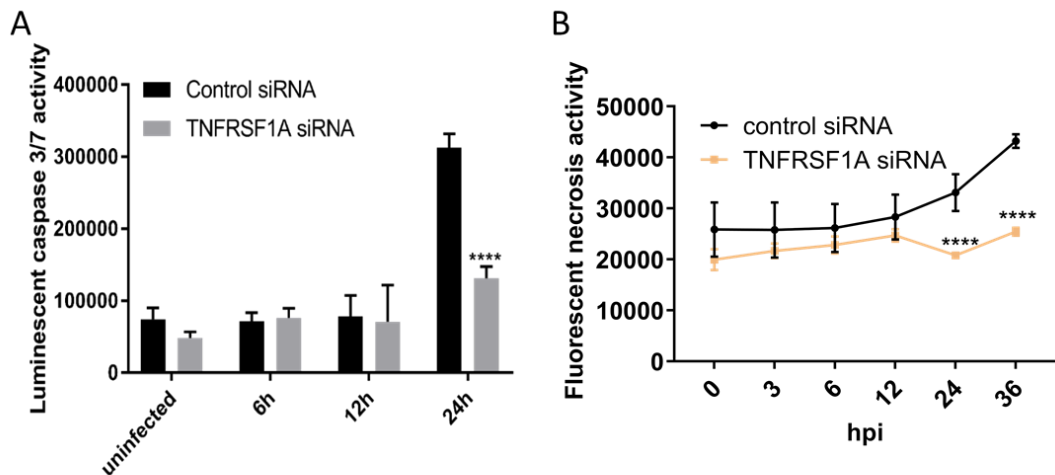


Figure 3-8. Knockdown of *TNFRSF1A* reduced apoptotic activity and necrosis activity in NHBE cells. NHBE cells were transfected with *TNFRSF1A* siRNA or control siRNA for two days. A) *TNFRSF1A* siRNA transfection reduced caspase 3/7 activity at 24 hpi. B) Necrosis assay time course during infection with 0.5 MOI of USSR H1N1 virus. *TNFRSF1A* siRNA significantly attenuated necrosis activity by 24 hpi. Significance is relative to corresponding control siRNA at each time point (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $P < 0.0001$ was given four asterisks.

3.3.4 Knockdown of *CFLAR* and *BIRC3* reduces virus production

To investigate the contribution of *CFLAR* and *BIRC3* in regulating virus production, *CFLAR* siRNA and *BIRC3* siRNA were separately transfected in NHBE cells from different donors. Knockdown of *CFLAR* and *BIRC3* resulted in reduced USSR H1N1 virus output (Figure 3-12), which suggests that *TNFRSF1A* knockdown down-regulates *CFLAR* and *BIRC3* leading to attenuation of apoptosis and necrosis

to reduce influenza virus production. Virus infection increased *BIRC3* and *CFLAR* expressions in comparison to control siRNA transfected uninfected cells, and *BIRC3* and *CFLAR* siRNAs impaired corresponding virus induced gene expressions.

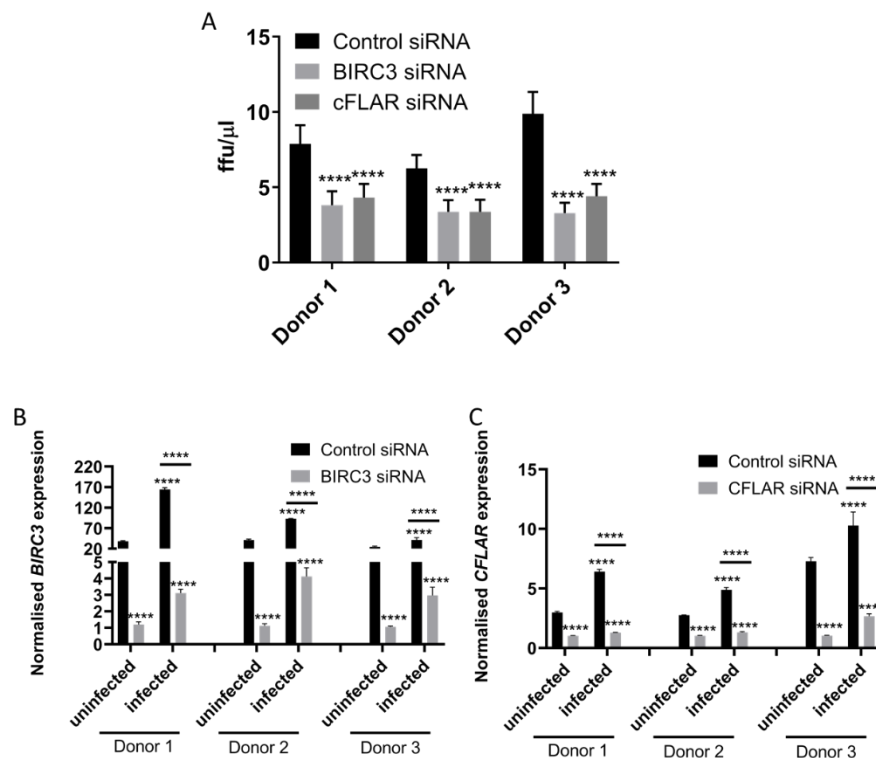


Figure 3-9. Knockdown of *CFLAR* and *BIRC3* in NHBE cells reduced virus production. (A) Transfection with *CFLAR* and *BIRC3* siRNAs reduced virus output; corresponding *BIRC3* gene expression (B) and *CFLAR* gene expression (C) in NHBE cells. Biological replicates of NHBE cells were transfected with *CFLAR* siRNA, *BIRC3* siRNA or control siRNA; 2 days post-transfection, cells were maintained in fresh media or infected with 1.0 MOI USSR for 24 hours. Supernatants of infected cells were collected for MDCK cell titration based of focus forming assay. Cell lysates were harvested for RNA extraction and cDNA conversion. Gene expression was normalised to *18S rRNA*. Significance is relative to corresponding control siRNA transfected cells within uninfected or infected group (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $P < 0.0001$ was given four asterisks.

3.3.5 Overexpression of *TNFRSF1A* increases virus output in PTECs but not in NHBE cells

We have found that basal *TNFRSF1A* expression in NHBE cells was significantly higher than in PTECs, and that *TNFRSF1A* knockdown in NHBE cells, but not PTECs, consistently reduced USSR H1N1 virus production (Figure 3-3). Next, viral effect of *TNFRSF1A* overexpression was compared between NHBE cells and PTECs. *TNFRSF1A* overexpression consistently increased USSR H1N1 virus progeny output in PTECs but had no effect on virus production in NHBE cells (Figure 3-10A, B and C). Viral M gene mRNA expression, and viral NP and M1 protein production were not affected by *TNFRSF1A* overexpression in PTECs which suggest that *TNFRSF1A* in PTECs indirectly facilitates increased progeny virus output post-translationally, possibly via viral protein transport or virus assembly (Figure 3-10D, E and F).

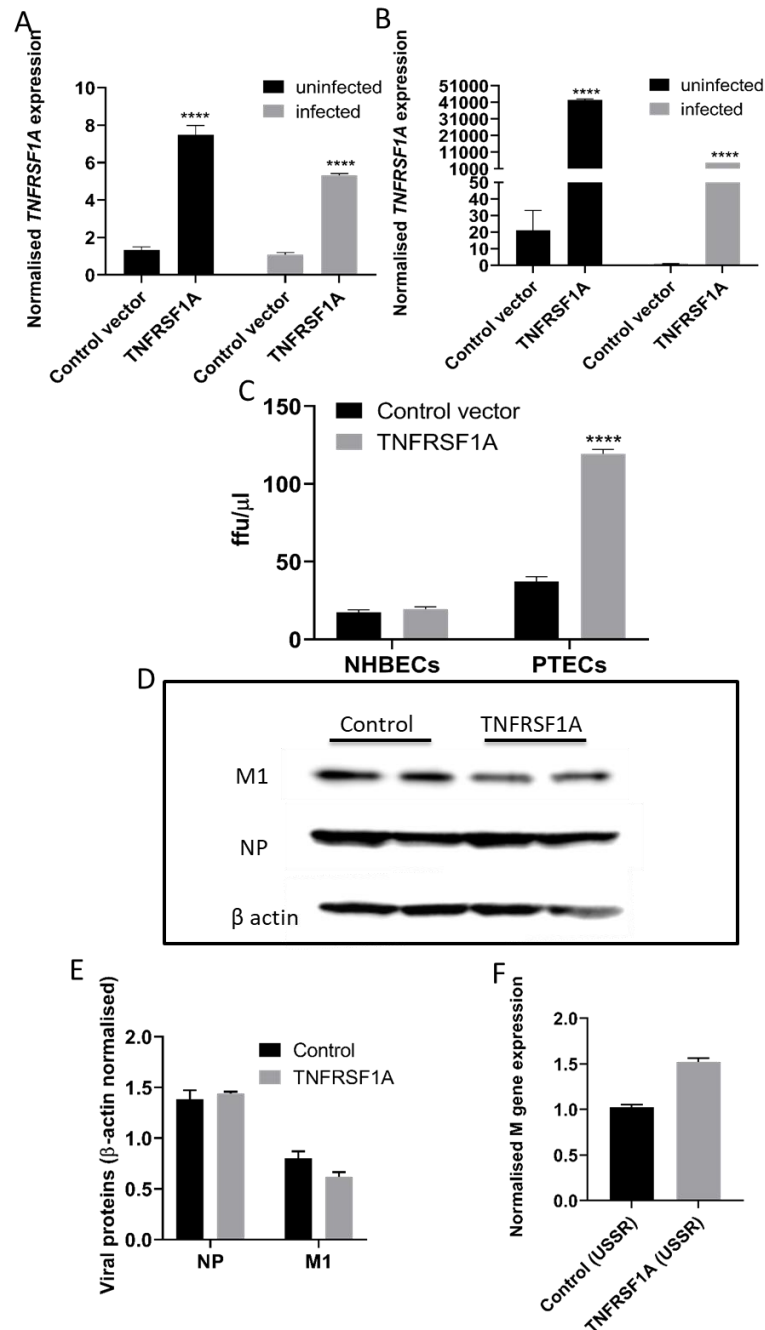


Figure 3-10. Overexpression of *TNFRSF1A* increased virus output in PTECs but not in NHBE cells. Cells were transiently transfected with *TNFRSF1A* expression control vector; 2 days post-transfection culture media were replaced with fresh media or cells were infected with 1.0 MOI USSR for 24 hours. Overexpression of *TNFRSF1A* RNA was evident in transfected NHBE cells (A) and PTECs (B). Supernatants of infected cells were collected for virus titration on MDCK cells by focus forming assay. Overexpression of *TNFRSF1A* in PTECs only increased progeny virus output (C). Results shown representative of 3 independent experiments. Significance from (A) to (C) is relative to corresponding control vector within each uninfected or infected group (2-way ANOVA with Sidak's multiple comparisons) (D) Detection of viral NP, M1 and β-actin in PTECs by

western blotting; each sample was loaded twice at 5 µg/well. (E) Viral NP and M1 protein expression in transfected PTECs normalised to β -actin. Significance is relative to corresponding control vector within NP or M1 group (2-way ANOVA with Sidak's multiple comparisons); (F) viral M gene RNA expression, normalised to *18S rRNA*. Significance is relative to corresponding control vector (unpaired t test with two-tailed P value). Levels of significance are shown in figures, $P < 0.0001$ was given four asterisks.

3.3.6 Overexpression of *TNFRSF1A* in PTECs elevates NF- κ B responsive gene expression during infection but does not affect the basal expression of NF- κ B- and type I IFN-associated genes

To evaluate host cell response to *TNFRSF1A* overexpression in PTECs, three NF- κ B responsive genes *IL-6*, *MCP1* and *TNF- α* and two type I IFN-associated genes *RIG-I* and *OAS1* were assessed as exemplars. In uninfected PTECs, overexpression of *TNFRSF1A* had no effect on the basal transcription of all selected NF- κ B responsive and type I IFN-associated genes (Figure 3-11). Infection of *TNFRSF1A*-transfected PTECs with USSR H1N1 virus significantly increased the expression of all three NF- κ B responsive genes and *RIG-I* relative to infected controls (Figure 3-11). Thus, increase in progeny virus output from *TNFRSF1A*-transfected PTECs is closely associated with the activation of NF- κ B responsive genes during infection.

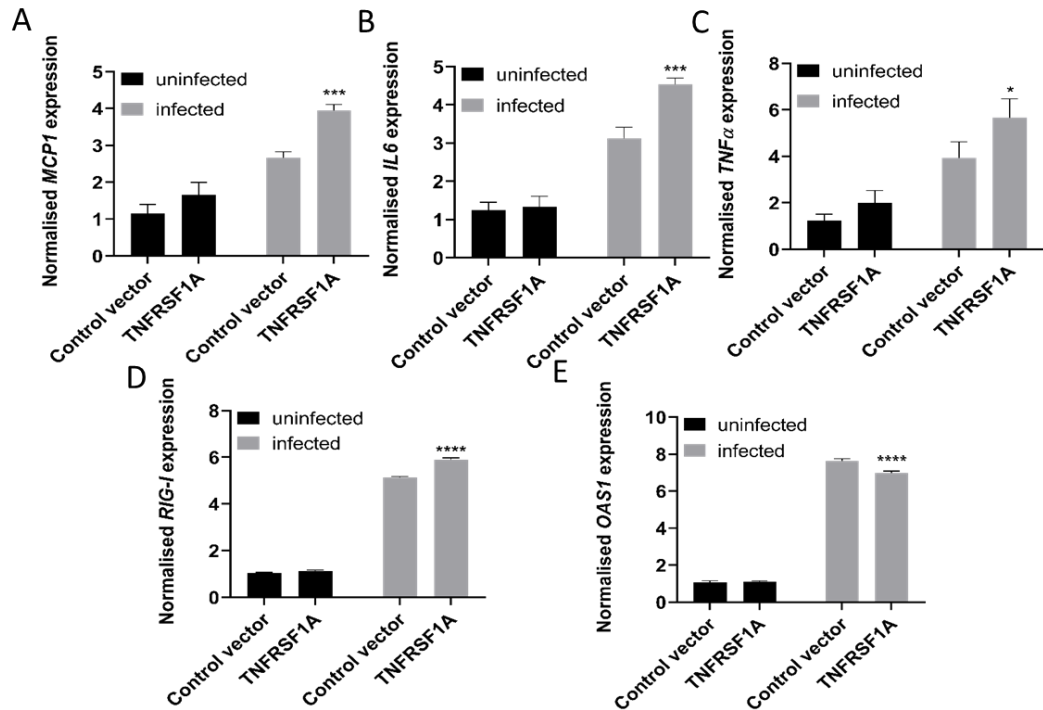


Figure 3-11. Overexpression of *TNFRSF1A* in PTECs elevated transcription of all three NF-κB responsive genes (*MCP1*, *IL-6* and *TNF-α*) during infection but not their basal (uninfected) expression. PTECs were transfected with *TNFRSF1A*-expression or control vector; 2 days post-transfection culture media were replaced with fresh media or cells were infected with 1.0 MOI USSR for 24 hours. Cell lysates were harvested for RNA extraction and cDNA conversion; gene expression was normalised to *18S rRNA*. *TNFRSF1A* overexpression had little effect on type I IFN responsive genes (*RIG-I* and *OAS1*) Significance is relative to corresponding control vector within uninfected or infected group (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, 0.01<P<0.05 was given one asterisk, 0.0001<P<0.001 and P<0.0001 were given three and four asterisks, respectively.

3.3.7 During infection *TNFRSF1A* overexpression in PTECs reduces necrosis activity and does not increase apoptotic activity

To assess possible effects of cell viability from *TNFRSF1A* overexpression in PTECs, expression of exemplar genes *BIRC3* and *CFLAR* was monitored pre- and post-infection. As earlier discussed, *BIRC3* and *CFLAR* are key checkpoints of apoptosis and necrosis, respectively, downstream of TNFR1 signalling pathway. Overexpression of *TNFRSF1A* reduced basal *CFLAR* expression but had no effect on *BIRC3* expression regardless of infection (Figure 3-12A and B), which suggests that overexpression of *TNFRSF1A* in PTECs could reduce necrosis activity. During infection, overexpression of *TNFRSF1A* in PTECs did not increase caspase 3/7 apoptotic activity (Figure 3-12C) but significantly reduced necrosis activity (Figure 3-12D), which indicates that reduced necrosis activity and the absence of increased apoptosis activation is closely linked to increased virus replication in PTECs. Overexpression of *TNFRSF1A* in NHBE cells did not affect virus output (Figure 3-10C), which shows different virus output outcome in comparison to overexpression of *TNFRSF1A* in PTEC. Collectively, altering basal *TNFRSF1A* expression prior to infection in NHBE cells and PTECs confers different outcomes in virus progeny production associated

with differential host cell response to apoptosis and necrosis (Table 3-2).

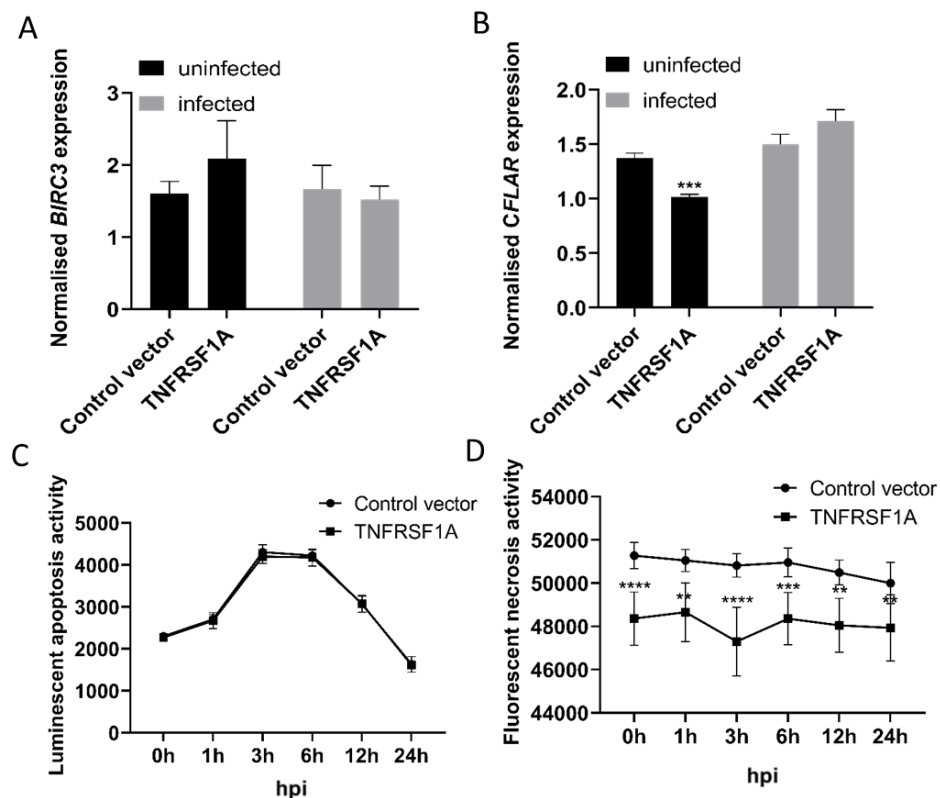


Figure 3-12. Overexpression of *TNFRSF1A* in PTECs reduced activity of necrosis but not apoptotic activity during infection. PTECs were transfected with *TNFRSF1A* expression or control vector; 2 days post-transfection culture media were replaced with fresh media or cells were infected with 1.0 MOI USSR for 24 hours. Cell lysates were harvested for RNA extraction and cDNA conversion; gene expression was normalised to *18S rRNA*. Significance is relative to corresponding controls (2-way ANOVA with Sidak's multiple comparisons.). (A) Expression of (A) *BIRC3* and (B) *CFLAR* post-*TNFRSF1A* transfection. (C) *TNFRSF1A* overexpression had no effect on apoptosis activity in PTECs. (D) *TNFRSF1A* overexpression reduced necrosis activity before and during infection. In apoptosis assay and necrosis assay, cells were transfected with indicated vectors for two days, and then were infected with 0.5 MOI USSR H1N1 virus. Measurements were taken at indicated time points. Indicated significance is relative to corresponding control vector at each time point (2-way ANOVA with Sidak's multiple comparisons). Levels of significance are shown in figures, $0.001 < P < 0.01$, $0.0001 < P < 0.001$ and $P < 0.0001$ were given two, three and four asterisks, respectively.

Table 3-2. Comparative impact of TNFRSF1A on virus output and host response in NHBE cells and PTECs.

Treatment	Virus output and Host response	NHBE cells	PTECs
TNFRSF1A knockdown	Virus output	Reduced	No effect
	NF-κB activity	No consistent effect before or during infection	ND
	Type I IFN activity	No consistent effect before or during infection	ND
	Apoptosis	Attenuated during infection	ND
	Necrosis	Attenuated during infection	ND
TNFRSF1A overexpression	Virus output	No effect	Increased
	NF-κB activity	ND	No effect before infection, but elevated NF-κB gene response during infection
	Type I IFN activity	ND	No consistent effect before or during infection
	Apoptosis	ND	No effect before or during infection
	Necrosis	ND	Reduced necrosis activity during infection

ND = not done

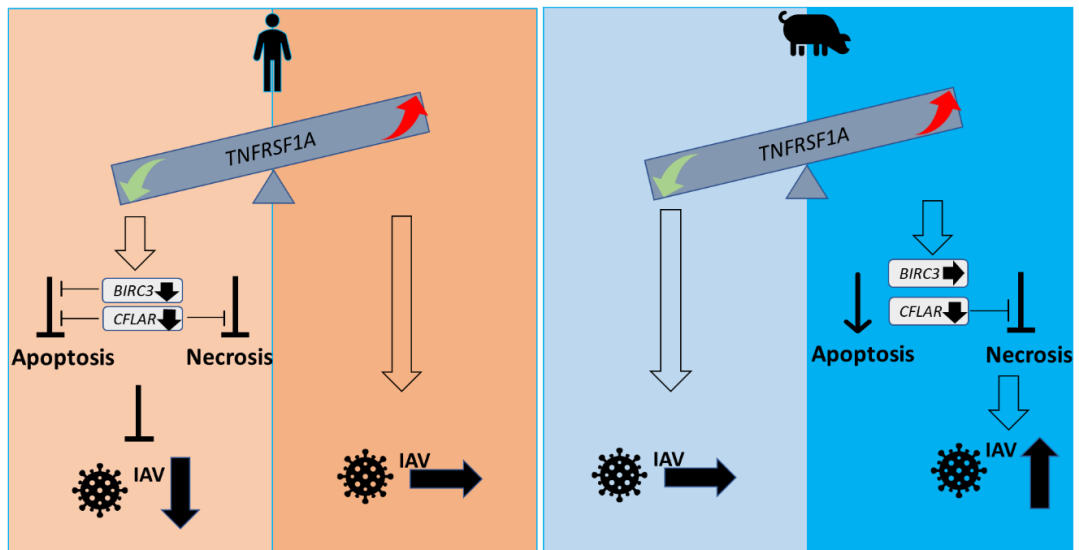


Figure 3-13. Schematic summary of the relative impact of *TNFRSF1A* on virus outcome in NHBE cells and PTECs. The relative basal expression of *TNFRSF1A* is higher in NHBE cells than in PTECs. *BIRC3* and *CFLAR* both serve as key regulators of apoptosis in the TNFR1 signalling pathway, and *CFLAR* also plays an important role in regulating necrosis, which can be dependent or independent of apoptosis. Reducing *TNFRSF1A* expression in NHBE cells reduces *BIRC3* and *CFLAR* expression, attenuates both apoptosis and necrosis activities, and indirectly reduces virus production. *TNFRSF1A* overexpression in NHBE cells has little effect on virus replication. In PTECs, *TNFRSF1A* overexpression reduces *CFLAR* expression but has no effect on *BIRC3* expression, reduces necrosis but not apoptosis activity, and indirectly increases virus replication. However, reduced *TNFRSF1A* expression in PTECs does not affect virus production. Solid black arrows indicate direction of change, bar-head arrows indicate inhibition.

3.4 Discussion

Basal *TNFRSF1A* expression, which encodes TNFR1, in NHBE cells is much higher than in PTECs. As TNFR1 is a key molecule that regulates the NF- κ B pathway, promotion of TNFR1 could activate NF- κ B to promote cell survival and induce apoptosis to cause cell death (Legler, Micheau et al. 2003). The ligation of TNFR1 with *TNF- α* can recruit downstream DD-containing adaptor protein (TRADD) to further recruit other regulators (Hsu, Shu et al. 1996). It was discovered that TNFR1 is important in influenza virus replication. TNFR1 knockout mice had delayed or reduced expression of genes associated with antiviral and innate immune signaling during influenza virus infection (Belisle, Tisoncik et al. 2010). Paradoxically, it was found that knockout of TNFR1 in mice had significantly reduced morbidity following HPAI H5N1 virus infection but no difference in viral replication and spread or ultimate disease outcome compared with wild-type mice (Szretter, Gangappa et al. 2007). All the above show that knockout of TNFR1 could benefit influenza virus replication.

Our study showed that changing the expression of *TNFRSF1A* prior to infection had different outcomes on influenza virus output between NHBE cells and PTECs. Reducing *TNFRSF1A* expression in PTECs, which have relatively low constitutive level of *TNFRSF1A* in comparison with NHBE cells, had little effect on virus progeny

production. However, in NHBE cells, where basal *TNFRSF1A* expression is relatively high, its knockdown significantly reduced virus output.

TNFR1 is typically activated by binding of TNF- α , and rapidly recruits TRADD, RIP1, TRAF2 and cIAP1/2 to form complex I and subsequently signals downstream to induce NF- κ B activity and cell survival or recruits TRADD, FADD and caspase 8/10 to induce apoptosis (Micheau and Tschopp 2003). We found no consistent transcriptional change of key molecules in complex I (TRADD, RIPK1, and TRAF2) and FADD in *TNFRSF1A* siRNA transfected NHBE cells (data not shown); and knockdown of *TNFRSF1A* in NHBE cells had no consistent effect on NF- κ B responsive and type-I IFN associated genes. Micheau et al. (2003) showed the formation of complex I from TNFR1 activation in HT1080 cells occurred at 5 mins post TNF- α treatment. As NHBE cells RNA samples were harvested at 2 days post-transfection, it is possible that transcriptional changes of these key molecules had occurred at an earlier stage.

The cellular inhibitor of apoptosis proteins cIAP1 and cIAP2 are E3 ubiquitin ligases encoded by *BIRC2* and *BIRC3* respectively (Zhang, Webster et al. 2019). Both cIAPs are recruited to the adaptor protein TRAF2 and subsequently bind to the adaptor TRADD, and serve as the key checkpoint of complex I of TNFR1 signalling pathway (Hsu, Xiong et al. 1995, Vince, Pantaki et al. 2009). The key function of

cIAP1 and cIAP2 proteins is to bind to caspases and to inhibit apoptosis. cIAP1 and cIAP2 share redundant actions in mediating TNFR1 signalling pathway (Silke and Brink 2010). Our study showed that knockdown of *TNFRSF1A* in NHBE cells reduced the mRNA expression of *BIRC3* consistently in all three donor cells with or without virus infection, but the knockdown had no effect on *BIRC2* expression (data not shown).

Complex I is rapidly formed upon ligand activation of TNFR1, followed by complex II formation containing TRADD, FADD, caspase-8 and cFLIP (Ting and Bertrand 2016). cFLIP is also known as and encoded by Caspase-8 and FADD Like Apoptosis Regulator (*CFLAR*), and a key molecule downstream of TNFR1. cFLIP can be induced by NF- κ B, and is a caspase-8 homolog that lacks catalytic activity but competes with caspase-8 for FADD binding (Ting and Bertrand 2016). cFLIP, like cIAPs, serves as part of a negative regulatory loop of the TNFR1 pathway to inhibit cell death (Irmeler, Thome et al. 1997). Therefore, one of the key roles of cFLIP in TNFR1 signalling is to inhibit apoptosis. Studies have also indicated the role of cFLIP in programmed necrosis. It was found that increased expression of the long isoform of cFLIP (cFLIPL) cleaved RIP1 to inhibit both apoptosis and programmed necrosis in mice (Ram, Ilyukha et al. 2016). Deletion of cFLIP in mouse intestinal epithelial cells resulted in enhanced apoptosis and programmed necrosis (Piao, Komazawa-

Sakon et al. 2012). cFLIP deletion in adult mice intestinal epithelium resulted in lethality with increased inflammation and robust caspase-8 and caspase-3 expression (Wittkopf, Gunther et al. 2013). In *TNFRSF1A* siRNA transfected NHBE cells, in addition to reduced *BIRC3* expression prior to and during influenza virus infection of NHBE cells, knockdown of *TNFRSF1A* NHBE cells also attenuated *CFLAR* expression during influenza virus infection. Based on real-time apoptosis and necrosis assays, knockdown of *TNFRSF1A* reduced apoptotic and necrosis activity in NHBE cells. Attenuated apoptotic activity was likely to be mediated by reduced expression of *BIRC3*, and the reduced necrosis activity could be possibly mediated by reduced *CFLAR* expression.

In the overexpression studies, increased expression of *TNFRSF1A* again resulted in different virus output outcomes between PTECs and NHBE cells. Overexpression of *TNFRSF1A* increased virus output in PTECs, but had no effect in NHBE cells. This host-species cell contrast indicates that the relative basal expression of NF- κ B and its associated genes could be a key factor that dictates the effectiveness of host cell response to influenza virus infection. The overexpression of *TNFRSF1A* in PTECs had little effect on type I IFN and NF- κ B responsive genes prior to infection, however, with increased *TNFRSF1A* expression, the expression of NF- κ B responsive genes (*IL-6*, *TNF- α* and *MCP1*) was upregulated during H1N1 virus infection.

TNFRSF1A overexpression did not affect *BIRC3* expression but reduced *CFLAR* prior to virus infection. Real-time apoptosis and necrosis assays indicated that apoptosis activity was unchanged by the *TNFRSF1A* overexpression, but the necrosis activity was significantly reduced in relation to corresponding control vector. This implies that the reduced necrosis activity in PTECs, which could be led by reduced *CFLAR* expression, is independent to apoptosis. *CFLAR* lacks catalytic activity but has high homology with caspase-8 can compete with caspase-8 for the binding with FADD to inhibit caspase-dependent apoptosis (Dondelinger, Darding et al. 2016). Apoptosis is generally facilitated by phagocytes, also refers to scavengers, to clear dead cells, but when the scavengers are absent, the occurrence of necrosis could become independent of apoptosis (Silva 2010). When apoptosis was blocked, cells were able to execute necrosis-like cell death in TNF-induced cells (Degterev, Huang et al. 2005, Degterev, Hitomi et al. 2008, Chen, Kang et al. 2018).

In this study, the apoptotic and necrotic activities were determined by real-time PCR, and apoptosis and necrosis assays. However, future characterisation, such as detection of caspases or necrosis responsive proteins, should include the use of western blotting or flow cytometry. RNA-seq will provide insights into global transcriptional changes from knockdown of *TNFRSF1A* in NHBE cells

or from overexpression of *TNFRSF1A* in PTECs. As changes in virus replication from knockdown of *TNFRSF1A* in NHBE cells or from overexpression of *TNFRSF1A* in PTECs occurred post-translationally, it will be important to elucidate specific post-translational modifications that regulate IAV replication.

In summary, based on microarray analysis of basal gene expression of human and pig epithelial cells, it was found that *TNFRSF1A*, a key modulator of NF- κ B and apoptotic activities, is more highly expressed in NHBE cells than in PTECs. Knockdown and overexpression of *TNFRSF1A* in the two cell types had different outcomes on H1N1 virus infection. *TNFRSF1A* knockdown reduced H1N1 virus output significantly, but had no effect to virus replication in PTECs. Real-time PCR, apoptosis and necrosis assays indicated that the virus reduction in NHBE cells was possibly led by the attenuated apoptosis and necrosis activities during influenza virus infection. With increased *TNFRSF1A* expression, virus replication in PTECs was increased but in human cells it remained unchanged in human cells; increased virus replication in PTECs might be related to apoptosis-independent necrosis.

4. Acetylsalicylic acid blocks influenza virus replication through activation of NF- κ B signalling in primary porcine tracheal epithelial cells, but attenuates virus-induced apoptosis in primary normal human bronchial epithelial cells

Abstract

Aspirin, also known as acetylsalicylic acid (ASA), is a widely used anti-inflammatory drug. It has a history of being used as an antiviral drug for influenza virus infection since the 1918 H1N1 virus pandemic. In this study, we show the potency of ASA as an antiviral drug that can inhibit influenza virus replication efficiently in human and pig airway epithelial cells. The most studied antiviral mechanism of ASA is its inhibition of IKK β activity in the NF- κ B pathway. Here, we found that ASA works through different mechanisms between human and pig airway epithelial cells to block influenza virus replication. In primary porcine tracheal epithelial cells (PTECs), ASA blocks influenza virus replication through a NF- κ B dependent mechanism, as the activation of NF- κ B diminishes the antiviral effect of ASA. However, the antiviral mechanism of ASA in primary normal human bronchial epithelial (NHBE) cells is possibly through the inhibition of virus-induced apoptosis. Moreover, virus replication is blocked by ASA at the transcriptional level in PTECs, but at post-translational level in NHBE cells. Taken together, ASA as an anti-influenza drug works through different antiviral mechanisms between human and pig airway epithelial cells.

4.1 Introduction

4.1.1 Overview of Aspirin

Aspirin is a widely used non-steroidal anti-inflammatory drug (NSAID) (Liu, Fang et al. 2017). The use of aspirin could possibly date back to 3500 years ago, when willow barks were used by ancient Sumerians and Egyptians as pain killer, and the active component of willow tree became the basis of aspirin development (Desborough and Keeling 2017). Aspirin is the trade name, which was given by the chief pharmacologist, Heinrich Dreser, from the Bayer laboratories (Sneader 2000). Acetylsalicylic acid (ASA) is the major active ingredient in aspirin that works through many different mechanisms of actions for a variety of clinical applications including cardiovascular diseases, stroke, pain relief and venom thromboembolism (Mekaj, Daci et al. 2015).

4.1.2 The antiviral effect of ASA

Aspirin was found to have antiviral effects against different viruses. The entry of hepatitis C virus (HCV) in Huh7.5.1 cells was blocked by aspirin through attenuated host cell expression of claudin-1 (Yin and Zhang 2016). Aspirin was found to reduce the replication of Zika virus in primary human fetal endothelial cells (Pan, Peng et al. 2018).

The use of ASA was found to be effective in reducing varicella zoster virus (VZV) (Primache, Binda et al. 1998). The increased resolvin D1 (RvD1) expression induced by aspirin reduced the replication of Herpes simplex virus (HSV) (Rajasagi, Bhela et al. 2017). Glatthaar-Saalmuller et al., (2017) showed that the use of aspirin inhibited the replication of influenza H1N1 virus, respiratory syncytial virus (RSV) and human rhinoviruses (HRV) (Glatthaar-Saalmuller, Mair et al. 2017). Most notably, aspirin was widely used to treat patients during 1918-1919 H1N1 influenza virus pandemic (Starko 2009).

4.1.3 The complication of ASA in influenza virus infection

The estimated death toll of 1918 H1N1 influenza virus was at least 50 million worldwide (Spreeuwenberg, Kroneman et al. 2018). The 1918 H1N1 virus was found to have a high mortality rate in the younger population. In 8 US cities, 30% of 25-29 adults were infected by the virus with 3% deaths caused by influenza or pneumonia (Starko 2009). It was shown that the high dosing recommendation of aspirin for influenza virus treatment could be one of the key factors in contributing to the high death rates during the pandemic (Starko 2009). According to the recommendation of the UK National Health Service (NHS), the maximum usage of aspirin per 24 h is 3600 mg and taken at every 4 h with 600 mg. However, several reports indicated aspirin was taken at much higher dose for

influenza virus treatment during 1918 influenza pandemic. The British Ministry of Health at the time suggested 15 to 20 grains of aspirin (1000 mg to 1300 mg) without indication of intake frequency, but a report revealed a doctor in London advised patients to take 20 grains every hour for 12 consecutive hours (Medicine 1918, French 1920, Starko 2009). In the US, the dosage of acetylsalicylic acid was set at 15 grains (1000 mg) for every three hours (Therapeutics 1918). Therefore, aspirin was abusively used during 1918, which could lead to toxicity and increased mortality in influenza virus infected patients.

The use of aspirin is known to cause Reye's syndrome, particularly in younger populations. Reye's syndrome is characterised by vomiting, fever, coma, liver failure and hepatic encephalopathy. Depending on the severity, Reye's syndrome can be fatal (Fitzgerald 2007). It was shown that 80% of the children diagnosed with Reye's syndrome had taken aspirin for three weeks (Chapman and Arnold 2020). One of the possible mechanisms of aspirin in causing Reye's syndrome is by damaging mitochondria integrity. In HEK293 cells and mouse embryonic fibroblasts (MEFs), the use of aspirin increased mitochondrial long-chain fatty acid oxidation (Uppala, Dudiak et al. 2017). The use of aspirin caused changes in host cell cytochrome P450 enzyme expression, which is the key enzyme to dysregulate cytokine (*IL-6*, *TNF- α* and *IL-1 β*) response, resulting in disruption of cytokine response and ultimately in Reye's syndrome

(Prandota 2002, Aitken and Morgan 2007). Calcium ion metabolism was found to be regulated by aspirin, and the regulation could lead to toxic consequences from liver damage. Studies have implied influenza virus infection could also cause Reye's syndrome. It was shown that influenza B virus infection could induce Reye's syndrome in mice (Davis, Green et al. 1985). A fatal case was reported on a two-year old boy who died from infection with influenza A virus, and Reye's syndrome was later found to be the primary cause of death (Noor and Gradidge 2018). Therefore, the use of aspirin and infection by influenza virus could be co-factors that caused deaths during 1918 H1N1 influenza virus pandemic.

4.1.4 ASA blocks IAV replication by inhibiting NF- κ B activity

One possible mechanism of aspirin in inhibiting influenza virus replication is through the NF- κ B pathway. Aspirin and sodium salicylate were first found to inhibit the NF- κ B signalling pathway; the inhibition prevents I κ B degradation, which retains NF- κ B in the cytosol (Kopp and Ghosh 1994). Aspirin binds to the helix-loop-helix domain of IKK2 to reduce ATP binding and phosphorylation *in vivo* and *in vitro* (Grilli, Pizzi et al. 1996, Yin, Yamamoto et al. 1998).

Mazur et al. (2007) showed the antiviral property of ASA is NF- κ B dependent, but did not seem to depend on cyclo-oxygenases (COX).

The use of a COX inhibitor, indomethacin, alone did not affect virus output of H7N7, PR8 (H1N1) and H5N1 viruses, which indicate that the anti-influenza activity of ASA is not due to the inhibition of the COX pathway in A549 cells (Mazur, Wurzer et al. 2007). ASA was also found to have no effect on influenza virus-induced mitogen-activated protein kinase (MAPK) cascades, but was found to inhibit I κ B α expression of NF- κ B. Mazur et al. (2007) further showed ASA delayed viral RNP export but did not affect viral protein accumulation. ASA blocks the synthesis of pro-apoptotic genes including TRAIL and FasL (Mazur, Wurzer et al. 2007).

4.1.5 ASA possibly inhibits influenza virus replication by blocking cyclo-oxygenases

In disagreement with what Mazur et al. (2007) suggested, that ASA may work through a COX independent mechanism to inhibit IAV replication, extensive studies suggest COX might be involved in the process. The other basic mechanism of ASA action is the inhibition of COX to inhibit prostaglandin synthesis (Vane 1971). The COX pathway is important in the regulation of immune response and is responsible for prostaglandin (PG) production. Three COX enzymes have been identified, constitutively expressed COX-1, inducible COX-2, and the recently identified but poorly understood COX-3 (Chandrasekharan, Dai et al. 2002). In A549 cells, ASA was found to fully inhibit COX-1 and COX-2 (Warner, Giuliano et al. 1999).

COX1 and COX2 have contrasting effects in response to influenza virus. Knockout of *COX-1* is detrimental, but *COX-2* deficiency is beneficial to mice during H3N2 influenza virus infection (Carey, Bradbury et al. 2005). *COX-1* deficiency led to a stronger inflammatory response, earlier proinflammatory cytokine production and higher mortality (Carey, Bradbury et al. 2005). However, viral titres of *COX-2* deficient mice were higher than *COX-1* deficient mice, which was inconsistent with the higher survival rate in *COX-2* deficient mice (Carey, Bradbury et al. 2005). In a follow-up study (Carey, Bradbury et al. 2010), comparison between the use of a *COX-1* inhibitor (SC-560) and *COX-2* inhibitor (celecoxib) in H3N2 influenza virus infected mice was conducted. *COX-1* inhibitor was detrimental and caused more mortality than *COX-2* inhibitor; *COX-2* inhibitor treatment, however, did not improve clinical outcome relative to infected WT mice; virus titres were comparable between treatment groups (Carey, Bradbury et al. 2010). Furthermore, another *COX-2* inhibitor (CAY10404) increased influenza virus production in A549 cells, and the host cell mRNA stability of *COX-2* was interfered with viral proteins (Dudek, Nitzsche et al. 2016).

All the above suggest that the role of *COX-1* and *COX-2* in influenza virus replication is far from clear. *COX-1* may play a protective role against influenza virus infection, and *COX-2* could contribute to virus replication.

Influenza virus replication could be more COX-2 dependent than COX-1 dependent. COX-2 was up-regulated during H3N2 virus infection in bronchial epithelial cell lines; however, the expression of COX-1 did not change (Mizumura, Hashimoto et al. 2003). The activation of COX-2 following influenza virus infection varies according to virus subtypes. H5N1 virus and H9N2 virus highly induced expression of COX-2, but not H1N1 virus (Lee, Gai et al. 2011). Proinflammatory cytokines and *TNF- α* were hyperinduced in H5N1 virus infected cells when COX-2 was highly expressed; relatively low inflammatory response was detected in H1N1 virus infected cells (Lee, Cheung et al. 2008). Subtypes of IAV that are high COX-2 inducers (H5N1, H9N2) are more sensitive to COX-2 inhibitors but subtypes that are low COX-2 inducers (H1N1, H3N2) are less sensitive to COX-2 inhibitors (Lee, Cheung et al. 2008).

To summarize, there are two major pathways mediated by ASA that could be involved in the inhibition of influenza virus infection. One is by inhibiting IKK2, and the other is by inhibiting COX-2. The antiviral property of ASA could involve both mechanisms. Studies have shown the activation of NF- κ B could lead to the induction of COX-2 (Rodriguez, Domingo et al. 2014). This implies a connection between NF- κ B and COX-2. Moreover, it was shown that induction of oxidative stress induced the phosphorylation of NF- κ B and MAPKs, and then activated NF- κ B to increase COX-2 expression, suggesting a complicated underlying mechanism (Garcia-Garcia, Mullol et al.

2012). Therefore, the mechanism of ASA-mediated inhibition of influenza virus replication needs to be clarified.

4.1.6 Aims and objectives

The primary aim of this study was to determine the antiviral effect of ASA in human and pig airway epithelial cells, and to identify the underlying mechanism of how ASA works to inhibit influenza virus replication in different host cells. The specific objectives were:

1. To examine the virus output outcomes between human and pig airway epithelial cells in response to ASA treatment.

Primary normal human bronchial epithelial cells (NHBE) and corresponding human cell line (A549 cells), primary porcine tracheal epithelial cells and corresponding porcine cell line (NPTr cells) were used to study the host cell response to ASA treatment prior to and during influenza virus infection. Cells were primed with ASA or treated with ASA during virus infection; virus output outcomes were assessed by focus forming unit (FFU) assay. Viral mRNA and protein expression were also assessed to determine which virus replication stage is inhibited by ASA.

2. To elucidate the antiviral mechanism of ASA in different types of host cells.

Host cell innate immune response was examined by real-time PCR and western blotting. RNA sequencing was performed to establish the transcriptional profile of the action of ASA during influenza virus infection.

4.2 Materials and Methods

4.2.1 Cytotoxicity assay

PTECs and NHBE cells were seeded into 96-well plates, and were treated with DMSO or ASA at desired concentrations (0.5 mM, 1.0 mM, 2.0 mM and 5.0 mM) for 24 h. CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used for the viability detection followed the product instruction. The CellTiter-Glo reagent was added to the 96-well plate, luminescence activity was measured by LUMIstar Omega Microplate Luminometer (BMG Labtech). The use of DMSO was not toxic, because the cells treated without DMSO showed similar viability with the cells treated with DMSO (data not shown).

4.2.2 Transfection and infection

The plasmids used for transfection were described in 2.2.2. In brief, kc2587 was used as a control (empty) vector, IKK2 CA (constitutively active I κ B kinase 2) and RelA-GFP were used to increase to expression of NF- κ B activity; IKK2 inactive was used to reduce the activity of IKK2 to further reduce NF- κ B activity. NHBE cells and PTECs were seeded into 12-well plates until 70% to 80% confluent, and cells were transfected with 1 μ g of each plasmid and

3 μ L of FuGENE HD Transfection Reagent (Promega) for 48 h. The transfected cells were washed with PBS for three times and infected with 1.0 MOI USSR H1N1 virus diluted in airway epithelial cell medium (PromoCell) supplemented with 500 ng/mL L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma) for 2 h. Cells were washed with PBS three times after 2 h. Cells were cultured in airway epithelial cell medium (PromoCell) containing DMSO or 2 mM ASA and 250 ng/mL TPCK for 24 h. Spun down supernatants were collected for progeny virus titration.

4.2.3 Chemical treatment and infection

For the priming cells with ASA experiment, A549, NPTr, PTECs and NHBE cells were seeded in 12-well plates, cells were treated with 2 mM ASA for 30 mins, 1 h, and 2 h, or with DMSO for 2 h. Cells were then washed with PBS for three times and infected with 1.0 MOI USSR H1N1 virus for 2 h. Cells were washed again with PBS for three times and cultured media for another 12 h. For the treatment with ASA during infection experiment, the four cell types were also seeded in 12-well plates and then cells were infected with 1.0 MOI USSR H1N1 virus for 2 h. Cells were then washed and cultured in media contain DMSO or 2 mM ASA for 12 h. Spun supernatants were collected for virus titration in MDCK cells.

4.2.4 Real-time PCR amplification

QuantiNova SYBR Green PCR Kit (QIAGEN) was used for real-time PCR amplification and performed by using LightCycler-96 (Roche) as described in 2.2.7. The gene expression was normalised by the *18S ribosomal RNA* for comparison. The primers were synthesized by Sigma-Aldrich, and the sequences for the primers are shown in the Table 4-1.

Table 4-1. Primers used in this study.

Gene	Human		Pig	
	Forward primer (5' to 3')	Reverse primer (5' to 3')	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>18s rRNA</i>	ACGGCTACCACATCCAAGGA	CCAATTACAGGGCCTCGAAA	Universal primer (same with human)	
<i>A20</i>	TGGGCTTAAGGGTGTCTGAG	CTTTCTAAGGCCACCTGGGA	CTTCAGCACCCCTCAAGGAGA	AGTCCAATCCGGGTGTCAT
<i>COX-1</i>	ACACCAACAGTGTGAAGTGC	ATCAGGCCTGGAAGTGAAGTC	CCAGTTTGTCAATGCCACCT	TCGCGTGTAGTAGCTCACAT
<i>COX-2</i>	AGGAGGTCTTTGGTCTGAG	TAGCCTGCTTGTCTGGAACA	GATGTATCCTCCGACAGCCA	GGAGGTGTTCAAGGAGTGTGA
<i>IL6</i>	CCAGGAGCCAGCTATGAAC	CCCAGGGAGAAAGCAACTG	CGCAGCCTTGAGGATTTCC	CAGGTGCCCCAGCTACATTATC
<i>OAS1</i>	TCCAAGCTCAGTCAGCAGAA	CAGGAGAGAGATGGCAACA	GAGCTGCAGCGAGACTTCCT	GGCGGATGAGGCTCTTCA
<i>RIG-I</i>	GAAGGCATTGACATTGCACAGT	TGGTTTGGATCATTCTTGATGACA	CCCTGGTTTAGGGACGATGAG	AACAGGAACTGGAGAAAAGTGA
<i>TNF-α</i>	GGAGAAGGGTGACCGACTCA	TGCCAGACTCGGCAAG	CCCGACTATCTGGACTTTGCT	CCAGCCCCTCATTCTCTTTCT
<i>Viral M gene</i>	AGATGAGCCTTCTAACCGAGGTCTG	TGCAAAAACATCTTCAAGTCTCTG	Universal primer (same with human)	

4.2.5 Caspase 3/7 assay

For the caspase 3/7 assay without infection, NHBE cells were seeded into 96-well plates and then were treated with DMSO or ASA at 0.5 mM, 1 mM and 2 mM. 100 µl Caspase-Glo 3/7 reagent (Promega) was added to the cells after 24 h treatment, the lysates were then transferred to white bottom 96-well plate and luminescence was recorded by spectrophotometer Fluostar Optima (BMG Labtech). For the caspase 3/7 assay during infection, NHBE cells were infected with 1.0 MOI USSR H1N1 virus for 2 h, and then were washed with PBS

and cultured for overnight. Cells were treated with the same reagent for the luminescence detection.

4.2.6 RNA-sequencing

NHBE cells were seeded into 12-well plates and were infected with 0.8 MOI USSR H1N1 virus at 80% confluence. Cells were then washed with PBS and cultured in media containing DMSO or 2 mM ASA for 12 h. The RNA samples were then generated for RNA-sequencing. A total of eight RNA samples was prepared including: two biological replicates of NHBE cell donors and each with two technical replicates treated with DMSO and ASA. The RNA-sequencing was performed by using HiSeq 3000 system (SCIF NGS facility). Skewer v0.2.2 was used to remove adaptor sequences, and the average quality score was set at ≥ 20 , the quality score of 3' ends was set at ≥ 3 . Human transcriptome and influenza virus sequences were acquired from Ensemble 92 (GRCh38.p12) and OpenFlu database (IDOFI_ISL_21018) respectively. Kallisto v0.43.0 (Pachter Lab) was used for sequence alignment and read quantification, and the differentially expressed transcripts were determined by Sleuth v0.28.1. Transcripts with a false detection rate (FDR) q-value < 0.05 and log₂ fold change (log₂FC) greater than 1 or less than -1 were selected as significantly regulated.

4.2.7 Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) software (QIAGEN) was used for microarray shortlisted gene analysis. Forty-four shortlisted transcripts with transcript IDs, symbols, q-values and log2 fold change were imported to IPA system for core analysis. Subsequent analysis included: canonical pathways, upstream regulators, diseases and functions, and other functions were performed by IPA.

4.3 Results

4.3.1 Cytotoxicity of ASA in NHBE cells and PTECs

To determine the non-toxic dose range of ASA, PTECs and NHBE cells were treated with 4 different concentrations: 0.5 mM, 1.0 mM, 2.0 mM and 5.0 mM for 24 h. At 5 mM, ASA showed cytotoxicity, based on cell viability, to both PTECs and NHBE cells ($P < 0.0001$). ASA at 2 mM had no effect on cell viability in both PTECs and NHBE cells. Therefore, ASA at 2 mM was chosen as the non-cytotoxic dose in PTECs and NHBE cells (Figure 4-1).

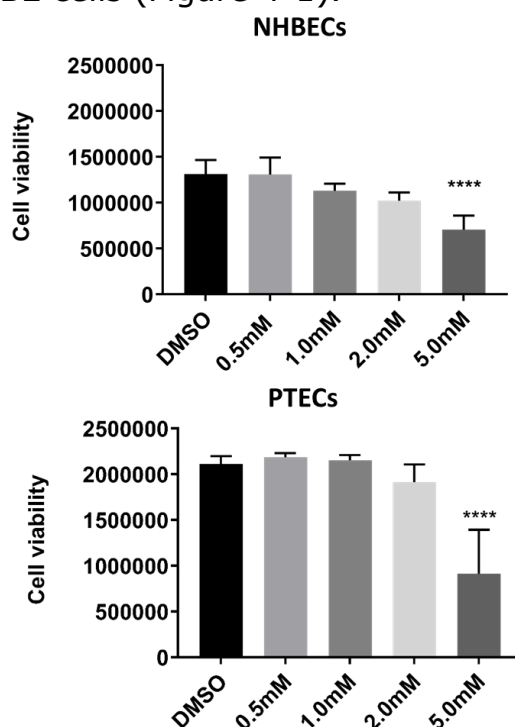


Figure 4-1. ASA at 2.0 mM was not cytotoxic to NHBE cells and PTECs.

Cell viability (CellTiter-Glo® Luminescent Cell Viability Assay kit, Promega) was determined on cells that were incubated with indicated concentrations of ASA or control DMSO for 24 h. Cell viability (based on ATP detection) assay was used to determine the cytotoxicity of ASA. (A) NHBE cells (B) PTECs. Significance is relative to the luminescence of DMSO treated cells (one-way ANOVA with Dunnett's multiple comparisons test). Levels of significance were shown in figures, $P < 0.0001$ was given four asterisks.

4.3.2 ASA reduces influenza virus output in human and pig epithelial cells

To determine the antiviral effect of ASA, human (primary normal bronchial epithelial [NHBE] and human alveolar adenocarcinoma A549 cells) and pig (primary pig tracheal epithelial cells [PTEC] and immortalised neonatal pig tracheal cells [NPTr]) cells were infected with 1.0 MOI USSR H1N1 for 2 h; cells were then washed with PBS and maintained in medium containing DMSO or 2 mM ASA for 12 h. In all four cell types, ASA treatment reduced virus output in comparison to their corresponding DMSO control (Figure 4-2). In the primary cells, ASA treatment at 2 mM reduced 87% and 99% virus output in NHBE cells and PTECs respectively (Figure 4-2A and 2B). In the cell lines, H1N1 virus output was reduced 75% and 76% by 2 mM ASA treatment in A549 and NPTr cells respectively (Figure 4-2C and 2D). This indicates that ASA inhibits influenza virus replication in human and pig airway epithelial cells.

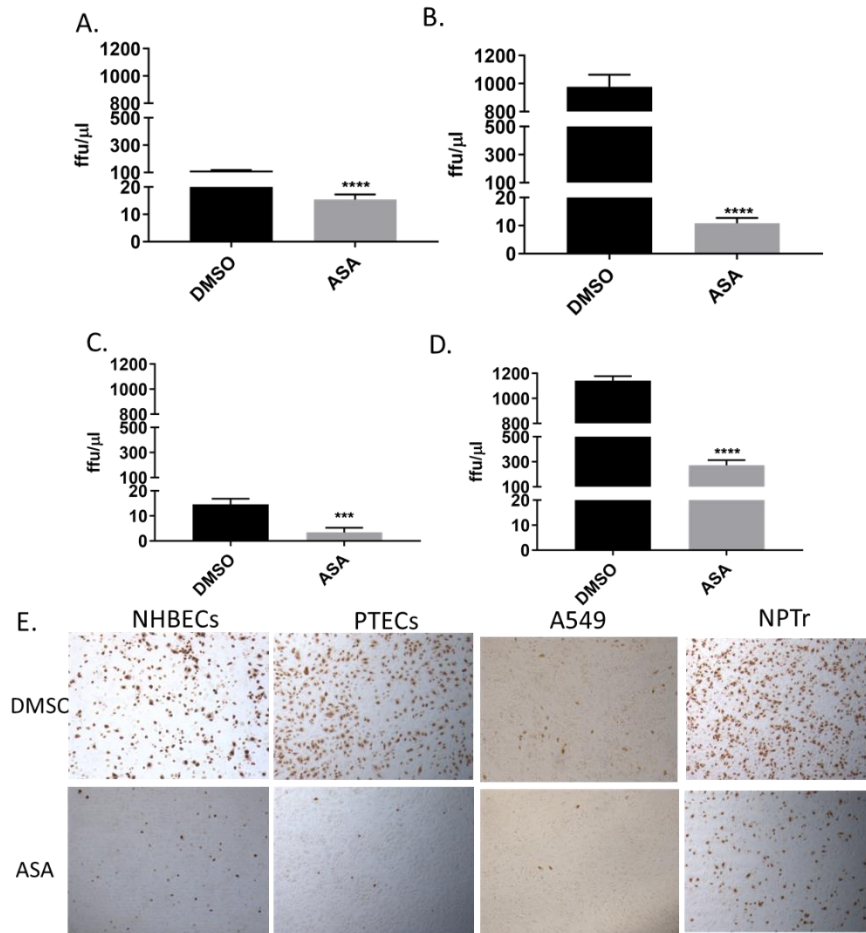


Figure 4-2. ASA reduces influenza virus output. Presence of ASA at 2 mM during infection reduced virus replication in (A) NHBE cells, (B) PTECs, (C) A549 and (D) NPTr cells relative to corresponding DMSO control. (E) Microscope imaging of virus output titration in different cell types. Cells were incubated with USSR H1N1 virus at 1.0 MOI for 2 h, washed with PBS and then maintained in fresh infection media containing DMSO or 2 mM ASA. Spun supernatants were collected for 6 h FFA on MDCK cells for the detection of viral NP. The significance is relative to the DMSO infected cells of each cell type (simple t-test). Levels of significance are shown in figures, $0.0001 < P < 0.001$ and $P < 0.0001$ were given three and four asterisks, respectively.

4.3.3 Pre-infection priming of PTECs (but not NHBE, A549 and NPTr cells) with ASA reduces virus output

It is possible that pretreat cells with ASA can potentially induce innate immune response to inhibit virus replication. NHBE cells, PTECs, A549 and NPTr cells were treated with 2 mM for three durations: 30 mins, 1 h and 2 h. Control cells were treated with DMSO for 2 h. Cells were then infected with 1.0 MOI USSR H1N1 virus for 12 h. There was no virus progeny reduction in NHBE cells, A549 and NPTr cells (Figure 4-3A, C and D). Surprisingly, ASA-pretreated PTECs showed incubation time-dependent reduction in virus output. In comparison with DMSO-treated PTECs, virus output was reduced 53%, 76% and 94% by priming with 2 mM ASA for 30 mins, 1 h and 2 h respectively (Figure 4-3B). This indicates that pre-infection priming of PTECs with ASA reduced virus output, but ASA pretreatment had no effect on virus replication in other three cell types.

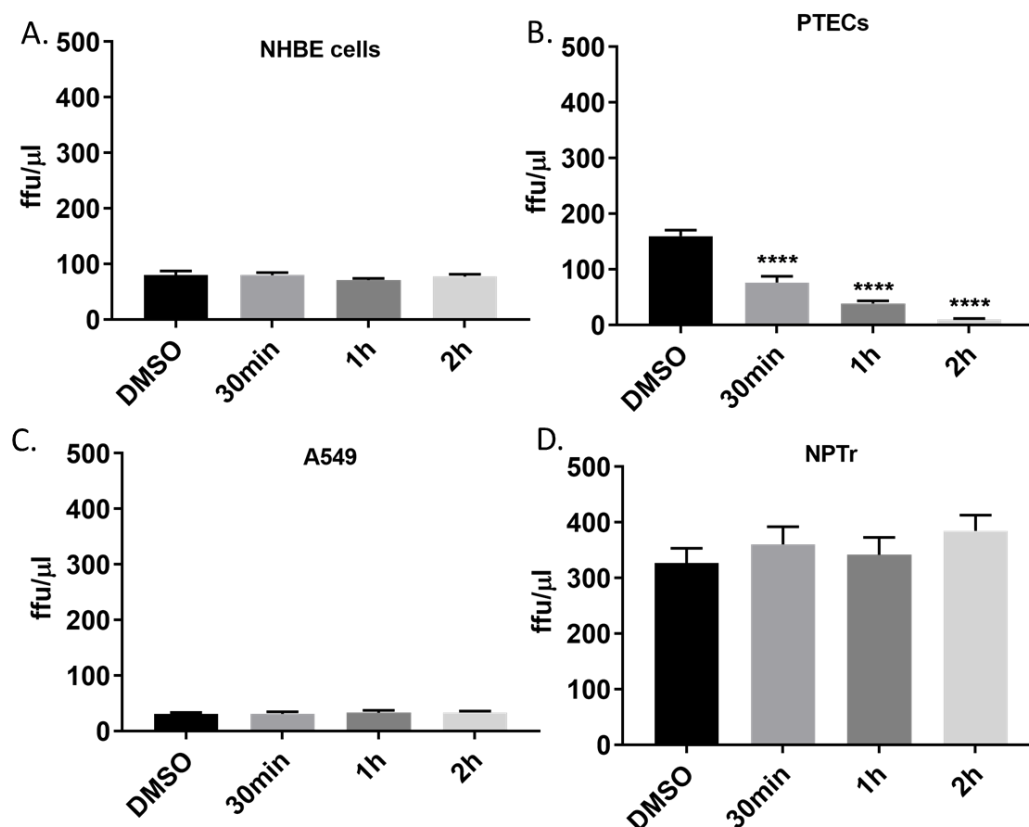


Figure 4-3. Pre-infection priming of PTECs (but not NHBE, A549 and NPTTr cells) with ASA reduced virus output. Cells were pretreated by 2mM ASA for 2 h, 1 h and 30 mins or DMSO for 2 h and then infected with 1.0 MOI USSR H1N1 virus for 2 h, cells were then washed with PBS and maintained with fresh infection media for a further 12 h. Spun supernatants were used in 6 h FFA on MDCK cells to detect viral NP in virus quantification. Virus output in (A) Priming of NHBE cells with ASA had no effect to virus output; (B) Priming of PTECs with ASA reduced virus output followed a time course-dependent manner; (C) Priming of A549 cells did not affect the virus output; (D) Priming NPTTr cells with ASA had no effect to virus replication. Significance is relative to DMSO infected cells of each cell type (one-way ANOVA with Dunnett's multiple comparisons test). Levels of significance were shown in figures, $P < 0.0001$ was given four asterisks.

4.3.4 ASA increases COX-2 and NF- κ B responsive gene expression in PTECs

Primary cells, PTECs and NHBE cells, were chosen for the following studies as primary cells are more biologically relevant to corresponding cells *in vivo*. As discussed earlier, one of the basic mechanisms of the action of ASA is through the inhibition of COX to inhibit prostaglandin synthesis (Vane 1971). ASA was discovered to inhibit NF- κ B signalling pathway, where it specifically binds IKK2 to reduce ATP binding and phosphorylation *in vivo* and *in vitro* (Grilli, Pizzi et al. 1996, Yin, Yamamoto et al. 1998). COX-2 was shown to induce inflammation; increase of COX-2 could lead to the activation of NF- κ B (Sobolewski, Cerella et al. 2010, Shi, Li et al. 2015). To determine if the COX pathway and NF- κ B pathway are involved in ASA treatment, two COX pathway responsive genes (*COX-1* and *COX-2*) and three NF- κ B responsive genes (*IL-6*, *A20* and *TNF- α*) were used as exemplars to assess host gene response. In the presence of ASA, uninfected and infected NHBE cells and PTECs showed different host gene response. ASA treatment of uninfected and infected PTECs had no consistent effect on *COX-1* mRNA expression, but increased *COX-2* gene expression (Figure 4-4A and 4-4B). In NHBE cells, ASA treatment also had little effect on *COX-1* mRNA expression regardless of infection but impaired *COX-2* mRNA expression in uninfected cells (Figure 4-4C and 4-4D). Expression of

IL-6, *A20* and *TNF- α* was significantly increased by ASA treatment in uninfected and infected PTECs, which indicates that ASA elevated expression of NF- κ B responsive genes in PTECs (Figure 4-5A to 4-5C).

Unlike PTECs, the NF- κ B response of NHBE cells to ASA treatment was inconsistent. ASA treatment reduced *IL-6* expression in uninfected NHBE cells from the second donor, but did not affect *IL-6* expression in uninfected cells of the first donor (Figure 4-5D). During infection, ASA had little effect on *IL-6* expression in the first donor NHBE cells, but increased *IL-6* expression in the second donor cells (Figure 4-5D). ASA treatment reduced *A20* expression in uninfected NHBE cells of both donors but had the opposite effects on *A20* expression between donors during infection (Figure 4-5E). ASA had little effect on *TNF- α* expression in uninfected and infected NHBE cells of the first donor, and *TNF- α* expression in the second donor NHBE cells was reduced by ASA without infection, and increased by ASA during infection (Figure 4-5F). All the above findings on NHBE cells suggest that ASA had marginal or no consistent effect on NF- κ B responsive genes regardless of infection.

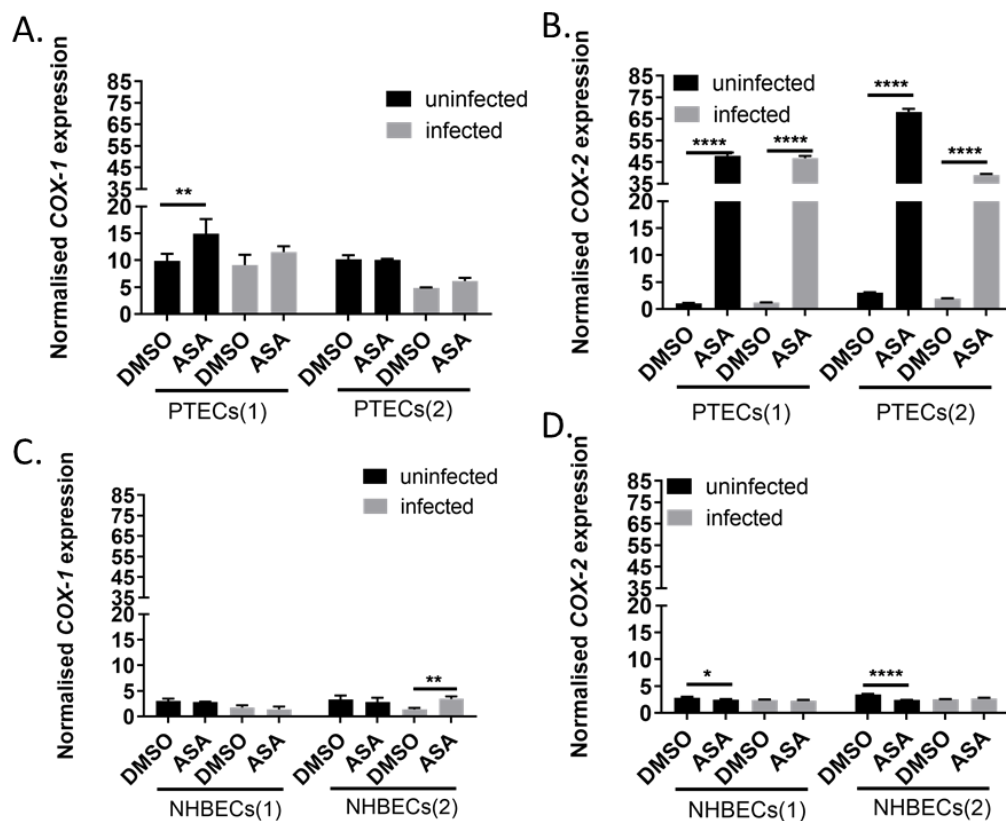


Figure 4-4. ASA induced transcription of COX-2 in uninfected and infected PTECs, but had little effect on NHBE cells. Cells were infected with 1.0 MOI USSR H1N1 virus for 2 h, and then were washed by PBS and maintained with infection media containing DMSO or 2 mM ASA for 12 h. Cell RNA lysates were harvested for gene expression analysis, normalised to *18S rRNA*. Significance (2-way ANOVA with Sidak's multiple comparisons test) is relative to corresponding uninfected or infected DMSO control. (A) COX-1 expression in PTECs and (B) COX-2 expression in PTECs. (C) COX-1 expression in NHBE cells and (D) COX-2 expression in NHBE cells. Two different donors of NHBE cells and PTECs were used. ASA had no consistent effect on COX-1 expression in PTECs and NHBE cells. Results shown representative of two independent experiments. Levels of significance are shown in figures, 0.01 < P < 0.05 was given one asterisk, 0.001 < P < 0.01 and P < 0.0001 were given two and four asterisks, respectively.

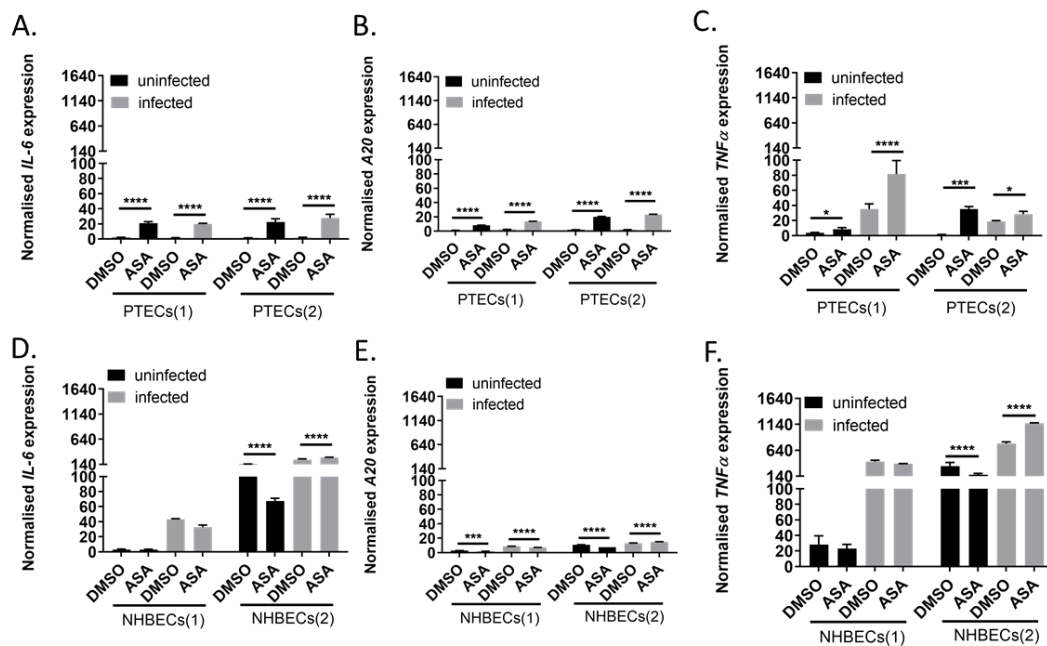


Figure 4-5. ASA induced transcription of *IL-6*, *A20* and *TNF-α* genes in (uninfected and infected) PTECs but not in NHBE cells. Cells from two donors were infected with 1.0 MOI USSR H1N1 virus for 2 h, washed with PBS and maintained with fresh infection media containing DMSO or 2 mM ASA for 12 h. Cell RNA lysates were harvested for gene expression analysis, normalised to *18S rRNA*. Significance (2-way ANOVA with Sidak's multiple comparisons test) is relative to corresponding uninfected or infected DMSO control. (A) *IL-6*, (B) *A20* and (C) *TNF-α* expression in PTECs. (D) *IL-6*, (E) *A20* and (F) *TNF-α* expression in NHBE cells. Results shown representative of two independent experiments. Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, $0.0001 < P < 0.001$ and $P < 0.0001$ were given three and four asterisks, respectively.

4.3.5 ASA has no consistent effect on the expression of type I IFN responsive genes in NHBE cells and PTECs

Two type I IFN responsive genes, *RIG-I* and *OAS1*, were chosen as exemplars to assess the effect of ASA treatment on their expression. ASA had no consistent effect on *RIG-I* expression in uninfected PTECs. ASA increased *RIG-I* expression in PTECs of both donors during infection (Figure 4-6A). *OAS1* expression was not affected by ASA in uninfected PTECs, and was reduced by ASA treatment during infection (Figure 4-6B). All the above indicates that ASA treatment of PTECs did not confer any consistent effect on type I IFN response in uninfected and infected cells.

In uninfected NHBE cells, ASA treatment also had little effect on the expression of *RIG-I* and *OAS1*; however ASA reduced *RIG-I* and *OAS1* gene response in NHBE cells during infection (Figure 4-6C and D).

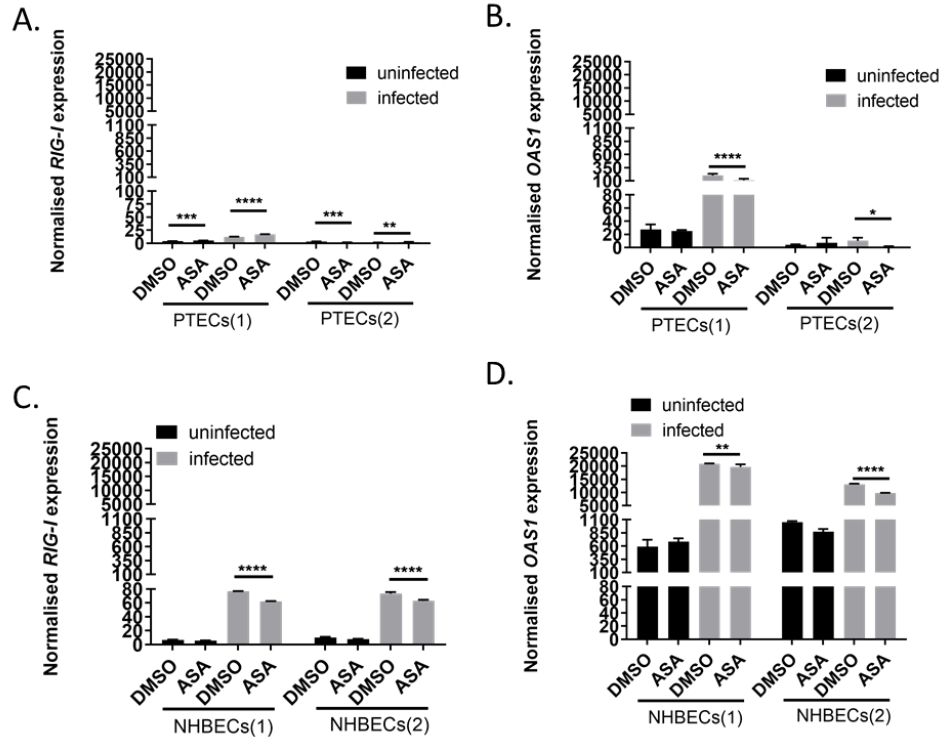


Figure 4-6. ASA had no consistent effect on type I IFN associated response in PTECs, but reduced type I IFN associated response in NHBE cells during infection. Cells were infected with 1.0 MOI USSR H1N1 virus for 2 h, washed with PBS and maintained with fresh infection media containing DMSO or 2 mM ASA for 12 h. Cell RNA lysates were harvested for gene expression analysis, normalised to *18S rRNA*. Significance (2-way ANOVA with Sidak's multiple comparisons test) is relative to corresponding uninfected vector control. (A) *RIG-I* and (B) *OAS1* expression in PTECs. (C) *RIG-I* and (D) *OAS1* expression in NHBE cells. Results shown representative of two independent experiments. Levels of significance are shown in figures, 0.01 < P < 0.05 was given one asterisk, 0.001 < P < 0.01, 0.0001 < P < 0.001 and P < 0.0001 were given two, three and four asterisks, respectively.

4.3.6 Pre-infection priming of PTECs with ASA increases phosphorylation of p65

We next examined the effect of ASA on NF- κ B protein activity in PTECs. Phospho-p65 is an indicator of NF- κ B activation. ASA treated uninfected cells showed significantly higher level of phospho-p65 (Ser536) (P < 0.05) than from DMSO treated uninfected cells,

indicating that ASA activated NF- κ B activity in PTECs (Figure 4-7A and 7B). ASA reduced total p65 protein in uninfected PTECs, but increased p65 during infection relative to corresponding DMSO controls (Figure 4-7C and 7D). All the above findings on PTECs suggest that ASA significantly increased NF- κ B activity prior and during H1N1 virus infection.

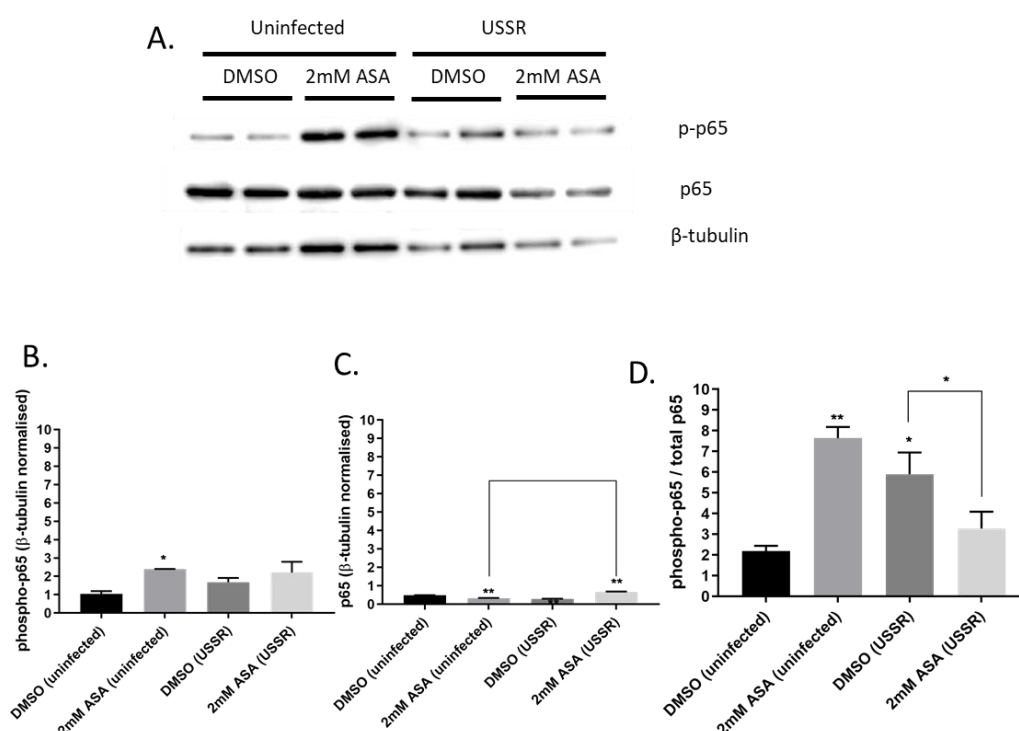


Figure 4-7. Pre-infection ASA increased phospho-p65 expression in PTECs. Uninfected PTECs were treated with DMSO or 2 mM ASA for 12 h. Infected PTECs were exposed to 1.0 MOI USSR H1N1 virus for 2 h and replaced with fresh media containing DMSO or 2 mM ASA for 12 h. Each sample (5μg) was assessed in duplicate. (A) western blotting; corresponding (B) phospho-p65 normalised to β-tubulin, (C) native p65 normalised to β-tubulin and (D) phospho-p65:native p65 ratio. Unless otherwise indicated, significance is relative to corresponding DMSO control (one-way ANOVA with Tukey's multiple comparisons test). Levels of significance are shown in figures, 0.01<P<0.05 was

given one asterisk, $0.001 < P < 0.01$ and $P < 0.0001$ were given two and four asterisks, respectively.

4.3.7 Pre-infection priming of NHBE cells with ASA does not promote phosphorylation of p65

The effects of ASA on NF- κ B activity in NHBE cells was examined by western blotting. Cells were first infected with 1.0 MOI USSR H1N1 virus for 2 h, and treated with 2 mM ASA or DMSO for another 12 h. Uninfected cells were treated with 2 mM ASA and DMSO for 12 h only. Proteins were extracted for western blotting to detect phospho-p65 (Ser536) and native p65 of the NF- κ B signalling pathway. Unlike elevated phospho-p65 protein expression in uninfected PTECs induced by ASA, in uninfected NHBE cells, ASA significantly reduced phospho-p65 (Ser536) protein detection ($P < 0.05$) (Figure 4-8A and 8B). Total p65 protein in uninfected cells was not affected by ASA (Figure 4-8C and 8D). Thus, ASA differentially affects the phosphorylation of p65 between host species of PTECs and NHBE cells.

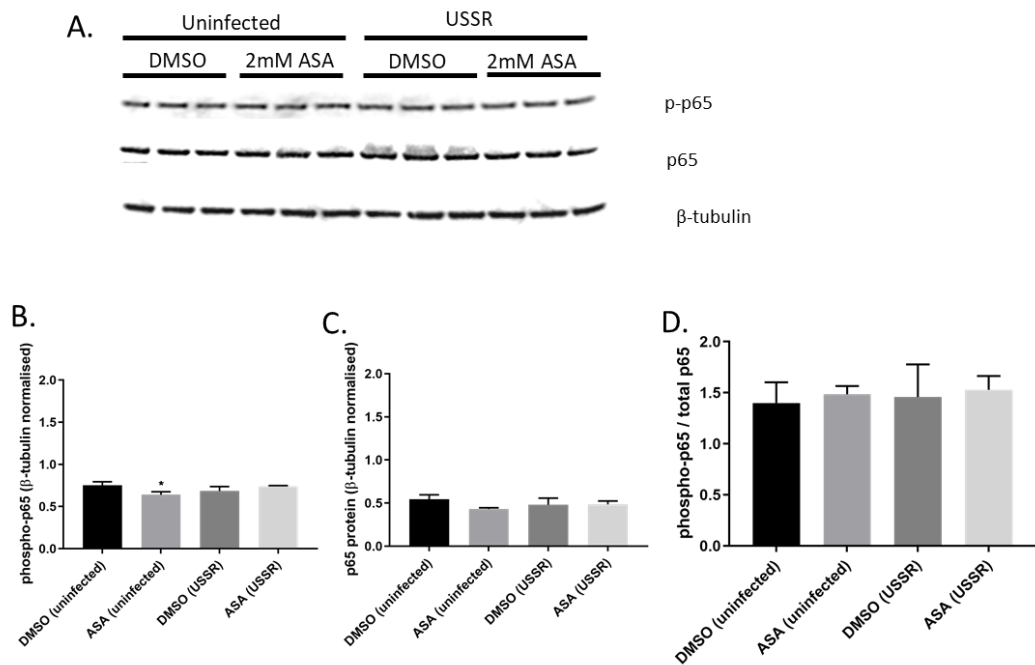


Figure 4-8. Pre-infection ASA priming of NHBE cells did not promote phosphorylation of p65. Uninfected NHBE cells were treated with DMSO or 2 mM ASA for 12 h; infected NHBE cells were exposed to 1.0 MOI USSR H1N1 virus for 2 h and replaced with fresh media containing DMSO or 2 mM ASA for 12 h. Each sample (5 μ g) was assessed in triplicate. (A) western blotting; corresponding (B) phospho-p65 normalised to β -tubulin, (C) native p65 normalised to β -tubulin, and (D) phospho-p65: native p65 ratio. Unless otherwise indicated, significance relative to corresponding DMSO control (One-way ANOVA with Tukey's multiple comparisons test). Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk.

4.3.8 ASA inhibits IAV replication post-translationally in NHBE cells, and transcriptionally in PTECs

NHBE cells and PTECs were infected with 1.0 MOI USSR H1N1 virus for 2 h, and cultured in the presence of DMSO or 2 mM ASA for 12 h. RNA lysates and protein lysates were then collected for viral RNA and protein detection. In NHBE cells, ASA treatment did not reduce viral NP and M1 protein production, and viral *M* gene expression was also not affected by ASA, which suggest that ASA inhibits IAV

replication post-translationally in NHBE cells (Figure 4-9A to 4-9D). In PTECs, ASA treatment clearly reduced viral NP and M1 protein production, as well as viral *M* gene expression, which indicate that ASA blocks viral transcription (Figure 4-9E to 9H). Thus, ASA targets IAV replication at different viral stages in PTECs and NHBE cells.

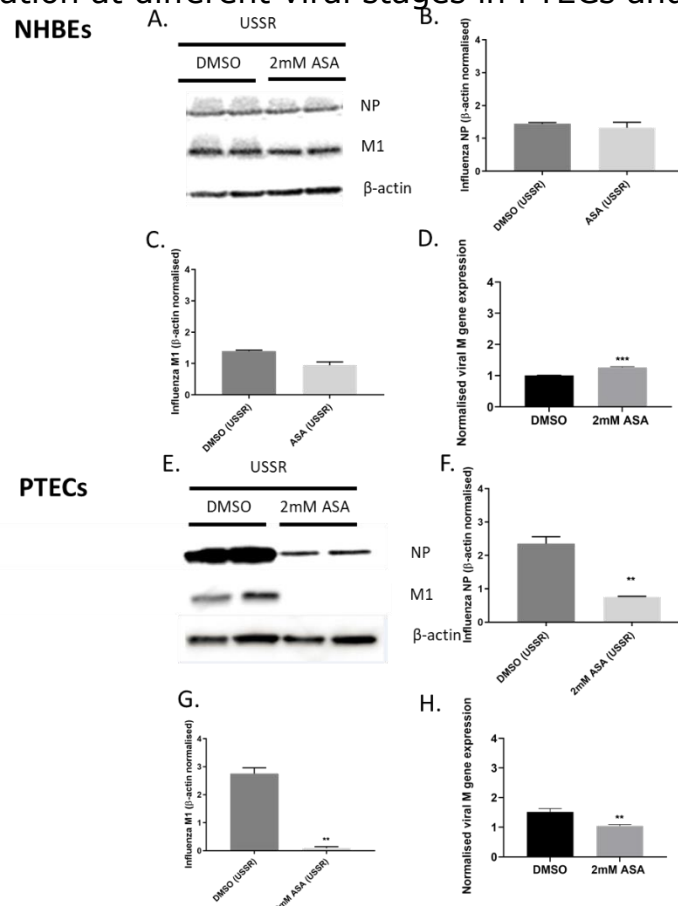


Figure 4-9. ASA targeted influenza virus replication post-translationally in NHBE cells, and transcriptionally in PTECs. Cells were infected with 1.0 MOI USSR H1N1 virus for 2 h and then replaced with media containing DMSO or 2 mM ASA for 12 h. Each sample was loaded 5 μ g with two replicates. Protein expression was normalised to β -actin. M gene expression was normalised to *18S RNA* expression. (A) western blotting of NHBE cells; (B) viral NP expression normalised to β -actin. (C) Viral M1 protein normalised to β -actin in NHBE cells. (D) *M* gene expression in NHBE cells. (E) western blotting of PTECs; (F) viral NP expression normalised to β -actin. (G) Viral M1 protein normalised to β -actin in PTECs. (H) Viral *M* gene expression in PTECs. Significance is relative to corresponding DMSO controls (Unpaired t test). Levels of significance are shown in figures, $0.001 < P < 0.01$ and $0.0001 < P < 0.001$ were given two and three asterisks, respectively.

4.3.9 Antiviral activity of ASA in PTECs appears NF- κ B dependent

Based on previous sections (4.3.4 and 4.3.6), ASA increased NF- κ B activity in PTECs regardless of virus infection but had marginal or no consistent effect on NF- κ B activity in NHBE cells. It is possible that the antiviral effect of ASA in PTECs is NF- κ B dependent and in NHBE cells is NF- κ B independent. To test this hypothesis, cells were transiently transfected with key modulators of NF- κ B pathway: IKK2 CA, RelA and the inactive form of IKK2. Two days post-transfection, cells were infected with 1.0 MOI USSR H1N1 virus for 2 h and then cultured in fresh media containing control DMSO or 2 mM ASA for 12 h. Supernatants were then collected for virus output titration. IKK2 CA and RelA but not IKK2 inactive activated NF- κ B. In control vector and IKK2 inactive transfected PTECs, ASA treatment reduced virus output compared with corresponding transfected and DMSO treated cells (Figure 4-10A). In PTECs that were transiently transfected with IKK2 CA and RelA, and subsequently treated with ASA, virus output was similar to those of transfected and DMSO treated PTECs. This suggests that ASA increases NF- κ B activity to inhibit IAV replication in PTECs. In NHBE cells, transfection of IKK2 CA and RelA had no effect on virus replication but subsequent treatment ASA reduced virus output (Figure 4-10B), indicating the antiviral effect of ASA in NHBE cells is NF- κ B independent.

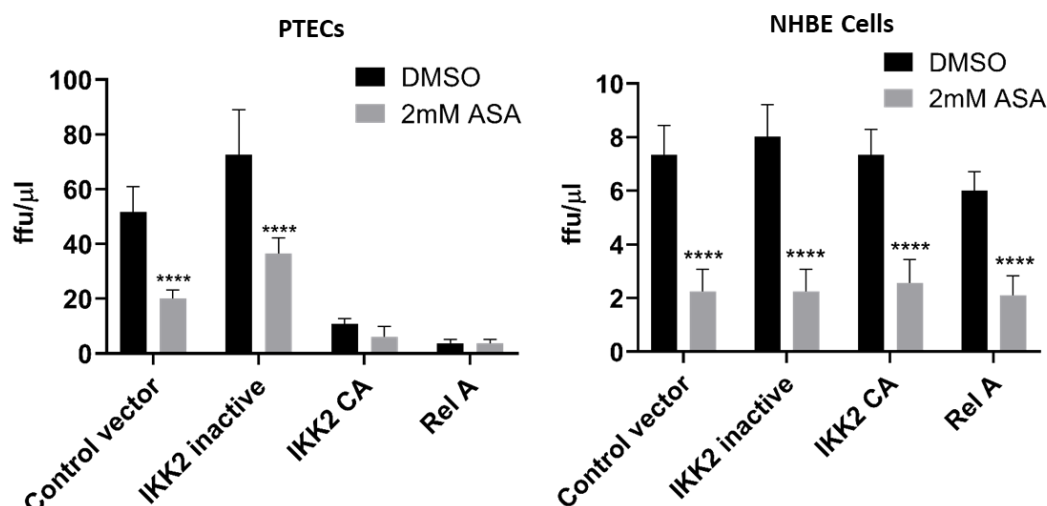


Figure 4-10. Antiviral effect of ASA on PTECs appears to be NF-κB dependent. (A) ASA had no antiviral effect in PTECs with activation of NF-κB (IKK2 CA and Rel A). (B) ASA blocked IAV replication in NF-κB activated NHBE cells. Cells were transfected with control vector, IKK2 inactive, IKK2 CA and Rel A expression constructs for 6 h; 48 h post-transfection cells were infected with 1.0 MOI USSR H1N1 virus for 2 h, washed with PBS and maintained with fresh infection media containing DMSO or 2 mM for 12 h. Supernatants were then collected to infect MDCK cells for 6 h in FFAs. Significance is relative to corresponding DMSO treated control (2-way ANOVA with Sidak's multiple comparisons test). Levels of significance were shown in figures, $P < 0.0001$ was given four asterisks.

4.3.10 RNA-seq analysis of antiviral mechanisms of ASA-treated NHBE cells

The antiviral effect of ASA in NHBE cells required continuous presence of ASA during infection; pretreatment of ASA only had no

antiviral effect in NHBE cells. To determine the transcriptional changes in NHBE cells treated with ASA during infection, next-generation-sequencing (HiSeq 3000 system, SCIF NGS Facility) was performed by using four replicates of NHBE cells with continuous presence of DMSO or 2 mM ASA and infected with 1.0 MOI USSR H1N1 virus for 12 h. Transcripts with a false detection rate (FDR) p-value <0.05 and log2 fold change (log2FC) greater than 1 or less than -1 were selected as significantly regulated. A total of 44 transcripts were identified between ASA-treated USSR infected NHBE cells and DMSO-treated USSR infected NHBE cells (Table 4-2). The shortlist of 44 transcripts were then imported into Ingenuity Pathway Analysis (IPA, QIAGEN) for functional analysis. Based on Upstream Regulators and Diseases and Function analysis within IPA, four groups were identified with valid z-score and prediction activation state. Z-score ≥ 2 predicts increased activity, and z-score ≤ -2 predicts reduced activity state (Figure 4-10). GnRH analog activity was predicted to be activated by ASA with a z-score of 2.24; 5 out of 44 genes were found to be directly or indirectly related with GnRH analog. There were 4 genes that were found to functionally reduce 5-azacytidine activity in ASA-treated NHBE cells, with a z-score of -2.00. Tretinoin activity was also predicted to be decreased by ASA treatment, and 8 out of 44 genes were identified to be related with tretinoin. Based on the Diseases and Function analysis, apoptosis was predicted to be more inhibited in ASA treated NHBE cells than

in DMSO treated NHBE cells, with 8 out of 44 genes connected with apoptosis.

Table 4-2. Top gene list of ASA USSR infected NHBEs vs. DMSO USSR infected NHBEs.

Symbol	Entrez Gene Name	q-value	Expr Log Ratio
ATP5F1A	ATP synthase F1 subunit alpha	0.0323	-6.137
CEBPZ	CCAAT enhancer binding protein zeta	0.0164	-1.038
CLPP	caseinolytic mitochondrial matrix peptidase proteolytic subunit	0.0294	1.276
CSNK2A1	casein kinase 2 alpha 1	0.0959	2.153
CYP51A1	cytochrome P450 family 51 subfamily A member 1	0.00159	-1.358
DDOST	dolichyl-diphosphooligosaccharide--protein glycosyltransferase non-catalytic subunit	0.00159	-7.936
DNTTIP2	deoxynucleotidyltransferase terminal interacting protein 2	0.00159	-1.212
DSTN	destin, actin depolymerizing factor	7.69E-06	-21.522
DYNLL1	dynein light chain LC8-type 1	0.0589	3.206
EBNA1BP2	EBNA1 binding protein 2	0.0589	-1.128
GNL2	G protein nucleolar 2	0.0308	-1.023
GPX4	glutathione peroxidase 4	0.0114	1.73
GSTO1	glutathione S-transferase omega 1	9.58E-07	-12.854
HARS	histidyl-tRNA synthetase	0.0439	-3.904
ITGA6	integrin subunit alpha 6	0.0703	-3.675
JPT2	Jupiter microtubule associated homolog 2	0.0164	-4.803
KHSRP	KH-type splicing regulatory protein	0.0764	-5.501
KRT17	keratin 17	0.063	-5.566
KRT19	keratin 19	0.0454	-4.821
KRT5	keratin 5	0.0308	3.784
MEAF6	MYST/Esa1 associated factor 6	0.0728	-3.241
NDUFAB1	NADH:ubiquinone oxidoreductase subunit AB1	0.0116	-5.485
NFU1	NFU1 iron-sulfur cluster scaffold	0.00159	-14.13
PKM	pyruvate kinase M1/2	0.0147	∞
PLA2G4A	phospholipase A2 group IVA	0.0764	-1.117
PTBP1	polypyrimidine tract binding protein 1	0.0648	-3.826
RACK1	receptor for activated C kinase 1	0.0365	4.922
RP11_217B12	chondroitin sulfate proteoglycan 4 pseudogene 3 Y-linked	0.00159	1.354
RP11_290D26	chondroitin sulfate proteoglycan 4 pseudogene 3 Y-linked	0.0413	1.566
RPA3	replication protein A3	0.0737	-4.842
RPS2	ribosomal protein S2	0.0873	1.258
RSRC2	arginine and serine rich coiled-coil 2	0.0237	-1.119
SLC39A10	solute carrier family 39 member 10	0.0294	-1.044
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	0.0439	-3.48
SNORA84	small nucleolar RNA, H/ACA box 84	0.00777	1.248
SQLE	squalene epoxidase	0.0439	-3.736
STK17A	serine/threonine kinase 17a	0.0385	-1.061
THBS1	thrombospondin 1	0.0866	-1.02
TNFAIP3	TNF alpha induced protein 3	0.0804	-4.154
TPM4	tropomyosin 4	0.086	2.656
TRAM1	translocation associated membrane protein 1	0.0114	-5.278
VTRNA1-1	vault RNA 1-1	0.0114	1.863
WDR43	WD repeat domain 43	0.0625	-1.186
WDR45B	WD repeat domain 45B	0.0405	-4.352

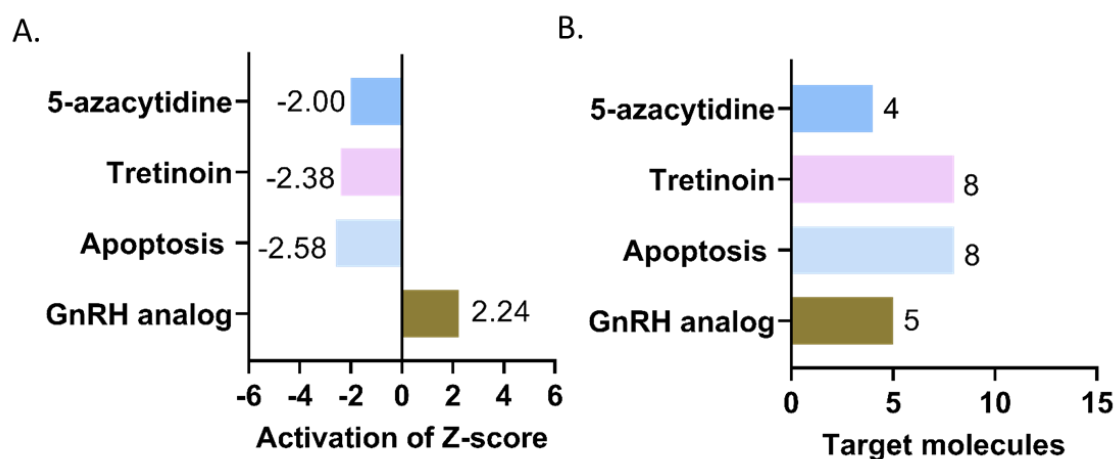


Figure 4-11. IPA predictive analysis for upstream regulators, diseases and functions. (A) IPA predicted 5-azacytidine is inhibited by ASA during infection with a z-score of -2.00; tretinoin was also predicted to be inhibited by ASA with a z-score of -2.38; Apoptosis was inhibited by ASA based on IPA prediction with a z-score of -2.58; GnRH analog was predicted to be activated by ASA with a z-score 2.24. (B) 4 out of 44 genes are associated with 5-azacytidine have expression direction consistent with inhibition of 5-azacytidine; and 8 out of 44 genes are associated with tretinoin and apoptosis have expression direction consistent with inhibition of tretinoin and apoptosis; 5 out of 44 genes are associated with GnRH analog have expression direction consistent with activation of GnRH analog.

4.3.11 ASA reduces apoptosis activity in NHBE Cells

Based on IPA analysis, eight genes were identified to be associated with inhibition of apoptosis in NHBE cells. The directional changes of increased expression of *CSNK2A1*, *DYNLL1*, *PKM* and *RACK1*, and reduced expression of *TNFAIP3*, *THBS1*, *STK17A*, and *SMARCE1* are predicted to be inhibitory to apoptosis in NHBE cells (Figure 4-12A). To further evaluate the effect of ASA on apoptotic activity in NHBE cells, we performed caspase 3/7 activity assay in uninfected and infected cells. ASA at 0.5 mM, 1.0 mM and 2 mM final concentrations were introduced to NHBE cells for overnight culture, followed by caspase 3/7 assays. ASA at 0.5 mM had little effect on apoptotic activity; ASA at 1.0 mM and 2 mM signed reduced apoptosis activity significantly in a dose-dependent manner (Figure 4-12B). We next examined the effect of ASA on apoptosis during infection. NHBE cells were infected with 1.0 MOI USSR H1N1 virus for 2 h, cells were then washed with PBS and maintained with media containing 2 mM ASA or DMSO for 12 h, after which caspase 3/7 assays were performed. During USSR H1N1 infection, 1 mM ASA treatment reduced apoptosis relative to infected DMSO- treated NHBE cells (Figure 4-12C). Collectively, ASA reduced apoptotic activity in uninfected and infected NHBE cells.

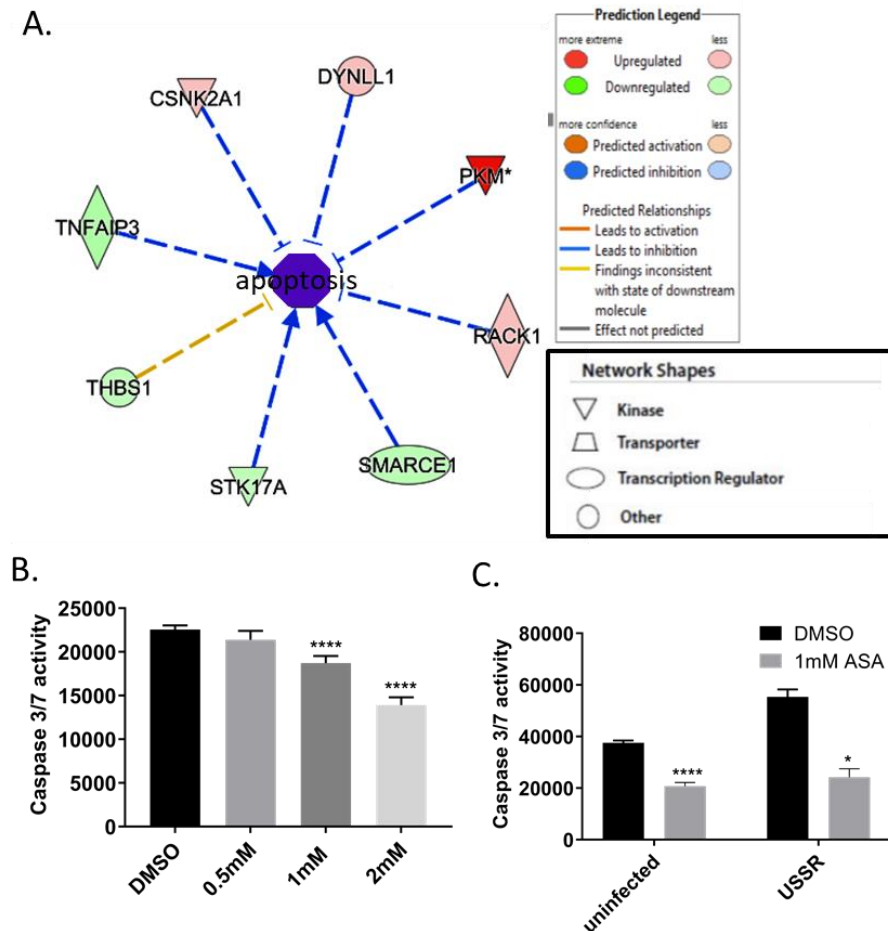


Figure 4-12. ASA treatment reduced IAV-induced apoptosis in NHBE cells. (A) Apoptosis was predicted by IPA to be inhibited in NHBE cells during IAV infection. Eight genes were found to associate with the inhibition of apoptosis, and the directional change in expression of the 8 genes is consistent with IPA-predicted inhibition of apoptosis. (B) Treatment of ASA inhibited apoptosis in uninfected NHBE cells. NHBE cells were treated with ASA at indicated concentrations or DMSO as control for 12 h for caspase 3/7 assays. Significance is relative to DMSO control (one-way ANOVA with Dunnett's multiple comparisons test). (C) ASA reduced apoptosis in IAV-infected NHBE cells. NHBE cells were infected with 1.0 MOI USSR H1N1 virus or left uninfected for 2 h; cells were then cultured in the presence of 2 mM ASA or DMSO overnight followed by caspase 3/7 assays. Significance is relative to corresponding DMSO controls (2-way ANOVA with Sidak's multiple comparisons test). Levels of significance are shown in figures, $0.01 < P < 0.05$ was given one asterisk, and $P < 0.0001$ was given four asterisks.

4.4 Discussion

Aspirin, also known as acetylsalicylic acid (ASA), has been shown to have antiviral effect in various types of virus infection, including influenza virus, HCV, Zika Virus, RSV and HSV (Grilli, Pizzi et al. 1996, Yin and Zhang 2016, Glatthaar-Saalmuller, Mair et al. 2017, Rajasagi, Bhela et al. 2017, Pan, Peng et al. 2018). Aspirin was extensively used as an antiviral drug during the 1918 H1N1 virus pandemic (Starko 2009). Here, we showed that ASA blocked H1N1 virus replication in human airway epithelial (NHBE and A549) and pig airway epithelial (PTEC and NPTr) cells, thus corroborating ASA as an antiviral compound. Interestingly, in NPTr, A549 and NHBE cells, the antiviral effect of ASA could be detected only if it was present throughout infection. Priming these cells with ASA prior to infection only did not affect virus replication. However, priming PTECs with ASA prior to infection did reduce virus output, which shows that ASA priming of PTECs induces a lasting antiviral effect in this particular host cell type, and suggests the antiviral mechanisms triggered by ASA are dissimilar between PTECs and other cell types. Immortalised cells are commonly used in research. It was reported that about 18% to 36% of cell lines may be contaminated or misidentified based on submissions to major cell repositories in the last decade (Hughes, Marshall et al. 2007). Over-culturing of cell lines can result in genotypic and phenotypic changes. Research showed that between

hepatoma cell line Hepa1-6 and primary hepatocytes, the resulting quantitative proteome of 4063 proteins had asymmetric distribution, with many proteins down-regulated in the Hepa1-6 cell line (Pan, Kumar et al. 2009). Therefore, immortalised cell lines like A549 and NPT_r cells are relatively poor models in comparison with corresponding primary cells. This might explain that pretreatment of NPT_r cells and PTECs showed different virus output outcomes.

Studies have shown the antiviral effect of ASA could be at the transcriptional, translational or post-translational stage of virus replication. Mazur et al. (2007) showed that ASA reduced H7N7 influenza virus replication in A549 cells, and that ASA treatment blocked the export of virus particles without affecting viral protein (including M, NS1, NP and PB1) expression, which indicates that ASA blocked influenza virus replication post-translationally in A549 cells. ASA was shown to inhibit HCV protein production in Huh7 replicon cells, which implies that the inhibition of HCV by ASA might be at a transcriptional or translational level (Trujillo-Murillo, Rincon-Sanchez et al. 2008, Sanchez-Garcia, Rios-Ibarra et al. 2013). Here, we showed that ASA treatment of PTECs inhibited the transcription of influenza virus replication, in contrast to viral post-translational inhibition in NHBE cells, which, collectively, is further evidence that the antiviral mechanisms triggered by ASA differ between host species.

It is a long held view that ASA inhibits NF- κ B activation. Yin et al. (1998) showed that the use of aspirin inhibited IKK- β activity in Jurkat, COS and Hela cells to inhibit NF- κ B activation. A similar study also indicated that aspirin blocked NF- κ B activity in primary rat cerebellar cells (Grilli, Pizzi et al. 1996). However, several studies also showed contrasting host cell responses to aspirin treatment. Aspirin was found to increase NF- κ B activity to induce apoptosis in human colorectal cancer cells (Stark, Din et al. 2001, Stark, Reid et al. 2007). Here, ASA was found to increase the expression of NF- κ B responsive genes and phosphorylation of p65, indicating ASA promotes NF- κ B activity in PTECs. The NF- κ B associated response to ASA in NHBE cells was less conclusive relative to PTECs. ASA impaired the phospho-p65 protein expression, and the mRNA expression of *COX-2* and *A20* in uninfected NHBE cells. No consistent ASA effect was observed in infected NHBE cells in the expression of *COX-2*, *A20*, *IL-6* and *TNF- α* ; this inconsistency could be accounted in part by genetic differences between donors. Another explanation could be that antiviral host response to ASA could have occurred transiently before 12 hpi. To examine whether the virus inhibition by ASA in PTECs is dependent on NF- κ B activation, we increased the NF- κ B activity in these cells by overexpressing positive regulators of NF- κ B, IKK2 CA and RelA. The results indicated that in IKK2 and RelA overexpressed PTECs, ASA did not further inhibit IAV replication. However, in corresponding NHBE cells, ASA treatment showed

antiviral effect in the IKK2 or RelA overexpressed cells. Collectively, the antiviral effect of ASA in PTECs is NF- κ B dependent, but in NHBE cells is NF- κ B independent, which implies that the underlying antiviral mechanisms of ASA are different between PTECs and NHBE cells.

Several studies also indicated ASA may act through inhibition of apoptosis or promotion of cell proliferation during virus infection. Cell proliferation associated response was increased by 24 h treatment of ASA in Huh7-HCV cells (Sanchez-Garcia, Rios-Ibarra et al. 2013). Nitric oxide synthases (iNOS) are key enzymes that regulate host cell immune response and promote apoptosis and angiogenesis in cells (Vakkala, Kahlos et al. 2000). ASA was shown to downregulate the activity of iNOS in Huh7-HCV cells, which suggests that its antiviral effect could be connected to anti-apoptotic function (Rios-Ibarra, Lozano-Sepulveda et al. 2014). ASA from 1 to 5 mM was shown to inhibit flavivirus replication through suppression of flavivirus-induced apoptosis in baby hamster kidney (BHK)-21 cells (Liao, Lin et al. 2001). Here, pretreatment of ASA showed no protection of the NHBE cells from IAV infection, and ASA must be present throughout infection to be antiviral. RNA-seq of NHBE cells provided insights of global transcriptional changes between ASA infected cells and control infected cells. IPA analysis predicted that ASA inhibited apoptosis activity during IAV infection. We validated the IPA prediction by showing that ASA reduced apoptosis in

uninfected and infected NHBE cells. This suggests that the primary antiviral effect of ASA in NHBE cells could be through inhibition of apoptosis.

In chapter two, it was shown that the relative basal transcriptional NF- κ B-associated activity was more highly regulated in NHBE cells than PTECs. Activation of NF- κ B exhibited strong antiviral effect on PTECs, but not on NHBE cells. Here we showed that ASA increased NF- κ B activity in PTECs to inhibit IAV replication, whereas the antiviral mechanism of ASA in NHBE cells was shown to be NF- κ B independent. Therefore, the relative transcriptional levels of NF- κ B associated genes prior to infection could be a key determinant of host species susceptibility or resistance to IAV infection and associated complications of cytokine dysregulation.

In summary, we showed that ASA can inhibit IAV replication in human and pig airway epithelial cells. The transcription of IAV replication was reduced by ASA in PTECs, whereas the virus replication process was blocked by ASA in NHBE cells occurred post-translationally. The underlying antiviral mechanisms of ASA between PTECs and NHBE cells were shown to be fundamentally different. The antiviral mechanism in PTECs appeared to be dependent on activation of NF- κ B, but in NHBE cells, ASA appears to operate through inhibiting apoptosis.

**5. Ledene, a naturally-occurring
sesquiterpene, blocks influenza virus
replication in normal human bronchial
epithelial cells**

Abstract

The emergence of drug-resistant influenza virus creates a challenge for antiviral therapy. Sesquiterpenes exist in natural oils and are composed of three isoprene units with a molecular formula $C_{15}H_{24}$. Sesquiterpenes are known for their antioxidant and antimicrobial activities, and have been extensively explored for antiviral application. Ledene is a sesquiterpene, found in the Australian tea tree (*Melaleuca alternifolia*). In this study, we found anti-influenza effect of ledene in primary normal human bronchial epithelial (NHBE) cells. Priming with 2 μ M ledene for 30 mins prior to infection significantly inhibited A/USSR/1977 (H1N1) virus replication. The selective index of ledene is 791.01, which is about 79 times higher than the anti-influenza drug oseltamivir, suggesting that ledene could be a safer and more potent antiviral. Further investigation showed ledene did not affect viral protein and mRNA expression, suggesting that it inhibits H1N1 virus replication post-translationally. RNA microarray analysis indicated that ledene inhibited host cell tumour protein p53 (*TP53*) activity, which could be an underlying antiviral mechanism.

5.1 Introduction

5.1.1 Overview of Sesquiterpenes

Sesquiterpenes with the molecular formula of $C_{15}H_{24}$ are terpenes that consist of three isoprene units (Montagnon, Kalaitzakis et al. 2014). There are thousands of different sesquiterpenes identified naturally in plants, insects and microbials (Kobayashi, Kondo et al. 1988, Chappell and Coates 2010). Sesquiterpene is a key compound of essential oil (Langenheim 1994). Essential oils are isolated from plants and used as pharmaceutical agents for disease treatment (Ali, Al-Wabel et al. 2015, Sharma, Rao et al. 2019). Fifty-eight sesquiterpene compounds were found in essential oil extracted from *Juniperus thurifera* wood alone (Barrero, Quilez del Moral et al. 2005). Four different types of sesquiterpenes were identified in essential oil of *Commiphora myrrha* (Xu, Guo et al. 2012).

5.1.2 Mechanism of action of sesquiterpenes

Extensive studies have reported functions of sesquiterpenes in regulating immune response in humans. One of the primary functions is believed to be antimicrobial activity. Sesquiterpenes isolated from *Juniperus thurifera* wood showed inhibitory effect

against bacterial (*Bacillus cereus* and *Proteus sp*) infections (Barrero, Quilez del Moral et al. 2005). Plant essential oils that contain sesquiterpenes showed significant bactericidal effects against several bacterial infections (Friedman, Henika et al. 2002). Sesquiterpenes were shown to have neuroprotective effects. Sesquiterpenes identified from *Commiphora myrrha* inhibited neuronal cell death in human dopaminergic SH-SY5Y cells (Xu, Guo et al. 2011, Xu, Guo et al. 2012). A sesquiterpene was found to have increased effect on *Nrf2* expression to further confer neuroprotective effect in neuron-like rat pheochromocytoma (PC12) cells (Peng, Hou et al. 2019). Members of sesquiterpenes were shown to have significant effects on lifespan extension. Beta-caryophyllene (BCP), a sesquiterpene, increased lifespan in *C. elegans* by 22% by maintaining redox homeostasis (Pant, Mishra et al. 2014, Proshkina, Plyusnin et al. 2020). Sesquiterpenes were also identified as promising antioxidants. Antioxidants are most commonly used to neutralise oxidative stress and protect host cell from the harm of oxidative stress (Baek and Lee 2016). Farnesol, a type of sesquiterpene, was found to attenuate oxidative stress and inflammatory dysregulation in Swiss albino mice (Santhanasabapathy, Vasudevan et al. 2015). Sesquiterpenes including helenalin, nerolidol and alantolactone were found to reduce oxidative stress in mice (Lin, Zhang et al. 2014, Javed, Azimullah et

al. 2016, Wang, Lan et al. 2018). Collectively, sesquiterpenes have been extensively characterised to have potent antioxidant properties.

5.1.3 Antiviral effect of sesquiterpenes

Sesquiterpenes were also found to have inhibitory effects on virus replication. Triptofordin C-2, a sesquiterpene, blocked the replication of herpes simplex virus type 1 (HSV-1) in HeLa cells (Hayashi, Hayashi et al. 1996). Two sesquiterpenes isolated from *Nicotiana tabacum* leaves inhibited approximately 20% of tobacco mosaic virus (TMV) progeny virus production by using anti-TMV assays (Shen, Xu et al. 2016). Sesquiterpenes most commonly exist as hydrocarbons, but also occur in oxygenated forms known as sesquiterpenoids (Awouafack, Tane et al. 2013). Sesquiterpenoids containing a lactone ring are known as a sesquiterpene lactone (SLs). Several studies showed that sesquiterpenes, sesquiterpenoids and SLs can inhibit IAV replication. Sesquiterpene fraction isolated from *Laggera pterodonta* blocked IAV replication of several subtypes (H1N1, H6N2, H7N3 and H9N2) through the inhibition of NF- κ B and p38 pathways (Gao, Han et al. 2017). Sesquiterpenoid cryptoporic acid E (CAE) inhibited IAV replication by interfering with IAV polymerase activity (Gao, Han et al. 2017). Sesquiterpene patchouli alcohol was found to inhibit H1N1 virus replication without affecting

viral NA activity (Kiyohara, Ichino et al. 2012). Influenza A viral RNA synthesis was inhibited by a sesquiterpene lactone, brevilin A, in MDCK cells (Zhang, Xia et al. 2019).

5.1.4 Overview of Ledene

Ledene is a sesquiterpene that is a dehydration product of ledol (Gaydou, Smadja et al. 1996). Ledol was isolated from the Australian tea tree (*Melaleuca alternifolia*) (Cain and Lynn 1934). The biological activities of ledene are not fully known. From previous compound screening, it was found that ledene inhibited progeny IAV production (unpublished data). Thus, we wish to further assess the antiviral activities of ledene.

5.1.5 Aims and objectives

The primary aim of this study was to assess the antiviral effect of ledene against IAV infection. The secondary aim is to characterise host cell responses to ledene treatment to have a better understanding of its antiviral mechanisms.

1. To examine the virus output outcomes in normal human bronchial epithelial (NHBE) cells treated with ledene.

NHBE cells were primed pre-infection with ledene, followed by infection with USSR H1N1 virus. Virus progeny output was analysed by focus forming unit (FFU) assay. Expression of viral mRNA and protein were detected by qPCR and western blotting.

2. To characterise host cell response to ledene.

A microarray transcriptomic approach was used to determine host transcriptional changes induced by ledene.

5.2 Materials and Methods

5.2.1 Cytotoxicity assay

NHBE cells were seeded into 96-well plates and incubated until fully confluent. Cells were then treated with DMSO or Ledene for 30 mins or 2 h at desired concentrations (5, 10, 20, 40, 60, 80 and 100 μ M). Cells were then washed with PBS for three times and cultured in airway epithelial cell media (Lonza) for 24 h. CellTiter-Glo Luminescent Cell Viability Assay (Promega) was then added to cells for luminescence detection measured by LUMIstar Omega Microplate Luminometer (BMG Labtech). The use of DMSO was not toxic, because the cells treated without DMSO showed similar viability with the cells treated with DMSO (data not shown).

5.2.2 Ledene pretreatment and infection

NHBE cells were primed with DMSO or 2 μ M ledene for 30 mins. Cells were then washed with PBS three times and infected with 1.0 MOI USSR H1N1 virus for 2 h, washed by PBS and cultured for 12 h. Spun supernatants were collected for virus output titration. Uninfected NHBE cells were maintained with airway epithelial cell medium for 12 h after 30 mins pre-treatment with DMSO or ledene.

5.2.3 Microarray analysis

NHBE cells were seeded into 12-well plates and incubated until 80% confluent. The cells were primed with DMSO or 2 μ M Ledene for 30 mins and washed three times with PBS. Uninfected cells were maintained with culture media for 12 h. Infected cells were infected with 1.0 MOI USSR (H1N1) virus for 2 h; cells were then washed and cultured for further 10 h. RNA samples with depleted ribosomal RNA were generated for RNA microarray analysis. Three different donors were used for the microarray analysis, in total of 12 samples:

DMSO uninfected NHBE cells (x3)	Ledene uninfected NHBE cells (x3)
DMSO H1N1 infected NHBE cells (x3)	Ledene H1N1 infected NHBE cells (x3)

RNA samples were labelled and processed using Human Gene 2.1 Array Strips (#902114; Affymetrix) on the microarray system, GeneAtlas from Affymetrix. Partek Genomic Suite 6.6 software was used for data analysis and figure generation. The RNA integrity number (RIN) was measured using Agilent 2100 Bioanalyzer. All RNA preps showed $RIN \geq 9.8$, where >7 is the accepted value.

5.2.4 Generation, fragmentation and hybridization of the sense cDNA on the microarray strips

All steps of sense cDNA synthesis, fragmentation and hybridization were performed according to the manufacturer's protocol (GeneAtlas WT Expression Kit User Manual). The resulting fragmented sense cDNA was assessed using the Agilent 2100 Bioanalyzer before hybridisation to the Array Strips. Hybridisation, washing, and scanning were carried out automatically in the GeneAtlas microarray system.

5.2.5 Microarray data analysis

Gene expression profile data were generated as CEL files and subjected to analysis with the Partek Genomics Suite 6.6 software. Genes of interest comprised those upregulated or downregulated by at least 2-fold with no FDR (false discovery rate), and filtered at p-value <0.2.

5.2.6 Ingenuity pathway analysis

Two groups of comparisons were made: Ledene uninfected NHBE cells vs. DMSO uninfected NHBE cells, and Ledene H1N1 virus infected NHBE cells vs. DMSO H1N1 virus infected NHBE cells. Two sets of shortlisted genes were imported into Ingenuity pathway

analysis (IPA) software (QIAGEN) for further canonical pathways, upstream regulators, and diseases & functions analyses.

5.3 Results

5.3.1 Cytotoxicity of ledene in NHBE cells

Ledene was assessed at 7 concentrations (5 μ M, 10 μ M, 20 μ M, 40 μ M, 60 μ M, 80 μ M and 100 μ M) for cytotoxicity in NHBE cells. NHBE cells were primed with ledene or DMSO for 30 mins or 2 h, washed with PBS and cultured for 24 h. Cell viability was not affected at 80 μ M ledene priming for 30 mins (Figure 5-1A). Priming NHBE cells with 40 μ M ledene for 2 h also showed no evidence of cytotoxicity (Figure 5-1B).

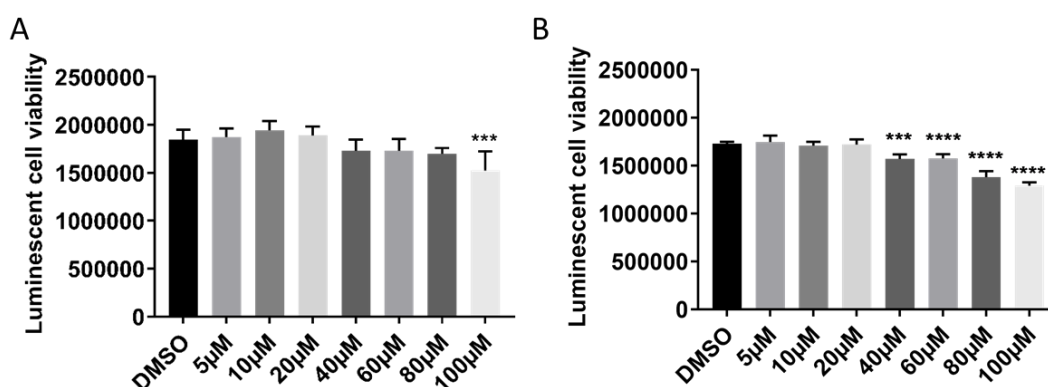


Figure 5-1. Cytotoxicity assessment of ledene in NHBE cells. Ledene priming at 80 μ M for 30 mins (A) or 40 μ M for 2 h (B) was non-cytotoxic to NHBE cells. Cell viability (CellTiter-Glo® Luminescent Cell Viability Assay kit, Promega) was determined on cells that had been pretreated with ledene or DMSO as indicated for 30 mins (A) or 2 h (B), washed with PBS and further cultured for 24 h. Significance is relative to corresponding DMSO control (one-way ANOVA with Dunnett's multiple comparisons test). Levels of significance are shown in figures, 0.0001 < P < 0.001 and P < 0.0001 were given three and four asterisks, respectively.

5.3.2 Priming NHBE cells with ledene blocks IAV replication

NHBE cells from three different donors were used to assess the antiviral effect of ledene. Cells were primed with ledene at a non-cytotoxic concentration of 2 μM for 30 mins and infected with 1.0 MOI USSR H1N1 virus for 12 h, the supernatants were then collected for virus output determination. Consistent across all three biological replicates, ledene reduced progeny virus by more than 50% indicating strong virus inhibition (Figure 5-2A, B and C). Next, the selective index (SI), an *in vitro* indicator of efficacy and safety, of ledene against influenza virus was determined. Ledene was serially diluted to prime NHBE cells for 30 mins, washed with PBS, and cultured overnight followed by cell viability assays to determine at 50% cytotoxic concentration (CC_{50}). A similar ledene priming process followed by 1.0 MOI USSR H1N1 virus infection for 12 h was performed to determine virus inhibition dose at 50% (IC_{50}), based on FFA. The CC_{50} of ledene was 438.3 μM and its IC_{50} was 0.5541 μM , giving an SI ($\text{CC}_{50}/\text{IC}_{50}$) of 791.01 (Figure 5-2D and E).

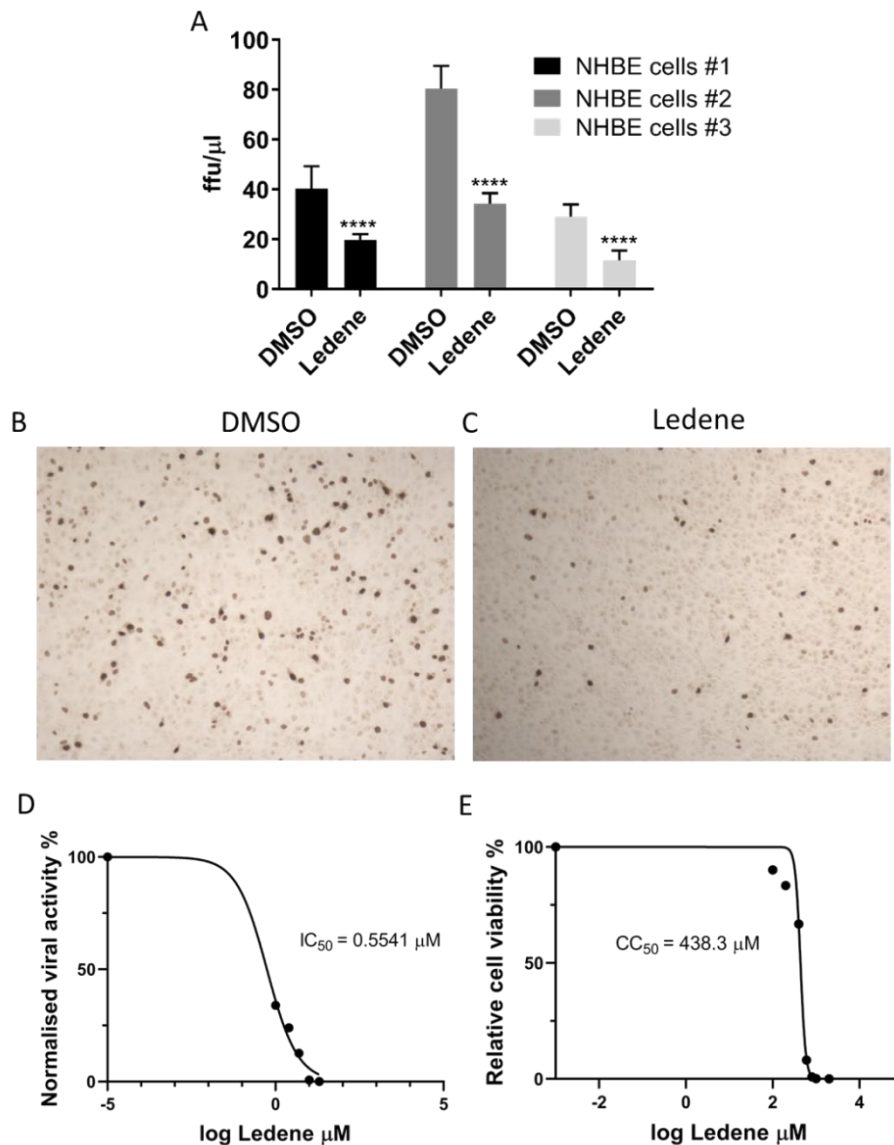


Figure 5-2. Priming NHBE cells with 2 µM ledene inhibited influenza virus replication. NHBE cells were treated with 2 µM ledene for 30 mins, cells were then washed with PBS three times and infected with 1.0 MOI USSR H1N1 virus for 12 h. Supernatants were collected for virus output titration in MDCK cells. (A) Ledene at 2 µM consistently reduced virus replication in NHBE cells in 3 donor cells. (B) Micrograph of virus output from DMSO treated NHBE cells. (C) Micrograph of virus output from 2 µM ledene treated NHBE cells. (D) IC_{50} of ledene is 0.5541 µM. NHBE cells were treated with DMSO or ledene at indicated concentrations for 30 mins, washed with PBS and infected with 1.0 MOI USSR H1N1 virus for 12 h, after which spun supernatants were used for virus titration. (E) CC_{50} of ledene is 438.3 µM. NHBE cells were primed with DMSO or ledene at indicated concentrations for 30 mins, cells were then washed and cultured for 24 h before cell viability assays. Levels of significance were shown in figures, $P < 0.0001$ was given four asterisks.

5.3.3 Ledene inhibits IAV replication post-translationally

To determine the stage of virus inhibition by ledene, we examined expression of viral NP and M1 protein, and viral M-gene RNA expression in ledene and DMSO primed NHBE cells. NHBE cells were pre-treated with 2 μ M ledene or DMSO for 30 mins, followed by 12 h infection for downstream processing of RNA or protein lysates for real-time PCR and western blotting. Ledene primed NHBE cells showed comparable production of viral NP and M1 proteins relative to corresponding DMSO primed cells (Figure 5-3A, B and C). There was also no consistent change in viral M-gene RNA expression in ledene primed cells relative to DMSO control cells (Figure 5-3D). Collectively, ledene appears to block influenza virus replication post-translationally.

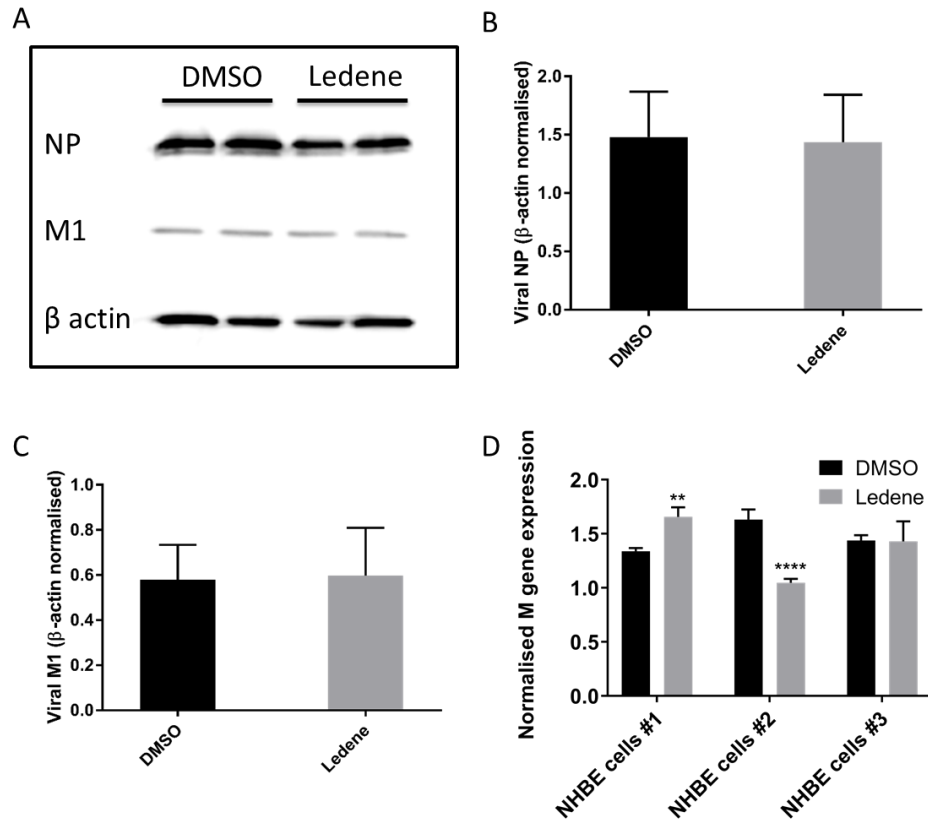


Figure 5-3. Ledene inhibits IAV replication in NHBE cells post-translationally. NHBE cells were primed with 2 μ M ledene or DMSO for 30 mins, washed with PBS and infected with 1.0 MOI USSR H1N1 virus. Cells were harvested for protein or RNA lysates at 12 hpi. Each protein sample was loaded at 5 μ g in duplicate. Protein expression was normalised to β -actin. (A) Viral NP, M1 and β -actin protein. (B) Viral NP protein expression normalised to β -actin. (C) Viral M1 protein expression normalised to β -actin in NHBE cells. (D) Viral M-gene RNA expression normalised to *18S rRNA*. Significance (2-way ANOVA with Sidak's multiple comparisons test) is relative to corresponding DMSO controls. Levels of significance are shown in figures, $0.001 < P < 0.01$ and $P < 0.0001$ were given two and four asterisks, respectively.

5.3.4 Priming of NHBE cells with Ledene has no consistent effect on expression of NF- κ B and type I IFN responsive genes

Two NF- κ B responsive genes (*IL-6* and *A20*) and two type I IFN responsive genes (*RIG-I* and *OAS1*) were selected to assess host gene response in NHBE cells to ledene priming. In the absence of virus infection, expression of NF- κ B responsive genes and type I IFN responsive genes was not affected by ledene treatment relative to corresponding DMSO treated cells (Figure 5-4A, B, C and D). In pre-infection ledene primed and infected NHBE cells, there was no consistent trend in the differential expression of of NF- κ B responsive genes across donor replicates (Figure 5-4A and B). *RIG-I* expression was increased by ledene priming during virus infection, but *OAS1* expression was unchanged in the first donor cells and was raised in the second donor cells (Figure 5-4C and D). Taken together, the antiviral effect of ledene appears to be not dependent on NF- κ B and type I IFN associated response.

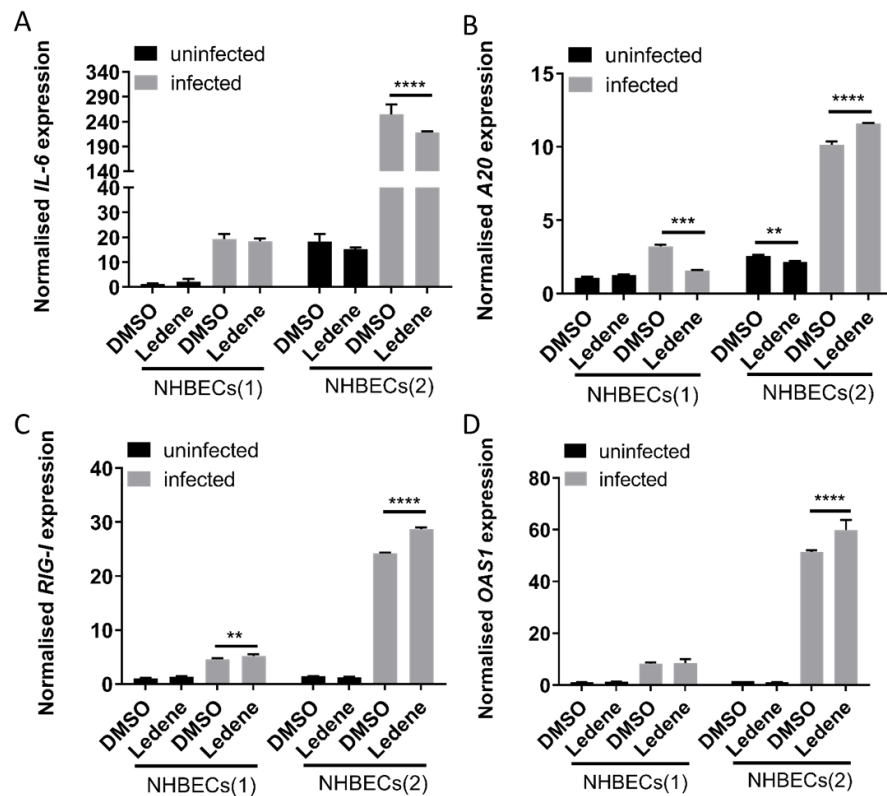


Figure 5-4. Ledene has no consistent effect on the expression of NF- κ B and type I IFN responsive genes. Two NF- κ B responsive genes, (A) *IL-6*, (B) *A20*, and two type I IFN associated genes, (C) *RIG-I* and (D) *OAS1*, were chosen to assess host gene response of NHBE cells to ledene priming. NHBE cells were primed with 2 μ M ledene or DMSO for 30 mins, washed with PBS and cultured in fresh media, or infected with 1.0 MOI USSR for 12 hours. Cell lysates were harvested for RNA extraction and cDNA conversion. RNA expression was normalised to *18S rRNA*. Significance is relative to corresponding DMSO treated cells (2-way ANOVA with Sidak's multiple comparisons test). Levels of significance are shown in figures, $0.001 < P < 0.01$, $0.0001 < P < 0.001$ and $P < 0.0001$ were given two, three and four asterisks respectively.

5.3.5 Profiling transcriptional changes of host genes in NHBE cells from ledene priming

The antiviral effect of ledene in NHBE cells appeared to be independent of NF- κ B and type I IFN response. To gain insight into transcriptional changes in NHBE cells induced by ledene, RNA microarray profiling was performed by using RNA samples from cells primed with 2 μ M ledene or DMSO for 30 mins. Cells were either infected with 1.0 MOI USSR H1N1 virus for 12 h or were uninfected. NHBE cells from three biological replicates (3 donors) were used to perform the microarray analysis (human Affymetrix Gene 2.1 ST Arrays); there was a total of 12 RNA samples in the array design.

The resulting PCA plot shows an overlapping area between ledene-primed uninfected and DMSO-primed uninfected NHBE cells, indicating that there were no significant transcriptional changes between the two groups (Figure 5-5). With filtering conditions set at fold-change (up and down) of at least 2, and p-value of <0.05 with false discovery rate (FDR), no significant gene was identified. When the selection condition was set at p-value of <0.2 with no FDR, 14 mapped transcripts were identified between uninfected ledene-primed cells and uninfected DMSO cells.

Transcriptional changes between infected ledene-primed cells and infected DMSO control cells were examined next. The resultant PCA

plot shows no overlap between the two groups (Figure 5-5), indicating that during infection the ledene-treated group had significantly regulated transcripts relative to DMSO treated group. Thirty-five transcripts were mapped with fold-change (up or down) of at least 2, p-value of <0.2 with no FDR applied.

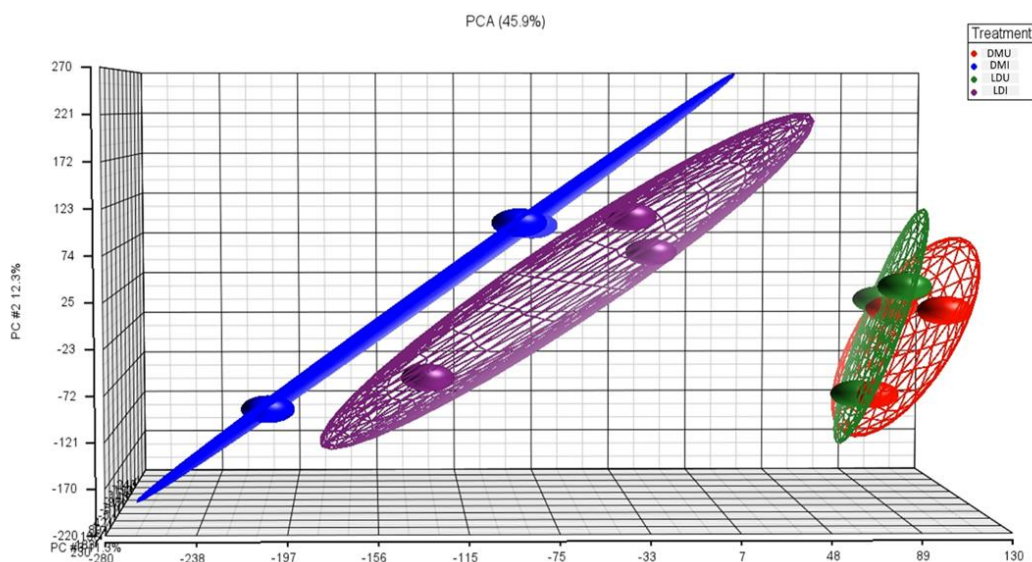


Figure 5-5. PCA plot of microarray RNA analysis of ledene primed NHBE cells. Each treatment group comprised three biological replicates (donors) and highlighted in different colours, each sphere represents one sample. NHBE cells were primed with 2 μ M ledene or DMSO control for 30 mins, washed with PBS and cultured uninfected, or infected with 1.0 MOI USSR H1N1 virus for 12 h upon which cells were harvested for total RNA. DMU: DMSO uninfected NHBE cells; DMI: DMSO USSR H1N1 virus infected NHBE cells; LDU: ledene uninfected NHBE cells; LDI: Ledene USSR H1N1 virus infected NHBE cells.

5.3.6 Ledene predicted to inhibit TP53 activity in NHBE cells

The 14 mapped transcripts (uninfected ledene group vs uninfected DMSO group) were imported into IPA for functional analysis (Table 5-2). Using the predictive tools of IPA, 5 out of 14 transcripts were found to be related to tumor protein 53 (*TP53*) of which directional changes of increased expression of *ACO1*, *AHSA1* and *TRAP1*, and reduced expression of *CTSD* and *NUSAP1* (all relative to controls) were predicted to have an inhibitory effect on *TP53* (Figure 5-6). *TP53*, which encodes protein p53, is a master gene that regulates cell stress, promotes cell cycle arrest, initiates apoptosis and DNA repair (Kastenhuber and Lowe 2017, Mantovani, Collavin et al. 2019). Thus, ledene priming could disrupt *TP53* function in NHBE cells. Using IPA, 6 out of 35 genes were differentially expressed between infected ledene-primed cells and infected DMSO control cells) were also found to be associated with *TP53* (Table 5-3). The directional changes of increased *PPP1CA*, *FOSL1*, and *MYO10*, and reduced expression of *CTSK*, *TRAP1* and *HMGA1* (all relative to control) are predicted to have an inhibitory effect of *TP53* (Figure 5-7). We then compared the shortlisted 14 genes and 35 genes, *ACTG1*, *TMEM45B*, *TRAP1* and *TUBB4B* were identified to be present on both gene lists as shown in table 5-4. Overall, based on IPA prediction, *TP53* was predicted to be inhibited by ledene in

NHBE cells, which could be an underlying antiviral mechanism of ledene blocking IAV replication in NHBE cells.

Table 5-1. Top gene list of ledene uninfected NHBE Cells vs. DMSO uninfected NHBE Cells.

Genes	Gene names	Cellular location	p value	fold change
ACO1	aconitase 1	Cytoplasm	0.13168	2.38941
ACTG1	actin gamma 1	Cytoplasm	0.12558	3.36952
AHSA1	activator of HSP90 ATPase activity 1	Cytoplasm	0.05467	2.7172
CCT7	chaperonin containing TCP1 subunit 7	Cytoplasm	0.03828	-2.10557
CHCHD2	coiled-coil-helix-coiled-coil-helix domain containing 2	Cytoplasm	0.06133	-2.92569
CTSD	cathepsin D	Cytoplasm	0.11395	-3.01782
G6PC3	glucose-6-phosphatase catalytic subunit 3	Cytoplasm	0.15953	-2.21212
NUSAP1	nucleolar and spindle associated protein 1	Nucleus	0.17209	-2.31797
PCYT2	phosphate cytidylyltransferase 2, ethanolamine	Cytoplasm	0.02056	-2.23214
SERBP1	SERPINE1 mRNA binding protein 1	Cytoplasm	0.08815	2.88055
SLC25A3	solute carrier family 25 member 3	Cytoplasm	0.00133	2.04124
TMEM45B	transmembrane protein 45B	Extracellular	0.13687	-2.09482
TRAP1	TNF receptor associated protein 1	Cytoplasm	0.12533	2.17672
TUBB4B	tubulin beta 4B class IVb	Cytoplasm	0.16741	-2.06784

Table 5- 2. Top gene list of ledene-H1N1 virus infected NHBE cells vs DMSO-H1N1 virus infected NHBE Cells.

Genes	Gene names	Cellular location	p value	fold change
ACTG1	actin gamma 1	Cytoplasm	0.146258	2.01811
ARF3	ADP Ribosylation Factor 3	Cytoplasm	0.122913	3.72691
ARPC5	Actin Related Protein 2/3 Complex Subunit 5	Cytoplasm	0.0525106	2.25177
CTSK	Cathepsin K	Cytoplasm	0.169628	-2.51687
CYP21A2	Cytochrome P450 Family 21 Subfamily A Member 2	Cytoplasm	0.0580055	2.12963
EIF1	Eukaryotic Translation Initiation Factor 1	Nucleus	0.105227	2.74551
EMC3	ER Membrane Protein Complex Subunit 3	Cytoplasm	0.0926895	-2.25681
Firre	Firre Intergenic Repeating RNA Element	Nucleus	0.0850872	2.00299
FOSL1	FOS Like 1, AP-1 Transcription Factor Subunit	Nucleus	0.0166072	2.97397
GRINA	Glutamate Ionotropic Receptor NMDA Type Subunit Associated Protein 1	Cytoplasm	0.020686	-2.38618
HMGA1	High Mobility Group AT-Hook 1	Nucleus	0.152692	-2.18883
IFT172	Intraflagellar Transport 172	Extracellular	0.153455	2.32974
IGSF9	Immunoglobulin Superfamily Member 9	Plasma membrane	0.0397496	2.11149
LRFN3	Leucine Rich Repeat And Fibronectin Type III Domain Containing 3	Plasma membrane	0.0037512	2.78723
LRRC59	Leucine Rich Repeat Containing 59	Nucleus	0.10728	2.20416
METTL8	Methyltransferase Like 8	Nucleus	0.0100884	-2.398
MYO10	Myosin X	Cytoplasm	0.190236	3.71198
MYO9B	Myosin IXB	Cytoplasm	0.0128702	4.36677
NOP14	NOP14 Nucleolar Protein	Nucleus	0.0412091	-2.04514
OGFR	Opioid Growth Factor Receptor	Nucleus	0.0513875	3.45375
PLEKHB1	Pleckstrin Homology Domain Containing B1	Nucleus	0.004	2.02189
PPP1CA	Protein Phosphatase 1 Catalytic Subunit Alpha	Plasma membrane	0.0985662	2.62153
PRKAG3	Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma 3	Cytoplasm	0.105439	2.71513
PRR14	Proline Rich 14	Nucleus	0.00409497	2.39647
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	Nucleus	0.172349	-3.05177
RPS16	Ribosomal Protein S16	Nucleus	0.109602	2.70275
SIL1	SIL1 Nucleotide Exchange Factor	Extracellular	0.176085	-2.66877
Svs2	Seminal vesicle secretory protein 2	Extracellular	0.00240307	-2.27512
TBL3	Transducin Beta Like 3	Nucleus	0.112152	2.75873
TMEM45B	transmembrane protein 45B	Extracellular	0.0528365	-2.74564
TRAP1	TNF receptor associated protein 1	Cytoplasm	0.0676455	-2.6113
TUBB4B	tubulin beta 4B class IVb	Cytoplasm	0.060954	-2.83673
VAMP5	Vesicle Associated Membrane Protein 5	Plasma membrane	0.0939444	-2.84002
YBX3	Y-Box Binding Protein 3	Nucleus	0.179418	5.93968

Table 5- 3. Overlapping genes between ledene-uninfected vs DMSO-uninfected, and ledene-H1N1 virus infected vs DMSO-H1N1 infected NHBE cells.

Genes	Gene names	Cellular location	LDU vs. DMU		LDI vs. DMI	
			p value	fold change	p value	fold change
ACTG1	actin gamma 1	Cytoplasm	0.1256	3.36952	0.146	2.018
TMEM45B	transmembrane protein 45B	Extracellular Space	0.1369	-2.09482	0.0528	-2.746
TRAP1	TNF receptor associated protein 1	Cytoplasm	0.1253	2.17672	0.0676	-2.611
TUBB4B	tubulin beta 4B class IVb	Cytoplasm	0.1674	-2.06784	0.061	-2.837

LDU: ledene uninfected NHBE cells.
DMU: DMSO uninfected NHBE cells.
LDI: Ledene USSR H1N1 virus infected NHBE cells.
DMI: DMSO USSR H1N1 virus infected NHBE cells.

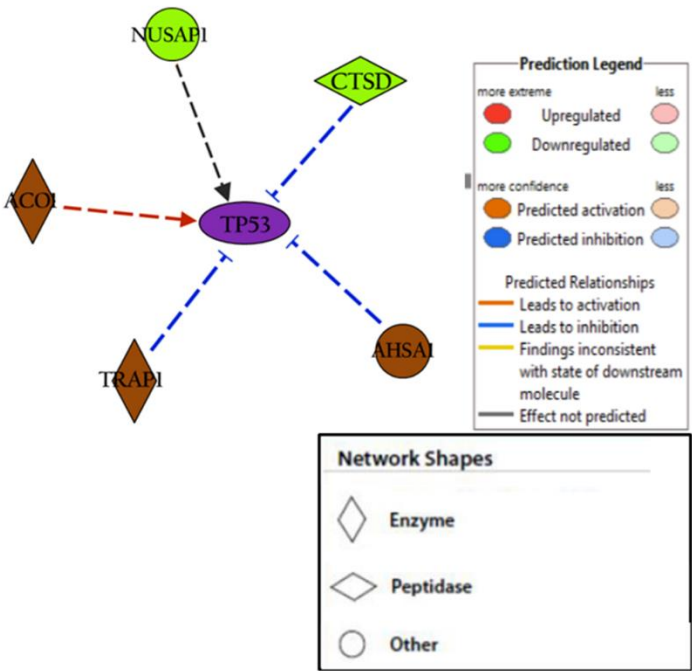


Figure 5-6. *TP53* was predicted by IPA to be inhibited in NHBE cells primed with ledene. Five (shown) out of the 14 genes found to be associated with *TP53*, and the direction of fold-change in expression of 5 genes, increased expression of *ACO1*, *AHSA1* and *TRAP1* and reduced expressions of *NUSAP1* and *CTSD* (all relative to DMSO), was consistent with IPA-predicted inhibition of *TP53*.

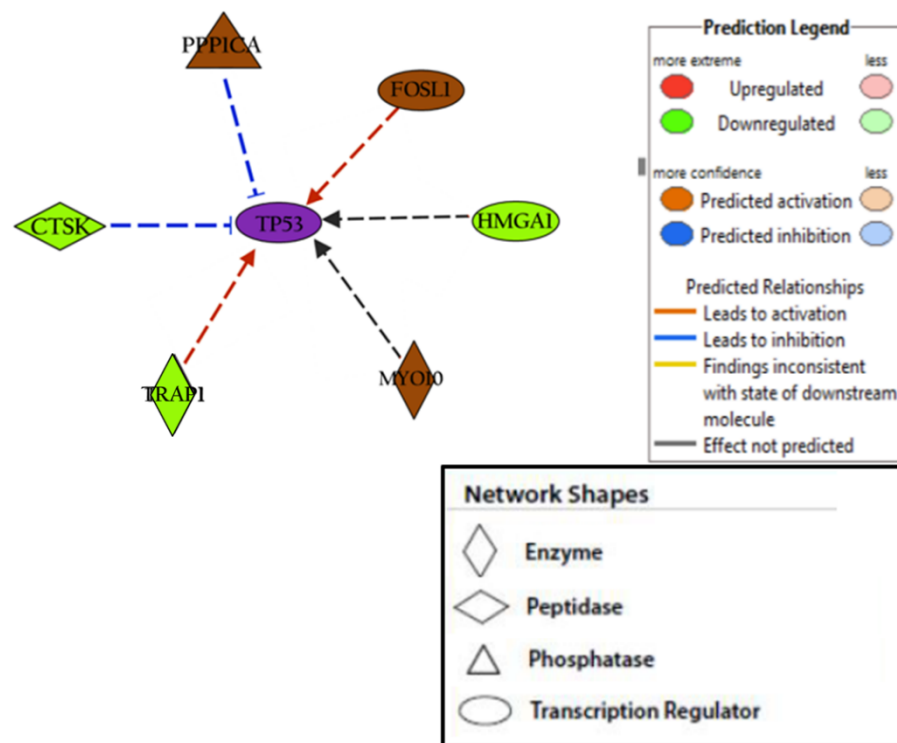


Figure 5-7. TP53 was predicted by IPA to be inhibited in NHBE cells primed with ledene during H1N1 virus infection. Six (shown) out of the 35 genes found to be associated with TP53, and the direction of fold-change in expression of 6 genes, increased expression of *MYO10*, *FOSL1* and *PPP1CA* and reduced expressions of *CTSK*, *TRAP1* and *HMGAI* (all relative to DMSO), was consistent with IPA-predicted inhibition of *TP53*.

5.4 Discussion

Several sesquiterpenes, sesquiterpenoids and sesquiterpene lactones have been shown to inhibit influenza virus replication (Hayashi, Hayashi et al. 1996, Li, Zhou et al. 2005, Nhiem, Van Kiem et al. 2014, Gao, Han et al. 2017, Wang, Zhou et al. 2017, Zhang, Xia et al. 2019). In this study, we showed that ledene effectively blocks H1N1 influenza virus replication, which added ledene as another sesquiterpene that can inhibit IAV replication. SI indicates the ratio between cytotoxicity and antiviral activity of a given chemical (Pritchett, Naesens et al. 2014). Oseltamivir (Tamiflu) a commonly used influenza antiviral. The SI of oseltamivir against 2009 pandemic H1N1 virus was reported as 10 ($CC_{50}=320.1\ \mu\text{M}$, $IC_{50}=32.0\ \mu\text{M}$) on MDCK cells (Chung, Chien et al. 2014). In our study, SI of ledene is 791.01 against H1N1 virus in NHBE cells ($CC_{50}=438.3\ \mu\text{M}$, $IC_{50}=0.5541\ \mu\text{M}$). This suggests that ledene is potentially more efficacious and safer than oseltamivir.

Studies have suggested sesquiterpenes may inhibit influenza virus replication at transcriptional or translational stage. Sesquiterpene CAE was shown to have an inhibitory effect on viral vRNA, mRNA and cRNA synthesis of H1N1/pdm09 virus in MDCK cells (Gao, Han et al. 2017). Sesquiterpene lactone brevilin A also inhibited IAV replication by reducing viral vRNA, mRNA and cRNA synthesis and

viral M2 protein expression (Zhang, Xia et al. 2019). H1N1 viral protein expression was inhibited by sesquiterpene triptofordin C-2 in HeLa cells, which indicates translational inhibition (Hayashi, Hayashi et al. 1996). Here, NHBE cells primed with ledene did not appear to affect viral gene and protein expression suggesting that viral inhibition occurred post-translationally.

Limited studies have described the antiviral mechanisms of sesquiterpenes. Want et al. (2017) reported that sesquiterpene of *Laggera pterodonta*, Fraction 14 (Fr 14), inhibited IAV replication in A549 cells through inhibition of p38 and NF- κ B pathways (Wang, Zhou et al. 2017). Sesquiterpene patchouli alcohol reduced mRNA expression of *IL-4* and *IFN- γ* and corresponding proteins during H1N1 infection in human respiratory epithelial (16HBE) cells (Wu, Ju et al. 2013). TNF- α level was impaired by patchouli alcohol in H1N1 infected mice, and patchouli alcohol provided mice protection against A/PR/8/34 (H1N1) virus infection, suggesting that the compound reduced inflammatory responses *in vivo* (Li, Peng et al. 2012). *TP53*, or its protein p53, plays a key role in influenza virus replication. Knockout of p53 in mice increased mortality rate in comparison with wild type mice, and inhibition of p53 activity in mouse embryo fibroblasts (MEF) promoted IAV replication (Turpin, Luke et al. 2005). The cytokine and interferon response was found to be delayed in p53^{-/-} mice, and the knockout of p53 significantly reduced the survival rate in H1N1 and H3N2 virus infection

(Munoz-Fontela, Pazos et al. 2011). Here, ledene had no consistent effect on the expression of NF- κ B and type I IFN responsive genes but appeared to reduce *TP53* activity to inhibit influenza virus replication, which is contradictory to previously reported results for ledene. At this stage, it is not clear about the functional relationship between ledene and p53, which should be a subject of future investigation.

Pre-infection priming of NHBE cells with ledene was able to block progeny virus production which implies that cellular changes, including presumably transcriptional changes, were triggered by the compound ahead of infection. However, microarray analysis found no significant differential expression between uninfected ledene-treated and uninfected DMSO control cells. Without the application of false discovery rate (FDR) (Rouam 2013), the identification of the 14 candidate differentially expressed genes might be unreliable. Indeed, eleven of the shortlisted genes (*ACO1*, *ACTG1*, *AHSA1*, *CCT7*, *CHCHD*, *TMEM45B*, *TRAP1*, *PCYT2*, *SLC25A3*, *SERBP1* and *TUBB4B* from ledene uninfected vs DMSO uninfected NHBE cells) could not be consistently validated by qPCR (data not shown). Four genes, *ACTG1*, *TMEM45B*, *TRAP1* and *TUBB4B*, were identified to be present on both gene lists (14 genes from ledene uninfected vs DMSO uninfected NHBE cells and 35 genes from ledene H1N1 virus infected vs DMSO H1N1 virus infected NHBE cells). Unfortunately, the qPCR results failed to

validate the microarray data (data not shown). In a future study, it would be more appropriate to use a higher non-cytotoxic dose of ledene, e.g. at 50 μ M, to accentuate differential gene expression. It is possible that ledene primarily regulates host cell immune response at the protein level. In future, it would be appropriate to perform quantitative proteomics to acquire in-depth insights into host protein regulation and post-translational modifications affected by ledene.

In summary, ledene blocks H1N1 virus replication at the posttranslational level. Preliminary RNA microarray analysis predicted that reduced host cell p53 activity could possibly be involved in virus inhibition. The precise antiviral mechanism of ledene remains to be determined.

6. General discussion

The first aim of the thesis was to understand the host innate immune response to IAV infection in human and pig airway epithelial cells. To this end, two projects were conducted. The first project characterised the differences in innate immune response to NF- κ B activation in human and pig airway epithelial cells to virus infection. The role of NF- κ B during virus infection appears to be contradictory. NF- κ B is the key transcriptional regulator that controls host cell inflammatory response and cell survival. NF- κ B activation induces cytokines, chemokines, *IFN- β* and its associated genes to inhibit virus replication. Knockout of NF- κ B inhibitory gene *A20* in mice elevated NF- κ B activity and increased survival rate against lethal H1N1 virus infection, which indicates that the activation of NF- κ B inhibits H1N1 virus replication (Maelfait, Roose et al. 2012). MEFs lacking NF- κ B component *RelA* showed reduced *IFN- β* response and increased NDV and SEV replications (Wang, Basagoudanavar et al. 2010). Therefore, NF- κ B is an important anti-viral factor.

The activation of NF- κ B is a rapid process that induces the transcription of early viral genes (Hiscott, Kwon et al. 2001). Activation of NF- κ B promoted the replications of H7N7, H1N1 and H2N2 viruses through NF- κ B-dependent viral induction of the proapoptotic factors (TRAIL and FasL) (Wurzer, Ehrhardt et al. 2004). p65 is a key regulator of NF- κ B pathway, knockdown of p65 in A549 cells reduced H1N1 virus vRNA synthesis to further reduce virus

replication(Kumar, Xin et al. 2008). In early cycle of H1N1 virus replication, it was shown that the activation of NF- κ B increased the expression of *SOCS3* to counteract the expression of type I IFN to increase virus replication (Pauli, Schmolke et al. 2008). Studies have also indicated that some viral genes may hijack NF- κ B pathway to enhance viral replication. HBV X protein (HBx) and HTLV-1 Tax protein were shown to activate the DNA-binding activity of NF- κ B and to hijack host cell NF- κ B pathway to enhance virus replication (Baeuerle and Henkel 1994). The non-structural protein NS5A of HCV activated NF- κ B pathway through calcium-dependent signalling pathways to promote virus replication in Huh 7 cells (Gong, Waris et al. 2001). Therefore, NF- κ B is also characterised as a pro-viral factor.

In pig NPTr cells and PTECs, it was obvious that over-expression of IKK2 CA and RelA inhibited USSR H1N1 and pdm H1N1 virus replication. This indicates that the activation of NF- κ B reduced IAV replication in pig airway epithelial cells. However, the effect of the NF- κ B activation to IAV infection in human A549 and NHBE cells was less conclusive. It was shown that the over-expression of IKK2 CA and RelA in A549 and NHBE cells had variable effects on IAV replication in comparison to consistent strong anti-viral effect in pig cells. The activation of NF- κ B induced strong type I IFN response in pig airway epithelial cells, but only had mild effect of type I IFN response in corresponding human cells. All together suggest that NF-

κB activation is an anti-viral factor in pig cells, but is less conclusive in human cells.

As described in section 2.4, the major discrepancy of this study is the failure of the microarray assay. The objective of the assay was to investigate the transcriptional changes led by IKK2 CA overexpression in NPTr cells. However, the chip failed to detect cytokines and chemokine response in NPTr cells. Therefore, it is encouraging to perform the similar study using proper pig microarray chips to determine the immune response led by IKK2 CA overexpression in NPTr cells.

The second project was to characterise the basal transcriptional levels of innate immune genes and to identify the key molecular factors that could play crucial roles in host innate immune response to influenza A virus infection between human and pig airway epithelial cells.

Pigs normally show mild or no clinical signs to IAV infection, whereas humans display severe clinical symptoms with strong pro-inflammatory response. Pigs inoculated with H5N1 virus showed 100% survival rate after 14 days, and no virus was detected from pig tissues at 3 and 14 days post infection (Isoda, Sakoda et al. 2006). Two- to three-week-old domestic pigs infected with HPAIV H5N1 virus showed no clinical signs and mortality in contrast to mice and ferrets, where the H5N1 was highly pathogenic (Lipatov, Kwon et al.

2008). Humans infected with HPAIV show high mortality rate. According to World Health Organisation (WHO) cumulative number, the death rate of H5N1 virus in human is 52.8% ([https://www.who.int/publications/m/item/cumulative-number-of-confirmed-human-cases-for-avian-influenza-a\(h5n1\)-reported-to-who-2003-2021-15-april-2021](https://www.who.int/publications/m/item/cumulative-number-of-confirmed-human-cases-for-avian-influenza-a(h5n1)-reported-to-who-2003-2021-15-april-2021)).

Pigs and humans pose significantly different immune response to IAV infection. Severe influenza virus infection causes humans to die from the aggressive pro-inflammatory response and dysregulation of anti-inflammatory responses. It was shown that human airway epithelial cells and macrophages mounted strong cytokines (*TNF- α* and *IL-6*) and chemokines (*CXCL9*, *CXCL10* and *CXCL11*) response in comparison to corresponding pig cells to HPAIV H5N1 infection (Nelli, Dunham et al. 2012). A similar study compared the innate immune response between pig and human bronchial epithelial cells. It was shown that the antiviral effect of pig cells was limited in comparison to human cells, and human cells posed stronger IFN responses than pig cells (Hauser, Dlugolenski et al. 2013).

The microarray analysis helped to profile the basal transcriptional level between NHBE cells and PTECs prior to virus infection. It was shown that the overall *TNF- α* and NF- κ B were more activated in NHBE cells, which suggests the basal transcription of NF- κ B-associated genes are highly primed in NHBE cells than PTECs. From

the microarray analysis, the expression of *TNFRSF1A* in NHBE cells was much higher than PTECs. *TNFRSF1A* is a key upstream regulator of NF- κ B signalling pathway. The binding of TNF- α to TNFR1 (encoded by *TNFRSF1A*) can activate NF- κ B to produce pro-inflammatory cytokines and chemokines. To this end, we examined the effects of *TNFRSF1A* on H1N1 virus replication and host cell innate immune response in NHBE cells and PTECs. Knockdown of *TNFRSF1A* reduced H1N1 virus replication in NHBE cells with attenuation of apoptosis and necrosis activity, but the knockdown had little effect in PTECs. The over-expression did not change the virus output outcome in NHBE cells, but increased H1N1 virus output in PTECs with increased necrosis. All above imply that the relative basal expression of innate immune genes in pigs and humans prior to infection could be a key determinant of host resistance or susceptibility to IAV infection.

Beside *TNFRSF1A*, some other key upstream genes of NF- κ B were shown to be differentially expressed between NHBE cells and PTECs from the microarray analysis (data not shown). *TNFRSF10B* and its ligand *TNRSF10* were shown to be highly expressed in NHBE cells than PTECs, and the basal transcription of *TNFRSF1B* in PTECs was much higher than NHBE cells. All these genes could be potential genes of interest in future study, it is worth investigating their roles in host innate immune response to influenza A virus infection between human and pig airway epithelial cells.

The second aim of the thesis was to identify potential anti-influenza drugs and to elucidate their antiviral mechanisms. To achieve the aim, two projects were undertaken. The first project was to understand the anti-influenza mechanism of acetylsalicylic acid (ASA) in human and pig primary airway epithelial cells.

ASA is a broad-spectrum antiviral drug that inhibits various types of virus infections including influenza A and B virus infections (Davis, Green et al. 1985, Glatthaar-Saalmuller, Mair et al. 2017). ASA is commonly used as an anti-inflammatory drug. It was shown that the use of ASA reduced the expressions of *IL-1*, *IL-6* and other cytokines (Vane and Botting 2003). ASA blocked NF- κ B activation by interfering the degradation of I κ B to further retain NF- κ B in cytosol (Kopp and Ghosh 1994). Other studies demonstrated that the binding of ASA with the key NF- κ B component, IKK2, prevented NF- κ B from activation (Grilli, Pizzi et al. 1996, Yin, Yamamoto et al. 1998). Mazur et al., (2007) showed that the use of NF- κ B inhibitor Bay 11-7085 significantly impaired the antiviral effect of ASA in H1N1, H5N1 and H7N7 infected A549 cells. All above suggest that the antiviral mechanism of ASA is through the inhibition of NF- κ B pathway most likely.

Studies have also suggested that ASA may act as COX inhibitor. ASA was found to acetylate C-terminus of COX, the acetylation could result in irreversible COX inhibition (Roth and Majerus 1975). Two

members of the COX family, COX-1 and COX-2, were shown to have important role in IAV replication. *COX-1*-deficient mice were showed to have higher mortality rate in comparison to *COX-2*- deficient mice during H3N2 virus infection, and *COX-1* deficiency resulted a stronger and earlier proinflammatory response (Carey, Bradbury et al. 2005). The inhibition of *COX-2*, unlike *COX-1*, was found to be beneficial to the host during IAV infection (Carey, Bradbury et al. 2005). Inhibition of *COX-2* led to higher survival rate in H3N2 infected mice with lower proinflammatory response and progeny virus production (Carey, Bradbury et al. 2005). Therefore, ASA may inhibits IAV replication through COX-dependent pathways.

However, the anti-influenza mechanism of ASA was shown to be neither through the inhibition of NF- κ B nor the inhibition of COX in this study. The use of ASA inhibited IAV replication at the post-translational level through the inhibition of virus-induced apoptosis in NHBE cells. However, in PTECs, the use of ASA blocked IAV replication at the transcriptional level through the activation of NF- κ B. All together suggests that the ASA works through different mechanisms to inhibit IAV replication in human and pig primary airway epithelial cells. It is possible that the anti-influenza mechanism could be host-species specific, which means that ASA may work through other mechanisms in host cells of other species. Therefore, as a interesting avenue, it is worth investigating the anti-influenza mechanism of ASA in host cell systems of different species,

including avian species (chicken and duck) and mammalian species (bat and mouse), to further elucidate the anti-influenza mechanism of ASA in response to different host-species cells.

The second project was to understand the anti-influenza mechanism of ledene in primary normal human bronchial epithelial (NHBE) cells.

Sesquiterpene and its derivatives (sesquiterpenoids and sesquiterpene lactones) are discovered to have anti-influenza effect. The synthesis of IAV vRNA, mRNA and cRNA were inhibited by sesquiterpene in MDCK cells and HeLa cells (Hayashi, Hayashi et al. 1996, Gao, Han et al. 2017). The anti-influenza mechanism of sesquiterpene was shown to be through inhibition of p38 and NF- κ B pathways in A549 cells (Wang, Zhou et al. 2017). The use of sesquiterpene reduced TNF- α expression in H1N1 virus infected mice (Li, Peng et al. 2012). Sesquiterpene was shown to reduce *IL-4* and *IFN- γ* expressions in H1N1 virus infected human respiratory epithelial (16HBE) cells (Wu, Ju et al. 2013). All above suggest that the anti-influenza mechanism of sesquiterpene is through the inhibition of pro-inflammatory response.

In this study, ledene was identified as a possible anti-influenza drug from our chemical compound screening (unpublished data). It was shown that the selective index of ledene was 79 times higher than oseltamivir, which indicates ledene could potentially be a much safer and potent anti-influenza drug. Viral protein and mRNA were not

affected by ledene, which indicates that ledene inhibits IAV replication at the post-translational level. However, due to the limitation of time, the study was only able to characterise the preliminary anti-influenza effect of ledene. The microarray experiment failed to provide the transcriptional changes with treatment of ledene. Therefore, to continue this study, it is worth repeating the microarray study by using a higher non-toxic dose of ledene in NHBE cells, by doing so, we can have a more in-depth understanding of the transcriptional changes of host cell affected by ledene.

7. References

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