

**Bat lung epithelial cells show
variable species-specific
susceptibility to human and avian
influenza viruses**

By

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Abstract

The recent identification of two novel influenza-like viruses in bats, H17N10 and H18N11 virus, and the discovery of serologically positive *Eidolon helvum* bats in Ghana for avian H9 virus prompted my hypothesis that, in addition to the large repertoire of zoonotic viruses hosted, bats may serve as asymptomatic reservoir species to conventional influenza A viruses found in birds and mammals.

To begin to test this hypothesis, the susceptibility of three bat cell lines, derived from lung epithelial cells of *Eidolon helvum*, *Carollia perspicillata* and *Tadarida brasiliensis* (TB1-Lu), to low pathogenicity avian viruses (H2N3 [A/mallard duck/England/7277/06] virus and H6N1 virus [A/turkey/England/198/09] virus), and human viruses (USSR H1N1 virus [A/USSR/77] and pandemic H1N1 2009 virus [A/California/07/2009]) was determined.

All three species of bat epithelial cells were found to be more robust and resistant to influenza virus infections than permissive MDCK cells. Infected bat cells produced lower levels of viral RNA and viral progeny, and were more viable than correspondingly treated MDCK cells. Interestingly, bat cells were more susceptible and replication permissive to avian than human influenza viruses. Among the bat cells, TB1-Lu cells were the least susceptible to influenza virus infection and that appears to be related to a lack of sialic acid α 2,6-Gal receptors, mammalian virus-preferred host receptors, which were present in the other two bat species.

The innate mechanisms underlying resistance to influenza virus infection in bats remains to be determined, however, inhibition of the JAK/STAT pathway was found not to affect virus production from infected bat cells suggesting that JAK/STAT signalling may not have a major role in influenza virus resistance in bat cells. Modulation of NF- κ B signalling was found to affect virus production suggesting that

tight regulation of NF- κ B may be key in controlling the pro-inflammatory response to influenza infection in bat cells and warrants further investigation.

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Declaration

I declare that I am directly responsible for the composition of this thesis and the work described here. The studies described in this thesis were carried out in the School of Veterinary Medicine and Science at the University of Nottingham. All work of other authors and materials from other sources has been accordingly acknowledged.

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List of abbreviations

%	=	Percentage
°C	=	Degrees Celsius
μM	=	Micromolar
μl	=	Microlitre
μg	=	Microgram
cDNA	=	Complementary DNA
COX 1/2	=	Cyclooxygenase protein 1/2
DAPI	=	4',6'-diamidino-2-phenylindole
DEPC	=	Diethyl pyrocarbonate
DMEM	=	Dulbecco's Modified Eagle's medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
dsRNA	=	Double stranded RNA
FCS	=	Fetal calf serum
FFA	=	Focus forming assay
FFU	=	Focus forming units
h	=	Hour(s)
HA	=	Haemagglutinin
HRP	=	Horse radish peroxidase
HPAI	=	Highly pathogenic avian influenza
IFN	=	Interferon
IKK	=	IκB kinase
IL	=	Interleukin
ISG	=	Interferon-stimulated gene

JAK	=	Janus kinase
LPAI	=	Low pathogenicity avian influenza
M	=	Matrix
MDCK	=	Madin-Darby canine kidney
MOI	=	Multiplicity of infection
min	=	Minute(s)
mg	=	Milligram
ml	=	Millilitre
NA	=	Neuraminidase
NEP/ NES	=	Nuclear export protein
NF- κ B	=	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	=	Nuclear localisation signal
nM	=	Nanomolar
NP	=	Nucleoprotein
NS1/2	=	Non-structural protein 1/2
P6	=	Pyridone 6
PA	=	Polymerase acidic protein
PB1/2	=	Polymerase basic protein 1/2
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
pi	=	Post-infection
qPCR	=	Quantitative polymerase chain reaction
RHD	=	Rel-homology domain
RIG-I	=	Retinoic acid-inducible gene I
rRNA	=	Ribosomal RNA

RNA	=	Ribonucleic acid
RT-qPCR	=	Real-time quantitative polymerase chain reaction
s	=	Second(s)
SA	=	Sialic acid
SDS	=	Sodium dodecyl sulphate
SOCS	=	Suppressor of cytokine signalling protein
STAT	=	Signal transducer and activator of transcription protein
ssRNA	=	Single-stranded RNA
TLR	=	Toll-like receptor
TB1-Lu	=	Lung epithelial cells from <i>Tadarida brasiliensis</i> bat (cell line)
TBS	=	Tris buffered saline
TBS-tween	=	Tris buffered saline-Tween 20
vRNA	=	Viral ribonucleic acid
vRNP	=	Viral ribonucleoprotein
x g	=	Relative centrifugal force

1. Introduction

1.1. Influenza A viruses

1.1.1. Classification of influenza A viruses

Influenza A viruses are part of the *Orthomyxoviridae* family, which cause influenza. These viruses are negative sense single-stranded (ss) RNA viruses. The subtypes of influenza viruses are defined by the combination of surface membrane proteins haemagglutinin (HA) and neuraminidase (NA). Most of the literature reports 16 HA sub-types and nine NA subtypes however the recent discovery of novel strains harboured in bats suggests there are additionally two more HA (H17 and H18) and NA subtypes (N10 and N11) (Tong et al. 2012; Tong et al. 2013).

Influenza A virus nomenclature is specified by the following: host of origin (if human, this is omitted), geographic location of first isolation, strain number and year of isolation (Memorandum 1980).

Influenza is usually a self-limiting disease yet it is estimated that between 250,000 and 500,000 fatalities arise from influenza every year (WHO., 2005). Those usually affected are vulnerable groups such as the young, the elderly and the immunocompromised. In the United States, 200,000 hospitalisations are a direct consequence of influenza virus infection costing over US\$10 million annually (Horimoto & Kawaoka 2001). These strains of influenza A viruses are classified as seasonal strains. Seasonal variants of the virus occur naturally over time due to antigenic drift- mutations in the genes of the surface antigens, HA and NA. With each

passing season, new mutations arise rendering the general population susceptible to infection.

Influenza A viruses are prone to mutation for a number of reasons. Firstly, the viral polymerase complex lacks proof-reading ability. This results in frequent substitutions, insertions and deletions (in the region of 1 in 10^4 bases) during replication (Holland et al. 1982; Steinhauer & Holland 1987). The segmented nature of the genome is also an important factor for creating diversity amongst the strains of influenza A virus as it allows for reassortment of the viral genome causing antigenic shift. The reassortment of a highly pathogenic strain with a strain capable of efficient human-to-human transmission can result in pandemic strain potentially affecting millions worldwide. Notable historical pandemics include Spanish flu in 1918, Asian flu in 1957 and Hong Kong flu in 1968 which resulted in millions of fatalities (as reviewed in Lipatov et al. 2004).

1.1.2. Structure of influenza A viruses

Influenza A viruses are enveloped, negative sense and single-stranded-RNA viruses. The phospholipid bilayer has three integral membrane proteins; HA, NA and matrix protein 2 (M2). The role of HA is to bind the virus and mediate fusion and subsequent entry into susceptible cells. The HA binds to receptors on host cells containing sialic acids attached to surface glycolipids or glycoproteins. Most of the literature reports 16 HA sub-types, however the recent discovery of novel strains harboured in bats suggests there are 2 more HA sub-types (Tong et al. 2012; Tong et al. 2013). The virus's genome is segmented, divided into eight RNA molecules which encode 14 known proteins. These proteins include HA, NA, matrix protein 1 (M1), M2,

nucleoprotein (NP), polymerase basic protein 1 (PB1), PB1-F2, PB2, polymerase acidic protein (PA), non-structural protein 1 (NS1), and nuclear export protein (NEP/NS2). Each viral RNA (vRNA) molecule is packaged into viral ribonucleoprotein (vRNP) complexes (**Figure 1.1-1**). Multiple copies of viral NP stabilises the complex as it forms a core which the vRNA wraps around. Each vRNP is also associated with one copy of a trimeric RNA polymerase complex composed of PA, PB1 and PB2. The vRNPs are considered to be the basic units of orthomyxoviruses as they can transcribe and replicate in absence of other viral proteins (Kemler et al. 1994; Pleschka et al. 1996; Mullin et al. 2004; Fodor et al. 2002) and a complete set of vRNPs can initiate infections (Fodor et al. 1999; Neumann et al. 1999; Hoffmann et al. 2000). Forming a shell around the vRNPs is the matrix 1 (M1) protein. PB1-F2 is encoded for by an alternate reading frame in the PB1 gene and localises to the inner and outer mitochondrial membranes and has been shown to induce cell death and contribute to viral pathogenesis (Zamarin et al. 2006).

The NS gene segment of influenza A viruses encodes NS1 and NS2 (also known as NEP). NS1 has many roles including the induction of apoptosis and suppression of the interferon (IFN) response. The function of NS2 is primarily to facilitate nuclear export of vRNPs, see 1.1.3.4 for further detail.

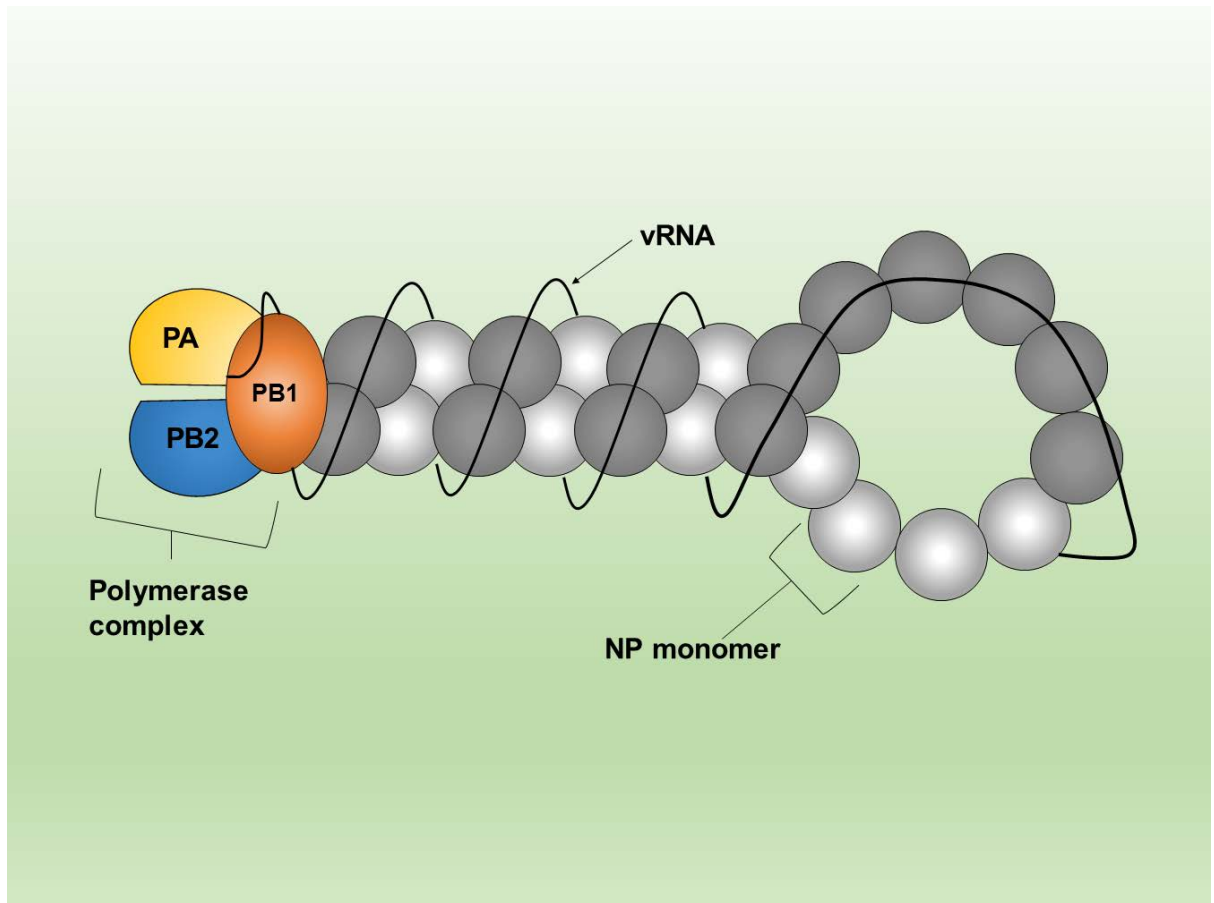


Figure 1.1-1 The influenza A virus ribonucleoprotein (vRNP) structure (Original)

Each viral RNA (vRNA) molecule is packaged into viral ribonucleoprotein (vRNP) complexes. Multiple copies of viral NP stabilises the complex as it forms a core which the vRNA wraps around. Each vRNP is also associated with one copy of a trimeric RNA polymerase complex composed of polymerase acidic protein (PA), polymerase basic protein 1 (PB1) and polymerase basic protein 2 (PB2).

1.1.3. Replication of influenza A viruses

1.1.3.1. Attachment and entry

Viruses hijack the host cell replication machinery in order to transcribe and replicate. The first stage of viral entry into the host cell is the recognition and subsequent binding of HA to host receptors containing *N*-acetylneuraminic acid, also known as sialic acid (SA) (Palese & Shaw 2007). It is the ability of HA proteins to bind to SA receptors which determines the susceptibility of the host cells to infection. Human strains of influenza A viruses are adapted to receptors with a α -2,6 linkage to the underlying galactose whereas avian strains are more adapted to α -2,3 linkage (Weis et al. 1988; Gamblin et al. 2004; Stevens et al. 2006). More recent evidence has suggested that the topological structure of SA also contributes to the binding affinity of HA to SA. Human influenza virus strains have been shown to have a higher affinity to the longer umbrella-shaped SA molecules with α -2,6 linkage whereas the shorter cone-like SAs are preferentially bound to by avian viruses (Chandrasekaran et al. 2008).

Although SA receptors are accepted as the main receptors for influenza virus entry (Edinger et al. 2014), it is unclear whether additional host factors are needed or other receptors exist as another route of entry (Edinger et al. 2014). Evidence to suggest other means of entry include desialylation studies whereby SA moieties are cleaved by a neuraminidase. These studies indicated desialylation of cells reduced virus binding and infectivity (Stray et al. 2000). However, despite this, virus can still bind to and subsequently enter desialylated cells and desialylated cells are no more protected from virus infection than untreated cells (Stray et al. 2000). These results have led to the proposal of the existence of an alternative pathway, independent of SA or a multistep pathway composed of SA receptors and nonsialylated receptors. Host

factors suggested to play an important role in virus binding and entry include C-type lectins (Londrigan et al. 2011), annexin V (Huang et al. 1996) and 6-sulfo sialyl Lewis X (Gambaryan et al. 2008).

After SA binding, the virus is subsequently internalised through receptor-mediated endocytosis into mainly clathrin-coated vesicles indicating the use of clathrin-mediated endocytosis routes (Patterson et al. 1979; Matlin et al. 1981; Yoshimura et al. 1982). It has been suggested that the route of internalisation of the virus may be dependent on the shape of virus particle (De Conto et al. 2011). Although most virus is packaged into clathrin-coated vesicles, macropinocytosis, the uptake of large sized cargo through the non-clathrin-coated vesicles known as macropinosomes, has been suggested as an alternative and preferred internalisation route for filamentous influenza virus (de Vries et al. 2011; Rossman et al. 2012).

Following internalisation, the virus progresses through the endosomal trafficking pathway from early endosomes to late endosomes in order to reach the fusion site whereby viral and endosomal membranes fuse to release vRNPs into the cytoplasm.

The fusion of viral and endosomal membranes is executed by the HA of the virus. The acidic environment of late endosomes induces the HA to undergo structural conformational changes revealing the fusion peptide (Carr & Kim 1993; Bullough et al. 1994; Chen et al. 1999). It has been shown that the optimal pH for fusion varies between different HAs of different subtypes; virus isolated from humans require a lower pH than virus derived from avian hosts of the same subtype (Galloway et al. 2013). The revealed fusion peptide of HA, positioned toward the target membrane, subsequently inserts into the endosomal membrane causing the membranes to fuse and fusion pore to be established (Spruce et al. 1989; Melikyan, Niles, Peeples, et al. 1993;

Melikyan, Niles & Cohen 1993). It is through this fusion pore that vRNPs can be released into the cytoplasm.

M1 and M2 proteins play a vital role in influenza A virus uncoating following fusion. The viral membrane and vRNPs are linked by M1 proteins thus these components must dissociate for the translocation of vRNPs into the nucleus. M2 ion channel activity facilitate this by mediating proton influx from the endosome into the virion resulting in the acidification of the virion interior (Wharton et al. 1994) and the subsequent detachment of M1 from vRNPs and their release into the cytoplasm (Zhirnov 1990). Host factors thought to be involved in the uncoating process include Itch, an E3 ubiquitin ligase, which ubiquitinates M1 and releases vRNPs (Su et al. 2013) however little is known about other cellular factors involved (Edinger et al. 2014).

1.1.3.2. Nuclear import

Once released from endosomes, vRNPs are transported to the nucleus where replication and transcription occur. The three polymerase subunits and NP possess nuclear localisation signal (NLS) motifs (O'Neill et al. 1995; Cros & Palese 2003) however the non-classical (not arginine- or lysine-rich) NLS of viral NP appears to be the most important for the nuclear import of RNPs (Cros & Palese 2003).

The vRNP nuclear transport utilises the karyopherin import pathway, with the main importins for vRNPs being karyopherins $\alpha 1$, $\alpha 2$ and $\alpha 5$ on the cellular side (O'Neill et al. 1995; Wang et al. 1997; Melén et al. 2003). Importin α adaptor proteins recognise the NLS encoded by NP and subsequently the importin β transport receptor recognises the adaptor proteins. NP and PB1 enter the nucleus as separate components whereas PA and PB2 dimerise and enter the nucleus together (Nath & Nayak 1990; Nieto et al. 1994; Neumann et al. 1997; Wang et al. 1997; Weber et al. 1998; Tarendeau et al.

2007). Currently, it is unknown whether vRNPs are transported in groups or separately (Eisfeld et al. 2014). The cellular factor CSE1L has also been shown to be important for vRNP nuclear import as it is involved in karyopherin cycling between the nucleus and cytoplasm (König et al. 2010).

1.1.3.3. Primary transcription and replication

The vesicles are uncoated and transported to the nucleus where replication and transcription take place. The polymerase complex composed of PA, PB1 and PB2 are responsible for transcription and replication. Both processes are required for new vRNP synthesis. During the early phase of influenza virus infection, vRNP transcription dominates whereas in late infection, replication is the more common process (Shapiro et al. 1987; Vreede et al. 2010).

Transcription is a single-step primer-dependent process whereas replication is a two-step process and does not require primers. Replication requires the production of a positive complementary strand of the negative vRNA, the cRNA, which acts as a template for the synthesis of new vRNA. Replication requires a full-length copy of vRNA, while transcription only requires a partial copy of the vRNA for the production of mRNA. During pre-initiation step of transcription, the partial copy of vRNA gains a 5' cap through 'cap-snatching' whereby the polymerase complex binds to and cleaves host RNA 5' caps, and is polyadenylated. Cap-snatching acts to prime for viral transcription and also serves to shut off host transcription and protein synthesis. The preferred substrates for cap-snatching have been found to be small nuclear RNAs and small nucleolar RNAs (Sikora et al. 2014; Gu et al. 2015; Koppstein et al. 2015). The capped RNA fragments are subsequently used in transcription initiation as primers to

transcribe viral mRNA. Viral mRNA is then exported into the nucleus for protein synthesis. Newly synthesised viral polymerase subunits (PB1, PB2, PA and NP) are then imported back into the nucleus to bind to vRNA and cRNA segments to assemble vRNPs and cRNPs.

The first step the replication process centres around the generation of a complementary strand of RNA (cRNA) from a full length copy of vRNA, catalysed by the resident polymerase (Vreede & Brownlee 2007; York et al. 2013). The cRNA intermediate assembles into a cRNP-NP complex, similar to vRNP structurally (York et al. 2013), and serves as a template for vRNA synthesis, the second stage of replication. This process requires a second polymerase, however, it has been found that this polymerase is not required to be catalytically active (York et al. 2013) implying that this second polymerase may act in a trans-activating, rather than trans-acting, role. In this trans-activating state, it has been speculated that the polymerase induces a conformational change in the resident polymerase causing the cRNA termini to be transferred to the active site (reviewed in te Velthuis & Fodor 2016). As transcription occurs, the newly synthesized vRNA is encapsidated by viral polymerase and free NP, forming vRNP (Moeller et al. 2012; Ye et al. 2013).

It is currently unclear what exactly triggers the resident polymerase to switch between replicase and transcriptase (Hutchinson & Fodor 2013). A number of viral proteins have been implicated as factors in this process, particularly NP (Beaton & Krug 1986; Shapiro & Krug 1988), however, recently evidence has shown that the regulation between transcription and replication may be stochastic (Vreede et al. 2004). Microarray analysis has identified hundreds of host factors which may regulate vRNP replication and synthesis however very few have been examined in detail (reviewed in te Velthuis & Fodor 2016).

1.1.3.4. Nuclear export

For infection to proceed, newly formed vRNPs need to be exported from the nucleus using several pathways. The viral proteins, M1 and NEP/NS2, which can pass through the nuclear pore without importin binding due to their small size, facilitate this exportation. These proteins, along with NP, encode nuclear export signals (NESs) recognised by the exportin, Crm1 (O'Neill et al. 1998; Neumann et al. 2000; Huang et al. 2013). NS2 binds to M1 and subsequently link vRNP to Crm1 in a 'daisy chain' conformation (Yasuda et al. 1993; Akarsu et al. 2003; Shimizu et al. 2011).

NS2 is a key regulator of vRNP export. NS2 protein synthesis is at a much lower rate compared with other viral proteins due to it being encoded at a weak splice site (Robb et al. 2010). As a result, NS2 slowly accumulates during infection and promotes nuclear export of vRNPs at the later stages. This is evidenced by the correlation of NEP levels with vRNP export (Chua et al. 2013).

Other signalling pathways involved in promoting vRNP exit include the mitogen-activated protein kinase (MAPK) which is activated by HA accumulation at the surface membrane (Marjuki et al. 2006) and the apoptotic pathway activated by caspase 3 (Hinshaw et al. 1994; Wurzer et al. 2003).

Recently, it has been hypothesised the Crm1 pathway may be inadequate to sustain the rate vRNP export and a caspase 3-mediated mechanism exists to complement and support vRNP export (Mühlbauer et al. 2015). Previous studies have revealed NF- κ B-induced TRAIL and FasL expression and infection-induced caspase activity promotes efficient nuclear export of vRNPs (Wurzer et al. 2003; Wurzer et al. 2004). Mühlbauer *et al.* (2015) showed that caspase activities degraded Nup153, a nuclear pore component, resulting in up to 20 nm increase in nuclear pore complex size allowing for passive diffusion to occur during late infection.

1.1.3.5. vRNP trafficking to surface membrane

After vRNPs are exported from the nucleus, they are transported to and accumulate at the perinuclear cytoplasm. Here, the vRNPs engage with Rab11 on recycling endosomes (reviewed in Hutchinson & Fodor 2013). During the late stages of infection, the vRNPs are transported to the apical membrane. Specific RNA segments are packaged together due to packaging signals in the termini of each segment.

1.1.3.6. Particle assembly

Virion assembly and budding is complicated and poorly understood. Surface glycoproteins, NA and HA, accumulate in lipid raft microdomains and span the membrane whereas M2 localises on the boundary of this microdomain. The mechanism for virion budding is unclear. Individual overexpression of HA, NP, NA, M1 and M2 can cause budding (Hutchinson & Fodor 2013). vRNP is known to interact and bind with M1; this causes M1 to undergo a conformational shape change. This shape may drive capsid formation due to M1 polymerisation or may promote elongation through reducing membrane curvature (Rossman & Lamb 2011). Once the glycoproteins are collected on the surface membrane and vRNPs are packaged in the virion, M2 mediates membrane abscission completing the virion assembly process (Rossman et al. 2011). For a more detailed review of virion assembly and budding, refer to Hutchinson & Fordor (2013) and Rossman & Lamb (2011).

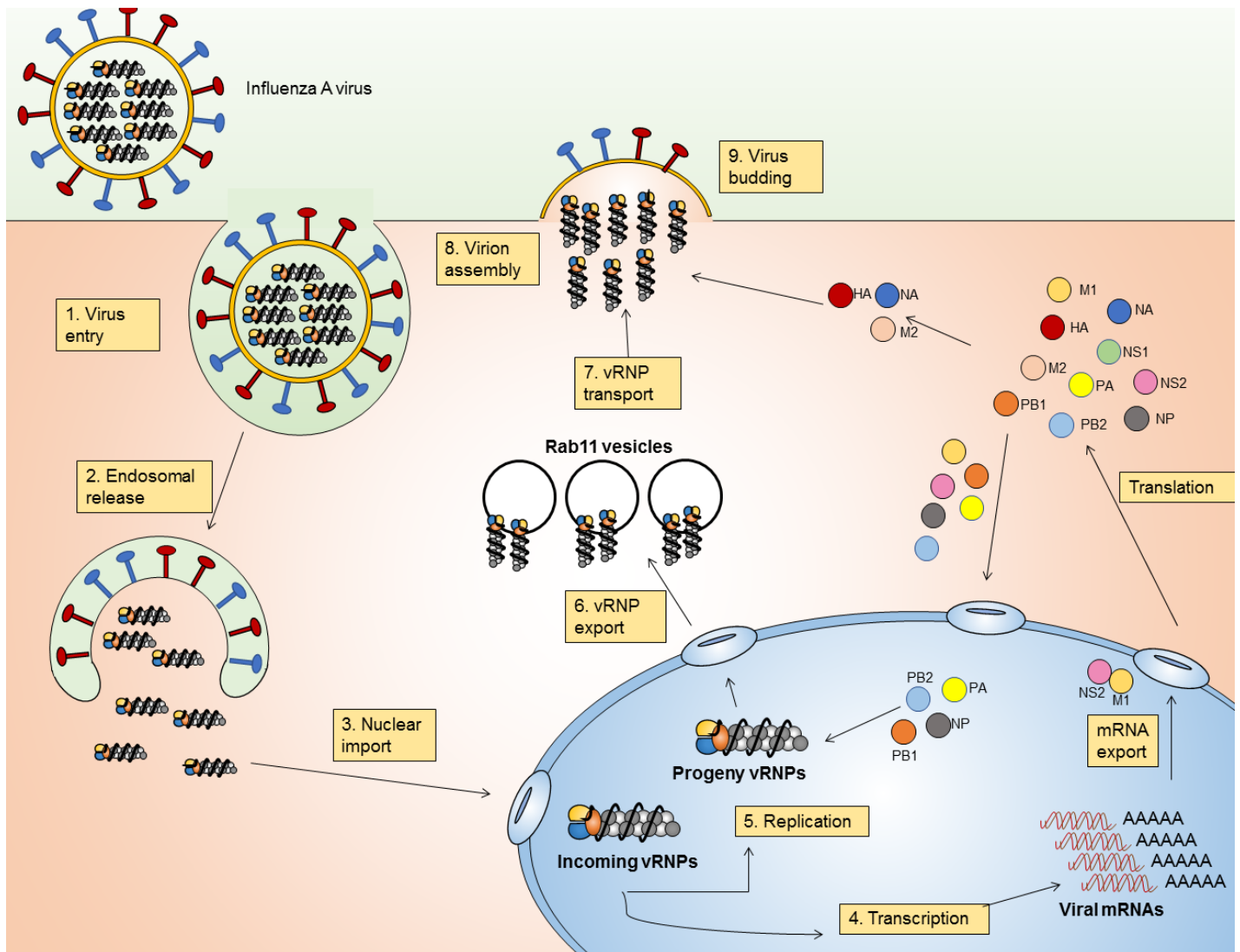


Figure 1.1-2 The influenza virus lifecycle (Original and adapted from Eisfeld et al. 2014; te Velthuis & Fodor 2016)

- (1) Haemagglutinin (HA) binds to host receptors at plasma membrane and the virus particle is subsequently internalised through receptor-mediated endocytosis into clathrin-coated vesicles. Following internalisation, the virus progresses through the endosomal trafficking pathway from early to late endosomes. Fusion between virion and endosomal membranes is catalysed by acidification of the endosome resulting in conformational changes in HA revealing the fusion peptide. Insertion of the fusion peptide causes the membranes to fuse and the establishment of a fusion pore.
- (2) Viral M2 ion channel also promotes acidification causing the dissociation of M1 from the virion and vRNPs allowing vRNPs to be released into the cytoplasm through the fusion pore.
- (3) Released vRNPs are transported into the nucleus through the nuclear pore complex where transcription and replication take place.
- (4) During pre-initiation step of transcription, vRNA gains a 5'cap through cap-snatching and is polyadenylated. Capped RNA fragment are used as primers to transcribe viral mRNA. Viral mRNAs are transported out of the nucleus and translated via cellular ribosomes. Newly translated viral proteins are transported to the nucleus or plasma membrane
- (5) Replication begins with the cRNA generation from vRNA catalysed by resident polymerase. cRNA serves as template for vRNA synthesis. Newly synthesised vRNA is encapsidated by viral polymerase and free NP forming RNP.
- (6) vRNPs are exported from the nucleus
- (7) Progeny vRNPs are transported via Rab11 vesicles to the plasma membrane
- (8) At the cell membrane, progeny virions are assembled and newly formed virus particle buds off (9).

1.1.4. Hosts and reservoirs of influenza A viruses

The mammalian and avian host range of influenza A viruses is very wide, including, but not limited to, humans, pigs, horses, sea mammals and birds (Webster et al. 1992). Aquatic birds have been implicated as the reservoir hosts for most influenza A viruses. In aquatic birds, influenza A viruses replicate in the gastrointestinal tract thus efficient transmission is achieved through the faecal-oral route as infectious faecal material is deposited in lakes and rivers (Webster et al. 1992).

It has been well characterised that aquatic birds such as waterfowl and shorebirds do not usually suffer any clinical symptoms when infected with influenza A viruses (Alexander et al. 1986). The absence of clinical signs has been reported even when infected with high pathogenic H5 virus subtypes (Alexander et al. 1986; Alexander 2000).

Influenza A viruses which infect poultry are classified into two groups, highly pathogenic and low pathogenicity avian influenza viruses. Highly pathogenic avian influenza (HPAI) viruses, typically limited to the H5 and H7 subtypes, cause virulent disease with a potential mortality of 100% in poultry. Low pathogenicity avian influenza (LPAI) viruses cause a milder infection in poultry and are generally not associated with high mortality. It is important to note that this classification only applies to poultry as a LPAI strain could cause severe disease to other species.

Aquatic birds, such as ducks, have been key in the generation and subsequent maintenance of the HPAI H5N1 virus in Asia (Kuchipudi et al. 2012). As such, it has been postulated that HPAI viruses may have evolved with aquatic birds. Virus adaptation to the host benefits both host and virus as it allows host survival and

continuous perpetuation of the virus. The efficient route of transmission supports this theory (Tong et al. 2013).

The innate resistance of aquatic birds is thought to be perhaps attributed to a superior immune system (Barber et al. 2010). Several studies have shown that there are fundamental differences in the innate host response to infection with influenza A viruses (Kuchipudi et al. 2012; Barber et al. 2010). The viral sensing RIG-I is thought to play an important role in the viral resistance of ducks as RIG-I was found to be present in ducks but absent in chickens (Barber et al. 2010). Furthermore, transfection of duck RIG-I into chicken cells enabled influenza virus sensing and induction of an anti-viral response (Barber et al. 2010).

Kuchipudi et al. (2012), found that, in ducks, rapid and accelerated cell death occurred along with reduced viral output. The cell death was mediated, at least in part, by apoptosis and thought to be a protective mechanism to contain the spread of infection. This caspase-induced cell death was not found to occur in chickens, which died 1-2 days following HPAI virus infection.

In contrast to aquatic birds, influenza A viruses of avian origin, such as those of the H5N1 virus subtype, can cause severe and often fatal disease in humans. Infection from the H7 and H5 virus subtype has been shown to progress to pneumonia, acute respiratory distress syndrome and dysfunction of multiple organs (Abdel-Ghafar et al. 2008; Zhou et al. 2013).

As acute respiratory distress syndrome and multiple organ dysfunction previously have been linked to the failure to regulate cytokine responses (Fisman 2000; Cheung et al. 2002), disease severity has been associated with hypercytokinemia. Hypercytokinemia or 'cytokine storm' is the excessive, uncontrolled release of cytokines and chemokines resulting in an extreme inflammatory response. It has been

suggested that hypercytokinemia is triggered by high viral loads. (de Jong et al. 2006; Abdel-Ghafar et al. 2008)

Patients infected with H5N1 virus have been found to have elevated levels of pro-inflammatory cytokines including TNF α , interleukin (IL)-6, IL-10 and the type II IFN, IFN- γ (To et al. 2001; de Jong et al. 2006). Additionally, *in vitro* experiments found HPAI H5N1 virus induced higher gene transcription of cytokines including inducible protein (IP)-10, IFN- β (a type I IFN), TNF α and IL-6 in primary human alveolar and bronchial epithelial cells compared to infection with H1N1 or H3N2 viruses (Cheung et al. 2002; Chan et al. 2005). It is postulated that high levels of pro-inflammatory cytokines contribute to the pathogenesis and severity of H5N1 disease through reactive hemophagocytosis, a cytokine driven condition and prominent feature of patients with severe H5N1 virus infection (To et al. 2001). Elevated levels of IL2, IL6 and TNF α have been associated with poor prognosis in patients with reactive hemophagocytosis (To et al. 2001). TNF α , specifically has been implicated in the pathogenesis of reactive hemophagocytosis by initiating the cascade of chemotactic cytokines which induces hyperinflammation (Cheung et al. 2002). Furthermore, it has also been reported that HPAI H5N1 viruses may be resistant to the anti-viral effects of cytokines (Seo et al. 2002), an advantageous adaptation considering the high induction of cytokines by HPAI H5N1 virus infection.

An outbreak of the LPAI H7N9 virus in March 2013 caused 44 deaths out of 134 cases across nine provinces in China (Wang et al. 2014). The mortality rate was estimated to be around 30% (Yu et al. 2013; Rongbao Gao, M.D., Bin Cao, M.D., Yunwen Hu, M.D., Zijian Feng, M.D. et al. 2013). Much like the HPAI H5N1 virus subtype, the virulence of H7N9 has been linked to hypercytokinemia. Lung samples showed cytokine and chemokine upregulation of up to 1000-fold greater than plasma levels

and higher concentrations of the pro-inflammatory cytokines IL-6 and IL-8 were found in patients infected with LPAI H7N9 virus subtype than those infected with HPAI H5N1 virus subtype (Zhou et al. 2013).

Pigs have been long presumed to be ‘mixing vessels’ for the reassortment of different strains of influenza A virus as they are susceptible to infection by subtypes of influenza A virus originating from avian and humans sources (Webster et al. 1992). In addition, pigs seem to have an innate resistance to the Eurasian HPAI H5N1 virus as they are asymptomatic or only mildly affected when infected with this strain (Nelli et al. 2012). It is postulated that the lung environment of the pig may be able to better regulate pro-inflammatory responses than other species, initiating a pro-inflammatory response without escalating to hypercytokinemia (Tisoncik et al. 2012).

Thus, although influenza A virus subtypes of pig origin are not easily transmissible to humans (Webster et al. 1992), pigs potentially play a role in the maintenance of pathogenic influenza A strains and the generation of novel subtypes, some of which may have pandemic potential, exemplified by the emergence of pandemic (H1N1) 2009 influenza virus (Yamada et al. 2012).

1.1.4A Egg/cell culture adaptations of viruses and effects on results

The influenza viruses used in this study were propagated in embryonated chicken eggs via the allantoic cavity, as described in 2.3.2. While, the allantoic cavity of embryonated chicken eggs is an efficient and therefore commonly used medium for influenza virus propagation (Robertson et al. 1995), virus propagated in embryonated

chicken eggs – egg-adapted- possess antigenically significant mutations differing from virus propagated through cell culture (Robertson et al. 1985; Roberston et al. 1987; Robertson et al. 1991; Katz & Webster 1988; Katz et al. 1990). These mutations specifically occur in the receptor binding site of the HA enabling the virus to replicate in embryonated chicken eggs (Roberston et al. 1987; Robertson et al. 1985; Katz & Webster 1992). Furthermore, egg-adapted human H1N1 virus has been shown to bind to and to be internalised by MDCK cells more efficiently compared with the same strain of influenza virus propagated in cell culture (Robertson et al. 1995).

Due to these properties of egg-adapted influenza viruses, it was considered that the outcomes of several of the *in vitro* infection experiments may have been different if the viruses were instead propagated in bat cells.

Poole et al. (2014) demonstrated that passaging WSN influenza virus in relatively resistant TB1-Lu cells resulted in increased virus replication, cytopathic effects and viral gene expression in TB1-Lu cells. Also, bat-adapted WSN virus was shown to replicate less efficiently in corresponding pig, human and bird cells compared to non-bat-adapted WSN virus (Poole et al. 2014).

In this study, the results of many experiments were true of egg-adapted viruses which showed both human viruses, USSR H1N1 and pdmH1N1 virus, and LPAI viruses, H2N3 and H6N1 virus, to infected and replicate efficiently in MDCK cells.

1.1.5. The host innate immune response to influenza

A virus infection

The innate immune system can be divided into the innate and adaptive immune systems. These two arms of the immune system work in conjunction to fight and

protect the body from infection. During influenza virus infection, the first line of defence is formed by the innate immune system which aims to control virus replication (Iwasaki & Pillai 2014). During the later stages of influenza virus infection, adaptive immune responses, mediated by virus-specific antibodies and T cells, clears the infection (Iwasaki & Pillai 2014). This study focuses on the innate host response to influenza virus infection.

In higher organisms, a typical host response begins with the recognition of the viral pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I) helicases (RLHs) and nucleotide oligomerisation domain (NOD)-like receptors (NLRs) (as reviewed by Iwasaki & Pillai 2014). The PRRs responsible for influenza A virus recognition are RIG-I, NLRs and TLR7 (Iwasaki & Pillai 2014). Signalling cascades are triggered by such PRRs resulting in the induction of pro-inflammatory cytokines including type I IFNs, chemokines and tumour necrosis factors (TNF). According to Iwasaki & Pillai (2014), host survival in the face of infection is mediated through either reducing pathogen burden, hence having strong viral resistance, or by increasing tolerance to the disease. Different classes of pro-inflammatory cytokines have been shown to mediate both these techniques for host protection. More recently, IFN- γ (type II IFN) and IFN- λ (type III IFN) have been shown to induce anti-viral activity (Mordstein et al. 2008; Iwasaki & Pillai 2014).

The upregulation of pro-inflammatory cytokines promotes viral clearance through the induction of interferon-stimulated genes (ISGs). In the case of influenza A virus, *Mx1* was one of the first ISGs to be characterised in mice. *Mx1* encodes Mx1 protein, a potent blocker of influenza virus infection in the nucleus (Iwasaki & Pillai 2014). MX1

was found to inhibit the transcription replication of influenza A virus through trapping essential components of the virus (Arnheiter et al. 1990; Papenfuss et al. 2012).

Since the discovery of *Mx1*, other ISGs have been identified including *Oas*, *Pkr*, *Ifitm3*, *Viperin* amongst many others (as reviewed by Iwasaki & Pillai 2014). The transcribed proteins of these ISGs are found in different intracellular locations and function to block different stages of the influenza A virus replication cycle and create an anti-viral state in the cell.

1.1.6. Treatment of influenza A virus infections

The primary form of intervention therapy for influenza is vaccination. Seasonal vaccinations are recommended to particularly vulnerable groups during the annual influenza season. Vaccinations aim to protect against the predicted seasonal strain of influenza A virus.

The efficacy of the vaccine is dependent on a number of factors including the age of the individual, their immune response and underlying conditions and degree of antigenic match but overall vaccinations have been shown to be safe and adequately effective in preventing influenza (Noh & Kim 2013).

The limitations of influenza vaccines lie in the method of production. Several techniques are used to produce vaccines but the majority use chicken eggs to grow and passage the influenza virus strains (Lipatov et al. 2004). This antiquated method is reliant on chicken egg availability and a minimum of several months is necessary to develop the vaccine (Reperant et al. 2014). This lag time makes the vaccination an ineffective and nonviable option at the height of new pandemic.

Due to the production limitations of influenza vaccines, in the face of a pandemic, the only form of treatment for novel influenza strains are anti-viral drugs.

The two classes of anti-viral drugs are adamantanes and neuraminidase inhibitors. The adamantanes, amantadine and rimantadine, have been used since the 1960s and 1980s, respectively, whereas the newer neuraminidase inhibitors, mainly oseltamivir and zanamivir have only been available since 1999-2000 (Hurt 2014). Although both classes are used to treat influenza A virus infections, they have different mechanisms of action at different stages of the viral life cycle. Adamantanes inhibit through blocking the M2 protein ion channel, stopping the life cycle at the uncoating stage. Neuraminidase inhibitors are analogous to the structure of sialic acid and therefore stop the viral neuraminidase from cleaving the sialic acid, preventing virion release through competition.

Unfortunately, the high mutation rate of influenza A viruses led to the development of drug resistant strains containing a number of mutations. The S31N amino acid substitution in the M2 protein lowered the binding affinity to adamantane anti-virals (Hay et al. 1986; Belshe et al. 1988). Cross-resistance occurred between compounds in the adamantane class; a strain resistant to amantadine was also resistant to rimantadine (Hurt 2014). Amantadine resistance rose rapidly between 2003-2004; the frequency of resistance in North America was 92.3% and by 2006, virtually all circulating seasonal human H3N2 viruses were resistant worldwide (Hurt 2014).

Resistance against neuraminidase inhibitors has also developed with little impact on viral fitness (Hurt 2014). Resistance was rife in particular to oseltamivir, whose structure more distant from sialic acid than that of other compounds in its class (Hurt 2014). Oseltamivir-resistant strains including the seasonal H1N1 virus and circulating 2009 pandemic H1N1 virus were found to contain a H275Y mutation (Lackenby et al.

2008; Hauge et al. 2009). This mutation and others associated with resistance have also been reported in highly pathogenic avian viruses, such as the H5N1 virus (Govorkova et al. 2009; Nguyen et al. 2013).

There is little to no cross-resistance between neuraminidase inhibitors (Hurt 2014), however, resistance to oseltamivir is particularly concerning as it is the most widely available anti-viral drug used to treat influenza worldwide.

1.1.7. Impact of anti-viral drugs on the environment and human health

Drug resistance is not the only issue surrounding anti-viral influenza drugs. Anti-virals, particularly oseltamivir, have been found to be an environmental pollutant which has the potential to interfere with natural biological systems.

The active metabolite of oseltamivir, oseltamivir carboxylate (OC), resists biological degradation and UV radiation resulting in incomplete removal during wastewater treatment (Kümmerer 2008). As such, the compound remains highly bioactive with the half-life of OC found to be 53 days in surface water (Accinelli et al. 2010). The presence of this compound concentrated in bodies of water further downstream has the potential to cause numerous unwanted effects on the environment and human health.

As oseltamivir is recommended to be used prophylactically and also as treatment during outbreaks of influenza by the World Health Organization (WHO), stockpiling oseltamivir is commonplace in many countries (WHO 2005). This practice could lead to high concentrations of OC in water sources, as demonstrated in Japan, the

highest per-capita- consumer of the anti-viral drug, where OC has been found present in the waterways and drinking water (Söderström et al. 2009).

OC is potentially toxic to certain organisms. Although lack of data available regarding total oseltamivir usage makes it difficult to assess the extent of the impact OC has had on non-target organisms, OC is predicted to be part of one the most hazardous therapeutic classes towards fish, algae and daphnids thereby having a wider impact on food chains and biological systems (Jain et al. 2013).

There is also the potential for OC to drive resistance to neuraminidase inhibitor drugs. Aquatic birds harbour influenza A viruses and as these viruses replicate in the bowel, ingestion of the active metabolite of oseltamivir could potentially drive anti-viral resistance, exacerbating the already concerning issue of resistance. A number of strains have already acquired resistance to oseltamivir and oseltamivir- resistant waterfowl were reported during the 2009 pandemic outbreak (Govorkova et al. 2009; Nguyen et al. 2013).

Like oseltamivir, newer neuraminidase inhibitors, zanamivir, peramivir and lanamivir have also been detected in river water (Järhult 2018). Zanamivir is considered as a major second line neuraminidase inhibitor treatment for influenza virus infection (Nykvist et al. 2017) and like OC, the active metabolite is not readily biodegradable (GlaxoSmithKline 2014). Due to the low rate of adsorption, it is assumed that 100% of the drug enters in the aquatic environment (GlaxoSmithKline 2014). However, a recent *in vivo* study using a Mallard duck model has shown the potential development and persistence of resistance to zanamivir is lower than OC in an environmental setting (Nykvist et al. 2017).

It is clear that due to the widespread resistance to both classes of anti-viral drugs, in conjunction with the residual environmental and health issues of antivirals, alternative

strategies to prevent and treat influenza need to be explored to prevent loss of life during a novel pandemic event.

1.2. Bats

1.2.1. Classification of bats

Bats are of the order Chiroptera and traditionally were classified into two subdivisions, Megachiroptera (megabats) and Microchiroptera (microbats). They account for a quarter of all mammal species with over 1200 species worldwide. Megabats, also known as fruit bats or new world bats, do not echolocation and feed on fruit, nectar or pollen whereas microbats, or new world bats, are generally smaller than megabats, mostly insectivorous and do use laryngeal echolocation. Recently, bats have been reclassified into two new suborders based on molecular data. It has been shown the microbat families *Rhinolophidae*, *Hipposideridae*, *Craseonycteridae*, *Megadermatidae*, *Rhinopomatidae* are more closely related to the megabat family, *Pteropodidae* (Tsagkogeorga et al. 2013). This new suborder has been termed Yinpterochiroptera. The other suborder, Yangochiroptera, encompasses the remaining 12 microbat families. This new classification suggests echolocation evolved separately in the two suborders or developed in a common ancestor and was subsequently lost in Yinpterochiroptera (Tsagkogeorga et al. 2013).

1.2.2. *The relationship between bats and viruses*

Due to their ability to migrate over large distances, bats have gained a global distribution and thus represent an important zoonotic reservoir for the transmission of viruses. Bats are very virus-rich mammals playing host to more virus families than rodent and avian species (Wang et al. 2011). The Indian flying fox (*Plecto giganteus*) harbours at least 58 viruses from 9 viral families (Anthony et al. 2013). Many highly pathogenic viruses to humans, such as Ebola virus, paramyxoviruses and SARs-Coronavirus, tend to persistently replicate in bats thus enabling them to transmit these viruses to livestock, domestic animal and humans (Wang et al. 2011). The viral richness and diversity of bats has prompted questions regarding the biological properties of bats and their suitability as reservoir hosts (Wang et al. 2011).

It has also been consistently observed that despite hosting a number of potentially lethal viruses, bats rarely succumb to any severe clinical disease (Baker et al. 2013). In fact, only rabies virus and Australian bat lyssavirus have been shown to severely affect bats although experimental results have been inconsistent (Mccall et al. 2000; Jordan et al. 2009). Compared to other mammalian models, little is known about bats and bat immunology (Papenfuss et al. 2012), mainly due to lack of funding and interest as bats were previously regarded as unimportant in the spread of viruses and diseases (Baker et al. 2013).

The observations of bats and their relationships with viruses have led to a growing interest in bat immunology and mechanisms that confer viral resistance, particularly to diseases that pose a threat to human health such as Ebola and Rabies.

1.2.3. *Bat host innate immune response to virus infection*

The observed asymptomatic nature of bats in response to viral infection has caused much speculation that bats have a ‘special’ relationship with viruses, due to a potentially superior immune system (Baker & Murcia 2014). Despite such speculations, as bats are the least studied of all mammalian taxa very little is known about bat immunology compared to other mammals (Papenfuss et al. 2012). Recent public availability of two bat genomes sequences in 2010, the large flying fox (*Pteropus vampyrus*), a megabat, and the little brown bat (*Myotis lucifugus*), a microbat, as part of project by the National Genome Research Institute, has opened up more opportunities to study the bat genome and provide new insights into potential mechanisms behind viral resistance. Since 2010, four more bat genomes have been sequenced by other groups: *Myotis brandtii*, *Rhinolopus sinicus*, *Eptesicus fuscus* and *Hipposideros armiger*.

There is much evidence to suggest bats possess a functioning innate immune system similar to other mammalian species. TLRs 1-10 have been identified in *Pteropus Alecto* which appear highly conserved with other mammalian species (Cowled et al. 2011). RIG-I and mda5 have also been characterised in *P. alecto* and shown to be functional (Cowled et al. 2012). The presence of the interferon system, an integral part of the innate immune system, has been characterised in several species of bats; type I IFNs have been described in the Egyptian rousette and the Malaysian flying fox (Kepler et al. 2010; Omatsu et al. 2008) whilst IFN- λ genes (type III IFNs), conserved with other mammalian IFN- λ sequences, have been characterised in *P. alecto* (Zhou et al. 2011). It has been suggested that type III IFNs in bats may be an alternative defensive mechanism induced during the suppression of type I IFNs during viral

infection (Zhou et al. 2011), although experimental results have been inconsistent (Virtue et al. 2011). A recent study into the immune gene repertoire of the *P. alecto* through transcriptome data sets revealed various innate immune genes such as PPRs, interferons, ISGs and natural killer cell receptors (Papenfuss et al. 2012). Consistent with Kepler *et al.* (2010) and Omatsu *et al.* (2008), type I IFN and its receptor were identified although type III IFNs were not. The transcriptome data set suggests the presence of similar ISGs in bats as in other species; it further revealed a number of genes orthologous to mammalian ISGs including Mx1, Mx2, OAS1, OAS2, OAS3, OAS-L, PKR, RNase L and ISG15 (Papenfuss et al. 2012). This is indicative of a comparable first line host defence to other mammals, triggered by viral infection. That being said, transcriptomic and genomic analysis indicate bats do not seem to possess important natural killer cell receptors and certain intracellular pattern recognition receptors, such as melanoma 2 (AIM2) a cytoplasmic receptor, responsible for recognising double-stranded DNA (Zhang et al. 2013).

A distinctive feature of the bat interferon system was the structure of the bat IFN- λ 1 gene as it contained a 4kb intron between exons 2 and 3 instead of the 1.1kb intron possessed by all other species examined in a study by Zhou et al. (2011). The relevance of this finding, in regards to viral immunity, has yet to be determined.

A theory exists to associate viral resistance in bats with their ability to fly. The theory postulates that as bats are the only mammals that possess the ability of flight, the evolved mechanisms to enable flight may also have driven selective pressure towards disease tolerance (O'Shea et al. 2014). Bats experience enormous metabolic changes and a great range of temperatures during flight. It is postulated that these changes increase the rate of immune response aiding in clearing the virus at the early stages of

infection (O'Shea et al. 2014). Compared to the metabolic changes which occur during flight, the costs associated viral infections are deemed negligible (O'Shea et al. 2014). In support of this, a number of immune genes have been found to be positively selected for in the common ancestor (Zhang et al. 2013). Of particular interest, is the positive selection of DNA damage-sensing and DNA repair genes and *c-REL*, vital member of NF- κ B family and regulator of type I and type III interferon response pathways (Zhang et al. 2013). It has been suggested that these genes are particularly favoured to in order to compensate for by products, such as reactive oxygen species (and additional DNA damage consequently) accumulated through increased metabolic rate required for flight. Other immune genes shown to be positively selected for in bats include those encoding TLR7, NOD, LRR and NLRP3 (Bean et al. 2013).

Wang et al. (2011) builds on the flight hypothesis by speculating that mitochondria efficiency may be responsible for the unique metabolic characteristics of bats, allowing for flight, body temperature range and longevity. In mouse and human cell lines, it has been recently recognised that mitochondria are involved in a number of signalling pathways including inflammation and innate immune responses suggesting a role in anti-viral activity, however, there are currently no published studies conducted in bats (Wang et al. 2011).

1.2.4. Bats as a potential reservoir for influenza A viruses

A high seroprevalence of influenza virus found in serum samples straw-coloured fruit bats (*Eidolon helvum*) in Ghana in 2009 and 2010 in conjunction with an antibody detection rate of 30% against HA-type H9 in a distantly related bat species from

another continent implicates bats are likely possible carriers of influenza virus (Freidl et al. 2015). Furthermore, as the bats appeared in a healthy condition during sample collection, this suggests bats may be asymptomatic carriers of the virus. Aquatic birds, the only postulated reservoir of influenza viruses, also do not present with symptoms. Interestingly, another feature of bats which has been likened to birds is tissue tropism of bats. In aquatic birds, virus replication occurs in the intestinal tract unlike in the majority of mammals whereby replication takes place in the respiratory tract. Virus replication has been suggested to also occur in the intestinal tract of bats as abundant quantities of genomic RNA have been found in intestine of bats (Mehle 2014).

Recently, isolation of two novel strains of influenza viruses in bats suggests that a sylvatic mammalian reservoir of influenza A viruses may exist in bats, challenging the notion of aquatic birds as the primary reservoir.

This followed the isolation of the A/Bat/Guat/09 (designated as ‘H17N10’) virus genome in yellow-shouldered bats (*Sturnira lilium*) in Guatemala (Tong et al. 2012), and the A/Bat/Peru/10 (designated as ‘H18N11’)-virus genome in the flat faced fruit-eating bat (*Artibeus planirostris*) in northern Peru, which were identified through next-generation sequencing (Tong et al. 2013).

The bat-derived H17 and H18, respectively, possess a 45% and 49.1% pairwise amino acid sequence identity to the other 16 HA molecules (Tong et al. 2012). This is comparable to the 49% similarity just between HA1-HA16. The amino acid sequences of both bat-derived HAs place them in group 1 of the HA molecules. The bat HA molecule sequences also possess conserved canonical sequence motifs such as the sialic acid receptor binding sites however the changes at certain positions in the sequence indicate a change in the binding specificity suggesting the HA molecules may have a different ligand preference than other HA molecules (Tong et al. 2012).

In accordance with the sequence data, *in vitro* experiments including surface plasmon resonance experiments, MDCK cell binding assays and glycan microarray assays have demonstrated that H17 and H18 are unable to bind to human or avian HA receptors due to the structural changes in the putative binding cavity, rendering it a ‘closed site’ (Wu et al. 2014). Thus it has been speculated that a different, likely protein-based receptor has been acquired (Wu et al. 2014). However, it was also noted that the detection methods used may not be sensitive enough to detect a very weak *in vitro* interaction and, perhaps, receptor clustering may enable a stronger interaction to occur *in vivo* (Wu et al. 2014).

The N10 and N11 sequences have much lower comparative pairwise amino acid sequence identity to influenza A and influenza B NA proteins (24% and 29.6% respectively) (Tong et al. 2013). Phylogenetically, these NA genes are more similar to the viral polymerase genes, PB1, PB2 and PA (Tong et al. 2013). The divergence of these amino acid sequences means they cannot be classified in the same groups as canonical influenza A virus NA molecules therefore they form their own a distinct group, proposed by Wu *et al.* (2014) as ‘group 3’. The poor conservation of residues at the sialic acid-binding site- the canonical catalytic active site, has resulted in a lack of sialidase activity when tested *in vitro* (Kowalinski et al. 2012; Zhu et al. 2012; Sun et al. 2013). Despite maintaining the same overall tetrameric structure as canonical NA molecules, the lack of neuraminidase function indicates these molecules should be regarded more as ‘NA-like’ proteins (Wu et al. 2014). It has been speculated that they may instead function as receptor-binding proteins due to their Ig-like folds (Wu et al. 2014).

Although bat-derived HA and NA molecules functionally differ from the canonical HA and NA of influenza A viruses, the reassortment between bat and human influenza A viruses is not an impossibility (Tong et al. 2013).

Phylogenetic analysis has shown that despite H17N10 being most closely related to H18N10, compared to the other influenza A virus strains, it forms a different lineage (Tong et al. 2013). The bat-derived viruses form a separate clade, and it is after this divergence from non-bat lineage that reassortment takes place, causing H18N10 to be part of a different lineage from H17N10. The separate bat lineage suggests that there is sustained transmission of influenza A viruses in bats (Tong et al. 2013). This is consistent with the high frequency of antibodies found in several species of bats widespread over the South American region indicating that influenza A viruses are well established in bats (Tong et al. 2013).

The phylogeny of bat-derived influenza A virus genomes in addition to the evidence of development of new virion entry mechanisms led Tong et al. (2013) to conclude that Influenza viruses have evolved in bats over an extended time period. Tong *et al.* (2013) hypothesised that the relationship between bats and influenza viruses is evolutionary similar to the postulated relationship between aquatic birds and influenza viruses. It is postulated that efficient transmission of multiple strains of influenza A viruses in aquatic birds is achieved through the faecal contamination of water and that a similar scenario may have occurred in bats (Tong et al. 2013).

1.3. Rationale and hypothesis

Bats have been found to harbour many viruses, a number of which are dangerous to humans, including Ebola virus, SARs-like coronavirus, rabies virus and Marburg virus without succumbing to severe disease. In light of the identification of H17N10 and H18N11 in *S. lilium* and *A. planirostris* and also the finding of 30% of *E. helvum* bats testing positive for avian H9 virus, it was hypothesised that bat cells are susceptible to conventional influenza A virus infection and are potential carriers of influenza viruses, constituting an alternative reservoir which has not been described previously due to asymptomaticity of bats to influenza of influenza viruses. The work carried out in this study aims to expand the current knowledge of the susceptibility and permissiveness of bat cells to influenza A virus infections and the potential ability of bats to transmit commonly circulating influenza A viruses. By examining the underlying innate immune mechanisms of resistance in infected bat cells, this will contribute to the current and limited knowledge of bat immunology.

Studying the innate immune response of bat cells to influenza virus infection could also provide a new approach for the development of much needed novel intervention therapies against influenza virus infection, as highlighted by the shortcomings and limitations of current influenza prevention and treatment options.

1.3.1. Aims and objectives

The aims and objectives of this study have been set out to try and address the hypothesis.

Aim 1

The first aim of this study is to characterise the host response of bat cells to conventional human and avian influenza A viruses.

Objectives

To achieve this aim, cells from several species of bat will be infected with endogenous and common circulating human and avian strains of influenza A virus to determine susceptibility, replicate permissiveness and robustness to influenza A virus infection. The key experiments to be undertaken to achieve this aim include determining viable progeny virus production using a focus-forming assay (FFA), quantifying virus gene expression by RT-qPCR and virus protein expression from infected bat cells by Western blot. Flow cytometry will be used to determine cell viability.

Aim 2

The second aim is to elucidate underlying mechanisms of viral susceptibility and permissiveness of bat cells by identifying major pathways involved in host innate immune resistance to influenza viruses.

Objectives

To achieve this aim, the JAK/STAT and NF- κ B pathways will be chemically inhibited in bat cells prior to influenza virus infection. Virus production will be quantified using FFA to determine the impact of JAK/STAT and NF- κ B inhibition.

2. Susceptibility and permissiveness of bat cells to influenza A virus infection

2.1. Chapter summary

Bats often harbour viruses of a highly pathogenic nature to humans without showing signs of severe clinical disease for reasons of which are currently known. The identification of two novel influenza-like viruses in bats, H17N10 and H18N11, has caused speculation that, in addition to the large repertoire of zoonotic viruses hosted, bats may serve as reservoir species to endogenous commonly circulating influenza viruses found in birds and mammals.

Epithelial cells derived from the lung of three species of bat, *Tadarida brasiliensis* (TB1-Lu), *Eidolon helvum* (E. helvum) and *Carollia perspicillata* (C. perspic) were infected with two human influenza viruses, USSR H1N1 and pdm H1N1 virus, and two low pathogenicity avian influenza (LPAI) viruses, H2N3 and H6N1 virus.

These findings found all three bat cell types were susceptible, to different degrees, to influenza virus infection but less susceptible than MDCK cells. All three bat cell types were also found to be more susceptible and replication permissive to avian than human influenza virus infections. Among the bat cell types, TB1-Lu cells were found to be the least susceptible and replication permissive to avian and human influenza virus infections suggesting the susceptibility and replication permissiveness to influenza virus infections may be species-specific.

2.2. Introduction

2.2.1. *Bats as a model organism*

Mouse models have provided much of our current knowledge of immune responses to bacterial and viral infections. This is due, in part, to similarities between mouse and human immune systems. Despite this, recent genomic studies have shown mice immune responses to several inflammatory diseases to diverge from human responses to the same diseases (Seok et al. 2013). Furthermore, there have been cases where clinical trials in humans have proven unsuccessful despite successful preclinical trails in mice (Benatar 2007; Tameris et al. 2013). Such issues have raised the need for the development of more appropriate animal models.

The observed relationship between bats and viruses has been described as ‘special’ relationship owing to the coexistence of viruses and bats without succumbing to severe clinical disease (Baker & Murcia 2014). This phenomenon makes bats a very useful model for studying innate immunity to virus infection.

Compared with other mammalian models, very little is known about bats. Dismissed historically as being unimportant in terms of being virus reservoirs or vectors, bats are among the least studied mammals (Baker et al. 2013). The existing knowledge of bats primarily consists of their ecology and physiology; bat immunology is largely unexplored (Kunz & Fenton 2003; Calisher et al. 2006).

Calisher et al. (200) reported that 390 species, over one-third of the 914 bat species catalogued in conservation lists, had not been assessed adequately. Furthermore, *Pteropodidae*, Old World fruit bats, which have been responsible for recent emerging viruses, made up 9.7% of these inadequately assessed species.

The current challenges surrounding the under studying of bat species are financial and ecological. Many of the countries affected by emerging zoonotic viruses suffer from a lack of resources, infrastructure, manpower and funding. Many bat species of interest are protected and cannot be kept or bred in captivity making it difficult to study host responses to viral infections and virus replication in bats (Eckerle et al. 2014). In many countries, studying and capturing wild bats for infection purposes is considered unethical (Eckerle et al. 2014) and poses a health risk as wild colonies carry diseases such as rabies (Calisher et al. 2006).

2.2.2. *Bat immune responses to virus infections*

This section reviews *in vivo* and *in vitro* experiments which have contributed to the current knowledge of the bat immune response to virus infections.

2.2.2.1. *Paramyxovirus*

Several studies have attempted to characterise bat immune responses to viral infection from viruses belonging to the *Paramyxoviridae* family. Hoffman et al. (2013) showed that several bat cell lines (RoNi/7, EpoNi/22.1, HypNi/1.1, RhiLu/1.1, CpLu and TB1-Lu), derived from different organs were susceptible to infection by Sendai virus and bovine respiratory syncytial virus.

The *Henipavirus* genus of the *Paramyxoviridae* family includes the highly pathogenic Nipah virus and Hendra virus. Bats act as natural reservoir to this family of viruses, while remaining asymptomatic, whereas in humans these viruses can prove fatal. In humans, Nipah virus has a mortality rate of 40-90% (Papenfuss et al. 2012). Virtue et

al. (2011) compared the innate response of both bat and human cells. IFN production in human epidermoid carcinoma cells and a range of cells derived from *P. alecto*, interferon production was antagonised by henipavirus; type I IFNs and type III IFNs were suppressed in bats. The lack of ISG induction also showed an inhibition of the IFN signalling pathway in bat. This inhibition was not reversed when treated with exogenous IFN, suggesting that lack of interferon was not responsible for absence of ISG induction (Virtue et al. 2011). This response contrasted with human cells where ISG induction was restored with exogenous ISG (Virtue et al. 2011). The suppression of both IFN production and IFN signalling was deemed as a universal and not cell-type specific response in bat cells. The findings show a remarkable difference between bat and human host innate response to henipavirus infection; the human response to henipavirus infection was shown to be controlled by the IFN pathway whereas the bat response was shown not to be controlled by the IFN pathway but a yet unidentified mechanism (Virtue et al. 2011).

Contrary to the results found by Virtue et al. (2011), Zhou et al. (2011) found differential expression of the type I IFNs and type III IFNs during infection of *P. alecto* splenocytes with Tioman virus, part of the *Paramyxoviridae* family closely related to Hendra and Nipah viruses. As with henipavirus infection, type I IFNs were not induced, however, IFN- λ 1 (type III IFN) was found to be upregulated during infection (Zhou et al. 2011). This contrasts with the down-regulation of type III IFNs found in response to henipavirus infection (Virtue et al. 2011). The differential expression pattern of type I and type III IFNs is unusual, as in other species the upregulation or induction of type I IFNs correlates with upregulation or induction of type III IFNs. It was hypothesised that this upregulation of type III IFNs may represent an alternative mechanism to control viral infection that is perhaps only implemented when type I

IFNs are suppressed (Zhou et al. 2011). Further experiments supported this hypothesis as supernatant from human 293T cells transfected with bat IFN- λ 2 was able to inhibit Pulau virus replication in infected PaFe05 cells in a dose-dependent manner and also induce antiviral activity through ISG induction; ISG56 and RIG-I were upregulated by 25-fold and 4-fold respectively.

The contrasting results between responses to henipavirus infection (Virtue et al. 2011) and Tioman virus (Zhou et al. 2011) shown the need to conduct more work to understand the role of type III IFNs in bats during viral infection.

2.2.2.2. Reovirus

Reoviruses are non-enveloped double-stranded RNA viruses which mainly infect the respiratory and gastrointestinal tracts of a range of hosts. Recently novel strains have been isolated in bats (reviewed by Sandekian et al. 2013).

The bat cell line, TB1-Lu, was infected with reovirus and compared with infected L929 cells (mouse fibroblasts), the classical model for reovirus replication (Sandekian et al. 2013). TB1-Lu cells were found to show resistance against the cytopathic effects of reovirus whereas L929 cells succumbed to cell death 3-5 days post-infection (Sandekian et al. 2013). In addition, persistent long-term infection of TB1-Lu cells with reovirus over a 2 month period did not hinder cell replication. This observed resistance was proposed to be attributed to a soluble antiviral factor such as IFN (Sandekian et al. 2013). In support of this hypothesis, the supernatant from reovirus-infected TB1-Lu cells seemed to confer protection against EMC virus as it decreased the EMC virus titre by more than 1000-fold whereas, supernatant from reovirus-infected L929 cells only reduced the titre by 4-fold (Sandekian et al. 2013).

2.2.2.3. *Ebola virus*

Ebola is one of the deadliest diseases known to humans, with a mortality rate of up to 90% (Kühl et al. 2011). It has been long reported that insectivorous bats and fruit bats are able to shed and continuously support Ebola virus replication without suffering any ill-effects implicating them as a reservoir for the virus (Swanepoel et al. 1996). Recent evidence in support of this includes the long-term survival (over thirteen months) of an seropositive bat (Olival & Hayman 2014).

Studies have shown that while bat cell lines, like TB1-Lu, were susceptible to Ebola virus infection, persistence was quickly established but little viral propagation (Strong et al. 2008). Persistence of Ebola virus was established as continued RNA production but suppression of RNA translation thus little viral propagation was expected (Strong et al. 2008).

A study conducted by Kühl et al. (2011) compared human cell lines with bat cell lines on the role of the Ebola glycoprotein (Ebola-GP) in viral resistance. Results of the study showed Ebola-GP functions as efficiently in bats as in humans. Ebola-GP was found to cleave efficiently, mediating viral entry, amplify virus particle budding and induce detachment of cells (Kühl et al. 2011). However, the interaction between in Ebola-GP and tetherin was found to differ between bat and human cells. Tetherin is an antiviral host cell factor that has been shown to prevent Ebola virus spread by inhibiting the release of virus particles (Kühl et al. 2011). The actions of tetherin have been shown to be counteracted by Ebola-GP. This study showed bat tetherin was more resistant to Ebola-GP antagonism than human tetherin potentially explaining the reduced viral propagation found in bat cells. Viral resistance to Ebola may be mediated, in part, by a similar mechanism as postulated in coronaviruses, whereby

viral pathogenicity has been linked to the interaction between the spike (S) protein and its receptor, ACE2 (Hoffmann et al. 2013).

2.2.2.4. *Lyssavirus*

Bats have been the source of much attention regarding lyssaviruses, as out of the 11 known lyssaviruses, they are the primary reservoir for 10 of these viruses including rabies virus (Turmelle et al. 2010). Bats are also the main source of rabies virus infections in humans (De Serres et al. 2008; Malerczyk et al. 2011). Rabies virus is a cause for concern as although a vaccine preventing rabies virus infection has been available for a number of decades, there is no treatment option available.

Despite reports of bat deaths from lyssaviruses and the isolation of live rabies virus from rabid bats (Jordan et al. 2009; McCall et al. 2000), several recent studies have suggested certain species may possess a level of innate immunity against rabies virus infection (Turmelle et al. 2010; Davis et al. 2013). Turmelle *et al.* (2010) found that multiple exposures to abortive rabies virus infections may have a protective effect in big brown bats (*Eptesicus fuscus*) by conferring immunological memory. In addition, a study undertaken by Davis *et al.* (2013) suggested that naïve bats (bats born in captivity) possess innate resistance against rabies virus as challenge with rabies virus resulted after inoculation correlated with a decrease in the development of rabies. Finally, the presence of rabies virus nucleoprotein and membrane glycoprotein antibodies in communities inhabiting the Peruvian Amazon with continuous exposure to bats suggests that abortive infection with rabies virus through vampire bat bites could confer immunity in humans against rabies virus infections (Gilbert et al. 2012).

2.2.2.5. *Coronavirus*

Infection by coronaviruses can cause gastroenteritis and affect the respiratory tract. Bats have been implicated as the principal source of alpha- and beta-coronaviruses (Vijaykrishna et al. 2007; Woo et al. 2009) and several theories have proposed the co-evolution of bats and coronavirus (Gouilh et al. 2011). Coronavirus RNA has been found in faecal samples derived from different species of bats, however, despite numerous attempts, no coronavirus has been successfully isolated from bats (as reviewed by Hoffmann et al. 2013).

The outbreak of SARS in 2003 and the identification of SARS-like coronavirus in horseshoe bats (Lau et al. 2005), in conjunction with evidence of SARS-coronavirus infection in healthy bats (Lau et al. 2005; Lau et al. 2010; Gouilh et al. 2011), prompted the relationship between bats and coronaviruses to be revisited.

Resistance to coronavirus infection has since been associated with the virus entry stage, as the absence of cell surface receptor for the coronavirus spike (S) protein prevents virus entry (Hoffmann et al. 2013). The role of the S protein is similar to that of influenza A virus HA protein; it mediates entry through recognition and binding to the cell surface receptor and internalisation through a fusion event. A number of bat cell lines were challenged with two coronaviruses, SARS-coronavirus and transmissible gastroenteritis coronavirus (TGEV) but were not susceptible to infection (Hoffmann et al. 2013). However, when transfected with the main receptor for SARS-coronavirus and TGEV, hACE2 and pAPN respectively, bat cells were readily infected (Hoffmann et al. 2013).

2.2.2.6. *Influenza A virus*

Only a small number of studies have been conducted on influenza A virus infection in bat cells. Hoffmann *et al.* (2013) was able to show that in the six bat cell lines tested, all cell lines were susceptible to the four strains of influenza A virus used (LPAI H7N7 and H9N2 and porcine H1N1 virus). It was also noted that the TB1-Lu and Rhi.Lu/1.1 cells, although still susceptible, were less susceptible than other bat cells lines (Hoffmann *et al.* 2013).

Similarly, another study demonstrated that TB1-Lu cells along with MyDauLu cells and BL5 cells were the least susceptible to infection of all the bat cell lines tested, with only 8% of cells infected. African fruit bats were found to be more susceptible to infection with R05T cells and EpoNi/22.1 cells infected at rates up to 100% (Poole *et al.* 2014). Using receptor destroying enzyme to remove host sialic acid receptors, this study also indicated TB1-Lu cells possess sialic acid receptors allowing for influenza virus binding and entry. Removal of receptors consequently inhibited influenza infection (Poole *et al.* 2014).

These results corroborated with a different study which focused on the susceptibility of *P. alecto* kidney (PaKi) cells to a number of strains of influenza A virus (A/WSN/33 H1N1, A/California/04/09 H1N1, A/Vietnam/1203/33 H5N1 and A/Swine/Illinois/02860/08 H1N1) (Dlugolenski *et al.* 2013). Dlugolenski *et al.* (2013) demonstrated that, not only were the cells able to support influenza A virus replication, but also allow the generation of two reassortment strains of the virus. Poole *et al.* (2014) showed bat cells were able to support the adaptation and evolution of influenza A viruses and investigated the capacity for reassortment of different virus strains in bat cells. While there was the potential for reassortment between strains, this was limited as RNA-dependent RNA polymerase (RdRP) activity only occurred with the

exchange of specific subunits in certain strains. Interestingly, the restriction of avian RdRPs in human cells was lessened when bat virus PA subunit was introduced in the reassortment.

In this chapter, bat cells derived from three bat species, *Tadarida brasiliensis* (TB1-Lu), *Eidolon helvum* and *Carollia perspicillata*, were subjected to influenza virus infection from two low pathogenicity avian influenza (LPAI) viruses, H6N1 and H2N3 virus, and two human viruses, USSR H1N1 and pandemic H1N1 2009 (pdm H1N1) to examine susceptibility and permissiveness of bat cells to virus infection by determining viable virus output, viral M-gene RNA expression and viral protein expression.

Cell line	Origin	Virus susceptibility	Comments	Reference(s)
RoNi	Epithelial cells from kidney of <i>Rousettus aegyptiacus</i>	Influenza virus Sendai virus Bovine respiratory syncytial virus SARS-coronavirus	Highly susceptible Highly susceptible Highly susceptible Infection restricted at level of cellular receptor	(Hoffmann et al. 2013) (Poole et al. 2014) (Hoffmann et al. 2013) (Hoffmann et al. 2013)
EpoNi	Epithelial cells from kidney of <i>Epomops buettikoferi</i>	Influenza virus Sendai virus Bovine respiratory syncytial virus SARS-Coronavirus	Highly susceptible Highly susceptible Highly susceptible Infection restricted at level of cellular receptor	(Hoffmann et al. 2013) (Poole et al. 2014) (Hoffmann et al. 2013) (Hoffmann et al. 2013) (Hoffmann et al. 2013)
HypNi	Epithelial cells from kidney of <i>Hypsignathus monstrosus</i>	Influenza virus Sendai virus Bovine respiratory syncytial virus SARS-coronavirus	Highly susceptible Highly susceptible Highly susceptible Infection restricted at level of cellular receptor	(Hoffmann et al. 2013) (Poole et al. 2014) (Hoffmann et al. 2013) (Hoffmann et al. 2013) (Hoffmann et al. 2013)
HypLu	Epithelial cells from lung of <i>Hypsignathus monstrosus</i>	Influenza virus	Highly susceptible	(Poole et al. 2014)
RhiLu	Epithelial cells from lung of <i>Rhinolopus alcyone</i>	Influenza virus Sendai virus Bovine respiratory syncytial virus SARS-Coronavirus	Less susceptible than other cell lines Highly susceptible Highly susceptible Unsusceptible- infection restricted at level of cellular receptor	(Hoffmann et al. 2013) (Hoffmann et al. 2013) (Hoffmann et al. 2013) (Hoffmann et al. 2013)
CpLu	Epithelial cells from lung of <i>Carollia perspicillata</i>	Influenza virus Sendai virus	Highly susceptible Highly susceptible	(Hoffmann et al. 2013) (Hoffmann et al. 2013)

		Bovine respiratory syncytial virus SARS-coronavirus	Highly susceptible Unsusceptible- infection restricted at level of cellular receptor	(Hoffmann et al. 2013) (Hoffmann et al. 2013)
TB1-Lu	Epithelial cells from lung of <i>Tadarida brasiliensis</i>	Reovirus Ebola virus Influenza virus Sendai virus Bovine respiratory syncytial virus SARS-Coronavirus Henipavirus	Resistance to cytopathic effects, viral persistence Persistence established, little viral propagation Less susceptible than other cell lines Highly susceptible Highly susceptible Infection restricted at level of cellular receptor Unsusceptible	(Sandekian et al. 2013) (Kühl et al. 2011) (Hoffmann et al. 2013) (Poole et al. 2014) (Hoffmann et al. 2013) (Hoffmann et al. 2013) (Hoffmann et al. 2013) (Virtue et al. 2011)
MyDau Lu	Epithelial cells from lung of <i>Myotis daubentonii</i>	Influenza virus	Less susceptible than other cell lines	(Poole et al. 2014)
BL5	Epithelial cells from lung of <i>Pipistrelle subflavas</i>	Influenza virus	Less susceptible than other cell lines	(Poole et al. 2014)
EidNi	Epithelial cells from kidney of <i>Eidolon helvum</i>	Influenza virus	Highly susceptible	(Poole et al. 2014)
PaKi	Epithelial cells from kidney of <i>Pteropus alecto</i>	Influenza virus	Susceptible and allows reassortment of virus strains	(Poole et al. 2014)(Dlugolenski et al. 2013)
	Splenocytes from <i>P. alecto</i>	Tioman virus	Differential expression of type I and type III IFNs (only type III upregulated)	(Zhou et al. 2011)

Table 2.2-1. Summary of bat cells infected/ challenged with different viruses and their responses

NB. 'Susceptibility refers to successful virus entry

2.3. Materials and methods

2.3.1. Cell lines

Madin-Darby canine kidney (MDCK) cells (ATCC-CL34) and human A549 cells and TB1-Lu cells (ATCC CCL-88), a commercial continuous epithelial cell line derived from the lung of *Tadarida brasiliensis*, were purchased from ATCC.

Immortalised lung epithelial cells derived from *Eidolon helvum* (*E. helvum*) and *Carollia perspicillata* (*C. perspic*) were kindly provided by Dr. Isabella Eckerle from Universitätsklinikum Bonn, Institute of Virology, Germany. These two cell lines were derived from the lung epithelium of an insectivorous South American microbat, *C. perspicillata*, and an African fruit bat, *E. helvum*. These two bat species reflect the two bat suborders, Yinpterochiroptera and Yangochiroptera. Both *E. helvum* and *C. perspicillata* have a broad distribution range and high population densities, properties relevant for zoonotic transmission of viruses (Eckerle et al. 2014). The isolation of these two bat cell lines have been detailed in Eckerle et al. (2014). Epithelial cells were chosen as the cell type for infection work because of their pivotal role in the viral infection process.

All cells were amplified from original vial, aliquoted and stored at -80 °C at passage 2, this was the stock used for all subsequent seeding.

Cell line	Origin	Purpose
MDCK	Epithelial cells from the kidney of dog	Comparison cell line for infection studies
A549	Epithelial cells from the lung of human	Comparison cell line for inhibitor (JAK/STAT, NF-κB) studies
TB1-Lu	Epithelial cells the lung of <i>Tadarida brasiliensis</i>	Infection studies, bat species-specific susceptibility and permissiveness
E. helvum	Epithelial cells from the lung of <i>Eidolon helvum</i>	
C. perspic	Epithelial cells from the lung of <i>Carollia perspicillata</i>	

Table 2.3-1 Summary of cell lines used in this study

2.3.2. Viruses

The strains of influenza A virus used in this study were low pathogenic avian influenza (LPAI) H2N3 virus (A/mallard duck/England/7277/06), LPAI H6N1 virus (A/turkey/England/198/09), human pandemic H1N1 2009 virus (A/California/07/2009(H1N1v)) and USSR H1N1 virus (A/USSR/77 [USSR]).

All viruses were propagated in 10-day old embryonated chicken eggs and 48 h post inoculation, the allantoic fluid was harvested. Subsequently, viruses were aliquoted and stored at -80°C until further use.

2.3.2.1. Virus titration

Virus titre was determined through immunodetection of viral NP on MDCK cells. MDCK cells were seeded at 90% confluency in 96-well plates and infected the

following day with 100 µl diluted virus in a serial dilution format and incubated at 37°C with 5% CO₂. Control samples were mock-infected with 1x phosphate buffered saline (PBS) (Invitrogen). 2 h post-infection (hpi), cells were washed three times with PBS and 100 µl fresh infection media was added to wells (see **Table 2.3-1**). Following this, cells were incubated for a further 4 h before being fixed in 1:1 methanol:acetone (cold) and subjected to immunocytochemical staining as described in 2.3.6. The volume of virus stock shown to result in 95-100% of MDCK cells positively stained for presence of viral nucleoprotein (NP) was established at an MOI 1.0.

2.3.3. *Maintenance of cell lines*

Cell lines were maintained in complete cell culture medium which was composed of Dulbecco's modified Eagle's medium (DMEM)-glutamax media (Invitrogen) with 10% fetal calf serum (FCS) (Invitrogen) and 1% penicillin-streptomycin (P/S) antibiotic (Invitrogen) at 37°C with 5% CO₂. Cells were passaged twice weekly using 1x trypsin (Invitrogen) for 5-10 min to detach cells. After cell detachment, an equal volume of complete cell culture medium was added to terminate the reaction. Cells were subsequently transferred into 50 ml tubes and centrifuged at 800 x g for 5 min at room temperature. After centrifugation, the supernatant was removed and the cells were re-suspended in PBS and seeded into T-75 flasks with fresh complete cell culture media at an appropriate concentration.

All cell lines were passaged a maximum of eight times after which they were discarded and were replaced with cells from the original stock (passage 2) stored at -80°C.

2.3.4. *Direct infection*

Cells were seeded into wells of either a 24-well or 96-well tissue culture plate in media for 90-100% confluency the following day. Confluent cells were infected at appropriate concentrations of virus in infection media (see **Table 2.3-1**) and incubated at 37°C with 5% CO₂. Medium was removed 2 hpi and replaced with fresh infection medium. At an appropriate time after infection, the supernatant was removed from the cells and stored at -80°C until further use. The cells were fixed in 1:1 methanol:acetone (cold) and subjected to immunocytochemical staining using DAKOenvision kit, as described in 2.3.6.

Type of Media	Complete cell culture media	Infection media
Medium	DMEM-glutamax (Invitrogen)	Ultraculture (Lonza)
Supplements	10% foetal calf serum (Invitrogen); 1% penicillin-streptomycin antibiotic (Invitrogen)	1% 2 mM glutamine (Invitrogen); 1% penicillin-streptomycin; 250 ng/ml tosyl phenylalanyl chloromethyl (TPCK) trypsin (Invitrogen)

Table 2.3-2 Media composition

2.3.5. Quantification of virus output

Confluent MDCK cells seed in either a 96-well plate or 24-well plate were infected by supernatants derived from direct infection assays (2.3.4). 2 hpi, supernatant was removed and the cells were washed with PBS. Subsequently, fresh infection media was added to the cells and incubated at 37°C for a further 4 h. The media was then removed, cells were washed twice with PBS and then fixed with cold 1:1 methanol : acetone. The cells were immunostained using the DAKOenvision kit and visualised through microscopy. Virus output was quantified according to Kuchipudi *et al.* (2012) focus forming assay (FFA).

The calculations are based on images captured at 100x microscope magnification and based on the following assumptions:

1. Fully confluent MDCK cells in a well (96-well plate) = 30 000
2. Number of MDCK cells in one microscopic field (as appear in captured image)
= 1000

$$\text{FFU } \mu\text{l} = \frac{30A}{X}$$

A = number of viral NP positive cells per captured field
X = μl of infectious supernatant

2.3.6. Immunocytochemical staining of viral nucleoprotein (NP)

Cells were washed and fixed in 1: 1 cold methanol:acetone (cold) for 10 min prior to staining using the Envision+ System-HRP (DAB) kit (Dako) according to manufacturer's instructions.

Fixed cells were pre-incubated with 1x Tris buffered saline (TBS) for 5 min and then blocked using a peroxidase block (Dako) for 5-10 min. Subsequently, cells were washed twice with 1x TBS and incubated with Influenza viral nucleoprotein (NP) antibody (Abcam), diluted at 1:8000 in 1x TBS, for 1 h. After incubation, cells were washed twice with TBS and incubated for 1 h with horse radish peroxidase (HRP)-labelled anti-mouse immunoglobulins. Cells were then washed twice with 1x TBS and incubated for 6-10 min with 3,3'-diaminobenzidin (DAB)-chromogen solution diluted in substrate buffer (1 drop of DAB solution per 1 ml buffer) (Dako). Finally, cells were washed thrice with 1x TBS before visualisation through microscopy.

2.3.7. *RNA extraction*

RNeasy Plus and QIAshredder kits (Qiagen) were used to extract RNA from cell lysates according to manufacturer's instructions. Quality of the extracted RNA and concentration was measured by Nanodrop ND1000 spectrophotometer (Thermo Scientific).

2.3.8. *cDNA conversion*

RNA was converted to cDNA using the Superscript III First Strand Synthesis kit (Invitrogen). Total RNA (1 µg) was incubated with 1 µl random primers (Invitrogen), 1 µl dNTPs (Invitrogen) and made up 13 µl with diethyl procarbonate (DEPC)-treated water (Invitrogen) at 65°C for 5 min and then placed on ice for 1 min. Subsequently, 4 µl First strand buffer (Invitrogen) was added with 1 µl DTT (Invitrogen), 1 µl RNase Out inhibitor (Invitrogen) and 1 µl Superscript III reverse transcriptase (Invitrogen) to

the mixture and incubated at room temperature for 5 min. This mix was then incubated at 50°C for 60 min. Finally, the mix was heated inactivated at 70°C for 5 min.

2.3.9. *Quantification of relative gene expression*

2.3.9.1. *TaqMan-based RT-qPCR*

For TaqMan based reactions, 10 µl LightCycler 480 probe mastermix (Roche) was mixed with 1 µl forward primer (10 µM stock) (Eurofins MWG Operon), 1 µl reverse primer (10 µM stock) (Eurofins MWG Operon), 0.2 µl probe (20 µM stock) (Sigma), 2.8 µl DEPC-treated water (Invitrogen) and 5 µl template cDNA at 1:100 or 1 : 50 dilution. **Table 2.3-2** shows the primers and probes used in this study. The PCR targets were USSR H1N1 M-gene and LPAI H2N3 M-gene (Baquero-Perez et al. 2015). The program used for TaqMan-based RT-qPCR reactions is stated in **Table 2.3-3**.

Genes	Primer/Probe	Sequence (5'-3')	Genbank Reference No.
18s rRNA	Forward	ACGGCTACCACATCCAAGGA	NR_003286.2 and AY265350.1
	Reverse	CCAATTACAGGGCCTCGAAA	
	Probe	CGCGCAAATTACCCACTCCCGA	
USSR	Forward	AGATGAGCCTTCTAACCGAGGTCG	M54941.1
H1N1 M	Reverse	TGCAAAAACATCTTCAAGTCTCTG	
	Probe	TCAGGCCCCCTCAAAGCCGA	
LPAI	Forward	AGATGAGTCTTCTAACCGAGGTCG	M63534.1
H2N3 M	Reverse	TGCAAAAACATCTTCAAGTCTCTG	
	Probe	TCAGGCCCCCTCAAAGCCGA	

Table 2.3-2. Primers and probes used

Step		Temperature (°C)	Time (s)
Initial denaturation		95	600
Melting	Denaturation	95	15
	Annealing	55	30
	Extension	72	1
Cooling		4	Hold

Table 2.3-4. Program for TaqMan-based RT-qPCR reactions

2.3.9.2. Normalisation of RT-qPCR

The “housekeeping” gene *18s rRNA* was chosen as the internal reference gene for normalisation and normalised Ct values were calculated using a standard curve (Figure 2.3-1).

To construct the standard curve, cDNA was pooled from all samples (uninfected and infected) and diluted several times. Final dilutions were 1:25, 1:50, 1:100, 1:200, 1:400 and 1:800. The relative concentration of the cDNA of interest and housekeeping gene was calculated through interpolation of the threshold value (Cq) of the standard curve. The gene of interest was subsequently normalised by dividing the concentration by the housekeeping gene concentration (Table 2.3-4).

LightCycler 96 SW 1.1 software was used to analyse the data.

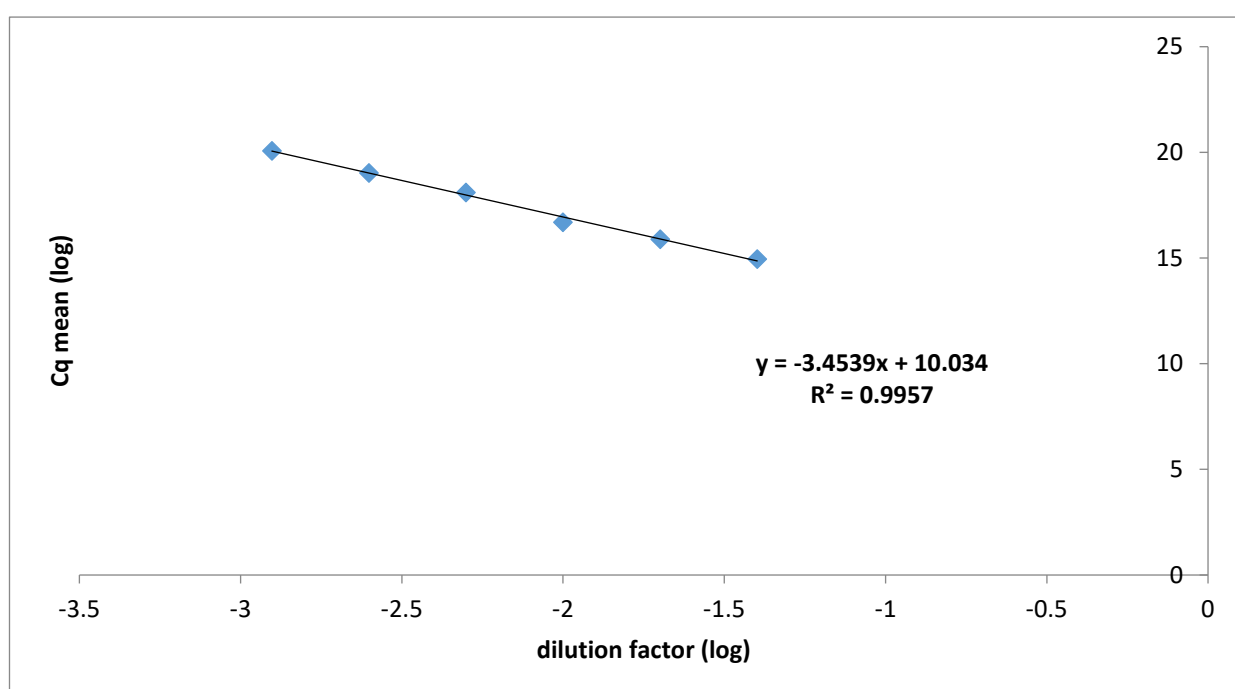


Figure 2.3-1. Standard curve for housekeeping gene *18s rRNA* expression

Slope of standard curve indicates PCR efficiency. As efficiency = $10^{(-1/\text{slope})} - 1$, a slope between -3.6 and -3.3 indicates an efficiency of 90-100%. R^2 value is another parameter to evaluate PCR efficiency. An R^2 of 1 indicates very good confidence.

Sample	USSR H1N1 M Ct	USSR H1N1 M Log Concentration	Anti-log USSR H1N1 M	Average 18sRNA standardised Ct value	M-gene USSR Ct/18sRNA ct	Average Normalised Ct
1	27.94	-2.367635149	0.004289087	0.001348686	3.180196531	3.049130268
1	28.25	-2.446092326	0.003580203	0.001348686	2.654585891	
1	27.87	-2.349919012	0.004467669	0.001348686	3.312608381	
2	24.45	-1.484359182	0.032782406	0.016096467	2.036621141	2.040588867
2	24.44	-1.481828305	0.032974005	0.016096467	2.04852432	
2	24.45	-1.484359182	0.032782406	0.016096467	2.036621141	
3	34.81	-4.106347439	7.82803E-05	0.003535728	0.0221398	0.024388541
3	35.7	-4.331595465	4.6602E-05	0.003535728	0.013180312	
3	33.89	-3.873506783	0.000133811	0.003535728	0.03784551	

Table 2.3-5 Example of normalisation of RT-qPCR results for gene of interest (USSR H1N1 M-gene) using 18s rRNA as internal reference gene

2.3.10. *Western blotting*

2.3.10.1. *Protein lysates preparation*

Infected cells were harvested from 12-well plates by lysing with 100 µl Radio-Immunoprecipitation Assay (RIPA) buffer (Santa Cruz Biotechnology) supplemented with 1% phenylmethylsulfonyl fluoride (PMSF) (Santa Cruz Biotechnology), 1% cocktail inhibitor and 1% sodium orthovanadate (Santa Cruz Biotechnology) per well. Using cell scrapers, lysates were transferred into a microcentrifuge tube, incubated for 30 min on ice and then centrifuged at 13 000 x g at 4°C for 10 min. Supernatant was removed and stored at -20°C until further use.

2.3.10.2. *Protein quantification*

Protein concentrations were determined using the Coomassie Plus the Better Bradford™ Assay Kit (Pierce) according to manufacturer's instructions. Briefly, a set protein standards ranging from 0 µg/ml – 2000 µg/ml were mixed and incubated with Coomassie Plus Reagent solution (Pierce) in a 96-well microtitre plate and the absorbance was measured at 595 nm. These readings were used to generate a standard curve to determine unknown concentrations of samples by extrapolation.

2.3.10.3. *Gel electrophoresis*

Protein (10 µg) was mixed with 5 µl NuPAGE lithium dodecyl sulphate (LDS) sample buffer (Invitrogen) and 2 µl NuPAGE sample reducing agent (Invitrogen). Water was

used to make up a final volume of 15 µl. The mixture was denatured by heating at 70°C for 10 min. Denatured proteins were separated according to their molecular weight by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using pre-cast 4-12% Bis-tris protein gels (Invitrogen) . The protein samples were loaded into the wells of the gel along with 5 µl HiMark pre-stain protein standard (Invitrogen). The gel was set up in a XCell SureLock Mini-Cell (Invitrogen) containing running buffer. The running buffer used 1x NuPAGE MOPS sodium dodecyl sulphate (SDS) Running buffer (Invitrogen) supplemented with NuPAGE antioxidant (Invitrogen). Samples were run at 200 V for 50 min. Subsequently, the gel was removed and immersed in cathode buffer for 5 min, see Table 2.3-5.

2.3.10.4. *PVDF membrane transfer*

Hybond-P polyvinylidene fluoride (PVDF) transfer membrane (Amersham) was rehydrated in methanol for 10 sec and subsequently rinsed in deionised water for 5 min. Next, one sheet of whatman filter paper, cut to appropriate size, was soaked in anode buffer 1, two sheets of filter paper in anode buffer 2 and three sheets of filter paper in cathode buffer (see Table 2.3-5). The filter paper soaked in anode buffer 1 was placed on a Trans-Blot[®] semi-dry transfer cell (Bio-Rad) and flattened ensuring all air bubbles were removed. This was followed by placing the two sheets of filter paper soaked in anode buffer 2 on top of the first filter paper. The PVDF membrane was placed next, followed by the gel and finally the three sheets of filter paper soaked in cathode buffer. The semi-dry transfer cell was assembled and run for 70 mA for 1 h. Once completed, the gel and filter paper was discarded and the PVDF membrane

was immersed in 1x Tris-buffered saline and Tween 20 (TBS-tween) and then washed 2-3 times with deionised water.

Buffer (1L)	Components
Anode buffer 1	36.34 g Tris 10% methanol
Anode buffer 2	3.03 g Tris 10% methanol
Cathode buffer	3.03 g Tris 3.003 g Glycine 10% methanol

Table 2.3-6 Buffers used for PVDF membrane transfer

2.3.10.5. *Antibody staining*

The PVDF membrane was blocked with 5% non-fat milk (Marvel) in TBS-tween for 1 h at room temperature prior to the addition of primary antibody. The primary antibody was added to the blocking solution at the appropriate concentration and the membrane was incubated overnight at 4°C in the dark. The list of primary antibodies used is stated in **Table 2.3-6**. Following this, PVDF membrane was washed three times for a minimum of 30 min in TBS-tween at room temperature. Secondary HRP-conjugated antibody was then added in 5% non-fat milk (Marvel) TBS-tween at an appropriate concentration for 1 h and subsequently washed three times in TBS-tween for 30 min. A list of secondary antibodies used is stated in **Table 2.3-7**. Finally, chemiluminenscent substrate, ECL Prime (Amersham), was applied to the PVDF membrane according to manufacturer's instructions. PVDF membrane was sealed in saran wrap and then exposed to X-Ray film in the dark.

Antibody	Product No.	Origin	Dilution	Predicted size	Supplier
PB1	SC-17601	Goat	1:10000	83 kDa	Santa Cruz
Beta-actin	A5316	Mouse	1:10000	42 kDa	Sigma
NP	PA5-32242	Rabbit	1:3000	56 kDa	Pierce
M1	ab20910	Goat	1:2000	27 kDa	Abcam

Table 2.3-6. Primary antibodies used for Western blot

Antibody	Product No.	Origin	Dilution	Supplier
Goat IgG	sc-2020	Donkey	1:10000	Santa Cruz
Mouse IgG	HAF007	Goat	1 to 1000	R&D systems
Rabbit IgG	P044802-1	Goat	1:100 000	Dako

Table 2.3-8. Secondary antibodies used for Western blot

2.3.10.6. Quantification of protein expression

To quantify protein expression from Western blotting, images were subjected to densitometry analysis using Image J software (version 1.48). Density of individual protein bands were normalised against corresponding density of internal reference gene, β -actin.

2.3.11. Statistical analysis

Student t-test was performed to compare the means between two groups. One-way ANOVA was conducted to compare the means between more than two groups. If more than one factor was involved, a two-way ANOVA was performed. Experiments were performed with a minimum of three biological replicates and four technical replicates and error bars represent standard deviation to show the variation of individual samples

from the sample mean. Stars (*) were used to denote statistical significance as shown below. All statistical analysis was conducted using Graphpad Prism 7.

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$
**** $P \leq 0.0001$

2.4. Results

2.4.1. *Phenotypic response of bat cells to influenza A virus infections*

MDCK cells and three bat cell types, TB1-Lu, E. helvum and C.perspic cells, were infected with two strains of human viruses, USSR H1N1 and pdm H1N1 virus and two strains of avian viruses, H2N3 and H6N1 virus, at MOI 1.0, as determined by focus forming assays (FFA) for 6 h, 12 h and 24 h.

Viral nucleoprotein (NP) was detected in the nuclei of all MDCK cells 6 h post-infection (hpi). By 12 hpi, viral NP had spread into the cell cytoplasm and 24 hpi., MDCK cells were enlarged and many cells had detached from the surface of the cell culture plate. This pattern of infection was consistent for MDCK cells infected with each of the four viruses (**Figure 2.4-1A**).

Contrastingly, TB1-Lu cells were not efficiently infected by any virus. 6 hpi., viral NP was detected in only a small proportion of cell nuclei (**Figure 2.4-1B**). Furthermore, viral NP did not spread into the cell cytoplasm, as found in MDCK cells, and there was no cell detachment even at 24 hpi. The majority of TB1-Lu cells appeared uninfected with all four viruses (**Figure 2.4-1B**). TB1-Lu cells appeared unchanged even at 72 hpi (data not shown).

Viral NP was detected in only a small fraction of *E. helvum* cell nuclei at 6 hpi with human USSR H1N1 and pdm H1N1 viruses. (**Figure 2.4-1C**). Viral NP remained localised in the cell nuclei 24 hpi, akin to the distribution pattern found in TB1-Lu cells. Unlike TB1-Lu cells, a high proportion of *E. helvum* cells infected with LPAI H2N3 and H6N1 viruses were positively stained for viral NP 6 hpi. By 24 hpi., viral NP had spread into the cell cytoplasm and widespread cell detachment was evident.

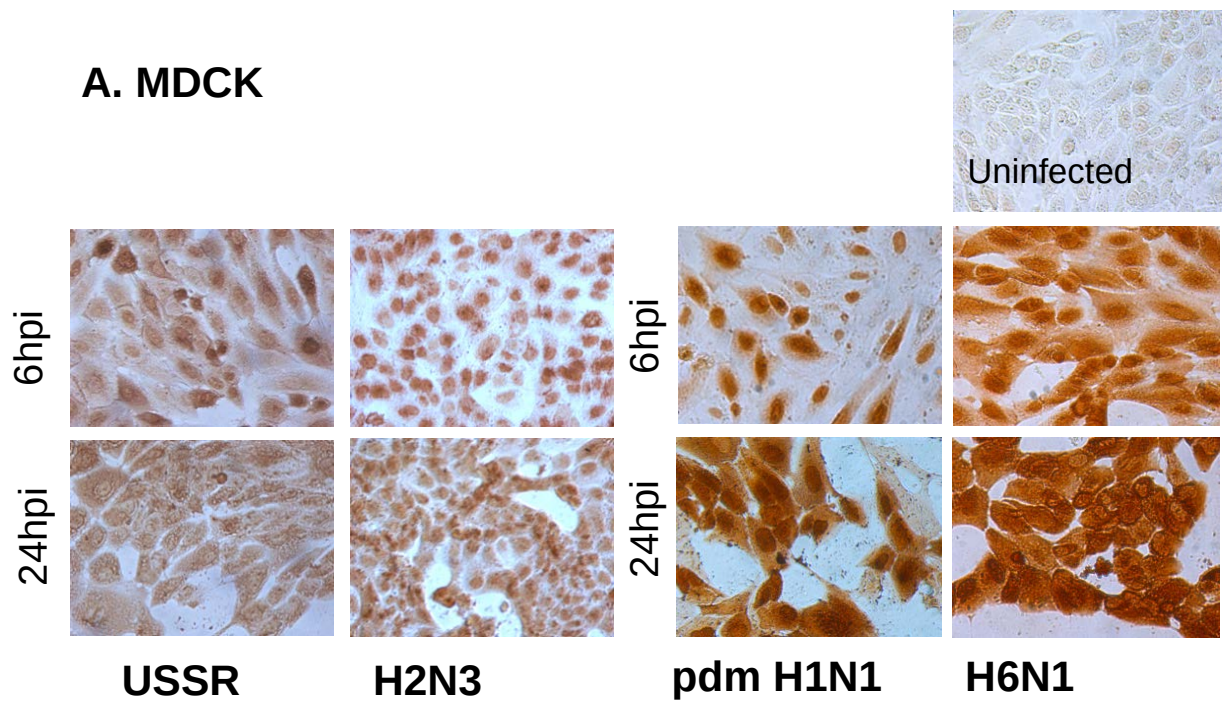
C. perspic cells readily infected with LPAI viruses but not human viruses. Viral NP was detected in most cell nuclei at 6 hpi. with H2N3 and H6N1 viruses (**Figure 2.4-1D**). Viral NP was widespread in the cell cytoplasm and most cells had detached from the cell culture surface 24 hpi. In contrast, the majority of *C. perspic* cells were uninfected when subjected to human virus infections. In the small fraction of cells infected with human viruses, viral NP remained intranuclear and there was no evidence of cell detached 24 hpi.

		USSR H1N1	Pdm H1N1	LPAI H2N3	LPAI H6N1
MDCK	6 h	< 90% nuclei infected	< 90% nuclei infected	< 90% nuclei infected	< 90% nuclei infected
	24 h	Cytoplasm infected Cell detachment	Cytoplasm infected Cell detachment	Cytoplasm infected Cell detachment	Cytoplasm infected Cell detachment
TB1-Lu	6 h	> 10% nuclei infected	> 10% nuclei infected	> 10% nuclei infected	> 10% nuclei infected
	24 h	> 10% nuclei infected No cell detachment	> 10% nuclei infected No cell detachment	> 10% nuclei infected No cell detachment	> 10% nuclei infected No cell detachment
<i>E. helvum</i>	6 h	> 10% nuclei infected	> 10% nuclei infected	< 90% nuclei infected	< 90% nuclei infected
	24 h	> 10% nuclei infected No cell detachment	> 10% nuclei infected No cell detachment	Cytoplasm infected Cell detachment	Cytoplasm infected Cell detachment
<i>C. perspic</i>	6 h	> 10% nuclei infected	> 10% nuclei infected	< 90% nuclei infected	< 90% nuclei infected
	24 h	> 10% nuclei infected No cell detachment	> 10% nuclei infected No cell detachment	Cytoplasm infected Cell detachment	Cytoplasm infected Cell detachment

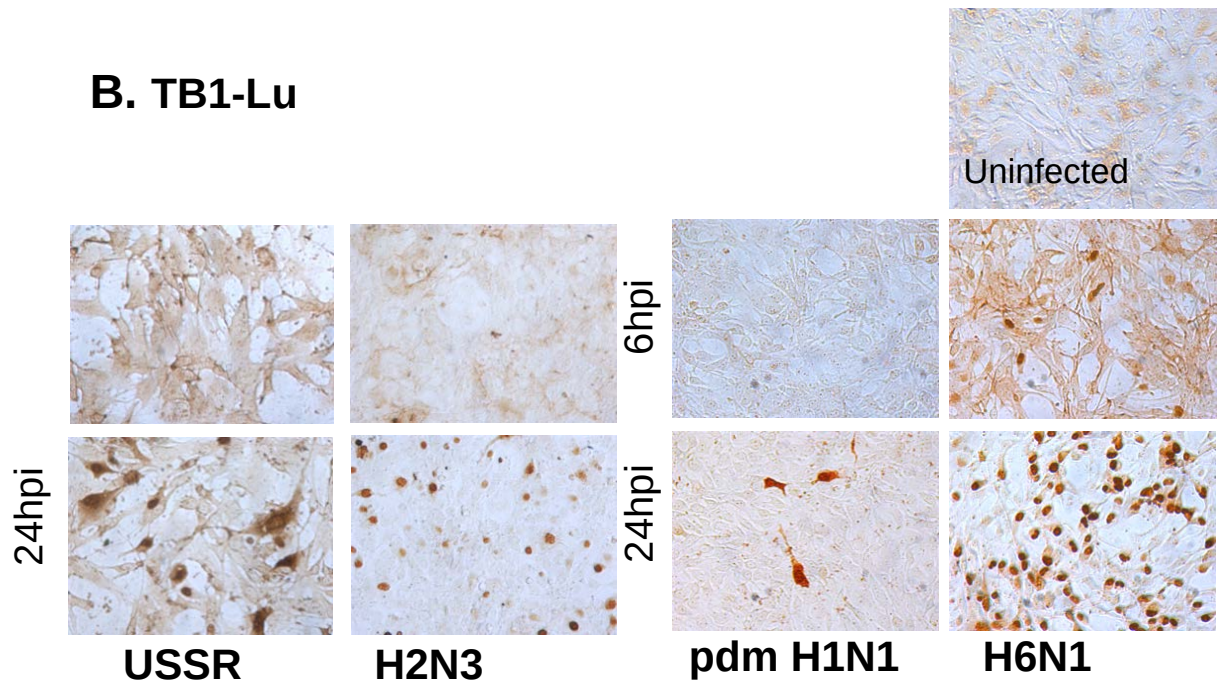
Table 2.4-1 Summary of phenotypic responses of MDCK, TB1-Lu, *E. helvum* and *C. perspic* cells to influenza A virus infections as shown in Figure 2.4-1.

Viruses were isolated and propagated in embryonated chicken eggs and subsequently titrated on MDCK cells, for further detail see...

A. MDCK



B. TB1-Lu



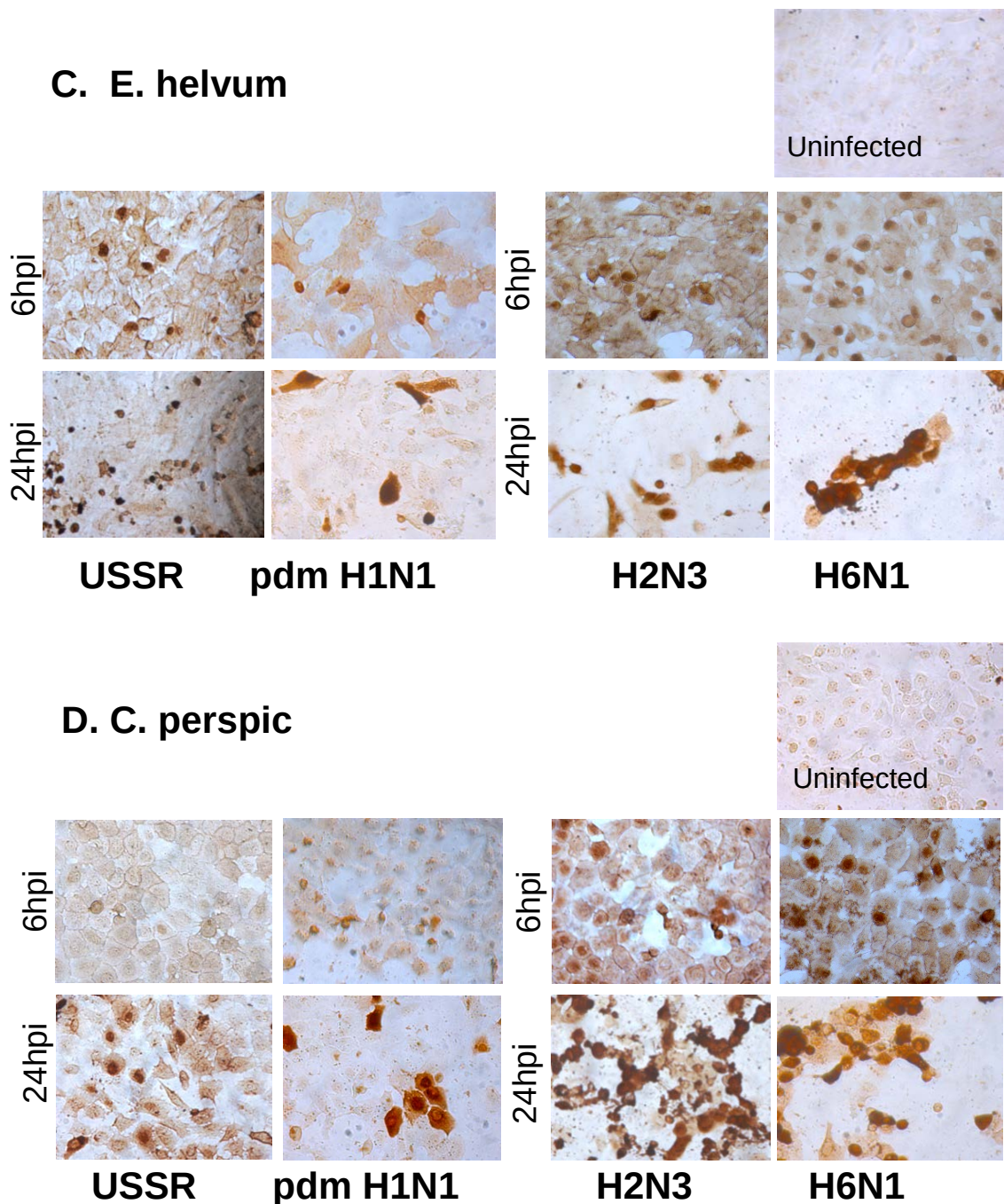


Figure 2.4-1 Bat respiratory epithelial cells were less susceptible than MDCK cells to influenza virus infections but showed differential susceptibility between human and avian influenza viruses.

TB1-Lu, *E. helvum*, *C. perspic* and control MDCK cells were separately infected with human (USSR H1N1 and pdm H1N1) and avian (H2N3 and H6N1) viruses at 1.0 MOI (titres based on 6h focus forming assays on MDCK cells) for 6h and 24h, and immunostained (brown) for viral NP. Expectedly, MDCK cells were extensively infected showing nuclear localisation of NP at 6hpi., and widespread cytoplasmic spread of NP at 24hpi. with each virus (A). TB1-Lu cells, by contrast, were not readily infected by human or avian viruses; even at 24hpi. only a limited number of cells showed intranuclear NP localisation (B). *E. helvum* and *C. perspic* showed differential infection susceptibility between human and avian viruses. Cells from the two bat species, like TB1-Lu cells, were not readily infected with human viruses (USSR H1N1 or pdm H1N1 virus) such that relatively few cells showed intranuclear NP localisation at 24hpi. (C and D). However, *E. helvum* and *C. perspic* cells were extensively infected by 6hpi. with avian viruses (H2N3 or H6N1 virus), and by 24hpi. exhibited extensive cell loss and cytoplasmic spread of NP (C and D).

2.4.2. *Influenza A virus output from infected bat cells*

Virus output is defined as progeny virus released in the supernatant of infected cells based on infection culture on confluent MDCK cells for 6 h and immunostained for viral NP (see 2.3.5).

Infected MDCK cells produced the highest virus output compared with bat cells, regardless of the virus used. Bat cells infected with human viruses produced less virus progeny (**Figure 2.4-2A and C**) than those infected with avian viruses (**Figure 2.4-2B and D**). Among bat cell types, infected TB1-Lu cells produced the lowest virus output; TB1-Lu cells infected with USSR H1N1 virus and pdm H1N1 virus produced less than 20% progeny virus compared with MDCK cells infected with respective viruses. E. helvum cells, infected with pdm H1N1 and H2N3 virus, produced the highest virus output of the bat cell types and C. perspic cells produced the highest virus output among bat cell types when infected with H6N1 and USSR H1N1 virus.

Despite marked variation in progeny virus produced by infected bat cells, virus output was significantly lower than from counterpart MDCK cells infected with the same virus.

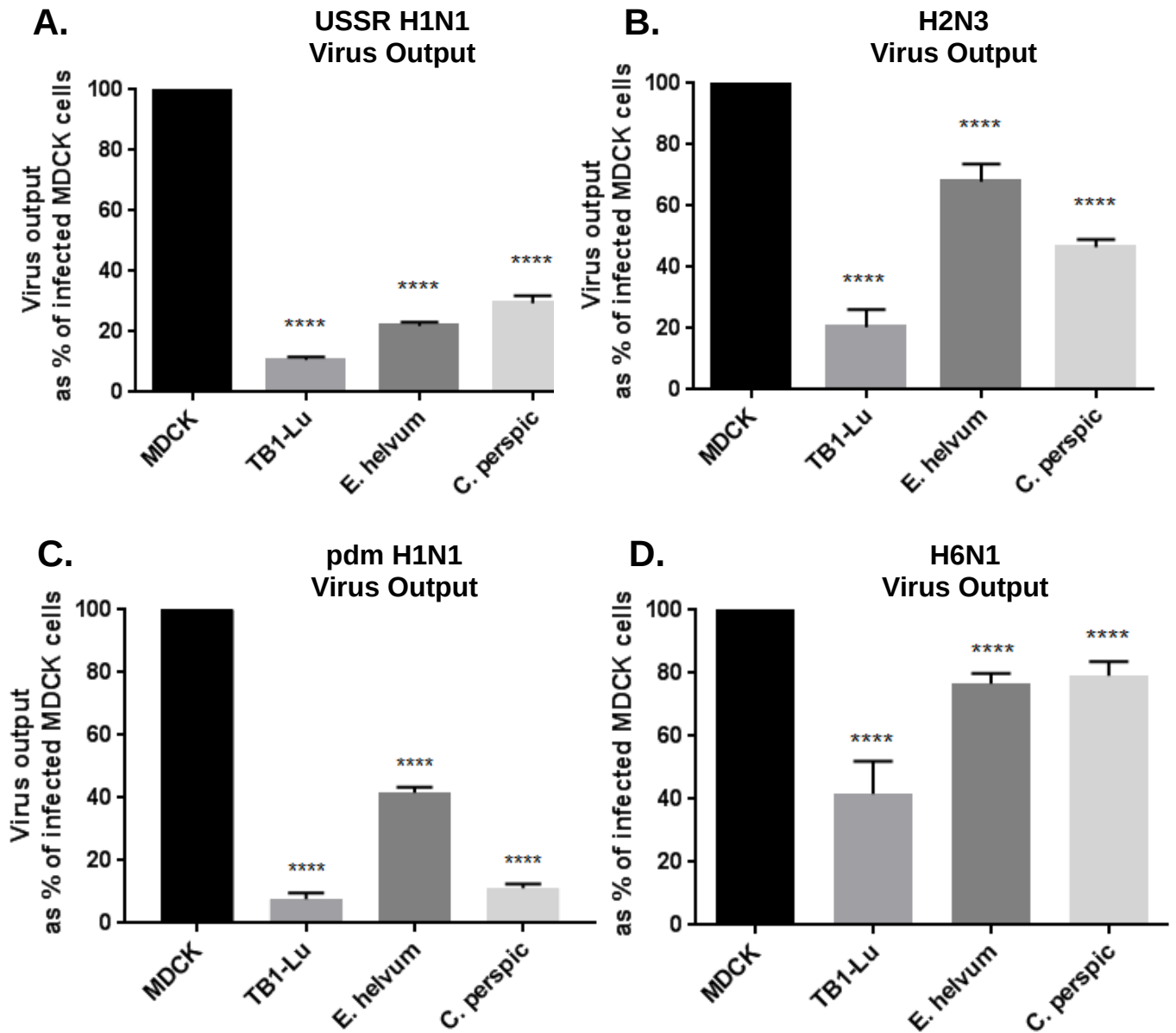


Figure 2.4-2 Infected bat cells produced significantly less progeny influenza viruses than corresponding MDCK cells.

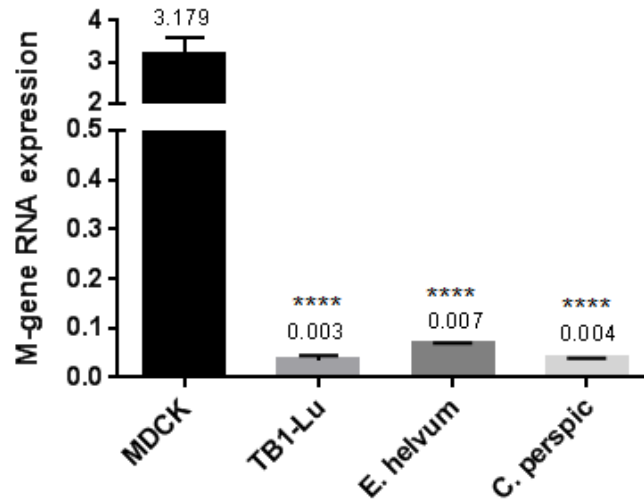
Cells of all three bat species (TB1-Lu, E. helvum and C. perspic) and MDCK cells were infected with USSR H1N1 or avian H2N3 virus at 0.5 MOI for 24h. (A and B) Supernatants of infected bat and MDCK cells were titrated on MDCK cells in 6h focus forming assays (FFA) to quantify progeny virus release. Infected bat cells of all three species produced significantly less (**** $P \leq 0.0001$) viable virus than correspondingly infected MDCK cells. Furthermore, more progeny avian H2N3 virus than USSR H1N1 virus was produced from each species of bat cells. TB1-Lu cells released the least progeny virus of the three bat species. (C and D) Supernatants from mammalian pdm H1N1 virus infected bat cells also produced significantly less (**** $P \leq 0.0001$) viable progeny compared to supernatants from avian H6N1 virus infected bat cells. It should be noted that propagation of viruses in bat cells may yield contrasting results from those achieved in these experiments. Results are representative of three combined biological replicates and six technical replicates for each biological repeat. Typical virus output from MDCK cells is 200 ffu/ μ l.

2.4.3. *Influenza virus M-gene RNA expression in infected bat cells*

Viral M-gene RNA expression was quantified in infected cells by RT-qPCR (see 2.3.9). M-gene RNA expression was significantly lower in all bat cell types compared with MDCK cells (**Figure 2.4-3**). Comparing M-gene RNA expression between bat cell types, the lowest expression was exhibited by TB1-Lu cells infected with H2N3 virus and USSR H1N1 virus. M-gene RNA expression in E. helvum and C. perspic cells was comparable to M-gene RNA expression in TB1-Lu cells infected with USSR H1N1 virus (**Figure 2.4-3A**) but notably higher when infected with avian H2N3 virus (**Figure 2.4-3B**). The pattern of M-gene RNA expression was remarkably similar to pattern of virus output among infected bat cells relative to MDCK cells (**Figure 2.4-2**).

A.

USSR H1N1
M-gene expression



B.

H2N3
M-gene expression

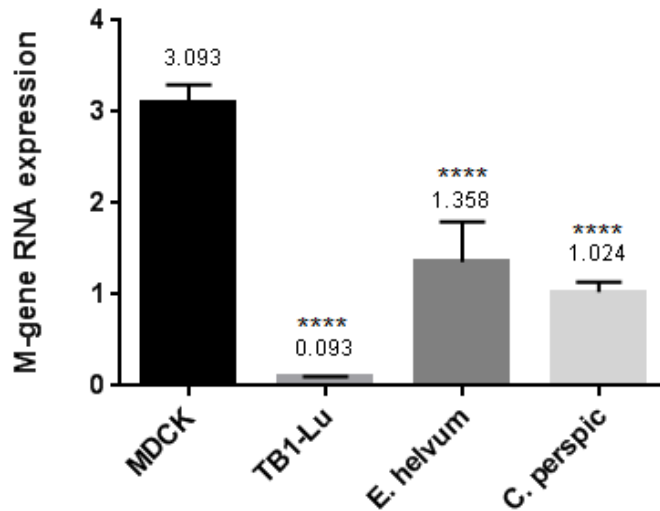


Figure 2.4-3 Infected bat cells produced significantly less viral M-gene RNA and progeny influenza viruses than corresponding MDCK cells.

Cells of all three bat species (TB1-Lu, E. helvum and C. perspic) and MDCK cells were infected with USSR H1N1 or avian H2N3 virus at 0.5 MOI for 24h. (A and B) Extracted total RNAs were quantified for viral M-gene expression normalised to 18s rRNA. Cells of all three bat species produced significantly (**** $P \leq 0.0001$) less viral M-gene RNA than MDCK cells for each virus. Each species of bat cells also generated more M-gene RNA from avian H2N3 virus than from USSR H1N1 virus infection. Results are representative of three biological replicates and six technical replicates for each biological repeat.

2.4.4. *Infected bat cells differentially expressed viral proteins*

Western blotting was carried out to characterise viral protein expression in the three species of bat cells infected with LPAI H23 or human USSR H1N1 virus (Figure 2.4-4). There was wide variation in viral protein production between infected cells. With each virus infection, all three viral proteins (PB1, NP and M1) were clearly expressed in MDCK and E. helvum cells. TB1-Lu cells exhibited the weakest viral protein expression for each virus. TB1-Lu cells displayed weak expression when infected with human USSR H1N1 virus (Figure 2.4-4B) and only NP was strongly expressed when infected with H2N3 virus (Figure 2.4-4A). C. perspic cells displayed an intermediate pattern of viral protein expression that was between that of corresponding E. helvum and TB1-Lu cells. Densitometry analysis of the Western blot bands using Image J confirmed the visual interpretation of the blots as it showed E. helvum and MDCK cells to have comparably high expression all three viral proteins, particularly PB1 and M1 when infected with both viruses. Unsurprisingly, TB1-Lu had the lowest relative band density of all cells.

C. perspic clearly expressed M1 and NP protein when separately infected with USSR H1N1 and LPAI HN3 virus. After extended exposure, PB1 protein was weakly expressed when infected with USSR H1N1 virus (Figure 2.4-4B) and protein expression could be detected in cells infected with H2N3 virus after extended exposure (Figure 2.4-4A).

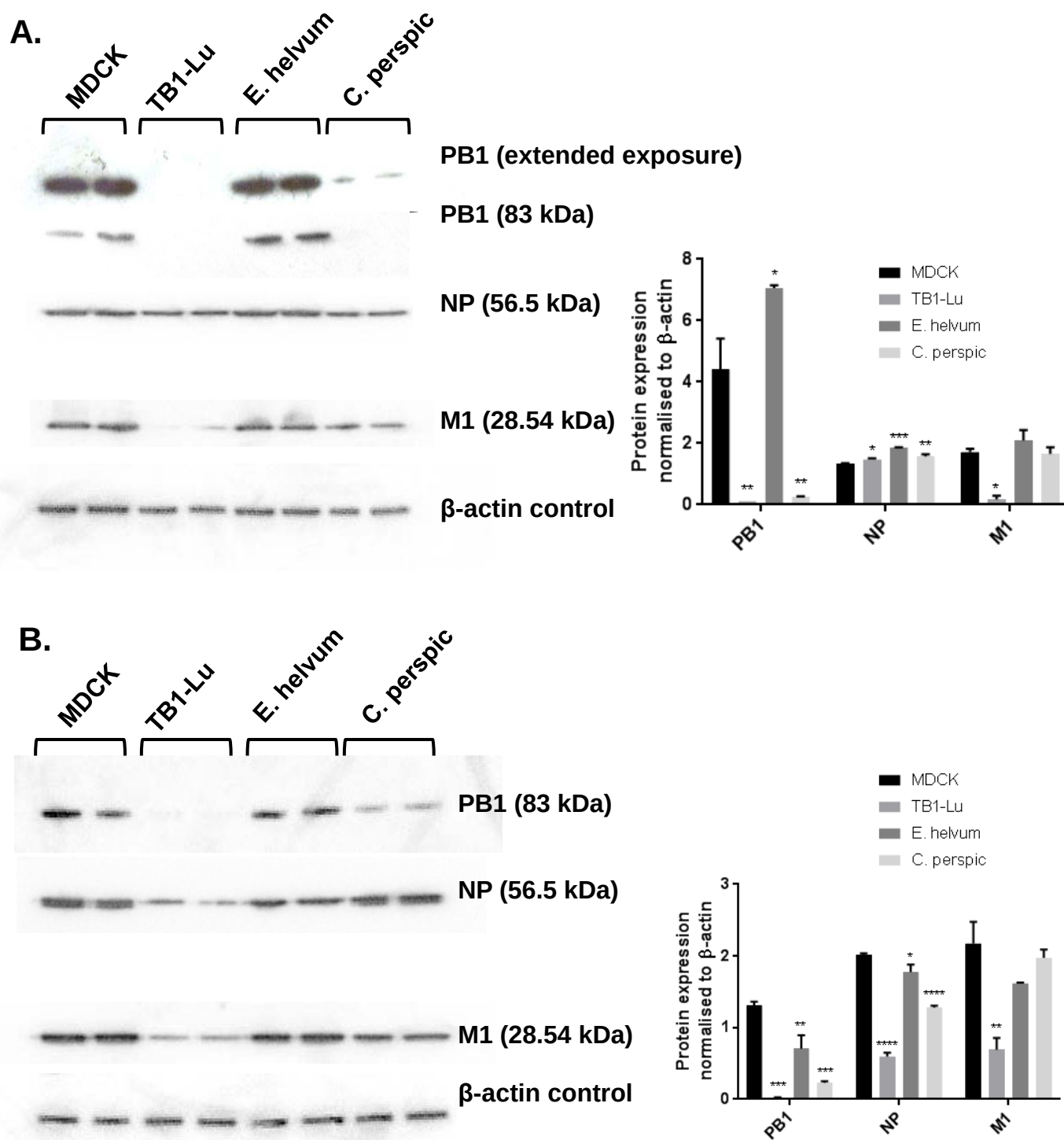


Figure 2.4-4 Variation in viral protein production between infected bat cells.

All three species of bat cells and control MDCK cells were infected with avian H2N3 (A) or USSR (B) at 0.5 MOI for 24h for the detection of viral PB1, NP and M1, with β -actin as loading control. Differential viral protein expression of PB1, NP and M1 was evident between the three bat cell types for each virus. Of the three bat species, *E. helvum* was most consistent and prominent in viral protein expression, in particular from avian H2N3 virus infection. *C. perspic* cells strongly expressed M1 and NP but showed weak expression of PB1. Infected TB1-lu cells showed the weakest viral protein expression for each virus.

2.5. Discussion

The well observed ‘special relationship’ between bats and viruses (Baker & Murcia 2014) enables bats to host many highly pathogenic viruses to other mammals while rarely succumbing to clinical disease (Baker et al. 2013). While this is a well observed phenomenon, mechanisms underlying this antiviral resistance are largely unknown.

The identification of two novel influenza-like viruses, H17N10 and H18N11 viruses, indicate that bats may also be a potential mammalian reservoir host for at least native bat influenza viruses. Further evidence to suggest bats may be asymptomatic carriers of influenza viruses is the recent discovery that around 30% of free ranging *Eidolon helvum* bats in Ghana were serologically positive for avian H9 virus while remaining clinically healthy (Freidl et al. 2015). This finding may not only be epidemiologically significant but also have implications to public health. The unusual biological properties of bats make them a valuable model organism to study innate immunity to viruses.

In this study, we used bat epithelial cells derived from the lung of three bat species, *Tadarida brasiliensis* (TB1-Lu), *Eidolon helvum* (E. helvum) and *Carollia perspicillata* (C. perspic). Currently there is only one cell line, TB1-Lu, derived from bat respiratory tract commercially available. Unfortunately, no additional information is available on its cell type or origin. As the first layer of cells to interact with the virus, the epithelium serves as a physical barrier which the virus is required infect for virus entry. Classically, the epithelium was considered as a purely physical barrier functioning to regulate water and ion transport (Proud & Leigh 2011). It is now known that epithelial cells are dynamic, multifunctional cells which contribute to host innate immunity and immune regulation (Proud & Leigh 2011).

Four influenza A viruses were used in this study. These viruses were chosen as they represented typical human and avian influenza viruses in circulation.

To begin to understand bat cell response to influenza virus infection, viral tropism was explored. Viral tropism is defined as the specificity of a virus for a particular host tissue and can be determined at each replication step (Nomaguchi et al. 2012). Viral tropism is dependent on both susceptibility and permissiveness. A susceptible host cell possesses specific receptors enabling the virus to enter the cell whereas permissiveness is focused on the ability of the virus to shut down host innate defences and replicate successfully. Recent studies have shown several bat cell lines as being susceptible to infection by conventional influenza viruses (Dlugolenski et al. 2013; Hoffmann et al. 2013; Poole et al. 2014). Using a single-cycle green fluorescent protein (GFP) reporter virus (human/WSN/33), 11 bat cell lines, derived from seven bat species, were positively infected (Poole et al. 2014). Another study found a number of infected cells among seven bat cell lines by immunocytochemical detection of viral NP (Hoffmann et al. 2013). Reassorted virus progeny from human H1N1 (A/WSN/1933) and HPAI H5N1 (A/Vietnam/1203/04) viruses was produced from *Pteropus alecto* kidney cells (Dlugolenski et al. 2013). Collectively, these findings appear to indicate that bat cells are susceptible to infection with conventional mammalian and avian influenza viruses. However, the permissiveness of bat respiratory epithelial cells to conventional influenza viruses to be able to produce viable virus progeny remains unclear.

By infecting bat cells for 24 h with two LPAI viruses and two human viruses, all three species of bat cells were found to be susceptible and permissive, to some degree, to influenza virus infection (**Figure 2.4-1**). Viral NP, an abundant protein in the virion, was positively detected in nearly all *E. helvum* and *C. perspic* cells infected with LPAI

H2N3 and H6N1 viruses and there was evidence of cell detachment 24 hpi (**Figure 2.4-1C** and **2.4-1D**). In contrast, viral NP was only detected in a small proportion of E. helvum and C. perspic cells infected with human H1N1 viruses and there was no evidence of cell detachment. TB1-Lu cells were not readily infected by either avian or human viruses denoted by the limited proportion of nuclei positively stained for viral NP (**Figure 2.4-1B**).

Focus forming assays (FFA) were used to determine the viable progeny virus output. While traditional methods notably plaque assays and end point dilution assays (TCID₅₀) are commonly used for virus quantification (Baer & Kehn-Hall 2014), critically these methods rely on cell lysis. As many influenza A virus strains do not lyse cells upon infection, such methods would not be appropriate. All three bat cell types produced significantly less ($P \leq 0.0001$) viable progeny virus output than from correspondingly infected MDCK cells (**Figure 2.4-2**). Among the three species of bat cells, infected TB1-Lu cells clearly produced the least progeny viruses (**Figure 2.4-2A-D**). Notably, virus output was higher from bat cells infected with avian viruses than corresponding cells infected with human viruses suggesting bat cells are more permissive to avian than human viruses.

The differences in permissiveness between the three bat species were reflected in RNA expression of viral M-gene, which encodes the abundant matrix and transmembrane proteins, M1 and M2. M-gene RNA expression was normalised to expression of internal reference gene or “housekeeping” gene, 18s rRNA, which was shown to be the most stably expressed gene among a number of other commonly used internal reference genes in influenza virus infection studies (Kuchipudi et al. 2012). The pattern of viral M-gene RNA expression from infected MDCK and bat cells closely matched the pattern of virus output as while All three bat cell types infected with LPAI

H2N3 and USSR H1N1 virus expressed significantly less viral M-gene RNA than correspondingly infected MDCK cells (**Figure 2.4-3**), among the three bat cell types, M-gene RNA expression was clearly lowest in TB1-Lu cells infected with USSR H1N1 and LPAI H2N3 virus. Furthermore, avian H2N3 virus produced higher M-gene expression (**Figure 2.4-3B**) than corresponding human USSR H1N1 virus infection (**Figure 2.4-3A**) in each species of bat cell. TB1-Lu cells exhibited the weakest expression of viral proteins (**Figure 2.4-4**), consistent with low virus production and low expression of viral M-gene RNA.

Similar to recent findings from examining susceptibility of bat cells to influenza virus infections (Hoffmann et al. 2013; Poole et al. 2014), TB1-Lu cells were shown as less susceptible and less efficiently infected than cells from other bat species. Our results also corroborated the finding of African fruit bat cells (such as *E. helvum* cells) being comparatively more susceptible to infection (Poole et al. 2014).

The finding of *E. helvum* cells as susceptible to avian influenza virus infection corroborated with the discovery of 30% of *E. helvum* bats testing serologically positive for avian H9 virus (Freidl et al. 2015). Despite positive detection of H9 virus, *E. helvum* bats were found to be clinically healthy, showing no signs of disease (Freidl et al. 2015) demonstrating that host susceptibility and permissiveness to virus infection does not necessarily result in disease. There have been several cases where fruit bat and fruit bat cells have been shown to be susceptible to infections by viruses which have failed to produce severe clinical disease in the host. *E. helvum* cells have been shown to be susceptible to Ebola virus infection yet not succumb to pathogenic disease during experimental and natural infection (Kühl et al. 2011). The glycoprotein (GP) of Ebola, responsible for mediating viral entry into host cells, was revealed to interact similarly with human and bat cells indicating virus entry was not the determining stage

for susceptibility to Ebola virus infection (Kühl et al. 2011). Supporting this, in an earlier study, fruit bats were shown to be highly susceptible and permissive to Ebola virus infection, producing high virus titres, but showed no sign of illness (Swanepoel et al. 1996). Similarly, high expression levels of viral proteins and high virus titres were found in *Rousettus aegyptiacus* cells subjected to Marburg virus infection (Krähling et al. 2010) despite fruit bats implicated as reservoir hosts to Filoviruses. Such studies implicate fruit bats as reservoir hosts to a number of viruses while also susceptible and permissive to virus infection. Our findings show lung epithelial cells from *C. perspic*, a microbat, as comparably susceptible and permissive to avian influenza virus infection as *E. helvum* cells. This suggests the susceptibility and permissiveness of bats to influenza virus infection may vary according to individual bat species rather than by bat groups (i.e. megabats and microbats).

In summary, these findings expanded upon recent susceptibility studies of bats to influenza viruses (Dlugolenski et al. 2013; Hoffmann et al. 2013; Poole et al. 2014) by examining virus replication permissiveness in bat cells. Collectively, the pattern of M-gene RNA expression and viable virus progeny production from infected bat cell are indicative of lower virus replication in bat cells than MDCK cells. Bat cells appear more permissive to avian than human influenza viruses and TB1-Lu cells are more resistant to influenza virus infections compared with *E. helvum* and *C. perspic* cells suggesting species-specific variation in replication permissiveness and susceptibility to influenza virus infection.

**3. Resistance of bat cells to
influenza A virus infection is
host species-specific**

3.1. Chapter summary

Susceptibility to influenza virus infection is determined by the distribution of sialic acid (SA) receptors on the membrane of host cells (Wang et al. 2015) whereas Permissiveness to virus infection is the ability of the virus to shut down host innate defences and replicate successfully.

In this chapter, the underlying the differences in susceptibility and replication permissiveness between bat cell types was explored by determining the presence of SA receptors on bat cells and the impact of SA receptor removal. All three types of bat cells were found to possess typical sialic acid receptors for the recognition and binding of influenza viruses, however, only the avian influenza virus-preferred SA α 2,3-Gal receptors were strongly detected on TB1-Lu cells. The lack of SA α 2,6-Gal receptors on TB1-Lu cells likely contributes to the reduced susceptibility of TB1-Lu cells to influenza virus infection compared with E. helvum and C. perspic cells.

All three bat cells were found to be more viable during infection with USSR H1N1 and LPAI H2N3 virus compared with correspondingly infected MDCK cells. The high viability of bat cells during influenza virus infection and replication permissiveness indicates bats may be potentially good hosts for influenza viruses. Apoptotic activity was greatly increased in C. perspic cells during influenza virus infection and inhibition of caspase activity resulted in an increased number of infected C. perspic cells. Taken together, these findings suggest C. perspic may use apoptosis as a defence mechanism to contain virus replication and spread.

3.2. Introduction

3.2.1. *Characteristics of resistant and susceptible hosts to influenza virus infection*

The host range of influenza A viruses is very broad, encompassing humans, pigs, horses, sea mammals and birds (Webster et al. 1992). However, the host range of a particular virus strain is often more restricted, partly due to receptor specificity. The distribution of sialic acid (SA) receptors on the membrane of host cells determines host susceptibility to influenza virus infection (Wang et al. 2015). Not only do avian viruses preferentially bind to SA α 2,3-Gal receptors whereas human viruses have an affinity for SA α 2,6-Gal receptors (Weis et al. 1988; Gamblin et al. 2004; Stevens et al. 2006), but human, swine and avian influenza viruses have individual receptor-binding specificities that give them, sometimes, almost species-specificity (Matrosovich et al. 2006). This receptor specificity is the main determinant of why human viruses do not replicate efficiently in birds and duck viruses do not replicate well in humans (Hatta et al. 2002). Ducks have been shown to predominantly express avian-type SA α 2,3 receptors in their intestinal tract, the major site of influenza virus replication, whereas chickens express high amounts of each receptor type (Wang et al. 2015) and are more susceptible to human virus infections. Pigs are believed to be ‘mixing vessels’ for the reassortment of influenza viruses as early studies indicated that there was co-expression of both SA α 2,3-Gal and SA α 2,6-Gal receptors on pig upper respiratory tract cells (Nelli et al. 2010). Indeed, the emergence of pandemic (H1N1) 2009 influenza demonstrated pandemic viruses can be generated in pigs (Yamada et al. 2012). However, more recent studies have failed to detect SA α 2,3-Gal receptors in the upper respiratory tract in pigs (Van Poucke et al. 2010; Trebbien et al.

2011) and have instead detected expression of SA α 2,6-Gal receptors in all areas of the respiratory tract and SA α 2,3-Gal receptors mostly on the alveolar epithelial cells in the lower respiratory tract (Trebbien et al. 2011). The lack of avian virus receptors implies avian and human viruses may not reassort as efficiently in pigs as previously suggested or, alternatively, other species with a similar sialic acid receptor distribution, such as chickens and humans may also act as mixing vessels (Shinya et al. 2006; Guo et al. 2007; Kuchipudi et al. 2009). Evidence for the latter includes the occasional transmission of avian viruses including H5N1, H7N7 and H9N2 from chickens and turkeys to humans (Yamada et al. 2012).

Although receptor specificity partly determines host susceptibility to infection, it does not imply permissiveness to virus infection. Some susceptible hosts can confer the necessary innate immune mechanisms to resist the ill-effects of infection. For example, pigs, susceptible to infection by both human and avian viruses, have been shown through experimental challenge studies and *in vivo* studies to be highly resistant to HPAI virus infections and to not go on to develop associated complications such as hypercytokinemia (Tisoncik et al. 2012). Hypercytokinemia, often described as the ‘cytokine storm’ is a notable complication of influenza virus infections seen in humans. This self-destructive and often fatal syndrome is caused by the hyperacute dysregulation of pro-inflammatory cytokines and chemokines (Nelli et al. 2012) often in response to HPAI viruses such as H5N1 virus. The sudden and strong induction of type I IFNs and IFN-inducible genes have been characterised in several hosts infected with H5N1 including humans (de Jong et al. 2006), ferrets (Cameron et al. 2008), and macaques (Baskin et al. 2009) suggesting an anti-viral activity mediated by IFN pathway may not be useful in combating highly virulent strains of influenza and may even be damaging by contributing to hypercytokinemia (Cameron et al.

2008). Expression of TNF α , a key pro-inflammatory cytokine, was shown to be at least 100-fold higher in human epithelial cells and macrophages infected with H5N1 virus than in similar pig cells. This finding was supported by *in vivo* studies which demonstrated a lack of TNF α induction in pigs challenged with H5N1 virus (Nelli et al. 2012). High constitutive expression of the regulator SOCS3 (suppression of cytokine signalling) was shown to be a major factor in modulating the pro-inflammatory host response to influenza virus infection. The SOCS family of proteins are inhibitors of the JAK/STAT signalling and SOCS3, in particular, has been well recognised for its anti-inflammatory properties, including reducing TNF α levels (Shouda et al. 2001; Jo et al. 2005). In HPAI H5N1 virus-infected pig cells, high levels of SOCS3 were accompanied by weak expression of IFN-inducible genes, such as Mx1 and OAS, whereas in infected human cells SOCS3 was downregulated and this coincided with strong induction of IFN-inducible genes (Nelli et al. 2012). Expression of pro-inflammatory chemokines, CXCL9, CXCL10 and CXCL10 was also shown to be either weak or absent in infected pig epithelial cells and macrophages. By contrast, the expression of such chemokines was greatly increased in human cells (Nelli et al. 2012). Interestingly, IL-1 β gene induction was found to be 100-fold higher in infected pig cells compared with infected human cells suggesting this gene may not be associated with the development of hypercytokinemia. Collectively, these results infer pigs can mount an appropriate, but not excessive, pro-inflammatory response to HPAI viruses. Infected pig cells were also shown to exhibit greater cellular and metabolic damage accompanied by reduced virus output compared with human cells. Cell death has been suggested as a mechanism to contain virus spread in duck cells infected with H5N1 virus (Kuchipudi et al. 2012a). Aquatic birds, such as ducks, harbour the 16 HA and 9 NA antigenic subtypes and are postulated as the natural reservoir hosts for all

strains of influenza virus. Aquatic birds either suffer mild clinical signs or do not exhibit clinical signs at all when infected with most influenza viruses including those highly pathogenic to other birds (Alexander et al. 1986). Conversely, it is well established that chickens infected with HPAI viruses result in nearly 100% mortality (Alexander et al. 1986). Surprisingly, cell death was found to be more apparent in resistant Pekin and mallard duck cells than in susceptible White Leghorn chickens infected with H5N1 virus (Kuchipudi et al. 2012). Cell death was attributed to caspase activity and was accompanied by reduced virus output. Early apoptosis was suggested to be beneficial for the avian host by containing virus replication (Kuchipudi et al. 2012).

Key differences in immune signalling pathways and immune-related genes have been established in ducks compared with other mammals, which may play a major role in resistance to influenza virus infection. Birds have been shown to have a more smaller repertoire of immune-related genes, than mammals (Hughes & Friedman 2008; Huang et al. 2013). Birds lack TLR8, TLR9 and TNF α , and IRF3 has yet to be identified (Yilmaz et al. 2005; Temperley et al. 2008). Recently, genes encoding interferon-induced membrane proteins (IFITM) have been identified in chicken and duck genomes. IFITM proteins have been described to limit influenza virus infection in mammals (Huang et al. 2011; Everitt et al. 2012). Specifically, IFITM3 restricts virus entry into host cells and also has been shown to have anti-viral effects in mouse knock-out models (Brass et al. 2009). Additionally, IFITM1 overexpression has been shown to restrict influenza infection *in vitro* in chicken cells (Smith et al. 2013). Duck cells infected with H5N1 virus were shown to mount and sustain induction of IFITM1, IFITM2 and IFITM3 whereas the induction of IFITM response in infected chicken cells was very weak. During H5N1 virus infection, chicken cells exhibited

upregulation of lipid metabolism gene expression whereas duck cells did not (Smith et al. 2015). This could be attributed to the differences between IFITM responses between duck and chicken cells as IFITM1 and IFITM3 function to block viral membrane hemifusion (fusion of outer membranes of the two lipid bilayers) through alteration of lipid composition decreasing membrane fluidity (Li et al. 2013).

The differences in expression of RIG-I, a pattern recognition receptor, may be a major factor in the different innate immune responses to HPAI infections between chickens and ducks. Chickens lack RIG-I expression and also lack the gene encoding RIG-I binding protein, *RNF125* (Magor et al. 2013) whereas ducks have intact RIG-I expression (Barber et al. 2010). Furthermore, the transfection of duck-derived RIG-I into chicken cells resulted in the induction of IFN- β promoter activity and limited replication of both HPAI and LPAI viruses (Barber et al. 2010).

RIG-I was also shown to be important in mediating anti-viral resistance in guinea pigs. Like ducks and pigs, guinea pigs infected with HPAI H5N1 virus do not typically show severe clinical signs. RIG-I expression was shown to be greatly increased during HPAI H5N1 virus infection and overexpression of RIG-I and RIG-I adaptor protein, MAVS, restricted H5N1 virus replication in guinea pig foetal lung fibroblasts and significantly reduced virus titres, respectively (Zhang et al. 2017). Transcriptomic analysis of guinea pig lung cells infected with HPAI H5N1 revealed altered gene expression of genes encoding cytokines and ISGs. Expression of ISGs, Mx1, STAT1, OAS1 and PKR gene expression was found to increase in infected cells. IFN α and IFN γ mRNA expression were also increased during infection. However, similarly to pig epithelial cells and macrophages infected with HPAI H5N1 virus, infection of guinea pig cells did not result in the significant production of pro-inflammatory cytokines (Zhang et al. 2017).

In summary, it appears that in order to successfully mitigate HPAI virus infections, such as H5N1 virus, the host needs to mount an appropriately robust yet moderate pro-inflammatory cytokine response to survive HPAI infection. An excessively strong pro-inflammatory response appears to have a negative impact on host survival as it may induce complications such as hypercytokinemia. Programmed cell death has also been shown to contain virus replication and spread (Kuchipudi et al. 2012), and differences in the repertoire of immune-related gene have been shown to be important triggering relevant signalling pathways to resist virus infection (Smith et al. 2015).

In this chapter, the susceptibility of bat cells was examined further through assessing the presence of two main types of SA receptors found in other mammalian species, The possibility of cell death is also explored as a mechanism for anti-viral resistance in bat cells was also examined by determining the viability of the three bat cell types post influenza virus infection.

3.3. Materials and methods

3.3.1. *Endosomal uptake of siRNA*

Cells were seeded in an 8-well chamber slide with 160 µl complete cell culture medium per well and incubated at 37°C overnight. The following day, complete medium was replaced with fresh complete cell culture medium. Control siRNA (12.25 µl) (10 µM) (Cell Signaling Technology) was added to Buffer F (Lipocalyx) to make a total of 18 µl in one tube. In a separate tube, 5.5 µl of Viromer Blue (Lipocalyx) reagent was added to 495 µl Buffer F. The second solution (162 µl) was added to the first solution to make a total of 180 µl. This was left to incubate for 10 min before adding 20 µl into each well of an 8-well chamber slide. The plate was incubated for 4 h at 37°C and subsequently washed twice with 1x PBS and fixed with 4% paraformaldehyde. After fixation, chamber sides were removed and mounted with Prolong Gold with DAPI (Invitrogen). This was left to cure for 24 h in the dark prior to imaging the following day. Cells were imaged using a fluorescent microscope (Leica Microsystems Ltd., Milton Keynes, UK).

3.3.2. *Detection of sialic acid receptors*

Cells were seeded and fixed with 4% paraformaldehyde on coverslips and blocked with biotin, streptavidin blocking kit (Vector labs) for 15 min then washed twice with 1x TBS.

Sialic acid (SA) receptors were detected using fluorescein isothiocyanate (FITC)-labelled *Sambucus nigra agglutinin* (SNA) (Vector labs) and another plant lectin, biotinylated-*Maackia amurensis leckoagglutinin II* (MAA-II) (Vector labs). Cells

were incubated with 10 µg/ml of each lectin at 4°C overnight in the dark. The following day, cells were washed three times with 1x TBS and then incubated with Alexa Fluor 594 conjugated Streptavidin at a dilution of 1: 500 for 2 h at room temperature in the dark. Cells were then washed three times with 1x TBS prior to careful removal of coverslips. Coverslips were left to air dry briefly before mounting on glass slides with Prolong Gold with DAPI (Invitrogen). These slides were left to cure for 24 h before imaging. Cells were imaged using a fluorescent microscope (Leica Microsystems Ltd., Milton Keynes, UK).

3.3.3. *Removal of sialic acid receptors*

SA receptors were removed generally with neuraminidase (Roche) derived from *Clostridium perfringens* (Ramos et al. 2011). Cells were treated with at a concentration of 0.05 U/ml added to cell culture media and incubated at 37°C for 4 h. Cells were subjected to lectin staining, as described in 3.2.2, to confirm the removal of SA moieties prior to further work.

3.3.4. *Caspase 3/7 assays*

Cells were seeded in 96-well format in complete cell culture media. Caspase 3/7 glo reagent (Promega) was prepared according to manufacturer's instructions and added to cells at equal volume. The plate was mixed briefly and left to incubate for 1 h at room temperature in the dark. Measurements were taken using a microplate luminometer (Orion L; Titertek Berthold; Germany).

To inhibit caspase 3 activity, caspase 3 inhibitor, Z-DEVD-FMK (R&D Systems) (Wurzer et al. 2003) was applied 30 min prior to infection at 10mM. Cells were rinsed twice with PBS and then infected with USSR H1N1 virus at MOI of 1.0. Simultaneously, DMSO-treated cells were infected with USSR H1N1 virus to be used as comparison controls. After 2 h, cells were rinsed twice with PBS and fresh infection media (see **Table 2.3-1**) was added with caspase inhibitor and incubated for a further 22 h before titration onto fresh confluent MDCK cells.

3.3.5. *Flow cytometry*

Cell viability was assessed using the LIVE/DEAD® Fixable Far Red Dead Cell Stain Kit (Thermofisher) according to manufacturer's instructions. Briefly, the reactive dye was mixed with dimethyl sulfoxide (DMSO) and added to washed cells suspended in 1 ml of 1x PBS. Prior to this step, positive (dead) control cells were heated at 60°C for 20 min. Cells were incubated at room temperature for 30 min in the dark to allow for the incorporation of the dye. Cells were then washed twice and resuspended in 1 ml 1x PBS with 1% bovine serum albumin (BSA). Finally, the cells were analysed through BD FACSCanto II (BD Biosciences).

3.4. Results

3.4.1. *TB1-Lu cells exhibited reduced endosomal uptake of siRNA*

The Viromer system (Lipocalyx) was used to examine endosomal uptake by bat cells as a means to explore the role of influenza virus attachment and entry. The Viromer system is based on active endocytic pathways mimicking influenza binding and entry (Panzer 2014). The siRNA was fluorescein-isothiocyanate (FITC)-conjugated enabling the visualisation of siRNA uptake through fluorescent microscopy.

Transfection of bat cells with FITC-labelled siRNA revealed differential uptake of siRNA between the three types of bat cells (**Figure 3.4-1**). A high degree of fluorescence was observed in transfected MDCK, E. helvum and C. perspic cells. However, relatively little fluorescence was observed in transfected TB1-Lu cells indicating less siRNA uptake compared with the other bat cell types.

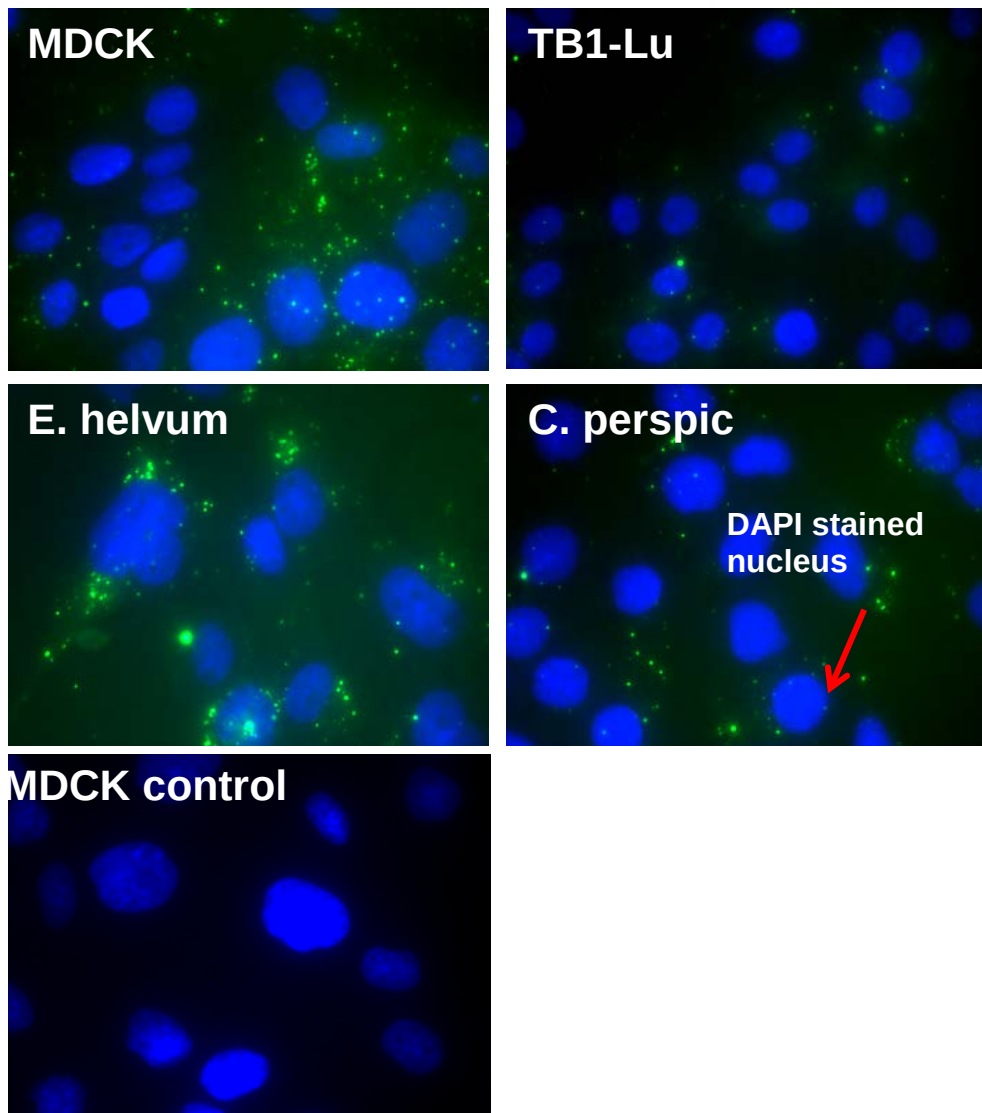


Figure 3.4-1 TB1-Lu cells exhibited the least uptake of siRNA in comparison with MDCK, E. helvum and C perspic cells.

Respiratory epithelial cells from the three bat species and MDCK cells were transfected with fluorescein conjugated siRNA with the use of Viromer Blue reagent (Lipocalyx) which mimics natural influenza viral entry and membrane fusion (Panzer, 2014). Fluorescence imaging at 4h showed detectable uptake of siRNA (green spots) in MDCK, E. helvum and C. perspic cells. However, TB1-Lu cells barely showed any fluorescence. Mock transfected MDCK cells showed complete absence of fluorescence. Results shown are representative of three experimental replicates.

3.4.2. *Bat cells possess canonical sialic acid receptors*

The influenza cell surface protein, haemagglutinin (HA) is responsible for the host cell entry by binding to SA receptors. SA moiety is linked to a galactose by either an $\alpha 2,3$ linkage or $\alpha 2,6$ linkage. It has been shown that avian viruses preferentially bind to SA $\alpha 2,3$ -Gal receptors whereas human viruses have an affinity for SA $\alpha 2,6$ -Gal receptors (Weis et al. 1988; Gamblin et al. 2004; Stevens et al. 2006). We examined the presence and spatial distribution of SA $\alpha 2,3$ -Gal and SA $\alpha 2,6$ -Gal receptors in the three species of bat and MDCK cells by binding with plant lectins *Maackia amurensis agglutinins* (MAA II) and *Sambucus nigra agglutinin* (SNA), respectively, to determine SA receptor contribution to differential susceptibility to influenza virus infection between bat cell types. The presence of the avian influenza SA $\alpha 2,3$ -Gal receptor was strongly detected on each cell type (**Figure 3.4-2**) denoted by the strong red fluorescence of the biotinylated MAAII. The human influenza SA $\alpha 2,6$ -Gal receptor was also clearly detected (green fluorescence) on MDCK, E. helvum and C. perspic cells. However, on TB1-Lu cells, there was little or absence of SA $\alpha 2,3$ -Gal receptor detection.

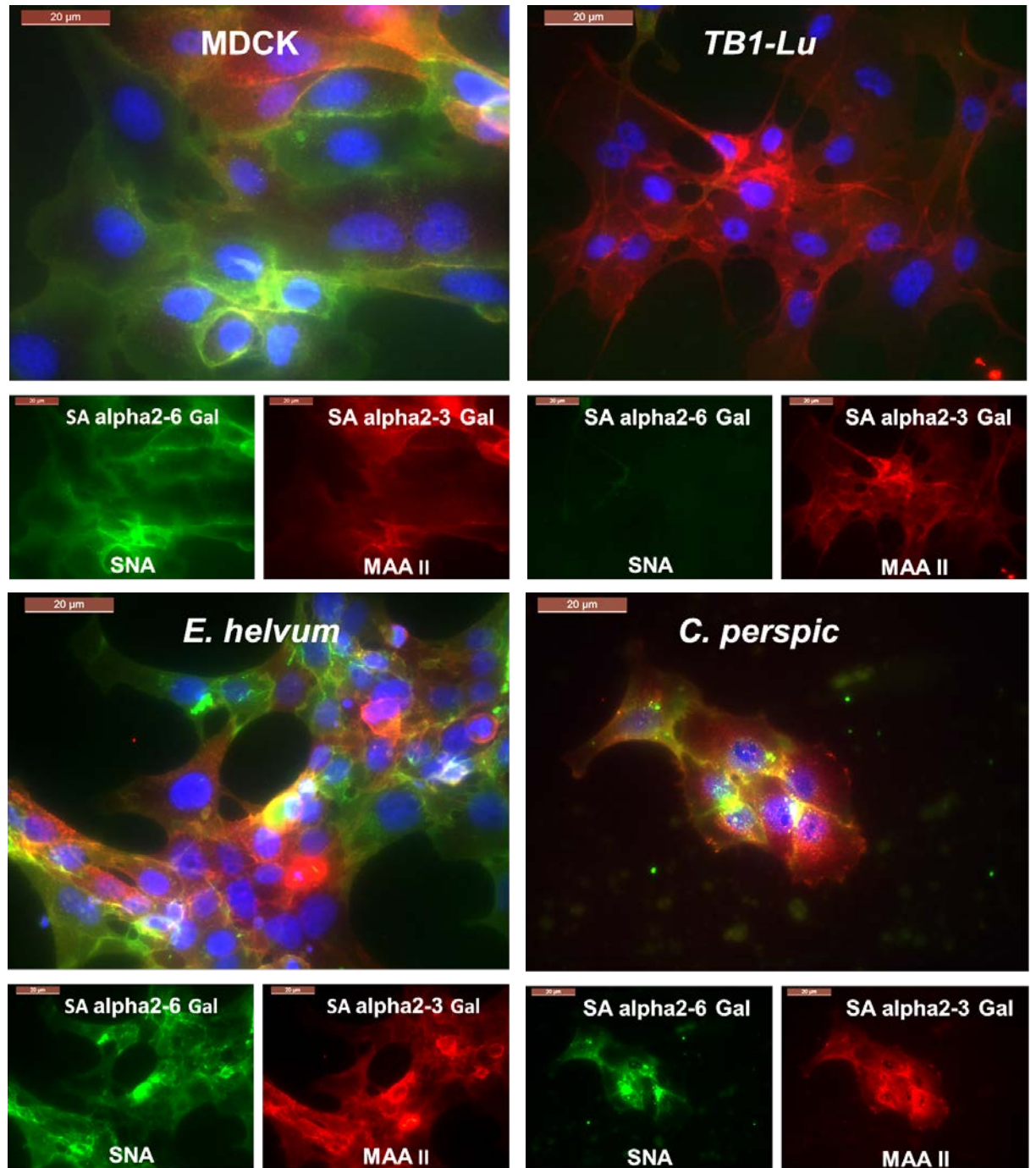


Figure 3.4-2 Cells derived from *E. helvum* and *C. perspic* but not TB1-Lu expressed human and avian sialic acid receptors.

Bat and MDCK cells were subjected to binding by specific lectins (SNA and MAA II) for the detection of human SA α 2,6 Gal (green) and avian SA α 2,3 Gal (red) receptors respectively. Nuclei were counterstained with DAPI (blue). Avian SA α 2,3 Gal and human SA α 2,6 Gal receptors were readily found in all cell types except for TB1-Lu cells where SA α 2,6 Gal receptors were not detected. Results shown are representative of three experimental replicates.

3.4.3. Removal of sialic acid receptors from bat cells resulted in reduced virus output

To further explore the functional impact of SA receptors on influenza virus infection, SA receptors were collectively removed from the cell surface using a generic neuraminidase derived from *Clostridium perfringens*, as previously described (Ramos et al. 2011). Following neuraminidase treatment, cells were subjected to plant lectin staining to visually confirm the removal of sialic acid moieties (**Figure 3.4-3**).

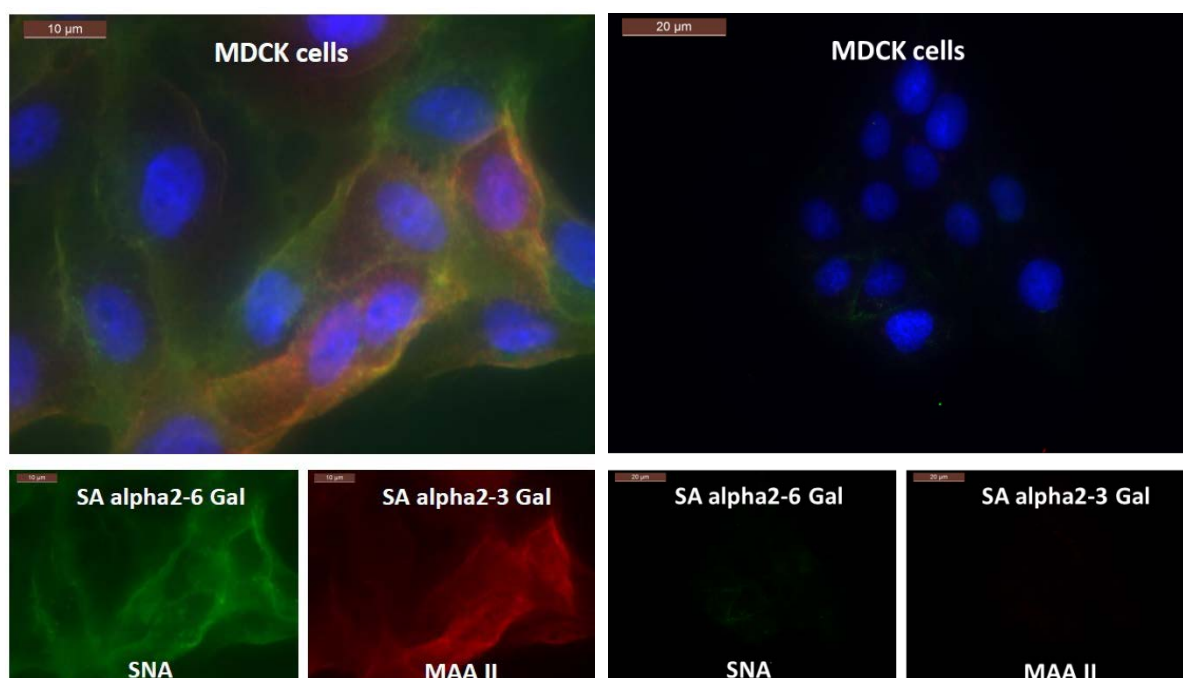


Figure 3.4-3 Removal of sialic acid (SA) receptors

SA receptors were clearly detected on MDCK cells prior to removal (A). (B) SA receptors were largely removed from MDCK cells treated with a general neuraminidase derived from *Clostridium perfringens* and subsequently fixed and stained using plant lectins, *Maackia amurensis* agglutinins (MAA II) and *Sambucus nigra* agglutinin (SNA).

Non-specific removal of SA receptors was shown to sharply reduce the number of infected cells and virus output from all cell types with each virus infection (**Figure 3.4-4**). The number of infected cells was shown to clearly decrease in MDCK, TB1-Lu, E. helvum and C. perspic cells infected with USSR H1N1 virus treated with

neuraminidase compared with untreated correspondingly infected cells (**Figure 3.4-4A**). Virus production from MDCK, TB1-Lu, E. helvum and C. perspic cells infected with all four viruses, separately, decreased sharply after removal of SA receptors (**Figure 3.4-4B**).

Despite extensive removal of SA receptors, some virus production remained suggesting the presence of an alternative SA-independent pathway for virus entry. In summary, SA α 2,6-Gal and SA α 2,3-Gal receptors appear important in bat cells for entry of conventional influenza A viruses, and the lack of SA α 2,6-Gal receptor in TB1-Lu cells could be contributing factor to resistance to entry of human influenza viruses.

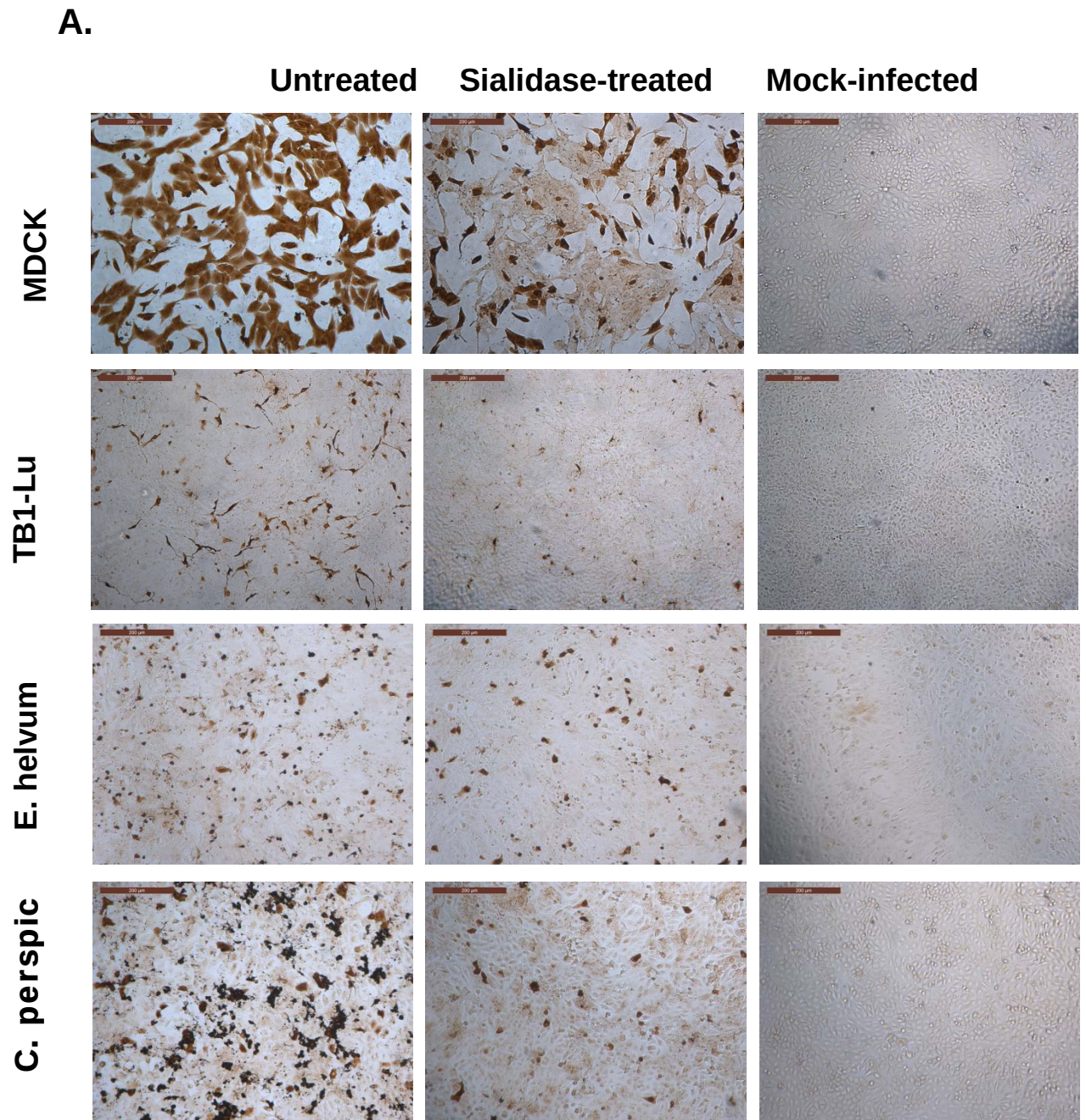
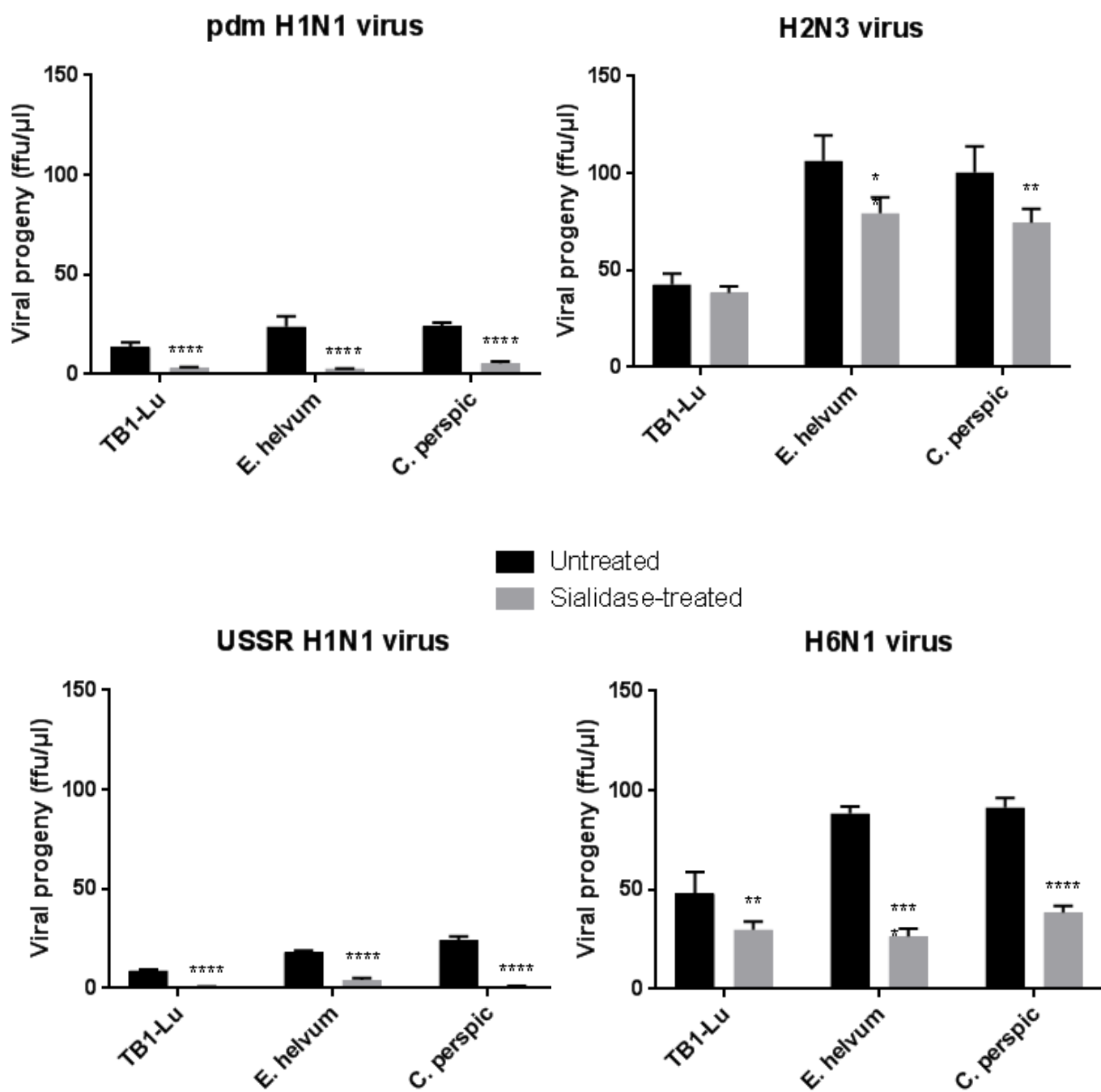


Figure 3.4-4 Non-specific removal of sialic acid (SA) receptors using neuraminidase derived from *Clostridium perfringens* resulted in a marked reduction in progeny virus released from all three bat species, particularly when subjected to human virus infection (pdm H1N1 virus and USSR H1N1 virus).

SA receptors were removed from bat cells using a neuraminidase derived from *Clostridium perfringens* and subsequently infected with LPAI viruses, H2N3 and H6N1 virus, and human viruses, USSR H1N1 and pdm H1N1 virus, at MOI 1.0. (A) Removal of SA receptors resulted in clear reduction in the number of USSR H1N1 virus infected cells as shown by viral NP detection 24 hpi. (B) SA receptor removal significantly reduced (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$) viable progeny production in each cell type. Each cell type was infected for 24 h. Results shown are representative of two biological replicates and six technical replicates for each biological repeat.

B.



3.4.4. *Bat cells appeared more viable than MDCK cells infected with influenza viruses*

Cell death was determined using flow cytometry measuring the percentage of infected cells with compromised cell membrane integrity. Flow cytometry sorts cells based on physical characteristics such as internal complexity of the cell and cell diameter which can be shown through forward-angle light scatter (FSC) and side-angle scatter (SSC) respectively. Cells can also be sorted according to their fluorescent characteristics by using fluorophores to bind to antibodies, DNA or amines. In this case, the fluorophore was designed to bind to intracellular free amines and amines on the cell surface. As such, cells with a compromised membrane will yield a much more intense fluorescent signal than viable cells as, in viable cells, reactivity is restricted to the cell surface amines. The fluorescent intensity signal generated by uninfected cells or 'live' control cells and by heat-killed 'dead' control cells was used to separate, or gate, viable infected cell populations from unviable infected cells (**Figure 3.4-5A**). Although viable and unviable populations were sorted primarily by fluorescent intensity, FSC and SSC were used to confirm distinct populations.

All three bat cell types infected with LPAI H2N3 virus were found to be more viable than correspondingly infected MDCK cells ($P \leq 0.0001$) (**Figure 3.4-5B**). Similar results were obtained when bat cells were infected with USSR H1N1 virus except there was less cell damage (**Figure 3.4-5B**). With the exception of *C. perspic* cells, LPAI H2N3 virus resulted in a greater cell death than human USSR H1N1 virus in all other cell types. This was consistent with earlier findings in virus output and viral M-gene RNA expression which indicated bat cells were more permissive to avian viruses than human viruses. Among the three bat cell lines, *E. helvum* cells showed the highest percentage of cell death whereas *C. perspic* cells were most viable post-infection with

1% and 2.1% cell death from infection with USSR H1N1 virus and LPAI H2N3 virus respectively.

A.

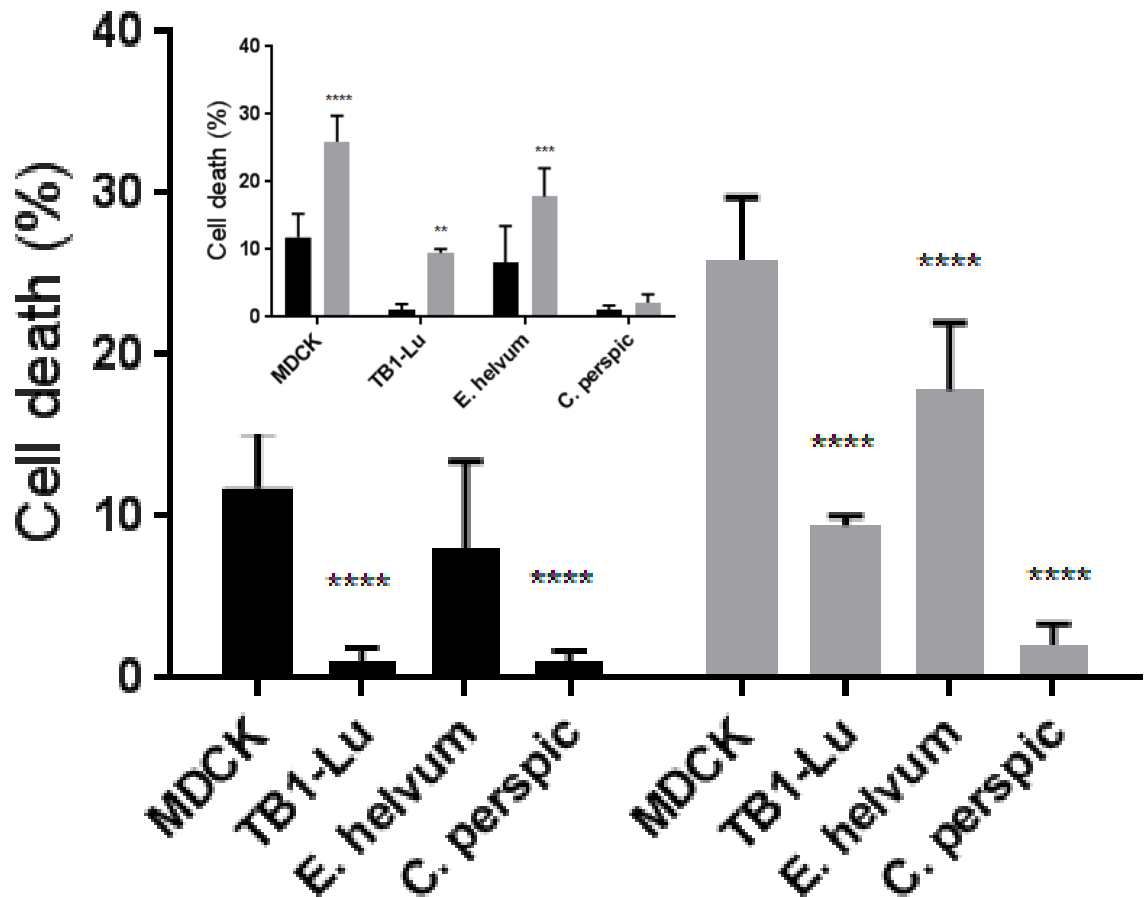


Figure 3.4-5- Influenza virus (LPAI H2N3 and USSR H1N1 virus) infected bat cells appeared more viable than correspondingly infected MDCK cells.

Cells were infected at 0.5 MOI with human USSR virus or avian H2N3 virus for 24 h and subsequently analysed by flow cytometry for cell membrane integrity with a LIVE/DEAD Fixable Far Red Dead Cell Stain kit (Molecular Probes). (B) Cells were primarily gated according to fluorescence intensity using heated cells and uninfected cells as 'live' and 'dead' controls. Data shown represents one biological sample set consisting of three technical replicates.

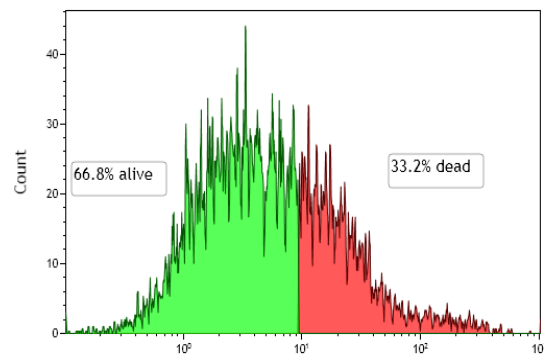
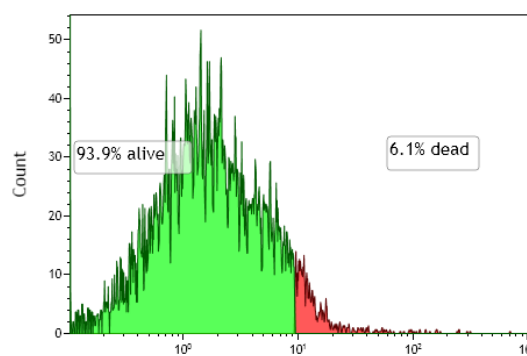
(A) Avian H2N3 virus elicited higher level of cell death than corresponding USSR virus for each cell type. In addition, with the exception of USSR virus infected *E. helvum* cells, each virus caused significantly higher (**** $P \leq 0.0001$) cell death in MDCK than bat cells. Inset figure is another representation of results for ease of comparison between viability of cells infected with USSR H1N1 and avian H2N3 viruses. Results shown represent four biological replicates and three technical replicates of each biological repeat.

B.

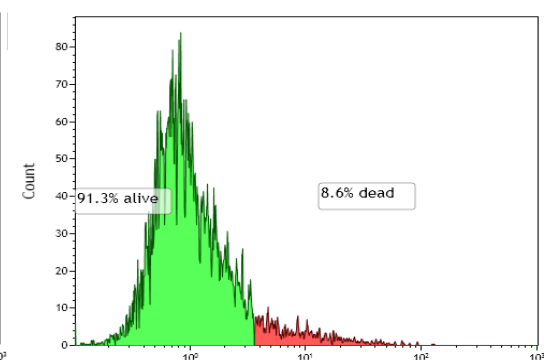
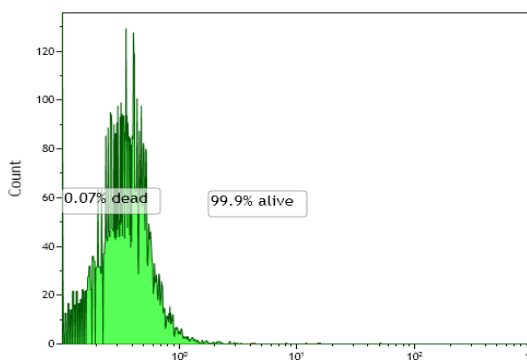
USSR H1N1

LPAI H2N3

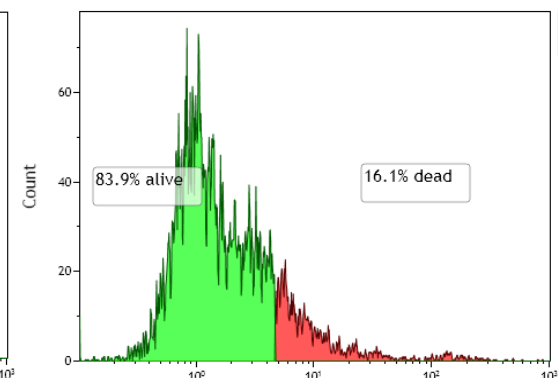
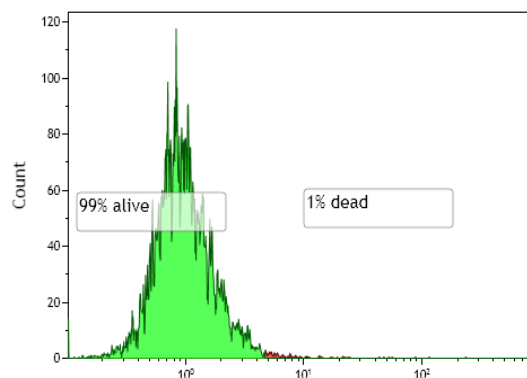
MDCK



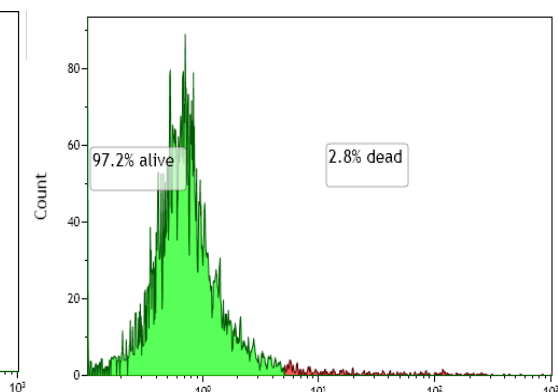
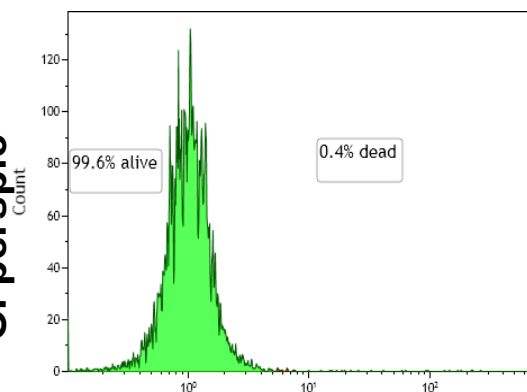
TB1-Lu



E. helvum



C. perspic



As influenza virus induced cell death is also mediated through programmed to cell death (Hinshaw et al. 1994), virus-induced apoptosis of bat cells was determined through the induction of caspase 3/7 induction. MDCK, *E. helvum* and *C. perspic* infected with USSR H1N1 virus showed increased apoptotic activity compared with uninfected counterparts (**Figure 3.4-6**). Apoptosis was found not to be induced in TB1-Lu cells infected with USSR H1N1 virus (**Figure 3.4-6**). During LPAI H2N3 virus infection, caspase activity was found only significantly increased in infected MDCK and *C. perspic* cells (**Figure 3.4-6**). Among the three bat cell types, the highest caspase activity, induced by infection with USSR H1N1 and LAPI H2N3, was found in *C. perspic* cells with an 8-10 fold increase in caspase 3/7 activity.

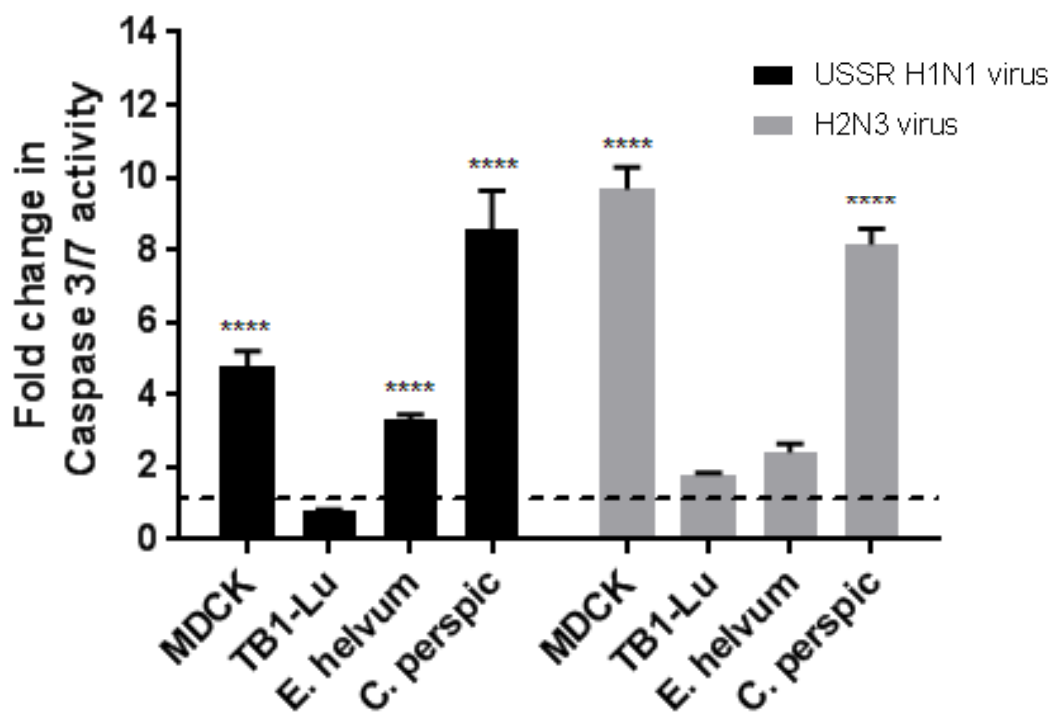


Figure 3.4-6 Caspase 3/7 activity in bat cells

Bat and MDCK cells were infected with USSR H1N1 or avian H2N3 virus at 0.5 MOI for 24h followed by the determination of caspase 3/7 activation using a Caspase-Glo 3/7 Assay kit (Promega). *C. perspic* cells exhibited the most pronounced increase in apoptosis among the three bat species. TB1-Lu and *E. helvum* cells showed modest increase in caspase 3/7 activation with each virus. Infected *C. perspic* cells showed low rate of cell membrane damage but clear significant elevation (**** $P \leq 0.0001$) in apoptosis. Results shown are typical of four independent repeats. Significance (*) is in relation to uninfected control cells. Dotted line (---) is equivalent to no change in activity.

3.4.5. *Inhibition of caspase 3 reduced virus output from MDCK and E. helvum cells but not C. perspic cells*

To further explore the relationship between apoptosis and influenza virus infection in bat cells apoptosis was inhibited in MDCK, A549, E. helvum and C. perspic cells using caspase 3 inhibitor, Z-DEVD-FMK. Z-DEVD-FMK-treated MDCK, E. helvum and A549 cells infected with USSR H1N1 virus produced less progeny virus compared with correspondingly infected DMSO control cells (**Figure 3.4-7A**). Virus production from infected C. perspic cells remained unchanged when treated with Z-DEVD-FMK (**Figure 3.4-7A**). Caspase inhibition was found to not affect the number of cells infected by USSR H1N1 virus in MDCK, A549 or E. helvum cells. The distribution of viral NP was unchanged when treated with Z-DEVD-FMK compared with DMSO-treated counterpart cells (**Figure 3.4-7B**). However, inhibition of caspase induction resulted in the increased number of infected C. perspic cells. Viral NP was detected in a much higher proportion of nuclei and cytoplasm of C. perspic cells treated with Z-DEVD-FMK compared with correspondingly infected DMSO-treated cells (**Figure 3.4-7B**).

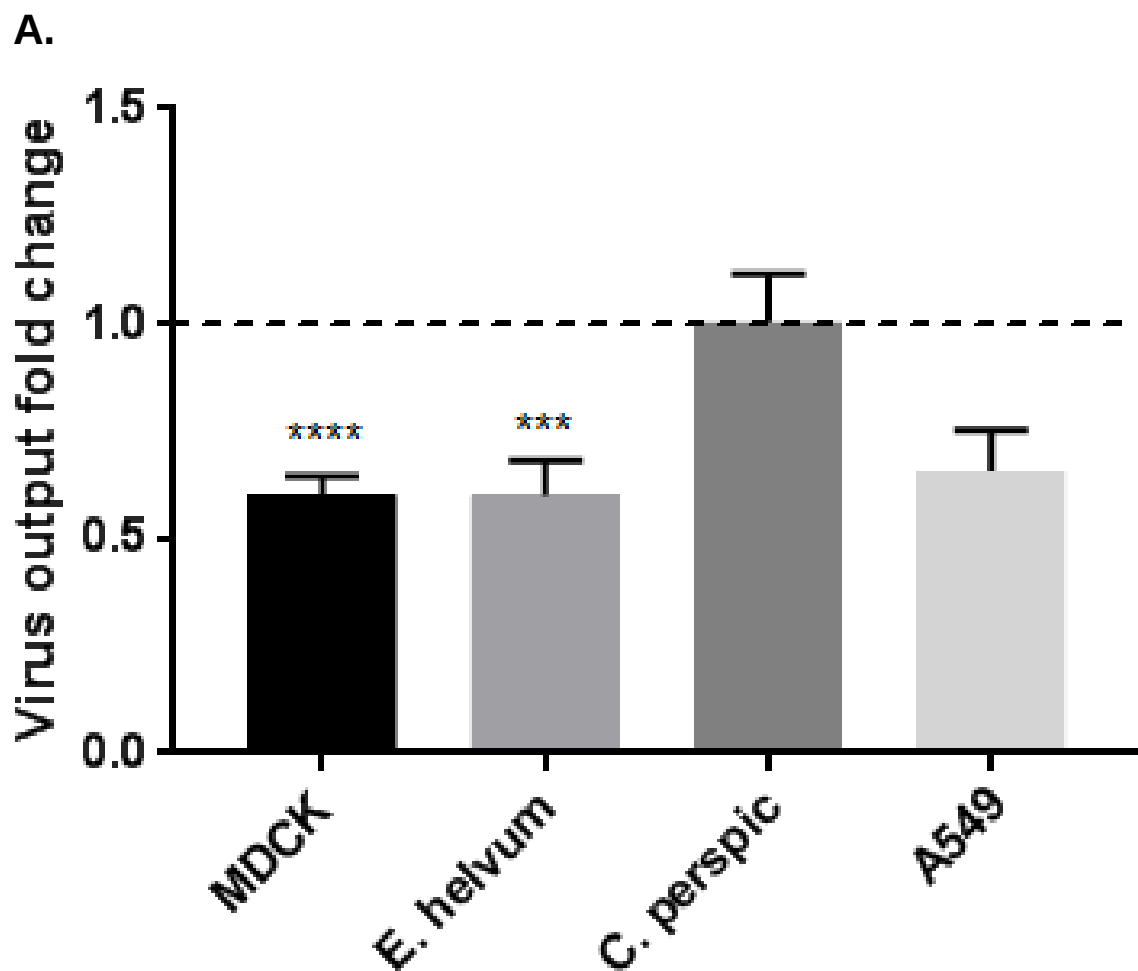
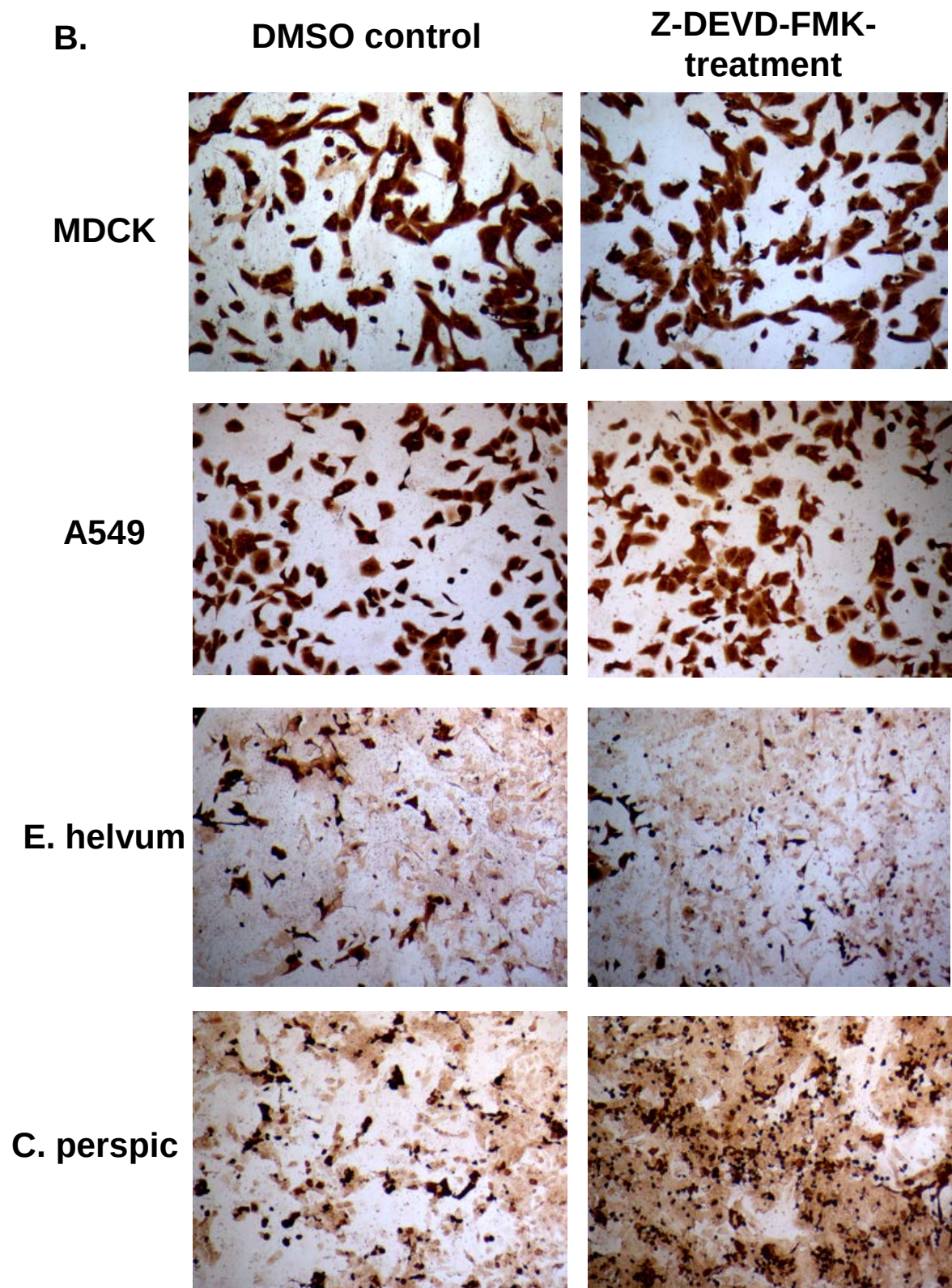


Figure 3.4--7 Inhibition of caspase 3 decreased the virus production from infected MDCK, A549 and E. helvum cells but not in infected C. perspic cells.

Caspase 3 inhibitor Z-DEVD-FMK, was applied for 30 min prior to infection with USSR H1N1 virus at MOI of 1.0 at 10 mM. (A) Inhibition of caspase 3 significantly decreased (***) $P \leq 0.001$, **** $P \leq 0.0001$) virus production from infected MDCK, E. helvum but did not affect the virus output from infected C. perspic cells compared with untreated counterpart cells. (B) Treatment with caspase 3 inhibitor resulted in the increase in infected C. perspic cells and the spread of viral NP into the cytoplasm. Caspase inhibition did not affect viral NP distribution in infected MDCK, A549 and E. helvum cells. Results are representative of three biological replicates and six technical replicates for each biological repeat. Dotted line (---) is equivalent to no change in activity.



3.5. Discussion

In Chapter two, lung epithelial cells of three diverse bat species, TB1-Lu from *Tadarida brasiliensis*, E. helvum and C. perspic, were found to be consistently more resistant to influenza virus infection than correspondingly infected MDCK cells in terms of reduced virus progeny, number of infected cells and reduced viral M-gene RNA expression. All bat cell types were shown to be more replication permissive to LPAI viruses, H2N3 and H6N1 viruses, than to human viruses, USSR H1N1 and pdm H1N1 viruses. These findings also found species-specific variation between the three species of bat cells: TB1-Lu cells were the least permissive to influenza virus infection, producing the least number of infected cells and progeny virus, whereas C. perspic and E. helvum cells were more susceptible to infection particularly with LPAI viruses.

Other studies (Dlugolenski et al. 2013; Hoffmann et al. 2013; Poole et al. 2014) have also shown variation in susceptibility between different species of bat, but there are no descriptions, prior to this study, of the mechanisms underlying this variation. TB1-Lu cells were consistently showed to be as one the least efficiently infected bat cell lines by several influenza viruses (Hoffmann et al. 2013; Poole et al. 2014) and fruit bat cells were as more susceptible to infection (Hoffmann et al. 2013).

To understand better variability in susceptibility and replication permissiveness between the three bat cell types, influenza virus entry was explored using the Viromer system. Bat cells were transfected with siRNA using Viromer system (Lipocalyx), developed to transfect cells by mimicking influenza virus endosomal escape mechanism. The acidification of virus HA results in a conformational change triggering the viral genome to release from the endosome to the cytoplasm. The viromers are synthetic polymers designed to assume the role of the HA fusion peptide.

Cells were transfected for 4 h, which allows time for siRNA to be taken up and accumulate near the cell nucleus. siRNA was prominent around the nuclei of MDCK, *E. helvum* and *C. perspic* cells (**Figure 3.4-1**), but, very little siRNA was found surrounding nuclei of TB1-Lu cells. This strongly suggests that the limited virus replication in TB1-Lu cells compared with the other bat cells tested, results from reduced virus attachment and entry.

Influenza virus attachment and entry is mediated by viral HA which recognises and binds to host receptors, and the specificity of such receptor-virus interactions is obviously an important factor in viral infection. For example, the interaction between the receptor for SARS-CoV, ACE2, and host receptors is considered to be a significant factor in susceptibility and permissiveness to infection, and also in viral pathogenicity (Ge et al. 2013). Accordingly, host receptors for avian and mammalian influenza viruses were examined for their presence on bat cells.

It is well established that host SA linked glycoproteins are the main receptors for influenza A virus attachment (Edinger et al. 2014). The HA of human influenza virus strains preferentially binds to SA α 2,6-Gal receptors whereas avian strains have an affinity for SA α 2,3-Gal receptors (Weis et al. 1988; Gamblin et al. 2004; Stevens et al. 2006). Pigs have been considered as a mixing vessel for the reassortment of influenza viruses as they possess both types of sialic acid receptor rendering them susceptible to both avian and human strains of influenza virus (Ma et al. 2008). Previous studies of TB1-Lu cells showed they possess canonical SA receptors (typical SA α 2,3-Gal and SA α 2,6-Gal receptors), functional for influenza virus infection (Poole et al. 2014) and removal of these SA receptors was shown to prevent influenza virus infection of TB1-Lu cells (Poole et al. 2014). Furthermore, desialylated TB1-Lu cells were shown to be successfully infected with vesicular stomatitis virus that bind and

attach host cells independent of SA moieties (Poole et al. 2014). While the study did show the functionality of SA receptors on TB1-Lu cells, it did not discriminate between different types of SA receptors and assumed that TB1-Lu cells were representative of all bat species. A recent study examined the SA receptors on the little brown bat (*Myotis lucifugus*) and showed abundant co-expression of SA α 2,3-Gal and SA α 2,6-Gal receptors in the respiratory tract (Chothe et al. 2017). Avian-type receptors were found to be predominantly expressed in the tracheal mucosa, similarly to in ducks (Kuchipudi et al. 2009). The tracheal mucosa in chickens, pigs and humans predominantly expresses human-type SA receptors (Kuchipudi et al. 2009; Nelli et al. 2010). Avian-type receptors were shown to be the main SA receptor type in luminal epithelial cells of the intestines in the little brown bat while other parts of the intestine including goblet cells, lamina propria, muscularis and serosa predominantly expressed SA α 2,6-Gal receptors. This pattern of expression is similar to the receptor pattern expressed in some families of wild birds (França et al. 2013) and in pigs (Nelli et al. 2010).

In the study described in this chapter, all three species of bat cells were examined for presence of both SA α 2,3-Gal and SA α 2,6-Gal receptors using plant lectins, MAAIL for SA α 2,3-Gal receptors and SNA for SA α 2,6-Gal receptors. *C. perspic* and *E. helvum* cells were shown to clearly possess both avian and human influenza virus receptors (**Figure 3.4-2**), however, only the SA α 2,3-Gal receptor (preferred by avian influenza viruses) could be strongly detected on TB1-Lu cells. While TB1-Lu cells were shown to strongly express avian-type SA receptors (**Figure 3.4-2**), the lack of SA α 2,6-Gal receptors likely prevents human viruses from efficiently binding to TB1-Lu cells. Indeed, despite being poorly infected by influenza viruses overall, TB1-Lu cells were found to be more susceptible to LPAI viruses than to human viruses. The

lack of human-type SA receptor presence on TB1-Lu cells likely contributes, at least in part, to the reduced endosomal uptake of siRNA by TB1-Lu cells (**Figure 3.4-1**) and to the reduced susceptibility of TB1-Lu cells to influenza virus infection compared with *E. helvum* and *C. perspic* cells, which were found to strongly express both SA receptor types.

SA receptors were collectively removed from the cell surface of bat cells to further explore the functional impact of SA receptors on influenza virus infection. Removal of SA receptors resulted in a clear reduction in production of viable virus progeny from all three species of bat cells infected with all four influenza viruses (**Figure 3.4-4B**), indicating the importance of SA α 2,3-Gal and SA α 2,6-Gal receptors for successful influenza virus infection. However, despite extensive removal of SA receptors, there remained some virus production suggesting the presence of an alternative SA-independent pathway for virus entry. SA receptors were previously considered the sole receptor for influenza virus infection (Stray et al. 2000), however the existence of an alternative pathway or multistep pathways has since been proposed. These findings are in line with previous studies (Stray et al. 2000) which showed that MDCK cells stripped of SA receptors could still be bind to and be infected with influenza viruses. It is unclear whether host factors are essential for virus binding and entry but those implicated in the process include C-type lectins (Londrigan et al. 2011), annexin V (Huang et al. 1996) and 6-sulfo-sialyl Lewis X (Gambaryan et al. 2008). The general removal of SA receptors was confirmed using lectin staining and fluorescent microscopy (**Figure 3.4-3**), however, this method may not be sensitive enough to target all SA moieties that may be present on the cell surface. Furthermore, plant lectin staining using MAAII and SNA did not measure the quantity of each receptor type on cells. It is possible that *C. perspic* and *E. helvum* cells may have more

SA receptors than TB1-Lu cells which potentially also contributes to reduced susceptibility of TB1-Lu cells to influenza virus infections. A useful extension of this study would be to characterise the SA receptor pattern in the other tissues and cell types, such as intestine and stomach, especially given the recent findings in *M. lucifugus* (Chothe et al. 2017) and also in other species such as pig and chicken (Kuchipudi et al. 2009; Nelli et al. 2010).

The discovery of two novel HA-like and NA-like glycoproteins possessed by influenza-like viruses, H17N10 and H18N11, has caused speculation that a novel, sialoside-unrelated, receptor might be possessed by bat cells. H17 and H18 were shown to possess 45% and 49.1% pairwise sequence identity, respectively, (Tong et al. 2012), comparable to the 49% sequence identity between H1-H16. These HA-like proteins contain conserved canonical sequences motifs. However, due to specific changes in sequence resulting in a distorted putative binding site and low thermostability (Sun et al. 2013), neither H17 or H18 were found to bind SA receptors including SA α 2,3-Gal, SA α 2,6-Gal or over six hundred other glycans (Sun et al. 2013). Similarly, N10 and N11 were shown to lack sialidase activity despite displaying the canonical sialidase fold (Zhu et al. 2012; Sun et al. 2013). The receptor for H17N10 and H18N11 virus attachment and entry has been speculated to be protein-based. Viruses such as paramyxoviruses have been shown to bind alternately between sialic acid receptors and protein receptors (Wu et al. 2014). Additionally, H17 and N10 contain Ig-like fold elements which provide a base to allow protein-based receptors interaction (Sun et al. 2013).

Receptors studies have revealed an important factor that likely contributes to the reduced susceptibility of TB1-Lu cells, compared with other bat cell types, to influenza virus infection. These findings have also highlighted the importance of SA

receptors in successful influenza virus infection and highlighted the possible existence of an alternative SA-independent pathway for infection.

Influenza viruses cause cell death following infection; however, the specific mechanism(s) and contribution of cell death pathways by which influenza virus triggers cell death is still not well understood (Hinshaw et al. 1994; Nogusa et al. 2016). Necrosis, caused by direct virus damage, is one of the major types of cell death during infection. The other well characterised mode of cell death is programmed cell death, or apoptosis, part of the cell's response to many virus infections. Cells undergoing necrosis swell and rupture whereas apoptosis is characterised by morphological changes including membrane blebbing, chromatin condensation, DNA fragmentation, cytoskeletal disruption and formation of membrane bound apoptotic bodies (Brydon et al. 2005). Apoptosis is also distinctly a non-inflammatory event, unlike necrosis (Brydon et al. 2005). Another mode of cell death, known as necroptosis or programmed necrosis, exhibits similar morphological characteristics as necrosis such as cell swelling and inflammation, organelle dysfunction and rupture of the plasma membrane (Weinlich et al. 2016). Despite being a regulated process induced by apoptosis-inducing stimuli, unlike apoptosis, necroptosis causes cell death independent of caspase activity. Both necroptosis and apoptosis can be induced by TNF α signalling. Complexes known as necrosomes, containing kinases receptor-interacting protein kinase 1 (RIPK1) and RIPK3, can activate either necroptosis or apoptosis depending on availability of effector proteins (Cho et al. 2009; Cook et al. 2014a; Chan et al. 2014). During necroptosis, RIPK3 activates pro-necroptotic pseudokinase mixed-lineage kinase-like protein (MLKL) inducing oligomerisation and translocation to membrane where MLKL is thought to form pores causing influx

of positively charged ions, an early event in TNF α -induced necroptosis (Cai et al. 2014; Chen et al. 2014; Wang et al. 2014).

Cell death in bat cells infected with USSR H1N1 virus and LPAI H2N3 virus was determined by flow cytometry by assessing membrane integrity. All three bat cell types were shown to be more viable during each infection than correspondingly infected MDCK cells (**Figure 3.4-5**). Cell death was greater in bat cells infected with LPAI H2N3 virus compared with cell infected with USSR H1N1 virus, corroborating with previous findings of bat cells being more replication permissive to avian viruses. *E. helvum* cells exhibited the highest cell death during influenza virus infection, among bat cell types, in line with replication permissiveness of *E. helvum* cells to infection. Interestingly, *C. perspic* cells, infected with USSR H1N1 virus, exhibited remarkably low cell death, comparable with cell death in TB1-Lu cells, despite appearing relatively more susceptible and permissiveness to influenza virus infection. Furthermore, *C. perspic* cells were the most viable cells when infected with LPAI H2N3 virus exhibiting less than 5% cell death. These findings suggest *C. perspic* cells may serve as reservoir hosts to conventional human influenza and LPAI viruses as virus replication is able to occur without damaging effects to the host cell.

As necrosis, necroptosis and late apoptosis all result in plasma membrane disruption, flow cytometry using amine-binding dye was unable to determine the mode of cell death. Influenza virus infection has been shown to induce parallel pathways apoptosis and necroptosis via stimulation of RIPK3 (Nogusa et al. 2016). Activation of RIPK3 in infected cells has been shown as a result of influenza virus infection rather than induced by surrounding uninfected cells. It is unclear whether the protein responsible for sensing and subsequently activating RIPK3 is a currently unknown host protein or an influenza virus-encoded protein (Nogusa et al. 2016).

Virus-induced apoptosis was investigated by measuring caspase 3/7 activity. Caspases are a family of cysteinyl proteins central to apoptosis (Ludwig et al. 2006). Caspases fall into two main groups, upstream initiator caspases, such as caspase 8 and caspase 9, and downstream effector caspases which include caspase 3, caspase 6 and caspase 7 (Ludwig et al. 2006). Initiator caspases, synthesised as inactive procaspases, or zymogens, once activated by appropriate stimuli subsequently cleave downstream effector caspases resulting in the induction of a caspase cascade degrading cellular components in a controlled manner. Caspase 3 has been shown to be crucial to the regulation of apoptosis as it drives a positive feedback loop in the apoptotic cascade. Furthermore, the level of inactive procaspase 3 has been shown to determine how much effect the apoptotic stimuli has on the cell (Jänicke et al. 1998).

Caspase 3/7 induction was shown to be lowest in TB1-Lu cells following infection with both USSR H1N1 virus and LPAI H2N3 virus (**Figure 3.4-6**). This was unsurprising due to the potential block in virus entry rendering TB1-Lu cells less susceptible to influenza virus infection. Caspase 3/7 levels were highest in infected C. perspic cells among bat cell types, with an increase of more than 8-fold compared with corresponding mock-infected control cells. The markedly high caspase 3/7 activity in infected C. perspic cells was unexpected as cell death in infected C. perspic cells was found to be very low (**Figure 3.4-5**). In the other two bat cell lines, the level of virus-induced apoptosis correlated with cell death percentage.

These findings suggest influenza virus infection induced high levels of necrosis or necroptosis in MDCK and E. helvum cells whereas infection induced apoptosis in C. perspic cells. Two models currently exist to explain the switch between apoptosis, necroptosis and necrosis. The stochastic model is based on cell type-specific differences in the abundance of MLKL compared to RIPK1 or RIPK3 along with other

effector molecules like caspase 8 and FADD (Cook et al. 2014b). Influenza virus infection was shown to activate MLKL without caspase 8 suppression (Nogusa et al. 2016), unlike during DNA virus infection whereby caspase 8 suppression is essential (Weinlich et al. 2016), supporting the stochastic model. The other model is based on a molecular switch mechanism where ubiquitinylation of RIPK1 and RIPK3 regulate the switch between modes of cell death (Vandenabeele et al. 2010).

Both necroptosis and apoptosis have been shown to function as a host defence mechanism to limit virus replication (Brydon et al. 2005; Weinlich et al. 2016). Necroptosis has been shown to aid viral clearance by limiting the infected cell lifespan preventing virus spread, inducing necroptosis-mediated inflammatory responses and activating the adaptive immune response through releasing immunogenic danger-associated molecular patterns (DAMPs) from dying cells (Nogusa et al. 2016; Weinlich et al. 2016). RIPK3^{-/-} mice were found to more susceptible to influenza virus infection and have diminished T-cell recruitment (Nogusa et al. 2016). Inherently resistant Pekin and mallard duck cells have been shown to undergo programmed cell death when infected with influenza viruses (Kuchipudi et al. 2012a). The induction of caspase activity was also shown to correlate with reduced virus production suggesting avian hosts may employ apoptosis to contain virus replication and reduce virus spread to secondary sites (Kuchipudi et al. 2012a). Inhibition of caspase induction in infected *C. perspic* cells was found not to affect virus production. However, a higher proportion of caspase-inhibited *C. perspic* cells were infected with USSR H1N1 virus compared with correspondingly infected DMSO-treated cells (**Figure 3.4-7B**). This result suggest that inhibition of caspase 3 increases susceptibility of *C. perspic* cells to virus infection but does not affect replication permissiveness. Collectively, speculatively, these findings suggest *C. perspic* cells may use apoptosis as a mechanism to mitigate

the damaging effects of influenza virus infection while still being replication permissive.

Although apoptosis upregulation can function as an anti-viral host response, it has been recognized that influenza viruses are able to induce apoptosis for their benefit through the activation of caspases in both permissive and non-permissive cultured cells and also *in vivo* (Takizawa et al. 1993; Fesq et al. 1994; Hinshaw et al. 1994; Mori & Komatsu 1995; Ito et al. 2002). Increased apoptosis has been linked to efficient replication of influenza viruses and increased virulence (Tripathi et al. 2013). Thus, it is still under debate whether apoptosis induction favours influenza viruses or the host (Ludwig et al. 2006). The exact mechanism for the induction of apoptosis by influenza viruses is not yet known but many influenza virus proteins including NP, NS1, M1, M2, NA and PB1-F2 have been shown to contribute to cell death by, likely, targeting host cellular factors (as reviewed by Tripathi et al., 2013). The overexpression of Bcl-2, an anti-apoptotic protein, lead to a reduction in virus production and correlated with the misglycosylation of HA (Hinshaw et al. 1994; Olsen et al. 1996) implicating HA in apoptosis induction. NS1 has been shown to induce apoptosis when ectopically expressed (Schultz-Cherry et al. 2001) and NP has been shown to induce apoptosis in human airway epithelial cells and modulate the protein kinase-R (PKR) pathway and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway, which may contribute to cell death (as reviewed by Tripathi et al., 2013).

The complex nature of apoptosis induction in the context of influenza virus infection makes it difficult to make any clear cut conclusions regarding whether bat cells use apoptosis as an anti-viral mechanism to limit virus replication and spread. Apoptosis is further explored in the next chapter in the context of NF- κ B and other innate immune system signalling pathways shown to confer anti-viral resistance.

4. Innate immune pathways in the modulation of influenza virus production in bat cells

4.1. Chapter summary

A key mechanism used to control influenza virus infection in resistant hosts is the mediation of a robust yet not overly excessive pro-inflammatory response to combat virulent virus infection (Kuchipudi et al. 2012; Nelli et al. 2012) without the risk of cytokine dysregulation.

This chapter explored the type I IFN and pro-inflammatory response and whether it contributed to resistance to influenza virus infection in bat cells. To this end, two pathways involved in the IFN and pro-inflammatory response, NF- κ B and JAK/STAT were inhibited to examine the effect on virus replication in infected bat cells.

Inhibition of JAK/STAT pathway was found not to affect virus production from all three types of bat cells infected with USSR H1N1 virus but significantly increased virus production from infected MDCK cells. This finding suggests the JAK/STAT pathway may not be involved in the resistance of bat cells to influenza virus infection. Inhibition of the NF- κ B using Sc75741 resulted in an increase in virus production from bat cells, however inhibition using a different compound, acetylsalicylic acid (ASA), resulted in a decrease in virus production. Furthermore, overexpression of IKK2 and RelA had no large effects on virus production. These conflicting findings potentially allude to tight regulation of NF- κ B signalling to control pro-inflammatory responses, a mechanism which may have arisen during flight evolution.

4.2. Introduction

This section provides an overview of the IFN and pro-inflammatory response- from receptor recognition to production of ISGs- and the JAK/STAT and NF- κ B signalling pathways.

4.2.1. *Recognition of viral pathogens*

Viral pathogens are sensed by PRRs which detect and recognise PAMPs on viral pathogens. There are three classes of PRRs: TLRs, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). TLRs recognise viral RNA in the endosomes while RLRs and NLRs detect virus in the cytoplasm. TLR7/8 detects viral ssRNA and TLR3 detects viral dsRNA, likely in currently unidentified RNA structures in influenza virus-infected cells (Schulz et al. 2005). RLRs, RIG-I and melanoma differentiation-associated protein 5 (MDA5) detect viral RNA in the cytosol through mitochondrial antiviral signalling (MAVS) signalling adaptor (Rehwinkel et al. 2010; Baum et al. 2010; Luo et al. 2011). MAVS contain caspase recruitment domain (CARD) which activates and associate with viral RNA PAMPs (Iwasaki & Pillai 2014) As influenza virus replicates in nucleus, it is currently unclear how RIG-I and viral RNA come into contact. However, it has been recently shown that RIG-I and viral RNA can localise in stress granules (Onomoto et al. 2012), aggregations of RNA and protein in the cytoplasm that accumulate during cellular stress, including oxidative, heat shock and endoplasmic reticulum stress (Onomoto et al. 2012). The NLRP3 inflammasome, expressed by myeloid cell types (Guarda et al. 2011) and human bronchial epithelial cells (Pothlichet et al. 2013), is stimulated by membrane damage, stress and infection (Martinon et al. 2009). Activation of the inflammasome is induced by signals provided

by influenza virus, such as ssRNA, and by TLR, IL-1 receptor and TNFR signalling (Martinon et al. 2009). Activation triggers the production of cytokines, IL1 β and IL18, and induces pyroptosis, an inflammatory form of cell death (Bergsbaken et al. 2009). TLR-dependent and TLR-independent (RIG-I) signalling result in the induction of type I IFNs through ‘master regulators’ interferon-regulatory factor (IRF) 3 and IRF7 (Hiscott 2007) which are in turn activated through TNF-receptor associated factors (TRAFs). RIG-I is linked by MAVs to TRAF3 which induces non-canonical I κ B kinases IKK ϵ and TBK1, to phosphorylate IRF3 and IRF7. Phosphorylated IRF3 and IRF7 form hetero- and homo-dimers and drive transcription. MAVS also activates the IKK α -IKK β -IKK γ complex and through a series of phosphorylation events leads to the activation of NF- κ B, described further in 4.1.4 (Kai Chan & Gack 2016). Activated NF- κ B translocates to the nucleus and, with activator protein 1 (AP1), drives transcription of type I and type III IFN and pro-inflammatory cytokines and chemokines. NF- κ B is postulated to have separate roles during distinct stages of infection, described further in 4.1.4.1. Secreted IFNs signal through their receptors in a paracrine and autocrine manner to establish an antiviral state.

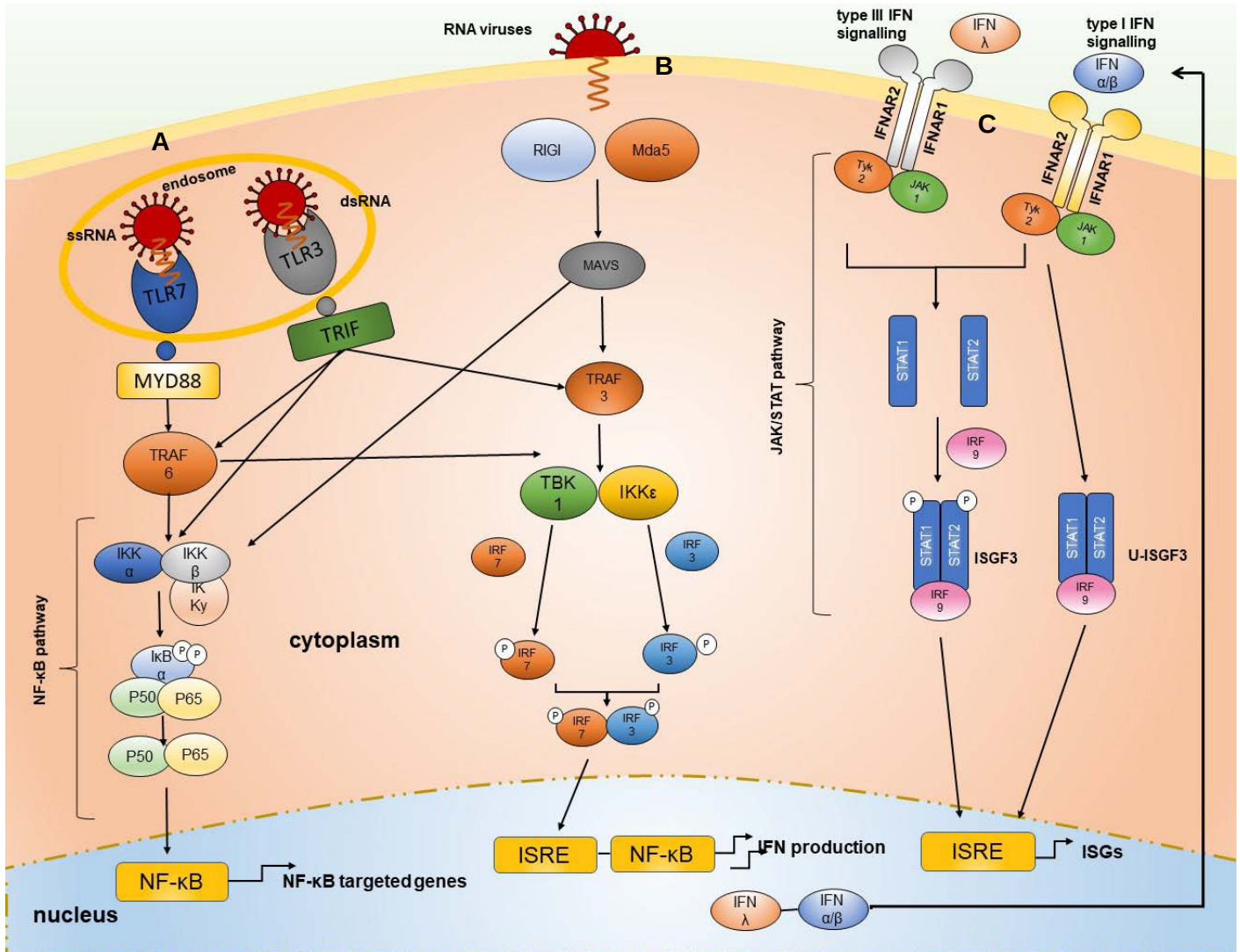


Figure 4.2-1 IFN response pathway (Original)

(A) Endosomal pathway: Double-stranded (ds)RNA viruses are recognised by Toll-like receptor 3 (TLR3) whereas single-stranded RNA recognised by TLR7. TLR7 induces NF-κB activation through tumour necrosis factors (TNF) receptor-associated factor 6 (TRAF6).

TLR3 recognition causes phosphorylation of tyrosine residues in TLR3 which leads to dimerisation of TLR3 and activation of signalling complex via TLR adaptor molecular 1 (TRIF). This leads to the emergence of two pathways: TRAF3 and non-canonical IκB kinases TANK-binding kinase (TBK1) and IKKε lead to phosphorylation of interferon-regulator factor 3 (IRF3). Phosphorylated IRF3 and IRF7 form homo- and hetero-dimers and translocate to the nucleus and drive transcription of type I and type III interferons (IFNs). The other pathway signals through TRAF6 causing the activation of NF-κB.

(B) Cytosolic signalling: RNA virus infection causes activation of retinoic acid-inducible gene I (RIG-I) pathway. RNA helicases, RIG-I and Mda5, recognise dsRNA and ssRNA viruses and use MAVS as an adaptor. MAVS functions similar to TRIF, activates same transcription factors (IRF3 and IRF7) causing induction of IFNs.

(C) IFN signalling: type I IFNs (IFNα/β) and type III IFNs (IFNλ) bind to type I IFN (IFNAR) and type III (IFNλ) receptors respectively. Upon binding, receptors associate with a Janus kinase (JAK) family member and subsequent phosphorylation and dimerisation of Signal Transducer and Activator of Transcription (STAT) proteins. Phosphorylated STAT dimers form a ternary complex with IRF9 (ISGF3) and drive transcription of ISGs in the nucleus.

Constitutive expression of type I IFNs leads to the unphosphorylated STAT dimers forming a complex with IRF9 (Unphosphorylated (U-)ISGF3) to maintain long term expression of ISGs.

4.2.2. *The IFN response*

4.2.2.1. *Classes of IFN*

The first line of host antiviral defence in response to virus infection is driven by the induction of IFNs, a multifunctional family of cytokines. There are three types of IFNs: type I IFNs, type II IFNs and the recently discovered type III IFNs.

Canonical type I IFN signalling is considered as the prototypical mechanism to viral resistance as it induces transcription of several hundred ISGs which serve to establish an antiviral state (Iwasaki & Pillai 2014). ISGs limit virus production through the inhibition of viral transcription, translation, replication and degradation of viral nucleic acids (MacMicking 2012; Saka & Valdivia 2012). There are many subtypes of type I IFNs, the most established being IFN α and IFN β . There are 13 IFN α isoforms which are encoded for by 14 genes but only a single gene encodes IFN β . Most cell types have been shown to produce IFN β whereas IFN α is predominantly produced by haematopoietic cells (Ivashkiv & Donlin 2013).

There is only one subtype of type II IFN, IFN γ , and this subtype of IFN is produced by T lymphocytes, NK cells and plasmacytoid dendritic cells (MacMicking 2012). IFN γ is very distinct from type I and also type III IFNs and does not share the same structural homology as type I IFNs (Pestka & Langer 1987; Chen et al. 2004; Pestka et al. 2004). However, due to the ability IFN γ to interfere with virus infection it is considered as an interferon (Pestka et al. 2004), despite limited direct anti-viral effects (Wack et al. 2015). Like, type I IFNs, type II IFN signalling induces the transcription of ISGs. However, although this subset of ISGs has been shown to overlap with type I IFN-induced ISGs, it is still a distinct subset of ISGs. For example, Mx and OAS

proteins are induced by type I IFN signalling and IRF1 is typically induced by type II IFN signalling (Der et al. 1998)

Type III IFNs are the most recent class of IFN to be discovered. In humans, the four types of type III IFN, IFN λ 1 (IL29), IFN λ 2 (IL28A), IFN λ 3 (IL28B) and IFN λ 4 (Prokunina-Olsson et al. 2013), are predominantly expressed by lung epithelial cells, mucosal tissue and intestinal epithelium. Type I and type III IFNs are usually co-expressed, such as by lung epithelium, however in the intestinal epithelia only responds to type III IFNs (Pott et al. 2011). Type III IFNs signal through many of the same pathways and utilise the similar mechanisms as type I IFNs, thus it is not surprising that type III IFNs also exhibit similar antiviral activity as type I IFNs (Wack et al. 2015). The viral tropism of type I IFNs and type III IFNs allow both systems to function non-redundantly to control virus infection. As gut cells respond solely to type III IFNs, these IFNs restrict virus replication in the intestines but type I IFNs mediate resistance systemically (Mahlakõiv et al. 2015). Lung epithelial cells produce both type I and type III IFNs during infection. During influenza virus infection, higher levels of type III IFNs were produced from mouse airway epithelial cells than type I IFNs (Jewell et al. 2010; Crotta et al. 2013), however, it has been speculated that the response may shift to type I IFNs once immune cells start producing IFN (Wack et al. 2015).

4.2.2.2. IFN signalling and activation of the JAK/STAT pathway

The JAK/STAT pathway has been described as a relatively uncomplicated and evolutionary pathway (Fleming 2016) as key factors are encoded by only several non-

redundant genes (Fleming 2016). Induction of JAK/STAT pathway is activated by the binding of IFN to its receptor. The three classes of IFN signal through different heterodimeric transmembrane receptors. All type I IFNs bind to a common receptor, IFN α receptor (IFNAR), composed of two subunits, IFNAR1 and IFNAR2 (Stark & Darnell 2012). The receptor for IFN γ is made up of IFNGR1 and IFNGR2 subunits. Type III IFNs bind to IFN λ receptor, which is composed of two chains, IFNLR1 (IL28R α) and IL10R2 (Boehm et al. 1997). Upon receptor binding, each chain associates with a member of the Janus activated kinase (JAK) family. IFNAR1 and IL10R2 associate with tyrosine kinase 2 (TYK2) (Darnell et al. 1994; Platanias 2003; Platanias 2005), IFNAR2, IFNGR1 and IFNLR1 associate with JAK1 and IFNGR2 associates with JAK2 (Bach et al. 1997; Platanias 2005). The activation of JAKs is followed by the phosphorylation of cytoplasmic transcription factors, Signal Transducer and Activator of Transcription (STATs) proteins. In the case of type I and type III IFN signalling, STAT1 and STAT2 dimerise and translocate to the nucleus (Stark & Darnell 2012). In the nucleus, the dimer binds with IFN-regulatory factor 9 (IRF9) forming IFN-stimulated gene factor 3 (ISGF3). The transcription of ISGs is induced by the binding of ISGF3 complex to DNA sequences known as IFN-stimulated response elements (ISREs) as they are present in certain ISG promoters (Ivashkiv & Donlin 2013).

Unlike type I and type III IFN signalling, ISGF3 is not induced by type II IFN and therefore IFN γ cannot induce transcription of ISGs with ISREs in their promoter. Instead, STAT1 homodimers translocate to the nucleus and bind to distinct IFN gamma-activated sequence (GAS) to initiate transcription of ISGs (Stark et al. 1998; Aaronson & Horvath 2002; Boehm et al. 1997). Type I IFNs can also induce

transcription of ISGs with GAS elements in their promoter region through other STAT heterodimers and homodimers (as reviewed in Platanias, 2005).

4.2.3. *NF- κ B pathway*

NF- κ B, a transcription factor, is a central mediator of immune response, mediating inflammation proliferation and apoptosis (Bonizzi & Karin 2004; Kumar et al. 2008a). Within minutes of exposure to pathogenic stimuli such as viral and bacterial infections, pro-inflammatory cytokines or UV radiation (Flory et al. 2000), the pathway is activated promoting the transcription of over 150 genes including pro-inflammatory cytokines and chemokines, adhesion molecules and enzymes involved in the production of apoptotic inhibitors (Flory et al. 2000; Hayden & Ghosh 2004).

There are five members of the NF- κ B family in mammals: NF- κ B1 (p105/p50), NF- κ B2 (p100/p52), RelA (p65), RelB and c-Rel. The NF- κ B proteins form dimers; p50 and p52 homodimers repress transcription whereas heterodimers containing RelA or c-Rel activate transcription. RelB can be either a transcriptional activator (Ryseck et al. 1992) and repressor (Ruben et al. 1992). All members of the NF- κ B family possess a Rel-homology domain (RHD) which contain an NLS (Ghosh et al. 1998). Inhibitory I κ B proteins bind to the RHD, interfere with the NLS and block translocation causing NF- κ B dimers to be retained in the cell cytoplasm (Bonizzi & Karin 2004).

The I κ B kinase (IKK) complex is made up of IKK α and IKK β catalytic subunits and the regulatory IKK γ subunit. Once activated, the IKK subunits dimerise and catalyse the degradation of I κ B proteins through phosphorylation and polyubiquitination (Ghosh et al. 1998). The degradation of the I κ B proteins allow the NF- κ B dimers to be translocation to the nucleus where gene transcription occurs (Ghosh et al. 1998).

4.2.4. *The role of NF- κ B in IFN response*

NF- κ B has been shown to have temporally distinct functions in mediating the type I IFN response. RelA, a subunit of NF- κ B, has been found to maintain constitutive basal autocrine *IFN β* signalling in uninfected cells (Basagoudanavar et al. 2011). During early virus infection, NF- κ B forms part of the enhanceosome on the *IFN β* promoter along with other transcription factors, ATF-2/c-Jun and IRFs (Thanos & Maniatis 1995; Thanos 1996). The enhanceosome is responsible for driving transcription of *IFN β* initiating the interferon response (Thanos & Maniatis 1995; Thanos 1996). During late stages of virus infection, *IFN β* expression is mediated by activated IRFs rendering NF- κ B redundant. The role of NF- κ B subsequently shifts to regulating pro-inflammatory responses and cell survival signals (Balachandran & Beg 2011; Basagoudanavar et al. 2011).

4.2.5. *NF- κ B as a pro-viral factor in influenza virus infection*

NF- κ B signalling is mainly regarded as an essential component of the innate immune system to induce the transcription of pro-inflammatory cytokines, chemokines to mediate an antiviral response against infection (Ghosh et al. 1998). NF- κ B has also been implicated in maintaining constitutive basal expression of *IFN β* in the early stages of virus infection as part of the *IFN β* enhanceosome (Balachandran & Beg 2011; Basagoudanavar et al. 2011). However, there have been numerous studies which have shown NF- κ B to function in a virus-supportive manner (Wurzer et al. 2004; Ludwig et al. 2006; Mazur et al. 2007; Kumar et al. 2008). NF- κ B has been shown to

support virus propagation of a several influenza viruses in various cell lines (Wurzer et al. 2004). Moreover, active NF- κ B signalling was shown to be a pre-requisite for successful influenza virus infection (Nimmerjahn et al. 2004). Cells with low levels of NF- κ B signalling, such as Epstein-Barr immortalised B cells, were shown to be resistant to influenza virus infection, however, upon activation of NF- κ B, these cells became susceptible to infection (Nimmerjahn et al. 2004). The supportive function of NF- κ B to influenza virus infection has been attributed, in part, to reduced NF- κ B-dependent tumour necrosis factor (TNF)-related apoptosis (Wurzer et al. 2004; Mazur et al. 2007). TNF-related apoptosis induces the expression of TNF superfamily ligands FasL and TNF-related apoptosis-inducing ligand (TRAIL). FasL and Fas were shown to be induced by influenza virus infection and *FasL* and *Fas* gene expression was found to be regulated by NF- κ B (Wurzer et al. 2004). Inhibition of FasL and TRAIL resulted in a decrease in virus production. Additionally, recombinant TRAIL and FasL were able to reverse the effects NF- κ B deficiency and increase virus production (Wurzer et al. 2004). TRAIL and FasL were proposed to promote vRNP export by inducing caspase activation (Wurzer et al. 2004b). It is well known caspases are induced by influenza virus infection (Schultz-Cherry et al. 2001; Lowy 2003; Ludwig et al. 2006) and other studies showed caspase 3 is required for efficient virus replication. Furthermore, inhibition of caspases, particularly caspase 3, caused nuclear retention of vRNP complexes (Wurzer et al. 2003). Mazur et al., (2007) corroborated these findings though the use of acetylsalicylic acid (ASA), a reported NF- κ B inhibitor (Yoo et al. 2000). Caspases, potentially, promote vRNP export through disruption of the nuclear membrane allowing for the release of vRNPs (Ludwig et al. 2006). However, it has also been shown that NF- κ B inhibition does not affect vRNP trafficking from the nucleus (Kumar et al. 2008). Immunofluorescence assays comparing infected

A549 cells treated with NF- κ B inhibitors, Bay11 and PDTC, with DMSO-treated control cells and cells treated with U0126, a known MEK inhibitor which cause vRNP nuclear retention (Pleschka et al. 2001), showed no discernible difference between NF- κ B inhibited cells and control counterpart cells (Kumar et al. 2008b). More conflicting findings resulted from another study which showed NF- κ B activity is not a requirement for influenza virus replication in several types of cells including MDCK, HEK293 and A549 cells (Bernasconi et al. 2005).

In this chapter, two key signalling pathways involved in IFN response, JAK/STAT and NF- κ B pathway, were examined in bat cells to explore the impact of these pathways on resistance to influenza virus infection.

4.3. Materials and methods

The roles of the JAK/STAT and NF- κ B pathways in virus resistance in bat cells were examined by inhibiting these pathways and determining the impact on virus production from infected bat cells. Bat cells were also transfected with activators and repressors of NF- κ B signalling to examine the effect of overexpression of NF- κ B on virus production from infected bat cells.

4.3.1. *Inhibitors*

Pyridone 6 (P6) (Merck), a JAK inhibitor, was applied at 5 μ M to cells for 20 h and incubated at 37°C prior to infection (Delgado-Ortega et al. 2014). NF- κ B inhibitors, Sc75741 (Abmole) (Ehrhardt et al. 2013) and acetylsalicylic acid (ASA) (Sigma) (Mazur et al. 2007) applied 30 min prior to infection. NF- κ B inhibitors were used at 5 mM. Cells were rinsed twice with PBS and then infected with USSR H1N1 virus at MOI of 0.5. Simultaneously, DMSO-treated cells were infected with USSR H1N1 virus to be used as comparison controls. After 2 h, cells were rinsed twice with 1x PBS and fresh infection media (see **Table 2.2-1**) was added with inhibitors and incubated for a further 22 h before titration onto fresh confluent MDCK cells.

4.3.2. *Expression plasmids related to NF- κ B signalling*

Cells were transfected with expression plasmids encoding constitutively active IKK2 (S177E and S181E), inactive IKK2 (K44M) cDNA encoding mutant forms of IKK2; kinase inactive IKK2 (K44M) (Mercurio et al. 1997) and RelA fused to EGFP reported gene (Chen et al. 2001). pBK-CMV (Agilent) was used as a control vector.

4.3.3. *Transient transfection*

Plasmids (see 4.3.2) were prepared using MaxiPrep kit (Qiagen) according to manufacturer's instructions and stored at -20°C. Cells were grown in 12-well plates

overnight and transfected with plasmids the following day. The transfection mixture consisted of 0.5 µg/µl plasmid and appropriate volumes of Lipofectamine 2000 and Opti-MEM (Invitrogen) according to manufacturer's instructions. The transfection mixture was added to cells and incubated for an appropriate duration at 37°C. After incubation, cells were rinsed with PBS and medium was replaced with fresh complete medium. Transfection efficiency was determined by fluorescence microscopy. Cells were infected with USSR H1N1 virus at MOI of 0.5 48 h post transfection.

4.4. Results

4.4.1. The JAK-STAT pathway did not appear to play a major anti-viral role in bat cells

To assess the role of JAK/STAT signalling during influenza virus infection, cells were treated with 5 µM pyridone 6 (P6), a JAK inhibitor, or with DMSO as control for 20 h prior (Delgado-Ortega et al. 2014) prior to infection with USSR H1N1 virus at MOI of 0.5.

There was no significant change in the virus output of all three bat cell types treated with P6 compared with counterpart DMSO controls (**Figure 4.4-1**) however, virus output of treated MDCK cells increased more than 2-fold compared with corresponding DMSO-treated cells.

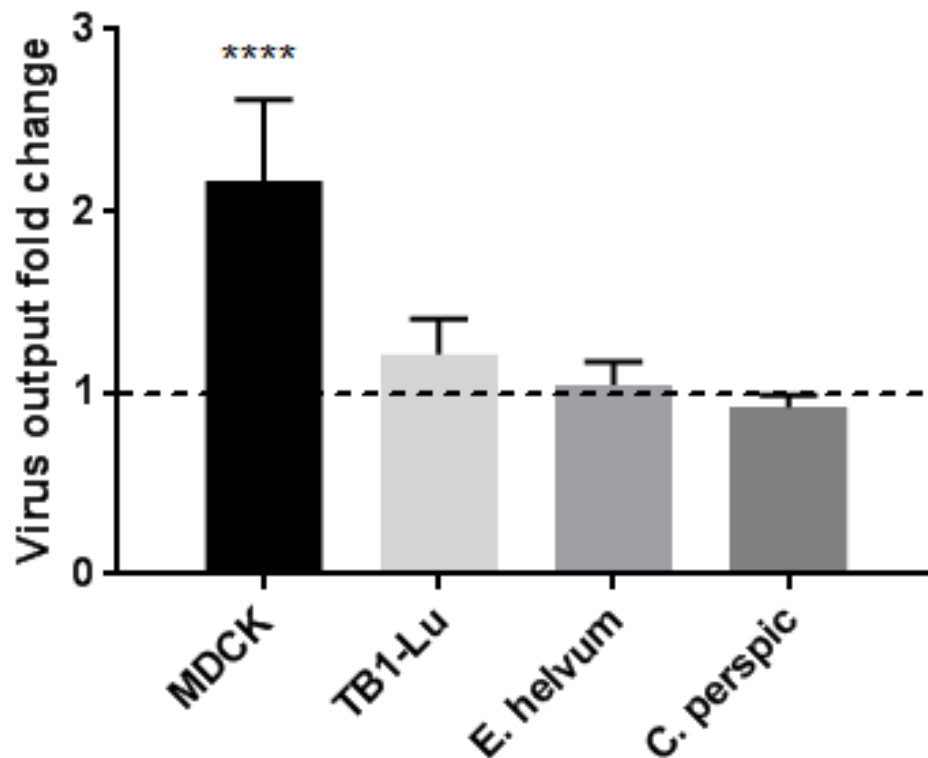


Figure 4.4-1 Inhibition of JAK/STAT pathway did not affect the virus output of infected bat cells.

Cells were treated with a JAK I inhibitor (P6) for 20h prior to infection with human USSR virus. There was no notable difference in virus output between inhibited *E. helvum* and *C. perspic* cells and their untreated counterparts. MDCK cells showed a marked significant (**** $P \leq 0.0001$) 2-fold increase in virus output when treated with the JAK I inhibitor. Results shown are representative of two biological replicates and six technical replicates for each biological repeat. Dotted line (---) is equivalent to no change in activity.

4.4.2. *Modulation of the NF- κ B pathway affected virus production in bat cells*

While the NF- κ B pathway has been regarded as anti-viral, involved in the expression of pro-inflammatory cytokines and chemokines, such as TNF α and IL1, adhesion molecules and enzymes involved in the production of apoptotic inhibitors (Ghosh et al. 1998), several studies have also shown NF- κ B have supportive functions for influenza viruses (Nimmerjahn et al. 2004; Wurzer et al. 2004; Ludwig et al. 2006; Mazur et al. 2007; Kumar et al. 2008; Ludwig & Planz 2008).

To assess the effect of the NF- κ B pathway on viral resistance in bats, the three species of bat cells were treated with two different NF- κ B inhibitors, acetylsalicylic acid (ASA) and novel compound Sc75741. Inhibitors were separately applied to cells at 5 nM prior to infection for 30 min. After 2 h of initial USSR H1N1 virus infection at 0.5 MOI, cells were rinsed with PBS and subsequently replaced with fresh infection media containing the same inhibitor.

The application of Sc75741 resulted in reduced virus output from human A549 cells and MDCK cells (**Figure 4.4-2A**). Treated bat cells responded differently: virus output from all three bat cell types was increased in response to Sc75741 treatment compared with untreated cells.

Inhibition of NF- κ B via ASA treatment resulted in a significant decrease in virus progeny of all cell types (**Figure 4.4-2B**).

Bat cells were transiently transfected with plasmids encoding human RelA/p65, a constitutively active form of IKK2/IKK β , and an inactive form of IKK2/IKK β . Control cells were transfected with an empty pBK-CMV. As a control, pig tracheal (NPTr) cells were transfected with the same plasmids as the effect on virus output has been well characterised in previous unpublished work (data not shown).

Multiple alignment of human and bat protein sequences of RelA and IKK2 showed there was a high percentage identity between bat and human for RelA and IKK2 proteins (**Table 4.4-1**) indicating transfection with human IKK2 and RelA in bat cells should result in similar functionality.

Transiently transfections revealed species-specific differences between bat cells (**Figure 4.4-3**). *C. perspic* cells produced fewer viral progeny when transfected with the inactive form of IKK2 and with RelA. Conversely, the constitutively active and inactive forms of IKK2 appeared to have no effect on the virus output of infected *E. helvum* cells, however, the overexpression of RelA did significantly increase virus output.

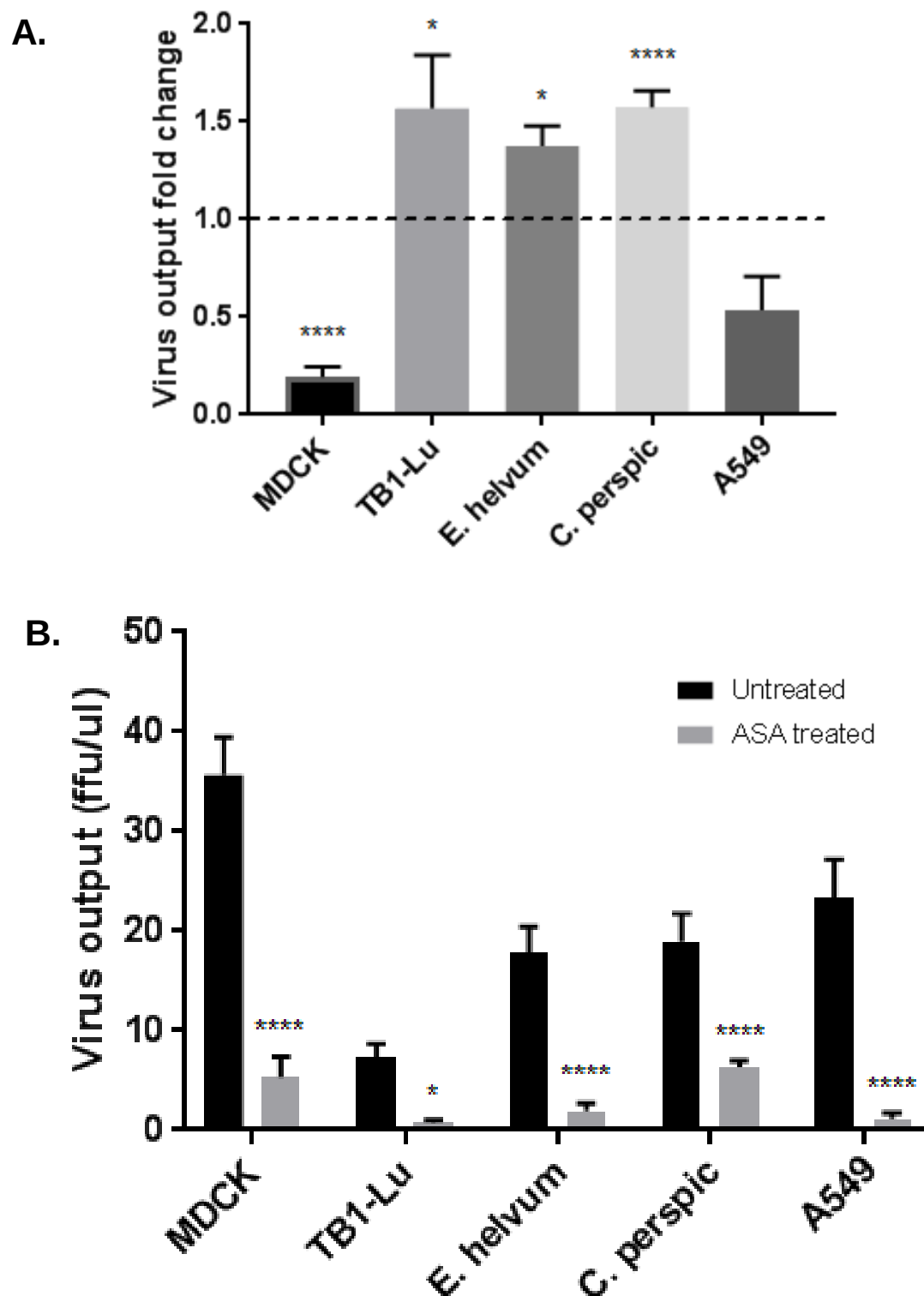


Figure 4.4-2 Modulation of the NF- κ B pathway affects the virus production of infected bat cells. Cells were treated NF- κ B inhibitors at 5 mM for 30 min prior to infection. The virus output of TB1-Lu, E. helvum and C. perspic cells treated with Sc75741 significantly increased 1.3-1.6-fold compared with untreated counterparts (A). Conversely, viral progeny of MDCK and A549 cells was reduced when the inhibitor was applied. Treatment with acetylsalicylic acid (ASA), another inhibitor of NF- κ B, resulted in the significant reduction (* $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$) of virus output in each of the cell types (B) compared with untreated controls. Results shown are representative of two biological replicates and six technical replicates for each biological repeat.

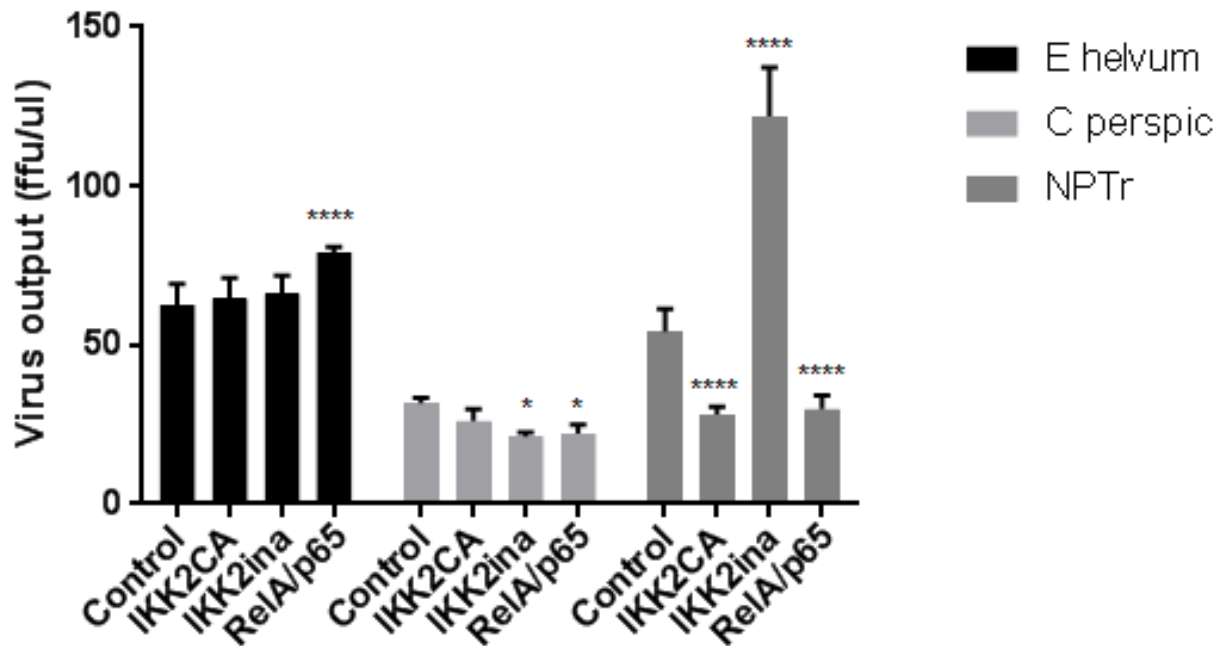


Figure 4.4-3 Transient transfection of bat cells with plasmids encoding components or activators/inhibitors of NF- κ B.

Cells were transiently transfected with plasmids encoding RelA, a constitutively active form of IKK2 and an inactive form of IKK2 and subsequently infected with USSR H1N1 at 0.5 MOI. Overexpression of RelA/p65 resulted in the significant increase (**** $P \leq 0.0001$) in virus output in infected *E. helvum* cells and the significant decrease (* $P \leq 0.05$) in virus output in *C. perspic* cells. The inactivation of IKK2 also decreased the virus output in *C. perspic* cells. NPTTr cells were used as a positive control as the effect of transient transfection of these cells with these plasmids has been well characterised (unpublished data). Results shown are representative of two biological replicates and six technical replicates for each biological repeat.

	IKK2 percentage identity to <i>Homo sapiens</i> IKK2 (%)	RelA percentage identity to <i>Homo sapiens</i> RelA (%)
<i>Myotis brandtii</i>	96.18	90.56
<i>Myotis lucifugus</i>	92.86	90.74
<i>Rhinolophus sinicus</i>	96.74	93.20
<i>Eptesicus fuscus</i>	95.74	90.51
<i>Hipposideros armiger</i>	96.03	91.59
Average similarity (%)	95.51	91.32

Table 4.4-1 Percentage protein identity between IKK2 of bats and human IKK2, and bat RelA and human RelA

While protein sequences of *E. helvum*, *C. perspic* and TB1-Lu are not available, RelA and IKK2 protein sequences of the six available bat species were aligned to corresponding human proteins. Multiple alignment indicated that there is a high sequence identity percentage between the bat and human for IKK2 and RelA proteins.

Protein sequences were obtained from the National Centre for Biotechnology Information. Sequence references: XP_006101621.1; XP_006097115.1; (*M. Lucifugus*) XP_014393535.1; XP_005856523.1 (*M. brandtii*) XP_008145266.1; XP_008142100.1 (*E. fuscus*) XP_019501147.1; XP_019504823.1 (*H. armiger*) XP_019593326.1; XP_019597663.1 (*R. sinicus*) NP_001138610.1; NP_001177649.1 (*H. sapiens*).

4.5. Discussion

The IFN system is an integral part of the innate immune system in the resistance to virus infection by conferring an antiviral state in cells (Schoggins et al. 2011; MacMicking 2012; Stark & Darnell 2012). As well as innate immune protection, the IFNs also have fundamental roles in controlling inflammation, acting as both driver and suppressor of inflammatory responses (as reviewed by Rauch et al. 2013).

The presence of an IFN system has been described in several species of bat including the Egyptian rousette (Kepler et al. 2010), the Malaysian flying fox (Omatsu et al. 2008), Australian black flying fox (Zhou et al. 2016) and has been inferred from low coverage whole genome sequences in *Pteropus vampyrus* (Kepler et al., 2010), *Eptesicus serotinus* and *Dobsonia viridis* (He et al. 2010; He et al. 2014).

The JAK/STAT pathway is the key signalling pathway of type I IFNs, cytokines and growth factors in the regulation of immune functions and, in particular, antiviral and pro-inflammatory responses (Stark & Darnell 2012).

The role of JAK/STAT in virus resistance in bat cells was examined by inhibiting JAK/STAT signalling and determining the impact on virus production from infected bat cells. The JAK/STAT pathway was inhibited in bat and MDCK cells using JAK inhibitor, P6. To ensure P6 could effectively inhibit JAK in bat and MDCK cells, a relatively substantial concentration of 5 μ M was used to treat cells. This dose was considered adequate due to the IC₅₀ (the inhibitory concentration where response or binding is reduced by 50%), being 15 nM against murine JAK1, 1 nM against murine JAK2 and 5nM against murine JAK3. In addition, the high sequence similarity of JAK proteins between bats, pig and dog (**Table 4.4-1**) suggests they share similar

functionality and therefore effectiveness of P6 inhibition would have been comparable in bat cells and MDCK cells.

Bat species	Percentage identity to <i>Canis lupus familiaris</i> (%)			Percentage identity to <i>Sus scrofa</i> (%)		
	JAK1	JAK2	JAK3	JAK1	JAK2	JAK3
<i>Myotis brandtii</i>	91.65	91.23	85.48	91.16	91.62	86.01
<i>Myotis lucifugus</i>	89.38	92.41	84.86	89.29	92.95	85.28
<i>Rhinolopus sinicus</i>	88.47	90.89	85.94	89.54	91.99	85.70
<i>Eptesicus fuscus</i>	92.39	91.00	88.63	92.30	92.83	88.43
<i>Hipposideros armiger</i>	88.63	91.62	86.28	89.22	92.35	86.62
Average similarity	90.10	91.43	86.24	90.30	92.35	86.41

Table 4.5-1 Percentage protein identity of JAK sequences between bats, dog and pig.

While JAK protein sequences of *E. helvum*, *C. perspic* or TB1-Lu are not available, JAK1, JAK2 and JAK3 protein sequences of the six available bats species were aligned to corresponding pig and dog JAK proteins. Multiple alignment indicated that there was a high sequence identity between all species (84-92.8%).

Protein sequences were obtained from the National Centre for Biotechnology Information. Sequence references: XP_014312230.1 XP_014315881.1; XP_006107112.2 (*M. Lucifugus*) XP_005868086.1; XP_014391164.1; XP_005873940.1 (*M. brandtii*) XP_008137617.1; XP_008143213.1; XP_008154637.1 (*E. fuscus*) XP_019502243.1; XP_019501466.1; XP_019497298.1 (*H. armiger*) XP_019587194.1; XP_019585320.1; XP_019592601 (*R. sinicus*) NP_999279.1; NP_999278.1; XP_005654964.1 (*S. scrofa*) NP_001274055.1; XP_013973307.1; XP_013977869.1 (*C. lupus*)

Inhibition of JAK/STAT was found not to affect virus progeny produced from of all three bat cells types infected with USSR H1N1 virus compared with correspondingly infected DMSO-treated control cells (**Figure 4.4-1**). In contrast to bat cells, the virus output of control MDCK cells increased by more than 2-fold when JAK was inhibited (**Figure 4.4-1**). Reduction in viral progeny in infected MDCK cells indicates the importance of the JAK/STAT pathway in controlling influenza virus infection in MDCK cells. As virus replication was unaffected in P6-treated infected bat cells it

suggests that induction of JAK/STAT signalling may not be involved in the resistance of bat cells to influenza virus infection.

Recent reports have provided evidence to suggest differences in JAK/STAT signalling and regulation between resistant and susceptible hosts to influenza virus infection. In resistant duck cells, *JAK1*, *IFNAR1*, *STAT3* were found to be upregulated during HPAI H5N1 virus infection whereas in susceptible chicken cells, these genes were found to be downregulated during infection (Kuchipudi et al. 2014). The inhibition of *STAT3* was shown to have no effect on virus replication in duck cells however, *STAT3* overexpression increased HPAI H5N1 virus production in chicken cells (Kuchipudi et al. 2014). JAK inhibition resulted in a decrease in virus replication in porcine NPTr cells accompanied by reduced type I and III IFN response and reduced expression of ISGs (Delgado-Ortega et al. 2014). Recent work infers the involvement of *SOCS3* in differential JAK/STAT regulation between hosts during influenza virus infection.

SOCS3 is a key mediator of inflammation and regulates over 30 cytokines by suppression of JAK/STAT signalling via the classical negative feedback loop (Crocker et al. 2008). The consistently high upregulation of *SOCS3* and association with reduced virus replication in pig epithelial cells infected with HPAI H5N1 virus suggests the JAK/STAT pathway may be a target of *SOCS3* in the modulation of pro-inflammation during virulent influenza virus infections (Nelli et al. 2012). An overly strong IFN-mediated pro-inflammatory response through JAK/STAT signalling has been shown to be ineffective against virulent virus infections and potentially hinder host survival. It is clear that there is a delicate balance between maintaining a robust host response but not an overly excessive one which could lead to dysregulation of pro-inflammatory cytokines leading to hypercytokinemia (Nelli et al. 2012).

Additional work is needed to further assess, chemically and genetically, the antiviral role of JAK/STAT signalling in bats.

The difference between bat cells and MDCK cells in their utilisation of the JAK/STAT pathway for mediating resistance may potentially be due differences in the upstream mediators and inducers, such as IFNs, of the JAK/STAT pathway. The type I IFN locus of *P. alecto* was found to be unusual compared with other eutherian (placental) mammals (Zhou et al. 2016). The type I IFN locus in *P. alecto* was shown to be smaller than other eutherian mammals, only 250 kb long compared with 350 kb in mouse and 1000 kb in pig. The smaller and contracted genome size found in bats and birds is speculated to be related to flight evolution (Hughes & Hughes 1995) and it has been shown the metabolic requirements for powered flight constrain genome size in both bats and birds (Kapusta et al. 2017).

The bat locus was also shown to be contracted with eight IFN α pseudogenes but only three functional conserved IFN α genes compared with 7-18 functional IFN α genes in other mammals (Zhou et al. 2016).

Functional tests revealed bat IFN α may induce antiviral resistance mechanisms independent of canonical JAK/STAT signalling (Zhou et al. 2016). Bat IFN α was found to have an unusual pattern of expression distinct from any other species. IFN α , but not IFN β , was shown to be constitutively expressed in the tissues and cells of *P. alecto* and *Carollia brachyotis*. Again unlike IFN β , IFN α was also shown not to be upregulated during poly I:C stimulation or in response to virus infection (Zhou et al. 2016). The constitutive expression of bat IFN α was shown to drive induction of a distinct subset of ISGs which includes tetherin and Mx1 through unphosphorylated ISGF3 (U-ISGF3).

U-ISGF3 is a ternary complex comprised of unphosphorylated (U)-STAT1 and U-STAT2 and IRF9 formed in the non-canonical JAK/STAT pathway. U-ISGF3 is thought to maintain prolonged ISG expression long after IFN stimulation (Cheon et al. 2013). Using accumulated STAT1, STAT2 and IRF9, produced during canonical JAK/STAT signalling, U-ISGF3 can induce ISG expression without needing tyrosine-phosphorylation (Cheon et al. 2013) (**Figure 4.2-1**). It is thought the non-canonical pathway may have evolved as a mechanism to enable ISG production in the event of the JAK/STAT pathway being targeted and shut down by viruses (Cheon et al. 2013). Prolonged exposure to IFN β expression has been shown to induce expression of U-ISGF3-dependent proteins in human cells (Cheon et al. 2013). Similarly, constitutive expression of bat IFN α was shown to induce ISGs via U-ISGF3. This finding suggests IFN α expression in bats may provide an alternative pathway to establishing an antiviral state and that inhibition of canonical JAK/STAT signalling does not critically affect the production of ISGs (Zhou et al. 2016).

The type III IFN system has also been suggested as an alternative defence mechanism to resist virus infection if type I IFNs are suppressed (Ank et al. 2006). Similar to the U-ISGF3 pathway, type III IFNs have been suggested to provide a slow acting defence to extend the antiviral state mediated by ISGs (Ank et al. 2008). Two conserved IFN λ genes, similar to human IFN λ 2 and IFN λ 3, were identified in *P. alecto* using a related, although low-coverage, *P. vampyrus* sequence as the genome of *P. alecto* had not been sequenced. There were a number of key differences between bat and human type III IFNs which suggest unique roles for bat type III IFNs. Bat IFN λ 1 was shown to contain a unique 4 kb intron between exons 2 and 3 which in place of a 1.1 kb intron present in other species (Zhou et al. 2011). This structural difference could potentially

translate to functional differences altering the role of bat IFN λ 1 during virus infection (Zhou et al. 2011).

	Structural differences	Functional differences
Bat type I IFNs (Zhou et al. 2016)	IFN α locus is smaller (250 kb compared to 350-1000 kb in other eutherian mammals)	IFN α constitutively expressed in tissues and cells
	IFN α locus is contracted: 3 functional IFN α genes compared with 7-18 in other mammals)	No upregulation of IFN α during infection
		IFN α signals via U-ISGF3 and induces distinct subset of ISGs
Bat type III IFNs (Zhou et al. 2011)	Unique 4 kb intron found in IFN λ 1 not found in other species	IFN λ 2 induced only by RIG-I
		Differential activation of type III IFNs depending on ds- or ssRNA

Table 4.5-2. Summary of functional and structural differences in bat interferons as shown in Zhou et al. (2011) and Zhou et al. (2016).

Bat type III IFNs were also shown to be differentially induced to mouse type I and III IFNs as bat IFN λ 2 only recognised cytosolic viral RNA and be induced by RIG-I whereas mouse type I and II IFNs were induced by both RIG-I and TLR recognition (Ank et al. 2006; Onoguchi et al. 2007). The functional and structural differences between bat type I and III IFNs and IFNs of other mammals are summarised in **Table 4.5-2**. These key structural and functional differences in type I and III IFN in bats suggest the bat IFN response and downstream signalling pathways may be induced and regulated differently from other mammals.

To further understand mechanisms that bat may use to confer resistance, another pathway with essential functions in innate immunity, NF- κ B, was examined. The NF-

κ B pathway has mainly been regarded as anti-viral, involved in the expression of pro-inflammatory cytokines and chemokines, such as $\text{TNF}\alpha$ and IL1 (Ghosh et al. 1998). $\text{TNF}\alpha$ has been shown to strongly inhibit virus replication (Seo & Webster 2002) and is a major ligand of TNFR1 signalling that leads primarily to the transcriptional activation of NF- κ B (Matikainen et al. 2006). NF- κ B has been shown to have roles in regulating *IFN* β expression at the early stages of infection. Before and during early infection, NF- κ B is involved as part of the *IFN* β enhanceosome mounting an early innate response to ensure timely induction of ISGs (Balachandran & Beg 2011; Basagoudanavar et al. 2011). Recently, several studies have reported NF- κ B to function in an influenza virus supportive manner (Wurzer et al. 2004; Ludwig et al. 2006; Mazur et al. 2007; Kumar et al. 2008) by upregulating caspase activity to promote endosomal transport of vRNP thereby increasing virus replication (Wurzer et al. 2004).

As with JAK/STAT signalling, NF- κ B was inhibited to examine the effects on virus replication in infected bat cells. The use of NF- κ B inhibitor, Sc75741, resulted in the clear increased production of virus (**Figure 4.4-2A**) in all three bat cell types, suggesting that NF- κ B activity in bat cells inhibited influenza virus production. The priming of porcine NPTr cells also resulted in increased virus output (data not shown) which corresponds with the transfection findings on NPTr cells (**Figure 4.4-3**). Activation of NF- κ B in NPTr cells, through constitutive expression of IKK2, reduced virus output whereas transfection with inactive of IKK2 resulted in the significant ($P \leq 0.0001$) increase in production of viral progeny. In contrast, priming MDCK and A549 cells with Sc75741 seemed to indicate that NF- κ B activity promoted production of influenza virus progeny (**Figure 4.4-2A**). These findings suggest the pro- and anti-viral function of NF- κ B may be dependent on specific species.

However, the use of a different NF- κ B inhibitor, ASA, resulted in significantly less progeny virus from all cells (bat, MDCK and A549) (**Figure 4.4-2B**). The contrast between the effects of Sc75741 and ASA on the virus production from infected bat cells could be due to unexpected effects of ASA and/or Sc75741 on different pathways.

ASA has been shown to competitively inhibit ATP binding to IKK β in several types of cells including COS, Jurkat, mouse PD31 and HeLa cells (Kopp & Ghosh 1994; Yin et al. 1998). Inhibition of IKK β kinase activity prevented phosphorylation and subsequent degradation of I κ B resulting in NF- κ B dimers being retained in the cytoplasm and prevented from translocating to the nucleus. ASA and another NF- κ B inhibitor, BAY11-7082, were shown to prevent cytopathic effects and block replication of HPAI H7N7 virus in A549 and MDCK cells (Mazur et al. 2007). However, ASA was shown, unexpectedly, to degrade I κ B and consequently induce apoptosis in colorectal cancer cells, but, had no effect on in A549 and HEK293 cells (Stark et al. 2001). Due to contrasting results, Stark et al. (2001) hypothesised the effects of ASA may be cell-type specific however inconsistent findings between different studies suggest the effects of ASA are even more complicated than first suggested.

Another complication of ASA as an inhibitor of NF- κ B, is its effects on cyclooxygenase (COX) 1 and COX2, important inflammatory mediators involved in prostanoïd synthesis from arachidonic acids (Morita 2002). COX1 is constitutively expressed whereas COX2 expression is induced by stimuli such as cytokines, including TNF α , growth factors and endotoxins (Mbonye & Song 2009). While, some studies have found the mechanism of action of ASA upon NF- κ B inhibition to be prostanoïd-independent (Yoo et al. 2000) and modulation of influenza virus

replication to be unrelated to COX1 or COX2 inhibition (Mazur et al. 2007), other studies have reported COX2 to significantly impact on influenza virus propagation (Carey et al. 2003; Dudek et al. 2016). During the early stages of infection, influenza virus was shown to induce COX2 expression in A549 cells (Dudek et al. 2016). COX2 and its product, prostaglandin E2, were found to decrease virus replication in A549 cells and primary human bronchial epithelial cells (Dudek et al. 2016). COX2^{-/-} mice, but not COX1^{-/-} mice, infected with influenza virus were found to have higher viral titres than corresponding wild type mice however infected COX2 knockout mice were also shown to have reduced morbidity and a diminished inflammatory response (Carey et al. 2003). Conversely, other studies reported COX2 deficiency in mice to result in an increased inflammatory response (Gavett et al. 1999; Carey et al. 2003). Furthermore, reduced morbidity was not found in mice treated with COX inhibitors (Carey et al. 2010). The conflicting results indicate the multiple actions of ASA can result in complex and off-target effects on cells. In an attempt to resolve the conflicting virus output results between Sc75741 and ASA, two other NF-κB inhibitors, Bay11-7085 and Bay11-7072, were used to treat cells, as shown in previous studies (Mazur et al. 2007; Kumar et al. 2008). However, at effective inhibitory concentrations, both inhibitors were found to be highly toxic to all cell types, resulting in cell death two hours after treatment (data not shown).

In contrast to the use of Sc75741 and ASA, overexpression of human IKK2 and RelA in bat cells resulted in modest effects on virus output (**Figure 4.4-3**). The difference in virus production between bat cells transfected with IKK2 or RelA and the control vector was relatively small compared with virus production differences from transfected NPTr cells (**Figure 4.4-3**). Thus, despite the difference in virus output between control and NF-κB overexpression in bat cells being statistically significant,

the relatively small effect size may mean there is little biological relevance. The overexpression of NF- κ B activators in bat cells may have had little effect on virus production due to a high basal level of NF- κ B activity in bat cells.

NF- κ B activity has been shown to be tightly regulated in bats to potentially cope with the risk of acute pro-inflammatory dysregulation from exposure to potentially pathogenic viruses (Zhang et al. 2013). As previously discussed, it is clear that there is a delicate balance between maintaining a robust host response to fight infection but not an overly excessive one which could lead to dysregulation of pro-inflammatory cytokines leading to hypercytokinemia.

Recently, the NF- κ B subunit, c-Rel, has been shown to under positive selection in bats potentially driven by the need to mitigate damaging consequences as result of overblown pro-inflammatory responses to virus infection (Banerjee et al. 2017; Enchéry & Horvat 2017). c-Rel has been implicated as a mechanism to control pro-inflammatory response in the big brown bat (*Eptesicus fuscus*) by repressing *TNF α* expression. Human cells were shown to express high levels of the pro-inflammatory cytokine, TNF α , whereas TNF α expression only slightly increased in *E. fuscus* kidney cells in response to poly I:C stimulation (Banerjee et al. 2017). Differential *TNF α* gene expression in human and *E. fuscus* kidney cells was attributed to a putative c-Rel binding site discovered in *E. fuscus* TNF α promoter, not present in the human counterpart, which when bound represses promoter activity (Banerjee et al. 2017). Similar potential c-Rel binding sites were also detected in other bats including *M. davidii* and *M. natalensis* (Banerjee et al. 2017). Furthermore, *c-Rel* was found to contain mutations in the RHD domain, potentially affecting binding of c-Rel to I κ B (Zhang et al. 2013; Banerjee et al. 2017). Banerjee et al. (2017) speculated that this

may allow nuclear transportation of NF- κ B and subsequent activation or repression of gene transcription without TLR stimulation.

Due to role of c-Rel in DNA repair, the positive selection of c-Rel has been linked to evolution of flight, a metabolically costly activity which produces DNA-damaging reactive oxygen species as cellular by-products (Adelman et al. 1988; Cooke et al. 2003). As c-Rel is known to be involved in activating the DNA damage response pathway, positive selection of c-Rel has been postulated to be induced to limit collateral DNA damage associated with oxidative stress (Zhang et al. 2013).

As number of genes involved in both innate immunity and DNA repair have been found under positive selection in bats (Zhang et al. 2013), it is possible that the repression of pro-inflammatory responses to infection in bats is a by-product of flight adaptation.

Flight adaptation has been speculated to the evolutionary driver of the loss of *PYHIN* genes in bats (Ahn et al. 2016). The PYHIN family are responsible for sensing of foreign DNA in cells and the subsequent activation of the inflammasomes (Goubau et al. 2010). Genomic analysis revealed the loss of the entire *PYHIN* gene family universally across ten bat genomes (Ahn et al. 2016). As this loss appears to be unique in bats, it points to potential differences in the inflammatory pathway between bats and other mammals. Inflammasomes play an important role in mediating mass inflammation in response to invading pathogens as well as in autoinflammation and autoimmunity (Salminen et al. 2012; Youm et al. 2013; Teijaro et al. 2014). As the PYHIN family are the only DNA sensors capable of activating inflammasomes (Paludan & Bowie 2013; Dempsey & Bowie 2015), the loss of *PYHIN* genes suggests this may be a mechanism evolved in bats to limit excessive pro-inflammatory

responses to infection and contribute to asymptomatic response to virus infection (Ahn et al. 2016).

The loss of *PYHIN* genes and positive selection of repressive c-Rel in bats indicate differences in the inflammatory pathways between bats and other mammals and suggest bats may have evolved mechanisms to control pro-inflammatory responses to virus infection potentially as a result of flight evolution.

This study found inhibition of JAK/STAT and NF- κ B signalling to affect virus replication differently in bat cells than MDCK cells. The JAK/STAT pathway was found not to be a major factor in limiting virus replication in bat cells potentially as a consequence of SOCS3 repression during the modulation of pro-inflammation during influenza virus infection. Functional differences in bat type I and III IFNs also suggest the presence of an alternative defence system that induces an antiviral state independent of JAK/STAT signalling (Zhou et al. 2011; Zhou et al. 2016).

While chemical inhibition of NF- κ B was found to clearly affect virus replication in bat cells, overexpression of NF- κ B via transfection did not largely affect virus replication suggesting tight control of NF- κ B potentially to prevent excessive pro-inflammation in response to infection. Further work will need to be carried out to consolidate current understanding of the role of NF- κ B in resistance to influenza virus infection in bat cells.

5. General Discussion

Bats often harbour viruses of a highly pathogenic nature to humans without showing signs of clinical disease (Baker et al. 2013). This phenomenon has resulted in a growing interest in bats and bat immunology, and speculation surrounding that a superior immune system in bats enables their coexistence with viruses (Baker & Murcia 2014).

Furthermore, the recent identification of two novel influenza-like viruses in bats, H17N10 and H18N11 viruses (Tong et al. 2012; Tong et al. 2013), led to the suggestion that bats might constitute a mammalian reservoir for conventional avian and human influenza viruses. This was supported by a later study that found of 30% of *E. helvum* bats in Ghana were seropositive for H9 antibodies (Freidl et al. 2015).

It was hypothesized that bat cells are susceptible to influenza virus infection and therefore possibly be potential carriers of influenza A viruses.

The main aim of this study was to determine the host response of bat cells to influenza virus infection. To this end, epithelial cells derived from the lung of three bat species, *T. brasiliensis* (TB1-Lu), *C. perspicillata* (*C. perspic*) and *E. helvum* (*E. helvum*), were examined for their host responses to influenza A virus infection. First, determining the susceptibility (ability to enter the cell) and permissibility (ability to replicate) of each bat cell type to two LPAI viruses and two human influenza viruses. All three types of bat cells were shown to be, to some degree, susceptible and replication permissive to influenza virus infection but much less than MDCK cells. All three species of bat cells were more susceptible and permissiveness to LPAI viruses than to human influenza virus infection. A recent study showed that influenza A viruses were able to adapt to replication in bat cells, even resistant bat cells such as TB1-Lu, with increasing passages (Poole et al. 2014). It is possible that wild bats

would have had more contact more with birds than humans and thus be more likely to become infected with avian influenza viruses. These avian viruses would adapt over time causing increasing susceptibility and permissiveness to avian influenza virus infection.

All three types of bat cells were more viable, as determined by flow cytometry, than MDCK cells when infected with USSR H1N1 or LPAI H2N3 viruses. The susceptibility and replication permissiveness of bat cells to influenza virus infection accompanied by low cell death suggest that bats could be reservoir hosts to avian and human influenza viruses.

Among bat cells, TB1-Lu cells were found to be the least susceptible and replication permissive to influenza virus infection corroborating with previous studies (Hoffmann et al. 2013; Poole et al. 2014). The reduced susceptibility of TB1-Lu cells to influenza virus infection is likely, at least in part, due to the SA receptor distribution. Only one other study has examined the expression of both SA α 2,3-Gal and SA α 2,6-Gal receptors on any species of bat (Chothe et al. 2017). *M. lucifugus* was shown to strongly express both human influenza and avian influenza virus-preferred receptors in the respiratory tract (Chothe et al. 2017). Like *M. lucifugus*, *E. helvum* and *C. perspic* cells strongly expressed avian SA α 2,3-Gal and human SA α 2,6-Gal receptors. However, TB1-Lu cells were found to only strongly express the SA α 2,3-Gal receptor. As receptor specificity partly determines host susceptibility to infection, the lack of SA α 2,3-Gal receptor expressed by TB1-Lu cells likely contributes to the reduced susceptibility and reduced virus entry observed in TB1-Lu cells.

Having looked at susceptibility and permissiveness, the next step was to elucidate the mechanisms which confer resistance to influenza virus infection in bat cells by identifying major pathways involved in host resistance. Two key innate immune

signalling pathways, JAK/STAT and NF- κ B, were examined for their ability to modulate influenza virus production in bat cells. Inhibition of JAK/STAT signalling was found not to affect virus progeny production from all three bat cell types infected with USSR H1N1 virus, suggesting that the JAK/STAT pathway may not play a major role in resistance to influenza virus infection in bat cells. A possible reason for this is that a highly upregulated pro-inflammatory response, mediated by JAK/STAT signalling has been shown as ineffective against virulent viruses such as HPAI H5N1 virus in pigs (Nelli et al. 2012). Furthermore, an overly strong pro-inflammatory response has been suggested to contribute to pathogenesis in chickens and humans (Nelli et al. 2012; Cornelissen et al. 2013; Kuchipudi et al. 2014) and also associated with reduced host survival (Cornelissen et al. 2013; Kuchipudi et al. 2014). Key differences in the structure and expression of bat type I and type III IFNs compared with other eutherian species (Zhou et al. 2011; Zhou et al. 2016) has suggested the existence of an alternative slow-acting defence system which can sustain an antiviral state without JAK/STAT signalling (Zhou et al. 2011).

Inhibition of the NF- κ B pathway through the use of Sc75741 and ASA resulted in conflicting effects on virus production from bat cells. While ASA treatment resulted in a clear reduction in virus production in all bat cell types, MDCK and A549 cells, virus production was significantly increased in bat cells treated with Sc75741. Although the mechanisms of action of Sc75741 and ASA has been reported to be overlapping (Yoo et al. 2000), other interactions of ASA, such as on TNF α , COX1 and COX2, may be more dominant than the inhibition of NF- κ B resulting in different response to the two NF- κ B inhibitors. Transfection of bat cells with active and inactive forms of IKK2 and RelA did not result in clear differences in virus production found in bat cells treated with NF- κ B inhibitors. It may be that bat cells express a high basal

level of NF- κ B activity (Zhang et al. 2013) such that overexpression of NF- κ B activators do not have much additive effect. Tight regulation of NF- κ B in bats may be important in the regulation of pro-inflammatory responses to prevent an excessive response causing inflammatory dysregulation during exposure to infectious stimuli. In particular, the c-Rel subunit of NF- κ B has been implicated as having an important role in innate immunity. c-Rel was found to be under positive selection in bats, potentially driven by flight evolution (Zhang et al. 2013) and to negatively moderate pro-inflammatory responses to infection (Enchéry & Horvat 2017). In a recent study, the c-Rel subunit of NF- κ B was shown to be a regulator of inflammatory gene expression by repressing activation of *TNF α* expression in *M. lucifigus* in response to poly I:C stimuli (Banerjee et al. 2017). In inherently resistant hosts such as ducks and pigs, the attenuation of an excessive pro-inflammatory gene expression appears to be a major factor to reduce pathogenicity of virulent influenza virus infections by reducing the risk of cytokine dysregulation. These findings suggest that NF- κ B signalling may contribute to the resistance of bats to influenza virus infection by regulating the pro-inflammatory response to virus infection.

This study established the three types of bats cells as susceptible and replication permissive to conventional human influenza and LPAI virus infection. The *in vitro* results, consistent with other susceptibility studies (Hoffmann et al. 2013; Poole et al. 2014), would suggest similar findings to those *in vivo* and support the hypothesis of Freidl et al. (2015) that bats might be mammalian carriers of conventional influenza A viruses which may have not been previously identified due to lack of symptoms. Due to the global distribution and migration patterns of bats, their potential ability to transmit conventional influenza viruses could have implications from a public health

perspective. The innate mechanisms behind the lack of symptoms to influenza virus infection in bats remains to be determined but the findings from this study suggest that tight regulation of NF- κ B signalling to control the pro-inflammatory response to influenza infection plays a key role in bats and warrants further investigation.

5.1. Limitations

The main limitation of the study was the lack of bat-specific reagents. The genome sequences of *T. brasiliensis*, *E. helvum* and *C. perspicillata* have not been published and sequence identities of the few bat species which are available are predicted sequences, poorly annotated and with low coverage. The redundancy of coverage or depth of coverage is the percentage of target bases that are sequenced a given number of times (Sims et al. 2014). The genomes of *M. lucifugus* and *P. vampyrus* were sequenced to low (2-fold) redundancy, with 22 other mammals, as part of a project by the National Genome Research Institute project to identify conserved elements in mammalian genomes in 2010. The decision to sequence to low redundancy was a compromise in order to include many species (Green 2007). However, at 2-fold coverage, there remain many gaps in the sequences and it is estimated that only 65% of bases are covered for the mammalian genomes (Green 2007). While there are types of biological information that can be gathered from low depth genomes, such as repeat content, partial sequences of most genes and identification of lineage specific families (Pontius et al. 2007), it is difficult to make inferences about lineage-specific losses of genes, and downstream analyses requiring complete features cannot be undertaken using low coverage genomes (Green 2007). Since 2010, four more bat genomes have been sequenced, however the majority of these sequences are also of low coverage

and have been annotated using different pipelines and to different degrees making comparisons between genomes challenging. The unavailability of the genomes of the three bat species used in this study made it challenging to quantify gene expression in these bat cells. Furthermore, using the low depth of the bat genomes that were sequenced to infer expression of different bat *IFNs* or ISGs produced inconsistent and therefore unreliable results.

5.2. Future work

To extend this study, it would be useful to examine the SA receptor distribution in the intestinal tract of *T. brasiliensis*, *C. perspic* and *E. helvum*, particularly given the recent findings of mainly avian influenza SA receptors in the intestinal tract of the little brown bat (Chothe et al. 2017). Future work would also need to address the conflicting NF- κ B inhibition results from the use of ASA and Sc75741. This could be potentially resolved using another inhibitor such as ammonium pyrolidinedithiocarbamate (PDTC). It would also be useful to determine the basal level of NF- κ B activity by stably transfecting bat cells with plasmid encoding *luciferase* in the NF- κ B promoter. Further exploration of the IFN and NF- κ B responses and the other signalling pathways involved in innate immunity would provide a deeper understanding of the innate mechanisms used by bats to confer viral resistance. It has been previously established that bats possess a similar innate immune repertoire to other mammals (Papenfuss et al. 2012), however, due to the lack of available sequences, the expression of key genes involved in the recognition and resistance to influenza virus infection could not be determined in the three types bat cells. To overcome this limitation, RNA-Seq could

be performed using the three types of bat cells infected with influenza virus at specific time points and compared with mock-infected cells and correspondingly infected MDCK cells. From the RNA-Seq data, genes that are differentially expressed infected bat cells compared with infected MDCK cells might be identified. Genes of particular interest would include those involved in the recognition of influenza viruses (e.g. RIG-I and TLR7), the IFN response (e.g. IFN α/β), establishment an antiviral state (e.g. ISGs) and pro-inflammatory response (e.g. TNF α). RNA-Seq might also identify other pathways that may be important in mediating viral resistance in bat cells such as the mitogen-activated protein kinase (MAPK) and PI3K/AKT pathway (Ludwig et al. 2006).

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Professional Internships for PhD Students

Reflection Form

Name of Organization

Qiagen

Details of Placement

Please describe your main activities during the placement

I was based in the Technology department and part of the Tech team. My role was to develop a diagnostic assay for the detection and diagnosis of different influenza strains using RT-qPCR and high resolution melt (HRM) analysis. The main activities during my placement were designing primers to amplify the M-gene region of influenza virus strains and carrying out test runs on the RGQ (qPCR) machines with influenza cDNA samples. I designed long oligonucleotides for the primers to act as positive controls. After the qPCR was performed, HRM was carried out to determine differences in the amplified sequences as different melt curves identified different strains of influenza virus. As a comparison, different Qiagen cDNA conversion kits were used and two different qPCR machines were used (Qiagen RGQ and Roche Lightcycler 480). My other responsibilities including rotational housekeeping duties and writing weekly reports.

Placement Achievements

Please detail all outcomes from the placement, including any publications, presentations given and reports written etc.

I gave a presentation at the beginning of my placement introducing influenza A viruses to the team. Each week, I would verbally present my findings during the weekly team meeting. Each qPCR run was documented in a laboratory book along with the design of the primers and long oligonucleotides.

Skill development

Has this Placement helped you developed any new skills or enhanced your previous skill set?

This placement has enhanced my primer designing skills and my problem solving ability. It has made me aware of other conversion kits and qPCR technologies. I have developed my report writing skills and presentation skills and made me more confident in presenting my ideas to a large group of people.

Future Work

Has this Placement influenced your future career aspirations? If so, in what way?

By undertaking a placement at Qiagen I have gained some experience what it is like to work in the biotechnology industry. I have previously worked in pharmaceutical and biotech companies previously and this placement has reaffirmed that I do enjoy this type of industry and would be consider a future career in the biotechnology or pharmaceutical industry.