Mechanism of infection of bronchial epithelial cells by human and avian influenza viruses

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Statement of originality

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Acknowledgement

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<tr>
<td>%</td>
<td>Percentage</td>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>BEGM</td>
<td>Bronchial Epithelial Growth Medium</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C-terminus</td>
<td>Carboxy-terminus</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FAM</td>
<td>Amine-reactive succinimidyl ester of 5`-carboxyfluorescein</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<td>HRP</td>
<td>Horse raddish peroxide</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<td>L</td>
<td>Litre</td>
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<tr>
<td>LB</td>
<td>Luria-bertani</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<td>mM</td>
<td>Milimolar</td>
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<td>Mililitre</td>
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<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>MGB</td>
<td>Minor groove binder</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<td>N-terminus</td>
<td>Amino-terminus</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>pBECs</td>
<td>Primary bronchial epithelial cells</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT-qPCR</td>
<td>Real-time quantitative PCR</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<td>UV</td>
<td>Ultraviolet</td>
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Abstract

Respiratory epithelial cells including bronchial epithelial cells (BECs) are the initial site of infection with influenza viruses, and the anti-viral responses elicited by these cells are a critical first line of defense against respiratory viral infection and can induce effective adaptive immune responses. However, relatively little is known about the importance of this innate anti-viral response, and the magnitude and effectiveness of this response is poorly understood. In addition to human influenza, avian influenza viruses are a potential source of future pandemics, therefore it is also critical to examine the effectiveness of the host anti-viral system to low and high pathogenic avian influenza viruses.

Anti-viral responses to a human influenza H3N2, a low pathogenic avian influenza H11N9 and a high pathogenic avian influenza H5N1 was profiled using Calu-3 cells and primary bronchial epithelial cells (pBECs) to model proximal airway cells and A549 cells to reflect alveolar cells. The susceptibility of BECs to influenza infection was not solely dependent on the sialic acid-bearing glycoprotein expression and was affected by the apoptosis and anti-viral responses after infection. The earlier and greater anti-viral signaling and protein production correlated with the control of infection. However the H3N2 strain resulted in a delay in anti-viral signaling and impaired release of type I and type III interferons (IFNs) compared to the H11N9 virus. The differences in anti-viral induction between H3N2 and H11N9 were partly due to the influenza non-structural (NS) 1 protein. The gene encoding for NS1 was transfected into the BECs and the H3N2 NS1 induced a greater inhibition of anti-viral responses compared to the H11N9 NS1. Regardless of this inhibition by the influenza viruses, the
constitutive secretion of IFN-β by BECs played a more critical role in inducing late anti-viral signaling via type I IFN receptors and was crucial in limiting viral infection by apoptosis. Infection with H5N1 resulted in a complete abolishment of this response in the infected BECs, including those induced by the constitutive IFN-β. This was due to the robust inhibition of host anti-viral responses by the NS1 protein of H5N1.

This study characterizes anti-influenza virus responses in airway epithelial cells and shows for the first time that constitutive IFN-β release plays an important role in initiating protective late IFN-stimulated responses during influenza infection in airway epithelial cells. In addition, the subversion of human anti-viral responses may be an important requirement for influenza viruses to adapt to the human host and induce disease. Furthermore by understanding both of these avenues will identify targets for potential therapeutics.
Chapter 1. Introduction
1.1 An overview

Influenza, also known as the flu, is a respiratory infection caused by the influenza virus, one of the major pathogens in human population that causes epidemics annually around the world, and which has been responsible for several severe pandemics in the 20th century and a novel influenza pandemic in 2009.

Influenza viruses are enveloped negative-sense single stranded RNA viruses, and are members of the genus Orthomyxovirus in the Orthomyxoviridae family. Within which influenza viruses are classified into three distinct genera, influenza A, B, and C viruses. The classification is based on the serological responses to their internal nucleoprotein, a major structural component that encapsulate the viral RNA [1], and the matrix protein (M1), which is important in viral component transportation within the host cells, and the virus assembly and budding [2]. The standard nomenclature for influenza viruses includes influenza type, location of initial isolation, the successive isolate number from that location, and year of isolation. For example, the influenza A virus strain used in this study was originally isolated in Wellington, New Zealand, in 2005, and there were three isolates in that year, hence it is designated as the influenza A/Wellington/3/2005.

While all three genera cause diseases in human, influenza A viruses are the most pathogenic and can result in the most serious illnesses, and are the main focus of these studies. Influenza A viruses are further sub-typed according to two major surface glycoproteins, haemaglutinin (HA) and neuraminidase (NA). Currently there are 16 HA and nine NA of influenza A subtypes serologically identified from birds, especially waterfowl [3], from which mammalian influenza viruses are directly or indirectly derived. At present, only H1, H2, and H3 type of HA and the N1 and N2 type of NA
have become established stably in human influenza lineage, and result in recurrent
annual epidemics.

The natural reservoir of influenza A viruses are aquatic birds such as shorebirds
and waterfowl [4]. Infections are usually asymptomatic and limited to the respiratory
and/or the gastrointestinal tract in these hosts [4]. Viruses are often excreted at high
titres from these natural hosts into the water supply where it can be transmitted to other
susceptible avian species such as turkey and chicken [5]. The viruses that are non-
virulent in natural hosts can be pathogenic in certain infected avian species. For
example, the recently emerged influenza H5N1 viruses isolated from infected humans
and chickens in Hong Kong in 1997 were highly pathogenic in chickens, but did not
cause diseases in infected ducks [6].

Infection with human influenza in healthy individuals usually results in
symptoms ranging from asymptomatic infection to serious illnesses with systemic
features. The acute infection is usually associated with an abrupt onset of illnesses
including fever (38 – 40°C), myalgias, malaise, anorexia, upper respiratory tract
congestion, and pharyngitis [7], and the severity of the disease differs between the virus
subtypes. The current circulating strain of influenza virus H1N1 subtype appears to be
less pathogenic and causes milder symptoms than H3N2, which lead to typical febrile
respiratory disease [8]. It was observed that people who were infected with H3N2
developed more severe symptoms than with then-novel H1N1 virus in 1977-78. In rare
cases, influenza infection can also lead to myocarditis, encephalitis, and other extra-
respiratory tract illness [7].
1.2 Epidemiology of influenza

1.2.1 Disease impact

Influenza epidemics lead to a significant medical and economic consequences as a result of increases in visits to the primary health care workers, use of prescription medicines and absenteeism from school or work [9]. Annual epidemics are regularly associated with increase in population morbidity and mortality, expressed in the form of excess rate of pneumonia and influenza-associated hospitalization [10, 11]. During the seasonal influenza epidemics, the impact on the population usually associates with the antigenic property of the virus, the immunity of the population, and the health conditions of certain groups of the population [12]. Excess mortality is primarily seen in elderly, children less than five years, and individuals with pre-existing cardiac or pulmonary disease, such as asthma and chronic obstructive pulmonary disease (COPD). These groups are more susceptible to infection or the complication of this infection [10], such as pneumonia or the worsening of chronic cardiac and pulmonary disease. These factors were thought to contribute in the United Kingdom to an excess in mortality of 5,000 – 29,000 deaths per annum [13].

1.2.2 Seasonality

In temperate climates, influenza outbreak concentrates in the winter months; the exact reason for this seasonality is unclear, though some studies have suggested that environmental conditions or behavioural changes of the population, such as increased humidity and indoor crowding, may increase viral transmissibility [14, 15].
1.2.3 Evolution of influenza viruses

The severity of annual outbreak may vary due to the inherent ability of the influenza viruses to alter its antigenic profile. The high antigenic variability of the virus is due to the relatively low proof-reading property of the influenza RNA-dependent RNA polymerase, which allows the virus to undergo frequent mutations (~1/10^4 bases per replication cycle) [16]. These genetic changes often occur in the genes encoding for its HA and NA, resulting in a new strain that is able to evade from the host neutralizing antibodies, the main immune response against influenza infection. This is the reason for the need of annual vaccination against influenza. While most of these mutations are non-viable, those that show improved growth potential are able to expand and dominate, leading to altered phenotypes to which the population has less immunity, this phenomenon is known as antigenic drift [17].

A second antigenic change happens when two different virus strains from two host species co-infect a single host, allowing genetic reassortment to occur in the host and generate a new strain of influenza virus to which the population may have little or no immune memory [17]. This process, known as antigenic shift, may result in a viral strain that has pandemic potential since the virus has a novel genetic makeups, which can have un-predictable pathogenicity in human [18]. Antigenic shift can also occur when influenza A virus is transmitted without reassortment from an animal reservoir to humans, and this direct adaptation may also lead to efficient human-to-human transmission [12].

1.2.4 The past, current and future influenza pandemics

Influenza pandemics usually occur approximately every 30 – 50 years, and those that happened before 19th century were recorded but were not well documented. There
were five influenza pandemics during the 19th century, though only the 1889 – 1890 pandemic was recorded in detail [19]. The virus strain that caused this pandemic is now known to have originated in Russia and spread throughout the world by 1890, with an estimated global death toll of one million. Although some have speculated that this Russian flu was an influenza A H2N2 subtype, the exact strain of this influenza remains inconclusive [19].

There were three major influenza pandemics that occurred in the 20th century, the 1918 Spanish flu, 1957 Asian flu, and 1968 Hong Kong flu, with the pandemic in 1918 being the most devastating in recorded history. These pandemics all occurred in three waves, with the first relatively mild wave and then followed by more severe second and third waves.

### 1.2.4.1 1918 Spanish influenza A H1N1 virus

The 1918 influenza virus, or the Spanish flu, caused unprecedented catastrophes in the recorded human history. This virus was a H1N1 strain of influenza A virus, which was estimated to have infected one third of the world’s population (500 million people), with an estimated death toll between 50 – 100 million people during the pandemic period [20]. The high mortality rate and fast spread of this virus was likely to be attributed by a combination of factors including mass migration/crowding, malnutrition, and lack of antibiotics associated with World War I in 1918 [20].

This 1918 pandemic occurred in three waves within a 12 months period [21]. The first wave started in March 1918, spreading through the United States and Europe with a high rate of illnesses but low mortality, and quietening down at the end of July 1918. The second wave arose in September 1918 and spread very rapidly around the world with lethal pneumonia. The third fatal wave started in January 1919 and ended at
the end of April 1919. The last two waves were characterized with high rates of severe viral pneumonia and also complicated with secondary bacterial super-infection. The combined influenza and pneumonia mortality seen in this pandemic showed that while people who were under five and above 65 years of age were more susceptible to influenza infection, as usually observed in seasonal human influenza, people who were between 15 to 45 years of age also exhibited high mortality [22].

![Figure 1. The three pandemic waves of 1918 Spanish flu. Figure taken from [21]](image)

This unique pattern could be partially explained by a theory hypothesizing that people who were born before 1880s were exposed to the same or similar strain as the 1918 strain, and this might provide some immune protection against the 1918 pandemic strain. However, this theory was only possible if there was a precursor virus that appeared and disappeared before 1889, re-appeared in 1918 and disappeared again without a trace [22].

The high pathogenicity of this H1N1 virus primarily caused a massive cytokine storm to the virus in the infected patients [23-25]. Cytokine storm is exaggerated immune responses to the invading pathogen, which in the case of 1918 H1N1 virus led
to massive hemorrhages, toxic shock, and fatal viral pneumonia, killing the infected patients directly. However, the majority of deaths were also observed with secondary bacterial infection following H1N1 infection [23, 26]. A unique feature of this pandemic was that the H1N1 infection simultaneously occurred in both human and swine [24]. It was found that there was no precursor virus circulating before 1918, and the 1918 H1N1 virus was antigenically novel in both human and swine. This raises the question of the source of this virus. The sequence and phylogenetic studies revealed that the genome of 1918 H1N1 strain retained a strong avian characteristics, indicating that this H1N1 virus was not a reassortment virus between avian and then-circulating human influenza virus. However, the genomes of this H1N1 virus are substantially different from the current avian influenza viruses [27-29].

1.2.4.2 1957 Asian influenza A H2N2 virus

The second influenza pandemic arose between 1957 and 1958 in Asia, where it was first isolated in southern China in late 1956 but the first wave actually started in Singapore in February 1957 and spread rapidly throughout the world. The fatal second wave arose in September and was followed by a third wave in December 1957. This virus was a new strain with H2 and N2 antigen, with the attack rate peaked in children aged between five and 19 [30]. The excess mortality during this pandemic was estimated to be one–four million [31].

1.2.4.3 1968 Hong Kong Influenza A H3N2 virus

In 1968, a new influenza A virus with new surface glycoproteins appeared and caused a global pandemic with approximately one million death. This strain only had a new HA, a H3, and retained the N2 from 1957 pandemic strain, thus this reduced mortality rate was likely to the result of the developed immunity in the population to N2
from previous 1957 – 1958 Asian pandemic [31]. This virus first appeared in July 1968 in Hong Kong where the first mild wave started and followed by a more severe second wave in August and a third wave in December 1968 to January 1969.

A novel influenza A virus H1N1 re-surfaced in the population in 1977, and was not considered as a true influenza pandemic since the highest attack rate was mostly observed in people born after 1950, and the older population had developed immunity to the H1N1 strains in 1918 [32]. The source of this H1N1 re-introduction into the population is still unclear, but it is now speculated that the live H1N1 vaccine trial in military soldiers in the Far East could be reason for this re-emergence [32, 33]. This H1N1 strain and the H3N2 strain have been circulating in the human population ever since [32].

1.2.4.4 1997 Human outbreak of avian influenza A H5N1 virus

In May 1997, an avian influenza strain was isolated from a 3-year-old boy right after an influenza outbreak among poultry in Hong Kong, whom died from severe pneumonia with acute respiratory distress syndrome (ARDS), multi-organ failure and Reye’s syndrome, a complication of influenza-associated with microvascular fatty infiltration in the liver [34]. A second outbreak occurred later the year among chickens in the markets and farms in Hong Kong and resulted in 17 additional human cases, five of which had fatal illness [35]. The virus strain isolated was then identified to be an avian influenza H5N1 virus with high pathogenicity. The outbreak was contained by massive slaughtering of chickens and intense influenza surveillance in Hong Kong, thereby successfully minimizing the outbreak and no further cases of human H5N1 infection were reported. In late 2003, an H5N1 influenza outbreak among poultry was reported in Korea, and subsequently outbreaks in poultry were also reported in Viet
Nam, Thailand, China, Cambodia, Laos, Malaysia, Indonesia, and Japan. The reason for these simultaneous H5N1 outbreaks in these Asian countries is still unknown, but it was speculated that the migratory birds carrying the H5N1 influenza virus might be involved in this series of outbreaks [36, 37]. As of March 2010 there are 507 confirmed cases of H5N1 infection around the world, which resulted in 302 deaths.

Clinical symptoms associated with H5N1 infection in human include viral pneumonia with diffuse, multi-focal infiltrates and lobular consolidations [38]. ARDS leading to respiratory failure and other common complications including ventilator-associated pneumonia, pulmonary hemorrhage, pneumothorax, and Reyes’s syndrome have also been described [35, 38, 39]. Multi-organ failure including renal dysfunction, gastrointestinal symptoms, and cardiac compromises is also common in patients with H5N1 [40]. The fatality rate of H5N1 infection in human is high, especially in people younger than 15 years of age with a mortality rate of approximately 90% [38, 39]. Death usually occurs at day nine or 10 after onset of illness with progression to respiratory failure.

The genetic analysis indicated that it crossed its species barrier without prior adaptation in an intermediate mixing host [41]. Nevertheless, it was shown that even though avian-to-human transmission of H5N1 influenza virus is observed, sustainable human-to-human transmission is still inefficient [42]. The accumulating number of human H5N1 infection cases has raised concerns that this could fasten the pace for the H5N1 virus to acquire necessary mutations to achieve sustainable human-to-human transmission in these Southeast Asian countries, where humans live very closely to live poultry. This allows both avian and human influenza viruses to co-circulate and possibly reassort in co-infected hosts such as pigs and chickens [43], making the adaptation of H5N1 in human much easier and faster.
As this highly pathogenic avian virus is a novel strain that causes extremely severe illnesses in humans, the mechanism of infection and human immune reaction to this virus is one of the main focuses in this study.

1.2.4.5 1999 Human infection of avian influenza A H9N2 virus

Outbreaks of this virus in poultry have been reported world-wide, but in 1999, “The Government Virus Unit” in Hong Kong isolated an influenza A virus from a girl which was confirmed by the WHO collaborating centre to be an avian influenza origin H9N2 virus. Molecular analysis of this strain indicated that six of the H9N2 internal genes are closely related to that of H5N1, however, whether it is H5N1 that donated its genes to H9N2 or vice versa could not be determined, since it is uncertain which strain appeared first [44].

1.2.4.6 2003 Human infection of avian influenza A H7N7 virus

A high pathogenic avian influenza H7N7 subtype emerged in The Netherland during 2003, and caused conjunctivitis and influenza-like symptoms in at least 82 infected individuals [45, 46].

1.2.4.7 2009 Human infection of swine influenza A H1N1 virus

In March 2009 an outbreak was detected in Mexico where an increase in cases of influenza-like illness was recognized. Centres for Disease Control and Prevention (CDC) subsequently confirmed this was caused by a novel swine origin H1N1 virus (S-OIVs). The virus quickly spread throughout the rest of the world. On June 11 2009, WHO declared influenza pandemic status in response to this rapid worldwide spread of this novel H1N1 influenza virus. This pandemic appears thus far to be following similar patterns as the ones in 1918 and 1957, as the first wave started in late March and early spring 2009, and followed by a second wave in July. The symptoms associated with this
virus are similar to that with seasonal human influenza including fever, headache and malaise, and infected individuals generally recover without medical attention. Diarrhoea and vomiting is also a common clinical feature of S-OIV infection [47]. Severe complications such as fatal pneumonia, lymphopenia leukopenia, and some degree of renal impairment have also been observed in hospitalized patients [47]. People who are under the age of five and over 65 years of age, pregnant women and people who have chronic illnesses are more susceptible to novel H1N1 infection. The mortality rate of swine H1N1 infection was particularly high in Mexico. The reason for this is currently unknown, it is speculated that only those with severe symptoms were admitted to hospitals and there may be a large number of undetected cases in Mexico. Australia has the third highest rate of infection (rate per 100,000 population) with 68% of total deaths being the vulnerable groups (Indigenous, pregnant and individuals with chronic illnesses) [48]. Vaccine for novel H1N1 virus was made available and approved by Food and Drug Administration (FDA) for mass vaccination in the United States and China in September 2009. In Australia, vaccine was made available in October 2009.

1.3 Influenza virus structure, genome, and replication

1.3.1 Influenza structure and genome

Influenza A virus has eight segmented, negative sense, single-stranded RNAs, and these are encapsulated within a viral envelope, and the structure of the virus particle is roughly spherical in shape with approximately 80 – 120nm in diameter [49]. The viral envelope contains two surface glycoproteins, haemaglutinin (HA) and neuraminidase (NA), at a ratio of four HA to one NA [50]. A small number of M2 ion channel is also embedded in the viral envelope at a ratio of one M2 channel to $10^1$ to $10^2$ HA molecule [51] (Figure 2).
Within the envelope are the eight segments of influenza RNA, each of which is numbered in an order of decreasing length. The segments are presented in a form of helical hairpin structure, each of which is coated with arginine-rich nucleoprotein (NP) [52-54], and is complexed with heterotrimeric RNA-dependent RNA polymerase, which consists of three subunits, polymerase basic (PB)1, PB2, and polymerase acidic (PA) [49]. Segment 1, 3, 4, 5, and 6 encodes for PB2, PA, HA, NP and NA, respectively. Segment 2 encodes for PB1, and via a different reading frame an accessory protein PB1-F2 is expressed in most influenza A strain. Segment 7 encodes for matrix 1 (M1) protein and also M2 ion channel via alternative splicing. Segment 8 encodes for non-structural protein (NS) -1 and nuclear export protein (NEP) or also known as NS-2. At both 3’ and 5’ end of each segment lies non-coding region of varying length that act as a promoter site for viral polymerase complex to initiate transcription. This region also contains an mRNA polyadenylation signal and a signal for virus assembly.

Figure 2. Components and structure of influenza A virus.
1.3.2 Influenza replication

The primary site of adherence to host cells during the initiation of infection by influenza viruses are epithelial cells of the respiratory mucosa. This occurs by the binding of the viral surface glycoprotein HA to the sialyl sugar chain receptors on the host cell surface, allowing the virus to be internalized by endocytosis into an endocytotic vesicle and finally an endosome in the host epithelial cell. The low pH environment of the endosome allows the viral HA to undergo conformational change. This results in the liberation and insertion of a fusion peptide from the amino terminus of HA into the endosomal membrane. This spring-loaded mechanism fuses the viral envelope and the membrane together, thereby allowing the release of viral RNP into the host cytoplasm [55, 56]. The M2 ion channel also allows an influx of H\(^+\) ions into the virion, and lowers the intra-virionic pH. This in turn disrupts viral RNP-M1 protein interaction and subsequently releasing viral RNP into host cellular cytoplasm [57-60].

Influenza viruses are different to other RNA viruses in that the transcription and replication of the viral genome takes place in the nucleus of the infected cells [61]. Therefore following the release of viral contents into the cytoplasm, the viral RNPs containing the viral RNA, nucleoprotein, and polymerase proteins are transported into the host nucleus. Within which the viral RNPs serve as templates for the synthesis of two forms of positive sense RNA, the viral messenger RNA (mRNA) and the complementary RNA (cRNA). Viral mRNA synthesis is initiated with primers that are cleaved and snatched from the 5\(^{\text{`}}\) end of host capped methylated cellular mRNA by the PB2 protein, the viral endonuclease. The combined functions of the PB2 protein with the PB1 protein, which is the RNA-dependent RNA polymerase, results in the generation of positive sense viral mRNA, which is then transported into the cytoplasm for translation into viral proteins. The segment encoding for M proteins and non-
structural proteins (NS) are alternatively spliced by host splicing machinery in the nucleus, giving rise to transcripts encoding for M1, M2, and NS1 and NEP proteins, respectively. The viral cRNA remains in the nucleus and serves as a template to synthesize the negative sense daughter virion RNA without the need for a primer. After replication, M1 re-associates with the newly formed daughter RNA strands and NP into daughter RNPs, which then re-associate with NEP for nuclear export.

While the viral RNA is being replicated, the envelope proteins including HA, NA, and M2 are synthesized by membrane-bound ribosomes and transported to the cell surface by the Golgi apparatus. During synthesis and transport, these proteins undergo further maturation, including the cleavage of the HA by host or bacterial proteases[62]. The newly formed viral segments traffic to a lipid raft on the plasma membrane and are then released from the lipid raft [63, 64]. A study by Takeda, et al. demonstrated that the lipid raft is not only used as a budding site, it also acts as a docking site for efficient HA concentration into budding virion and to facilitate efficient virus-host cell fusion [65]. NA has also been shown to concentrate in the lipid raft [66]. Since the viral envelope is derived from the host membrane, which contains sialic acid glycoproteins, the newly formed virus remains intact on the host cell surface. The viral NA cleaves the host cell surface sialic acid residues, releasing the newly formed virions free from the host cell surface (Figure 3).
Figure 3. The replication cycle of influenza virus in the host epithelial cell.

1.4 Influenza pathogenicity and virulence factors

1.4.1 Haemaglutinin (HA)

Influenza HA is responsible for the entry of the virus in the host cells and the HAs of different strains of influenza virus bind to their receptors on the surface of host cells with different specificities, which are thought to be important determinants for the host range of influenza virus.

1.4.1.1 HA binding to host receptors

The binding target for influenza virus is the glycoproteins with terminal sialic acid residues of specific configuration. Sialic acids (SAs) are sugar molecules found ubiquitously as terminal residues on the glycan chain of many polysaccharide and glycoproteins on human epithelial cell surface, secreted glycoproteins, as well as surface glycolipids [67]. Diversity in SA presentation include the position of SA
branching from the carbon backbone of glycoproteins, modification of hydroxyl group, and different α-linkages from the 2-carbon to the sugar chain.

Influenza of different strains has preference to binding different configuration of SA residues. Human influenza viruses preferentially binds to host epithelial cell surface glycoprotein containing the terminal SAα2,6Gal linkage [68-70], whereas avian influenza viruses binds to that with terminal SAα2,3Gal linkage [68-71]. The difference in their binding specificity can be explained by the amino acid residue at position 226 of HA glycoprotein. HA of human influenza viruses contains a leucine residue at position 226 that results in the preferential binding to the surface glycoprotein containing the terminal SAα2,6Gal linkage. In contrast, HA of avian influenza viruses have a glutamine at position 226, which binds to terminal SAα2,3Gal linked glycoproteins [72, 73]. The human upper airway epithelium predominantly express glycoproteins with SAα2,6Gal in the upper respiratory system, and those with SAα2,3Gal linkage in the lower respiratory system [74], whereas wild birds express more glycoproteins with SAα2,3Gal linkage in the respiratory tract and gastrointestinal tract [68]. Human gastrointestinal tract epithelium contains both SAα2,6Gal and SAα2,3Gal residues [75]. This difference in binding specificity and distribution of sialic acid residues may in part explain why highly pathogenic avian influenza virus H5N1 is currently incapable of transmitting from human to human in a sustainable fashion. In contrast pig trachea was found to contain both SAα2,6Gal and SAα2,3Gal linkages, indicating that pigs can act as a intermediate mixing host, allowing the reassortment of both avian and human viruses to occur, and possible leading to the generation of new influenza strain with efficient human-to-human transmission [76-78]. However there is also evidence contradictory to the above findings, that human airways can also act as a mixing bowl in which influenza reassortment can occur [79]. Human tracheobronchial epithelial cells
contain a mixture of non-ciliated and ciliated cells, which express glycoproteins with SAα2,6Gal and SAα2,3Gal linkages, respectively. In addition non-ciliated and ciliated cells supported human and avian influenza replication, respectively. While this contradicts with the notion that SAα2,3Gal residues are predominantly expressed in the distal airway epithelial cells, this may suggest that viral reassortment could allow a shift in cellular tropism of avian influenza or generation of a new influenza strain if the human respiratory epithelia are infected simultaneously with both human and avian influenza viruses [79].

Regardless of these findings other studies reported that influenza viruses can still cause infection in the absence of its respective receptors. Human influenza A virus was found to infect and replicate to the similar titre in the lung of the mice lacking receptors with SAα2,6Gal linkages compared to wild type mice [80]. Hence despite the fact that SAα2,6Gal and SAα2,3Gal terminated glycoproteins are considered to be the predominant receptors for influenza virus entry, this study demonstrated that SAα2,6Gal linkages may not be absolutely essential in influenza virus entry. It is possible that influenza infection can be established via alternative moieties, such as sulfonated, fucosylated or other sialylated glycoproteins that may fit into the binding cleft on influenza HA protein [81, 82].

1.4.1.2 HA activation

Influenza virus is only infective when HA becomes activated [83, 84]. HA is synthesized as a precursor HA₀ form, and the activation requires post-translational cleavage by the proteases into two subunits, HA₁ and HA₂, rendering the viruses infectious [83, 84]. The cleavage site of HA₀ is located in the external loop linking HA₁ and HA₂, and the composition of this loop determines the sensitivity to the host
proteases, thereby affecting the virus tissue tropism and virulence [85]. This conserved loop contains either a single arginine or lysine residue, forming a monobasic cleavage site, or several arginine and/or lysine residues with a R-X-K/R-R motif, creating a multi-basic cleavage site [83].

All influenza viruses, except for highly pathogenic H5 and H7 strains, contain this monobasic cleavage site, and the proteases involved belong to a family of serine proteases, which are mainly encountered in limited number of cells such as respiratory epithelial cells. As a result infections are usually restricted to the respiratory tract in human. One such protease found to be involved in HA cleavage is the tryptase Clara in human epithelial cells [86]. In contrast, the H5 and H7 of highly pathogenic avian influenza strain have multibasic cleavage site that can be recognized and cleaved by ubiquitously expressed proteases, hence infection can become systemic, resulting in more severe diseases [87, 88]. In addition, the activating proteases involved in the HA cleavage of pathogenic avian influenza viruses can also be subtilisin-like proteases. This type of proteases are able to recognize the polybasic amino acid sequences, but are unable to cleave at the single arginine residue in the HA of non-pathogenic avian influenza viruses. Subtilisin-like proteases such as furin and PC6 are found to be ubiquitously distributed in birds and human, thereby allowing the HAs to be activated in virtually all cell types, therefore replication can occur at multiple sites in the avian hosts [88, 89]. Plasmin, a serine protease [90] and a factor Xa-like protease [91] involved in blood coagulation were shown to be the potential candidates for the HA activation in mammalian host. This characteristics of HA activation and the availability of proteases are the main determinants of influenza infection tropism and pathogenicity.

The presence of bacteria at the site of influenza infection can also contribute to the activation of influenza HA. Bacteria including *Staphylococcus aureus, Haemophilus*
*influenzae*, and *Streptococcus pneumoniae*, express proteases such as staphylokinase from *S. aureus* can directly bind and activate HAs. Bacterial proteases may contribute indirectly to activation by generating plasmin from plasminogen, which in turn activate influenza viruses for infection [92, 93]. This may be one of the factors that contribute to the elevated mortality rate commonly seen in secondary bacterial infection in humans [26, 94, 95].

Interestingly the genetic analysis of archival lung tissue from a young soldier who died of influenza in 1918 revealed that the 1918 H1N1 virus does not possess a polybasic cleavage site as seen in recently emerged highly pathogenic avian H5N1 virus, indicating that other virulence factors might have contributed to its high pathogenicity [96]. Indeed, the presence of carbohydrate moieties in the stalk region of HA has been found to associate with the infectivity of influenza viruses. Low pathogenic influenza viruses contain carbohydrate moieties in the stalk region of HAs that interferes with the accessibility of host proteases into the cleavage site. As a result only a limited range of proteases can act activate the HAs [97]. On the other hand the removal of carbohydrate side chains adjacent to the receptor binding site increased the influenza HA binding affinity to its sialic acid residues on the host, and this would require a very active NA to free the newly formed virions [98]. HAs of pathogenic strains do not possess such carbohydrate chains in the vicinity, allowing proteases greater accessibility to the cleavage sites and greater replication rate [98].

1.4.2 Neuraminidase (NA)

Influenza virus NA is important in the cleavage of the HA on the newly formed virus from the sialic acid residues on the infected cell surface, hence it has the same binding specificity as HA. For example, the N2 of avian origin can only act on sialic
acid residues with $\alpha_2,3\text{Gal}$ linkage [99]. This binding specificity of NA was found to be correlated with the amino acid composition at or near the active site [100]. An isoleucine at position 275 near the enzymatic active site in N2 is only found in avian influenza virus and is associated with high binding specificity for glycoprotein with SA$\alpha_2,3\text{Gal}$ linkage. A mutation at this position to a valine residue showed an increase in the NA binding specificity to SA$\alpha_2,6\text{Gal}$ linkage without affecting its activity to SA$\alpha_2,3\text{Gal}$ linkage. This mutation and the resulting binding specificity is maintained in the sequences of all human H2N2 and H3N2 viruses isolated after 1962, hence its importance in the host range determination in influenza viruses.

NA has also been described to indirectly activate HA for the release of new virions. Influenza virus A/WSN/33 is a strain derived from A/WS/33 (H1N1), a descendant of the virus responsible for 1918 pandemic, that does not possess a polybasic cleavage site in the HA, making it distinct from other pathogenic avian influenza viruses, and suggest other mechanism must be involved in the HA activation [96].

The carboxy-terminal lysine residue in the NA of A/WSN/33 virus is able to bind to the human plasminogen and convert it to its active form plasmin, which can subsequently activate HA without the need of additional proteases [101]. Another structural requirement for NA to affect the activation of HA is the lack of oligosaccharide side chain at position 146 of NA. When present, its glycosylation after translation inhibits the accessibility of proteases to facilitate HA activation. These two structural features are both required for the successful cleavage of HA, therefore any strains of influenza with a NA that contains a lysine at the carboxyl-terminal of NA and lack of the oligosaccharide side chain at position 146 are considered potentially pathogenic [101].
1.4.3 Viral polymerase

Influenza vRNA is always packaged in a complex form with PB1, PB2, PA, and NP, and upon successful entry into epithelial cells, this complex translocates to the nucleus where replication occurs. This polymerase complex therefore is critical in the survival of influenza virus, and has been implicated by many studies to be involved in the host range determination as well. Clements et al. demonstrated that the reassortment virus containing the PB2 gene of avian origin and all other genes from human influenza virus is able to replicate efficiently in the avian host, while showing restricted replication in the mammalian respiratory tract [102]. This reassortment virus was then progressively passaged to generate mutant reassortment viruses that are able to replicate in mammalian tissues, which was then compared with the normal reassortment viruses. Nucleotide sequence analysis revealed that this phenotype of the reassortment virus is due to the amino acid at position 627 of PB2 gene [103]. A single amino acid substitution from glutamic acid to a lysine residue allowed this mutant reassortment virus PB2 to replicate in the mammalian system. Furthermore, all the avian influenza A viruses analyzed to date has a glutamic acid at this position in the PB2, whereas human influenza viruses have a lysine residue, demonstrating that this residue at position 627 of PB2 is an important host range determinant of influenza A viruses [103].

The compatibility of other subunits of polymerase complex is also involved in the host range specificity [104, 105]. Reassortment viruses with human PA and avian PB1 and PB2, or with human PA and PB2 and avian PB1 showed a restricted viral replication in mammalian cells, indicating that specific constellation of polymerase genes may be involved in the host range specificities [105].
In addition, human reassortment influenza virus with an avian NP protein was found to attenuate viral replication in primates, and this phenotype was able to be transferred to avian-human reassortment viruses [106]. This indicates that NP is also involved in the determination of host range specificity; however, the specific nucleotide sequence in NP responsible for this attenuation is not clear [105].

PB1-F2 is an accessory protein that is found not involved in the influenza viral replication in in vitro studies [107, 108]. It contains a mitochondrial targeting sequence at the carboxy-terminus and has a pro-apoptotic effect on immune cells [109]. By binding to the inner and outer membrane transport protein it disrupts mitochondrial integrity, thereby leading to apoptosis. PB1-F2 was also shown to enhance the pathogenesis of influenza viral pneumonia in a mouse model [110], as well as the secondary bacterial pneumonia [94]. Furthermore bactericidal effect has also been described with PB1-F2. It was hypothesized that the carboxyl terminal region of PB1-F2 can break down bacteria cell wall and release lipoteichoic acids and peptidoglycan, thereby enhancing the inflammatory response [94]. This feature of PB1-F2 may explain why the 1918 influenza A H1N1 was efficient at enhancing secondary bacterial pneumonia [94]. In support of this notion, the same study found that 1918 PB1-F2 protein enhanced viral replication, increased pulmonary inflammation with increases in neutrophils, macrophages, and T cells in mice compared to those without PB1-F2 or with a mutant PB1-F2.

### 1.4.4 Non-structural protein 1

NS1 protein of the influenza virus is encoded by the NS gene together with nuclear export protein (NEP) via alternative splicing. Sequence homology study had identified two alleles of influenza NS gene, allele A and B [111-113]. The NS gene of
human influenza A viruses belong to the A allele, and both alleles are present in the circulating avian influenza viruses [112, 113]. The current pandemic strain S-OIVs on the other hand has a NS gene of allele B [114]. NS1 is predominantly expressed rapidly to high levels in influenza virus-infected epithelial cells [115, 116]. NS1 has a length of 230-237 amino acids depending on the influenza strain, and an approximate molecular mass of 26,000 dalton. It has a RNA-binding domain at its amino-terminus (residue 1 – 73), and an effector domain (residue 74 – 230) at the carboxy-terminus [115, 117, 118]. The RNA-binding domain of NS1 recognizes different dsRNA sequences and blocks host RNA detection system. The effector domain of NS1 can stabilize the RNA-binding domain, but it predominantly interacts with host cellular protein and interferes with host mRNA processing [119], nuclear transport [120-123], and translation [119]. In addition to those mentioned above, the major function of NS1 is to antagonize host innate immune responses during infection. The inhibitory effect of NS1 in the host mRNA processing and translation allows influenza virus to rapidly establish infection, and by inhibiting the anti-viral responses will ensure the survival of influenza in the host. The detailed immune suppression by influenza NS1 is elaborated in section 1.5.3.
1.5 Host immune responses and immune evasion by influenza

The epithelial cells of the respiratory tract are the primary site of infection for influenza viruses. As the first barrier to pathogens the innate immune responses elicited by the epithelial cells will limit viral replication. It also has a profound effect on development of adaptive immune system, thereby affecting the outcome of infection.

1.5.1 Innate immune response to influenza

During infection, viral RNAs are generated as influenza replicates inside the epithelial cells and the viral pattern recognition receptors (PRRs) in the cytoplasm can recognize viral RNA and initiate immune response to this viral invasion.

Three important PRRs are currently found to be important in mediating innate immune responses during influenza infection. These are toll-like receptor 3 (TLR3), retinoic acid-inducible gene - I (RIG-I), and melanoma-differentiation-associated gene - 5 (MDA-5) [124, 125]. TLR3 situates within endosomal membrane inside host cells, and is involved in the recognition of viral dsRNA [126]. Upon binding TLR-3 initiates a signalling cascade that activate cytoplasmic nuclear factor κB (NF-κB), which is then translocated to the nucleus where it induces the expression of pro-inflammatory cytokines such as interleukin (IL) -6, IL-8, and tumour necrosis factor (TNF) –α (Figure 4). TLR3 was also known to induce the expression of type I interferons (IFNs) via IRF3-mediated pathway, however the precise anti-viral property of TLR3 remains inconclusive. Le Goffic et al. 2006 demonstrated that human influenza infection in TLR3-deficient mice had a reduced inflammatory response that led to a lowered clinical manifestation of influenza-induced pneumonia [127]. The same author later showed that influenza-induced inflammatory response is largely via TLR3 signalling pathway, and RIG-I is the primary mediator for both type I IFNs and inflammatory responses [128].
However other studies showed that TLR3 activation may only lead to clearance of specific RNA viruses such as encephalomyocarditis (EMCV) [129] but not influenza virus and West Nile virus. [127, 130-132]. Although the role of TLR3 in anti-viral response to influenza infection remains inconclusive, the inflammatory response elicited via TLR3 signalling in influenza infection is still an important factor in viral pathogenesis and disease symptoms. This is especially true in people infected with H5N1 virus as patients normally develop massive cytokine storm that lead to toxic shock [35, 38, 39]. Interestingly a study by Salomon et al. 2007 showed that mice deficient in IL-6, TNF-α, or CCL2 also succumb to infection with high pathogenic avian influenza H5N1 compared to wild type mice [133].

RIG-I and MDA-5 on the hand recognize viral RNA and initiate IFNs expression. RIG-I preferentially recognizes ssRNAs with 5’ triphosphate (5’ppp ssRNA) [128, 134-137], which is distinct from the host RNAs and only generated during influenza replication [134]. MDA-5 on the other hand binds to dsRNA depending on its length instead of its 5’ modification. However, studies have also reported that MDA-5 signalling can be partially triggered in ssRNA virus infection such as West Nile virus. MDA-5 in this case only appeared to be amplifying the anti-viral response while RIG-I binds and initiates the IFN response. RIG-I and MDA-5 contains two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. Once RIG-I binds to influenza RNA, the tripartite motif protein 25 (TRIM25) ubiquitinates the CARD domain of RIG-I, leading to a conformational change that allows the second CARD domain to associate with the adaptor protein IFN-β promoter stimulator 1 (IPS-1; also known as MAVS, VISA or CARDIF) [138-142], which is found on the mitochondrial membrane [140]. This complex then associates with TNF-receptor-associated factor (TRAF) -3 [143-145], which then recruits and activates TRAF family
member-associated NF-κB activator (TANK) -binding kinase 1 (TBK1) and inducible IκB kinase [146, 147]. These kinases phosphorylate IRF3 and IRF7, which then translocate to nucleus where they act as transcription factors for the induction of type I and III IFNs [146-149], as well as chemokines such as CXCL-10 that attracts CXCR3-expressing leukocytes to the site of infection and also directs effector T cell generation [150-152]. Type I IFNs (IFN-α/β) and type III IFNs (IL-29 (IFN-λ1), IL-28A (IFN-λ2), and IL-28B (IFN-λ3) are potent innate anti-viral cytokines induced by epithelial cells and also antigen-presenting cells (APCs) upon viral infection.

Figure 4. TLR3 and RNA helicases pathway leading to the expression and release of inflammatory cytokines and IFNs in epithelial cells. RIG-I/MDA-5 recognizes viral RNA and signals for type I and type III IFNs expression via the phosphorylation of IRF3. TLR3 also binds to viral RNA and induce the expression of inflammatory cytokines and chemokines. TLR3 has also been proposed to cause activation of IRF3, leading to IFNs expression.
The synthesized and secreted IFNs bind to their respective receptor (IFNAR1/2 for type I IFNs and IFN-λR1 for type III IFNs) on the same or neighbouring cells [149, 153, 154]. This binding results in the phosphorylation of the receptors that leads to the recruitment of signal transducer and activator of transcription (STAT) -1 and -2 [155-158]. This forms a transcription factor complex named ISG factor (ISGF)-3 with IRF9, and subsequently acts on interferon-stimulated response element (ISRE). This will initiate the expression of over 300 interferon-stimulated anti-viral genes (ISGs), allowing the establishment of full anti-viral state of epithelial cells and provide positive feed-back regulation to the IFN system [159, 160].

Figure 5. Type I and type III IFNs initiation of ISGs expression in epithelial cells. IFNs bind to the respective receptors on the same and/or neighbouring cells and initiates the expression of a wide range of ISGs such as positive regulators RIG-I and anti-viral protein PKR.
The critical anti-viral ISGs include protein kinase RNA-activated (PKR), MxA, 2’, 5’ – oligoadenylate synthetase (OAS), and ISGs such as ISG15 and ISG56. ISGs that are positive regulators of type I IFNs include RIG-I, MDA-5, and IRF7, which further amplify the entire anti-viral system.

PKR is a dsRNA-activated cellular enzyme that not only induces a rapid and potent expression of IFN proteins [161, 162], but also induces apoptosis upon viral infection [163-165]. PKR also phosphorylates the initiation factor eIF2, which results in a rapid inhibition of viral mRNA translation [158]. The OAS and MxA protein elicit an anti-viral state in the infected cells by inhibiting the viral replication. OAS can be activated by viral RNA, which in turn activates an endoribonuclease RNase L that can cleave extensively viral RNA [166]. MxA also interferes with viral protein synthesis and inhibits viral replication by promoting host cell apoptosis [167-170].

Despite the central role of IFN in the anti-viral system, a study by Paladino et al have identified a pathway that leads to an induction of a subset of ISG response in an IFN-independent and IRF3-dependent fashion [171, 172]. This response was observed against both a mutant herpes simplex virus type I (HSV-1) that cannot release viral RNA content into the cytoplasm, and HSV-1 particles without viral RNA genome, indicating that this response requires viral particle entry but not exposure to viral RNA and viral replication. RIG-I/MDA-5 and TLR-3 are primarily involved in the initiation of anti-viral response via IRF3 activation, however this newly identified induction pathway although required IRF3 activation but was not initiated by these PRRs. The cellular mediator involved in this pathway is still unknown [173-176]. This group proposed a model that explains this observation [173]. Epithelial cells may be constantly challenged by low levels of viral infection. By inducing subsets of ISG response upon exposure to low levels of invading viral pathogen via IRF3
independently of IFN may restrict viral replication within infected cells without causing unnecessary cellular damage due to the release of pro-inflammatory cytokines. If the virus particle entry threshold is exceeded, a full IFN response via RIG-I/MDA-5 and TLR3 will be initiated to limit viral replication.

1.5.2 Adaptive immune response to influenza

If intracellular viral infection becomes established and persistent, viral clearance is largely dependent on the adaptive immune response. The role of type I IFNs also extends to its ability to enhance the development of an adaptive response to influenza. APCs such as dendritic cells are important in mediating the antigen presentation process. As lung DCs acquire viral antigens presented on the infected epithelial cells surface, these cells undergo maturation as it migrates to lymph nodes, where they present the antigen to naïve CD8 T lymphocytes, also known as cytotoxic T lymphocytes (CTLs). Type I IFNs expressed by epithelial cells and DCs can enhance the expression of MHC class I molecules and co-stimulatory molecules such as CD40, CD80, and CD86, thereby promoting generation of influenza-specific CTLs (Rosa et al. 1983; Guadagni et al. 1989; Luft et al. 1998). CD8 T lymphocytes then undergo activation and division in lymph nodes, and subsequently home to the lungs to kill influenza-infected cells. CTLs are the main effector that recognizes the infected epithelial cells via the binding of MHC class I molecules associated with viral antigen on the surface of the cells [172]. Following binding CTLs then eliminate the infected cells by releasing highly effective cytotoxins including granzymes and perforin [172].

CD4 T lymphocytes or helper T lymphocytes are also activated in the lymph node. Upon contact with antigen-bearing DCs, CD4 T lymphocytes are activated to express CD40 ligand, which through binding to CD40 on DCs enhances the activation
of CTLs by DC [177-180]. The up-regulation of co-stimulatory molecules CB80, CD86, and cytokines including IL-12 also enhance this interaction [181-185]. CD4 T lymphocytes also express IFN-γ and TNF-α that exerts a direct anti-viral effect on infected cells, and at the same time attracting DCs to accumulate in the lungs for antigen uptake [174, 186, 187]. In addition, CD4 T lymphocytes can aid the generation of neutralizing antibody from B cells [188]. The specific antibody is directed against influenza surface antigen HA and NA, but are shown to have limited effect in the clearing of influenza. However, this neutralizing antibody does provide protection against strain-specific influenza virus following influenza vaccination.

1.5.3 Immune suppression by influenza NS1

NS1 protein has a molecular mass of 26,000, and is a multi-functional protein expressed very rapidly to help the establishment of viral infection by interfering with host mRNA processing and translation, as well as inhibiting host immune responses, especially the anti-viral system. The binding targets of influenza NS1 protein currently known are summarised in Figure 6.
Figure 6. Known binding targets of influenza NS1 proteins in relations to amino acids involved.

NS1 protein binds to RIG-I with RNA-binding domain, and R38/K41 is found to be critical in this binding. CPSF30 was bound with the effector domain, and amino acids 103, 106, 144 – 186 are important in this binding. PKR and PABII binding involves amino acids 123 – 127 and 189, respectively.
Host pre-mRNA undergoes endonucleolytic cleavage, and is polyadenylated at the 3’ end [189]. This maturation process requires a cleavage and polyadenylation specificity factor (CPSF) to act on a conserved AAUAAA hexamers sequence upstream of the cleavage site. CPSF has a 160kDa subunit and a 30kDa subunit, both of which are important in pre-mRNA 3’ end processing and subsequent polyadenylation [190-193]. CPSF, together with poly A binding protein (PAB) II, facilitate a rapid polyadenylation of host mRNAs. The effector domain of NS1 is able to bind to the 30kDa subunit of CPSF (CPSF30) (residue 189) and interact with PABII, thereby inhibiting host cellular mRNA processing [166, 194, 195]. By shutting down the host cellular protein synthesis in the infected cells will help the virus to gain control of the host machineries required for viral protein synthesis (Figure 7).

Figure 7. Influenza NS1 inhibition of host mRNA processing.

The IFN antagonistic property of NS1 occurs at multiple stages of the IFN signalling cascade. NS1 interacts with the viral sensor RIG-I, and can also inhibit the downstream activation of MAVS and nuclear translocation of IRF3, thereby inhibiting
type I IFNs [196, 197]. The NS1 protein specifically inhibits TRIM25-mediated RIG-I ubiquitination, which is crucial for maximal type I IFNs expression during viral infection [198]. This NS1-TRIM25 binding event is dependent on the arginine and lysine at position 38 and 41 respectively in the RNA binding domain, and glutamic acid at position 96 and 97 in the effector domain of NS1 [123, 199].

Beside the inhibitory role in type I IFNs expression, NS1 protein also inhibits cellular proteins that establish the anti-viral state of infected cells. The RNA-binding domain of NS1 can bind to viral RNA to prevent detection by PKR [200]. It also binds to PKR itself via the effector domain (residue 123 – 127) and inhibits PKR-mediated viral mRNA suppression and PKR-induced apoptosis [201-204]. OAS, which detects and cleaves viral RNA by activating RNase L, can also be blocked by influenza NS1. The RNA-binding domain of NS1 can out-compete the RNA binding capacity of OAS, thereby augmenting anti-viral response [205].
Interestingly, patients who died of H5N1 infection had extensive apoptosis in the alveolar epithelial cells [206]. This was thought to be part of a host defence mechanism since NS1 was known to be anti-apoptotic, however recent studies suggested that apoptosis is induced by avian H5N1 NS1 protein [207]. This pro-apoptotic property of NS1 is due to its interaction with phosphoinositol-3-kinase (PI3K). PI3K is a kinase that, when activated, generates a second messenger that recruits a serine/threonine protein kinase Akt, which subsequently regulates a wide range of cellular activities including anti-apoptosis, proliferation, and cytokine signalling and production. During influenza infection, NS1 can induce PI3K activation and Akt phosphorylation, thereby inhibiting PI3K/Akt-mediated anti-apoptosis effect. The exact mechanism of this inhibition at the molecular level is still unclear, but the effector domain of NS1 is shown to specifically bind to the p85β regulatory subunit of PI3K and inhibits its function. Several studies have identified the amino acid residues that play a role in this binding event. Tyr 89/Met 93 [208], Leu 141/Glu 142 [209], and Pro 164/Pro167 [210] of the NS1 effector domain are all located adjacent to each other within a cleft between the two NS1 monomers and are currently found to be involved in p85β binding.

The multiple roles of influenza NS1 protein are not limited to innate immune regulation but also extend to the adaptive arm of immunity, especially its inhibitory role in DC function. DCs are efficient APCs that activate influenza-specific T cells for viral clearance, and also have the same viral PRRs and signalling pathways found in the epithelial cells [211-213]. Using human DCs it was found that influenza NS1 can efficiently reduce the expression of IL-6, IL-8, TNF-α, RANTES, type I IFNs, and co-stimulatory molecule CD86, thereby inhibiting DC activation [211]. Furthermore, a lack of IFN-γ induction from naïve T cells was observed when DCs were infected with
influenza virus, indicating an inhibition by the NS1 protein in T cell priming towards Th1 anti-viral response [211].

The effector domain of the NS1 mainly involved in the inhibition of host cellular proteins, however it is likely that this domain also support the inhibition of anti-viral response by the RNA-binding domain.

Highly pathogenic avian H7N7 strain that lacks NS1 or that encodes an NS1 with large carboxyl-terminus deletion showed impaired IFN suppression activity and attenuated replication in mammalian and avian epithelial cells [214]. Other studies also observed similar results with influenza of different species. Both swine and turkey influenza virus with NS1 carboxyl-terminal truncation showed attenuated replication and an increase in IFN response compared to the wild type infection in its respective epithelial cells [215, 216].

In addition, when the NS1 with a natural deletion of residues 191 - 195 from low pathogenic swine influenza H5N1 was used to replace the NS1 of a highly pathogenic strain, the resulting virus was attenuated in replication and its IFNs inhibition in chickens [217]. The deleted residues were then engineered into this NS1 and the resulting virus gained virulence, demonstrating the importance of these residues at 191-195 are important in IFN inhibition. Although this region is not in a protein-binding motif, but is shown to be important in CPSF30 binding, which then leads to IFN suppression [216-218].

Further studies revealed that while human influenza is sensitive to IFN pre-treatment regardless of the inhibition by NS1, the avian H5N1 strain (A/HK/97) was able to survive regardless of IFN pre-treatment [219]. Normally anti-viral proteins can degrade NS gene and other viral genes of other circulating influenza, but NS1 of
A/HK/97 is able to bind and inactivate these anti-viral proteins [220]. This anti-viral resistance of NS1 was found to require a glutamic acid at position 92. Human influenza engineered with NS1 of A/HK/97 strain showed an enhanced replication in the presence of cytokines, whereas the wild type virus and the mutant virus containing a mutation at residue 92 failed to replicate under the same condition [220]. However, highly pathogenic avian influenza viruses isolated since then are not found to carry this residue anymore [221]. Influenza NS1 is no doubt a major virulence factor during infection due to its anti-viral suppression. This allows influenza virus to replicate inside the host with minimal interference from the host immune system, nonetheless the exact mechanisms with which NS1 exerts its function needs to be further investigated before a novel therapeutics can be discovered.

### 1.6 Influenza vaccines and anti-viral drugs

Influenza vaccination is the most effective way to prevent influenza infection and reduce potential severe complications following infection. The vaccine currently available is a trivalent inactivated vaccine (TIV) that contains killed viruses. TIV is administered intramuscularly in a single dose [222]. A trivalent live attenuated influenza vaccine (LAIV) of influenza A H3N2 and H1N1 subtypes and influenza B strain is also licensed for use and is given as a nasal spray [223]. Following vaccination influenza A specific antibody is generated from B cells with the help from CD4 T lymphocyte via CD40-ligand [188]. TIV is the approved vaccine in Australia.

It is recommended that children over six months of age, those who are working at health care facilities, people over 50 years of age, and those with chronic illnesses should have influenza vaccine every year. TIV but not LAIV can be given to those who are pregnant as well. Vaccines should not be given to people who are less than six
months of age, who have severe allergy to chicken eggs or severe reaction to influenza vaccine. Influenza vaccine may cause some minor side effects including aches, redness or swelling where shot was administered, or mild fever. In rare occasions, severe allergic reaction to influenza vaccine may occur following administration.

Influenza vaccine contains three human inactivated strains, one influenza A H3N2, one influenza A H1N1, and one influenza B virus. Each year the circulating influenza viruses and information on the severity of the disease are collected by 122 national influenza centres in 94 countries. This information is then combined and analyzed by the four World Health Organization (WHO) Collaborating Centres for Reference and Research on Influenza around the world. Based on the analysis scientists attempt to predict which influenza viruses are likely to circulate in the following season and recommendation for specific strains for vaccine can then be made. The WHO recommendation is made in February for the Northern Hemisphere, and in September for the Southern Hemisphere. Each country can use these predictions and decide which strains are more suitable for the country [224]. In Australia, the Australian Influenza Vaccine Committee (AIVC) receives recommendation every year and decides which strains should be incorporated in the influenza vaccine [225]. As soon as these recommendations are made private manufacturers can start producing vaccines. It usually takes at least six months to produce influenza vaccines in large quantities.

Recently a novel vaccine was developed using cell culture-derived trivalent inactivated influenza vaccine (CCIV) technology. Optaflu®[^], manufactured by Novartis Vaccine, is the first influenza vaccine manufactured using mammalian cell line, and was shown to be equally immunologic, safe and well-tolerated as the current vaccines made in chicken eggs. This provides an alternative choice of influenza vaccine as this is
beneficial for people who are allergic to chicken eggs from which current vaccines are made and can be manufactured in a relative short period of time [226].

GlaxoSmithKline (GSK) recently developed two vaccines that showed a protective immunity to highly pathogenic avian influenza virus. GSK-1562902A and GSK-1557484A contains inactivated A/H5N1 Vietnam strain as a vaccine virus adjuvanted with GSK’s proprietary oil-in-water emulsion AS03 compound. After influenza-naïve ferrets were vaccinated with these vaccines, it induced protective immunity against homologous A/Vietnam and a heterologous A/Indonesia strain. In clinical trials, the vaccines were well tolerated and were effective in inducing protection in over 90% of the vaccinees, and can be used in both pre-pandemic and pandemic period. GSK-1562902A and GSK-1557484A were recently approved in Europe as Prepandrix and Pandemrix, respectively. Phase II/III trials are also ongoing at the time of publication for both GSK-1562902A and GSK-1557484A. With the enormous demand for an effective vaccine in the event of an H5N1 pandemic, this vaccine is likely to be a promising additional choice of vaccine for future pandemics.

In addition to vaccines, anti-viral drugs against influenza are also available for prophylaxis and are more likely to be effective against new strains of influenza. There are currently two types of anti-viral drugs targeting two influenza proteins, M2 ion channel and NA. Adamantane (Symmetrel®) and rimantadine (Flumadine®) are M2 ion channel blockers that inhibits viral un-coating after entry, while zanamivir (Relenza®) and oseltamivir (Tamiflu®) are neuraminidase inhibitors that prevent newly formed virions from being released on epithelial cells.

Both adamantane and rimantadine are 70 – 90% effective when given prophylactically against influenza A, and are more effective in the presence of pre-
existing antibody immunity [227, 228]. However, adamantane-resistant strain have appeared and become prevalent due to increased use of this drug. This has resulted in an increase from 15% of adamantane resistant influenza A H3N2 in 2004/2005 to over 90% at the end of 2005 [229, 230]. From a phylogenetic analysis, this resistance is due to 17 single amino acid mutations in the M2 protein [231].

This rapid widespread of adamantane-resistant strain in countries previously shown to have little resistant virus suggests that this increase is due to the genetic reassortment of the resistant strain with other circulating non-resistant strain, instead of direct drug selection pressure [231]. As a result adamantane and rimantadine may have limited effect in the future pandemics.

The NA inhibitor zanamivir is given as inhaled form and oseltamivir is taken orally. They have similar efficacy when given in seasonal prophylaxis and post-exposure against both influenza A and B, including highly pathogenic avian strain H5N1 [232-234]. The actual effectiveness of oseltamivir in patients infected with H5N1 however is hard to analyse since by the time patients are admitted to hospital treatments are usually too late. A recent study have demonstrated that oseltamivir is effective when treatment starts early, and a higher dosage may be required if patients are infected with the lethal H5N1 Vietnam isolate [235, 236].

Oseltamivir and zanamivir targets the highly conserved active site in NA, and therefore resistance is less common than adamantane. The occurrence of oseltamivir resistance remains below 1% in adults [237], but is higher in children [238, 239]. Resistance is attributed to mutations in the highly conserved active site, and this may attenuate the virus and therefore reduce its fitness, which may explain this low occurrence rate.
Arginine at 292 to lysine mutation (R292K) and glutamate 119 to valine (E119V) is the most common mutation amongst H3N2 strains of clinical isolates and has shown to be the most effective amongst mutations in decreasing sensitivity to oseltamivir [237-239]. Histidine 274 to tyrosine (H274Y) is found in the N1 subtypes including human influenza A H1N1 [237, 239-241] and high pathogenic avian H5N1 strain [242, 243]. Both E119V and H274Y but not R292K mutant virus showed efficient transmissibility between ferrets [244, 245], but how closely the ferret model mimics human to human transmission is unknown.

Nonetheless, both oseltamivir and zanamivir have been the choice for stockpile in many countries due to the high oral bioavailability of oseltamivir, the effectiveness in decreasing symptoms and complications, and also because the current N1 viruses that are oseltamivir-resistant are still sensitive to zanamivir [239, 246].

Due to the rate of emergence of drug-resistant influenza viruses, new anti-viral drugs are also in progress. A multimeric form of zanamivir showed a higher efficacy in viral inhibition both in vitro and in vivo [247, 248]. Peamivir is a neuraminidase inhibitor derived from cyclopentane and has shown to be effective in both animal human studies [249]. Novel NA inhibitor, A-315675, was found to have similar or superior inhibition effect than oseltamivir against multiple strains of influenza [250]. This drug was also able to inhibit recombinant H1N1 oseltamivir-resistant mutant virus and also clinically isolated oseltamivir-resistant H3N2 and H5N1.

T-705 is a viral polymerase inhibitor that was shown to inhibit influenza replication and the mortality rate in mice. This inhibition was influenza specific since it does not influence the host cellular DNA or RNA processing; therefore it may be a useful compound for the treatment of influenza infection [251, 252]. In addition, the
discovery of RNA interference (RNAi) has opened up a new field of application that allows the silencing of gene of interest, especially in studying gene function and in therapeutic medicine. By using siRNA to influenza NP and PA, Ge, Q., et al. has shown that these siRNA can inhibit the accumulation of NP and PA mRNA, and also all other influenza genes [253, 254]. The silencing of M2 gene has also been shown to significantly decrease not only the level of M2 mRNA but also the NP mRNA, and potently inhibits the replication of H1N1 as well as high pathogenic avian H5N1 virus [255]. The silencing of additional genes of these siRNAs may be due the IFN response such as PKR elicited to the introduced siRNAs, thereby inhibiting viral replication. RNAi is an attracting technology in therapeutics due to its ability to silence gene of interest temporarily so infectious agent cannot replicate in the host. However, this may cause the emergence of siRNA-resistant virus due to selection pressure. An example of such resistance was observed in siRNA based treatment in HIV and HCV infection [256, 257]. Therefore the siRNA-based therapeutics and the rise of resistant influenza virus in treatment must be evaluated.
1.7 Research aims

Influenza virus is a major respiratory pathogen that causes significant medical and socio-economical consequences. As a result, all aspects of influenza infection in humans and other hosts have attracted extensive investigation.

Continuous focuses on the influenza genetics, virulence factors and pathogenesis have advanced the understanding of influenza evolution and infection in humans and other hosts. Recent progress on the immune signalling pathways and immune suppression strategies has also revealed the complexities of the human immune system as well as the elegance of influenza in anti-viral antagonism. This includes the molecular mechanism of viral RNA recognition and its downstream signalling by the RNA helicases, and other alternative routes of type I and type III IFNs initiation. The adaptive immune responses and novel targets for therapeutic design are also being explored extensively. Conversely, BECs as the primary site of infection for influenza viruses, the kinetics and magnitude of anti-viral responses activated during infection remain poorly characterized. In addition, as influenza viral infection can also cause primary viral pneumonia in the lower airways, it remains unclear as how distal airway epithelial cells respond to influenza infection.

Immune evasion is a common feature of all pathogenic micro-organisms in humans, and in addition to the frequent mutations of influenza surface glycoprotein HA and NA that evades the immune detection, influenza viruses also encode NS1 protein that suppresses human immune response. It is well-characterized that NS1 can inhibit host mRNA processing and inhibit the signalling events that lead to IFN response, the effectiveness of this suppression in human BECs however is still unclear. In addition as human influenza viruses continue to cause major health problems every year, avian
influenza viruses carried by the migratory birds are a potential source of future pandemics. For that reason it is important to investigate the infectivity of low pathogenic and high pathogenic avian influenza virus as well as the effectiveness of human anti-viral responses to both human and avian influenza viruses.

With these questions in mind this study aimed to investigate the mechanism of infection with human and avian influenza viruses of low and high pathogenicity in the immortalized cell lines of proximal and distal airways, and the kinetics and magnitude of the innate immune responses during infection.
Chapter 2. Materials & methods
2.1 Materials

2.1.1 Influenza virus

Table 1. List of influenza virus strains used in this study.

<table>
<thead>
<tr>
<th>Influenza strains</th>
<th>Description</th>
<th>Reference / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Wellington/43/2006 (H3N2)</td>
<td>A currently circulating human influenza virus</td>
<td>WHO Collaborating Centre for Reference and Research on Influenza (Vic, Australia)</td>
</tr>
<tr>
<td>A/Sharp-tailed Sandpiper/Australia/6/2004 (H11N9)</td>
<td>A low pathogenic avian influenza virus isolated in Carrington Wetland, Newcastle, NSW.</td>
<td></td>
</tr>
<tr>
<td>A/Vietnam/1203/04 (H5N1)</td>
<td>A high pathogenic avian influenza virus isolated in Vietnam</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2 Bacterial strain

Table 2. Bacteria used in this study

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Description</th>
<th>Reference / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>OneShot® TOP10 chemically competent E. coli for cloning purpose</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

2.1.3 Medium for bacterial growth

Luria-Bertani (LB) medium: 1% (w/v) Bacto-Tryptone (Oxoid), 0.5% (w/v) Bacto-Yeast Extract (Oxoid), and 1% (w/v) NaCl

LB agar: 98.5% LB and 1.5% (w/v) Bacto-agar (Difco)

2.1.4 Antibiotics

Table 3. List of antibiotics and its concentration used in this study.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Abbreviation</th>
<th>Concentration (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Amp</td>
<td>50</td>
</tr>
</tbody>
</table>
2.1.5 Tissue culture

2.1.5.1 Immortalised cell lines

Calu-3 A human broncho-trachael epithelial cell line acquired from patient with lung adenocarcinoma.

A549 A human alveolar basal epithelial cell line acquired from a patient with lung carcinoma.

MDCK A canine kidney epithelial cells used for plaque assay.

2.1.5.2 Primary bronchial epithelial cells (pBECs)

pBECs pBECs obtained from second and third generation of bronchiole of healthy volunteers by endobronchial brushings.

2.1.5.3 Media for tissue culture

MEM Minimum Essential Medium (Invitrogen). 1% penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5g/litre sodium bicarbonate, and 10% fetal bovine serum was added for subsequent cell culture.

DMEM Dulbecco’s Modified Eagle Medium (Invitrogen). 1% penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5g/litre sodium bicarbonate, and 5% fetal bovine serum was added for subsequent cell culture.

L-15 Leibovitz’s L-15 Medium (Invitrogen). Medium used in plaque assay on MDCK cells without additional supplements.
BEGM  Bronchial Epithelial Growth Medium (Cambrex). 1% penicillin-streptomycin, 0.25µg/ml amphotericin B, 10µg/ml insulin, 40µg/ml bovine pituitary extract, 1.0µg/ml recombinant human epidermal growth factor, 1.0µg/ml epinephrine, 20µg/ml transferrin, 3.7µM triiodothyronine, 0.0001µg/ml retinoic acid, and hydrocortisone was added for subsequent pBECs culture.

Opti-MEM-I  Modified Eagle’s Minimum Essential Medium with reduced serum for plasmid transfection

### 2.1.6 Molecular biology reagents

#### 2.1.6.1 Common buffers for genetics

TAE buffer:  40mM Tris.acetate, 2mM EDTA

TE buffer:  10mM Tris.HCl pH 8.0, 1mM EDTA

#### 2.1.6.2 Primers

Table 4. List of primer-probes and sequences used in RT-qPCR.

<table>
<thead>
<tr>
<th>Primers RT-qPCR</th>
<th>Probes (5’ fluorescent dye and 3’ quencher)</th>
<th>Sequence (5’- 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIG-I</td>
<td>FAM – MGB</td>
<td>CCTAGACCATGCAGGTTATTCTGGA</td>
</tr>
<tr>
<td>MDA-5</td>
<td>FAM – MGB</td>
<td>AGACAGAAACCGGATTGCTGCTGCA</td>
</tr>
<tr>
<td>TLR3</td>
<td>FAM – MGB</td>
<td>GTCATCCAACAGAATCATGAGACAG</td>
</tr>
<tr>
<td>PKR</td>
<td>FAM – MGB</td>
<td>TAAGGAAAAAGAAGGCAGTTAGTCCT</td>
</tr>
<tr>
<td>IFN-β</td>
<td>FAM – MGB</td>
<td>CCTCCGAACACTGAAGATCTCCTAGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FAM – MGB</td>
<td>GGGCGCCTGGTCACCAGGGCTGCTT</td>
</tr>
<tr>
<td>18S</td>
<td>VIC – MGB</td>
<td>Proprietary</td>
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Table 5. List of primers and sequences used in PCR.

<table>
<thead>
<tr>
<th>Primers for influenza NS1</th>
<th>Sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>NS forward primer</td>
<td>TATTCGTCTCAGGGAGCAAAAGCAGGGTG</td>
</tr>
<tr>
<td>NS reverse primer</td>
<td>ATATCGTCTCGTATTAGTAGAAACAAGGGGTGTTT</td>
</tr>
<tr>
<td>NS1-SAM forward primer</td>
<td>CCATTGCCTTTTTTTCCCGGACATACTATTGAGGATG</td>
</tr>
<tr>
<td>NS1-SAM reverse primer</td>
<td>CATCCTCAATAGATGTCCGGGAAAAGAAGGCAATGG</td>
</tr>
</tbody>
</table>

2.1.6.3 Plasmids

Table 6. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>References / Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA 3.3-TOPO vector, TOPO adapted</td>
<td>Cloning vector containing SC40 promoter and origin, and AmpR</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pcDNA:H3N2-NS1</td>
<td>pcDNA 3.3 with H3N2-NS1 insert</td>
<td></td>
</tr>
<tr>
<td>pcDNA:H11N9-NS1</td>
<td>pcDNA 3.3 with H11N9-NS1 insert</td>
<td></td>
</tr>
<tr>
<td>pcDNA:H3N2-NS1-SAM</td>
<td>pcDNA 3.3 with H3N2-NS1-SAM insert</td>
<td></td>
</tr>
<tr>
<td>pcDNA:H11N9-NS1-SAM</td>
<td>pcDNA 3.3 with H11N9-NS1-SAM insert</td>
<td></td>
</tr>
<tr>
<td>pUC19 control DNA</td>
<td>Control plasmid for transformation purpose</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pcDNA 3.3 TOPO/LacZ expression control plasmid</td>
<td>Cloning control plasmid containing LacZ</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
Table 7. List of antibodies and dilutions used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIG-I</td>
<td>Rabbit polyclonal anti-RIG-I (Prosci, Inc)</td>
<td>1:4000</td>
<td>Donkey polyclonal to rabbit IgG:HRP (Abcam)</td>
<td>1:6000</td>
</tr>
<tr>
<td>MDA-5</td>
<td>Rabbit polyclonal anti-MDA-5 (Prosci, Inc)</td>
<td>1:4000</td>
<td>Donkey polyclonal to rabbit IgG:HRP (Abcam)</td>
<td>1:6000</td>
</tr>
<tr>
<td>PKR</td>
<td>Rabbit polyclonal anti-MDA-5 (Abcam)</td>
<td>1:4000</td>
<td>Donkey polyclonal to rabbit IgG:HRP (Abcam)</td>
<td>1:6000</td>
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<tr>
<td>pIRF3</td>
<td>Rabbit polyclonal anti-pIRF3 (Ser396) (Milipore)</td>
<td>1:2000</td>
<td>Donkey polyclonal to rabbit IgG:HRP (Abcam)</td>
<td>1:2000</td>
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<tr>
<td>HA</td>
<td>Goat polyclonal anti-HA (Santa Cruz)</td>
<td>1:500</td>
<td>Anti-goat IgG:HRP (RnD System)</td>
<td>1:1000</td>
</tr>
<tr>
<td>NS1</td>
<td>Goat polyclonal anti-NS1 (Santa Cruz)</td>
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<td>Anti-goat IgG:HRP (RnD System)</td>
<td>1:1000</td>
</tr>
<tr>
<td>IFN-β</td>
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<td>1:4000</td>
<td>Donkey polyclonal to rabbit IgG:HRP (Abcam)</td>
<td>1:6000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Goat polyclonal anti-GAPDH (Abcam)</td>
<td>1:4000</td>
<td>Anti-goat IgG:HRP (RnD System)</td>
<td>1:6000</td>
</tr>
</tbody>
</table>

2.1.6.4 Common buffers for protein analysis

PBS: 120mM NaCl, 2.7mM KCl, 10mM phosphate salts pH 8.0

TBS: 120mM NaCl, 10mM Tris.Cl pH 8.0

TBS-T: 120mM NaCl, 10mM Tris.Cl pH 8.0, 0.05% (v/v) Tween 20

Towbin transfer buffer: 192mM Glycine, 25mM Tris.HCl pH8.3, 20% Methanol

Radioimmunoprecipitation assay (RIPA) buffer: 50mM Tris.HCl pH 7.4, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 2% sodium deoxycholate, 0.1% SDS
2.2 Methods

2.2.1 Tissue culture

2.2.1.1 Immortalized cell lines

Calu-3 cells were grown in MEM medium complete with 10% FCS at 37°C and 5% CO_2 in Hera 240 incubator (Heraeus) to approximately 95% confluence. The flask in which the cells were grown was trypsinized off the surface of the flask with trypsin. The flask was placed in the incubator for 15 – 20 minutes and was taken out. Medium containing FCS was added to the cells to inactivate the trypsin. The cells were then pelleted down at 15,000 rpm for five minutes. The supernatant was decanted and the cells were re-suspended in 10ml MEM / 10% FCS. Cells were counted using a hemocytometer, and appropriate number of cells was then sub-cultured into a fresh tissue culture flask. Medium in which the cells were grown was changed with fresh medium every two days. A549 and MDCK cells were grown in DMEM / 5% FCS and sub-cultured as detailed above.

2.2.1.2 pBECs

2.2.1.2.1 Subjects

Volunteers with no history of smoking and no evidence of airflow obstruction were recruited. A clinical history, examination and spirometry were performed on all individuals. They were questioned about the previous severity of cold symptoms. At the time of recruitment no subjects had symptoms of an acute respiratory tract infection for the preceding 4 weeks.

2.2.1.2.2 Bronchoscopy

All subjects underwent fibreoptic bronchoscopy in accordance with standard guidelines. These procedures were performed by A/Prof. Peter Wark who has extensive
experience in research bronchoscopy. pBECs were obtained using a single sheathed nylon cytology brush applied under direct vision. Approximately 5-10 brushings were taken from second to third generation bronchi. All volunteers gave written informed consent.

2.2.1.2.3 pBEC culturing

pBECs that was taken directly after bronchoscopy was washed with Dulbecco’s PBS and then cultured in tissue culture flask with BEGM complete supplemented with hydrocortisone. Medium was changed every two days with fresh medium. Cells were typsinized as detailed in 2.2.1.1.

2.2.2 Influenza infection

2.2.2.1 Virus preparation

Influenza viruses were propagated in MDCK cells. The viruses were inoculated onto MDCK cells and were incubated at 37°C and 5% CO₂ incubator. After five days infection supernatants were collected, and centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernatants were removed from pellet, aliquoted into 500μl aliquots, and stored at -80°C until use. The titre of virus stock was determined by plaque assay on MDCK cells.

All works with human influenza A/Wellington/43/2006 (H3N2) and avian influenza A/Sharp-tailed Sandpiper/Australia/6/2004 (H11N9) were carried out in Biosafety Level (BSL) II containment facility at the University of Newcastle.

All works with highly pathogenic avian influenza A/Vietnam/1203/04 (H5N1) were carried out in BSL-III facility available at The Commonwealth Scientific and Industrial Research Organization (CSIRO) - Australian Animal Health laboratory,
according to strict WHO Collaborating Centre for Reference and Research on Influenza standard operating procedures.

2.2.2.2 Plaque assay

MDCK cells were grown to approximately 70% confluence before infection. The cells were washed with Dulbecco’s PBS three times and were submerged in L-15 medium supplemented with HEPES and N-p-tosyl-L-phenylalanine chloromethyl ketone treated trypsin (trypsin-TPCK). Samples were serially diluted in L-15 medium (supplemented with HEPES) and were added to the cells. After one hour of incubation at 37°C / 5% CO₂ and inoculum removed, 1.8% agarose in L-15 medium containing trypsin-TPCK was overlayed on to the cell monolayers. After 48 hour of incubation at 37°C / 5% CO₂, plaques were stained with 0.1% crystal violet and counted.

2.2.2.3 Virus infection

BECs were trypsinized and seeded into a 24 well tissue culture plate as detailed in 2.2.1.1. BECs were infected with influenza virus when the cells reached approximately 80% confluence. Virus was diluted in the appropriate serum-free medium and added to cells in a volume of 200μl at multiplicity of infection (M.O.I.) of 5. After one hour incubation at 37°C / 5% CO₂, the virus inoculum was removed and replaced with appropriate serum-free medium. For infection with highly pathogenic avian influenza H5N1 strain, BECs were infected at M.O.I. of 0.1. Cells were treated with 100μg/ml of polyinosinic:polycytidylic acid (Poly I:C) (Sigma-Aldrich) as positive control since it is known as TLR3 and RIG-I/MDA-5 agonist.

2.2.3 Apoptosis & viability assay

The level of apoptosis and viability of BECs after infection with influenza virus was measured using PE Annexin V Apoptosis Detection kit I (Becton Dickinson)
according to manufacturer’s instruction. After infection at 48 hours BECs were trypsinized off the surface of tissue culture plate as detailed in 2.2.1.1. After the trypsin was inactivated and the cells pelleted down, the cells were washed with PBS once and with 1X staining buffer. After centrifuging at 1,500 rpm for 5 minutes, the cells were re-suspended in 1X staining buffer with annexin V-PE stain and vital dye 7-amino-actinomycin (7-AAD) for 15 minutes at room temperature in dark. The cells were then analyzed by FACSCanto II (Becton Dickinson) and analysed on FACSDiva software.

2.2.4 RNA analysis

2.2.4.1 RNA harvesting and extraction

After influenza infection and supernatant removed, 350μl of RLT buffer containing 300mM β-mercaptaethanol was added to the cell monolayer and incubated at room temperature for 20 minutes. The RNAs were extracted from the RLT samples using RNeasy Mini Kit (Qiagen). Equal volume of 70% ethanol was added to the samples and mixed by pipetting. The mixture was transferred into an RNeasy mini spin column and centrifuged for 15 sec at 10,000 rpm. Flow-through was discarded and 700μl of RNeasy Buffer RW1 was added to the column and centrifuged 15 sec at 10,000 rpm. The spin column was transferred to a new collection tube and 500μl of RNeasy Buffer RPE and centrifuged for two minutes at 14,000 rpm. Flow-through was discarded and re-centrifuged for one minute at 14,000 rpm to remove all trace of ethanol. The spin column was transferred to a 1.5ml eppendorf tube and 50μl of RNase-free water was added and centrifuged for 1m minutes at 10,000 rpm to elute the RNA. The concentration and quality of eluted RNA was determined by NanoDrop 1000 (Thermo Scientific).
2.2.4.2 Reverse transcription of RNA to cDNA

Extracted RNA (1μg) was treated with 2U of DNase-1 using thermocycler set at 65°C for 10 minutes. DNase was inactivated by adding 2μl of 25mM EDTA to the RNA and cooled for 5 minutes at -20°C. The RNA was mixed with a master-mix containing 600ng of random primer, 2μl of 10mM dNTP, and 2.8μl of nuclease-free water. The samples were incubated at 65°C for five minutes in thermocycler, and followed by chilling for five minutes at -20°C. A second master-mix containing 8μl of 5X PCR buffer, 4μl of 100mM DTT, 40U RNaseOUT, and 1ul water was added to each RNA sample and incubated at 25°C for 10 minutes and followed by 42°C for two minutes in the thermocycler. Following the third run 200U of Superscript II reverse transcriptase (Invitrogen) was added to each sample and incubated at 42°C for 50 minutes and followed by 70°C for 15 minutes.

2.2.4.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

cDNA products (1μg) from the reverse transcription step were used as template for RT-qPCR using Taqman Gene Expression (Applied Biosystems). Reaction was performed in duplicate with 10μl of 10X Eppendorf mix (Eppendorf), 0.375μl of 18S primer-probe (VIC-MGB), and 1.25μl of the primer-probes targeting the genes of interest (Table 4). Ribosomal RNA (18S) was used as the reference gene, and each target was performed separately using ABI 7500 sequence detection system (Applied Biosystems). The cycles performed in the RT-qPCR reaction is described below.

Table 8. The temperature, time, and cycles used in RT-qPCR reaction.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (Minutes)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>95</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
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</tr>
<tr>
<td>3</td>
<td>60</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Raw data was presented as the threshold cycle at which the fluorescence emission is significantly above baseline, and was then normalized by dividing the mean value of the Ct value of 18S RNA and was expressed as an exponent of 2 ($2^{Ct}$) by the mean value of $2^{CT}$ for the target gene. The fold induction over the medium control was then calculated by dividing the normalized target value by the normalized control value.

2.2.5 RNA interference (RNAi)

Transfection reagent was prepared by diluting siPORT NeoFX transfection agent (Ambion) (1µl) with 25µl of OPTI-MEM I (Invitrogen) and incubated at room temperature for 10 minutes. Small interfering RNA (siRNA) to RIG-I (Applied Biosystem) was diluted to 10nM with OPTI-MEM I, mixed with the transfection reagent by inversion, and incubated at room temperature for 10 minutes. This transfection complex was mixed with freshly trypsinized pBECs and added to the tissue culture 24 well plate. The transfectant cells were incubated at 37°C and 5% CO$_2$ incubator for 24 hours before influenza infection. GAPDH was silenced with siRNA in a separate well as a positive control. Successful knockdown was considered when mRNA was knocked down to $\geq 70\%$ measured by RT-qPCR (2.2.3.3) with $\leq 15\%$ cell death assessed as described in 2.2.2.4.

2.2.6 Microarray analysis

RNA was extracted from Calu-3 at 24 hours after infection using RNeasy Mini Kits (Qiagen) as detailed in section 2.2.4.1. Total RNA was amplified using Illumina TotalPrep RNA Amplification Kit (Ambion) according to manufacturer’s instruction. RNA (500ng) was first reverse transcribed to synthesize first strand cDNA using reverse transcription master mix, and was incubated at 42°C for two hours. Second strand cDNA was synthesized by using second strand master mix and incubated at 16°C for
another two hours. The cDNA product was purified and used as template for in vitro transcription to synthesize cRNA at 37°C for 14 hours. cRNA product was then purified, concentrated to 750ng, and hybridized onto Illumina Sentrix Human Ref-8_v3_Beadchip (Illumina) according to manufacturer’s instruction. Briefly, hybridization buffer was added to each samples and then pipetted onto the beadchip for incubation at 58°C for 20 hours. The chip was washed and stained with streptavidin-Cy3 for detection. Following detection the chip was washed, dried, and scanned using the Illumina Bead Station and captured using BeadScan 3.5.11 (Illumina). Data were imported into BeadStudio 3.0 (Illumina) and normalized to the median hybridization intensity of all genes on the beadchip, and exported to GeneSpring GX 10 (Agilent Technologies) for further analysis. Data were first normalized to the un-infected medium control, and several filtering steps were performed to reveal any genes whose expression was significantly changed during influenza infection. Filtering on flags was performed to remove genes that are not expressed at significant level in any conditions, and filtering on expression intensity (20^{th} – 100^{th}) percentile removed genes whose expression intensity was not above the 20^{th} percentile, which corresponds to genes that were not expressed. A one-way analysis of variance (ANOVA) was used with Benjamini-Hochberg correction to identify genes that were differentially expressed in H3N2 and H11N9 infection. A p value \leq 0.05 was considered to be significant and a fold change \geq 2 in expression was defined as the cut-off for significant change in expression. Hierarchical clustering was performed using centroid linkage clustering with Euclidean Distance. Data sets were submitted to the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under the accession number GSE19580.
2.2.7 Protein analysis

2.2.7.1 Flow cytometric analysis of surface influenza receptors

BECs were grown in the appropriate medium at 37°C and 5% CO₂ in Hera 240 incubator (Heraeus) to approximately 95% confluence. The flask in which the cells were grown was trypsinized off the surface of the flask with trypsin. The flask was placed in the incubator for 5 – 15 minutes and was taken out. Medium containing fetal calf serum (FCS) was used to inactivate trypsin, and then pelleted down at 1,500 rpm for 5 minutes. The supernatant was decanted and the cells were washed in 1ml of PBS pH 7.4. Following centrifuging at 1,500 rpm for 5 minutes, the cell pellet was washed in 1ml of lectin buffer (10mM HEPES, pH 7.0, 0.15M NaCl), pelleted and re-suspended in 1.5ml of lectin buffer. The cells were split into three FACS tubes of 500μl each. Biotinylated lectin Maackia amurensis lectin II (MAL-II) and FITC labelled Sambucus Nigra Bark lectin (SNA) was purchased from Vector Laboratories. One tube of cells was stained with MAL-II while another tube with SNA, and the remaining one was used as negative control. The cells were stained in the dark for 50 minutes at 4°C. Streptavidin-PerCP (RnD System) was added to biotinylated samples and incubated for another 30 minutes at 4°C. The cells were centrifuged, washed with 1ml lectin buffer for three times, and the pellet was re-suspended in 500μl lectin buffer. The incubation mixture was then counted by FACSCanto II (Becton Dickinson) and analysed on FACSDiva software. Results were expressed as median fluorescence intensity.

2.2.7.2 Harvesting of infection supernatant and whole cell lysate

After infection supernatant was taken off the cell monolayer and kept at -80°C until use. The cells were washed with ice-cold Dulbecco’s PBS once and decanted, followed by the addition of ice-cold RIPA buffer supplemented with protease inhibitor cocktail (Roche). The cells were incubated at room temperature for 20 minutes, after
which the whole cell lysates were collected. The lysates were sonicated on ice (4x 15 seconds burst set at 200W output with a 20KHz converter) using the Misonix ultrasonic processor XL-2000 (Qsonica). The lysates were then centrifuged at 3,000 rpm for 5 minutes at 4°C, and stored at -80°C until use.

2.2.7.3 Cycloheximide treatment in BECs in influenza infection

Cycloheximide (100μg) was used to pre-treat the cells 30 minutes before infection with influenza, and after one hour of viral inoculation, cycloheximide (100μg) was added in the appropriate medium to the cells. At six hours after infection cells collected for apoptosis/viability assay. Supernatants were also collected at six hours and 48 hours for plaque assay and protein analysis.

2.2.7.4 Z-DEVD-FMK treatment in BECs in influenza infection

Z-Asp(D)-Glu(E)-Val(V)-Asp(D) – Fluoromethylketone (FMK) was diluted to 100μM with dimethyl sulfoxide (DMSO), and was used to pre-treat the cells three hours before infection with influenza. After one hour of viral inoculation, Z-DEVD-FMK (100μM) was added in the appropriate medium to the cells. At 48 hours after infection, supernatants were collected for plaque assay, protein analysis and cells collected for apoptosis/viability assay.

2.2.7.5 IFNAR2 neutralization in influenza infection

IFNAR2 on the BECs was blocked with 1μg/ml of mouse monoclonal neutralizing antibody in the appropriate media (PBL InterferonSource) for one hour prior to infection. The media containing the antibody was taken off and replaced with virus inoculum for 1 hour at 37°C and 5% CO₂, and replaced with appropriate media. At 48 hours after infection supernatants were collected for plaque assay and protein analysis.
2.2.7.6 Exogenous IFN-β and IFN-λ1 priming of BECs in influenza infection

Human recombinant IFN-β (PBL InterferonSource) and IFN-λ1 (RnD System) at 1 ng was used to pre-treat the cells either alone or in combination at 37°C and 5% CO₂ for six hours prior to infection. The cytokines were removed and replaced with virus inoculum for one hour at 37°C and 5% CO₂ and replaced with appropriate media. At 48 hour after infection supernatants were collected for plaque assay.

2.2.7.7 Total protein concentration quantification

The concentration of proteins in infection supernatants and whole cell lysates were determined using BCA Protein Assay Kit (Thermo Scientific). Samples and BCA standards were vortexed and centrifuged, and 10 μl from which was loaded in wells of 96 well plate. Solution A and solution B was mixed and 200 μl of which was added into each well. The plate was placed on a plate shaker and mixed at 300 rpm for 30 seconds, and incubated at 37°C for 30 minutes. The plate was then read at absorbance of 560 nm in a microplate reader (BMG Labtech) and the concentration of samples was calculated by the software FLUOstar OPTIMA.

2.2.7.8 SDS-PAGE electrophoresis and western blotting

Protein samples were mixed with an equal volume of 2x loading buffer (125 mM Tris.Cl pH 6.8, 4.1% (w/v) SDS, 0.001% (w/v) Bromophenol Blue, 20% (v/v) glycerol, and 300 mM β-mercaptaethanol for reducing gels. The samples were boiled at 95°C for 10 minutes and loaded into the wells of the NuPAGE 4 – 12% Bis-Tris gel (Invitrogen). The gel was electrophoresed in 1X MOPS SDS running buffer using XCell SureLock Electrophoresis cell (Invitrogen) at 200V for one hour. After electrophoresis the gels was equilibrated in towbin transfer buffer for 30 seconds and placed on to a pre-soaked whatman filter paper (Whatman). A pre-soaked nitrocellulose membrane (Hybond) was
then placed on top of the gel and followed by another piece of filter paper. The Gel-membrane was then sandwiched between pre-soaked sponges and placed in the XCell II Blot Module (Invitrogen). The transfer was performed at 30V for one hour. The membrane was blocked with 5% (w/v) BSA in TBS-T for one hour at RT, after which was washed three times for 10 minutes each in TBS-T. The membrane was probed for the following targets at the appropriate dilution with the respective antibody listed in Table 7.

After 1 hour incubation at room temperature on an orbital shaker, the membrane was washed three times for 10 minutes each in TBS-T. The membrane was then incubated with appropriate secondary antibody in TBS-T for 1 hour at room temperature on an orbital shaker, and washed three times for 10 minutes each in TBS-T. The membrane was then developed by chemiluminescence using SuperSignal West Femto substrate (Thermo Scientific) and visualized using Fujifilm LAS-3000 Intelligent Dark Box with analysis software Fujifilm Image Reader LAS-3000. The densitometry on the blot was performed using the software Image J (http://rsbweb.nih.gov/ij/index.html).

2.2.7.9 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of CXCL-10 and IFN-λ1 was measured by ELISA (RnD System) according to manufacturer’s instruction. Microplate 96 well flat bottom (NUNC) was pre-coated with 100μl of capture antibody in PBS overnight at room temperature. The plate was washed with 400μl of wash buffer (0.05% Tween 20 / PBS pH 7.2) three times, and was inverted and blotted to remove remaining wash buffer. Reagent diluent (1% BSA / PBS pH 7.2, filtered) (100μl) was added to each well to block unspecific sites for one hour at room temperature. After three washes with wash buffer 100μl of samples was added to the wells and incubate at room temperature for
two hour. Three washes of the plate were repeated and 100μl of secondary detection antibody was added to each well. After two hour incubation at room temperature, the washing step was repeated and 100μl of streptavidin-HRP was added to each well and incubated at room temperature in dark for 20 minutes. Three washes were performed again and 100μl of substrate solution was added to the wells and incubated at room temperature in dark for 20 minutes. Stop solution (2NH₂SO₄) (50μl) was added to stop the reaction. The plate was then read by the microplate reader (BMG Labtech) at 450nm with wavelength correction at 540nm, and the concentration of target proteins were calculated by the software FLUOstar OPTIMA.

2.2.8 Influenza NS1 study

2.2.8.1 Influenza viral RNA extraction and reverse transcription

Influenza viral RNA was extracted using RNease Mini Kit (Qiagen) as detailed in section 2.2.4.1. The concentration of extracted RNA was measured using NanoDrop 2000 (Thermo Scientific), and used for subsequent reverse transcription.

SuperScript III First-Strand synthesis system (Invitrogen) was used to generate viral cDNA. Viral RNA (1μg) was mixed with 50ng random primer, 10mM dNTP, and incubated at 65°C for five minutes in the thermocyclor. The mix was then added to the cDNA synthesis mix containing 10X RT buffer, 25mM MgCl₂, 0.1M DTT, 40U RNaseOUT, and 200U SuperScript III reverse transcriptase, and incubated in the following cycles.

Table 9. The temperature, time, and cycles used in reverse transcription reaction.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature (°C)</th>
<th>Time (Minutes)</th>
<th>Cycles</th>
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2.2.8.2 Polymerase Chain Reaction (PCR)

PCR reaction was performed with 10μl of 10X PCR buffer, 2.5mM MgCl₂, 0.1mM dNTP, 1μl of influenza NS forward and reverse primers, 0.5U Taq polymerase, 2μl reverse transcribed cDNA, in a total volume of 25μl. The reaction was performed in cycles described below.
Table 10. The temperature, time, and cycles used in PCR reaction.

<table>
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2.2.8.3 DNA gel electrophoresis and gene extraction

10x loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% Ficoll, 0.1M Na2EDTA, and 1% SDS, in H2O) was added to DNA at a ratio of 1:10 and ran on 1% agarose in TAE buffer at 90V for 30 minutes. The gel was stained in TAE buffer containing 5μg/ml ethidium bromide for 10 minutes and the DNA was visualized using DigiDoc-It Imaging system (UVP). The band representing NS gene in the gel was excised out of the gel and purified by freeze-thaw-smash method. 10μl of TE was added to the gel containing the inserts, and was frozen at -80°C for three minutes. Following removal from the freezer gel pieces were placed at 37°C for three minutes. During this time the gel was smashed into smaller pieces. This freeze, thaw, smash cycle was repeated four times then centrifuged at 13,000 rpm for five minutes. The DNA-containing supernatant was pipetted into a new eppendorf tube.

2.2.8.4 Addition of 3’ A-Overhangs post-amplification

After amplification 1U of Taq polymerase was added to the genes, and incubate at 72°C for 10 minutes, and was used for ligation into the expression vector pcDNA.

2.2.8.5 Ligation of NS gene into pcDNA 3.3-TOPO vector

The extracted genes with 3’ A-overhangs (2μl) were mixed with pcDNA 3.3-TOPO vector (Invitrogen) and incubated at room temperature for five minutes. The reaction was then placed on ice for subsequent transformation.
2.2.8.6 Transformation

Ligation mixture (2μl) was added into a vial of OneShot Chemically Competent *E. coli* (Invitrogen), mixed gently and incubated on ice for 30 minutes. The mixture was then heat shocked at 42°C for 30 seconds, immediately placed on ice immediately for five minutes and 250μl LB medium added. The mixture was shaked at 200rpm at 37°C for one hour. The mixture (100μl) was plated on LB agar plate containing appropriate antibiotics and incubated at 37 °C overnight.

2.2.8.7 Site-directed mutagenesis

Internal primers were designed to generate a silent point mutation at the nucleotide 514 in the cDNA sense (A to C). This prevents this splice acceptor site to produce NEP proteins via alternative splicing during translation. By using the pcDNA:NS1 as a template, 5μl of 10X PCR buffer, 50mM MgCl₂, 10mM dNTP, 1μl of NS1-SAM forward and NS1 reverse primer, 0.5 units *Taq* polymerase, 2μl of the pcDNA:NS1 were mixed in a total volume of 50μl. Amplification was as previous, except run for 10 cycles only. A second PCR reaction was performed using NS1-SAM reverse primer and NS1 forward primer for 10 cycles. The two products were joined and amplified by performing a third PCR reaction using NS1 forward and reverse primer for 15 – 20 cycles (Figure 9). The PCR product was then confirmed by DNA gel electrophoresis, and sent for sequencing to ensure that the desired mutation was successful.
Figure 9. Site-directed mutagenesis of influenza NS gene.

2.2.8.8 DNA purification and preparation for sequencing

Single colony PCR reactions were performed to screen for positive clones, which were then inoculated into 25ml LB culture with appropriate antibiotics and incubated overnight at 37°C with shaking. Plasmid in the bacterial culture was then extracted using PureLink HiPure Plasmid FP Maxiprep kit (Invitrogen) as instructed in manufacturer’s protocol. Briefly, the bacterial culture was centrifuged at 4,000 rpm for 10 minutes, after which supernatant was decanted and bacterial pellet was re-suspended in lysis buffer. The lysate mixture was mixed to homogenous before passing the lysate through a pre-equilibrated maxiprep column that binds to the plasmid in the lysate. The flow-through was discarded, and column was washed with wash buffer. Plasmid on the column was then eluted with elution buffer, and precipitated with isopropanol to de-salt and concentrate the plasmid. Isopropanol was added to the samples and pelleted at
13,000 rpm for 30 minutes at 4°C. With supernatant decanted, the pellet was re-suspended with 70% ethanol and centrifuged at 13,000 rpm for five minutes at 4°C. The supernatant was removed and the pellet was air-dried to remove any remaining ethanol. DNA was then re-suspended in TE buffer and sent to Australian Genome Research Facility, University of Queensland, Queensland, Australia) for sequence confirmation.

2.2.8.9 Transfection

Total RNA was extracted from purified influenza virus using RNeasy Mini Kit (Qiagen) as detailed in section 2.2.4.1. Plasmid was transfected into Calu-3 using Lipofectamine LTX and PLUS reagent (Invitrogen). pcDNA:H3N2-NS1-SAM and pcDNA:H11N9-NS1-SAM (1µg) was diluted in 100µl of Opti-MEM I reduced serum medium and mixed by inverting up and down. PLUS reagent (1.5µl) was added to the diluted DNA, mixed, and incubated for 15 minutes at room temperature. Lipofectamine LTX (3µl) was added to the DNA and mixed gently before incubating at room temperature for 30 minutes. The DNA-LTX complex (100µl) was added to the Calu-3 cells and 400µl of MEM / 5% FCS without antibiotics was added. After six hours, media was removed and polyinosinic:polycytidylic acid (Poly I:C) of 100 µg/ml (Sigma) was added to the transfectant cells. At 42 hour following stimulation supernatants and whole cell lysates were collected for NS1-SAM expression and protein induction analysis.

2.2.9 Data analysis

All results were obtained from three independent experiments and are presented as standard error of the mean (SEM).

Densitometry in western blotting was performed and for intracellular proteins the values were expressed as protein/GAPDH ratio for RIG-I, MDA-5, pIRF3, and
influenza HA, and were presented as fold induction from medium control. For secreted protein the values were presented as fold induction from medium control.

Statistical analysis was performed using student’s $t$ test on virus replication. A one-way analysis of variance (ANOVA) was performed on mRNA relative fold change. A p-value of <0.05 was considered significant.

### 2.2.10 Ethics and funding source

This study was approved by Hunter New England Human Research Ethics Committee for the protocol *Mechanisms of Inflammatory airways disease* (05/08/10.3.9).

This study was funded by National Health and Medical Research Council (NHMRC project grant #510762).
Chapter 3. Results
Anti-viral response is a critical component of innate immune systems that limits viral replication at the site of infection, at the same time allows appropriate adaptive immune response to develop for efficient viral clearance. The pathways from viral RNA detection to amplification of anti-viral responses in BECs to general viral infections are well-known, however kinetics and effectiveness of the anti-viral response to influenza infection, especially when faced with influenza-induced IFN inhibition, remain poorly characterized. In addition, avian influenza viruses are a potential source of future pandemics as they have the ability to undergo mutations and reassortment that allow the mutant virus to infect and transmit from human to human in a sustainable manner. As the natural reservoir of influenza viruses are birds, an avian influenza virus of low pathogenicity was isolated from a trans-hemispheric migratory bird sharp-tailed sandpiper (A/Sharp-tailed Sandpiper/Australia/6/2004), and was used as a comparison to the human influenza H3N2 strain.

To assess the anti-viral responses of human BECs to influenza infection the proximal airway epithelial cells were modelled using Calu-3 cells and primary BECs (pBECs) obtained from the proximal bronchi of healthy volunteers. The response of distal airway epithelium was also assessed by examining the effect of infection in A549 cells. The anti-viral response to infection was determined by assessing the induction kinetics and magnitude of anti-viral signalling proteins and subsequent IFN responses during human influenza virus infection.
3.1 Influenza replication of H3N2 and H11N9 and the involvement of sialic acid-linked glycoprotein

The infectivity of H3N2 and H11N9 in proximal and distal airways cells were not well-defined, especially the avian H11N9 strain. Calu-3 cells, A549 cells, and pBECs were infected with H3N2 or H11N9, and viral replication was measured by plaque assay at 48hr after infection. H3N2 and H11N9 replicated to a high titre at 48hr after infection, and the titre of H3N2 was significantly higher than that of H11N9 in Calu-3 cells (Figure 10). While both viruses also replicated to a high titre in A549, the titre of both viruses was significantly lower compared to that in Calu-3 cells. Replication was the lowest in pBECs for both viruses.

![Influenza viral replication](image.png)

Figure 10. H3N2 and H11N9 replication at 48hr after infection in Calu-3, A549 cells, and pBECs.

H3N2 virus release into the supernatants of infected cells was determined by plaque assay on MDCK cells. At 48hr after infection, H3N2 replicated to a significantly higher titre in Calu-3 cells compared to A549 (p = 0.002), and to healthy pBECs (p < 0.001). A549 also supported a greater viral replication than pBECs (p = 0.003). Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
As glycoproteins with sialic acid residues of specific configuration on the surface of BECs are the binding targets for influenza viruses, viral replication could have been influenced by the expression level of these residues. To determine if there was a correlation between the level of SAα2,6Gal and SAα2,3Gal residues with H3N2 and H11N9 replication, respectively, these sialic acid residues were assessed on Calu-3 cells, A549 cells, pBECs. Viral entry was also measured in infected cells 2hr after infection by western blotting.

Calu-3 cells showed a significantly higher level of SAα2,6Gal residues compared to SAα2,3Gal residues (Figure 11). However, both viruses initially infected the cells equally well, with evidence of influenza HA protein present in similar levels at 2hr post infection (Figure 12). The level of SAα2,6Gal linked glycoprotein expression on Calu-3 cells and A549 cells was significantly lower than that found on pBECs, which is in contrast with their viral replication titre (Figure 10). The expression level of SAα2,6Gal residues on pBECs was the highest but showed the lowest viral titre. This indicates that factors other than these sialic acid residues are involved in the susceptibility of pBECs to influenza infection. Therefore influenza infection and replication might not be dependent alone on the level of sialic acid residues on the surface of the BECs, and other factors were involved in the susceptibility to influenza infection.
Figure 11. SAα2,6Gal and SAα2,3Gal linked glycoprotein levels on the BECs.

FITC-labelled SNA and MAL were used to stain BECs and was analysed by flow cytometry. All cell types showed significantly higher levels of SAα2,6Gal than SAα2,3Gal linked glycoproteins. In addition, pBECs had a significantly higher level of SAα2,6Gal linked receptor expression compared to Calu-3 cells (p < 0.001), A549 (p = 0.003). Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
Influenza virus internalization following infection of BECs.

Viral entry was analyzed by measuring the level of HA inside the infected cells at 2hr after infection by western blotting. The HA level was similar between H3N2 and H11N9 in all infected cells, however, the level was higher in Calu-3 cells, and lowest in pBECs, indicating other binding sites may be involved in infection. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).

Figure 12. Influenza HA protein at 2hr after infection
These results indicate that influenza entry and subsequent viral replication was not dependent on the expression level of SAα2,6Gal or SAα2,3Gal linked glycoprotein on the surface of BECs alone, and other factors were involved in influenza infection. Traditional theory suggested that glycoproteins with SAα2,6Gal linkage are the primary binding target for human influenza viral entry into BECs for efficient viral replication, however, it appears that influenza viruses could replicate efficiently with low levels of these residues. In addition, A549 cells as a model for distal airway cells had less sialic acid linked glycoprotein however both influenza strains could replicate to a high titre compared to that in pBECs. This indicates other factors may be involved in the host susceptibility to influenza infection.
3.2 Innate anti-viral response to influenza infection in proximal and distal airway epithelial cells

Human innate anti-viral response to influenza infection in the infected BECs is critical in restricting viral replication and spread, at the same time providing signals for the appropriate adaptive immune response to develop for efficient viral clearance. IFN responses are important elements of innate immune response to influenza infection as these proteins. The pathways leading to these responses are well-characterized however the induction kinetics are poorly elucidated, therefore important anti-viral molecules were examined during influenza infection at mRNA and protein level.

3.2.1 Anti-viral signalling kinetics to influenza infection in Calu-3 cells

To assess the anti-viral responses in infected cells, anti-viral gene induction was measured by RT-qPCR at 6h, 12h, 24h, 48h, and 72h after influenza infection and Poly I:C stimulation. Poly I:C (100µg/mL) is a known agonist for RIG-I/MDA-5 and TLR3, and was used as a positive control in this study. In all BECs Poly I:C stimulation and influenza infection resulted in a significant induction of anti-viral genes compared to the medium control. UV-inactivated virus was no different to the medium control (Data not shown). Poly I:C induced an early induction of RIG-I mRNA at 6hr and 12hr in Calu-3 cells and pBECs, respectively (Figure 13A). MDA-5 mRNA was up-regulated only late in infection at 48hr and 72hr (Figure 13B). The induction of RIG-I was followed by inductions of TLR3, PKR, and IFN-β mRNA from 12hr to 48hr (Figure 13C-E).

Infection with H3N2 and H11N9 led to an early induction of RIG-I (25 fold) by 6hr, with a more modest increase in MDA-5 mRNA (5 fold) by 12hr (Figure 13A, 13B). This was followed by a second late induction of both genes at 72hr. H11N9 induced
similar responses, although the later rise in RIG-I from 24-72hr was more modest and gradual. Infection induced TLR3 mRNA at 6hr with a second rise from 48-72hrs, which occurred to a greater extent than for H1N9 (Figure 13C). Both viruses induced PKR and IFN-β mRNA from 48hr to 72hr (Figure 13D, 13E), with H1N9 inducing an earlier IFN-β response.
Figure 13. Induction of anti-viral response genes to influenza infection in Calu-3 cells. H3N2 and H11N9 induced an early up-regulation of (A) RIG-I mRNA at 6hr, whilst (B) MDA-5 mRNA was not increased until 48hr. (C) TLR3 mRNA expression was increased at 12-72hr. (D) Both viruses expressed (D) PKR and (E) IFN-β mRNA from 48 to 72hr. H3N2 induced earlier and greater RIG-I and TLR3 responses, whereas H11N9 induced an earlier IFN-β response. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
The protein expression of RIG-I and MDA-5, as well as IFN-β was also assessed after infection. As there are no quantitative detection methods (ELISA) available for intracellular proteins detection, western blotting was performed to measure RIG-I and MDA-5 proteins in cell lysates, and IFN-β measured in the supernatants. In contrast to observations made with mRNA, H3N2 induced delayed RIG-I protein expression until 48hr, and this induction was significantly lower compared to H11N9 (Figure 14A). Similarly MDA-5 protein was only detected at 48hr after H11N9 infection (Figure 14B) but not at earlier time points by both viruses (Data not shown).
Figure 14. RIG-I and MDA-5 protein production in response to influenza infection in Calu-3 cells.

The protein induction of RIG-I and MDA-5 was detected by western blot. (A) H3N2 only induced the significant production of RIG-I at 48hr, whereas RIG-I was detected at 6hr, 24 and 48hr after H11N9 infection, and the induction was significantly up-regulated compared to medium control. (B) Similarly, H11N9 but not H3N2 infection resulted in a detectable MDA-5 expression at 48hr. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
As inductions were mostly observed at 48hr after infection, IFN-β protein induction was measured at 48hr. Infection with H3N2 induced a minimal IFN-β at 48hr compared to the medium control, and the induction by H11N9 infection was significantly higher than that by H3N2 (p = 0.011) (Figure 15).

Figure 15. IFN-β protein production in response to influenza infection in Calu-3 cells. The protein induction of IFN-β was measured by western blot. IFN-β protein was not induced above medium control, whereas H11N9 infection caused a significant up-regulation of IFN-β (p = 0.001), and this induction was also significantly higher than that observed in H3N2 infection (p = 0.011). Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
3.2.2 Anti-viral signalling kinetics to influenza infection in pBECs

In pBECs the mRNA induction of RIG-I, MDA-5, TLR3, and PKR generally occurred earlier compared to that in Calu-3 cells. RIG-I mRNA was induced to 25 fold at 12hr and sustained throughout the time course (Figure 16A). MDA-5 mRNA was also induced earlier than in Calu-3 cells and to a greater magnitude of 60,000 fold at 48hr (Figure 16B). TLR3 mRNA was minimally induced throughout the course of infection (Figure 16C). RIG-I expression was followed by an induction of PKR (5 fold) and IFN-β mRNA (150 fold) at 24hr (Figure 16D, 16E).
Figure 16. Type I IFN signalling and responses to influenza infection in pBECs.

The relative induction of mRNA was measured by RT-qPCR, normalized to housekeeping gene 18S ribosomal RNA, and expressed as fold change from medium control. (A) RIG-I mRNA was induced early (25 fold) at 12hr and sustained throughout the course of infection. (B) MDA-5 mRNA was also induced earlier than in Calu-3 cells and to a greater magnitude of 60,000 fold at 48hr. (C) TLR3 mRNA was minimally induced throughout the time course, and (D) PKR mRNA was induced to 5 fold at 24hr. (E) IFN-β mRNA was up-regulated 175 fold at 24hr pi and sustained throughout the time course. Results were obtained from three independent experiments are presented as standard error of the mean (SEM).
Protein productions were similar to that observed in Calu-3 cells. RIG-I protein levels were detected and maintained at 6 and 24hr and again were only significantly induced at 48hr after H3N2 infection (Figure 17A). MDA-5 protein was again not detected after H3N2 infection (Figure 17B) and IFN-β protein levels remained at baseline levels (Figure 18). In contrast, H11N9 infection resulted in a significantly higher induction of all proteins measured compared to H3N2.

Figure 17. RIG-I and MDA-5 protein induction in pBECs at 48hr after influenza infection.
(A) RIG-I protein levels were maintained at 6 and 24 hrs during infection and a significant induction of RIG-I protein was only detected at 48hr (p < 0.001). (B) MDA-5 protein levels were also non-detectable after H3N2 infection. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
Figure 18. IFN-β protein induction at 48hr after influenza infection.

IFN-β protein was maintained at 48hrs compared to medium control, and the induction by H11N9 infection was significantly higher than that by H3N2 (p = 0.002). Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
3.2.3 Anti-viral signalling kinetics to influenza infection in A549 cells

Influenza viruses are also known to infect epithelial cells of lower airways and can lead to viral pneumonia, therefore the anti-viral responses was also examined in A549 cells, a cell line modelling distal airway epithelial cells.

Following influenza infection A549 cells had intermediate immune responses between Calu-3 cells and pBECs. These cells had a relatively late induction of RIG-I mRNA (45 fold) at 24hr (Figure 19A). MDA-5 mRNA was also induced at 48hr with a 4 x 10^3 fold increase (Figure 19B). TLR3 and PKR mRNA was induced 10 fold 24hr after infection (Figure 19C and 19D). Interestingly, IFN-β mRNA could not be detected except in the un-infected medium control (Data not shown).
Figure 19. Type I IFN signalling and responses to influenza infection in A549 cells.

The relative induction of mRNA was measured by RT-qPCR, normalized to housekeeping gene 18S ribosomal RNA, and expressed as fold change from medium control. (A) RIG-I mRNA was up-regulated to 45 fold at 24hr after H3N2 infection and then declined, whereas (B) MDA-5 mRNA was induced early at 24hr after H3N2 infection and increased 5,000 fold at 48hr. (C) TLR3 and (D) PKR mRNA was up-regulated to 10 fold at 24hr and then declined. IFN-β mRNA was not detected at any time point after infection or Poly I:C stimulation. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
In contrast to Calu-3 cells and pBECs, RIG-I protein expressions in A549 cells were induced above baseline at 6hr ($p = 0.024$) and remained at a similar level up to 48hr after H3N2 infection (Figure 20A). While MDA-5 protein was not detected in any samples (Data not shown), IFN-β protein levels were significantly decreased after H3N2 infection ($p = 0.034$) (Figure 20B). PKR protein was also not induced above baseline level in all conditions (Figure 20C).

**Figure 20.** Protein expression of RIG-I, IFN-β, and PKR after infection in A549 cells. (A) A significant induction of RIG-I protein was observed at 6 ($p = 0.024$) and 48hr ($p = 0.042$) after infection. (B) Infection and Poly I:C stimulation induced a significant decrease in IFN-β protein levels at 48hr. (C) In sharp contrast PKR was not induced above media control in all conditions. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
Intermediate levels of influenza infection occurred in alveolar epithelial cells and correlated with intermediate immune responses compared to upper airway epithelial Calu-3 cells and pBECs, although A549 appeared to be incapable of maintaining IFN-β protein expression after infection and Poly I:C stimulation.

3.2.4 IRF3 phosphorylation status after influenza infection and Poly I:C stimulation

Following viral RNA recognition by RIG-I, IRF3 is phosphorylated at serine 293, leading to the dimerization and nuclear translocation of IRF3. The phosphorylated IRF3 acts as a transcription factor for expression of IFNs, which in turn amplify the ISGs expression including RIG-I. The delayed responses observed in Calu-3 cells and pBECs could be due to an inefficient activation of IRF3. As the magnitude of anti-viral responses were similar in Calu-3 cells, pBECs, and A549 cells following infection, whole cell lysates of Calu-3 cells were collected at 6hr after infection and were homogenized to release pIRF3 from nucleus, and was measured by western blotting.

Phosphorylated IRF3 was not observed at 6hr after H3N2 and H11N9 infection whereas Poly I:C induced a 6 fold induction of pIRF3 by 6hr (p < 0.001) (Figure 21).
Figure 21. Phosphorylation of IRF3 during influenza infection of Calu-3 cells. Whole cell lysates were sonicated to release pIRF3 from nucleus, which was detected with anti-pIRF3 antibody. pIRF3 was increased at 6hr after Poly I:C stimulation (p < 0.001) but not after influenza infection. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).

Taken together these results showed that control of influenza infection is related to the early induction of RIG-I and subsequent induction of IFN response. Influenza infection showed a delayed and a reduced IFN response compared to the synthetic dsRNA Poly I:C control. Infection in A549 cells induced an earlier RIG-I induction compared to other cells tested however this early induction did not result in an increase in IFN response, and subsequently led to a high viral replication. This delay in IFN responses in the host cells was due to a lack of IRF3 phosphorylation downstream of RIG-I/MDA-5 signalling pathway during infection, which subsequently led to a low level of IFN-β protein production. The impaired anti-viral response may result from the anti-IFN suppressive properties of influenza NS1 protein [196-198].
3.2.5 Type III IFN and ISGs induction after influenza infection

Type III IFNs are a recently discovered family of IFNs that shares similar antiviral property as type I IFNs, whose induction might also be inhibited by the influenza viral infection, leading to subsequent reduction of ISGs response. As the IFN-β protein induction in Calu-3 cells was similar to that in pBECs and A549 cells following infection, IFN-λ1 protein induction and an important downstream ISG CXCL-10, a chemokine that attracts T cells to the site of infection, was examined in Calu-3 cells at 48hr after infection by ELISA. PKR, an anti-viral ISG that is involved in apoptosis induction, was also measured after infection by western blotting.

Infection with H3N2 induced minimal IFN-λ1 (p = 0.005) and a significant decrease in CXCL-10 protein production (p = 0.002) compared to the medium control at 48hr, and were also significantly reduced compared to induction by H11N9 (IFN-λ1, p < 0.001; CXCL-10, p = 0.001) (Figure 22A, C). PKR protein on the other hand although was significantly induced in all conditions (Figure 22B), and the induction by H11N9 infection appeared to be higher than that by H3N2 (p = 0.055).
Figure 22. IFN-\( \lambda \)1, CXCL-10, and PKR protein production in response to influenza infection in Calu-3 cells.

IFN-\( \lambda \)1 and CXCL-10 protein was measured by ELISA. (A) IFN-\( \lambda \)1 production was minimally induced by H3N2 infection, but was significantly up-regulated by H11N9 infection compared to media treated controls. (B) CXCL-10 production was significantly induced after H11N9 but not H3N2 infection. (C) Similarly, while PKR expression was significantly higher in all conditions, PKR protein induction also appeared to be higher in H11N9 infection compared to that in H3N2. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
These results indicate that human BECs respond poorly to human influenza virus compared to the low pathogenic avian influenza virus. This low induction of RIG-I with a lack of MDA-5 expression in H3N2 infection, despite its high mRNA induction, resulted in a reduced type I and type III IFN as well as ISG and chemokine response, which ultimately led to the high H3N2 viral titre as observed in this study.
3.3 RIG-I-mediated signalling in protection against influenza infection

The early RIG-I detection observed in pBECs could have resulted in the low influenza replication compared to Calu-3 cells (Section 3.2.2), and to determine if RIG-I signalling is involved in this resistance to infection in pBECs, RIG-I was suppressed in pBECs using siRNA before and during H3N2 infection or Poly I:C exposure (Figure 23A). RIG-I knockdown without stimulation or during Poly I:C exposure led to decreased IFN-β but increased PKR production compared to media treated controls (Figure 23B). In contrast RIG-I knockdown during H3N2 infection did not alter IFN-β and PKR protein production. Critically suppression of RIG-I also did not alter the replication efficiency of H3N2 influenza (Figure 23C). This suggested that while RIG-I may be critical in influenza viral RNA recognition and downstream signal transduction, this may not be important in the control of influenza infection due to inhibition by the virus, and other host factors are more vital in anti-viral responses to influenza infection.
Figure 23. RIG-I knockdown in pBECs did not protect against influenza infection.

(A) RIG-I signalling was suppressed using siRNA in pBECs during H3N2 infection. (B) This resulted in a decrease in IFN-β and an increase in PKR production in RIG-I-suppressed and media or Poly I:C exposed controls compared to that in intact cells. However, IFN-β levels remained unchanged and PKR was up-regulated in H3N2 infection in RIG-I-suppressed cells. (C) Critically, RIG-I knockdown did not affect H3N2 replication compared to RIG-I-intact cells. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM). * - **** indicates significant induction of from medium control.
3.4 Apoptosis induction in protection against influenza replication

While inefficient antiviral responses were induced following H3N2 infection, both Calu-3 cells and pBECs had similar magnitudes of anti-viral responses to infection, but support different levels of influenza replication. As RIG-I-initiated signalling was shown not to influence IFN-β production in influenza infection (Section 3.3), other factors could be more important in controlling influenza replication. Apoptosis is an important component of anti-viral responses that limit viral replication in the infected cells and can be triggered by IFN-β. Therefore to investigate the mechanisms of differential replication the level of apoptosis accompanied with H3N2 and H11N9 infection was assessed in Calu-3 cells, A549 cells, and pBECs by flow cytometry.

Reduced viral titres in pBECs (Figure 9) were accompanied by a higher level of apoptosis compared to Calu-3 cells (Figure 24A), which explained the lower viral replication in pBECs compared to that in Calu-3 cells. A549 cells exhibited significant reduction of apoptosis after influenza infection compared to the control, and the induction was dramatically lower compared to the other cells.

To determine if apoptosis directly impaired viral replication, the caspases inhibitor z-DEVD-Fmk was used to inhibit apoptosis before and during influenza infection. H3N2 was chosen in this experiment to demonstrate the role of apoptosis in influenza infection in Calu-3 cells and pBECs. A549 cells was not assessed due to the similarly high viral replication and lower apoptosis induction between Calu-3 and A549 cells. Inhibition of apoptosis during H3N2 infection (Figure 24B) led to significantly higher viral replication in Calu-3 cells and pBECs (Figure 24C), suggesting that apoptosis is a critical factor in limiting influenza replication.
Figure 24. The effect of apoptosis on H3N2 replication in Calu-3 cells and pBECs. (A) There was a significant induction of apoptosis in pBECs compared to Calu-3 cells 6h after H3N2 infection. (B) Inhibition of apoptosis with Z-DEVD-Fmk led to (C) enhanced viral titres in both cell types. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM). * - **** indicates significant induction from medium control. § and §§ indicates a significant reduction compared to medium control.
3.5 Role of type I and type III IFN in protection against influenza infection

3.5.1 Constitutive IFN-β release

Throughout the time course studied in all cell types IFN-β was detected in the supernatants of un-infected medium controls, indicating that there was low-level constitutive release of IFN-β even in un-stimulated cells. This constitutive release of IFN-β may be important in the anti-viral response to influenza infection, even when the virus inhibits the induction of type I IFNs following infection. To investigate this possibility the de novo protein synthesis was inhibited by treatment of Calu-3 cells and pBECs prior to and during influenza infection and Poly I:C stimulation with the protein synthesis inhibitor cycloheximide (100μg/mL). H3N2 was used in this experiment to demonstrate that while the inducible IFN-β responses was lower in the host cells after H3N2 infection, constitutive IFN-β could play an important role in the induction of late RIG-I and PKR response as observed previously.

Cycloheximide treatment led to loss of GAPDH 6hr after infection, indicating successful inhibition of host protein synthesis (Figure 25A), and H3N2 did not replicate in the presence of cycloheximide (Figure 25B). However IFN-β protein release was significantly increased after cycloheximide treatment compared to un-treated Calu-3 cells 6hr after influenza infection. In cells stimulated with Poly I:C and media only there was also an increase in IFN-β protein expression in both Calu-3 cells and pBECs (Figure 25C). As IFN-β is known to induce apoptosis during viral infection as a means of limiting influenza replication [258, 259], the enhanced IFN-β release could also be associated with an increase in host cell apoptosis during infection. Indeed, while
cycloheximide treatment alone caused a significant induction of apoptosis in the BECs, H3N2 infection and Poly I:C stimulation enhanced this induction compared to treated media control, and also compared to those without cycloheximide treatment (Figure 25D). This increase in apoptosis correlated with the enhanced IFN-β release observed in Figure 25C. In comparison to Calu-3 cells, pBECs were also more sensitive to apoptotic signals.
Figure 25. Influenza replication and IFN-β release in the presence of cycloheximide.

(A) Cycloheximide treatment inhibited protein synthesis in airway epithelial cells, demonstrated by the loss of GAPDH production after treatment and Poly I:C exposure or H3N2 infection after 6h. (B) Cycloheximide treatment abolished viral replication, and (C) enhanced IFN-β release, which (D) correlated with enhanced apoptosis. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM). * - **** indicates significant induction of from medium control. § indicates significant reduction compared to medium control.
This confirms that IFN-β can be released when host protein synthesis was inhibited, and influenza-induced inhibition of host protein synthesis may act as a signal for the release of IFN-β that is presumable pre-formed. To determine if the constitutive release of IFN-β is functionally important to initiate or amplify ISG expression following influenza infection, the type I IFN receptor (IFNAR2) on Calu-3 cells and pBECs was blocked prior to infection using neutralizing antibodies. H3N2 replicated to a significantly higher titre in IFNAR2 neutralized cells compared to un-treated cells (Calu-3, p < 0.001; pBECs, p = 0.004) (Figure 26A). There was also a significant reduction of IFN-β protein production in all conditions following IFNAR2 neutralization (Figure 26B). IFNAR2 neutralization almost completely inhibited induction of IFN-β after H3N2 infection, and substantially reduced IFN-β expression following Poly I:C and in the medium only control. The reduced IFN-β expression also correlated with reduced induction of apoptosis following infection (Figure 26C).
Figure 26. Influenza replication and IFN-β production in IFNAR2 neutralized Calu-3 cells.

(A) Blocking of IFNAR2 before infection resulted in significant increases in influenza titres 48h after infection compared to no treatment. The rise in titre correlated with (B) significant decreases of IFN-β in all conditions involving neutralization of the IFNAR2 compared to untreated controls. (C) Decreased release of IFN-β after IFNAR2 neutralization resulted in significant decreases in apoptosis after H3N2 infection. Results were obtained from three independent experiments and presented as standard error of the mean (SEM). * — **** indicates significant induction from medium control. § indicates a significant reduction compared to medium control.
### 3.5.2 The effect of type I and type III IFN priming on BECs

The constitutive IFN-β release and the minimal induction of type I and type III IFN after H3N2 infection may have led to the increase in H3N2 replication compared to H11N9. To determine if IFN-β and IFN-λ1 played a role in limiting infection and whether both H3N2 and H11N9 was sensitive to IFN treatments, cells were pre-treated with 1ng/ml of either IFN-β, IFN-λ1, or in combination before infection. Pre-treatment with IFN-β or IFN-λ1 alone significantly decreased the viral replication of H3N2 and H11N9 by 3 fold, and the combination of both IFNs further reduced the replication of both viruses (Figure 27).

![Figure 27](image)

Figure 27. The anti-viral effect of IFN-β and IFN-λ1 against influenza infection in Calu-3 cells.

IFN-β and IFN-λ1 was used to prime Calu-3 cells before influenza infection, and viral replication was analyzed by plaque assay at 48hr after infection. Both H3N2 and H11N9 had a 3–4 fold decrease in viral replication when Calu-3 cells were pre-treated with IFN-β and IFN-λ1 alone, and replication was further reduced when IFN-β and IFN-λ1 was used in combination. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
These data indicate that while de novo IFN-β production was inhibited, the release of constitutive IFN-β is important in amplifying IFN signalling during influenza infection and suppressing viral replication in BECs. The priming of Calu-3 cells with type I and type III IFN before infection helped the host cells to rapidly establish antiviral response to viral infection. This suggests that both types of IFNs could act cooperatively to limit viral replication during influenza infection.
3.6 Global anti-viral genes induction in BECs after influenza infection

To investigate if the differences in anti-viral responses by H3N2 and H11N9 are widespread across different anti-viral signalling pathways, gene expression in infected Calu-3 cells was examined at 24hr by microarray analysis.

Infection with H3N2 and H11N9 resulted in the altered expression of 13,388 genes after normalization to media treated controls. ANOVA analysis with the more stringent Benjamini-Hochberg correction identified 2,155 genes that were differentially expressed (p ≤ 0.005). Of these 330 genes were altered by ≥2 fold with 41 genes differentially expressed in response to H3N2 infection, 300 by H11N9 and 35 genes were altered by both H3N2 and H11N9 infection (Figure 28)
ANOVA analysis with Benjamini-Hochberg correction showed that the expression of 330 genes were changed by ≥2 fold. Of these 330 genes 41 were differentially expressed in response to H3N2 infection, 300 by H11N9, and 35 genes were altered by both H3N2 and H11N9 infection.

Gene ontology analysis revealed that H3N2 infection only induced the ISG OAS2, whereas in contrast H11N9 infection up-regulated many immune and virus responses genes including IFN-β, IFN-λ1, and a wide range of ISGs (Figure 29, Figure 30). This confirms the results observed previously that human BECs respond better to low pathogenic avian influenza virus compared to the human influenza virus.
Figure 29. H3N2- and H11N9-induced genome-wide gene expression was examined at 24hr after infection by microarray analysis.

The cDNAs from infection were hybridized onto the beadchip, scanned, and imported into GeneSpring GX 10 (Agilent Technologies) for analysis.
Figure 30. Induction of anti-viral-associated genes is widespread after influenza infection in Calu-3 cells.

Gene ontology analysis showed that H3N2 infection only induced OAS2 gene above the 2 fold change cut-off point. H11N9 infection resulted in altered expression of 300 genes, which are largely associated with immune responses and are anti-viral-associated genes such as IFN-β, IFN-λ1, IRF, OASL, RIG-I (DDX58) and MX2 genes.
3.7 Influenza NS1: The level of anti-viral inhibition

The influenza NS1 protein is a known immune antagonist expressed by influenza viruses. It not only inhibits host mRNA processing to halt host protein synthesis, it also enhances viral protein synthesis for efficient viral replication. In addition, a major role of influenza NS1 protein is the suppression of host anti-viral responses, which was achieved by binding to multiple host targets such as TRIM25 and IRF3 [120, 121, 166, 197, 201, 211, 260, 261].

The difference in anti-viral responses observed in the infected cells therefore is likely to be due to the effect of influenza NS1 protein. The level of anti-viral suppression by the NS1 may be variable in different strains of influenza, and in this case, the NS1 protein of human influenza H3N2 could be more competent in the IFN suppression compared to that of low pathogenic avian H1N9 strain.

To examine the effectiveness of the NS1 protein from these viruses, the NS gene encoding for the NS1 protein was isolated from viral RNAs, amplified and cloned into a mammalian expression vector, which was then transfected into Calu-3 cells for functional study.

3.7.1 Cloning of NS gene into pcDNA 3.3-TOPO

Total viral RNA was extracted and NS gene was reverse transcribed to cDNA by reverse transcription PCR using the designed primers. The NS gene was separated on a 1% DNA agarose gel (Figure 31), and the band corresponding to the NS gene was gel extracted.
3.7.2 Generation of splice acceptor mutation in NS and by site-directed mutagenesis

Influenza NS gene encodes for protein NS1 and NEP via alternative splicing, therefore a silent mutation was created at (A to C at nucleotide 541 in the cDNA sense) by site-directed mutagenesis to inhibit the expression of NEP using internal NS1-SAM primers, generating splice acceptor mutation (SAM) (Figure 32).

Two parallel primary PCR was performed to amplify the fragment upstream and downstream of the mutant site with the designed specific PCR primers containing its mutation. The PCR product of upstream and downstream of the mutant site was gel extracted and purified (Figure 33A – 33B). The complete NS sequence was then created by overlaying first and second PCR products with a third PCR reaction (Figure 33C).
Figure 32. Site-directed mutagenesis of influenza NS gene.

The first PCR reaction was performed with NS1 forward primer and NS1-SAM reverse primer, and the second PCR reaction was performed with NS1 reverse primer and NS1-SAM forward primer. The two PCR products were then overlaid by the third PCR reaction to generate the full length \textit{NS1-SAM}. 
Figure 33. Site-directed mutagenesis of NS1-SAM by PCR reaction.

(A) Amplified products of first (lane 1) and second (lane 2) PCR reaction of H3N2 NS1. The expected size is approximately 500 and 350 bp for first and second PCR product, respectively. (B) Amplified products of first (lane 1) and second (lane 2) PCR reaction of H11N9 NS1. (C) The overlapping PCR reaction generating the full length H3N2 (lane 1) and H11N9 NS1-SAM (lane 2).
The products were cloned into a mammalian expression vector pcDNA3.3-TOPO (Invitrogen) (Appendix I). The construct was then transformed into *E. coli* DH5α cells, and plated on LB containing Amp for selection. Single-colony PCR was then performed to test for positive clones (Figure 34).

![Figure 34](image-url)

**Figure 34.** Single colony PCR reaction of *E. coli* colonies carrying NS1-SAM gene after transformation.

Lane 1 – 5 are colonies of *E. coli* carrying **H3N2 NS1-SAM** insert, with lane 1, 2, and 4 were tested positive. Lane 6 – 10 are positive colonies of **H1N9 NS1-SAM**.

Positive clones were sent to the Australian Genome Research Facility Ltd. to confirm the correct mutation had been made to the target nucleotide (Figure 35).

![Figure 35](image-url)

**Figure 35.** Sequencing results of NS1-SAM.

The arrow indicates the position of successful mutation of nucleotide A to C, creating **NS1-SAM**.
3.7.3 Anti-viral suppression by influenza NS1 protein

The NS1-SAM gene of H3N2 and H11N9 were transfected into Calu-3 cells, and at 6hr after transfection Poly I:C (100µg/mL) was added to the transfectant cells. After 42hr of stimulation the level of NS1-SAM expression and induction of IFN-β, IFN-λ1 and CXCL-10 protein was measured.

NS1 of both H3N2 and H11N9 were expressed to similar levels in Calu-3 cells (Figure 36).

![Image]:

Figure 36. Influenza NS1 expression after transfection in Calu-3 cells.
H3N2- and H11N9-NS1 was transfected into Calu-3 cells, which was then stimulated with Poly I:C at 6hr after transfection. The level of NS1 protein expression was measured at 48hr after Poly I:C stimulation. H3N2 and H11N9 NS1 proteins were expressed to a similar level at 48hr after transfection and levels were maintained after Poly I:C stimulation. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
IFN-β, IFN-λ1, and CXCL-10 protein production in response to Poly I:C was inhibited by NS1 of both viruses but the suppression of these anti-viral proteins were significantly greater by the NS1 protein of H3N2 compared to that of H11N9 virus. Vector alone did not induce these proteins above media treated controls (Figure 37A – C).

This clearly demonstrates that the NS1 protein from H3N2 is more effective in the inhibition of type I and type III IFNs expression than that from H11N9. This enhanced suppression allows H3N2 virus to replicate more efficiently with less interference from the host, thereby leading to a higher replication titre in human BECs.
Figure 37. Induction of IFN-β, IFN-λ1, and CXCL-10 protein production after NS1 transfection and Poly I:C stimulation in Calu-3 cells.

The protein induction of IFN-β was measured by western blot, and IFN-λ1, and CXCL-10 protein was measured by ELISA. (A) H3N2- and H11N9-NS1 transfection significantly down-regulated IFN-β production after Poly I:C stimulation, with a significantly greater effect of H3N2-NS1. (B) IFN-λ1 and (C) CXCL-10 production was also significantly reduced by H3N2-NS1 compared to H11N9-NS1. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
3.8 High pathogenic avian influenza H5N1 infection in BECs

Highly pathogenic avian influenza virus (H5N1) is currently a serious threat to the human populations as it results in severe and usually lethal diseases in human. The prevalence of this virus is continuing to climb around the world, especially in South East Asia, and is a concern as the virus may acquire the necessary mutations for sustainable human-to-human transmission. As the importance of the innate immune response of the BEC in limiting viral replication was demonstrated in the previous sections, the effect of H5N1 as an example of a highly pathogenic influenza strain was examined in both Calu-3 cells and pBECs.

3.8.1 H5N1 infection and replication in Calu-3 cells

Infection with H5N1 (A/Vietnam/1203/04) was performed at M.O.I of 0.1 as infection with M.O.I of 5 destroyed the cell monolayer completely within 12hr of infection (Data not shown).

Infection in Calu-3 cells resulted in a tremendous amount of cytopathic effect (CPE) (>50% CPEs) at 24hr after infection compared to the medium control and replicated to 1 x 10^5 PFU/mL (1 log increase in titre) (Figure 38, Figure 39). This level of CPE was not seen in Calu-3 cells with both H3N2 and H11N9 infection (Data not shown).
Figure 38. H5N1 infection in Calu-3 cells at 24hr after infection.
Infection with H5N1 resulted in high levels of CPE in Calu-3 cells (>50% CPE) at 24hr after infection.

H5N1 replication in Calu-3 cells at 48hr after infection

Figure 39. H5N1 replication in Calu-3 cells at 48hr after infection.
H5N1 replicated to $1 \times 10^5$ PFU/mL in Calu-3 cells at 48hr after infection. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).
3.8.2 Anti-viral induction to H5N1 infection in Calu-3 cells

Anti-viral responses are clearly important in limiting viral replication in BECs as shown previously (Section 3.2 and 3.4 – 3.5), and as differences in the responses were mostly observed at 48hr anti-viral protein inductions were measured at 48hr after infection.

H5N1 infection resulted in a significant reduction of RIG-I and MDA-5 protein induction at 48hr after infection compared to medium control as well as H11N9 infection (Figure 40). This reduction in viral RNA sensory protein led to a significant decrease in PKR, IFN-β and IFN-λ1 induction after infection (Figure 41A – C).

![Figure 40. RIG-I and MDA-5 protein induction in Calu-3 at 48hr after infection.](image)

(A) RIG-I and (B) MDA-5 protein expression was not detected and reduced after H5N1 infection compared to medium control and H11N9 infection. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
Figure 41. PKR, IFN-β and IFN-λ1 protein induction in Calu-3 cells at 48hr after infection.

(A) PKR, (B) IFN-β, and (C) IFN-λ1 protein were not induced above medium control and was significantly reduced compared to H11N9 infection. PKR and IFN-β was measured by western blotting. IFN-λ1 was measured by ELISA. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
3.8.3 H5N1 infection and replication in pBECs

Infection in pBECs was also conducted at M.O.I of 0.1 though did not result in CPE that was as severe as that observed in Calu-3 cells at 24hr, and monolayer remained intact throughout the course of infection (Figure 42). This was different to the pBECs infected with H3N2 and H1N9, which induced minor CPEs (Data not shown) during infection.

Figure 42. H5N1 infection in pBECs at 24hr after infection.
Infection with H5N1 resulted in minimal levels of CPE in pBECs (<5% CPEs) at 24hr after infection.

The intact monolayer with minimal CPEs after infection could have resulted from the NS1 protein that inhibited the host-induced apoptosis, therefore allowing the virus to replicate more efficiently in the cells [200, 203, 204, 262] (Figure 43).
H5N1 replication in pBECs at 48hr after infection

![H5N1 replication in pBECs at 48hr after infection](image)

Figure 43. H5N1 replication in pBECs at 48hr after infection. H5N1 replicated to a high titre at $8 \times 10^6$ PFU/mL in pBECs at 48hr after infection. Results were obtained from three independent experiments and are presented as standard error of the mean (SEM).

3.8.4 Anti-viral induction to H5N1 infection in pBECs

Induction of anti-viral proteins was similar to that observed in Calu-3 cells. RIG-I and MDA-5 protein was not detected at all after infection (Figure 44A – B), which led to an undetectable PKR and IFN-β protein at 48hr after infection (Figure 45A – B). However, in contrast to that observed in Calu-3 cells, IFN-λ1 protein was induced above medium control, and was significantly higher than that in H11N9 infected pBECs (Figure 45C).
Figure 44. RIG-I and MDA-5 protein induction in pBECs at 48hr after infection. 
(A) RIG-I and (B) MDA-5 protein expression was not detected and reduced after H5N1 infection compared to medium control and H11N9 infection. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).
Figure 45. PKR, IFN-β and IFN-λ1 protein induction in Calu-3 cells at 48hr after infection.

(A) PKR, (B) IFN-β protein was not induced above medium control and was significantly reduced compared to H11N9 infection. (C) IFN-λ1 protein however was induced and the induction was higher than that in H11N9 infection. Results are representative of three independent experiments, and are presented as standard error of the mean (SEM).

Infection with H5N1 showed a potent anti-viral suppression in both Calu-3 cells and pBECs, and this was related to a high replication titre. Although the infection M.O.I of H5N1 was lower than that of H3N2 and H11N9, it resulted in relatively higher CPEs
in Calu-3 cells after infection. Together with superior immune suppression infection led to a high replication titre. Infection in pBECs however did not result in CPEs, this may be due to the anti-apoptotic effect of the NS1 protein that slowed down the rate of apoptosis so viral replication can be maximized in these cells. Nevertheless an increase in IFN-λ1 was observed in pBECs after H5N1 infection, and this induction was also higher than that observed in H11N9 infection. The exact reason for this observation is unknown, and the high induction of IFN-λ1 did not appear to be sufficient to limit viral replication.
Chapter 4. Discussion
4.1 Research aims and overall findings

Influenza virus is a major human pathogen of enormous importance due to its intrinsic nature, which results in serious social and economical consequences around the world every year. Frequent mutations of the influenza genome not only reduce the effectiveness of the immune response in both innate and adaptive arm, this also complicates novel therapeutics designs.

Innate anti-viral system in BECs is vitally important in providing immediate protection against influenza infection, and concomitantly provides signals for appropriate adaptive immune system to develop for viral clearance. The viral recognition and anti-viral initiation pathway leading to a full anti-viral response to general viral infection including influenza virus is well-characterized. However as the virulence factors of influenza virus interact with multiple anti-viral components, the kinetics and effectiveness of human anti-viral response as a result of these interactions are poorly understood. In addition how these anti-viral responses may be altered in response to different virus strains is also yet to be defined. More importantly, while human influenza viruses continue to cause major problems every year, avian influenza viruses are a potential source of future influenza pandemics. The ability of these influenza viruses to mutate and acquire necessary mutations to infect humans in a sustainable fashion is likely to pose a serious threat to the human population. The novel swine origin influenza H1N1 2009 is a clear example of this phenomenon. Therefore the effectiveness of human innate anti-viral responses to both human and avian influenza viruses is critical in determining the outcome of influenza infection, hence the focus of this study.
Human and avian influenza viral entry and replication efficiency in airway epithelial cells is not dependent on the abundance of sialic acid residues or RIG-I-mediated pathways as previously thought. Both human and avian influenza viruses used in this study could enter into BECs with similar efficiency and also replicate efficiently even with lower levels of these sialic acid residues. Innate anti-viral response, including those exerted by the constitutive IFN-β release via IFN-β-IFNAR2 signalling, are more critical in the control of influenza replication, especially during heavy IFN suppression by human influenza virus. The NS1 protein of H3N2 virus was more effective in inhibiting the anti-viral responses in BECs compared to that of H11N9, thereby aiding the human virus to replicate to high titre. Regardless of this antiviral suppression, there was an early induction of RIG-I and a greater proportion of infected cells underwent apoptosis that was associated with reduced viral replication in pBECs. However this RIG-I responses was not required to suppress viral replication. Importantly, the constitutive release of IFN-β played a critical role in triggering apoptosis and limiting viral replication in infected BECs. Furthermore, blocking the type I IFN receptor and apoptosis enhanced viral replication. In contrast infection of BECs with H5N1 resulted in inhibition of both inducible and constitutive IFN-β release, and impaired apoptosis that led to highly efficient viral replication.

4.2 Influenza viral entry and replication

Calu-3 cells and pBECs are representative of human proximal airway epithelial cells and are known to predominantly express SAα2,6Gal residues. These residues are the binding site for human influenza virus on epithelial cells, while avian influenza viruses appear to bind more readily to SAα2,3Gal, that are not as abundant in Calu-3 cells or the upper respiratory tract but are mainly found in the lower airway [70, 71, 77].
H3N2 and H11N9 viruses were found to readily infect these human airway epithelial cells to a similar extent based on HA levels inside the infected cells immediately after infection, and replicated to a similar titre after infection. The differential expression of these sialic acid residues however did not appear to influence the in vitro infectivity of influenza viruses. It is possible that low levels of sialic acid residues are sufficient to facilitate viral entry or influenza HAs can bind to both sialic acid residues to gain entry. The HA protein of 1918 H1N1 strain showed binding preference to both sialic acid residues and it is possible that some of the strains have retained the binding specificities to certain extent [263]. Alternatively influenza viruses may utilize other moieties for entry such as caveolae and other lipid-linked glycoproteins [80, 81]. HA proteins have been found to have binding affinity towards glycoproteins with fucosylation, sulfation and sialylation at the terminal trisaccharide [264]. However it is unclear as whether influenza binds to these glycoproteins directly for viral entry or if these glycoproteins serve as co-receptors that aid viral endocytosis.

Primary BECs were most resistant to H3N2 and H11N9 infection due to higher levels of apoptosis after infection, which led to reduced viral replication. In contrast Calu-3 and A549 cells did not readily undergo apoptosis, which resulted in more efficient replication. The reason for this difference in apoptosis is probably due to the nature of immortalized cell lines, and results from pBECs provide a better insight into the natural human host cell response to influenza infection.

H5N1 infection on the other hand resulted in a more efficient viral replication in both cells, although replication was relatively lower in Calu-3 cells compared to that in pBECs. This lower viral titre in Calu-3 cells was associated with a high level of CPEs that was not observed in pBECs, the sustained monolayer of which could have helped the virus to replicate more efficiently in the pBECs than in Calu-3 cells. The underlying
reason for this difference in CPEs between these two cells is unclear as it seemed unlikely that the CPEs observed were host-induced, and viral factors of H5N1 were possibly involved in this phenomena.

NS1 protein of H5N1 has dual effects on apoptosis induction, that it either inhibited the host-induced apoptosis process more efficiently in pBECs to achieve high viral replication [201-204], or the NS1 protein itself caused the apoptosis in the infected Calu-3 cells [207]. Inhibition of apoptosis is achieved by NS1 suppressing IFN-β expression, leading to a dramatic reduction in ISG PKR expression, which in turn results in a reduced induction of apoptosis by PKR [200, 201, 203, 221, 265-267]. NS1 can also directly bind and inhibit PKR-induced apoptosis [203, 204]. On the other hand studies have shown that the induction of apoptosis by H5N1 NS1 protein is required for efficient influenza mRNA synthesis [268-270], however the exact mechanism underlies this observation remains unknown.

In addition to the effect of NS1 in the infected cells, PB1-F2 protein, an apoptosis-inducing protein that was not examined in this study, might have also caused a more severe apoptosis in Calu-3 cells after H5N1 infection [107, 109]. PB1-F2 binds to voltage dependent anion channel (VDAC) 1 and adenine nucleotide translocator (ANT) 3 on the outer and inner mitochondrial membrane, respectively, and promote apoptosis by forming membrane pores in mitochondrial membrane [109, 271]. PB1-F2 protein might have exerted a more potent pro-apoptotic effect on the Calu-3 cells [109, 110], although it is unclear as why PB1-F2 did not have the same effect in pBECs as it did in Calu-3 cells. The complicated interaction between NS1, PB1-F2 and host factors such as mitochondria and PKR therefore determines the viability and/or apoptosis of the infected cells, and as discussed previously pBECs could be a better model that provides more realistic results.
4.3 Anti-viral response during influenza infection

Immediately after endocytosis into BECs, cytosolic RNA helicase RIG-I recognizes influenza viral RNA and signals through IRF3 to induce the expression and production of type I IFNs. The secreted IFN-β then binds to the IFNAR2 on the same and/or neighbouring cells and amplifies the anti-viral responses by inducing ISGs expression via JAK1/STAT1 pathways.

The kinetics of anti-viral response varied between different airway epithelial cells. Calu-3 cells exhibited a relatively late anti-viral induction compared to pBECs as the mRNA for most anti-viral genes were induced earlier and some sustained throughout the infection in pBECs. Late induction was also observed for another RNA helicase, MDA-5, as well as TLR3. The later gene inductions probably represent the second phase of anti-viral responses that are up-regulated by the constitutive release of IFN-β. However, at the protein level RIG-I protein was produced at 48hr but not 6hr or 24hr, and together with lack of MDA-5 protein expression and low level induction of type I and type III IFNs, correlated with a higher viral replication. The minimal IFN responses observed in H3N2 infection was most likely the result of the lack of IRF3 phosphorylation, which is a critical transcriptional factor downstream of RIG-I signalling pathway [156, 272]. This lack of activation may be due to the activity of the influenza NS1 protein, which was shown to inhibit the activation of IRF3, as well as NF-kB [197, 273, 274], and RIG-I [196, 260, 275]. As a result, the reduced IFN responses led to a lower ISGs expression including CXCL-10.

In pBECs, while the induction of MDA-5, IFNs, and PKR was again not induced above baseline in pBECs after H3N2 infection, RIG-I protein was detectable early in infection and was also significantly increased late in infection. This early RIG-I
detection and late induction however did not correlate with the low viral titre in pBECs, as RIG-I knockdown surprisingly affected neither IFN-β nor viral replication in pBECs as previously shown by another study [260]. This indicates that while RIG-I is important in the recognition of influenza viral RNA during infection [135, 260, 275], its signalling pathway was likely inhibited by the virus, and the constitutive IFN-β release and host cell apoptosis was more important in the control of influenza infection. This difference in RIG-I detection early in infection between Calu-3 cells and pBECs could be due to the lack of apoptosis induction, which allowed for a sustained expression of NS1 and its inhibition in Calu-3 cells. In comparison pBECs elicited a higher apoptosis induction that limited viral replication, which was mediated by the constitutive IFN-β release after infection.

TLR3 is also part of the viral PRRs recognition network that binds to viral dsRNA and initiates NF-κB-mediated inflammatory response such as IL-6 and IL-8 [126]. In our study TLR3 was up-regulated at the mRNA level but TLR3 protein could not be detected by western blotting and by flow cytometry. This may be due to technical difficulties associated with the antibody specificity and biochemical properties of TLR3. Human TLR3 protein contains a leucine-rich repeat (LRR), which makes up 16.2% of entire protein and may contribute to the insolubility of this protein. In addition, TLR3 protein is also made up of 18% amino acid residues of asparagine, glutamine, and threonine residue, which are also known to contribute to the insolubility of proteins [276]. This suggests that the TLR3 protein could remain insoluble and precipitate as pellets after cell lysis, and a more potent solubilisation detergent such as higher percentage ratio between sodium deoxycholate and triton X-100 may be required to solubilize TLR3 protein. However influenza virus generates 5′ppp ssRNA during replication that is recognized by RIG-I and not TLR-3 [134], and it has been shown that
TLR3 may not play as important role in type I IFN responses compared to RIG-I [129, 277]. Indeed, accumulating evidence suggests that the contribution of TLR3 to influenza viral clearance is variable and that it may only have limited ability to initiate type I IFN responses [129, 277].

The anti-viral response in A549 distal airway epithelial cells largely occurred at 24hr after infection. However despite of the poor IFN responses after infection and Poly I:C stimulation, RIG-I was also induced early and late in infection. This was similar to that observed in pBECs that this induction was most likely to be initiated by constitutive IFN-β as part of ISGs induction. In addition, although the efficient viral replication correlated with low anti-viral responses, other mechanisms may be involved in the relative inefficiency in viral entry compared to that in Calu-3 cells. Furthermore, the poor anti-viral induction in A549 cells may indicate that the lower airway cells are not able to respond to viral infection with type I IFNs as efficiently as proximal airway cells, and may potentially lead to severe complications such as viral pneumonia. It is known that infection with influenza, including H3N2, lead to severe tracheobronchitis and alveolitis [278, 279], however the difference in the anti-viral responses between upper and lower respiratory tract were not detailed in these studies. Using primary alveolar epithelial cells could provide more insight into the anti-viral responses in the lower airways.

In sharp contrast infection with H5N1 in Calu-3 cells and pBECs resulted in a complete demolition of host anti-viral responses, leading to a very efficient replication in these cells. This is in agreement with another study that demonstrated a delayed IFN-β response in H5N1 infection compared to H3N2 infection, leading to an efficient replication in Calu-3 cells [280]. Another study by Chan, MC. et al. 2009 also showed high viral replication by H5N1 in both well-differentiated and un-differentiated normal
human bronchial epithelial (NHBE) cells, albeit IFN-β protein expression was undetectable by ELISA due to ELISA sensitivity issue [281]. Furthermore the same group also demonstrated the highly efficient H5N1 replication in polarized human alveolar epithelial cells [282], which further strengthening that highly pathogenic avian H5N1 virus was able to replicate very efficiently in both upper and lower respiratory tract regardless of SAα2,6Gal and SAα2,3Gal residues.

4.4 Critical role of constitutive IFN-β release in protection against influenza infection

Despite the low IFN-β response induced after H3N2 infection, a selective induction of RIG-I and PKR protein during late infection was still observed, and the constitutive IFN-β release played a pivotal role in this late antiviral signalling after infection. Cycloheximide was used as a general inhibitor of host protein synthesis to determine if the IFN-β was being induced in response to infection or was pre-existent. Treatment of the BECs with cycloheximide effectively inhibited protein synthesis as demonstrated with loss of GAPDH expression and resulted in cell death. However treatment in H3N2 infection led to an enhanced release of IFN-β, which correlated with a higher induction of apoptosis following infection. Influenza, as well as many other RNA viruses such as herpes simplex virus are known to effectively inhibit host cell protein synthesis in preference to viral protein synthesis to promote replication [283]. This inhibition of protein synthesis may acts as a trigger for an enhanced release of pre-formed IFN-β, and a signal to induce apoptosis in the infected host cell, despite the ability of the virus to prevent the induction of IFN-β through RIG-I mediated pathways. Interestingly a similar response has been observed in cells infected with Newcastle Disease virus (NDV). Cells treated with cycloheximide during NDV infection enhanced IFN-β release, which was thought to occur through enhanced stability of IFN mRNA.
This however does not explain the constitutive release of IFN-β protein when host protein synthesis was blocked at the translational step by cycloheximide. While the exact mechanism by which IFN-β protein was released when protein synthesis was blocked is unclear, this study demonstrated that this potentially pre-formed IFN-β is functionally important in limiting viral replication via IFN-β-IFNAR signalling. Furthermore, Ethuin, et al. 2004 showed that human neutrophils contain a pre-existing pool of IFN-γ, a cytokine involved in immuno-regulations beyond the scope of this study, and this pre-formed IFN-γ was released rapidly upon stimulation with cycloheximide and other de-granulating agents [285]. This further strengthens the possibility of such IFN-β store inside BECs and is released upon virus-induced immune inhibition via mechanisms that are yet to be discovered.

The constitutive release of IFN-β shown here has also been reported previously in the absence of viral infection in vitro and in vivo [286-289], and has been suggested to play a role in the priming and enhancement of IFN response, as proposed in a “revving-up model” [289, 290]. A constitutive weak signal induced by IFN-β-IFNAR signalling allows epithelial cells to elicit a more robust response toward viral infection, while in the absence of this signal epithelial cells become hypo-responsive to this stimulus. Despite the critical role of constitutive IFN-β in late RIG-I, MDA-5, PKR induction to H3N2 infection, H5N1 inhibited all antiviral signalling more effectively in the infected cells, including those induced by the constitutive IFN-β via its potent NS1 protein. In support of this revving-up hypothesis, the priming of BECs with IFN-β and IFN-λ1 effectively reduced viral replication, which was even more profound when both of these IFNs were administered concurrently, a finding also demonstrated by Wang et al 2009 in type II human alveolar cells [291]. This is in agreement with the revving up
model and also indicates that both type I and type III IFNs act co-operatively to limit viral replication during influenza infection.

4.5 Influenza NS1 protein and anti-viral inhibition

Type I and type III IFNs as well as the subsequent ISG responses are well-known for their function in the establishment of an anti-viral state in infected and neighbouring cells, however influenza viruses continue to replicate and spread efficiently in BECs. Influenza NS1 protein is a potent virulence factor that was able to delay and suppress RIG-I signalling pathway and subsequent IFN responses. The NS1 protein of H3N2 was able to inhibit the expression of type I and type III IFNs more effectively than that of H11N9, thereby leading to a wide inhibition of ISGs expression. This anti-viral inhibition was even more potent in H5N1 infection compared to other influenza viral infections, and although NS1 of H5N1 was not cloned and tested in this study, numerous reports have demonstrated the robustness of NS1 protein of H5N1 in the suppression of host innate anti-viral responses [199, 207, 217, 292-297]. Collectively, these results indicate that there are differences in the level of immune suppression by different strains of influenza, that may be related to the NS1 activity, and this appears to be an important factor that determines whether influenza viruses have high or low pathogenic properties in human airway cells.

Influenza NS1 protein can inhibit the host anti-viral system at both pre- and post-transcriptional levels. During transcription NS1 binds to CPSF30 that is required for polyadenylation of host mRNA, thereby suppressing host mRNA maturation [166, 194, 195]. At the post-transcriptional level, the IFN antagonistic property of NS1 occurs at multiple stages of the IFN signalling cascade. NS1 interacts with TRIM25 that associates with RIG-I and inhibits its association with IPS-1 on mitochondria [198].
NS1 also inhibits the activation of IRF3, thereby suppressing type I IFNs expression [196, 197]. It is possible that as influenza circulates in the human population the H3N2 NS1 has evolved to effectively suppress the human innate immune response. Alternatively influenza viruses that develop a NS1 protein that is more effective in human cells are more likely to lead to sustained infection in humans and this may be an important factor in the zoonotic transmission of avian influenza to humans. Many studies have used the highly pathogenic avian influenza H5N1 to analyze the function of its NS1 protein and have mapped the active site for IFN suppression to a number of amino acid residues including R38/K41 [198] and amino acids at 123–127 [201, 202]. Nevertheless, it remains unclear which set of residues differentiates the NS1 of high or low pathogenic influenza viruses although a change in one residue (92E) has been implicated in increased disease severity with H5N1 NS1 in pigs [220]. The residue at position 92 in the NS1 protein of H3N2 and H11N9 is an aspartate, which is also found in H5N1 strain (A/Vietnam/1203/2004). The genetic sequences of H3N2, H11N9 and H5N1 strain also showed a difference at the position 41, which together with conserved R38 is involved in RNA binding [298]. While R38 and K41 are conserved in H11N9 and H5N1, an arginine was found at position 41 in H3N2. K41 was also found in another LPAI strain H4N8 (Data not shown). There are also differences at the effector domain that differentiates human H3N2 strain from avian strains, and are specifically found in the region implicated in the CPSF30 binding including residues at 106, 144 and 145. This study demonstrates that differences in NS1 at the genetic level may potentially serve as a marker for influenza pathogenicity.

The immune antagonistic property of H5N1 NS1 directly compromises the antiviral defences in the host cells and enhances the pathogenesis of this virus. The NS1 protein of different influenza strains may possess variable levels of immune antagonism,
and the outcome of this intracellular warfare between the host and these viral virulence factors determines the outcome of infection.

### 4.6 Conclusion and future directions

Innate immune responses of infected BECs are important in determining the level of influenza replication, which correlated poorly with the level of sialic acid residues expression. This indicates that a low level of BEC expression of sialic acid residues is sufficient to allow infection and does not appear to be a major rate-limiting factor for influenza susceptibility. In BECs the anti-viral responses initiated after endocytosis have a more important role in limiting viral replication. In the early phase of infection epithelial cells have limited ability to produce inducible IFN-β, which may be a result of the suppression of the immediate RIG-I signalling cascade by the influenza NS1 protein. However constitutive IFN-β response counter-balances some of these suppressive effects of the influenza virus, via IFNAR2 signalling to induce expression of ISGs including RIG-I and PKR. These ISGs are important in establishing an antiviral state in BECs as they are the effector antiviral proteins that inhibit viral mRNA translation, inducing apoptosis and amplify the entire IFN response.

On the other hand, infection with influenza impairs these IFN responses through the action of the NS1 protein. The NS1 protein from different strains of influenza clearly has different levels of IFN suppression. The effectiveness of NS1 protein clearly has an important impact on the efficiency of viral replication in the infected host, especially the NS1 protein of H5N1 that allowed for efficient replication in the hosts.

Deciphering the host innate anti-viral mechanisms and the interactions between anti-viral response and influenza virulence can progress the understanding of viral
pathogenesis and may facilitate the discovery of novel therapeutic targets. In particular, the exact mechanism of anti-viral suppression by the NS1 and PB1-F2 protein as well as other virulence factors such as viral polymerase complex may assist in the discovery of currently unknown immune pathways. In addition, anti-viral enhancement may be a novel potential therapeutics for influenza treatment. RIG-I/MDA-5 agonist may activate IFN pathways, thereby aiding the establishment of anti-viral state in the host during infection. The recent emergence of pandemic influenza strain and highly pathogenic avian strains emphasizes the need for research in this area. During influenza pandemic vaccines are not likely to be available and the efficacy of current anti-viral drugs is also questionable to novel viruses, therefore exploring of host-virus interactions at the molecular level will aid the discovery of more effective therapeutic targets.
Appendix
Appendix I.  pcDNA™ 3.3-TOPO map

The pcDNA™ 3.3-TOPO vector map was taken from http://products.invitrogen.com/ivgn/product/K830001.
Manuscripts arising from this study
Journal of Experimental Medicine

Critical role of constitutive type I interferon in innate immunity to pathogenic human and avian influenza infection

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Running title: Constitutive IFN-β and influenza infection

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ABSTRACT

Innate antiviral responses in bronchial epithelial cells (BECs) provide the first line of defence against respiratory viral infection and the effectiveness of this response is critically dependent on the type I interferons (IFNs). However, the magnitude of the antiviral responses in BECs during early and late influenza infection is not well understood. We profiled the antiviral responses to H3N2 and H5N1 influenza infection using Calu-3 cells and primary BECs to model proximal airway cells. The susceptibility of BECs to influenza infection was not solely dependent on the sialic acid-bearing glycoprotein expression and was affected by the antiviral responses after viral endocytosis. The early antiviral protein and apoptosis induction correlated with the control of infection, and importantly the constitutive secretion of IFN-β by BECs and not RIG-I-initiated signalling had a critical role in inducing late antiviral signalling via type I IFN receptors, and was crucial in limiting viral infection. This study characterizes anti-influenza virus responses in airway epithelial cells and shows that constitutive IFN-β release plays an important role in initiating protective late IFN-stimulated responses during human influenza infection in airway epithelial cells.
INTRODUCTION

The recent highly pathogenic bird flu and swine flu influenza pandemics demonstrate the danger that this virus poses, with its capability to evade our immune response and rapidly be transferred throughout populations and across the globe. Much attention has focused on the ability of the virus to evade the host adaptive immune response through antigenic drift and antigenic shift of the virus (Fraser et al., 2009). However, the ability of the virus to initially infect humans and evade early innate immune responses is less well defined.

Influenza first gains entry into humans via the airway epithelium yet little is known about this interaction and how it may vary between strains of influenza viruses, especially those that are more pathogenic to humans. As influenza viruses enter the airways the haemagglutinin (HA) glycoprotein on the virus attaches to airway epithelial cell surface glycoproteins terminating with specific configurations of sialic acid (SA) residues. Human influenza preferentially binds to SA\(\alpha_2,6\)Gal linkages that are predominantly found in the upper respiratory tract, while avian influenza viruses bind to the SA\(\alpha_2,3\)Gal residues in the lower airway (Ito et al., 2000; Ito et al., 1997; Ryan-Poirier et al., 1998; Suzuki, 1998).

The airway epithelium is an important contributor to the early innate immune response to virus infection. Type I interferons (IFN-\(\alpha/\beta\)) and the recently discovered type III IFNs (IFN-\(\lambda_1\), -\(\lambda_2\), -\(\lambda_3\)) are central players in innate antiviral responses, since IFNs initiate signalling cascades that lead to the containment of viral spread and subsequent activation of the adaptive immune response (Durbin et al., 2000; Sheppard et al., 2003).
Following successful entry into the cells influenza RNAs are recognized by the intracellular RNA helicase retinoic acid-inducible gene–I (RIG-I), which leads to the production of type I and type III IFNs via transcription factors interferon regulatory factor (IRF) 3 and IRF7 (Fitzgerald et al., 2003; Kotenko et al., 2003; Sharma et al., 2003). The released type I and type III IFNs then bind to their respective receptors IFNAR2 and IL-28Rα/IL-10Rβ on the same and/or neighbouring cells, and initiate the expression of over 300 IFN-stimulated genes (ISGs) (Levy and Darnell, 2002). Many ISGs such as IFN-inducible protein kinase R (PKR) and 2, 5 oligoadenylate synthetase (OAS) directly degrade viral RNAs and also initiate apoptosis within the infected host cell, thereby limiting viral replication (Chen et al., 1999; Garcia et al., 2006; Gil and Esteban, 2000; Stark et al., 1998; Zhang and Samuel, 2007).

Little is known about the kinetics and effectiveness of antiviral responses to influenza infection in primary bronchial epithelial cells (pBECs) and how these may be altered in response to different virus strains. We have previously shown that BECs mount an effective innate antiviral response mediated by RIG-I with the robust release of IFN-β, IFN-λ1 and ISGs to the low pathogenic avian influenza virus H11N9 (Hsu et al., 2010). In contrast this response was markedly impaired to a human influenza H3N2 virus. These different responses were the result of differential effects of viral non-structural protein 1 (NS1). NS1 from the pathogenic H3N2 strain effectively inhibited RIG-I mediated signalling and the inductions of type I and III IFNs, whereas the NS1 from the H11N9 strain did not. Several studies have also shown that the NS1 protein of high pathogenic avian H5N1 virus is more potent in the host antiviral suppression (Bornholdt and Prasad,
2008; Jackson et al., 2008; Jiao et al., 2008). Intriguingly, we also observed that IFN-β protein release occurred even in the absence of influenza infection (Hsu et al., 2010). If and how this constitutive release of IFN-β contributes to antiviral responses to influenza infection remains unknown.

In this study we assessed the antiviral responses of human BECs to human influenza, H3N2, and highly pathogenic avian influenza, H5N1, using a proximal airway epithelial cell line, Calu-3 cells, and primary BECs (pBECs) obtained by bronchoscopy from healthy volunteers. We have defined, for the first time, the critical role of the constitutive IFN-β release, but not the induction of RIG-I, in BECs in innate responses to pathogenic human and avian influenza infection. A pathogenic human H3N2 strain replicated less effectively in pBECs compared to Calu-3 cells. This was due to the effects exerted by the constitutive release of IFN-β. In pBECs constitutive IFN-β production was associated with the induction of RIG-I and PKR and resulted in host cell apoptosis that inhibited viral replication, even when the inducible IFN response was suppressed. In stark contrast the pathogenic avian H5N1 strain severely impaired the effectiveness of the constitutive IFN-β, completely suppressed the inducible IFN response and prevented host cell apoptosis, which led to substantially higher viral replication in both Calu-3 cells and pBECs. Our work demonstrates the importance of constitutive IFN-β release from BECs that promotes the early induction of ISG expression and apoptosis and may control influenza infection, even when inducible IFNs are inhibited by NS1 protein.
RESULTS

Influenza viral replication in bronchial epithelial cells is independent of sialic acid residues expression. We first investigated the importance of sialic acid residue expressions in H3N2 infection of BECs. H3N2 replicated to a significantly higher viral titre in immortalised Calu-3 cells compared to pBECs (Figure 1A). However the high viral titre in these cells was not related to the expression of SAα2,6Gal glycoproteins. SAα2,6Gal residues on pBECs was significantly higher than that on Calu-3 cells (Figure 1B), but pBECs supported lower viral replication. This indicates that viral binding and entry into airway epithelial cells is not dependent on the expression level of SAα2,6Gal residues alone, and that the post-endocytotic events are more important in limiting influenza infection.

Antiviral responses to influenza viruses are reduced compared to the IFN agonist Poly I:C. To assess the antiviral response of infected cells, antiviral gene induction was measured by RT-qPCR at 6h, 12h, 24h, 48h, and 72h after Poly I:C stimulation and influenza infection. Poly I:C stimulation and H3N2 infection resulted in a significant induction of antiviral genes compared to the medium control in Calu-3 cells (Figure 2) and pBECs (Figure 3). UV-inactivated virus was no different to the medium control (data not shown). In both Calu-3 cells and pBECs Poly I:C induced early increases in RIG-I mRNA expression (6h and 12h, Figure 2A and Figure 3A), which was followed by later induction of IFN-β and PKR mRNA (Figure 2B – C and 3B – C). RIG-I, IFN-β, and PKR protein induction was also significantly up-regulated after Poly I:C stimulation in both cells (Figure 2D – F and 3D – F).
Similarly, infection of Calu-3 cells with H3N2 resulted in early induction of RIG-I at 6h and was followed by a substantial second rise in RIG-I mRNA (25 fold) at 72h (Figure 2A). IFN-β and PKR mRNA was significantly and substantially induced only late in infection respectively (Figure 2B – C). Infection also resulted in the significant production of RIG-I and PKR but not IFN-β protein after 48h (Figure 2D – F).

In pBECs, H3N2 infection again induced an early and sustained induction of RIG-I mRNA (Figure 3A), which followed by a significant and substantial induction of IFN-β and PKR mRNA by 24h (150 and 5 fold, Figure 3B – C, respectively). Infection induced significant production of RIG-I and PKR but not IFN-β protein expression after 48h (Figure 3D – F).

These results suggest that while type I IFN responses were minimally up-regulated after H3N2 infection in pBECs, the early up-regulation of RIG-I in pBECs may be important in resistance to infection.

**RIG-I induction does not protect against influenza infection.** To determine if RIG-I signalling is involved in resistance to infection in pBECs, RIG-I was suppressed in pBECs using siRNA before and during H3N2 infection or Poly I:C exposure (Figure 4A). RIG-I knockdown without stimulation or during Poly I:C exposure led to decreased IFN-β but increased PKR production compared to media treated controls (Figure 4B). In contrast RIG-I knockdown during H3N2 infection did not alter IFN-β and PKR protein production. Critically suppression of RIG-I also did not alter the replication efficiency of H3N2 influenza (Figure 4C). This suggested that the constitutive release of IFN-β may play a more important role in antiviral response to H3N2 than RIG-I-mediated inducible IFN-β.
Constitutive IFN-β release plays a critical role in protection against influenza infection. During this study IFN-β was detected in the supernatants of medium exposed controls (Figure 2E, 3E, 4B), indicating that there was constitutive release of IFN-β even in un-stimulated cells. To determine if constitutive release of IFN-β has a role in the antiviral response to influenza infection, we inhibited protein synthesis in the airway epithelial cells using cyclohexamide prior to and during infection with H3N2 and Poly I:C stimulation. GAPDH was not detected after treatment with cycloheximide, indicating successful inhibition of protein synthesis (Figure 5A). In the presence of cycloheximide H3N2 was unable to replicate, consistent with the complete inhibition of protein synthesis (Figure 5B). However there was an enhanced release of IFN-β protein in response to both H3N2 infection and Poly I:C stimulation with an increase even in the cycloheximide-treated media control (Figure 5C). Therefore IFN-β release occurred in the absence of protein synthesis, which presumably represents the release of pre-formed IFN-β protein.

Since IFN-β induces apoptosis during infection as a means of limiting influenza replication (Chawla-Sarkar et al., 2003; Clemens, 2003), we determined if enhanced IFN-β release was associated with an increase in BEC apoptosis during infection. While cycloheximide treatment alone caused a significant induction of apoptosis, H3N2 infection and Poly I:C stimulation further enhanced apoptosis of pBECs compared to treated media control, and in the absence of cycloheximide treatment (Figure 5D). This increase in apoptosis correlated with enhanced IFN-β release (Figure 5C). In comparison to Calu-3 cells, pBECs were more sensitive to apoptosis.
To determine if the constitutive release of IFN-β is functionally important in the antiviral response of epithelial cells to H3N2 infection, we then blocked the type I IFN receptor (IFNAR2) prior to infection using IFNAR2 neutralizing antibodies. This blockade resulted in significant increases in H3N2 titres compared to un-treated cells (Figure 6A). There were significant reductions in IFN-β protein production in all conditions following IFNAR2 neutralization (Figure 6B). IFNAR2 neutralization almost completely inhibited the production of IFN-β after H3N2 infection, and substantially reduced IFN-β expression following Poly I:C stimulation and in the medium only control (Figure 6B). The decrease in IFN-β was accompanied by reduced apoptosis following infection (Figure 6C). This provides further evidence that while RIG-I-initiated IFN-β production was inhibited by H3N2, the release of constitutive IFN-β and IFN-β signalling during infection is more important in suppressing viral replication in BECs.

Apoptosis is critical in limiting H3N2 replication in pBECs. While inefficient antiviral responses were induced following H3N2 infection, both Calu-3 cells and pBECs had similar magnitudes of antiviral responses to infection, but support different levels of H3N2 replication. Apoptosis is an important component of antiviral responses that can be triggered by IFN-β. Therefore, to investigate the mechanisms of differential replication we measured the levels of apoptosis during H3N2 infection in Calu-3 cells and pBECs. Reduced viral titres in pBECs (Figure 1A) was accompanied by a higher level of apoptosis compared to Calu-3 cells (Figure 7A). To determine if apoptosis directly impaired viral replication, the caspases inhibitor z-DEVD-Fmk was used to inhibit apoptosis before and during H3N2 infection. Inhibition of apoptosis during H3N2 infection
(Figure 7B) led to significantly higher viral replication in Calu-3 cells and pBECs (Figure 7C).

H5N1 infection led to high replication titre with complete abolishment of antiviral responses and impaired apoptosis in Calu-3 cells and pBECs. H5N1 infection resulted in a high viral replication in both cell types (Figure 8A), regardless of limited SAα2,3Gal residues on the BECs (Figure 1B). Viral titre was significantly higher in pBECs than that in Calu-3 cells (Figure 8A), which is in contrast to that observed with H3N2 infection (Figure 1A). Infection was accompanied with no mRNA induction of RIG-I, PKR, and IFN-β from 6h to 72h (data not shown), and also protein induction at 48h after infection (Figure 8B).

Owing to constraints in working with H5N1 infection it was not possible to measure apoptosis using flow cytometry with AxV/7AAD staining. Therefore apoptosis was measured by assessing Bax protein expression. In agreement with AxV/7AAD staining during H3N2 infection (Figure 6A), Bax was not up-regulated above baseline in Calu-3 cells, but was significantly induced in pBECs during H3N2 infection (Figure 7D). H5N1 infection reduced Bax expression in Calu-3 cells and did not affect expression in pBECs (Figure 7D). This indicates that while host cell apoptosis is a critical factor in restricting influenza replication, H3N2 and H5N1 infection effectively inhibited the inducible IFN-β response, thereby the apoptosis induction. H5N1 also is a more pathogenic virus than H3N2 that completely reduced all antiviral signalling, including those exerted by constitutive IFN-β and host cell apoptosis, leading to highly efficient replication.
DISCUSSION

Here we demonstrate that the extent of pathogenic human and avian influenza virus replication in airway epithelial cells is dependent on constitutive IFN-β release and IFN-β signalling rather than the abundance of sialic acid residues or RIG-I-mediated pathways as previously thought. H3N2 infection was able to limit inducible IFN-β release in BECs, however, this did not prevent the induction of ISG and an effective innate immune response was mounted that limited viral replication. In pBECs there was an early induction of RIG-I and a greater proportion of infected cells underwent apoptosis that was associated with reduced viral replication. However the RIG-I responses was not required to suppress viral replication. Importantly, the constitutive release of IFN-β played a critical role in triggering apoptosis and limiting viral replication in infected BECs. Furthermore, blocking the type I IFN receptor and apoptosis enhanced viral replication. In contrast infection of BECs with H5N1 resulted in inhibition of both inducible and constitutive IFN-β release, and impaired apoptosis that led to highly efficient viral replication.

Calu-3 cells and pBECs were used as proximal airway epithelial cells, which showed important differences in antiviral responses to infection and affected their susceptibility and outcome of influenza infection. Lower levels of SAα2,6Gal linked glycoprotein expression were observed on Calu-3 cells compared to pBECs, however H3N2 replication was greater in Calu-3 cells. Avian H5N1 virus also replicated to a high level in BECs despite of the low levels of SAα2,3Gal residues. These observations are in accordance with our previous work that demonstrated similar HA levels immediately after human and avian influenza viral infection in Calu-3 cells and pBECs irrespective of these
sialic acid residues (Hsu et al., 2010). Hence these sialic acid-bearing glycoproteins may only have a minor role in susceptibility, and that post-endocytosis antiviral responses are more important determinants of replication.

Antiviral responses are critically important in suppressing viral spread early in infection. In our study pBECs were more resistant to H3N2 infection due to higher levels of apoptosis after infection, which led to reduced viral replication. In contrast Calu-3 cells did not readily undergo apoptosis, which resulted in more efficient replication. The reason for this difference in apoptosis is probably due to the nature of immortalized cell lines, and results from pBECs provide a better insight into the natural human host cell response to influenza infection.

Primary BECs showed early and late increases in RIG-I expression after H3N2 infection. While numerous studies demonstrated the importance of RIG-I in influenza RNA recognition and subsequent signal transduction to induce IFN responses (Hausmann et al., 2008; Hornung et al., 2006; Kato et al., 2006; Loo et al., 2008; Matsukura et al., 2007; Opitz et al., 2007; Takeuchi and Akira, 2008), suppressing RIG-I affected neither IFN-β protein expression nor H3N2 replication efficiency in pBECs. This suggests that while RIG-I is critical in viral RNA recognition, RIG-I-signalling may not be important in influenza infection as its signalling pathway is interfered by NS1 proteins, and other factors are critical in controlling viral replication.

The lack of IFN-β induction after H3N2 infection is known to result from the suppression by the influenza NS1 protein. We have previously shown that the NS1 protein of the H3N2 strain was more potent in inhibiting the antiviral type I IFN response than that
of a low pathogenic H11N9 avian influenza virus (Hsu et al., 2010). The host binding
targets of NS1 include IRF3 phosphorylation (Collins et al., 2004), TRIM25 (Gack et al.,
2009), NK-kB (Ludwig et al., 2002; Talon et al., 2000; Wang et al., 2000), and cellular
mRNA translation processes (Qiu and Krug, 1994). This results in impaired innate immune
responses in infected host cells.

Despite the ability of H3N2 virus to inhibit inducible type I IFN response in BECs,
we still observed a selective induction of RIG-I and PKR protein late in H3N2 infection,
and the constitutive release of IFN-β played a pivotal role in this late antiviral signalling.
Cycloheximide was used as a general inhibitor of host protein synthesis to determine if the
IFN-β was induced in response to infection or was pre-existing. Treatment of BECs with
cycloheximide effectively inhibited protein synthesis, as demonstrated by the loss of
GAPDH expression, and resulted in cell death. However, treatment during H3N2 infection
led to an enhanced release of IFN-β, which correlated with increased apoptosis. Influenza,
as well as many other RNA viruses such as herpes simplex virus are known to
preferentially and effectively inhibit host cell protein synthesis and promote viral protein
synthesis and replication (Gale et al., 2000). Our results suggest that the inhibition of
protein synthesis may acts as a trigger for the enhanced release of pre-formed IFN-β and
apoptosis in the infected host cell, which can occur despite the ability of the virus to
prevent the induction of IFN-β through RIG-I mediated pathways. Interestingly a similar
response has been observed in cells infected with Newcastle Disease virus (NDV). Cells
treated with cycloheximide during NDV infection released greater amounts of IFN-β,
which was thought to occur through enhanced stability of IFN mRNA (Ringold et al.,
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1984). This, however, does not explain the constitutive release of IFN-β protein when host
protein synthesis was blocked at the protein translational level by cycloheximide. While the
exact mechanism by which IFN-β protein is released when protein synthesis is blocked is
unclear, we have demonstrated that this constitutively released IFN-β is functionally more
important in limiting viral replication via IFN-β-IFNAR signalling than the initial RIG-I
initiated signalling.

The constitutive release of IFN-β demonstrated here has been reported previously in
vitro and in vivo (Bocci et al., 1985a; Bocci et al., 1985b; De Maeyer-Guignard et al., 1988;
Sato et al., 2000), and was suggested to play a role in the priming and enhancement of IFN
response, as proposed in a “revving-up model” (Sato et al., 2000; Taniguchi and Takaoka,
2001). A constitutive weak signal induced by IFN-β-IFNAR signalling allows epithelial
cells to elicit a more robust response toward viral infection, while in the absence of this
signal epithelial cells become hypo-responsive to this stimulus.

Nevertheless despite the critical role of constitutive IFN-β in late RIG-I and PKR
induction to H3N2 infection, H5N1 inhibited all antiviral signalling more effectively in
infected cells, including signalling induced by constitutive IFN-β. This presumably
occurred as results of the potent suppressive properties of the H5N1 NS1 protein
(Bornholdt and Prasad, 2008; Cheung et al., 2002; Garcia-Sastre, 2006; Garcia-Sastre and
Biron, 2006; Jackson et al., 2008; Jiao et al., 2008; Lam et al., 2008; Twu et al., 2007; Zhu
et al., 2008), leading to a more efficient viral replication in these cells.

Collectively our results demonstrate that the constitutive type I IFN response of
infected BECs is important in determining the level of influenza replication. The level of
sialic acid-bearing glycoproteins does not correlate with the level of virus replication. This indicates that even low-level SA residue expression is sufficient to support infection and does not appear to be a major rate-limiting factor for influenza susceptibility. By contrast, the antiviral responses initiated after endocytosis have an important role in limiting virus replication, particularly the effect of constitutive release of IFN-β during infection. In the early phase of infection epithelial cells have limited ability to produce IFN-β, as a result of the suppression of the immediate RIG-I signalling cascade by the influenza NS1 protein. However constitutive IFN-β response counter-balances some of these suppressive effects of the influenza virus, via IFNAR2 signalling to induce expression of ISGs including RIG-I and PKR. These ISGs are important in establishing an antiviral state in BECs as they are the effector antiviral proteins that inhibit viral mRNA translation, inducing apoptosis and amplify the entire IFN response.
MATERIALS AND METHODS

Influenza virus. Human influenza A/Wellington/43/2006 (H3N2) strain and A/Vietnam/1203/04 (H5N1) was obtained from the WHO Collaborating Centre for Reference and Research on Influenza (Vic, Australia). Influenza viruses were propagated and virus titres determined by plaque assays on Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection (ATCC), USA) (Huprikar and Rabinowitz, 1980). All work with H5N1 was performed in the level 4 containment facility at The Commonwealth Scientific and Industrial Research Organization (CSIRO) - Australian Animal Health laboratory, Geelong, Victoria, Australia.

Cell culture and viral infection. Calu-3 and MDCK cells (ATCC) were maintained in minimum essential medium supplemented with 10% fetal bovine serum and Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum, respectively (Hsu et al., 2010). Human pBECs were obtained from healthy individuals by endobronchial brushing during fibre-optic bronchoscopy (Hurd, 1991). Subjects had no history of smoking or lung disease and had normal lung function. All subjects gave written consent. pBEC were cultured as described (Hsu et al., 2010; Wark et al., 2005).

H3N2 and H5N1 were diluted in the appropriate serum free medium and added to cells at multiplicity of infection (M.O.I.) of 5 and of 0.005, respectively. After 1 h of incubation, the inocula were removed and replaced with serum-free medium. Cells were treated with 100μg/ml of polyinosinic:polycytidylic acid (Poly I:C, Sigma-Aldrich) a known agonist of IFN response as a positive control. Cycloheximide (100ug/ml, Sigma-Aldrich) was used to inhibit protein synthesis by pre-treatment of cells (30min, 37°C, 5%
CO2) and was added to medium after virus inoculation. In experiments involving the blockade of IFNAR2, cells were incubated with 1µg/ml of mouse monoclonal antibody to IFNAR2 (CD118, PBL Laboratories) for 1h prior to virus inoculation.

siRNA. siRNA to RIG-I was purchased from Applied Biosystem, and was complexed with siPORT NeoFX transfection agent (Applied Biosystem) and diluted to 10nM with OPTI-MEM-I (Invitrogen). The siRNA was added to the cells for 24h prior to infection with H3N2.

Flow cytometry. FITC conjugated Sambucus niger agglutinin (SNA) and Maackia amurensis (MAA) lectins (Vector Laboratories) were used to identify SAα2,6Gal and SAα2,3Gal residues, respectively. BECs were stained with FITC-SNA at 10µg/ml and incubated at 4°C for 1h. The cells were then analysed using flow cytometry (FACSCanto II, Becton Dickinson) and results were expressed as median fluorescence intensity (Hsu et al., 2010).

Apoptosis. Apoptosis was measured using PE Annexin V Apoptosis Detection kit I (Becton Dickinson) according to manufacturer’s instruction. Cells were harvested and stained with annexin V-PE (AxV) stain and vital dye 7-amino-actinomycin (7AAD) and then analyzed using a FACSCanto II (Becton Dickinson) and FACSDiva software.

RT-qPCR. RNA was extracted from infected Calu-3 cells using RNeasy Mini Kits (Qiagen) according to the manufacturer’s instructions. RNA (1µg) was reverse transcribed to cDNA and was used for RT-qPCR assays (Applied Biosystem). Ribosomal RNA (18S)
was used as the reference gene. The cycle threshold (Ct) value obtained was normalized to that of the 18S gene, and expressed as fold induction over the medium control.

**Immunoblotting.** Calu-3 cells and pBECs were lysed in RIPA buffer, and proteins (10µg) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for detection of IFN-β in supernatants, RIG-I, PKR, and Bax in cell lysates. GAPDH was detected as a loading control for proteins in cell lysates. Protein estimation was determined by densitometry and the values were expressed as protein/GAPDH ratio and presented as fold induction from medium control. As there is no loading control available for secreted proteins, densitometric values for IFN-β was presented as fold induction from medium control.

**Data analysis.** Data were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using student’s t test for analysis A p-value of < 0.05 was considered significant. The study was approved by The University of Newcastle Human Research Ethics Committee.

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FIGURE LEGENDS

Figure 1. H3N2 replication, SAα2,6Gal and SAα2,3Gal linked glycoprotein levels on Calu-3 cells and pBECs. (A) H3N2 replicated to a higher titre in Calu-3 cells compared to pBECs at 48h after infection. (B) Despite the higher H3N2 viral titre in Calu-3 cells, the level of SAα2,6Gal linked glycoproteins was lower than that on pBECs. Results were derived from three independent experiments and are presented as standard error of the mean (SEM).

Figure 2. Type I IFN signalling and responses to influenza virus infection in Calu-3 cells. H3N2 infection induced increases in mRNA of (A) RIG-I early at 6h and late at 72h, (B) IFN-β (40,000 fold) at 72h, and (C) PKR (4 fold) at 48 and 72h. H3N2 infection induced increases in protein induction of (D) RIG-I and PKR (F) but not IFN-β (E) at 48h compared to medium control. Results were derived from three independent experiments and are presented as standard error of the mean (SEM).* and ** indicates significant induction from medium control. § and §§ indicates a significant reduction compared to medium control.
Figure 3. Type I IFN signalling and responses to influenza infection in pBECs. (A) RIG-I mRNA was induced early (25 fold) at 12h and sustained throughout the course of infection. (B) IFN-β mRNA was up-regulated 175 fold at 24h, which was sustained throughout the time course, and (C) PKR mRNA was induced to 5 fold at 24h and 48h. (D) RIG-I protein levels were maintained at 6 and 24h during infection, and was significantly up-regulated at 48h (p < 0.001). (E) IFN-β protein was again not induced compared to medium control, and (H) PKR protein was significantly up-regulated compared to medium control (p < 0.001). Results were derived from three independent experiments and are presented as standard error of the mean (SEM). * - **** indicates significant induction from medium control. § indicates a significant reduction compared to medium control.

Figure 4. RIG-I knockdown in pBECs did not protect against influenza infection. (A) RIG-I signalling was suppressed using siRNA in pBECs during H3N2 infection. (B) This resulted in a decrease in IFN-β and in increase in PKR production in RIG-I-suppressed and media or Poly I:C exposed controls compared to that in intact cells. However, IFN-β levels remained unchanged and PKR was up-regulated in H3N2 infection in RIG-I-suppressed cells. (C) Critically, RIG-I knockdown did not affect H3N2 replication compared to RIG-I-intact cells. Results were derived from three independent experiments and are presented as standard error of the mean (SEM). * - **** indicates significant induction of from medium control.
Figure 5. H3N2 replication, IFN-β release, and apoptosis in the presence of cycloheximide in Calu-3 cells and pBECs. (A) Cycloheximide treatment inhibited protein synthesis in airway epithelial cells, demonstrated by the loss of GAPDH production after treatment and Poly I:C exposure or H3N2 infection after 6h. (B) Cycloheximide treatment abolished viral replication, and (C) enhanced IFN-β release, which (D) correlated with enhanced apoptosis. Results were derived from three independent experiments and are presented as standard error of the mean (SEM). * - **** indicates significant induction of from medium control. § indicates significant reduction compared to medium control.

Figure 6. H3N2 replication, IFN-β production and apoptosis in IFNAR2 neutralized Calu-3 cells and pBECs. (A) Blocking of IFNAR2 before infection resulted in significant increases in influenza titres 48h after infection compared to no treatment. The rise in titre correlated with (B) significant decreases of IFN-β in all conditions involving neutralization of the IFNAR2 compared to untreated controls. (C) Decreased release of IFN-β after IFNAR2 neutralization resulted in significant decreases in apoptosis after H3N2 infection. Results were derived from three independent experiments and are presented as standard error of the mean (SEM). * - **** indicates significant induction from medium control. § indicates a significant reduction compared to medium control.
Figure 7. The effect of apoptosis on H3N2 replication in Calu-3 cells and pBECs.

(A) There was a significant induction of apoptosis in pBECs compared to Calu-3 cells 6h after H3N2 infection. (B) Inhibition of apoptosis with Z-DEVD-Fmk led to (C) enhanced viral titres in both cell types. Results were derived from three independent experiments and are presented as standard error of the mean (SEM). * and ** indicates significant induction from medium control. § indicates a significant reduction compared to medium control.

Figure 8. The antiviral induction after H5N1 infection in Calu-3 cells and pBECs.

(A) H5N1 replicated to a significantly higher titre in pBECs compared to that in Calu-3 cells (p < 0.001), and (B) infection led to a complete inhibition of RIG-I, PKR, and IFN-β in both cells. (C) Bax was measured to indicate the level of apoptosis at 6hr after infection. While Bax was not up-regulated in H3N2 infection, it was significantly reduced in H5N1 infection in Calu-3 cells. On the other hand in pBECs Bax was significant induced by H3N2 but again not by H5N1 at 6hr after infection. Results were derived from three independent experiments and are presented as standard error of the mean (SEM). * and ** indicates significant induction from medium control. § indicates a significant reduction compared to medium control.
Figure 1

A

Virus titre (pfu/ml)

\[
\begin{align*}
\text{Calu-3} & : 4.0 \times 10^4 \\
\text{pBECs} & : 1.0 \times 10^3 \\
\end{align*}
\]

\[p < 0.001\]

B

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<tr>
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\[p < 0.001\] \hspace{1cm} \[p < 0.001\] \hspace{1cm} \[p < 0.001\] 

\[p > 0.002\]
Figure 3

A

RIG-I mRNA fold change

0 10 20 30 40 50

Time post infection (hr)

6h 12h 24h 48h 72h

B

IFN-β mRNA fold change

0 50 100 150 200 250 300

Time post infection (hr)

6h 12h 24h 48h 72h

C

PKR mRNA fold change

0 12 24 36 48

Time post infection (hr)

6h 12h 24h 48h 72h

D

RIG-I

H3N2 Poly I:C Medium

6h

GAPDH

E

IFN-β

H3N2 Poly I:C Medium

p = 0.001

24h

GAPDH

p < 0.001

48h

GAPDH

F

PKR

H3N2 Poly I:C Medium

GAPDH

Field change of PKR from medium

p < 0.001

0 0.5 1 1.5 2

H3N2 Poly I:C

Field change of RIG-I from medium

p < 0.001

0 1 2 3

H3N2 Poly I:C
Figure 4

A

- - - + + + sRIG-I
+ - - + - H3N2
. + - - + - Poly I:C

B

- - - + + + sRIG-I
+ . . + - - H3N2
. + . . + - Poly I:C

IFN-β

PKR

GAPDH

C

Virus titre (pfu/ml)

0 5x10^4 1x10^4 1.5x10^4

H3N2 H3N2 + sRIG-I

p < 0.001

p < 0.001

p < 0.001

p < 0.001
Figure 6

A

B

Calu-3 cells

pBECs

H3N2 Poly I:C Medium

IFN-β

IFN-β

Fold change of IFN-β from medium

p < 0.001

p = 0.004

p < 0.001

p = 0.023

Fold change of IFN-β from medium

p < 0.001

p = 0.033

p < 0.001

p = 0.023

C

Calu-3 cells

pBECs

H3N2 Poly I:C Medium

Apoptosis (%)

p < 0.002

p < 0.001

p < 0.001

p < 0.001

p < 0.001

p < 0.001

p < 0.001

p < 0.001

p < 0.001

p < 0.001
Figure 7

A

![Graph showing apoptosis](image)

B

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C

![Graph showing virus titre](image)
Figure 8

A

![Virus titre (pfu/ml) comparison](image)

B

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![Fold change of Bax from medium](image)
American Journal of Respiratory Cell and Molecular Biology

Human influenza is more effective than avian influenza at antiviral suppression in airway cells

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2. WHO Collaborating Centre for Reference and Research on Influenza

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Running title: Influenza infection and IFN responses

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Abstract

Airway epithelial cells are the initial site of infection with influenza viruses. The innate immune responses of airway epithelial cells to infection are important in limiting virus replication and spread. However, relatively little is known about the importance of this innate antiviral response to infection. Avian influenza viruses are a potential source of future pandemics, therefore it is critical to examine the effectiveness of the host antiviral system to different influenza viruses. We used a human influenza (H3N2) and a low pathogenic avian influenza (H11N9) to assess and compare the antiviral responses of Calu-3 cells. After infection H3N2 replicated more effectively than the H11N9 in Calu-3 cells. This was not due to differential expression of sialic acid residues on Calu-3 cells but was attributed to the interference of host antiviral responses by H3N2. H3N2 induced a delayed antiviral signaling and impaired type I and type III interferons (IFNs) induction compared to the H11N9. The gene encoding for non-structural (NS) 1 protein was transfected into the BECs and the H3N2 NS1 induced a greater inhibition of antiviral responses compared to the H11N9 NS1. While the low pathogenic avian influenza virus was capable of infecting BECs, the human influenza virus replicated more effectively than avian influenza virus in BECs and this was due to a differential ability of the two NS1 proteins to inhibit antiviral responses. This suggests that the subversion of human antiviral responses may be an important requirement for influenza viruses to adapt to the human host and cause disease.

Keywords: Influenza, innate immune response, antiviral, interferon
Introduction

Influenza A viruses are major respiratory pathogens that primarily infect human bronchial epithelial cells (BECs), and as the initial barrier to the virus the immune response elicited by these cells is important in determining the outcome of infection. Type I IFNs (IFN-α/β) and type III IFNs (IFN-λ1, IFN-λ2, and IFN-λ3) are critical components of the human innate immune system that provide immediate responses to limit viral infection and help initiate an appropriate adaptive immune system response to clear infection.

Influenza infection of airway epithelial cells is initiated by the binding of influenza haemaglutinin (HA) to sialic acid residues. Human influenza viruses have been found to preferentially bind to glycoproteins containing sialic acid residues with a terminal α2,6Gal linkage (SAα2,6Gal), which are thought to predominate in the upper airway and proximal bronchial tree [1-3]. In contrast, avian influenza viruses bind to SAα2,3Gal linkages mainly found in the lower airways [1, 2]. These differences in binding preference and sialic acid distribution in the respiratory system are thought to account for the reduced ability of avian influenza viruses to infect humans [2-4]. Once bound, viruses are endocytosed and replicate in BECs. The cytoplasmic RNA helicase retinoic acid-inducible gene (RIG) -I recognizes the viral RNA and associates with tripartite motif protein 25 (TRIM25). This enables RIG-I to associate with the adaptor protein IFN-β promoter stimulator 1 (IPS-1) on the mitochondria [5, 6], which leads to the subsequent phosphorylation of interferon regulatory factor (IRF) 3. pIRF-3 then translocates to the nucleus where it initiates the expression of type I IFNs and chemokines such as CXCL-10 [7-10]. After secretion type 1 IFNs bind to the type I interferon receptor (IFNAR2) on the same and/or neighbouring cells and then signal via...
JAK1/STAT1 to induce the expression of over 300 IFN-stimulated genes (ISGs), including antiviral proteins such as PKR and OAS, and positive regulators RIG-I, MDA-5, and IRF7 [11-16]. These ISGs are able to inhibit viral replication, amplify the antiviral responses, and also induce an antiviral state in neighbouring airway epithelial cells [17, 18]. Type III IFNs are newly discovered IFNs that, although bind to different receptors, have similar mechanisms of inducing antiviral activity as type I IFNs [19]. Viral infection including influenza viruses and herpes simplex viruses (HSV) were shown to induce type III IFNs via RIG-I-IRF3 signalling pathway, and once released bind to IFN-λR1 and subsequently activate JAK1/STAT1 and initiate ISG expression [15, 16, 20].

In spite of these host immune responses influenza viruses are known to replicate efficiently in host epithelial cells and have developed virulence factors to subvert these responses. The influenza virus NS1 protein is a multi-functional protein that when expressed inhibits host mRNA processing and immune responses, particularly antiviral responses, that are activated against influenza [21-26]. NS1 protein binds to TRIM25-RIG-I complex and disrupts downstream RIG-I signalling [21, 22]. NS1 also binds to IRF3 and prevents the initiation of IFN responses [23].

Avian influenza viruses are a potential source of future pandemics as they have the capacity to undergo genetic mutation and reassortment with other strains of influenza from different hosts and subsequently cause diseases in humans. The recent pandemic caused by the novel H1N1 influenza virus is a clear example of this phenomenon and this virus is the result of triple reassortment between human, avian, and swine influenza viruses [27]. Therefore, it is critically important to understand the mechanisms of pathogenesis of human and avian influenza viruses and the effectiveness
of human antiviral responses to these viruses. In this study we used a low pathogenic avian influenza virus strain H11N9 from a trans-hemispheric migratory bird. This strain is not known to cause disease in either avian or human hosts, therefore we evaluated the ability of human H3N2 and avian H11N9 to infect BECs Calu-3 cells and primary bronchial epithelial cells (pBECs), and investigated the antiviral responses to infection with these viruses.
Materials and methods

Viruses. Human influenza A/Wellington/43/2006 (H3N2) strain and a low pathogenic avian strain A/Sharp-tailed Sandpiper/Australia/6/2004 was obtained from the WHO Collaborating Centre for Reference and Research on Influenza (Vic, Australia) [28]. Influenza viruses were propagated and virus titre determined by plaque assays on Madin-Darby canine kidney (MDCK) cells (ATCC, USA) [29].

Cell culture and viral infection. Calu-3 and MDCK cells (ATCC) was maintained in minimum essential medium supplemented with 10% fetal bovine serum and Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum, respectively. Human pBECs were obtained from healthy individuals by endobronchial brushing during fibre-optic bronchoscopy [30]. Subjects had no history of smoking or lung disease with normal lung function. All subjects gave written consent. pBEC were cultured as described [31]. Virus infection was performed at multiplicity of infection of 5. Cells were treated with 100μg/ml of Poly I:C (Sigma-Aldrich) as a positive control. For IFN-β and IFN-λ1 pre-treatments, Calu-3 cells were incubated with 1ng/ml of IFN-β and IFN-λ1 for 3hr prior to infection.

Flow cytometry. FITC conjugated Sambucus niger agglutinin (SNA) and Maackia amurensis (MAA) lectins (Vector Laboratories) were used to identify SAα2,6Gal and SAα2,3Gal residues, respectively. Calu-3 cells were stained with 10μg/ml of lectins, analysed using flow cytometry (Becton and Dickinson), and results expressed as median fluorescence intensity.

RT-qPCR. RNA was extracted from infected Calu-3 cells using RNeasy Mini Kits (Qiagen) according to the manufacturer’s instructions. RNA (1μg) was reverse
transcribed to cDNA and was used for RT-qPCR assays (Applied Biosystem). Ribosomal RNA (18S) was used as the reference gene. The cycle threshold (Ct) value obtained was normalized to that of the 18S gene, and expressed as fold induction over the medium control.

**Immunoblotting and ELISA.** Calu-3 cells were lysed in RIPA buffer, and proteins (10µg) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for detection of IFN-β in supernatants, RIG-I and MDA-5 in the cell lysates. GAPDH was detected as a loading control. CXCL-10 and IFN-λ1 concentration was assessed using human CXCL-10 and IFN-λ1 ELISA kits (RnD) according to the manufacturer’s instructions.

**Microarray analysis.** RNA was extracted from Calu-3 cells 24hrs after infection using RNeasy Mini Kits (Qiagen). RNA was amplified, hybridized onto beadchips, and scanned for gene expression analysis. Data sets were submitted to the NCBI Gene Expression Omnibus (GEO) database under the accession number GSE19580.

**Expression plasmids and transfection.** Viral RNA was extracted from influenza viruses, reverse transcribed to cDNA, and used as templates to amplify NS genes by PCR. The gene was then cloned into an expression vector pcDNA3.3-TOPO (Invitrogen) and transfected into Calu-3 cells. The transfectant cells were stimulated with Poly I:C (100 µg/ml, Sigma) and NS1, IFN-β, IFN-λ1, CXCL-10 expression was measured.

**Data analysis.** Data were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using student’s t test on virus replication. A one-way
analysis of variance (ANOVA) was performed on mRNA relative fold change and a p-value of < 0.05 was considered significant. The study was approved by the University of Newcastle Human Research Ethics Committee.
Result

Human influenza H3N2 replicates more efficiently than avian influenza H11N9 independently of influenza receptor expression on Calu-3 cells. We first examined the sialic acid residues expression on Calu-3 cells, a cell line representing epithelial cells of the proximal lower respiratory tract. By staining the cells with lectin SNA and MAL Calu-3 cells showed a significantly higher SAα2,6Gal than SAα2,3Gal residues, which is similar to primary proximal airway epithelial cells (Figure 1A). Both H3N2 and H11N9 initially infected Calu-3 cells equally well as evident with similar of HA content immediately after infection (Figure 1B), and replicated with similar kinetics. By 48hr however H3N2 had replicated to a significantly higher titre compared to H11N9 (p = 0.009) (Figure 1C). This indicates that although there was only a low level of SAα2,3Gal residues H11N9 was still able to infect Calu-3 cells and initially replicated as efficiently as H3N2. However H3N2 appeared to be able to establish infection with greater replication at 48hr.

Induction of antiviral responses after H3N2 and H11N9 infection. We then investigated the kinetics of the induction of antiviral responses to see if there were differences in the response of Calu-3 cells to infection by the two viruses. Poly I:C was used as a positive control of antiviral responses, and UV-inactivated virus was used as a negative control, which had no significant effect compared to the medium control (Data not shown).

H3N2 led to an early induction of RIG-I (25 fold) by 6 hr, with a more modest increase in MDA-5 mRNA (5 fold) by 12hr (Figure 2A, 2B). This was followed by a second late induction of both genes at 72hr. H11N9 induced similar responses, although
the later rise in RIG-I from 24-72hr was more modest and gradual. H3N2 induced TLR3 mRNA at 6hr with a second rise from 48-72hrs, which occurred to a greater extent than for H11N9 (Figure 2C). Both viruses induced PKR and IFN-β mRNA from 48hr to 72hr (Figure 2D, 2E), with H11N9 inducing an earlier IFN-β response.

The protein expression of RIG-I and MDA-5, as well as IFN-β, IFN-λ1, and CXCL-10 was also assessed after infection. In contrast to observations with mRNA, H3N2 induced delayed RIG-I protein expression until 48hr, and this induction was significantly lower compared to H11N9 (Figure 3A). Similarly MDA-5 protein was only detected at 48hr after H11N9 infection (Figure 3B) but not at earlier time points by both viruses (Data not shown). As inductions were mostly observed at 48hr after infection, IFN-β, IFN-λ1, CXCL-10, and PKR protein induction was measured at 48hr and H3N2 induced a minimal IFN-β, IFN-λ1 (p = 0.005), CXCL-10 (p = 0.002), and PKR protein production (p = 0.05) at 48hr compared to medium control, and were significantly reduced compared to induction by H11N9 (Figure 3D, 3E, 3F). Furthermore, infection in pBECs also showed similar IFN induction pattern. H11N9 infection induced significant higher levels of IFN-β and CXCL-10 protein compared to H3N2 infection (IFN-β, p = 0.002; CXCL-10, P = 0.011) (Figure 5), further confirming the difference in anti-viral responses between the two influenza infections.

**IFN-β and IFN-λ1 have major roles in limiting influenza viral replication in Calu-3 cells.** To determine if the minimal induction of type I and type III IFNs, after H3N2 infection led to the increase in replication compared to H11N9. Cells were pre-treated with either IFN-β, IFN-λ1 alone or in combination prior to infection, and viral replication was measured at 48hr. Pre-treatment with IFN-β or IFN-λ1 alone at 1ng/ml
significantly decreased the viral replication of H3N2 and H11N9 by 3 fold, and the combination of both IFNs further reduced the replication of both viruses (Figure 6).

**H3N2 virus infection leads to widespread reductions in antiviral responses in Calu-3 cells.** To investigate if the difference in antiviral responses by H3N2 and H11N9 was widespread across different antiviral signalling pathways, gene expression in infected cells was examined at 24hr by microarray analysis.

Infection with H3N2 and H11N9 resulted in the altered expression of 13,388 genes after normalization to media treated controls. ANOVA analysis with the more stringent Benjamini-Hochberg correction identified 2,155 genes that were differentially expressed (p ≤ 0.005) (Figure E1). Of these 330 genes were altered by ≥2 fold (either up or down regulated) with 41 genes differentially expressed in response to H3N2 infection, 300 by H11N9 and 35 genes were altered by both H3N2 and H11N9 infection. Gene ontology analysis revealed that H3N2 infection only induced the ISG OAS2, whereas in contrast H11N9 infection up-regulated many immune and virus responses genes including IFN-β, IFN-λ1, and numerous ISGs.

**H3N2 NS1 is more effective in suppressing IFN responses in Calu-3 cells compared to H11N9 NS1.** The influenza NS1 protein is known to inhibit antiviral responses in Calu-3 cells, therefore, we determined if the NS1 of H3N2 was more effective in suppressing immune responses compared to that of H11N9. The NS1 genes of H3N2 and H11N9 were transfected into Calu-3 cells, which were then stimulated with Poly I:C. NS1 of both H3N2 and H11N9 were expressed to similar levels in Calu-3 cells (Figure E2), and significantly down-regulated IFN-β, IFN-λ1 and CXCL-10 protein production compared to media treated controls (Figure 7A, 7B, 7C). IFN-β,
IFN-λ1, and CXCL-10 protein production in response to Poly I:C was inhibited by NS1 of both viruses but the suppression of these antiviral proteins were significantly greater by the NS1 protein of H3N2 compared to that of H11N9 virus. Vector alone did not induce these proteins above media treated controls. This clearly demonstrated that human Calu-3 cells respond poorly to H3N2 due to the enhanced NS1-induced suppression of antiviral responses, which was less effective in H11N9 NS1.
**Discussion**

The innate antiviral response to influenza by the airway epithelial cells is the first line of defence against infection and influences the subsequent adaptive immune response and the effective clearance of the virus. In this study we have demonstrated that human influenza H3N2 replicated more efficiently than avian H11N9 influenza virus in human Calu-3 cells. The increase in replication of H3N2 was not due to the greater expression of SAα2,6Gal residues on the Calu-3 cells as similar levels of H3N2 and H11N9 titre were observed shortly after infection, demonstrating a similar ability for both viruses to enter into airway epithelial cells. Instead differences in replication were attributed to the ability of H3N2 to impair host cellular innate immune responses, with reduced induction of RIG-I and downstream release of IFN-β, IFN-λ1, PKR, and CXCL-10, compared to H11N9. We have also demonstrated that this impaired antiviral response was induced by the NS1 protein of H3N2, which more effectively inhibited these antiviral responses compared to that of H11N9.

Calu-3 cells are representative of human proximal airway epithelial cells and predominantly express SAα2,6Gal residues. These residues are the binding site for human influenza virus on epithelial cells, while avian influenza viruses appear to bind more readily to SAα2,3Gal, the level of which was found not as abundant as SAα2,6Gal residue in the proximal airway epithelial cells [2-4]. Despite this we demonstrated that both H3N2 and H11N9 viruses were able to readily infect these human airway epithelial cells to a similar extent based on HA level and similar initial replication titre. This suggests that either only a low level of SAα2,3Gal is sufficient to allow viral entry to establish infection or that other receptors may also play a role in influenza infection such as caveolae and other lipid-linked glycoproteins [32, 33]. A study has shown that a
SAA2,6Gal deficient mice could in fact allow influenza virus to replicate to similar extent as in the wild type mice, supporting the theory that other factors are in play in the susceptibility to influenza infection [34].

Post-endocytosis events clearly play a key role in determining the success of infection. After endocytosis into Calu-3 cells, cytosolic RNA helicase RIG-I recognize influenza viral RNA and signals through IRF3 to induce the expression and production of type I IFNs. The secreted IFNs then bind to the IFNAR2 on the same and/or neighbouring cells and amplify the antiviral responses by inducing ISGs expression via JAK1/STAT1 pathways. Type III IFNs are also induced through similar pathways [15, 16, 20], and via a distinct receptor IFN-λ1 [19], they signal through the same JAK1/STAT1 pathway to induce ISGs responses [15, 16, 35].

H3N2 induced a more robust expression of RIG-I mRNA compared to H11N9 and late induction was also observed for MDA-5 as well as TLR3, again with a greater induction by H3N2 infection. Paradoxically, at the protein level RIG-I and MDA-5 were more potently suppressed by H3N2 infection, and this correlated with the higher level of H3N2 replication. The disparity between mRNA and protein induction could be explained by the NS1 protein that inhibits the host mRNA processing thereby minimising the IFN responses in the infected cells [36-38].

Type I and type III IFNs are critically important in establishing the anti-viral state in the infected micro-environment [39, 40]. While priming of Calu-3 cells with either IFN-β or IFN-λ1 alone reduced viral replication by two fold, concurrent administration of both types of IFNs further limited viral infection by five fold. Despite the anti-viral effect of IFNs H3N2 still replicated to a higher extent than H11N9,
suggesting that one type of IFN alone may not be sufficient to counteract the suppressive effect of H3N2 NS1 protein. The NS1 protein of H5N1 has been shown to be resistant to IFN pre-treatment in vitro via un-identified pathways [41], and it is possible that H3N2 NS1 also has similar ability to certain extent. The mechanism in which NS1 confers resistance to anti-viral cytokines are still unclear, however it is known that a glutamic acid at position 92 is required for this activity.

We also observed residual levels of IFN-β in the absence of infection. The release of IFN-β may be constitutive and has also been hypothesized to play a major role in epithelial cell priming for a more robust anti-viral response to infection [12, 42]. In our case Calu-3 cells and pBECs appeared to be unable to sustain this constitutive release in H3N2 infection, which was at least in part due to the suppressive activity of its NS1 protein.

Human H3N2 influenza virus was able to inhibit the host IFN response and replicate more efficiently in Calu-3 cells than the H1N1N9 virus. We have demonstrated that the NS1 protein of human influenza more effectively inhibited IFN responses than the avian strain, and this at least in part is responsible for the enhanced replication of H3N2. This further demonstrates that the enhanced replication of H3N2 may result from the activity of the NS1 protein in suppression of antiviral responses. Collectively, these results indicate that there are differences in the level of immune suppression by the NS1 protein of different strains of influenza, and this appears to be an important factor that determines whether influenza viruses have high or low pathogenic properties in human airway cells.
The potent antiviral suppression by human influenza NS1 protein may be due to the selection pressure that drives the evolution of the virus for better adaptation in human populations. In addition influenza viruses that develop a NS1 protein that is effective in human cells are more likely to lead to sustained infection in humans and this may be an important factor in the zoonotic transmission of avian influenza to humans. Nevertheless, it remains unclear which set of residues differentiates the NS1 of high or low pathogenic influenza viruses although a change in one residue (92E) has been implicated in increased disease severity with H5N1 NS1 in pigs [41]. Our study demonstrates that differences in NS1 at the genetic level may potentially serve as a marker for influenza pathogenicity and are an important determinate for replication in human Calu-3 cells.

We can not rule out that other influenza virulence factors may also be involved in the antiviral suppression, such as PB1-F2 protein. By targeting inner mitochondrial membrane PB1-F2 is able to cause mitochondrial membrane permeabilization, leading to apoptosis of the infected cells and thereby increasing the virulence of the virus [43-45]. However we did not observe a significant level of apoptosis at 48hr after infection (both early apoptosis and late apoptosis/necrosis), which could be due the nature of immortalized cell line.

In summary, we have demonstrated that a human influenza virus that has had a long period of adaption in man replicates to a higher level in human airway cells than a low pathogenic avian virus in Calu-3 cells. This is not determined by the ability of the virus to enter the host cell but rather the ability of the virus to inhibit the post endocytotic host innate immune response. Both type I and type III IFNs can limit influenza replication. We have shown that infection with H3N2 impairs these IFN
responses, through the action of its NS1 protein, whereas the NS1 protein from H1N9 virus was far less effective at inhibiting these responses. This study demonstrates that the ability of an influenza virus to inhibit the early innate immune response in human Calu-3 cells is an important factor in promoting its ability to infect humans. Therefore factors that either impairs this early response could enhance infection, while factors that enhance the antiviral response may have quite profound effects on limiting infection. Both of these avenues should be further explored to look for reasons why individuals may be susceptible to infection and to explore this as a potential therapeutic pathway.

Acknowledgements

We thank Ms. Kristy Parsons and Ms. Melina Tooze for technical assistance, and Dr. Katherine Baines for assistance in microarray analysis.
References


Figure legends

Figure 1. SAα2,6Gal and SAα2,3Gal residue levels, HA level at 2hr and 6hr after infection, and H3N2 and H11N9 replication kinetics in Calu-3 cells. (A) SAα2,6Gal residue level is significantly higher than SAα2,3Gal residue on Calu-3 cells, similarly found in cells of upper respiratory tract. (B) After infection with H3N2 and H11N9 in Calu-3 cells, both viruses initially infected the cells equally well, as evident with similar HA level of H3N2 and H11N9 inside infected cells. (C) In addition both viruses replicated to similar levels until 48hr when H3N2 replicated to a significantly higher titre than H11N9 (p = 0.009). Results were obtained from three independent experiments. Sialic acid residue levels were assessed by staining with fluorescein-labelled SNA and MAL, analysed by flow cytometry and expressed as median fluorescence intensity. Results are representative of three independent experiments. Densitometry on the results from HA western blot was expressed as PKR/GAPDH ratio and presented as fold induction from medium controls. Viral replication was measured by plaque assays and the results are presented as standard error of the mean (SEM).

Figure 2. Induction of anti-viral response genes to influenza infection in Calu-3 cells. The relative induction of mRNA was measured by RT-qPCR, normalized to the housekeeping gene 18S ribosomal RNA, and expressed as fold change from media treated controls. H3N2 and H11N9 induced an early up-regulation of (A) RIG-I mRNA at 6hr, whilst (B) MDA-5 mRNA was not increased until 48hr. (C) TLR3 mRNA expression was increased at 12-72hr. Both viruses expressed (D) PKR and (E) IFN-β
mRNA from 48hr to 72hr. H3N2 induced earlier and greater RIG-I and TLR3 responses, whereas H11N9 induced an earlier IFN-β response.

Figure 3. RIG-I and MDA-5 protein production in response to influenza infection in Calu-3 cells. The protein induction of RIG-I and MDA-5 was detected 48hr after infection by western blot. (A) H3N2 only induced the significant production of RIG-I at 48hr, whereas RIG-I was detected at 6hr, 24 and 48hr after H11N9 infection, and the induction was significantly up-regulated compared to H3N2 at 48hr. (B) Similarly, H11N9 but not H3N2 infection resulted in a detectable MDA-5 expression at 48hr. Results are representative of three independent experiments, expressed as protein/GAPDH ratio and presented as fold induction from media treated controls. § and §§ indicates a significant reduction from medium control.*, **, and *** indicates significant induction from medium control.

Figure 4. IFN-β, IFN-λ, CXCL-10, and PKR protein production in response to influenza infection in Calu-3 cells. The protein induction of IFN-β and PKR was measured by western blot, IFN-λ1, and CXCL-10 protein was measured by ELISA. (A) IFN-β and (B) IFN-λ production was not induced by H3N2 infection, but was significantly up-regulated by H11N9 infection compared to media treated controls. (C) CXCL-10 production was significantly induced after H11N9 but not H3N2 infection. (D) PKR protein was induced by both viruses, and induction was also higher in H11N9 infection compared to that in H3N2 (p = 0.055). Results are representative of three independent experiments, and densitometry of the results from IFN-β western blot is
expressed as fold induction from media treated controls. PKR densitometry was expressed as PKR/GAPDH ratio and presented as fold induction from media treated controls. *, **, and *** indicates significant induction from medium control.

Figure 5. IFN-β and CXCL-10 protein production in response to influenza infection in pBECs. The protein induction of IFN-β and CXCL-10 was measured by western blot at 48hr after infection. (A) IFN-β and (B) CXCL-10 protein induction was significantly higher in H11N9 infection compared to that in H3N2 infection at 48hr. Results are representative of three independent experiments, and densitometry of the results from IFN-β western blot is expressed as fold induction from media treated controls. * and ** indicates significant induction from medium control.

Figure 6. The antiviral effect of IFN-β and IFN-λ1 against influenza infection in Calu-3 cells. IFN-β and IFN-λ1 (1ng/mL) was used to prime Calu-3 cells before influenza infection, and viral replication was analyzed by plaque assay at 48hr after infection. Both H3N2 and H11N9 had a 3–4 fold decrease in viral replication when Calu-3 cells were pre-treated with IFN-β and IFN-λ1 alone, and replication was further reduced when IFN-β and IFN-λ1 was used in combination. Results are representative of three independent experiments.

Figure 7. Induction of IFN-β, IFN-λ1, and CXCL-10 protein production after NS1 transfection and Poly I:C stimulation in Calu-3 cells. The protein induction of IFN-
β was measured by western blot, and IFN-λ1, and CXCL-10 protein was measured by ELISA. (A) H3N2 and H11N9-NS1 transfection significantly down-regulated IFN-β production after Poly I:C stimulation, with a significantly greater effect of H3N2-NS1. (B) IFN-λ1 and (C) CXCL-10 production was also significantly reduced by H3N2-NS1 compared to H11N9-NS1. Results are representative of experiments performed in three independent experiments, densitometry on the results from the IFN-β western blot was performed and presented as fold induction from medium controls. * and ** indicates significant reduction from medium control. § and §§ indicates a significant reduction compared to induction by Poly I:C.

Figure E1. Induction of antiviral-associated genes is widespread after influenza infection in Calu-3 cells. (A) H3N2- and H11N9-induced genome-wide gene expression was examined at 24hr after infection by microarray analysis. The cDNAs from infection were hybridized onto the beadchip, scanned, and imported into GeneSpring GX 10 (Agilent Technologies) analysis. (B) Gene ontology analysis showed that H3N2 infection only induced OAS2 gene above the 2 fold change cut-off point. H11N9 infection resulted in altered expression of 300 genes, which are largely associated with immune responses and are antiviral-associated genes such as IFN-β, IFN-λ1, IRF, OASL, RIG-I (DDX58) and MX2 genes.

Figure E2. Influenza NS1 expression after transfection in Calu-3 cells. H3N2- and H11N9-NS1 was transfected into Calu-3 cells, which was then stimulated with Poly I:C at 6hr after transfection. The level of NS1 protein expression was measured at 48hr after
Poly I:C stimulation. H3N2 and H11N9 NS1 proteins were expressed to a similar level at 48hr after transfection and levels were maintained after Poly I:C stimulation. Results are representative of experiments performed in three independent experiments, densitometry was performed and the values were expressed as NS1/GAPDH ratio, and presented as fold induction from media treated controls.
Figure 1

A

![Graph showing median fluorescence intensity](image)

B

![Graph showing fold change from medium](image)

C

![Graph showing virus titre](image)
Figure 3

A

B

Fold change from medium

6hr
GAPDH
24hr
GAPDH
48hr
GAPDH

H3N2 H11N9 Poly I:C Med

H3N2 H11N9 Poly I:C Med

p = 0.005
p = 0.025
p = 0.005

p = 0.005
Figure 4

A

B

C

D

PKR

GAPDH

p = 0.011

p < 0.001

p = 0.001

p = 0.055
Figure 5

A

H3N2  H11N9 Poly I:C  Medium

Fold increase over medium

p = 0.002

H3N2  H11N9  Poly I:C

B

Fold change from medium

p = 0.011

H3N2  H11N9
Online Data Supplement

Human influenza is more effective than avian influenza at antiviral suppression in airway cells

Alan Chen-Yu Hsu¹, Ian Barr², Philip M. Hansbro¹, and Peter A. Wark¹,³*

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Figure E2

![Image of a figure with a blot and a bar graph showing fold change from medium for different treatments.]


202. Dauber, B., J. Schneider, and T. Wolff, *Double-stranded RNA binding of influenza B virus nonstructural NS1 protein inhibits protein kinase R but is not


