EFFECT OF ALFERON N ON REPLICATION OF INFLUENZA A VIRUSES IN CELL CULTURES

by

JINGQUN MA

B.A., CHINA AGRICULTURE UNIVERSITY, 2009

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Approved by:

Major Professor
Dr. Juergen A Richt

Abstract

Influenza A virus is an important respiratory pathogen with the potential to affect both humans and animals, thereby creating the conditions for public health disasters, especially during pandemic episodes. At present, two primary strategies to combat influenza are vaccination and antiviral drugs. Since influenza viruses mutate rapidly and constantly via antigenic drift and shift, vaccines can become quickly outdated; and resistance to antiviral drugs can readily result. Interferon alpha (IFN-α) plays an important role as a first line of innate antiviral immunity. To investigate the antiviral potential of exogenously applied IFN-α on the replication of different subtypes of influenza A viruses, three subtypes of influenza A virus, i.e. swine H3N2, pandemic H1N1 and avian H9N2 were chosen. Their replication kinetics in the presence of Alferon N (human Interferon alpha) on human epithelium (A549) cells and swine testis (ST) cells was evaluated. In these tests of the three subtypes of influenza A viruses, it was found that the replication ability of all three viruses was inhibited when ST cells were treated with Alferon for four hours before infection. The ability of Alferon to inhibit influenza A viruses replication was found to be dose-dependent. Similar results were obtained when A549 cells were used; however, pretreatment of A549 cells with Alferon for more than 16 hours was necessary before infection. Furthermore, the expression of some ISGs (Interferon stimulated genes) between ST and A549 cells was also investigated. The differences in response of the ISGs between the two cell lines provided an explanation of the disparity towards exogenous interferon treatment. In summary, these results demonstrated that Alferon N has the ability to inhibit replication of different subtypes of influenza A viruses in cell cultures. This study provides a foundation for future *in vivo* studies using exogenous IFN-α treatment as an alternative approach to combat influenza A virus infection.

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Acknowledgements

I would like to thank my major professor, Dr. Juergen Richt for his support and guidance. I also thank my committee members Dr. Wenjun Ma and Dr. Carol Wyatt for their suggestions and expertise. I am also very grateful to Dr. Robert Kahn who has advised me how to improve the English presentation of these results. Thanks to all of the members in the Richt Lab and other members of the KSU DMP for their help during this journey.

Chapter 1 - **Influenza Virus Literature Review**

1.1 Introduction

Influenza is an infectious disease caused by RNA viruses of the family *orthomyxoviridae*. The symptoms in humans include fever, coughing, sneezing, sore throat, muscle pain and fatigue. This disease affects various species such as humans, pigs, birds, ferrets, dogs, etc. It circulates constantly among susceptible species as seasonal epidemics causing significant economic loss and health burdens. Around 30,000 to 50,000 influenza-related deaths have been estimated to occur each year in the United States. It also causes a pandemic every 10 to 50 years, with a significant number of human deaths (72). Three main influenza pandemics occurred in the 20th century—1918 Spanish flu, 1957 Asian flu, 1968 Hong Kong flu—and one in the 21st century, the 2009 swine flu. There were over 20 million human deaths because of the 1918 Spanish flu alone. Clearly, influenza still poses a significant public health threat.

1.2 *Orthomyxoviridae* Family

The *Orthomyxoviridae* family is a family of RNA viruses that consist of five genera: Influenza A, Influenza B, Influenza C, Isavirus and Thogotovirus. Influenza is caused by influenza A, influenza B and influenza C genera among birds and mammals. Each genus has one type of species which is influenza A virus, influenza B virus and influenza C virus respectively. Influenza A draws the most attention among them because of its ability to cause influenza pandemics. Influenza B viruses can cause the same spectrum of diseases as influenza A viruses and can cause substantial morbidity. Therefore, the trivalent seasonal influenza vaccine contains an influenza B virus component. However, influenza B viruses do not cause pandemics, mainly

because they infect only humans and occasionally seals (57); and reassortment between new strains is less likely to happen. Influenza C viruses are isolated from and infect humans and pigs (24), causing mild upper respiratory illness rather than severe lower respiratory tract complications. There is currently no vaccine for influenza C virus.

1.3 Influenza A Virus

1.3.1 Virus Structure and Genome

The influenza A virus genome consists of 8 negative sense, single strand RNA segments; and each segment encodes one or two proteins. Thus up to 11 or 12 proteins are encoded by the 8 viral genes (49): segment 8 encodes the host antiviral response antagonist non-structural protein (NS1) and the nuclear export protein (NEP, also named NS2); segment 7 encodes the matrix protein M1 and the ion channel M2; segment 4 encodes the receptor-binding protein heamagglutinin (HA); segment 6 encodes the sialic acid destroying enzyme neuraminidase (NA); segment 5 encodes nucleoprotein (NP); and segments 1, 2 and 3 encode components of polymerase complex polymerase basic (PB)2, polymerase basic (PB)1 and polymerase acid (PA) respectively. Besides PB1, segment 2 has been reported to encode N40 whose function is still unknown (80). Moreover, PB1 in some viruses encodes a pro-apoptotic virulence factor named PB1-F2 by using an alternative open reading frame (ORF) in the PB1 segment (12).

Influenza A viruses are pleomorphic viruses with a lipid envelope derived from the plasma membrane of infected cells. These viruses are either spherical with a diameter around 100nm or filamentous with a length of more than 300nm by electron microscopy. There are three proteins located in the envelope: HA, NA, M2 proteins. HA and NA proteins, which are glycoproteins, form spikes which protrude from the surface of the influenza virion in a ratio of

approximately four to one. HA proteins are present as rod-shaped spikes while NA proteins are present as mushroom-shaped spikes, with both proteins anchored in the viral envelope by a short sequence of hydrophobic amino acids (13, 55). HA, and to a lesser extent NA, are highly variable and represent major targets for the host humoral immune response. The small amount of M2 protein integrated in the envelope serves as an ion channel, playing an important role in the virus uncoating process. Beneath the envelope is the matrix protein (M1) which encloses the virion core and supports the morphology of influenza particles. Inside the matrix proteins are nuclear export proteins (NEPs) (also called NS2s) and the ribonucleoproteins (RNPs) complex which represent the virion core. The RNP complex consists of a viral genome RNA in association with nucleoproteins (NP) and the RNA-dependent RNA polymerase complex, represented by PA, PB1 and PB2 proteins. The RNPs are the fundamental units which are responsible for viral replication and transcription (55).

Based on the two glycoproteins on the surface of the virus, namely hemagglutinin (HA) and neuraminidase (NA), Influenza A viruses are divided into different subtypes. Before 2012, 16 different HA and 9 different NA subtypes had been described in various combinations; and a novel HA subtype (H17) has recently been isolated from fruit bats (73). Theoretically, different combinations between HA and NA can occur resulting in a multitude of different subtypes. Reverse genetics enables the generation of all of these subtypes under laboratory conditions. However, the viability of the generated viruses or their virulence remains unknown.

Different subtypes might have different host tropisms; however, H17 has been found in fruit bats which might constitute a potential mammalian host for influenza A viruses (73). All 16 HA and 9 NA have been found among wild waterfowl and seabirds, which are believed to be the natural reservoir for influenza A viruses (IAVs) (56). Some subtypes preferentially circulate in

certain species. For example, H1, H3, N1 and N2 are predominantly isolated from humans and pigs worldwide, while H3N8 and H7N7 circulate preferentially in horses. The host barrier for influenza A viruses is restricted under normal conditions; however, the barrier can be broken due to antigenic drift or antigenic shift. Antigenic drift is the random mutation that happens constantly within individual genes due to the lack of proofreading. Antigenic shift occurs when a virus acquires new HA, and possibly NA segments from a different subtype, resulting in a novel virus strain. Antigenic shift can occur when a host is infected by two or more different subtypes of influenza A viruses. Since the resulting new virus strains encode antigenic proteins (HA and to a less extent NA) to which the host has no pre-existing immunity, pandemic influenza can occur.

1.3.2 Replication

1.3.2.1 Virus Attachement

Virus attachment to the target cells is the first step for infection. Influenza A viruses recognize N-acetylneuraminic (sialic) acid which is ubiquitously expressed on many host cell types and in many species. The surface sialic acid terminal binds through its carbon-2. Based on whether the carbon-2 binds to carbon-3 or carbon-6 galactose, there are two type of surface sialic acid: α-2, 3-linkake and α-2, 6-linkage (54). Different virus strains have different preference to bind either α-2, 3-linkage or α-2, 6-linkage via the HA. Normally, human and swine influenza viruses preferentially bind to α-2, 6-linkage, while avian and equine influenza viruses preferentially bind to α-2, 3-linkage (74). Therefore, the distribution of different types of sialic acids will determine the susceptibility to certain types of influenza A viruses (IAVs). In humans, the α-2, 6-linkaged sialic acid is predominantly expressed in the upper respiratory tract, whereas the α-2, 3-linkage is predominantly expressed in intestinal tissues in poultry (54). To a certain

extent, α-2, 3-linkaged sialic acid can be expressed in the upper respiratory tract in humans which explains why this species can become infected by avian influenza viruses, even though with less efficiency than by human influenza strains. In addition, the concentration of α-2, 3-linkaged sialic acid receptors is higher in the lower respiratory tract (bronchioles and alveoli) which might explain the high pathogenicity of some avian influenza strains for humans. Replication of highly pathogenic avian influenza H5N1 viruses in the lower respiratory tract of humans is most likely the reason for its high virulence. Humans are less susceptible to avian influenza; however, more severe clinical symptoms will result once they become infected.

1.3.2.2 Virus Entry

After attachment and being recognized by the host receptors, influenza viruses enter host cells by receptor mediated endocytosis. During entry, the viruses are encapsulated inside of the endosome and translocated to the places near the nucleus. The acidification in the mature endosome (PH around 5) facilitates the uncoating process of viruses and helps release vRNPs into the cytoplasm (42). This process has two significant steps. First, low pH triggers a conformational change in the HA, exposing a fusion peptide that mediates the fusion of viral envelope with the host membrane, thus opening a pore through which the viral RNPs are released into the cytoplasm. Second, before the membrane fusion happens, the hydrogen ions from the endosome enter the viral particle via the M2 ion channel, creating an acid environment which disrupts M1 Matrix proteins polymerization and interaction between M1 and RNPs, allowing uncoating, i.e., the release of the RNPs from the viral matrix into the cellular cytoplasm (8).

1.3.2.3 Synthesis of Viral RNA

Unlike most other single stranded RNA viruses, the replication and transcription of IAVs take place in the nucleus (8). After release from the virion, RNPs and the viral proteins are imported into the nucleus directed by nuclear localization signals (NLSs) (8). The viral RNA-dependent RNA polymerase uses the negative sense vRNA as a template to synthesize positive sense mRNAs and cRNAs. mRNA serves as a template for viral protein synthesis, while cRNA is transcribed to produce more copies of negative sense, genomic vRNA immediately. The regulation process between the replication and transcription is still not well-known. Influenza viruses have unique ways to add 5'cap and 3' poly (A) tail to the mRNA. Generally, the virus can snatch the host mRNA 5' methylguanosine cap with 10-15 nucleotides downstream by the endonuclease activity of its PB2 and use it as template to transcript viral mRNA (13). For the 3' poly (A) tail, the vRNA encodes five to seven uracil residues approximately 17 bases from its 5' end. This is where the transcription stops and the viral polymerase transcribes the poly U tract into the positive sense as a string of adenosines that form the poly (A) tail. So the mRNA is an incomplete copy with around 17 bases less than the complementary vRNA (13). Once polyadenylated and capped, the viral mRNA is ready to be translocated to the cytoplasm and translated like the host mRNA, while the vRNA segments are exported from the nucleus by the NEP and M1 proteins.

1.3.2.4 ***Synthesis of Viral Proteins and Assembling***

The polymerase subunit proteins PA, PB1 and PB2 are translated using viral mRNA as a template and then transported into the nucleus by the NLS signals they contain after trimerization. A portion of M1 proteins is transported to the nucleus while the remaining stay in the cytoplasm. Also, the NEP proteins are imported into the nucleus through free diffusion due to their small size (8). Both M1 and NEP are needed for the RNPs exportation to form new viral

particles (9). Viral protein synthesis is closely related to the amount of corresponding mRNA which is transcribed in a selective process. Different quantities of vRNAs are preferentially present at different stages of infection (13). The membrane proteins HA, NA, and M2 proteins are synthesized during the late stage of infection (13). Using viral mRNA as template, they are synthesized on membrane bound ribosomes in the endoplasmic reticulum with further maturation in the Golgi apparatus then directed to the cell membranes for subsequent assembly. The remaining viral proteins are synthesized in the cytoplasm in preparation for the virion assembly. Two different models have been proposed for viral assembly. One is random packaging model which predicts that the viral segments will be randomly packaged into virions, the other is specific packaging model which predicts that the viral segments will be selectively packaged into virion by signals on each RNA segments (65).

1.3.2.5 Virus Budding and Release

Influenza virus budding occurs at the cell membrane. The progeny virions bind to sialic acid on the cell membranes and accumulate in the budding sites. This aggregation may reduce viral spreading. This process can be reversed by NA proteins, which function as sialidases by cleaving terminal sialic acid residues from cell-surface glycoproteins and gangliosides, releasing progeny virus from the host cells. The antiviral drug, Oseltamivir (Tamiflu) is a NA inhibitor which blocks the NA activity, preventing the spread of viral particles and inhibiting viral replication.

1.3.3 Pathogenicity and Transmission

Different influenza A virus strains possess different capabilities to infect and transmit among host species. The seasonal influenza A virus transmits very well among humans; however, the infection in most cases is relatively mild. The highly pathogenic H5N1 virus causes

severe pneumonia resulting in systematic infection and multiple organ failure; however, human-to-human transmission is very limited. Meanwhile, the range of severity of disease caused by genetically similar influenza A viruses in humans is extremely wide. The same virus infection will result in different disease severity among individuals. Thus understanding the factors determining the pathogenicity and transmission of a virus is pivotal for understanding influenza A viruses in order to control and prevent future influenza epidemics and pandemics. Substantial amounts of research have been done to investigate these factors, indicating that both virus and host factors contribute to the pathogenicity and transmission of IAVs (22, 49) . The establishment of reverse genetics for influenza viruses provides a significant tool to elucidate the role of each viral protein (22). In addition, animal models (including mice, guinea pigs, ferrets, and pigs) are being used in experiments to study virus and host factors related to pathogenesis and transmission (49).

1.3.3.1 Virus Factors

HA: In order for influenza A viruses to infect cells, their HA has to bind to certain types of sialic acid. As noted previously, human influenza A viruses preferentially bind to α-2, 6- sialic acid and avian influenza A viruses bind to α-2, 3-sialic acid. HA is responsible for targeting cells for infection and determining the organ and host tropism. In humans, the α-2, 6- sialic acid is predominantly expressed by the epithelial cells in the upper respiratory tract (67), which explains why seasonal IAVs transmit so easily between humans but with less severity. The HA of highly pathogenic H5N1 influenza virus binds to α-2, 3 sialic acid which is expressed relatively more in the lower respiratory tract of humans; therefore, it can cause severe pneumonia. However, the poor transmission ability of H5N1 is partially due to the sparse spread of α-2, 3 sialic acid in the upper respiratory tract. Mutations on the HA genes may change the

receptor binding preferences, pathogenicity and transmission ability of influenza A viruses. The D222G mutations in HA pandemic H1N1 show an increased ability to bind to avian-like (α-2, 3) sialic acid and decreased avidity for human-like (α-2, 6) sialic acid (6). These facts remind us to pay close attention to the HA mutations in H5N1 as well as seasonal H1N1 for their pandemic potential.

HA influences pathogenicity in another way by its susceptibility to host proteases. The requirement for a virus infection to occur is that its HA proteins become cleaved to HA1 and HA2 to expose the fusion protein. The HA of seasonal IAV and most low pathogenic influenza viruses possesses a single arginine at the cleavage site that can be cleaved by extracelluler trypsin-like protease that is present at respiratory surfaces or in the intestines of the host (22, 49); however, the HAs of highly pathogenic H5N1 process multiple basic amino acids within the cleavage site can be cleaved by furin and PC6 which are ubiquitously expressed in many organs (13). This might explain why the HPAIV can cause severe systematic infection (29). HA with multiple basic amino acids near a cleavage site can be viewed as one sign of high pathogenicity (13).

The RNP complex: HA is not the only factor to determine influenza virus pathogenicity and transmission ability, since the 1918 Spanish flu didn't have the multibasic amino acids at the cleavage site but still had devastating results. It is believed the viral polymerase complex (PA, PB2, PB1), the fundamental requirement for virus replication and transcription, is also responsible for determining virus pathogenicity. By replacing the genes except for the polymerase complex and NP from the 1918 influenza virus with those from the a seasonal influenza strain, the virulence property showed similarity with the wild type 1918 in both the upper and lower respiratory tracts of ferrets, indicating that the polymerase complex is the major

determinant of the pathogenicity of the 1918 pandemic virus (79). Substituting lethal H5N1 polymerase genes with genes from nonlethal H5N1 reduced the virus virulence and polymerase activity (64). Several mutations in the PA and PB2 genes also changed the virus tropism, adapting the avian strains to infect mammals (10, 41, 69). In addition, the post-translational modification of the polymerase gene complex and NP genes will also alter the virus infectivity (59).

PB1-F2: This 11th protein of influenza A viruses, discovered approximately 10 years ago, is a 90-amino acid protein preferentially located in the mitochondria of infected cells (12). Researchers have shown that the pleiotropic function of PB1-F2 contributes to an enhanced pathogenicity. The PB1-F2 protein induces immune cell apoptosis in response to influenza virus infection. The PB1-F2 protein also works as a virulence factor and increases mortality and pathogenicity and promotes secondary bacterial infection (47, 83). The S66 residue in PB1-F2 is found in both 1918 pandemic virus and HAPI H5N1 and substitution of this residue will result in reduced virulence for both viruses (15). Also, by directly interacting with PB1, PB1-F2 regulates polymerase activity through phosphorylation, resulting in an increased virus replication (46). In some viruses, it has been found that PB1-F2 can work together with NS1 protein to inhibit the host IFN system (77). New data suggest that some functions of PB1-F2 are strain and host specific (36). More research needs to be done to elucidate how PB1-F2 contributes to pathogencity (36).

NS1: The influenza A virus NS1 gene encodes a protein about 26.8 kD, which works as a homodimer during virus infection. NS1 has been shown to be an alpha/beta interferon antagonist both in vitro and in animal systems (68). It enhances viral pathogenicity by counteracting the innate immune system. The N terminus of NS1 binds to and sequesters dsRNA, and thereby

blocks the activation of RIG-1, 2’-5’ OAS, PKR, or other dsRNA-activated proteins during virus infection. The C terminus of NS1 can block the activity of the nuclear proteins PABPII and CPSF, preventing the processing and export of mRNA transcripts. The whole NS1 gene is required to ensure optimal interferon antagonist function *in vitro* and *in vivo.* The ability to counter IFN-alpha/beta *in vitro* was shown to be decreased in NS1 Carboxy-truncated Tx/98 virus compared to wild Tx/98 with the whole NS1 segment (68). The severe lung pathology caused by 1918 influenza was likely due in part to its NS1 gene. The 1918 NS1 protein induced numerous pro-inflammatory chemokine and cytokine genes. The product of many of these genes served to attract and activate infiltration of immune cells with inflammatory functions. Also, the 1918 NS1 down-regulated the expression of IFN-stimulated genes which are the first line of host response against virus infection (7).

1.3.3.2 Host Factors

Host receptors: Influenza A virus replication starts by interaction between virus HA proteins and the host cell receptors. Sialic acid has been identified as a receptor determinant for influenza A viruses. The distribution of this receptor varies between host species and different organs in the same host species, resulting in the different pathogenicity and transmission characteristics of influenza viruses. The use of sialic acid linkage-specific lectins, like *Maackia amurensis* (MAA, specific for α2-3-linkage) and *Sambucus nigra* (SNA, specific for α2-6-linkage), enables the study of sialic acid localization in organs and species. As previously noted, in humans, mammalian like (α2-6-linkages) sialic acid is predominantly expressed in the upper respiratory tract, which supports the binding of seasonal influenza and supports its easy transmission. The avian like (α2-3 linkages) sialic acids are mainly expressed in the human lower respiratory tract (alveoli) which supports infection by avian H5N1 viruses (54). Pig respiratory

tract express a substantial amount of both avian-like and mammalian-like sialic acid in their tracheal epithelial cells (54), making them susceptible for both avian and human influenza viruses co-infection. Pigs are believed to work as a "mixing vessel", where virus reassortment can happen and result in novel influenza virus strains posing a pandemic threat (49).

Host immune system: The host immune system determines the outcome of viral infection by interacting with the influenza viruses to form an acute infection, normally lasting one to two weeks (16), so the innate and humoral immune responses play a major role in combating influenza virus infections, while T cell mediated immunity is responsible for virus clearance (16).

Innate immunity: Innate immunity is the first line of defense against invading pathogens. It recognizes the pathogen associated molecular patterns (PAMP) and endogenous danger signals by pattern recognition receptors (PRR), sending the infected cells and their neighboring cells into an altered state by inducing secretion of interferon to upregulate interferon-stimulated genes (ISGs) to build an anti-viral status and secretion of chemokines and cytokines which attract immune cells (35).

TLR pathway: TLR3 and TLR7 have been reported to recognize influenza virus RNA during virus replication. TLR3 recognizes double stranded RNA while TLR7 recognizes single stranded RNA. Activation of TLR3 and TLR7 results in the activation of IRF3 and IRF7, respectively, along with AP1, and p50/p65. IRF3 and IRF7 will eventually initiate transcription of IFN beta and alpha which have strong antiviral effects by limiting host and viral protein synthesis (35). These IFNs can also induce downstream ISGs' expression and inhibit viral replication. On the other hand, AP1 and p50/65 can induce pro-inflammatory cytokines. TLR

deficient mice show enhanced mortality along with reduced inflammatory reaction by influenza A virus infection (39, 75).

The RLR (RIG-I like receptors) pathway: When an influenza virus infects a cell, the 5'-triphosphate single stranded RNA accumulated during virus replication is recognized by retinoic acid-inducible gene-I (RIG-I). The activation of RIG-I induces the production of type I IFNs and activates an antiviral response (35). Knockout of RIG-I will result in increased mortality (75). IAV can suppress the RIG-I activation using its NS1 protein, thus escaping the innate immunity. RIG-I activation also induces the production of inflammatory proteins which are mediated by NF-kB activation.

IFN signaling: Influenza virus infection results in increased production of type 1 interferon including interferon alpha and beta (IFN- α/β) which plays an important role in innate immunity by infected cells. Upon secretion, type I interferon can act in an autocrine and paracrine fashion by binding to the IFN-α receptor (IFNAR) complex on the infected cells or on its neighboring cells respectively. This process, following signal transduction, leads to upregulation of INF-stimulated genes (ISGs), such as Protein Kinase R (PKR), 2'5'-oligoadenylated synthetase (OAS), and Mx genes, which mediates the biological function of IFN, such as inhibition of viral replication, cellular growth inhibition and apoptosis to build a antiviral state and limit the viral replication and prevent the spread of invading viruses (63).

Humoral response: The humoral response against influenza A viruses plays an important role in protecting a host from infection. Neutralizing antibodies are mainly produced against the viral surface proteins HA, NA, and M2 (35). Among them, the HA-specific antibody is the most potent neutralizing antibody for disease prevention (16). The main antibody isotypes that can be detected during the primary influenza infection are IgA, IgM, IgG (16, 35). Secretory

IgA is secreted in the mucus of respiratory tract, protecting the local cells from being infected with influenza viruses. IgM can be viewed as a sign of primary infection which peaks after two weeks; serum IgG together with IgA are predominant in secondary infection and provide long-live protection against the same virus infection (16, 35, 75). This immunity can last for several years or even up to several decades, as indicated by the remarkable evidence that the neutralizing antibodies against 1918 influenza were found in the survivors 90 years after first exposure (82). The influenza A viruses outsmart the immune responses by their high genetic variability resulting from antigenic drift or antigenic shift. Under the pressure of neutralizing antibody, the virus HA mutates its amino acid residues, resulting in the decreased efficiency or failure of the existing antibody (35).

Cellular immunity: T cell mediated immune response is responsible for virus clearance in infected cells and for limiting virus spread in cooperation with antibodies (16) . Cytotoxic T lymphocytes (CTL) are recruited to infection sites and lyse or induce apoptosis of the infected cells (35). The viral targets for CTL are mainly the internal proteins, such as M1, NP, PA and PB2 proteins (16, 21, 35). CTL responses have been shown to display cross-protection between different subtypes of influenza A viruses (16, 19)

1.3.4 Prevention and treatment

1.3.4.1 Vaccination

Currently, vaccination is still the most effective way to prevent and control influenza among humans and other species. Isolation of influenza virus from embryonated chicken eggs has enabled massive vaccine production since the 1930s. In the U.S., the current licensed human flu vaccine is trivalent inactivated (TIV) and live-attenuated influenza vaccine (LAIV) to combat seasonal and pandemic influenza outbreak (28). Because of the antigenic drift property when the

viruses go through point mutation during viral replication, vaccines for seasonal influenza are modified annually. A pandemic can begin through antigenic shift in which the gene segments reassort between different species, resulting in a dramatic change of the virus. Thus surveillance and prediction has to be done prior to vaccine production in anticipation of the next year's strain of pandemic. Vaccination is suggested for every individual, especially for vulnerable populations which suffer complications from influenza, such as people older than 50 years or health care workers (16). Many systematic reviews and meta-analyses show that vaccination decreases the illness or mortality caused by influenza among different populations (17, 26, 30, 53, 78), indicating the substantial benefits of vaccination. Despite the general benefits of vaccination, it is not perfect. First, constant antigenic drift variants pose a challenge for any vaccine, as the virus goes through antigenic mutation very quickly; and mismatch between the changing virus and the vaccine can impair the vaccine effectiveness (11), resulting in less effective vaccine protection through vaccines or even complete failure of vaccine protection. Second, the time line for producing real-time protection via vaccination for an emerging pandemic is challenging because there is not sufficient time for massive vaccine production and delivery when a pandemic influenza strain emerges quickly.

It is important to vaccine both humans and animals (especially pigs) since pigs play an important role in influenza reassortment. As previously noted, pigs serve as a "mixing vessel" in which novel influenza strains can emerge, raising significant food safety and public health concerns (44). The commercially available influenza vaccine used for swine worldwide is inactivated whole virus whose response to new strain emergence in time is still limited (45).

New technology and research has been applied to improve the efficiency of many vaccines, such as DNA vaccine, subunit vaccine, vectored vaccine and vaccines with improved

adjuvants. An ideal goal for a vaccine is to produce a universal flu vaccine to solve the problems that occur with the current vaccine situation.

1.3.4.2 Antiviral Drugs

In addition to vaccination, antiviral drugs are an important method for the control and prevention of influenza infections. Two classes of licensed influenza antiviral drug agents are available in the United States: adamantanes and neuraminidase inhibitors (61).

1.3.4.2.1 Adamantanes

Adamantanes such as amantadine and rimantadine were the first approved antiviral drugs for influenza treatment in clinics. By interfering with the function of the viral M2 protein, which is an ion channel for hydrogen ions to facilitate the viral RNP complex to dissociate from the matrix protein (M1), adamantanes can impact viral replication at an early stage. The therapeutic benefit of these drugs has been described as reduced duration of fever by one day and the prevention of clinical influenza cases up to 23% (31). Side effects associated with adamantane have been identified such as anxiety, depression, insomnia and gastrointestinal disorders (31, 32). Besides these side effects, another important factor that has limited the use of adamantane has been the prevalence of drug-resistance mutations. A single substitution at one of five codons (L26F, V27A, A30V, A30T, S31N, and G34E) in the transmembrane region of the M2 protein is sufficient to result in drug resistance (18, 37, 81) The prevailing emergence of adamantine resistant strains has been reported worldwide since 2004 (61). Because of these reasons, the clinical use of adamantines is now discouraged.

1.3.4.2.1 Neuraminidase Inhibitors

Neuraminidase inhibitors function by inhibiting the Neuraminidase (NA) enzyme activity of the virus. NA enzyme cleaves the N-acetyl sialic acid moieties and releases the budding influenza virions from the cell, allowing the virus to spread to other sites. In the United States, Zamavir and Oseltamivir (Tamiflu) are approved for both influenza prophylaxis and treatment. The FDA has also approved the use of Peramivir, an intravenous neuraminidase inhibitor for the treatment of patients with severe influenza (4). The antiviral effect of Neuraminidase inhibitors have been demonstrated (2, 20, 28, 70). Nevertheless, there are also issues in the use of Nueraminidase inhibitor. *In vitro* studies have shown that viruses go through mutation in both NA and HA under the pressure of Neuraminidase inhibitors, resulting in drug resistance (1, 48). Thus the extensive use of Neuraminidase inhibitors for the prophylaxis and treatment of pandemic H1N1 can result in the emergence of drug-resistant variants (76). Resistance to Zanamivir is less frequent than resistance to Oseltamivir; however, whether this difference in drug resistance is because of the drug property or just because Zanamivir is simply less used than Oseltamivir still needs to be clarified (61).

1.4 Purpose of the Research

As previously noted, the currently applied methods for IAV prevention and treatment are vaccination and antiviral treatments. However, both methods have their limitations. Vaccines can lose their efficiency to protect the host due to influenza viruses' constant evolutionary adaptation through either antigenic drift or antigenic shift. Furthermore, the emergence of antiviral resistant strains causes the failure of antiviral drugs such as Amantadine and Oseltamivir.

Alferon N contains at least 14 subspecies of alpha interferon molecules and is a natural interferon alpha product derived from human leukocytes. This drug has been approved by the U.S. Food and Drug Administration for the treatment of refractory *condylomata acuminata* (genital warts). The key issue that emerges from this literature review of influenza viruses is to investigate whether Alferon N is able to inhibit influenza A virus replication in cell cultures. Furthermore, it is necessary to explore the efficiency of using exogenous IFN-α treatment *in vivo* as an alternative approach to combat infection with influenza A viruses, especially in patients infected with influenza strains which are resistant to common antiviral treatments.

Chapter 2 - **Materials and Methods**

2.1 Viruses and Reagents

Swine-origin pandemic H1N1 (Alb09; A/swine/Alberta/25/2009), isolated from Canadian pigs, was used in this experiment. The genome of this virus exhibited 99.6%-100% identity at the nucleotide level with human isolated pandemic H1N1 (A/CA/04/09, CA09) (43). Alb09 was amplified in 9-day-old embryonated chicken eggs. Avian H9N2 (A/quail/Hong Kong/G1/97) was a gift by Dr. Peiris (University of Hong Kong) and generated in the Richt lab using a reverse genetic system and amplified on 9-day-old embryonated chicken eggs. Swine H3N2 (A/swine/Tx98/4199-2/98) was amplified in MDCK cells. All viruses were titrated in MDCK cells by detection of Nucleoprotein (NP) protein in the cells after 72 hours infection via immunohistochemistry assay, using influenza NP protein specific monoclonal antibody. The virus stocks had titers of $10^{6.5}$ $TCID_{50}$/ml, $10^{8.5}$ $TCID_{50}$/ml, $10^{7.5}$ $TCID_{50}$/ml in MDCK cells using the method of Reed and Muench for Alb09, H9N2, Tx98 respectively.

Diluting solution: PBS with 0.05% Tween (w/v) (Acros, New Jersey, USA) and 1% BSA (w/v) (Sigma, St. Louis, MO); AEC diluet0.1M acetic acid mix with 0.1M sodium acetate at a ratio of 21:79 (v/v); AEC solution: 3-Amino-9-ethyl-Carbazole (AEC; sigma, St. Louis, MO) dissolved with dimethylformamide at a ratio of 4:1 (w/v). AEC substrate: AEC diluent mixed with AEC solution at a ratio of 19:1.

2.2 Cells and Alferon N

Swine Testis (ST) and Madin-Darby canine kidney (MDCK) cells were cultured in minimum essential medium (MEM; Mediatech, Manassas, VA) with 5% fetal bovine serum (FBS; HyClone, Logan, UT), 1% L-glutamine (Invitrogen, Grand Island, NY), 1% MEM

vitamins (Invitrogen, Grand Island, NY) and 1% antibiotics (Invitrogen, Grand Island, NY). For ST and MDCK cells, these virus infections were performed in an infection medium containing MEM, 0.3% bovine serum albumin (BSA; Sigma, St Louis, MO), L-glutamine (Invitrogen, Grand Island, NY), MEM vitamins (Invitrogen, Grand Island, NY), and 1% antibiotics (Invitrogen, Grand Island, NY).

Adenocarcinomic human alveolar basal epithelial cells (A549) were cultured in Dulbecco's modified eagle's media (DMEM; Mediatech, Manassas, VA) with 10% fetal bovine serum (FBS; HyClone, Logan, UT) 1% L-glutamine (Invitrogen, Grand Island, NY), 1% MEM vitamins (Invitrogen, Grand Island, NY) and 1% antibiotics (Invitrogen, Grand Island, NY). Virus infections using A549 cells were performed in an infection medium containing Opti-MEM (Invitrogen, Grand Island, NY), 0.2% bovine serum albumin (BSA; Sigma, St Louis, MO).

The Alferon N injection (Hemispherx, New Brunswick, NJ) was provided by Hemispherx Biopharma, INC.. It is a natural interferon alpha product derived from human leukocytes which contains at least 14 subspecies of alpha interferon molecules that has been approved by the U.S. Food and Drug Administration in the treatment of refractory *condylomata acuminata* (genital warts). Alferon N was diluted with infection media respectively for ST and A549 cells treatment before use.

2.3 Alferon N treatment

Confluent ST and A549 cells were grown in 48-well plates. Immediately before Alferon N treatment, the cells were washed with warm MEM (Mediatech, Manassas, VA) or opti-MEM (Invitrogen, Grand Island, NY) and subsequently treated with 10000 U/well, 1000 U/well, 100 U/well, 10 U/well of Alferon N (Hemispherx, New Brunswick, NJ) in 250 μl MEM containing 0.3% bovine serum albumin (BSA; Sigma, St Louis, MO), L-glutamine (Invitrogen, Grand

Island, NY), MEM vitamins (Invitrogen, Grand Island, NY), and 1% antibiotics for ST cells and in Opti-MEM (Invitrogen, Grand Island, NY), 0.2% bovine serum albumin (BSA; Sigma, St Louis, MO) for A549 cells. For real-time RT-PCR, 4h, 8h, 16h, 24h treated cells were digested with Typsin-Versene mixture (Lonza, Walkersville, MD) until cells detached from the plates. The resulting cell suspension was centrifuged at 1200rpm for 5 minutes and cell pellets were collected and stored in -80°C for later use. For the cell viability test, four hours of Alferon N treated ST cells and 16 hours of Alferon N treated A549 cells were collected every 12 hours over 48 hours period. Cells were detached from the plates by incubating with typsin-Versene mixture. Collected cell suspension was mixed very well by pipetting up and down for several times and was stained with 0.4% trypan blue (Sigma, St Louis, MO); and live cells versus damaged cells were counted under a microscope using hemacytometer (Fisher, Pittsburgh, PA). For infection, cells were infected with A/swine/Alberta/25/2009, A/quail/Hong Kong/G1/97, A/swine/Tx98/4199-2/98 after incubated at 37°C, 5% CO_2 for 4h, 8h, 16h, 24h with Alferon N. The cells were infected at a multiplicity of infection (MOI) of 0.01 and the final volume was 500µl/well. Viruses were diluted in fresh infection medium supplemented with 1µg/ml TPCK [l-(tosylamido-2-phenyl) ethyl chloromethyl ketone] trypsin (Worthington Biochemical, Lakewood, NJ). Supernatants were collected at 12, 24h, 36h, 40h, 48h post infection and stored at -20°C.

2.4 Titrations

Quadruplicate titration of each supernatant sample was performed in 96-well plates in tenfold serial dilutions from 10^{-1} to 10^{-8}. The dilutions were transferred to confluent monolayers of MDCK cells in 96-well plates which were washed once using blank MEM (Mediatech, Manassas, VA) or Opti-MEM (Invitrogen, Grand Island, NY) before titration. The cells were

then incubated at 37°C, 5% CO_2 for 3 days; and the titer of virus in the supernatants was evaluated by detecting the cytopathic effect (CPE) induced by the virus in the cell culture every day. At 72 hours post-infection, cells were fixed with methanol at room temperature for ten minutes and immunohistochemistry was performed to detect viral NP protein in the infected cells. First, mouse anti-nucleoprotein (NP) serum (ATCC#HB-65) diluted by diluting solution (see above "viruses and reagents") at a ratio of 1:500 was added to the cells and incubated for 30 minutes. Second, after washing plates with washing medium (PBS, 0.05% Tween 20 (Acros, New Jersey, USA)), cells were incubated with Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP (sigma, St Louis, MO) which was diluted by diluting solution at a ratio of 1:300 and incubated for 30 minutes. Finally, after washing the plates 3 times with the washing medium, cells were incubated with AEC dilution (see above "viruses and reagents") containing 1% Hydrogen Peroxide (sigma, St. Louis, MO) for 45 minutes before stopping the reaction with distilled water. Positive signals were detected as red color under the microscope. The 50% tissue culture infective dose ($TCID_{50}$)/ml was calculated by the Reed and Muench method (38). The detection limit for the virus load was 10 $TCID_{50}$/ml.

2.5 RNA extraction

RNA was extracted using RNeasy® Mini kit (Qiagen, Maryland, USA). Briefly, cell pellets were thawed thoroughly and disrupted by adding Buffer RLT, then homogenized with 1 volume of 70% ethanol. Samples were centrifuged through RNeasy spin column for 15sec at 13,000rpm. The RNA was eluted by adding RNase-free water directly to the spin column membrane after washing the membrane with RW1 (reagent in kit) once and RPE (reagent in kit) twice. RNA concentration was detected using NanoDrop 1000 spectrophotometer (Thermo Scientific) and diluted to 100ng/µl using RNase-free water.

2.6 Quantitative RT-PCR

Quantitative RT-PCR was established to detect the Interferon Stimulated Genes (ISG)-Mx, 2'5'OAS1. The following primers used were: Mx Forward: 5'-CGCATCTCCAGCCACATCC-3'; Mx Reverse: 5'AGCCGCTCCTTCAGGAACTT-3'. 2'5' OAS1 Forward: 5'-ACCAAGCTGAAGAGCCTCATCC-3'; 2'5' OAS1 Reward:5'-GCTCCCATGCATAGACTGTCAG-3'and control housekeeping gene (β-actin) Forward: 5'-CTCGATCATGAAGTGCGACGT-3', β-actin reverse: 5'-GTGATCTCCTTCTGCATCCTGTC-3'. The primers were designed to detect conserved regions within Mx, OAS1 and β-actin of *sus scrofa* and *homo sapiens*. Primers (IDT, San Jose, CA) were diluted in distilled water to 25pmol for final use. Quantitative RT-PCR was performed using QuantiFast SYBR Green RT-PCR Kit (Qiagen, Maryland, USA) on the SmartCycler (Cepheid, Sunnyvale, CA) in a total volume of 25 µl containing 1 µl of each primers, 0.25 µl QuantiFast RT Mix, 12.5 µl 2X QuantiFast® SYBR® Green RT-PCR Master Mix, 1 µl template RNA, 9.25 µl RNase-free water. The cycling program consisted of a reverse transcription step at 50°C for 10min, PCR initial activation step at 95°C for 5min, followed by 40 cycles of 95°C for 10sec, 60°C for 30sec. Real time RT-PCRs for housekeeping gene (β-actin) were run in parallel with target genes. Gene mRNA level (ΔCt) was normalized by using Ct value of target genes subtracting the Ct value of β-actin. The ΔCt value of Alferon N non-treated sample was than subtracted from the Alferon N treated sample to obtain ΔΔCt. The fold change of target genes in response to Alferon N treatment was calculated by $2^{-\Delta\Delta Ct}$.

2.7 Statistical Analysis

Statistics are expressed as the mean and standard error for each mean. Results are represented as the mean of three independent experiments under each condition. A two-tail t-test was used to determine the differences between means, and a significant difference was considered as $P<0.05$.

Chapter 3 - **Results**

3.1 ST and A549 cells are susceptible to influenza virus infections

In order to study the susceptibility of ST cells and A549 cells to influenza A virus infection and replication, both cell types were infected with swine-origin pH1N1 (A/swine/ Alberta/25/2009), Avian H9N2 (A/quail/Hong Kong/G1/97) or Swine H3N2 (A/swine/Tx98/ 4199-2/98) respectively at a MOI of 0.01. Supernatants were collected 48 hours post infection and titrated in MDCK cells. All three strains of influenza A viruses replicated in ST cells at 48h pi; as shown in Figure 3-1, Pandemic H1N1 replicated to 5.7 ± 0.15 log $TCID_{50}$/ml; Swine H3N2 replicated to 5.8 ± 0.25 log $TCID_{50}$/ml; Avian H9N2 replicated to 6.33 ± 0.17 log $TCID_{50}$/ml. As shown in Figure 3-2 below, in A549 cells both H9N2 and H3N2 reached to titers around 7 log$TCID_{50}$/ml (Swine H3N2 replicated to 6.8 ± 0.11 log $TCID_{50}$/ml; Avian H9N2 replicated to 7.5 ± 0.29 log $TCID_{50}$/ml). Pandemic H1N1 also infected A549, albeit with a lower virus titer of 5.18 ± 0.18 log $TCID_{50}$/ml. These data show that ST cells and A549 cells are susceptible to infection and replication of the influenza A viruses used in this experiment.

Figure 3-1 ST cells are susceptible to influenza A viruses infection

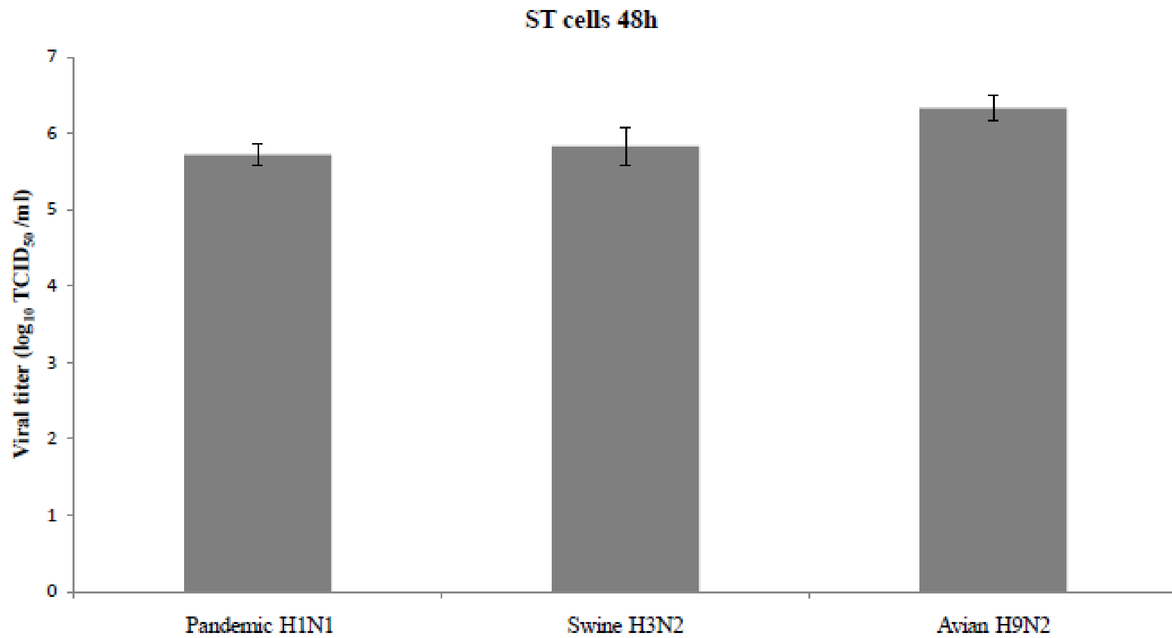

Fig 3-1. ST cells were infected with Pandemic H1N1, Swine H3N2 and Avian H9N2 at a MOI of 0.01. After 48h, progeny virus titer in the supernatant was determined by titration on MDCK cells. Each data indicates the means from three independent experiments, and error bars indicate the standard errors of the means.

Figure 3-2 A549 cells are susceptible to influenza A viruses infection

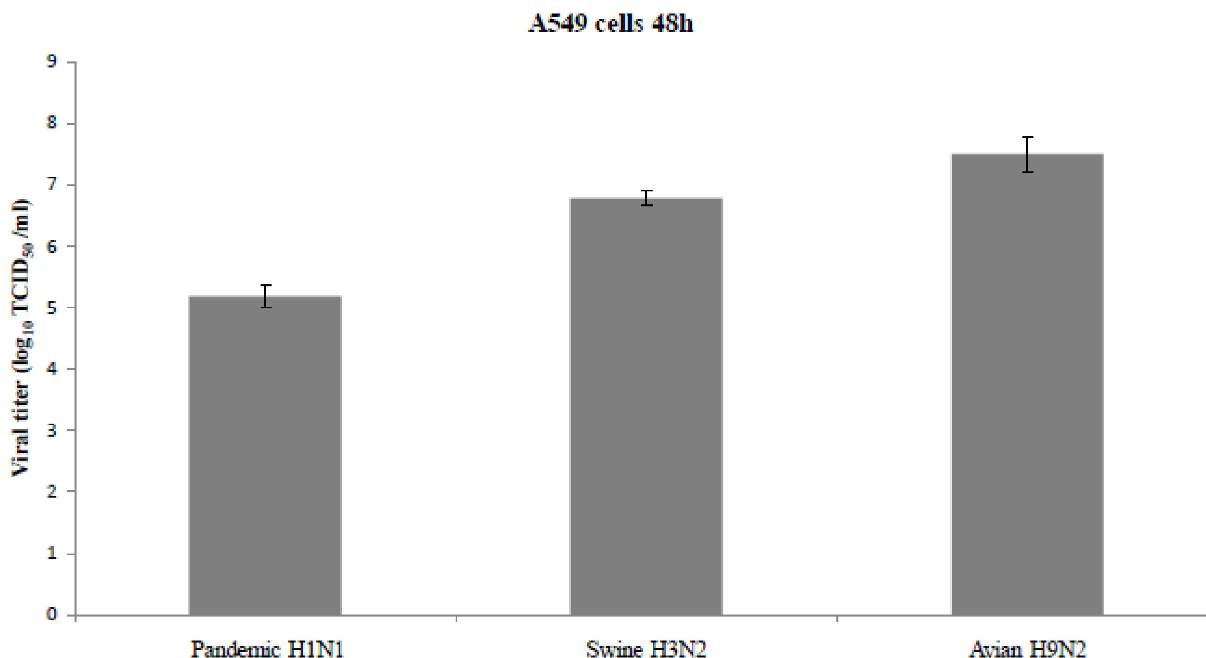

Fig 3-2. A549 cells were infected with Pandemic H1N1, Swine H3N2 and Avian H9N2 at a MOI of 0.01. After 48h, progeny virus titer in the supernatant was determined by titration on MDCK cells. Each data indicates the means from three independent experiments, and error bars indicate the standard errors of the means.

3.2 Pretreatment with Alferon N Inhibits Influenza A Virus Replication in ST cells

3.2.1 Alferon N Inhibits Pandemic H1N1

To examine the antiviral effect of Alferon N against Pandemic H1N1 infection and replication, confluent ST cells in 48-well plates were treated with 10000 IU/well, 1000 IU/well and 500 IU/well of Alferon N for four hours prior to infection with pH1N1 (A/swine/Alberta/25/2009) at a MOI of 0.01. Viral titer was determined 48 hours after infection. Reduced viral titer was observed in all Alferon treated samples compared to mock treated samples. In mock-treated

ST cells, the virus replicated to $10^{6.5}$ $TCID_{50}$/ml at 48h pi, whereas viral titer was $10^{3.33}$ $TCID_{50}$/ml in 1000 IU/well Alferon N treated samples or even lower in the other Alferon N-treated cells, as set out in Figure 3-3. The results showed that Alferon N inhibited pH1N1 replication in ST cells.

Figure 3-3 Alferon N inhibits pH1N1 replication in ST cells

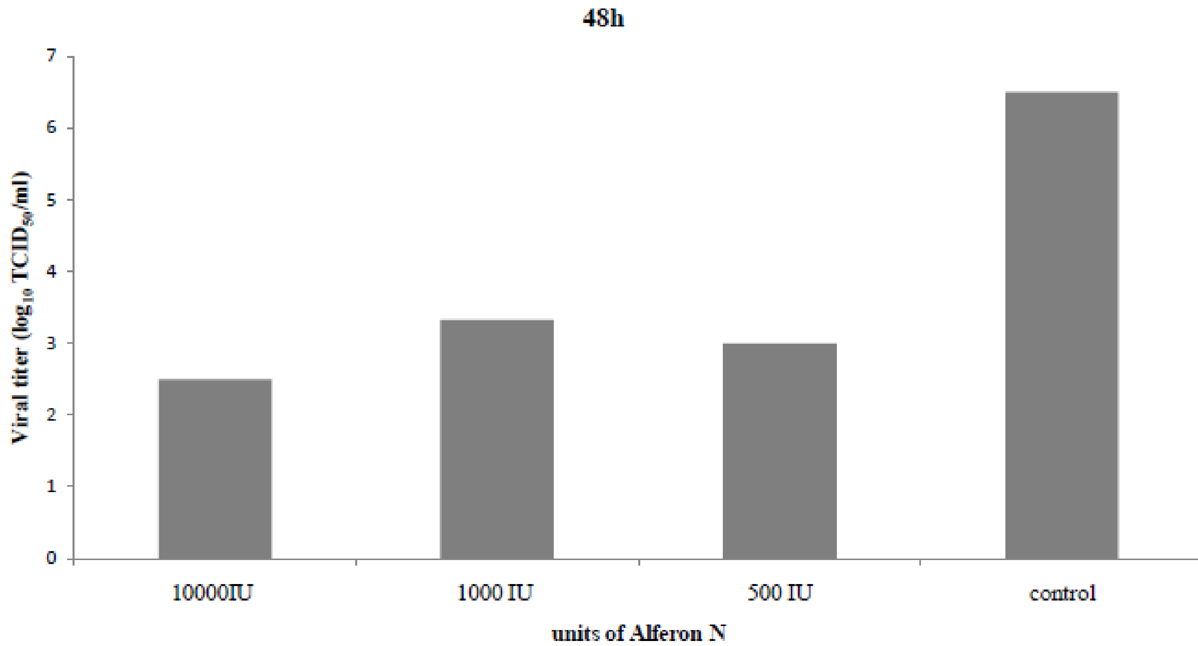

Fig 3-3. ST cells were treated with 10000 IU, 1000 IU, 500 IU of Alferon N for 4h before Pandemic H1N1 inoculation. After 48h, progeny virus titers in the supernatant was determined by titration on MDCK cells.

To examine the antiviral effect of Alferon N against Pandemic H1N1 infection and its replication, confluent ST cells in 48-well plates were treated with 10000 IU/well, 1000 IU/well, 100 IU/well, 10 IU/well of Alferon N for four hours prior to infection with pH1N1

(A/swine/Alberta/25/2009) at a MOI of 0.01. Virus replication kinetics were measured over 48 hours. As shown in Figure 3-4, at 12h pi viruses with similar titers were detected from the mock-treated and 10 IU Alferon N treated samples, while no viruses were detected from samples treated with 100 IU or higher doses of Alferon N. At 24h pi, there was approximately 1 log $TCID_{50}$/ml of virus detected in all Alferon N treated samples which was significantly reduced compared to in mock treated cell cultures which was 4.44 log $TCID_{50}$/ml ($P<0.05$). At 36h pi, there was more than 1.5 log $TCID_{50}$ difference in virus titer between the Alferon N treated and mock treated groups, while virus replication was significantly reduced in all Alferon N treated samples compared to mock treated samples ($P<0.05$). Virus titers reached a similar titer in 10 IU of Alferon N-treated samples as the mock treated samples at 48h pi; virus titer of samples treated with higher units of Alferon N was significantly reduced ($P<0.05$) up to more than 2.5 log $TCID_{50}$ than that of mock treated samples. These results indicated that Alferon N is able to inhibit replication of the pH1N1 virus in ST cells and that 100 units of Alferon N is the minimal amount for efficient inhibition of pH1N1 virus replication.

Figure 3-4 Dose dependent inhibition effect of Alferon N on the replication of Pandemic H1N1 in ST cells

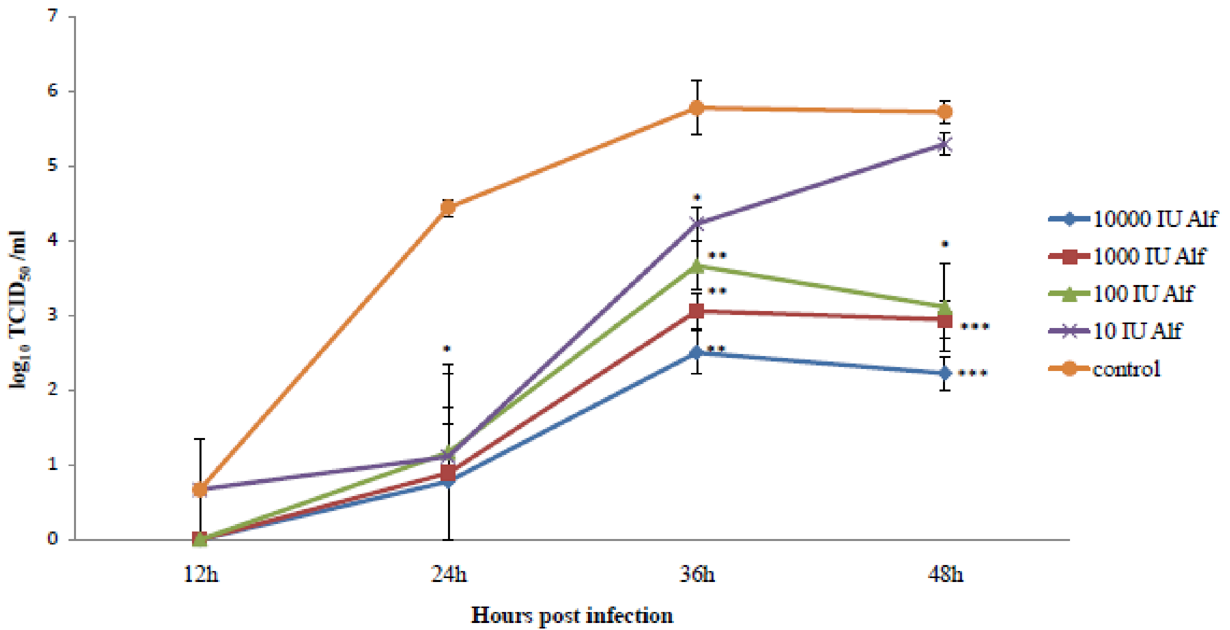

Fig 3-4. ST cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 4h followed by infection with Pandemic H1N1 virus at a MOI of 0.01. Supernatants were collected every 12 hours over 48h period and titrated on MDCK cells. Each data point on the curve indicates the means from three different independent experiments, and error bars indicate the standard errors of the means(*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.2.2 Alferon N Inhibits Avian H9N2

Avian H9N2 was also used to determine the antiviral effect of Alferon N on avian influenza virus infection in ST cells. Confluent ST cells in 48-well plates were treated with 10000 IU, 1000 IU, 100 IU, 10 IU of Alferon N before infection with Avian H9N2 (A/quail/Hong Kong/G1/97) at a MOI of 0.01. Virus growth kinetics were measured over 48 hours. As show in Figure 3-5, reductions in virus titers were observed in Alferon N treated samples between 12h pi and 48h pi. At 12h pi, more than 1 log $TCID_{50}$ reduction in virus titer was observed in 100 IU or a higher dose of Alferon treated samples compared to mock treated samples, with no virus was detectable in 10000 IU treated samples. The virus titer in 10 IU of

Alferon N treated samples was similar to mock treated samples ($P<0.05$). At 24h pi, virus replicated to $10^{5.4}$ $TCID_{50}$/ml in mock treated cells, but only to $10^{4.3}$ $TCID_{50}$/ml in 10 IU Alferon N treated cells. 100 IU of Alferon N treatment led to a reduction of 1.9 log $TCID_{50}$ compared to controls ($P <0.05$); 1000 IU and 10000 IU of Alferon N treatment resulted in a virus titer reduction of up to 3.7 log $TCID_{50}$ at this time point ($P <0.05$). A more obvious inhibition effect of Alferon N was found at 36h pi, as described below. At this time point, virus replicated to 10^7 $TCID_{50}$/ml in mock treated cells; and a significant reduction of virus titers was found in all Alferon N treated cells ($P<0.05$) compared to controls. 10 IU of Alferon N treatment resulted in a virus titer reduction of 1.5 log $TCID_{50}$ ($P <0.05$), while 100 IU and 1000 IU of Alferon N treatment resulted in a reduction of 3 and 5.5 log $TCID_{50}$, respectively ($P <0.05$). Meanwhile, no virus was detectable in the supernatant of 10000 IU treated samples. Virus replicated to a similar titer in 10 IU of Alferon N treated samples as in the mock treated samples at 48h pi; and the virus titer of samples treated with higher units of Alferon N was significantly reduced up to more than 3 log $TCID_{50}$ compared to that of mock treated samples ($P<0.05$). Thus enhanced inhibition was observed when a higher dose of Alferon N was applied, with 10000 IU of Alferon N treatment leading to undetectable virus in the supernatants. These results demonstrate that Alferon N is able to inhibit replication of avian H9N2 virus in ST cells in a dose dependent manner with 100 units of Alferon N the cutline for efficient inhibition of avian H9N2 virus replication.

Figure 3-5 Dose dependent inhibition effect of Alferon N on the replication of Avian H9N2 in ST cells

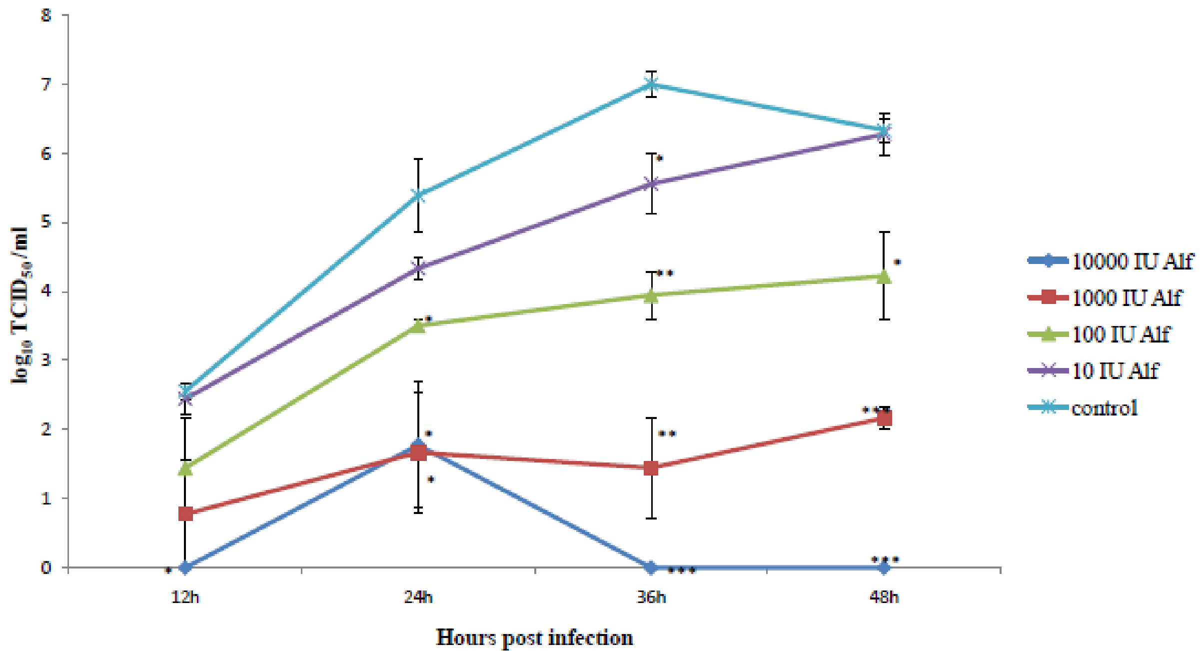

Fig 3-5. ST cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 4h followed by infection with Avian H9N2 virus at a MOI of 0.01. Supernatants were collected every 12 hours over 48h period and titrated on MDCK cells. Each data point on the curve indicates the means from three different independent experiments, and error bars indicate the standard errors of the means(*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.2.3 Alferon N Inhibits Swine H3N2

To further explore the broad spectrum of antiviral potential of Alferon N against IAV infection, swine H3N2 virus was then used in this experiment. Confluent ST cells in 48-well plates were treated with 10000 IU, 1000 IU, 100 IU, 10 IU of Alferon N before infection with swine H3N2 (A/swine/Tx98/4199-2/98) at a MOI of 0.01. Virus growth was measured over 48 hours. As shown in Figure 3-6, at 12h pi, no reduction of virus titers was found in 10 and 100 IU of Alferon N treated samples compared to mock treated ones. Furthermore, viruses were barely detectable in the supernatant of 1000 IU or 10000 IU treated cells. At 24h pi, the virus replicated to $10^{5.5}$ $TCID_{50}$/ml in mock treated cells, while 10 IU of Alferon N treatment reduced the virus

titer to $10^{4.0}$ $TCID_{50}$/ml ($P<0.05$). Higher doses of Alferon N treatment resulted in virus titer reduction of up to 3.9 log $TCID_{50}$, significantly inhibiting the virus growth ($P<0.05$) compared to mock treated cells. 10 IU of Alferon N treatment did not reduce the virus titer at 36 and 48h pi, while 100 IU of Alferon N treatment led to approximately 1 log unit of viral titer reduction compared to controls at these time points, respectively. 1000 IU or 10000 IU of Alferon N treatment resulted in viral titer reduction of up to 3 log $TCID_{50}$ at 36h pi ($P<0.05$). There was a 3 log $TCID_{50}$ reduction in 1000IU treated samples and a 5 log $TCID_{50}$ reduction in 10000IU treated samples at 48h pi ($P<0.05$). These results suggest that Alferon N can inhibit replication of swine H3N2 virus in ST cells. The inhibition effect is dose dependent with 100 units of Alferon N the minimum dose for efficient inhibition of the swine H3N2 virus replication.

Figure 3-6 Dose dependent inhibition effect of Alferon N on the replication of Swine H3N2 in ST cells

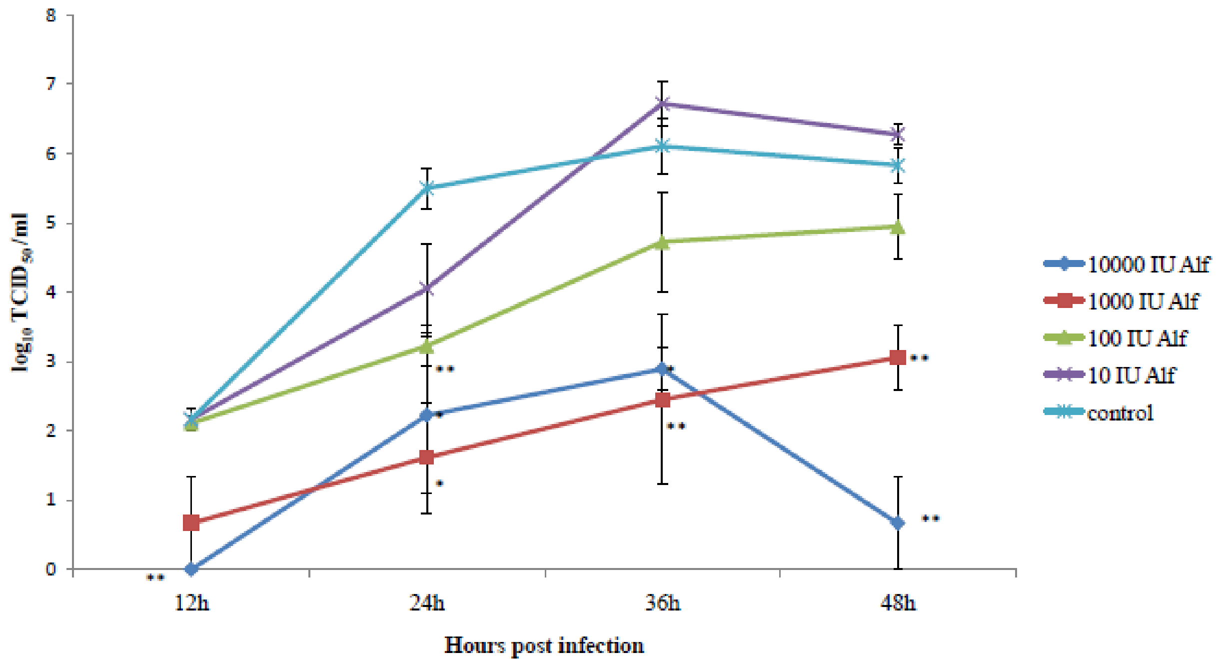

Fig 3-6. ST cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 4h followed by infection with Swine H3N2 virus at a MOI of 0.01. Supernatants were collected every 12 hours over 48h period and titrated on MDCK cells. Each data point on the curve indicates the means from three different independent experiments, and error bars indicate the standard errors of the means(*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.3 Treatment of Alferon N for 4h Inhibits IAVs Replication in A549 cells

In addition to ST cells as an *in vitro* study model, A549 (human lung adenocarcinoma epithelial) cells were also used to determine the antiviral potential of Alferon N against IAVs infection and replication. Confluent A549 cells in 48-well plates were treated with 10000 IU, 1000 IU, 100 IU, 10 IU of Alferon N before infection with pandemic H1N1, avian H9N2, swine

H3N2 at a MOI of 0.01. Supernatants were collected 36 and 48 hours post-infection; and virus titers were determined by titration on MDCK cells.

3.3.1 Alferon N Inhibits Pandemic H1N1

As shown in Figure 3-7A, pandemic H1N1 replicated to $10^{4.7}$ $TCID_{50}$/ml in mock treated A549 cells. 10 IU of Alferon N treatment led to a virus titer reduction of 0.6 log $TCID_{50}$, while higher doses of Alferon N treatment (100 IU, 1000 IU and 10000 IU) resulted in approximately 1 log $TCID_{50}$ virus titer reduction compared to mock treatment. At 48h pi (Figure 3-7B), there was approximately 1 log $TCID_{50}$ difference between 10 IU, 100 IU Alferon treated samples and mock treated samples. Virus replicated to a titer of 3.5 log $TCID_{50}$/ml in 1000 IU and 10000 IU Alferon N treated cells, which was 2 log $TCID_{50}$ lower than in mock treated cells ($P<0.05$). These results demonstrated that Alferon N can inhibit the replication of pandemic H1N1 in A549 cells, albeit to a lesser extent than in ST cells.

Figure 3-7 Effect of Four hours of Alferon N treatment of A549 cells on the replication of Pandemic H1N1

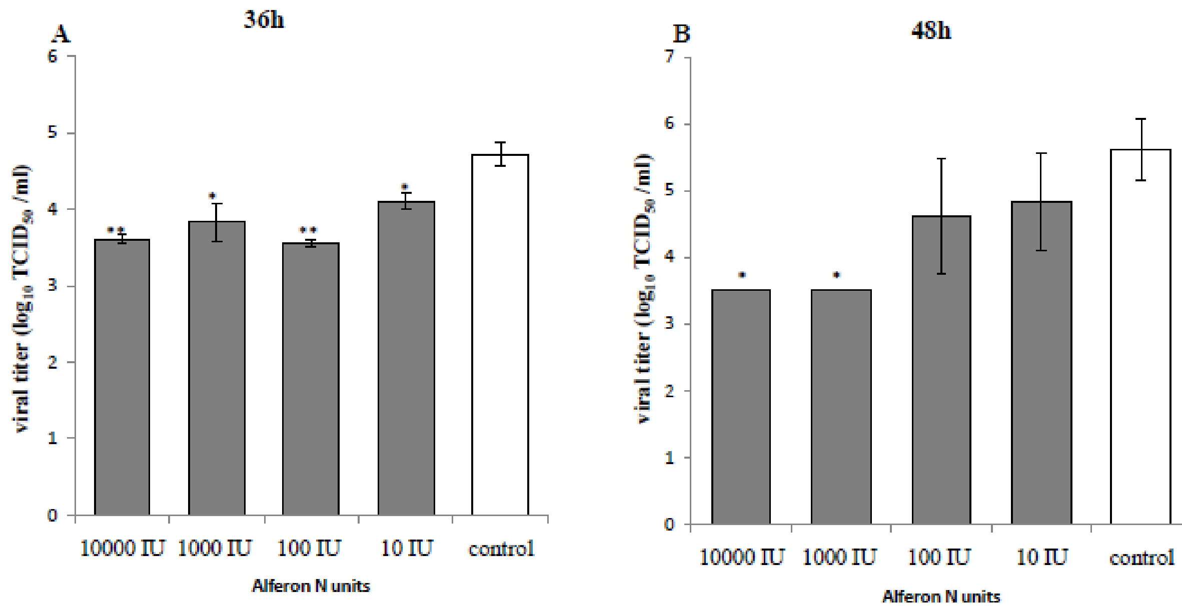

Fig 3-7. A549 cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 4h followed by infection with Pandemic H1N1 virus at a MOI of 0.01. Supernatants were collected 36h pi (A) and 48h pi (B) and titrated on MDCK cells. Each data point indicates the means from three different independent experiments, and error bars indicate the standard errors of the means(*, $P < 0.05$; **, $P < 0.01$).

3.3.2 Alferon N Inhibits Avian H9N2

As shown in Figure 3-8A, at 36h pi, avian H9N2 replicated to 6.4 log $TCID_{50}$/ml in mock treated A549 cells and virus titer reduction was observed in Alferon N pretreatment cells. 10 and 100 IU of Alferon N pretreatment resulted in 0.8 and 1.4 log $TCID_{50}$ reductions compared to mock treatment, respectively, while 1000 IU of Alferon N treatment led to 2 log $TCID_{50}$ reduction compared to mock treatment. Virus replicated to the lowest titer ($10^{3.3}$$TCID_{50}$/ml), which is 3.2 log lower than mock treated samples at 36h pi ($P<0.05$). At 48h pi (Figure 3-8B), maximum inhibition effect was observed in 10000 IU of Alferon N treated samples, virus

replicated to1.6 log $TCID_{50}$ lower than in mock treated samples. There was approximately 1.3 log $TCID_{50}$ reduction between 1000 IU Alferon N treated samples and mock treated ones, whereas only 0.6 log $TCID_{50}$ reduction was observed between 100 IU or 10 IU Alferon N treated and mock treated samples. This data suggested that Alferon N can inhibit the replication of pandemic H1N1 in A549 cells, albeit to a lesser extent than in ST cells. Interestingly better inhibition was achieved at 36h pi compared with 48h pi.

Figure 3-8 Effect of four hours of Alferon N treatment of A549 cells on the replication of Avian H9N2

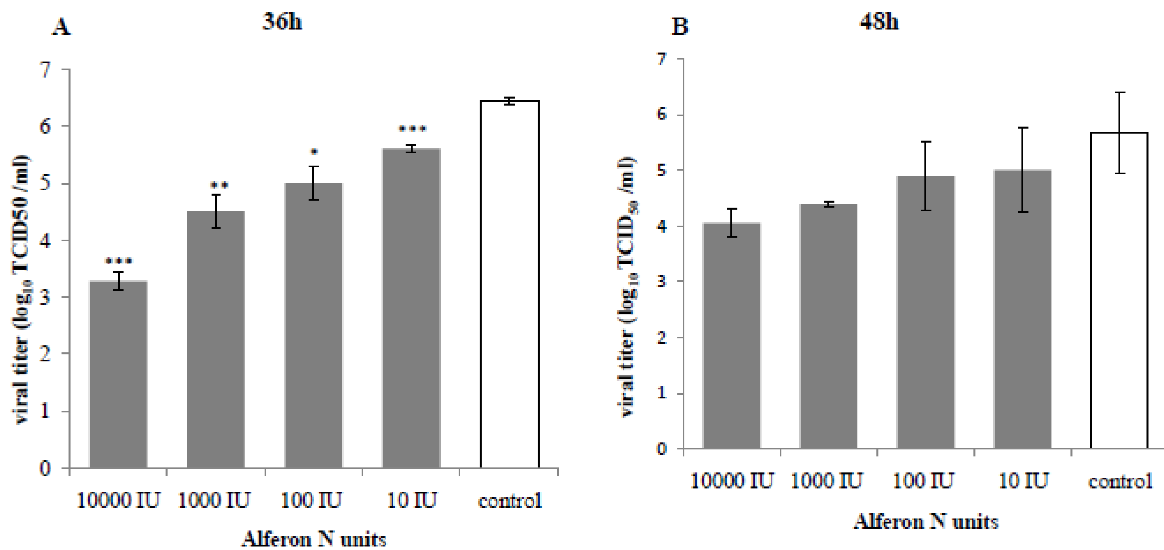

Fig 3-8. A549 cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 4h followed by infection with Avian H9N2 virus at a MOI of 0.01. Supernatants were collected 36h pi (A) and 48h pi (B) and titrated on MDCK cells. Each data point indicates the means from three different independent experiments, and error bars indicate the standard errors of the means (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.3.3 Alferon N Inhibits Swine H3N2

As shown in Figure 3-9A, at 36h pi, virus replicated to the similar level with control in 10 IU and 100 IU of Alferon N treated samples. There was a 0.6 log $TCID_{50}$ difference between1000 IU of Alferon N treated samples and mock treated samples. A significant inhibition was observed in 10000 IU treated samples ($P<0.05$), where the virus titer was 1.5 log $TCID_{50}$ lower than in mock treated samples. At 48h pi (Figure 3-9B), 10000 IU of Alferon N treatment led to 1 log $TCID_{50}$ reduction of virus titer compared to mock treatment ($P>0.05$). There were no obvious differences between the other doses of Alferon N treatment and mock treatment. These results showed that a much higher dose is required to reach inhibition against swine H3N2 in A549 cells than in ST cells for 4 hours of Alferon N pretreatment.

Figure 3-9 Effect of Four hours of Alferon N treatment of A549 cells on the replication of Swine H3N2

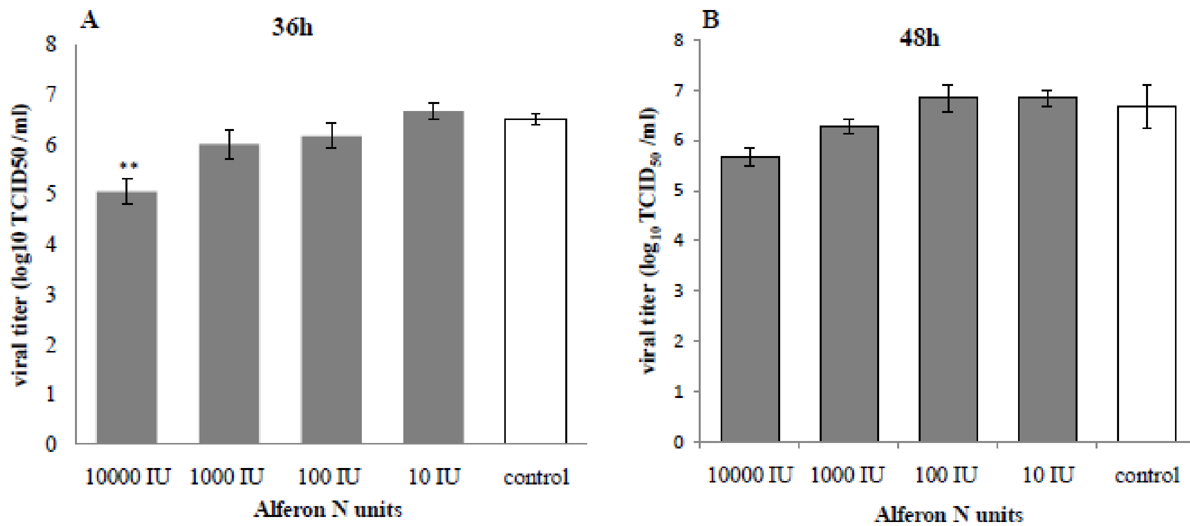

Fig 3-9. A549 cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 4h followed by infection with Swine H3N2 virus at a MOI of 0.01. Supernatants were collected 36h pi (A) and 48h pi (B) and titrated on MDCK cells. Each data point indicates the means from three different independent experiments, and error bars indicate the standard errors of the means (**, $P < 0.01$).

3.4 Longer Time of Alferon N Pretreatment Enhances the Antiviral Potential in A549 Cells

As can be seen in chapter 3.3, the inhibition of 4 hour Alferon N of A549 cells pretreatment is not as obvious as was observed in ST cells. Therefore, it was appropriate to consider whether a longer time of Alferon pretreatment could enhance the antiviral effect. In order to answer this question, confluent A549 cells in 48-well plates were treated with 1000 IU of Alferon N before infection for 4, 8, 16 and 24 hours. Cells were then infected with pandemic H1N1, avian H9N2 or swine H3N2 at a MOI of 0.01. Supernatants were collected 40h pi and virus titers were determined by titration in MDCK cells. As shown in Figure 3-10, pandemic H1N1 in mock treated cells replicated to $10^{4.3}$ $TCID_{50}$/ml at 40h pi, while 4 hour Alferon

pretreatment resulted in around 1.4 log $TCID_{50}$ compared to mock treatment ($P<0.05$). Enhanced viral titer reduction was observed in longer Alferon N pretreated samples. The virus titers in longer Alferon N (more than 8 hours) treated samples were more than 2.5 log $TCID_{50}$ lower than in controls ($P<0.05$).

Avian H9N2 replicated to $10^{5.8}$ $TCID_{50}$/ml in mock treated samples at 40h pi. Virus replication was significantly inhibited in Alferon N treated samples ($P<0.5$) compared to mock treated samples. An enhanced inhibition effect was observed if a longer time of Alferon N pretreatment was applied. The strongest inhibition was reached in 16 hours of Alferon N treated samples with up to a 5 log $TCID_{50}$ reduction of virus titer compared to mock treated samples.

Swine H3N2 replicated to 10^{6} $TCID_{50}$/ml in mock treated cells at 40h pi. Virus titer was reduced to $10^{5.3}$ $TCID_{50}$/ml in 4h Alferon N ($P<0.05$). There was a even more significant difference of virus titer between eight or more hours Alferon N pretreated samples and mock treated sample ($P<0.05$), while 8 and 16 hours of Alferon N pretreatment led to up to 2.2 and 2.4 log $TCID_{50}$ reduction respectively compared to mock treatment. The highest viral titer reduction was achieved in 24 hours with Alferon N pretreated samples which were up to 3.2 log $TCID_{50}$ reduction.

Figure 3-10 Time effect of Alferon N treatment on the inhibition result achieved in A549 cells

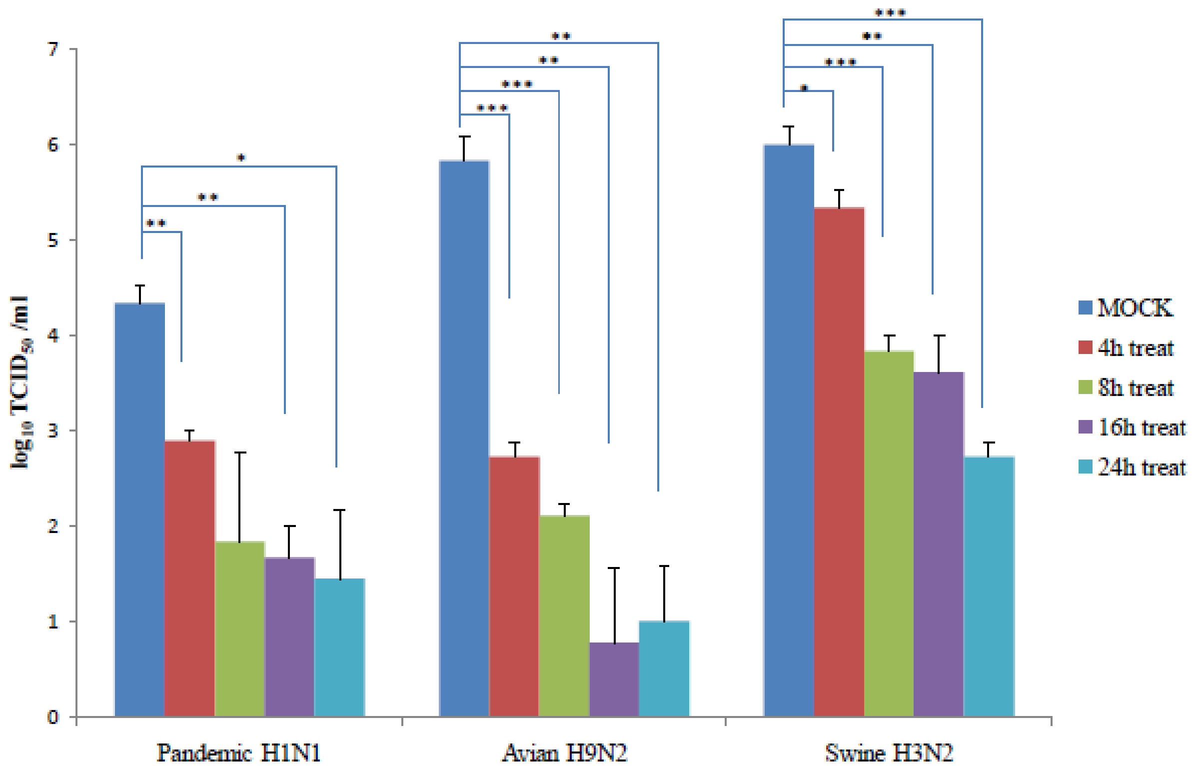

Fig 3-10. A549 cells were treated with 1000 IU of Alferon N for 4h, 8h, 16h, 24h followed by infection with Pandemic H1N1 virus, Avian H9N2 virus or Swine H3N2 virus at a MOI of 0.01. Supernatants were collected 40 h pi and titrated on MDCK cells. Each data point indicates the means from three different independent experiments, and error bars indicate the standard errors of the means (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

These data suggested that a longer time of Alferon N pretreatment can enhance the antiviral effect in A549 cells—an important issue that is investigated further below.

3.5 16 Hours of Alferon N Pretreatment Inhibits Influenza A Virus Replication in A549 Cells

To explore the antiviral effect of longer time Alferon N pretreatment against IAV infections in A549 cell, confluent A549 cells in 48-well plates were treated with 1000 IU, 100 IU and 10 IU for 16 hours before infection with pandemic H1N1, avian H9N2 or swine H3N2 at a MOI of 0.01. Virus replication kinetics were monitored over 48 hours by titration in MDCK cells.

3.5.1 Pandemic H1N1 Replication after 16 Hours of Alferon N Pretreatment

As shown in Figure 3-11, at 12h pi, pandemic H1N1 replicated to $10^{2.9}$ $TCID_{50}$/ml in mock treated A549 cells; virus reduction was found at 1000 IU treated samples, which were 0.5 log $TCID_{50}$ lower than mock treated ones. At 24h pi, 10 IU treatment resulted in 0.3 log $TCID_{50}$ reduction, while 100 IU and 1000 IU treatment result in approximately 1 log $TCID_{50}$ reduction compared to mock treatment ($P<0.05$). Inhibition was observed at 36h pi, when 1 log $TCID_{50}$ reduction was found in 10 IU treated samples, and approximately 2 log $TCID_{50}$ reduction was observed in 100 and 1000 IU treated samples compared to mock treated controls ($P<0.05$). Inhibition of 1 and 2 log $TCID_{50}$ was found in 10 IU and 100 IU Alferon N pretreated samples at 48h pi ($P<0.05$); and 1000 IU of Alferon N pretreatment led to a reduction of 2.5 log $TCID_{50}$ in virus titer compared to the mock treatment ($P<0.05$). These results indicated that pandemic H1N1 replication was susceptible to inhibition by 16 hours Alferon N pretreatment as low as the dose of 10 IU.

Figure 3-11 Dose dependent inhibition effect of 16 hours Alferon N treatment on the replication of Pandemic H1N1 in A549 cells

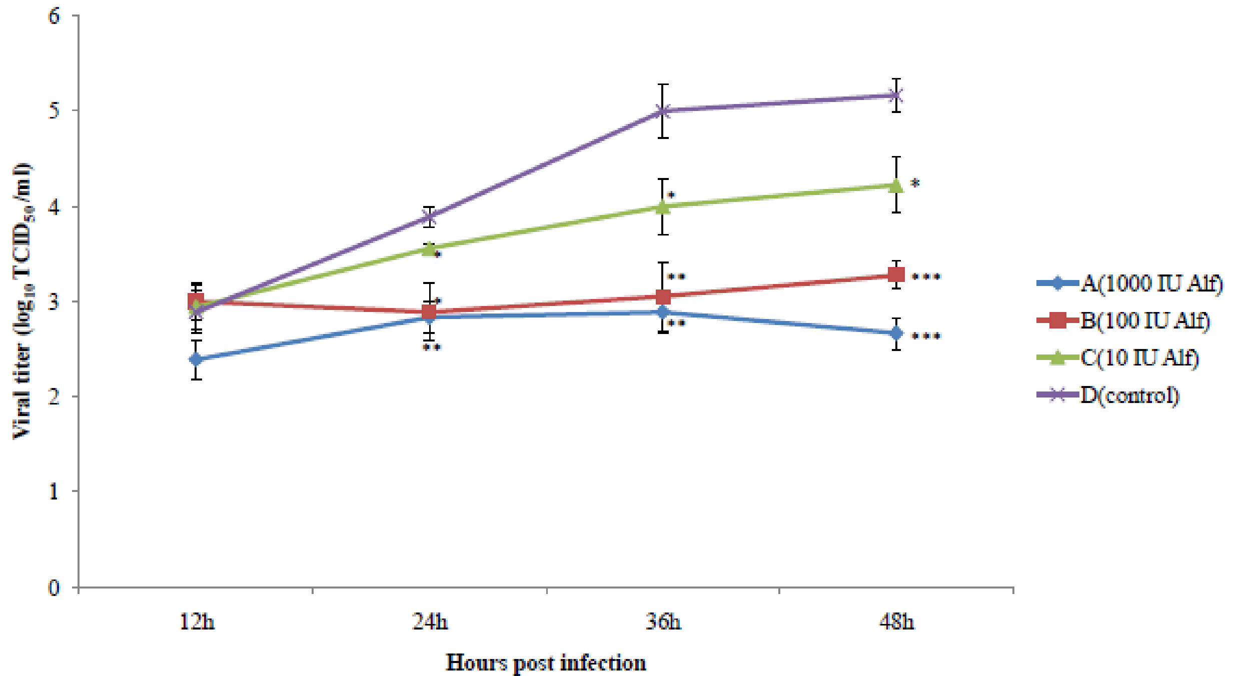

Fig 3-11. A549 cells were treated with 1000, 100, 10 IU of Alferon N for 16h followed by infection with Pandemic H1N1 virus at a MOI of 0.01. Supernatants were collected every 12 hours over 48h period and titrated on MDCK cells. Each data point on the curve indicates the means from three different independent experiments, and error bars indicate the standard errors of the means (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.5.2 Avian H9N2 Replication after 16 Hours of Alferon N treatment

As shown in Figure 3-12, pandemic H1N1 replicated to $10^{2.9}$ $TCID_{50}$/ml in mock treated A549 cells. Furthermore, pretreatment with Alferon N for 16 hours reduced the virus titer from 0.7 to 1 log $TCID_{50}$ depending on the dose applied at 12h pi. At 24h pi, virus replicated to a similar titer in 10 IU treated samples with mock treated samples. Significant virus titer reduction was observed in 100 IU and 1000 IU treated samples with titers 2 and 2.5 log $TCID_{50}$ lower than control samples ($P<0.05$). A 10 IU treatment led to a titer reduction of 1.1 log $TCID_{50}$ at 36h pi compared to mock treatment, while 100 IU and 1000 IU of Alferon N treatment resulted in 2.5 and 4.8 log $TCID_{50}$ of virus titer reduction compared to mock treatments. At 48h pi, virus replicated to $10^{6.6}$ $TCID_{50}$/ml in 10 IU treated cells, which was 0.9 log $TCID_{50}$ lower than mock

treated cells. 2.3 and 3.5 log $TCID_{50}$ reductions were found in 100 IU and 1000 IU treated samples respectively compared to mock treated controls. These results indicate that avian H9N2 replication was susceptible to inhibition by 16 hours of Alferon N pretreatment with a dose as low as 100 IU.

Figure 3-12 Dose dependent inhibition effect of 16 hours Alferon N treatment on the replication of Avian H9N2 in A549 cells

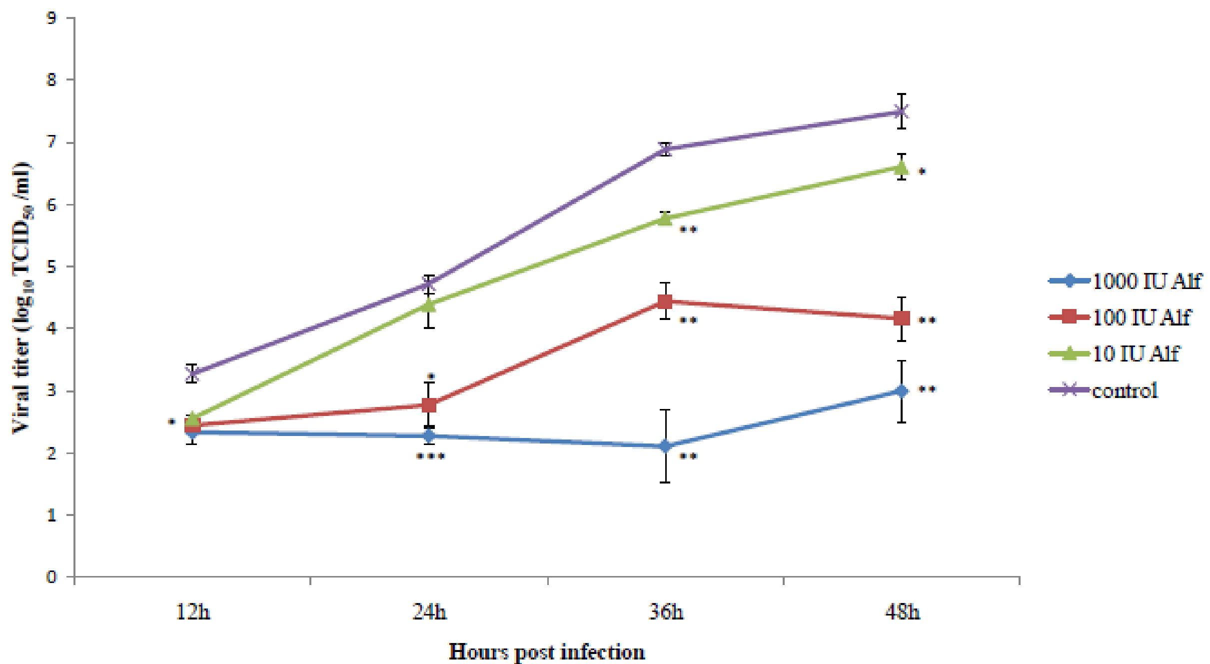

Fig 3-12. A549 cells were treated with 1000, 100, 10 IU of Alferon N for 16h followed by infection with Avian H9N2 virus at a MOI of 0.01. Supernatants were collected every 12 hours over 48h period and titrated on MDCK cells. Each data point on the curve indicates the means from three different independent experiments, and error bars indicate the standard errors of the means (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.5.3 Swine H3N2 Replication after 16 Hours of Alferon N Pretreatment

As shown in Figure 3-13, there were no observed differences between mock controls and 10 IU of Alferon N treatments from 12 to 48 h pi. There was no difference of virus titer between

100, 1000 IU Alferon treated samples and mock treated controls at 12h pi. At 24h pi, 100 IU Alferon N treatment led to 0.9 log $TCID_{50}$ reduction compared to mock treatment, while 1000 IU Alferon N significantly reduced virus replication by reducing virus titer up to 2 log $TCID_{50}$ compared to mock treatment ($P<0.05$). Similar results were found at 36h pi with at 24h pi for 100 IU and 1000 IU Alferon N treatment compared to its mock treatment respectively. At 48h pi, virus replicated to the same titer ($10^{2.9}$ $TCID_{50}$/ml) in 100 and 1000 IU treated cells which were significantly lower than in mock treated cells ($P<0.05$). These results demonstrated that swine H3N2 replication was susceptible to inhibition by 16 hours of Alferon N pretreatment at as low a dose as 100 IU starting at 48h pi.

Figure 3-13 Dose dependent inhibition effect of 16 hours Alferon N treatment on the replication of Swine H3N2 in A549 cells

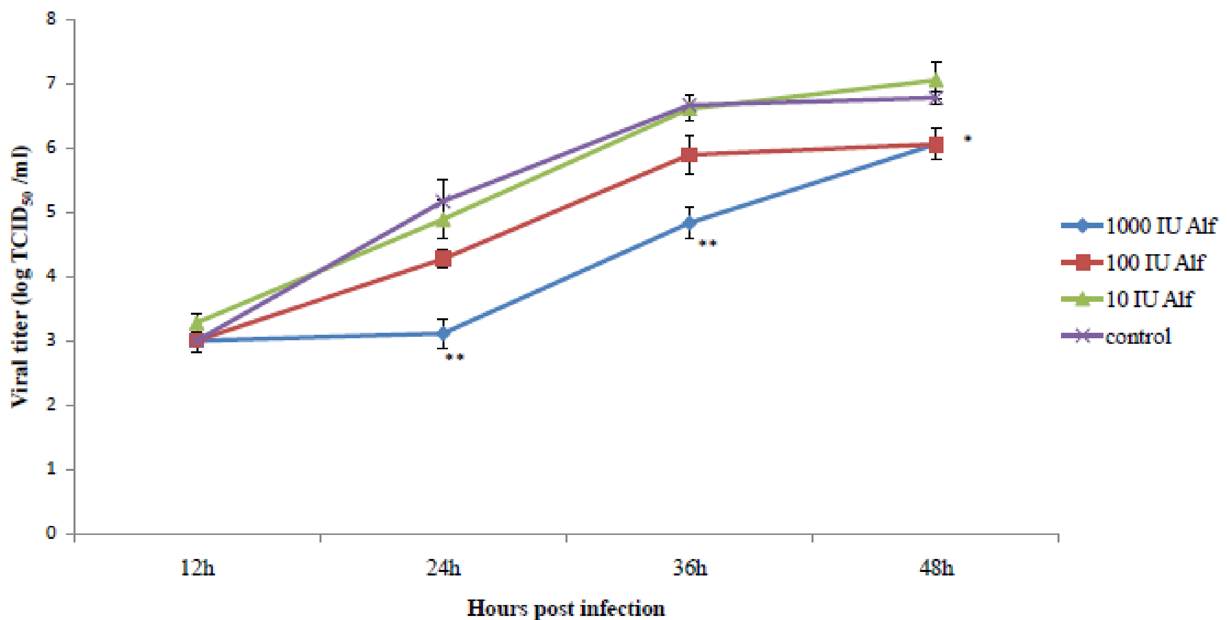

Fig 3-13. A549 cells were treated with 1000, 100, 10 IU of Alferon N for 16h followed by infection with Swine H3N2 virus at a MOI of 0.01. Supernatants were collected every 12 hours over 48h period and titrated on MDCK cells. Each data point on the curve indicates the means from three different independent experiments, and error bars indicate the standard errors of the means (*, $P < 0.05$; **, $P < 0.01$).

3.6 Cellular toxicity of Alferon N

To determine the cellular toxicity of Alferon N, cell viability assays were performed in both ST and A549 cells. Generally, both cells were treated with 10000 IU/well, 1000 IU/well, 100 IU/well, 10 IU/well of Alferon N for 48 hours. Cells were collected every 12 hours and mixed with 0.4% trypan blue. Viable versus nonviable cells were counted under the microscope using a hemacytometer. As shown in Figure 3-14 (A), there was no obvious cellular toxicity in the ST cells treated with different doses of Alferon N; the 10000 IU treated samples had the highest nonviable cell rate (from 1.6% to 3.6%) when compared to controls (1.1% to 2.3%), however, the difference was not significant ($P>0.05$). As shown Figure 3-14 (B), no cellular toxicity was detected in Alferon N treated A549 cells; the nonviable cell rate in 10000 IU Alferon N treated samples was around 5% (from 3.2% to 5.2%) which was similar to the cellular toxicity in mock treated controls (from 2.8% to 5.1%). There was no significant difference between Alferon N treated samples and controls ($P>0.05$). These data indicate a low cellular toxicity for Alferon N employing the concentrations used in the above described experiments.

Figure 3-14 Cellular toxicity of Alferon N in ST and A549 cells

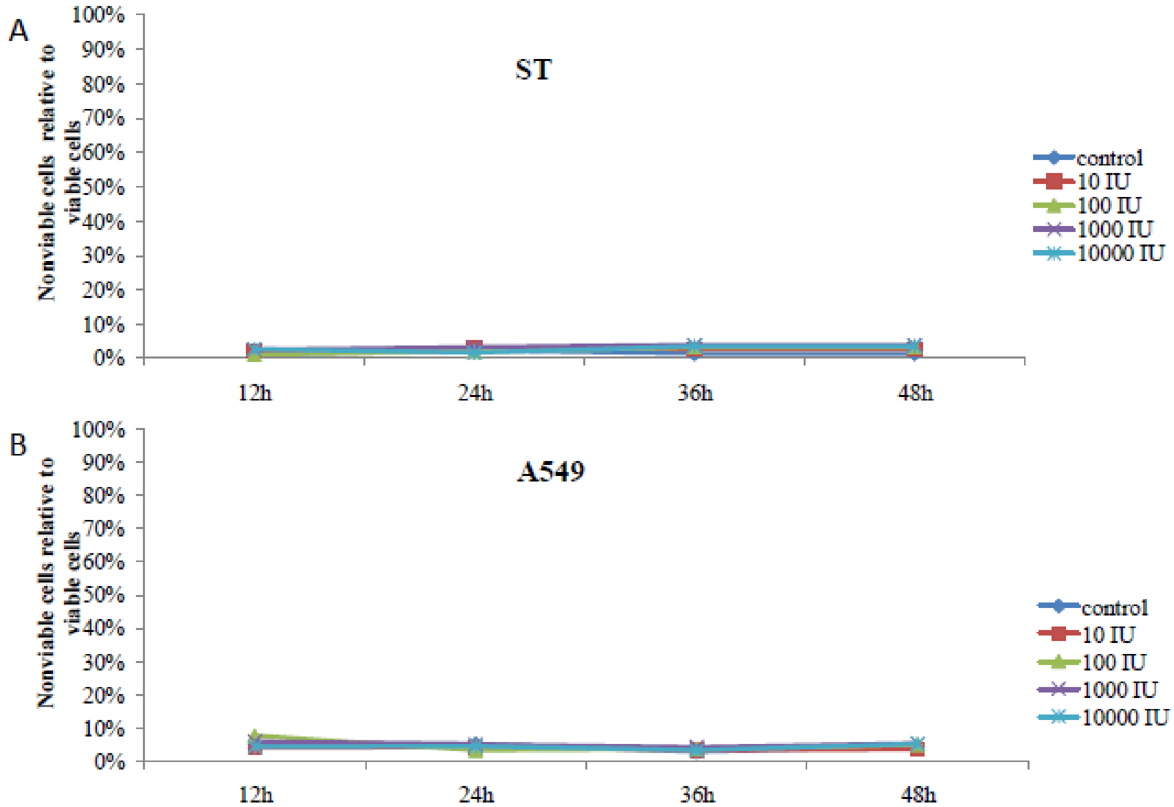

Fig 3-14. ST (A) and A549 (B) cells were treated with 10000, 1000, 100, 10 IU of Alferon N for 48 hours. Cells were collected every 12 hours and cell viability assays were performed using 0.4% trypan blue. Each data point on the curve indicates the means from three different independent experiments.

3.7 Analysis for Antiviral Gene Expression under 1000 IU Alferon N Treatment in ST and A549 Cells

To explain the antiviral effect resulting from Alferon N pretreatment and the disparity of response to treatment between the two cell lines, confluent ST cells and A549 cells were lysed and collected after treatment with 1000 IU Alferon N for 4, 8, 16 and 24 hours. RNA was extracted from the samples, and real time RT-PCR was performed to examine the expression of Interferon Stimulated Genes (ISGs) Mx1/A (defined as Mx1 in swine and MxA in humans) and OAS1. Figure 3-15 (blue bar) showed that Mx1 expression was ten-fold higher in four hour

treated ST cells compared to mock treatment. An increasing trend for the upregulation of this gene was observed if ST cells were treated for a longer time. The highest Mx1 gene expression in ST cells was found in 24 hour Alferon N treated samples, which were approximately 40 fold up-regulated compared to controls. In A549 cells, MxA gene was enhanced 40 fold in four hour Alferon N treated samples compared to mock treatment. Longer time of treatment resulted in higher MxA expression in A549 cells. Up to 298 fold upgrade of MxA gene expression was observed in 24 hour Alferon N treated A549 cells compared to mock treatment (Figure 3-15, red bar). When comparing gene up-regulation between ST cells and A549 cells under the same treatment condition, Mx1/A gene expression was upregulated more in Alferon N treated A549 cells than in ST cells.

Figure 3-15 The relative up-regulation of Mx1/A mRNA due to Alferon N treatment in ST and A549 cells

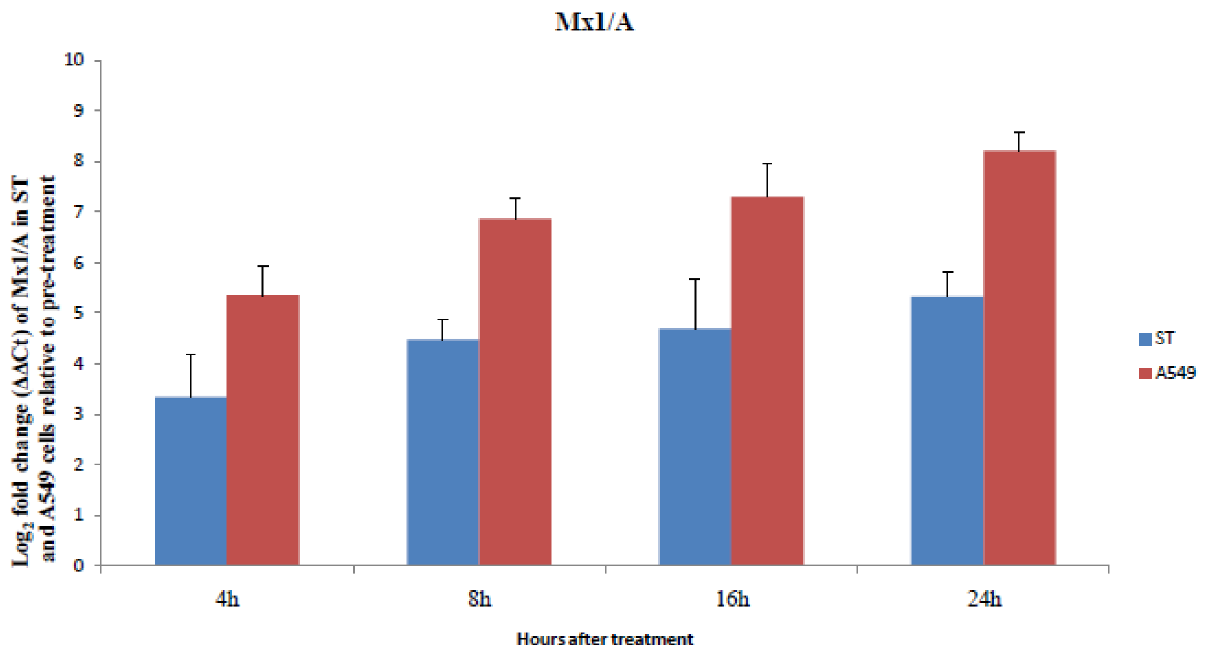

Fig 3-15. ST and A549 cells were incubated with Alferon N (1000IU/well) for time indicated. All data are depicted as the rate of $\log_2$ fold change of Mx1/A mRNA from samples after and before Alferon N treatment. Each data point indicates the means from three different independent experiments, and error bars indicate the standard errors of the means.

OAS1 gene expression was upregulated dramatically in Alferon N treated ST cells, with the highest fold change in 16 hour Alferon N treated samples, which were up to 480 fold higher than in mock treated controls (Figure 3-16, blue bar). In A549 cells (Figure 3-16, red bar), OAS1 was also upregulated by Alferon N treatment, with maximum up-regulation of 32 fold found in the 16 hour Alferon N pretreated samples. OAS1 was enhanced to a significant level during treatment in ST cells compared to A549 cells. When upregulation was compared between ST cells and A549 cells, OAS1 gene expression was upregulated more in Alferon treated ST cells than in A549 cells under the same conditions.

Figure 3-16 The relative up-regulation of OAS1 mRNA due to Alferon N treatment in ST and A549 cell

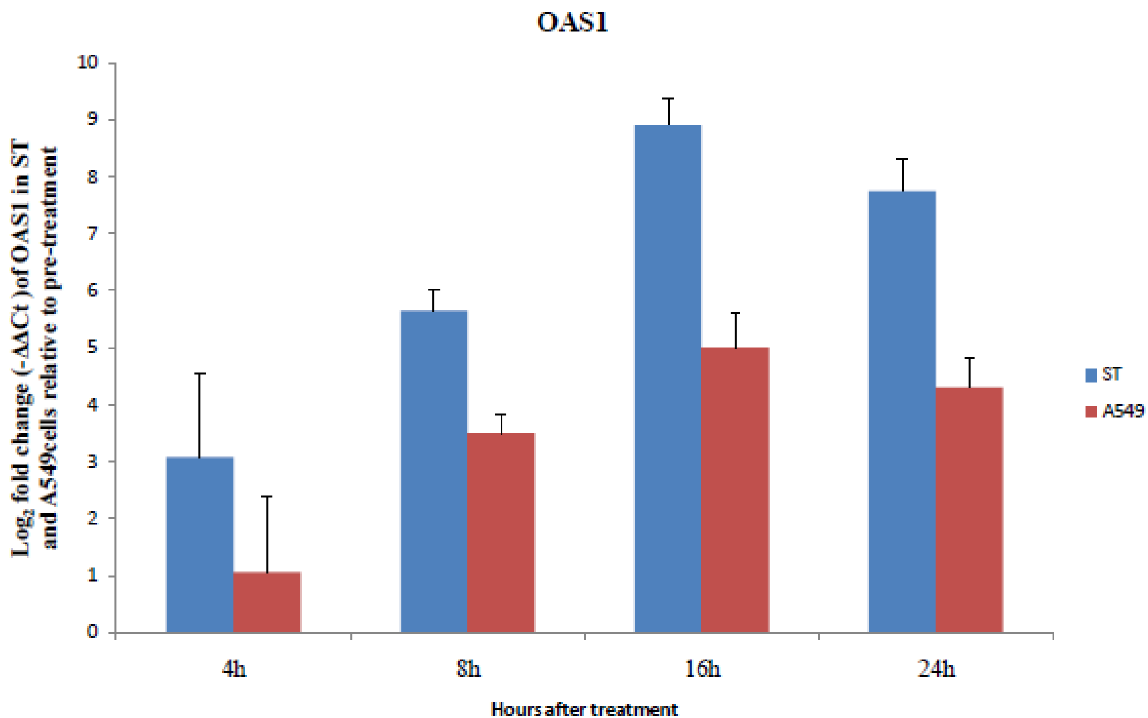

Fig 3-16. ST and A549 cells were incubated with Alferon N (1000IU/well) for time indicated. All data are depicted as the rate of log_2 fold change of OAS1 mRNA from samples after and before Alferon N treatment. Each data point indicates the means from three different independent experiments, and error bars indicate the standard errors of the means.

In summary, ST cells and A549 cells were shown to sustain replication and infection of multiple influenza A viruses and were susceptible to the pre-treatment of Alferon N. The replication of different subtypes of IAVs was inhibited in a dose dependent manner after pretreatment of Alferon N: four hours of pretreatment was sufficient for the antiviral effect of Alferon N in ST cells, whereas a longer time of pretreatment was needed for detectable inhibition in A549 cells. The ISGs, such as Mx1/A and OAS1, contributed to the antiviral effect; and the different responses to Alferon N treatment could explain the different responses between ST cells and A549 cells.

Chapter 4 - **Discussion and Conclusions**

Influenza has been a public health threat for over a century throughout the world. The evolution and variability of influenza viruses create significant hurdles for treatment and prevention. The 2009 pandemic H1N1 influenza was the first pandemic outbreak in the 21st century. It was a novel reassorted influenza virus with genes introduced from different avian, human and swine influenza viruses (58). Swine serve as important mixing vessels, mediating such reassortment from different species of influenza viruses. After adaptation in pigs, these viruses could infect humans, resulting in pandemic emergence. Attention has been focused on the avian H9N2 virus as a possible cause of a future pandemic, since this avian virus is now endemic in poultry and has been shown to infect both humans and pigs. Moreover, the avian H9N2 A/quail/Hong Kong/G1/97 was shown to share the same six internal genes with the lethal H5N1 virus which has caused disease in animal and human disease since 1997 (40).

To investigate novel antiviral drugs in addition to current methods for better preparing for the next pandemic, I studied the growth of pandemic H1N1, avian H9N2 and swine H3N2 influenza viruses after pretreatment with the FDA approved drug Alferon N (human interferon alpha). My experiments demonstrated that pretreatment of swine and human cell lines with Alferon N can inhibit the growth of different subtypes of influenza A viruses. Alferon N pretreatment presented a broad spectrum of inhibition effects on different subtypes of Influenza A Viruses, including pandemic H1N1, swine H3N2 and avian H9N2. The experimental results also demonstrated that these inhibition effects are related to the upregulation of Interferon Stimulated Genes (ISGs) such as Mx and OAS1; and the disparity of response between cell lines

can result from the differential up-regulation levels of these genes after interferon alpha pretreatment.

A549 cells have been used previously as a human lung cell model for influenza A viruses infection, because human lung cells are the primary target for influenza infection and A549 cells generate the complete signaling pathway associated with innate immunity upon influenza virus infection (62, 71). I demonstrated here that pandemic H1N1, avian H9N2 and swine H3N2 can readily infect both a swine cell line and a human cell line; however pandemic H1N1 replicated to a lower level in human A549 cells compared with other subtypes and with its replication in ST cells. This finding underlines the susceptibility of both swine and humans to different species of influenza virus infections and underscores the threat of novel influenza strain emergence by reassortment when two different strains infect the same host. All three subtypes of influenza viruses replicated to a similar level in ST cells, including pandemic H1N1, which supported the theory that pigs can serve as mixing vessels for influenza infection. Interestingly, the swine origin pandemic H1N1 replicated to a lower level in human A549 cells compared with swine ST cells, likely because this pandemic H1N1 was a swine isolated strain which is better adaptive to swine than to human.

This research demonstrated that the replication of pandemic H1N1, avian H9N2, swine H3N2 was susceptible to pretreatment with Alferon N in a dose dependent manner, with an effective dose as low as 100 IU in ST cells. The broad spectrum of the antiviral effect of interferon alpha was similar to results from previous studies (25, 51). Furthermore, in this research, I found that a similar effect of pretreatment of Alferon N could be achieved in human A549 cells; however this effect required longer treatment time, such as 16 hours. Interferon alpha is the first line of innate immunity to combat influenza virus infection. Upon infection, cells will

secrete interferon to alert themselves and neighboring cells to induce the expression of ISGs which together will result in protection from the virus. The pathogenicity of influenza A viruses are substantially controlled by the interferon system. Exogenous interferon alpha could induce the expression of many ISGs. Upon binding to the type I interferon receptor, interferon alpha activates the JAK/STAT pathway. The phosphorylated STAT1 and STAT2 proteins form a heterodimer which then associates with IFN-regulatory factor 9 (IRF-9). The new heterotrimer translocates to the nucleus and binds to ISRE (interferon stimulated response element) which resides in the promoter region of many ISGs and enhances the transcription of these genes (63). The upregulation and expression of cellular ISGs establishes the antiviral status of the host. Previous studies have shown that Mx and OAS play primary roles in mediating responses to foreign antigens (23, 62). Mx proteins (Mx1and Mx2 in swine, MxA and MxB in humans) are interferon induced GTPase involved in intracellular vesicle trafficking (27). MxA and Mx1 localize in cytoplasm and possess strong capacity to inhibit the replication of influenza A virus via interfering with different steps of virus life cycle (27, 52). Human MxB is found both in nucleus and cytoplasm but fails to display antiviral function (50); Porcine Mx2 localizes in the nucleus and has been shown to possess antiviral activity against influenza viruses (52). By interacting with viral RNP proteins such as PB2 and NP, MxA or Mx1 can block the trafficking of viral proteins between cytoplasm and nucleus, resulting in the inhibition of viral transcription (63); The endocytic trafficking of incoming influenza A virus particles can also be blocked by porcine Mx1 (60). OAS proteins help synthesize 2’,5’-ogligoadenylates which will activate latent RNaseL, leading to RNA degradation, thus inhibiting the viral replication (63). The results in this study showed upregulation of Mx1/A and OAS1 in both ST cells and A549 cells, however the time course and quantity of upregulation is different between the two cells with MxA being

higher in A549 cells and OAS1 being higher in ST cells. The upregulation of both genes correlated with the decreased viral growth after Alferon N pre-treatment in both cells. In future experiments it would be interesting to study whether or not a longer time of Alferon N treatment or repeated Alferon N pretreatment would increase antiviral ability in cells. The different responses of cells following Alferon N treatment could be explained by the different levels of ISG gene expression with Alferon N treatment between the two cell lines. The rapid induction of OAS1gene expression in ST cells rather than in A549 cells after 4h of Alferon N treatment might account for the better inhibition effect in ST cells under these conditions. It still needs to be determined whether other ISGs may play a role in combating influenza A viruses and account for the different response in these two cell lines after Alferon N treatment.

This research demonstrated that to counteract the interferon system, influenza A viruses have adapted methods to ensure a successful infection in the host. This interferon antagonizing function is mediated mainly by the nonstructural protein (NS1) through blocking the activation of interferon regulatory factor 3 (IRF3) and post translational processing of cellular mRNA (33). It has been shown that different NS1 genes will exhibit different capacities to counter the interferon system, thus resulting in different levels of pathogenicity (33) . Further, replication of viruses lacking interferon antagonistic NS1 gene have been shown to be significantly reduced (34). In this study, under the same conditions of IFN treatment, swine H3N2 replicated to a higher titer than either pandemic H1N1 or H9N2 especially in A549 cells, suggesting swine H3N2 expresses a NS1 protein with a stronger interferon antagonist compared with the other two strains. A previous study showed that a lethal H5N1 virus was resistant to the exogenous interferon treatment *in vitro* due to a glutamic acid at position 92 of its NS1 protein (66). However, none of the viruses in this experiment possess glutamic acid at position 92 of their

NS1 proteins, suggesting the interferon resistance of the swine H3N2 is due to other factors. Interestingly, the swine H3N2 A/swine/Tx98/4199-2/98 used in this experiment possesses glutamic acid, phenylalanine and methionine at position 186, 103, 106 of its NS1 protein, respectively, which have been shown to be critical for the viral NS1 binding to the host factor CPSF and thus inhibiting the host mRNA processing (33). The inhibition of host mRNA processing will result in decreased expression of antiviral proteins and therefore allowing viral replication even in interferon treated cells. To control swine H3N2 influenza virus replication to a desired low level, high dosage and repeated (14) interferon pretreatment might be necessary in order to override its NS1 interferon antagonistic ability.

As previously indicated, the *in vivo* experiment indicating administering interferon alpha to prevent influenza A viruses infection has shown promise in ferrets (5). Orally administrated chicken interferon-alpha has also been shown to inhibit avian H9N2 influenza virus replication in chickens (51). In humans, intranasal interferon was used successfully for prevention and treatment of influenza infection successfully in Moscow (3); and long term therapy with high dose of IFN alpha in conjunction with other drugs is being used to treat hepatitis B and C in humans (14) . However, the side effects of applying high doses of IFN limit its use in the treatment of influenza in humans. Studies have also shown that combating influenza is possible by using low dosage of interferon *in vivo* without adverse effects (3, 25). In summary, both this study and earlier research have demonstrated that using interferon could become a promising alternative for the treatment of influenza infections.

In conclusion, this research has demonstrated the efficient antiviral effect of Alferon N (human interferon alpha) pretreatment of cells for different subtypes of influenza viruses has the

potential to cause future pandemics. Significantly reduced virus replication was observed with the treatment of Alferon N in a dose dependent manner; and low doses of interferon pretreatment were sufficient to cause significant inhibition. In general, by pretreating the cells for a longer time or by repetitive treatment, better inhibition was seen even with low treatment doses of treatment. These results increase understanding about the replication of various influenza A viruses subtypes, as well as demonstrate the efficacy of using interferon to inhibit the replication of viruses in cell cultures. Furthermore, the data support the idea that interferon can be used as a potential antiviral drug to prevent a host from being infected by novel and lethal influenza viruses, especially when those viruses are drug resistant strains. This research has demonstrated the effect of exogenous IFN-α treatment on the replication of different influenza A viruses *in vitro* and provides a foundation for future *in vivo* studies on using exogenous IFN-α treatment as an alternative approach to combat influenza A virus infection.

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