Development of Pre-pandemic Influenza Vaccines against Highly Pathogenic H5 Strains

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ABSTRACT

The emergence of highly pathogenic avian influenza A viruses (HPAI) and their potential to cause human infections poses substantial public health threats. Among HPAI, H5 viruses are of particular concern given their global spread in avian species, zoonotic infection to humans and the high mortality rate in humans. While HPAI H5N1 human infections have typically been reported in Asian countries, Canada reported a case of fatal human infection by the HPAI H5N1 virus in 2014. The genome of the causative virus A/Alberta/01/2014 (H5N1) (AB14 (H5N1)) has been reported; however, the isolate had not been evaluated for its pathogenicity in animal models.

In the first part of my thesis study, I characterized the pathogenicity of AB14 (H5N1) in mice and found that AB14 (H5N1) is highly lethal in mice. The virus caused systemic viral infection and erratic proinflammatory cytokine gene expression in different organs, including lung, spleen, and brain. This study not only provided experimental evidence to complement the specific human case report but also established an animal model for HPAI H5N1 virus, which is valuable and essential for evaluating vaccine and antiviral candidates against the potential influenza pandemics.

Vaccination is the most effective intervention to prevent possible pandemic outbreaks. The conventional egg-based vaccine production strategy takes up to six months for vaccine development, during which the antigenically distinct pandemic viruses are allowed to spread in the naïve population leading to a rapid disease progression. Traditional inactivated vaccines against zoonotic avian influenza viruses often suffer from low immunogenicity due to the intrinsic poor antigenicity of avian influenza viruses, or they are poor inducers of cellular immunity. In my second part of the study, I aimed to develop a new subunit H5 influenza vaccine to achieve better immune protection against HPAI H5N1 virus infection. I expressed and purified hemagglutinin (HA) derived from AB14 (H5N1) using both mammalian (m) and bacterial (b) expression systems. The purified recombinant proteins were formulated with a proprietary adjuvant (TriAdj); their efficacy as vaccine candidates was then evaluated in the mouse model I established in the first part of the study. Intramuscular vaccination of two doses of TriAdj-formulated mammalian-expressed HA (m-HA/TriAdj) provided full protection against a lethal challenge of AB14 (H5N1) in mice. In contrast, bacterially expressed HA with TriAdj (b-HA/TriAdj), b-HA without adjuvant or m-HA without adjuvant did not result in protection in immunized mice. Furthermore, I analyzed the immune responses and found that m-HA/TriAdj elicited significantly higher levels of balanced

Th1 and Th2 responses and neutralizing antibody. All of the mice in this group survived a lethal AB14 (H5N1) challenge and showed no signs of disease or infection as demonstrated by no loss of body weight or detectable virus in the lungs. My results suggest that m-HA formulated with TriAdj has the potential to protect against pandemic H5N1 in the event of its cross over to the human host.

Overall, the two parts of this thesis provide an animal model for the HPAI AB14 (H5N1) virus and a highly effective vaccine candidate against the HPAI H5N1 virus. Understanding the pathogenicity of HPAI H5N1 virus in an animal model and developing the anti-H5 vaccine candidate will all contribute to the preparedness for a potential influenza pandemic caused by HPAI H5N1 viruses.

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LIST OF ABBREVIATIONS

AB14 (H5N1)	A/Alberta/01/2014 (H5N1)
ARDS	Acute respiratory distress syndrome
AREB	Animal Research Ethics Board
AS03	Adjuvant System 03
b-HA	Bacterial expressed HA
b-HA/TriAdj	TriAdj formulated bacterial expressed HA
BC15 (H7N9)	A/British Columbia/1/2015 (H7N9)
CCL	Chemokine (C-C motif) ligand
CD	Circular dichroism spectroscopy
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CPE	Cytopathic effects
cRNA	Complementary RNA
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
d.p.i	Days post-infection
EBNA1	Epstein-Barr virus nuclear antigen 1
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
Epstein-barr virus	EBV
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	Food and Drug Administration
H&E	Hematoxylin and eosin
HA	Hemagglutinin
HI	Hemagglutination inhibition
HPAI	Highly pathogenic avian influenza
hRSV	Human respiratory syncytial virus
IAV	Influenza A virus

IDR	Innate defense regulator
IFN	Interferon
IIV	Inactivated influenza vaccine
IL	Interleukin
IP-10	Interferon-gamma-inducing protein 10
LAIV	Live attenuated influenza vaccine
LD100	Lethal dose 100
LPAI	Low pathogenic avian influenza
M	Matrix protein
m-HA	Mammalian expressed HA
m-HA/TriAdj	TriAdj formulated mammalian expressed HA
M1	Matrix protein 1
M2	Matrix protein 2
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
NA	Neuraminidase
NEP	Nuclear export protein
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NLSs	Nuclear localization signals
NP	Nucleoprotein
NS	Nonstructural protein
PA	Polymerase acid
PB1	Polymerase basic 1
PB2	Polymerase basic 2
PCEP	Polydi(p-oxyphenylpropionate) phosphazene
PFU	Plaque forming unit
PHAC	Public Health Agency of Canada
PI3K	Phosphoinositide 3-kinase

PNPP	P-nitrophenyl phosphate substrate
Poly(I:C)	Polyriboinosinic acid-polyribocytidylic aci
PR8	A/Puerto Rico/8/34
PRR	Pattern recognition receptors
RBS	Receptor binding site
RDE	Receptor destroying enzyme
RdRp	RNA dependent RNA polymerases
rHA	Recombinant HA
RIG-I	Retinoic acid-inducible gene I
SD	Standard deviation
T	Threonine
$TCID_{50}$	50% tissue culture infective dose
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	Tissue plasminogen activator
TriAdj	Triple adjuvants
UACC	University Animal Care Committee
vRNA	Viral genomic RNA
vRNP	Viral ribonucleoprotein
WHO	World Health Organization
WIV	Whole inactivated virus

CHAPTER 1 INTRODUCTION

1.1 Influenza A virus (IAV) biology

1.1.1 IAV genome and proteins

Influenza virus belongs to the family of *Orthomyxoviridae*, which is composed of seven genera, including *Alphainfluenzavirus, Betainfluenzavirus, Gammainlfuenzavirus, Deltainlfuenzavirus, Thogotovirus, Isavirus, and Quaranjavirus* (Hause et al., 2014; Hause et al., 2013). Most of the seasonal epidemics and outbreaks are associated with influenza A and influenza B viruses, whereas influenza C viruses only cause mild or asymptomatic infections in humans (Hause et al., 2013; Mosnier et al., 2015; Poon et al., 2016). Additionally, Influenza D viruses have not been isolated from humans but from pigs and cows (Hause et al., 2014). Salmon anemia virus belongs to the genus of *Isavirus,* which causes infectious salmon anemia in farmed salmon (Aamelfot, Dale, & Falk, 2014). Unlike influenza viruses, which commonly replicate in the respiratory tract, *Thogotovirus* replicates systemically in the vertebrate host and is transmitted by ticks (Kochs et al., 2010). Quaranfil virus which belongs to the genus of *Quaranjavirus,* is also transmitted by ticks and is the only virus that could cause human infections in this genus (Presti et al., 2009).

The IAV contains eight single-stranded negative-sense RNA segments encoding at least 17 viral proteins. The capacity of one viral segment coding for more than one protein results from either splicing or using an alternative open reading frame. In the order of length, the eight segments are: polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acid (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M), NS (nonstructural protein) (Figure 1.1) (Krammer et al., 2018). In each viral segment, the coding region is flanked by a segment-specific non-coding region at both ends. The self-complementary terminal genomic regions of viral genomic RNA (vRNA) could form a double-stranded panhandle structure, which serves as the promoter of viral replication. The panhandle structure on each vRNA segment is bound to the trimeric RNA dependent RNA polymerase (RdRp) complex comprising the PB2, PB1, and PA proteins, while the rest of the segment is encapsidated with multiple NP proteins. The vRNA, NP protein, and the three polymerase proteins together assemble into the viral ribonucleoprotein (vRNP) complex, which is the minimal functional unit for viral transcription and replication (Figure 1.2). Virion core including eight vRNP complexes is surrounded by the matrix protein 1 (M1), which lies right underneath a host-derived lipid membrane harboring a large

number of spike proteins hemagglutinin (HA) and neuraminidase (NA) at a ratio of approximately four to one along with a low abundance of matrix protein 2 (M2) (Figure 1.1) (Palese P, 2013).

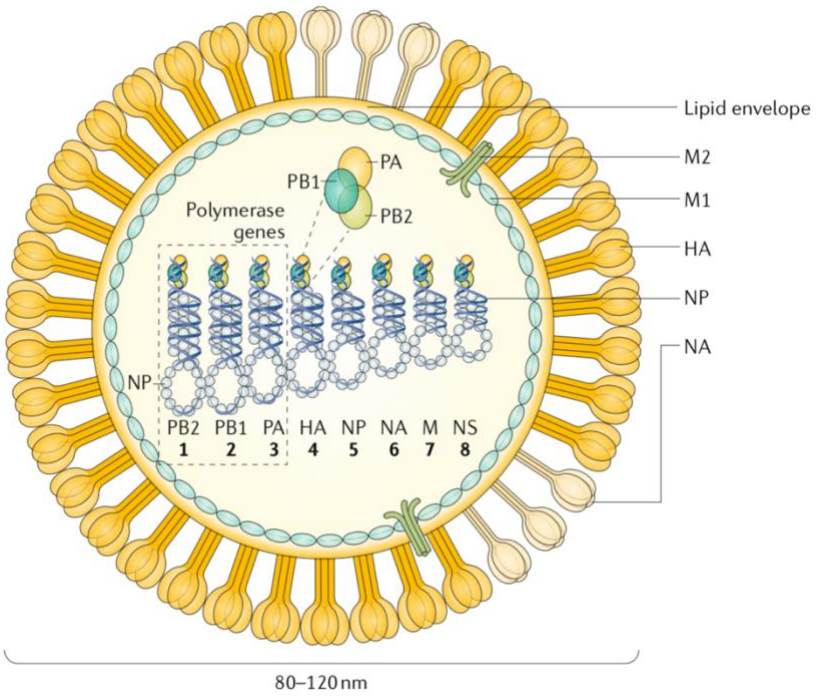

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Figure 1.1 The structure of influenza A virus.

The two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and the iron-channel protein M2 are anchored on the lipid envelope derived from the host cell membrane, beneath which the matrix protein (M1) forms a matrix layer. Within the matrix layer are eight viral ribonucleoprotein complexes, each of which comprises one viral RNA genome associated with nucleoprotein (NP) and the three polymerases (polymerase basic protein 1(PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA)). M, matrix protein; NS, nonstructural protein.

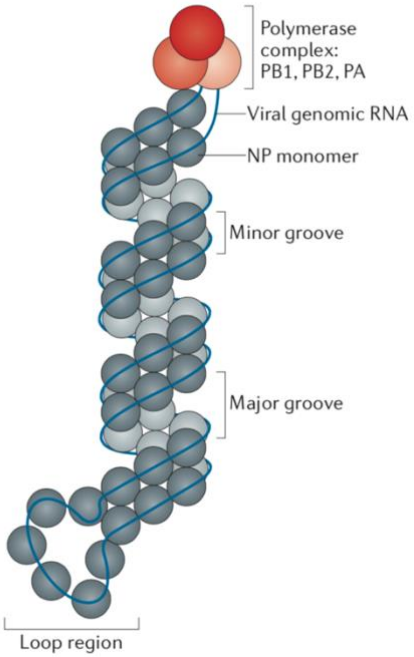

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Figure 1.2 The influenza vRNP complex.

Viral ribonucleoprotein (vRNP) complex is comprised of a single-stranded negative-sense viral genomic RNA associated with multiple nucleoprotein (NP) and a trimeric polymerase complex consisting of polymerase basic protein 1(PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA). The 5' and 3' end of vRNA forms a partially complementary panhandle structure and bind by the polymerase complex at one end of the vRNP filament. The vRNA associated with NP is organized into an antiparallel double helix structure with a loop at the opposite end of the vRNP filament to the complex of three polymerases.

IAV encodes 12 major viral proteins, and they each have diverse functions. Each viral polymerase core is a compact of three polymerase units interacting with each other (Hemerka et al., 2009; Vasin et al., 2014). PB2 and PA not only play vital roles in viral replication, but also are essential in viral mRNA transcription due to their unique roles in catching the cap of host RNAs and cleaving the capped RNA off; which is then used in the initiation of viral mRNA synthesis (Boivin, Cusack, Ruigrok, & Hart, 2010; Dias et al., 2009; Guilligay et al., 2008; Liang, Danzy,

Dao, Parslow, & Liang, 2012). The viral mRNA elongation is catalyzed by PB1, the RNA-dependent RNA polymerase, which could also replicate vRNA in a primer-independent manner by synthesizing complementary RNA (cRNA) acting as replicative intermediate (Fodor, 2013; Te Velthuis & Fodor, 2016). PB1 alone has also been shown to be able to transcribe the RNA template in vitro (Kawaguchi, Naito, & Nagata, 2005). There is an alternate open reading frame on the PB1 segment encoding the PB1-F2 protein: an accessory protein expressed in most of the human influenza A viruses. Although PB1-F2 is dispensable in viral transcription and replication, it is critical in regulating the virus pathogenesis and disrupting the host immune response towards influenza viruses by inducing the death of immune cells, interfering with interferon induction, and stimulating the secondary bacterial infection (James et al., 2019; Kamal, Alymova, & York, 2017; Vidy et al., 2016). PA segment also encodes a viral protein PA-X by ribosomal frameshift, which has been proved to be involved in the degradation of host mRNA in order to reduce general host protein expression in virus infected cells. (Chaimayo, Dunagan, Hayashi, Santoso, & Takimoto, 2018; Jagger et al., 2012). The host shutoff effect of PA-X contributes to the modulation of viral pathogenesis and facilitates virus replication (Hayashi, MacDonald, & Takimoto, 2015).

Besides the three polymerases, NP as an RNA-binding protein is also a major component of the vRNP. NP encapsulates the RNA genome, binds to the viral RdRp, and has a vital role in viral replication and transcription. The template-associated NP as an elongation factor is essential in completing the processivity of RdRp during the replication of the vRNA (Turrell, Lyall, Tiley, Fodor, & Vreede, 2013).

The primary viral membrane proteins HA and NA are protruding on the surface of the viral envelope, which is derived from the host lipid membrane. The genetic and antigenic properties of the HA and NA are used to classify the IAV into subtypes. There are 16 HA (H1-H16) and 9 NA (N1-N9) subtypes identified in IAVs in aquatic birds (Yoon, Webby, & Webster, 2014). In addition, two HA and NA subtypes have been isolated from bats (Tong et al., 2013). The constant mutations on HA and NA proteins are referred to as antigenic drift, which is in response to the pre-existing antibody-mediated immunity in the host due to the selection pressure posted by previous natural infections or vaccinations. The direct changes altering the antigenicity of HA or NA of currently circulating IAV is known as antigenic shift, and these changes are often due to the reassortment between two different strains of the same type of virus, or between two more diverse viruses. (Krammer et al., 2018). Antigenic shift is likely to result in influenza A pandemics

because of the lack of pre-existing antibodies in the population to the antigenically distinct influenza viruses. Antigenic drift and shift are the two processes responsible for the frequent updates of influenza vaccines to ensure the matching of the vaccine strains and the newly emerging viruses. Aside from the antigenicity of HA and NA, they each have unique roles in the virus lifecycle. Specifically, HA is responsible for the binding to the sialic acid receptor located on the cell surface and fusion of the viruses to the host cellular endosomal membrane. The sialidase enzymatic activity of NA allows the cleavage of the sialic acids on both host cells and the viral glycoproteins, which results in the proper release of the newly synthesized virions and the prevention of aggregated nascent virus particles, respectively (Taubenberger & Kash, 2010).

1.1.2 IAV life cycle

Influenza virus enters the cells through binding to the sialic acid receptor on the cell surface, followed by uptake into the cell through receptor-mediated endocytosis. After the fusion of the viral membrane to the host endosomal membrane, the vRNP complexes are released into the cytoplasm and are imported into the nucleus, where the viral transcription and replication takes place. Viral mRNA is exported into the cytoplasm for viral protein translation, and some nascent viral proteins are imported back to the nucleus to assemble new vRNP complexes. Newly-synthesized vRNPs are exported from the nucleus along with other viral proteins remaining in the cytoplasm to assemble new viral particles and bud out from the host cells (Figure 1.3).

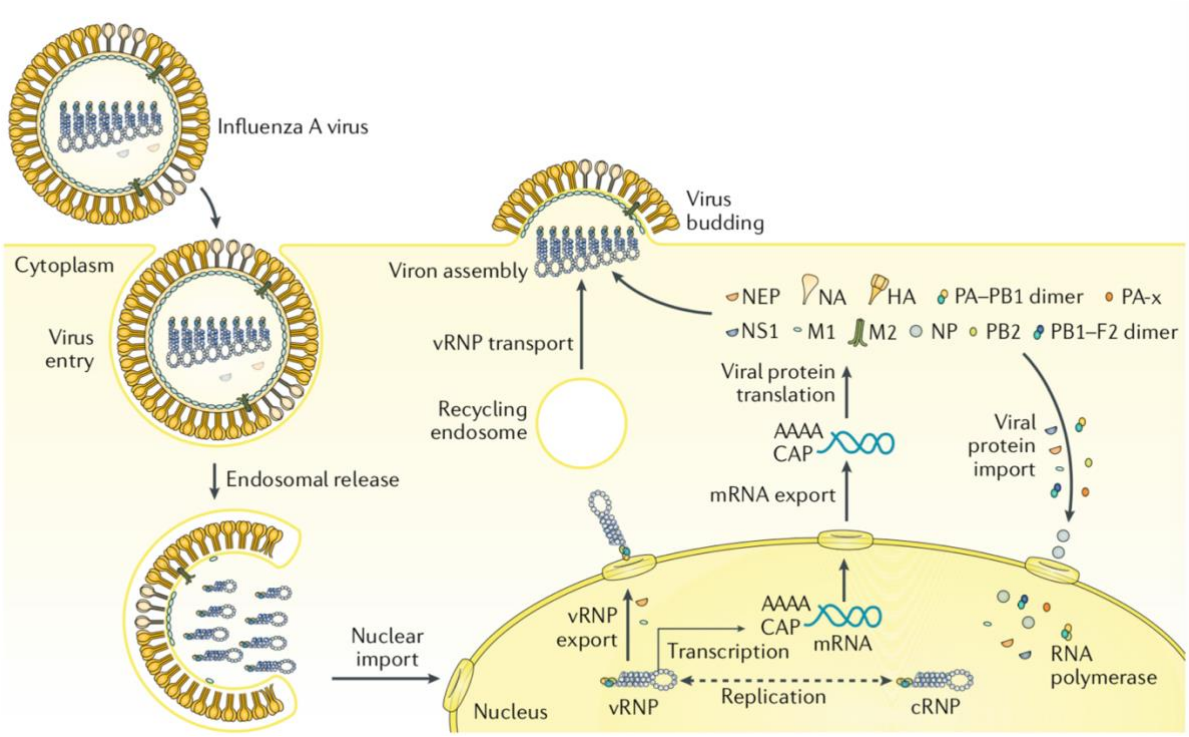

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Figure 1.3 Influenza life cycle.

Influenza virus enters the cell through receptor-mediated endocytosis, followed by the release and the import of viral genome into the nucleus for the transcription and replication of the viral genome. Viral mRNAs are exported into the cytoplasm for viral protein translation, and the translated viral proteins along with the newly synthesized vRNPs are assembled into new virions. NEP, nuclear export protein; NA, neuraminidase; HA, hemagglutinin; M1, matrix protein; M2, membrane protein; NS, nonstructural protein; NP, nucleoprotein; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2; PA, polymerase acidic protein.

1.1.2.1 Entry and endosomal fusion

IAV initiates the infection by binding of the viral glycoprotein HA to cell surface glycoconjugates containing sialic acid residues. This binding might not be sufficient for further internalization of the virus particle, because multiple HA-adhering events on the cell surface are required for the stable association between influenza viruses and host cells. This insufficient

binding event leads the individual HA to "browse" across the neighboring receptors, and this "browsing" behavior is referred to as the receptor exchange mechanism of HA. The receptor exchange mechanism mediated by the sialidase activity of NA directly drives the translocation of the influenza virus particles on the cell surface between receptors until enough number of HA-receptor complexes are formed. In this way, the virus is tightly bound to the cell surface (Sakai, Nishimura, Naito, & Saito, 2017)

Influenza viruses' replication efficiency in specific species depends on their specificity towards different sialic acid (SA) linkages. Human influenza viruses have a preference for the receptors with α-2, 6-linked SA, while HAs from avian influenza viruses have a higher affinity for the α-2, 3-linkage (Matrosovich et al., 2000; Rogers & Paulson, 1983). In agreement with the findings of the viral specificity, the α-2, 6-linked SAs are abundant in human tracheal epithelial cells, while α-2, 3-linked SAs are presented in the lower respiratory tract. Pigs have similar α-2, 6-linked and α-2, 3-linked SA distribution as humans in the respiratory tract (Rajao, Vincent, & Perez, 2018). In ducks and chickens, α-2, 3-linked SAs mostly exist in the gut epithelium, and both 2, 6-linked and α-2, 3-linked SAs are presented at the respiratory and intestinal tract in all birds (Franca, Stallknecht, & Howerth, 2013; Nelli et al., 2010; Shinya et al., 2006). Mouse, as a commonly used laboratory model to study influenza pathogenesis, has α-2, 3-linked SA receptors on the apical membrane of the mouse, which greatly resembles the receptor distributions on human ciliated airway epithelial cells (Ibricevic et al., 2006). While in the lower respiratory tract, unlike the human, mouse showed a significant amount of α-2, 3-linked receptors but few 2, 6-linked receptor in the lung (Wasik et al., 2017). The abundance of 2, 3-linked receptor in mice makes it an ideal model to evaluate the pathogenesis of influenza viruses of avian origin.

The expression of avian influenza virus receptor (α-2, 3-linked SA) or human influenza virus receptor (2, 6-linked SA) in the human brain also explains systematic diseases associated with some influenza infections. Kim et al. described that the majority of neutrons and astrocytes in the human cerebral cortex, brainstem, hippocampus, and cerebellum showed expression of both avian and human virus receptors (M. Kim et al., 2013).

The receptor association mediated by HA leads to the endocytosis of the virus particles. Once the viruses enter the endosome, the low pH conditions in the endosome lead to a drastic conformational change in pre-cleaved HA proteins on the viral surface. The cleavage of HA allows the exposure of the fusion peptide, and the fusion peptide inserts into the endosomal membrane

upon pH change. Meanwhile, the C-terminus of HA anchors in the viral membrane. The fusion peptide aligns antiparallel to the membrane-anchored HA domain and brings the endosomal membrane and the viral membrane in close proximity leading to fusion (Bullough, Hughson, Skehel, & Wiley, 1994). After the fusion, the low pH condition inside the virus enables the influx of H^+ ions through the ion channel M2 protein into the virus particle, which results in the release of vRNPs to the cytoplasm (Shimbo, Brassard, Lamb, & Pinto, 1996).

1.1.2.2 Genome transcription and replication

Once the vRNP complexes are released into the cytoplasm, they have to rely on an active nuclear import mechanism due to their large sizes. The import of the vRNPs is adequately and efficiently dependent on the nuclear localization signals (NLSs) on NP, although the NLS is located in all polymerase proteins. The importin α binds directly to the NLS on NP and then recruits importin β to form a complex; importin β docks at the nuclear pore and facilitates the translocation of the vRNPs from the cytoplasm into the nucleoplasm (Gorlich, Vogel, Mills, Hartmann, & Laskey, 1995). Inside the nucleus, vRNA transcription and replication are accomplished by RdRp. The transcription is initiated with the stealing of the 5'-cap, which is around 10 to 13 nucleotides in length, from the host noncoding RNAs, preferentially promoter-associated capped small RNAs and pre-mRNAs, and this process is known as cap-snatching (Gu et al., 2015; Koppstein, Ashour, & Bartel, 2015). The cap-snatching process relies on the cap-binding function of the PB2 protein and the endonuclease function of the PA protein. The transcription of vRNA to mRNA depends on the polymerase function of PB1 protein. The transcription starts on the 3' end of the vRNA and elongates until approximately 16 nucleotides before the 5' end, where the polyadenylation signal is generated. One of the widely accepted models proposed that RNA polymerase synthesizes mRNA from 3' to 5' end and is unable to read beyond the poly-uridine stretch due to the spatial hindrance, which results in the addition of poly(A) tail at the 3' end of the newly synthesized mRNA (Palese P, 2013).

Different from the primer-based initiation mechanism of mRNA synthesis, vRNA replication intermediate cRNA is synthesized in a primer-independent process, which relies on the accurate complementation of free rNTP to the 3' end of the vRNA. Nascent cRNA associates with newly synthesized polymerases and NP protein to incorporate into the cRNP complex. The cRNA then serves as the template to generate progeny vRNA complex (York, Hengrung, Vreede, Huiskonen, & Fodor, 2013).

1.1.2.3 Genome packaging and budding

Besides viral polymerase proteins and NP protein, which are imported into the nucleus for vRNP assembly, NS1, M1, and nuclear export protein (NEP) are also translocated into the nucleus. NEP and M1 play essential roles in vRNP nuclear export and prevention of re-import of vRNP into the nucleus through blocking the NLS signal on NP protein (Bui, Whittaker, & Helenius, 1996; York et al., 2013). Other nascent viral proteins, including HA, NA, and M2, are transported to the endoplasmic reticulum (ER), processed by post-translational modification in the Golgi apparatus, and translocated to the plasma membrane by translocon, which refers to the protein complex that facilitates the translocation of polypeptide across the membrane (Schnell & Hebert, 2003). The transmembrane domain of HA and NA both contain the apical targeting information which directs the two glycoproteins to the plasma membrane (Mora, Rodriguez-Boulan, Palese, & Garcia-Sastre, 2002). The export of the vRNP complex is mediated by M1 protein that associates with both the vRNP complex and the transmembrane domain of HA and NA. Thus, M1 protein is crucial in recruiting the viral components to the viral assembling site at the host plasma membrane (Nayak, Hui, & Barman, 2004).

Upon viral packaging, the precise mechanism of packaging eight vRNPs in each viral particle is vital to further viral replication. The widely acknowledged model is *the selective incorporation model*, which suggested that there were unique packaging signals located on the coding region and noncoding region at both the 3' and 5' end of each vRNA segment; thus every virion is able to incorporate a full set of 8 vRNPs (Hutchinson, von Kirchbach, Gog, & Digard, 2010). Virus budding starts with the outward curvature of the lipid raft region on the plasma membrane, where viral HA and NA are located. The final step of viral budding is the scission of virion off the plasma membrane. Besides viral glycoproteins HA and NA, M2 also plays a vital role in virus budding and scission. By interacting with M1 protein, M2 is recruited to the viral budding site, where the membrane-proximal amphipathic helix domain on M2 alters the membrane curvature, and this process further leads to the budding and releasing of the virus particle (Rossman & Lamb, 2013).

1.2 IAV HA

HA is the most abundant glycoprotein in the influenza virus and is responsible for viral attachment and subsequent fusion of the viral membrane to cellular membranes. Among the four

types of influenza viruses (A, B, C, and D), influenza A viruses are divided into subtypes based on the two viral glycoproteins: HA and NA. There are 18 known HA subtypes (H1-H18) and 11 known NA subtypes (N1-N11). Based on the primary sequence of HA, IAV could be categorized into two phylogenic groups, group I and group II , and each group contains various viral subtypes: group I includes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; and group II contains H3, H4, H7, H10, H14, and H15 subtypes (Gamblin & Skehel, 2010; Y. Wu, Wu, Tefsen, Shi, & Gao, 2014).

1.2.1 HA structure and antigenic sites

HA is a trimeric rod-shaped molecule with its C-terminus inserted into the viral membrane. The first X-ray crystallographic structure of the HA ectodomain derived from H3N2 virus (A/Aichi/68) was solved in the early 1980s and was the largest biological molecule that had a resolved structure(Wilson, Skehel, & Wiley, 1981). Crystal structures of numerous HAs of different subtypes of influenza A virus have been solved now. Although the amino acid sequence similarity is less than 50% between them, the structure and even the function of these HAs are unbelievably conservative. Moreover, the structure and function of HA in influenza A and B virus is also similar though with only 25% of amino acid sequence identity (Palese P, 2013).

HA is synthesized as a polypeptide HA0, and the cleavage of HA0 into HA1 and HA2 is essential for activating the infectivity of influenza virus, because the cleavage of HA0 exposes HA2, which plays a vital role in the virus intake process after absorption (Figure 1.4). The initiation of the HA cleavage occurs extracellularly after the release of viruses from the cells. The cleavage of HA is essential for virus infectivity owing to the exposure of the N- terminal fusion peptide located at the N-terminus of the HA2 peptide, which allows the fusion between cells and viruses during infection (Steinhauer, 1999). HA protein is synthesized as a precursor protein (HA0), and it requires to be cleaved at a single arginine or rarely a single lysine site in order to be activated for most avian and mammalian influenza viruses (Bottcher-Friebertshauser et al., 2010). HA0 is cleaved into two subunits HA1 and HA2, which are linked by a disulfide bond. The identified HA activators are trypsin or various trypsin-like proteases, such as plasmin, the tryptase Clara in murine bronchiolar epithelial cells, the tryptase from mast cells of porcine lung, and the protease from chicken allantoic fluid (Gotoh, Yamauchi, Ogasawara, & Nagai, 1992; Kido, Okumura, Yamada, Le, & Yano, 2007; LeBouder et al., 2008). Besides the HA activators from animals, proteases in human airways includes TMPRSS2 (transmembrane protease, serine 2, also

known as epitheliasin) and HAT (human airway trypsin-like protease) that are capable of HA cleavage at a single arginine site (Bottcher et al., 2006). Due to the characteristics of these proteases, virus replication is restricted to the respiratory tract in the mammalian host, where these proteases are located. Some of the highly pathogenic avian influenza (HPAI) viruses have multiple basic cleavage sites that could be cleaved by ubiquitously expressed subtilisin-like proteases including furin and PC6. This adaptation change increased the pathogenicity of HPA1 viruses significantly, allowing them to spread systematically in susceptible poultry and humans, and even jump from poultries to mammalian hosts (Horimoto & Kawaoka, 2001).

HA1 is the antigenic region of HA and is highly susceptible to develop mutations under the selection pressure of pre-existing antibodies. However, the receptor binding site (RBS) located on HA1 is highly conserved among different strains of influenza A viruses. HA RBS consists of four loops named after their position on the amino acid sequence: 130-loop, 150-loop, 190-helix, and 220-loop. Besides the RBS, the HA stem region, which is primarily the HA2 region with a small part of the C-terminus of HA1, is also conserved among influenza strains (N. C. Wu & Wilson, 2017).

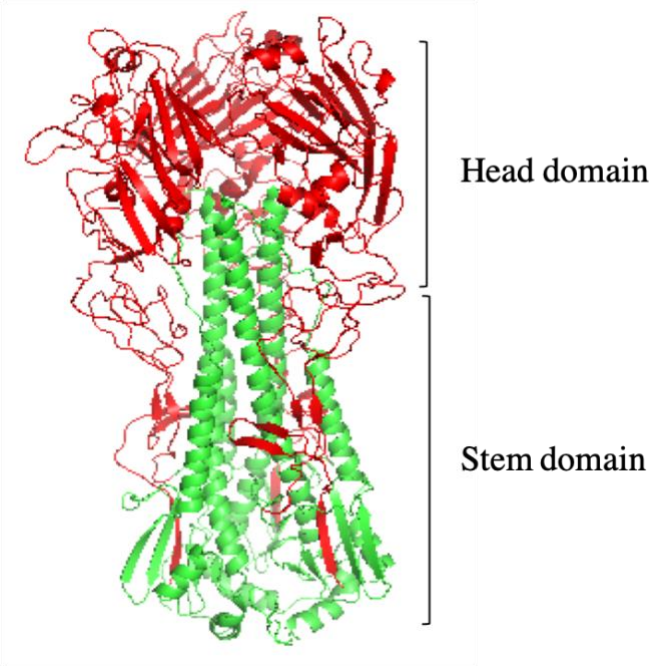

Figure 1.4 The 3D structure of influenza HA.

The trimeric structure of influenza Globular head region (red) and stem region (green). Structure was derived from the HA sequence of A/Alberta/01/2014 (H5N1) virus by SWISS Model and was plotted by PyMOL software.

There are five major antigenic sites in the globular head for H1 HAs: Sa, Sb, Ca1, Ca2, and Cb, and these antigenic sites are all surrounded by the sialic acid binding sites. The locations of Sa, Sb are on the distal tip of each HA monomer, while the remaining three antigenic sites are located near the stalk region (Xu, McBride, Nycholat, Paulson, & Wilson, 2012) (Figure 1.5). The five major antigenic sites have been discovered and mapped by locating the mutations on HA of the mutant virus that can escape the neutralization by a monoclonal anti-HA antibody. The antigenic sites on A/Puerto Rico/8/34 (PR8) virus were mapped with 34 antigenically unique PR8 mutant viruses, 58 monoclonal anti-HA antibodies, and the resolved crystal structure of H3 HA as a reference (Gerhard, Yewdell, Frankel, & Webster, 1981). These five regions have been designated as antigenic sites of HA because epitopes on the monoclonal antibodies elicited by full-length HA antigen were mostly mapped to those regions. Besides, these regions also fulfill the definition of discrete antigenic sites as any antibody binding to one site is not affected by the mutations that occurred in neighboring antigenic sites (Caton, Brownlee, Yewdell, & Gerhard, 1982).

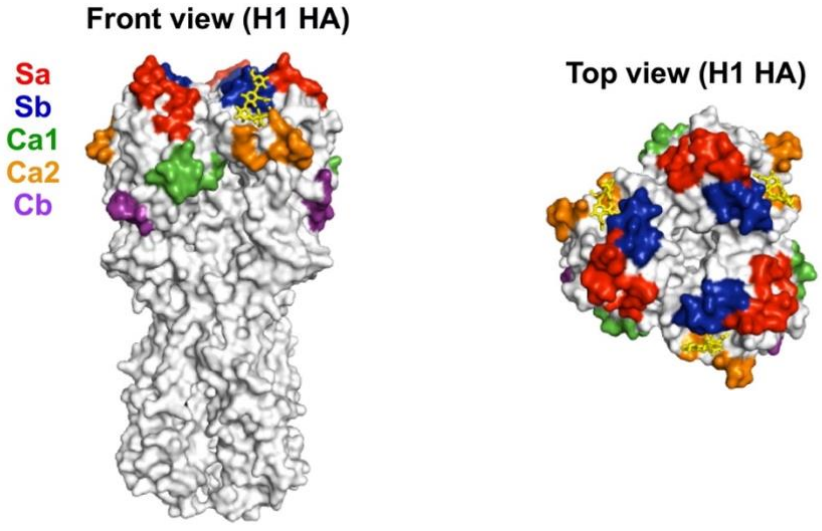

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Figure 1.5 Major antigenic sites on H1 HA.

The front and top view of the location of major antigenic sites Sa, Sb, Ca1, Ca2, and Cb on HA of H1 influenza viruses. The trimeric HA structure model is based on the HA of A/California/04/2009 virus. The location of each antigenic sites is color-coded: Sa, red; Sb, blue; Ca1, green; Ca2, yellow; and Cb, purple.

Antigenic site Sa and Sb are slightly overlapping in a spatial arrangement on the HA globular head; thus, they cannot be associated with antibodies simultaneously. The site Sa extends down along the β sheet structure on the opposite side of the HA to the RBD, while the site Sb occupies the region beside Sa in close proximity to the RBD. Site Sa includes residues 128, 129, 158, 160, and 162 to 167 (excluding residue 164), while site Sb comprises residues 156, 159, 192, 193, 196, and 198. Subsites Ca1 and Ca2 are located on the opposite side of the HA monomer. Subsite Ca1 contains residues 169, 173, 207 and 240, and the other subsite, Ca2, includes residues 140, 143, 145, 224, and 225. Cb as a region near the bottom of the HA globular head is comprised of residues 78 to 83 (excluding residue 80) and residue 122 (Caton et al., 1982).

The induction of different types of monoclonal antibodies targeting different antigenic sites on HA varies between influenza subtypes and host species (Matsuzaki et al., 2014; W. Sun et al., 2019). The HA inhibition active monoclonal antibody of the antiserum obtained from H1 influenza-infected mice is targeting Sb and Ca2 region of the HA. However, HA inhibition active antibody of infected ferrets is predominantly towards site Sa. In the adult human, the antibody pattern is yet different from in mice and ferret, the HA inhibition activity is significantly affected by site Sb and Sa on the antibody. The association of HA inhibition activity of the antigenic sites targeted by monoclonal antibodies has been defined to guide vaccine development (S. T. H. Liu et al., 2018).

1.2.2 HA glycosylation

To ensure the efficiency of the subunit influenza vaccine, it is critical to obtain functional viral antigens. Posttranslational modifications regulate functions of proteins by modulating their synthesize process. Among post-translational modifications, glycosylation is considered as one of

the most common and important modifications during protein synthesis (Jayaprakash & Surolia, 2017; Moremen, Tiemeyer, & Nairn, 2012). Glycosylation plays a significant role in protein conformation, secretion, stability, solubility, and antigenicity (Mimura et al., 2018; Ryan & Cobb, 2012; Scott & Panin, 2014). Although glycosylation seems to have profound effects on protein characteristics, it is controversial how glycosylation of HA in a subunit influenza vaccine would affect vaccine immunogenicity. On the one hand, glycosylation facilitates HA maturation and trimerization (Tate et al., 2014). Non-glycosylated HA antigen induced significantly lower virus-specific antibodies *in vivo* comparing to the N-glycosylated HA immunogen, indicating glycosylation plays a crucial role in the immunogenicity of HA-based vaccine (Hutter et al., 2013). Furthermore, high titres of neutralizing antibody and superior protection activity were observed after immunization with HA antigens expressed in yeast, insect and mammalian cells, which proved that the glycosylated viral HAs could be high efficacious immunogens (Hu et al., 2019; Kopera et al., 2019; Y. Wang, Zhang, et al., 2017).

On the other hand, antibodies induced by HA antigen with only a single N-linked glycan at each glycosylation site had better receptor binding affinity and greater neutralizing ability comparing to antibodies raised against fully glycosylated HA protein (C. C. Wang et al., 2009). Moreover, HA antigens produced in a bacterial system (*E. coli*) that are commonly believed to have no glycosylation, were proved to be strongly immunogenic and used as promising candidates for supplying vaccine stocks against newly emerging influenza viruses, especially pandemics. HA antigen of A/H1N1/2009 virus produced in *E.coli* appeared to be correctly folded and exerted protective activity against the homologous virus challenge in the ferret model (Aguilar-Yanez et al., 2010).

1.2.3 Current HA expression strategies

A recombinant HA (rHA) vaccine as a promising subunit vaccine candidate could largely shorten the vaccine manufacturing time comparing to the egg-based approach. It requires lower standards of facility infrastructure during vaccine production. Particularly in the production of HPAI vaccine, the rHA strategy avoids the requirement for level 3 facilities and equipment. rHA proteins have mainly been produced in three expression systems: bacterial cells, insect cells and mammalian cells.

Currently, HA1 subunit, HA2 subunit, and HA ectodomain have all been expressed in bacterial cells (Bommakanti et al., 2010; Farsad, Malekzadeh-Shafaroudi, Moshtaghi, Fotouhi, &

Zibaee, 2016; Saczynska et al., 2017). Relatively short production time and low maintenance cost have made bacteria-produced antigens a novel competitive way for vaccine production, while the potential concerns of antigens produced in prokaryotic cells are the proper folding and the glycosylation of antigens. HA1 of 2009 pandemic H1N1 produced in bacterial cells fused with bacterial flagellin (a TLR5 ligand) elicited robust neutralizing antibodies response and protected mice from a lethal challenge with homologous virus (Liu et al., 2011). Similarly, recombinant flagellin-HA1 vaccine of H5N1(A/Vietnam/1203/04) induced high levels of HA-specific antibody and reduced nasal virus shedding in ferrets, and this flagellin-HA1 vaccination protected mice and ferrets against homologous viral infection (Liu et al., 2012; Song et al., 2009). Besides the HA1 subunit vaccine, bacterially expressed HA ectodomain of H1N1 (A/California/07/2009) significantly reduced viral loads in the upper respiratory tract after homologous viral challenge in the ferret (Khurana et al., 2010). Recombinant HA0 of H5N1 (A/Vietnam/1203/2004) virus expressed in *E.coli* elicited high levels of HA-specific IgG after three injections of 15 μg rHA antigen (Biesova et al., 2009) in mice.

Moreover, recombinant proteins produced in mammalian cells acquired similar posttranscriptional modifications to that generated in the egg-based production system, which results in generation of fully glycosylated proteins. Various research has reported that eukaryotic HA head or ectodomain induced robust antigen- specific immune responses and provided superior protection rates against viral challenges (H. Lu et al., 2011; Pua et al., 2017). HA1 of H5N1 (A/Anhui/1/2005) virus fused with Fc fragment induced high neutralizing antibody titres against heterologous H5N1 strains and provided complete protection against lethal dose of heterologous H5N1 challenges in mice (Du et al., 2011). Immunization with HA ectodomain of H5N1 virus produced in a mammalian system (A/Thailand/KAN-1/2004) with Ribi adjuvant in mice induced similar levels of serum IgG antibodies to that generated with inactivated influenza H5N1 inactivated split vaccine (Wei et al., 2008). HA0 and HA1 proteins of H5N1 (A/Vietnam/1203/04) were produced in 293 Flp-In cell lines and were able to elicit homologous and heterologous H5N1 neutralizing antibodies in rabbits (H. Lu et al., 2011). The overall manufacturing time of a rHA antigens from cloning, transfection, stable cell line generation and protein purification could be accomplished within 5-6 weeks, suggesting that mammalian rHA vaccines have the potential to become a modern approach to pandemic influenza outbreaks (H. Lu et al., 2011).

Similar to the mammalian expression system, antigens produced in insect cells are scalable, reproducible and were minimum endotoxin level, and the manufacturing process does not require high-levels of bio-containment facilities (M. M. Cox & Hollister, 2009). Vaccine antigens produced in insect cells also have posttranslational modifications, which may contribute to a better antigenicity of the protein. However, the HA antigen alone have a relatively low immunogenicity as a vaccine candidate and may require a prime-boost process or additional adjuvants to achieve protective immunity. FluBlok® as the first non-egg-based vaccine licensed by World Health Organization (WHO) is produced in a baculovirus-based insect cell expression system (M. M. Cox & Hollister, 2009). Clinical data of FluBlok® showed a stronger protection against influenza infection, especially in people with relatively weak immune responses. Besides the efficient commercialized recombinant subunit influenza vaccine, a study also proposed that a baculovirus-based vaccine containing the rHA of the highly pathogenic H7N9 strain (A/Chicken/Jiaxing/148/2014) significantly reduced viral shedding and viral replication in vaccinated chickens compared to the inactivated whole virus of the same strain; the rHA antigen also fully protected immunized chickens from homologous virus challenge (Hu et al., 2019).

1.3 Overview of influenza vaccines

It has been 100 years since the first pandemic of influenza happened in 1918. Due to the constant mutation of the virus, it is impossible to eliminate the disease completely. Currently, the best intervention is to prevent the infection and spread of the virus by vaccination. The first influenza vaccine has been developed in the Soviet Union in 1936 attempting to vaccinate with a live-attenuated influenza virus. This vaccine has been produced by continuous passage of the virus in eggs for over 30 times; as a result, the virus is only capable of causing mild infection, and the vaccinated subjects were protected from the reinfection of the same virus (Hannoun, 2013). Ever since then, the live-attenuated vaccines have been used for over 50 years in the Soviet Union. In the meantime, the inactivated virus vaccines have been developed in England and in the USA. For the development of inactivated virus vaccines, a large quantity of viral antigen is obtained from the virus propagated in allantoic fluid followed by formalin inactivation. All influenza vaccines were produced in an egg-based manufacturing system until 2012, when the cell-based vaccine Flucelvax® was approved by the US Food and Drug Administration (FDA) (Harding & Heaton, 2018). Live attenuated vaccines use reassortment techniques to produce recombinant viruses with

six segments from a well-adapted PR8 virus with high growth rate and the HA and NA segments from the circulating viruses. These recombinant viruses possess a high yield property to facilitate the mass production and bear the antigenic property targeting the circulating viruses (Wong & Webby, 2013).

Although the influenza vaccines are provided annually to the public, the variation of the effectiveness of the vaccines is attributed to two main reasons: the characteristic of the recipient (age, health conditions) and the matching of the vaccinated strains to the circulating strains. The overall effectiveness of the influenza vaccines, judging by the risk of influenza illness reduced by vaccines, is between 40% to 60% when the circulating viruses are well-matched to the vaccinated ones (CDC, 2019d). However, influenza vaccines may have reduced effectiveness in immune-naïve young children or the elderly with decreased immune functions.

To minimize the mismatches between the vaccine viruses and the circulating viruses and to improve the efficacy of seasonal vaccines, the WHO established a surveillance system to monitor the circulating influenza strains worldwide in several countries in 1952. This system allows the detailed analysis of influenza A (H3N2 and H1N1) viruses and influenza B viruses circulating in human or animal species, particularly in birds and pigs, which could facilitate the cross-species viral transmission. Upon the discovery of new viral strains, the WHO will evaluate the antigenicity and the epidemiological behavior of the viruses, followed by the analysis of the viral sequences. The surveillance data are reviewed by the WHO investigators twice a year to determine which strains would possibly be circulating in the next flu season. Based on these reviews, the experts in WHO will decide which strains would be the best match and would be incorporated in this year's seasonal flu vaccine (Gerdil, 2003).

1.3.1 Types of influenza vaccines and commercial production

The ultimate goal of influenza vaccines is to protect individuals from influenza infection and to provide an overall immunity to limit virus spreading within the population. There are three commonly used vaccines for influenza virus: the inactivated vaccine, the live attenuated vaccine and the recombinant subunit vaccine. Current seasonal vaccines on the market are mainly inactivated vaccines and live attenuated vaccines.

1.3.1.1 Inactivated influenza vaccine (IIV)

There are three main types of inactivated influenza vaccine: whole inactivated virus vaccine, split virus vaccine and inactivated subunit vaccine.

In whole inactivated virus (WIV) vaccine, the virus-containing fluid is chemically inactivated by formaldehyde or β-propiolactone, whereas in split virus vaccine, the virus is split by reagent such as ether, de-oxy-cholate, and detergents, such as Triton X-100 (Kon et al., 2016). In WIV vaccines and split virus vaccines, the chemical inactivators or splitting reagents are removed before sterile filtration and formulation. For inactivated subunit vaccines, the production also starts with the amplification of influenza viruses in embryonated eggs, followed by the inactivation of the virus and the stripping of the HA and NA protein from the lipid membrane by non-ionic detergents like Triton N101. After stripping, HA and NA could be efficiently separated from the viral core by density gradient centrifugation (Brady & Furminger, 1976). This additional purification step is required during the production of the subunit vaccine to remove the components necessary for viral replication, while the viral glycoproteins, which act as the major immunogens, would be purified out. Because of this purification step, a subunit vaccine contains no internal viral protein in contrast to WIV and split virus vaccine, which makes the inactivated subunit vaccines less reactogenic than the two inactivated vaccines (Talbot et al., 2015). Among the three types of inactivated vaccines, considering immunogenicity and adverse effects of the vaccines, and complicity of the vaccine production procedures, split virus vaccine is superior to the others. Thus, split virus vaccines have commonly been used as seasonal influenza vaccines.

The adverse events of vaccines may affect a considerable number of recipients since vaccine coverage in many countries is increasing rapidly. Although the WIV vaccines are capable of inducing a robust immune response, they are also very reactogenic, which results in mainly local injection site infection and febrile illness (Tong et al., 2013). Thus, WIV vaccine now is mostly replaced by split virus vaccine; the disruption of influenza virions significantly reduces the adverse reactions in the recipients and increases the safety of the flu vaccine. However, mild local reactions, including pain and redness in the injection site, are still associated with split virus vaccine, and they occur in over 25% of the elderly recipients and approximately 50% of the healthy adults (R. J. Cox, Brokstad, & Ogra, 2004).

The most widely applied seasonal influenza vaccine is the split virus vaccine in quadrivalent form, which is composed of 4 influenza strains that are predicted to be circulating in the upcoming flu season. Quadrivalent inactivated vaccine was licensed in 2012 and contains four types of virus strains: two influenza A strains (H1N1 and H3N2) and one or two influenza B types (Yamagata and Victoria). These vaccines are standardized by the quantity of hemagglutinin

contained in the vaccine. The initial splitting reagent is ether; however, ether is reported to have local toxicity (skin or eyes irritation) and to interfere with the HA quantification procedure. Consequently, ether is replaced by other splitting reagents in commercial vaccine production, such as de-oxy-cholate (Afluria, Fluarix®, and Flulaval®) and Triton®X-100 (Fluarix®, Flucelvax®, and Fluzone®) (Prevention, 2015). For adults, there are commonly 15 µg of HA per strain in seasonal flu vaccines; whereas for the elderly, higher doses (60 µg of HA per strains) of vaccines are licensed (DiazGranados et al., 2014). Inactivated vaccines have an excellent safety record, and it is recommended to a broad range of recipients. Different inactivated vaccines are approved to apply to different age groups because certain age groups are at high risk of developing severe complications from influenza infection. Children aged 6 month to 8 years who have never been vaccinated before or people aged 65 years and older, due to their weak self-immunity, requires high doses of vaccines or adjuvanted vaccines that could provide stronger immune responses following vaccination (CDC, 2019b, 2019c). The most frequently used quadrivalent inactivated vaccine Fluzone® (Sanofi Pasteur) is applied to children older than six months of age, the elderly, pregnant women, and people with chronic health conditions.

IIV vaccines are capable of inducing serum antiviral responses, which is predominantly the increase of serum IgG levels. The serum IgG level is detected by enzyme-linked immunosorbent assay (ELISA), and viral-specific neutralizing antibody in the serum is identified by the hemmaglutination inhibition (HI) assay. The serum antibody appears to be effective in neutralizing the upcoming viruses in the infection site, which is on the mucosal layer of the respiratory tract (Wagner et al., 1987).

1.3.1.2 Live attenuated influenza vaccine (LAIV)

Although LAIV has been used in the early 1930s in the Soviet Union, the vaccine was licensed in the US in 2003 for 2 - 49 years old recipients, and not until 2012 the vaccines were recommended to be used in Europe for children aged 2 -17 years (R. J. Cox, 2013; Sridhar, Brokstad, & Cox, 2015). The development of LAIV is based on HA and NA from the specific virus strains predicted by WHO to be circulating in the next flu season and the backbone from an attenuated, cold-adapted virus. There are two different cold-adapted virus development technologies: First, the backbones used in Russia (Ultravac®) and India (Nasovac-S®) are A/Leningrad/134/17/57 (H5N2) and B/USSR/60/69 for influenza A and B viruses, respectively (Rudenko, Yeolekar, Kiseleva, & Isakova-Sivak, 2016). Second, Ann Arbor backbone produced

by AstraZeneca in the UK by serial passaging the WT A/Ann Arbor/6/60 strain in primary chicken kidney cells at successively low temperature (Maassab, 1967). LAIV using the Ann Arbor backbone is commercialized in North America and Europe under the names FluMist® and Fluenz®, respectively.

The master donor virus, which provides the six internal gene segments except HA and NA segments, in LAIV is temperature sensitive. This virus could only replicate at 33°C, but its growth is restricted in temperature condition above 37.8°C; therefore, the replication of the attenuated virus incorporated in the vaccine is restricted to the human upper respiratory tract and does not replicate in lower respiratory tracts, where the temperature condition is over 37.8°C (Trombetta, Gianchecchi, & Montomoli, 2018). For this reason, there is less chance to develop severe influenza infections after immunization with LAIV. The master donor virus has a significantly low reversion rate after multiple passages in infected hamsters (Jin et al., 2003). The of LAIV is more efficacious than IIV vaccine, with 80% protection in children aged younger than six years old and 40% efficacy in adults (Sridhar et al., 2015).

LAIV is administrated intranasally (needle-free), and it elicits a longer-lasting and broader immune responses (humoral and cellular). Comparing to the conventional intramuscular delivery of seasonal influenza vaccines, the administration method of LAIV not only largely benefits young children with possible fear of needles, but also closely resembles the natural infection scenario of influenza virus. The LAIV immunization is capable of inducing various adaptive immune responses including high levels of serum antibody, strong mucosal responses, and cytotoxic T cells targeting influenza virus (Isakova-Sivak & Rudenko, 2015).

In comparison to IIV-induced serum IgG responses, LAIV immunization mainly elevates local immune responses associated with the secretion of antiviral IgA antibody. Virus-specific IgA is believed to protect the mucosal surface against viral infection by neutralizing the upcoming viruses or preventing viral attachment to the mucosal epithelium. Although plasma antibody is capable of preventing viral infection in lower respiratory organs, such as lung, it is not proved to be effective in protecting mice and ferrets from upper respiratory tract viral infection. In terms of disease transmission, mucosal IgA antibodies, but not plasma IgG antibodies, are more efficient in reducing viral shedding in nasal secretions after influenza infection (Renegar, Small, Boykins, & Wright, 2004).

However, LAIV has been reported to cause slightly more adverse reactions compared to IIV. Thus, LAIV is not recommended to young children under 2 years of age, the elderly, and the immunosuppressed people. Besides, an increased risk of asthma is reported to be associated with LAIV immunization in young children prone to develop asthma; thus, LAIV should not be administrated to any individual with asthma or children under 5 years of age with recurrent wheezing (Bergen et al., 2004; Kelso, 2012). Upon LAIV immunization, viruses could be isolated from nasal swabs in young children until seven days post-immunization, but it is undetectable over 14 days post-vaccination (R. Belshe, Lee, Walker, Stoddard, & Mendelman, 2004). The adverse effects associated with LAIV are not severe; these include running nose and sore throat on the day of immunization, and slight fever on 2 to 9 days post-vaccination. Moreover, research showed that the LAIV exerted lower effectiveness against H1N1 virus in some studies over several flu seasons since 2013 in the USA. Due to these adverse effects and inconsistent vaccine efficacy in the USA and Europe, the use of LAIV was not recommended for the 2016-2017 and 2017-2018 flu seasons in the US. The recommended use of LAIV was resumed for the 2018-2019 flu season (Caspard et al., 2016; CDC, 2019a; Trombetta et al., 2018).

In the case of seasonal influenza vaccines, IIV has been administrated annually as the injectable vaccine and LAIV has been recommended as the nasal spray on and off in the market. However, both IIV and LAIV are based on the egg-based manufacturing system, which takes up to 6 months to produce the vaccines. Upon emerging influenza virus explosions, egg-based manufacturing system would need a response time of several months, which would create a gap in the immunogenically naïve population and allow the spread of the emerging viruses. Thus, there is a need to generate influenza vaccines in a short amount of the time to respond to the antigenically distinct influenza virus explosions.

1.3.1.3 Recombinant subunit influenza vaccine

The majority of commercial influenza seasonal vaccines are inactivated or live-attenuated vaccines, but there are a few recombinant subunit influenza vaccines in the market. Recombinant subunit influenza vaccine contains purified viral glycoprotein HA or NA from recombinant protein expression systems. Recombinant proteins are expressed and purified from various systems including mammalian cells, insect cells, bacteria, and yeast; thus, the production of recombinant flu vaccine does not require chicken eggs (Gadalla, El-Deeb, Emara, & Hussein, 2014; Lei, Jin, Karlsson, Schultz-Cherry, & Ye, 2016; H. Lu et al., 2011). There are several advantages of

recombinant subunit vaccines over the current seasonal vaccines produced in embryonated eggs. Firstly, the recombinant proteins are produced in a scalable and reproducible process in various types of cells. Secondly, the manufacturing process is more rapid and costly effective than the conventional egg-based system, in the event of the emerging pandemic strains. Thirdly, recombinant subunit vaccine avoids the adaptation step required in the egg-based vaccine production system. This additional viral adaptation step in eggs is aim to acquire high levels of recombinant virus, which does not commonly replicate in avian species, for vaccines manufacture. Fourthly, the production of recombinant proteins does not require chemical inactivation or organic extraction procedures, which reduces the chance of residual toxicity in the vaccine (M. M. Cox, Patriarca, & Treanor, 2008).

FluBlok® is a recombinant HA vaccine developed by Protein Sciences Corporation. This rHA vaccine is a trivalent vaccine formulated with HA antigens from three influenza strains comprised of two influenza A viruses and one influenza B virus, which are selected to be included in the annual flu vaccine by the WHO around February every year. The three HA proteins incorporated in the recombinant subunit vaccine are produced in a continuous insect cell line derived from Sf9 cells of fall armyworm using baculovirus as vectors. After the expression, HA antigens are purified from serum-free media by column chromatography. The HA antigens in the FluBlok® vaccine are full-length HA proteins containing both HA1 and HA2 regions. FluBlok® contains 45 μg HA of each virus, which is three times the amount of antigen incorporated in a common inactivated influenza vaccine (M. M. Cox et al., 2008). Clinical data proved that FluBlok® provided stronger protection against influenza infection in the elderly and immuno-compromised people comparing to the inactivated influenza vaccine. Meanwhile, FluBlok® also induced an enhanced antibody response towards the influenza virus, which is comparable to that induced by IIV, in healthy adults. However, the increased amount of antigen in FluBlok® may also associate with a higher rate of local injection site pain, headache, muscle pain and fatigue (W. A. Keitel et al., 1996). Thus, FluBlok® vaccines are licensed by the FDA to be used in adults over 18 years old.

Despite the advantages in recombinant subunit vaccine production, drawbacks of the vaccine manufacture process remain. Comparing to the egg-based vaccine manufacture system that was established over 50 years, large-scale infrastructure for recombinant subunit vaccine production does not exist worldwide. Moreover, the subunit vaccine has relatively low

immunogenicity compared to conventional vaccines; therefore, prime-boost strategy and the use of adjuvants are necessary steps to improve the efficacy of recombinant subunit vaccines.

1.3.2 Universal vaccines

Although current influenza vaccines are efficacious and are the best countermeasures to influenza infection, the decline of existing neutralizing antibodies and the constant mutations in influenza viruses necessitates the annual updates of the seasonal influenza vaccines. Current influenza vaccines induce neutralizing antibodies towards the main antigen of influenza HA, which changes continuously due to the error-prone viral polymerases and the selection pressure posed by pre-existing antibody in the human population (human herd immunity). The development of a universal vaccine, which covers broader ranges of influenza strains, provides long-lasting protection and avoids the needs for annual vaccination. In the universal influenza vaccine strategic plan of the National Institute of Allergy and Infectious Diseases, they stated that a universal flu vaccine should provide protection against both group I and II influenza A viruses, and the vaccine needs to provide protection that lasts at least one year in all age groups (Coughlan & Palese, 2018).

To design a universal influenza vaccine providing broad cross-reactive immunity, the conservative proteins or domains of influenza virus are the promising candidates for vaccine development. Several approaches to develop universal influenza vaccines focused on the conserved region of HA protein, glycoprotein NA, the ectodomain of ion channel protein M2, and the combination of nucleoprotein NP and matrix protein M1 (Estrada & Schultz-Cherry, 2019; Nachbagauer & Krammer, 2017). There are three strategies to develop universal vaccines inducing immune responses against the conservated stem region of HA protein: the production of headless HA stem immunogens, immunization with chimeric HA viruses, and sequential immunization with the combination of live-attenuated vaccine and inactivated vaccine.

The reason for generating headless HA stem immunogens is that the stem region of HA is highly conserved among different influenza strains, which makes it an ideal candidate to induce broadly protective immune responses (Figure 1.4). However, HA stem in neutral pH is not exposed to the viral surface due to the steric hindrance of the HA head, which is an immunodominant region on HA. Thus, it is difficult to mount immune responses against HA stem region in a natural infection, and this necessitates the expression of HA stem alone *in vitro*. Stable mini-HA stem antigen based on HA from H1N1 (A/Brisbane/59/2007) derived from a mammalian system was capable of forming intermolecular trimers and binding to the known broadly neutralizing

antibodies, which confirms the proper folding of the protein. This mini-HA immunogen also completely protected mice from lethal challenge with heterologous and heterosubtypic viruses. Moreover, mini-HA stem immunogen expressed in a mammalian system significantly reduced fever after heterologous viral challenge in cynomolgus monkeys and reached a comparable effect compared to vaccination with trivalent seasonal influenza vaccine (Impagliazzo et al., 2015). However, there are some limitations of stem-based immunogens. The mini-HA stem immunogen has been reported to be effective for the group I influenza virus, but not group II or influenza B viruses (Krammer, 2015). Besides the relatively narrow protection ranges, antibodies induced by mini-HA stem might promote virus fusion, facilitate the intake of the virus, and enhance virus-associated respiratory disease (Khurana et al., 2013).

In the chimeric HA virus immunization strategy, viruses with chimeric HAs, consisting of the stem region of the same virus and the head domain of various heterologous or heterosubtypic viruses within the group I influenza A virus, are generated (Isakova-Sivak et al., 2018; T. T. Wang et al., 2010) (Figure 1.5). Repeated immunization with viruses containing chimeric HAs that express the same HA stem could induce enhanced immune responses against the stem region (Nachbagauer et al., 2017). In this study, mice were electroporated with a DNA vaccine encoding cH9/1 (HA head from an H9 isolate and stem from H1N1 virus) and were boosted with a baculovirus expressed cH6/1 (H6 head and H1 stem) protein followed by a repeated boost of baculovirus expressed cH5/1 (H5 head and H1 stem) protein. Immunization with the sequential chimeric HA vaccines protected mice from heterologous and heterosubtypic influenza viral challenges, suggesting that chimeric HA vaccination elicited broad immune responses against group I influenza viruses. However, chimeric HA vaccines could not protect mice from challenge with the H3N2 virus, which is an HA group II virus, suggesting that the chimeric HA failed to provide intergroup protection against influenza viruses (Krammer, Pica, Hai, Margine, & Palese, 2013).

Another strategy to develop immune responses towards conserved epitopes on HA is through a sequential immunization regime (Zhou et al., 2017). Researchers proved that initial immunization with LAIV vaccine, which significantly resembles the natural influenza infection, along with a boost with subunit vaccine would elicit a protective stem-derived antibody response (Nachbagauer et al., 2017). Moreover, ferrets vaccinated with LAIV vaccine expressing cH8/1 (H8 head and H1 stem), followed by a boost of inactivated whole virus vaccine of cH5/1 (H5 head

and H1 stem) developed reduced viral titres in the upper respiratory tract after H1N1 viral challenge, suggesting that the combination of prime-boost and chimeric HA strategy generated superior protection against heterologous viral challenges (Nachbagauer, Krammer, & Albrecht, 2018). One advantage of this strategy is that previous natural influenza infections in the host, which served as prime vaccinations, already induced some baseline stem-directed antibodies; thus, a prime vaccination might not be required within this strategy (Margine et al., 2013).

1.3.3 H5N1 vaccines

Since the first human infection case of avian influenza H5N1 virus reported in 1997 in Hong Kong, the avian-origin H5N1 virus has rapidly developed into various subclades and spread to more than 16 countries during 2003 to 2019 with over 50% motility rate in humans (Subbarao et al., 1998; WHO, 2019b). There has been a global effort since 2005 to develop vaccines against the avian-origin H5N1 virus and other influenza viruses of pandemic potential (K. R. Talaat et al., 2014). Both conventional and novel strategies have been used to develop effective anti-H5 influenza vaccines. There are 21 licensed human anti-H5 pre-pandemic vaccines available in the market until 2013; most of them are inactivated virus vaccine, and one of them is a live-attenuated vaccine (WHO, 2013).

Aside from the licensed H5 vaccines, many new pre-pandemic H5 vaccines are under development. Research showed that LAIVs might be more beneficial in providing protection against influenza pandemics due to their high replication efficiency and yield in eggs and their rapid induction of antiviral responses (Manuell, Co, & Ellison, 2011). One study on the production of the LAIV H5N1 vaccine showed that the cold-adapted H5N1 (A/Vietnam/1203/2004 and A/Hong Kong/213/2003) vaccines bearing avian H5 HA antigens were significantly attenuated and able to induce serum antigen-specific antibodies and neutralizing antibodies in healthy adults (Karron et al., 2009). Moreover, another study on LAIV H5N1 vaccine also stated that cold-adapted influenza vaccine with HA and NA from another H5N1 virus (A/Anhui/2/2005) could provide protective immunity against the highly pathogenic H5N1 challenge in a nonhuman primate model (Fan et al., 2009).

Other studies on inactivated H5N1 vaccines showed that adjuvanted H5N1 inactivated vaccine (Sanofi®) based on A/Vietnam/1203/2004 induced high levels of HA-specific IgG, but relatively low levels of serum neutralizing antibodies (Wong et al., 2017). A clinical study also demonstrated that an inactivated whole-virion vaccine derived from A/Chicken/Astana/6/05 of

H5N1 virus formulated with the aluminum hydroxide adjuvant was safe and well tolerated in the immunized individual, and a single immunization of adults with this inactivated vaccine appeared to be immunogenic (Sansyzbay et al., 2013).

New vaccine technologies, which utilize non-egg-based production methods, intended to rely on the production of recombinant HA of the avian H5N1 virus are being developed. Clinical data suggested that MF59-adjuvanted, cell culture-derived, subunit H5N1 vaccine with 7.5 μg of HA antigen derived from A/turkey/Turkey/1/05 (H5N1)-like strain (NIBRG-23) was strongly immunogenic in healthy adults or elderly subjects in 2 phase II studies (Frey et al., 2019).

These efforts in providing an economical, safe, inexpensive and effective pre-pandemic influenza vaccine will significantly broaden the scope of pandemic preparedness and create high-performance vaccines.

1.4 Common vaccine adjuvants

An adjuvant is a component used in some vaccines that could enhance the immune response induced by antigens in vaccinated people; in other words, the adjuvants significantly increase the immunogenicity of the vaccine. Traditional adjuvants such as aluminum salts have been used in human vaccines since 1932, and aluminum salts was the only adjuvant approved to be used in licensed vaccines for over 70 years (Marrack, McKee, & Munks, 2009).

Currently, the development of novel adjuvants for vaccine use is an area of high interest. Several factors need to be considered in the selecting of an ideal vaccine adjuvant, such as safety, tolerance, manufacturing process, shelf life, compatibility with viral antigen, and feasibility. Among these crucial factors, the benefit to risk ratio of adjuvants in specific vaccines is a prioritized criterion in adjuvants development (Brito, Malyala, & O'Hagan, 2013).

1.4.1 Functions of adjuvants

Several reasons are listed below for the use of adjuvants in vaccines.

Firstly, an adjuvant serves as an additional component that boosts the immune response in specific recipients with low reactions to regular vaccinations. This recipient population includes immune-compromised patients, young children who are immunogenically naïve to viral infections, and the elderly suffering from reduced immune responses. Adjuvant System 03 (AS03) and MF59 have been widely used as adjuvants in commercial influenza vaccines. AS03 adjuvant enhanced the antibody responses induced by the H1N1 2009 pandemic influenza vaccine (Arepanrix, GSK)

in patients with HIV infection (Yam et al., 2014). The MF59-adjuvanted trivalent subunit vaccine was more effective and induced higher antibody titres in children than the non-adjuvanted vaccine (Nolan et al., 2014).

Secondly, besides boosting the relatively weak immune responses in specific hosts, adjuvants are also capable of improving the immunogenicity of weaker antigens that are naturally less immunogenic. The conventional egg-based influenza seasonal vaccines, including LAIV and IIV, are based on viral antigens derived from the whole virions; however, the new approaches of subunit vaccines development often utilize recombinant proteins as antigens. The possible reason that some subunit vaccines are less immunogenic than the whole virion-derived vaccines is that pathogen-derived vaccines have other viral proteins in addition to the highly purified viral glycoprotein (subunit vaccines), and these additional proteins could enhance the immune responses generated by a single viral antigen.

Thirdly, adjuvants could efficiently reduce the frequency of vaccine administration and the doses of antigen incorporated in the vaccine. During pandemic influenza strikes, most vaccines need multiple administrations to reach ideal efficacy and provide protection to recipients. However, the addition of an efficacious adjuvant is capable of reaching the protectable threshold after only one vaccination. MF59-adjuvanted inactivated H9N2 virus induced similar levels of serum antibody to 2 doses of non-adjuvanted vaccine (Atmar et al., 2006).

Fourthly, adjuvants could also change the types of immune responses induced by viral antigen via altering the secretion pattern of cytokines and chemokines, which leads to differences in recruiting immune cells. Particularly, T cell responses could be changed drastically by formulation of different adjuvants with the same antigen. HA antigen pairing with MF59 or Alum adjuvant induced higher levels of serum IgG1 antibody and increasing numbers of IL-5 secreting T cells, suggesting the activation of Th2 biased cellular response, whereas vaccination consisting of the same influenza antigen combined with the cationic liposomal adjuvant CAF01 generated a Th1 and Th17 biased subpopulation of T helper cells (Knudsen et al., 2016).

Although adjuvants all have the ability to improve the efficacy of viral antigens by boosting the host immune system, the mechanism of individual adjuvant action is drastically different due to their diversified compositions. Some adjuvants could help soluble antigens to be retained at the injection site to prevent quick clearance of antigens by lymphatics. Insoluble adjuvants like Alum or oil-in-water emulsions including MF59, AS03, and AF03 could all lead to the retention of

antigens (Hutchison et al., 2012). MF59, AS03 could also elevate the local immune responses by increasing the recruitment of immune cells to the injection site either directly or indirectly through the release of cytokines and chemokines (Calabro et al., 2011; Morel et al., 2011). Moreover, adjuvants such as TLR agonists are able to stimulate the innate immune responses by activation of antigen-presenting cells; particularly pattern recognition receptors (PRR) (Choi et al., 2012; McCarron & Reen, 2009). PRRs, including toll-like receptors, rig-like receptors, and inflammasomes, are crucial in recognizing foreign antigens and inducing immune responses. Adjuvants trigger PRR recognition either directly by interactions or indirectly by inducing local damage that would be detected by inflammasomes (Tregoning, Russell, & Kinnear, 2018).

1.4.2 Adjuvants in commercial influenza vaccines

Many researchers have developed various adjuvant candidates that are capable of actively enhancing the immune responses induced by viral antigens, and a few of these discoveries have been commercialized and incorporated in licensed vaccines. There are three licensed adjuvants in the USA including aluminum salts, MF59, and AS03 (Shah, Hassett, & Brito, 2017). Each of the adjuvants have its advantages and disadvantages, and the study of current adjuvants provides valuable insights into the development of future adjuvants.

Aluminum salts are the name for two different adjuvants: aluminum phosphate and aluminum hydroxide, which have different properties but the same product name in the vaccine community. Alum mainly functions as an antigen delivery system by associating with antigens through electrostatic interactions or ligand exchanges. The interaction of antigens and aluminum salts enables the retention of antigens at the injection sites for an extended period, which promotes the antigen uptake by antigen-presenting cells including monocytes and macrophages (Bastola et al., 2017). Besides that, the injection of alum adjuvants has been shown to stimulate immune responses by activating the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome and triggering PRRs (Awate, Babiuk, & Mutwiri, 2013; Tregoning et al., 2018). However, alum could not efficiently induce mucosal IgA antibodies, and adverse effects of alum adjuvants include the potential of granulomas, allergenicity, neurotoxicity induced by immunization. In addition, aluminum salts shift the immune response towards a Th2 biased phenotype, which leads to the secretion of IgE antibodies that possibly associate with allergic reactions in some research subjects (Petrovsky & Aguilar, 2004). Aluminum is the most widely

used adjuvant, and various researchers stated that the safety and potency profile of alum adjuvant might associate with the types of antigen co-formulated in the vaccine.

MF59 is the second adjuvant approved for human use after aluminum salts, and it was first approved for human use in 1997 in Italy. Shortly after the first approval, MF59 has been used as a component for the influenza vaccine for seniors (Rambe, Del Giudice, Rossi, & Sanicas, 2015). MF59 is an oil-in-water emulsion and composed of squalene, Span 85, and Tween 80 in sodium citrate buffer. MF59 is also a potent adjuvant to compensate for the lower antigen concentration in vaccines (O'Hagan, Ott, Nest, Rappuoli, & Giudice, 2013). The mechanism of MF59 is that it does not create depot at the injection site but induces the influx of antigen-presenting cells at the site of injection either directly or indirectly by upregulating the production of cytokines and chemokines (O'Hagan, Ott, De Gregorio, & Seubert, 2012). The recruitment of antigen-presenting cells further leads to an increase in antigen uptake by neutrophils and monocytes and promotes the delivery and translocation of antigens to immune-competent cells (N. C. Wu & Wilson, 2017). Clinical trials with MF59-adjuvanted vaccines have been completed in different age groups with different types of antigens, and most of these MF59-adjuvanted vaccines proved to enhance the immunogenicity induced by co-vaccinated antigens with a high safety and tolerance profile. Particularly, in 2009, the safety profile of the seasonal flu vaccine Fluad® in children has been identified with 89 subjects, indicating that the MF59-adjuvanted influenza vaccine is safe and highly immunogenic in young children (Vesikari, Groth, Karvonen, Borkowski, & Pellegrini, 2009). However, granuloma formulation, injection site pain, and inflammatory reactions are the most common adverse effects of MF59-adjuvanted vaccine.

AS03, developed by GlaxoSmithKline Biologicals (GSK; Rixensart, Belgium), is also an oil-in-water adjuvant, which is composed of squalene, DL-α-tocopherol and Tween80 in phosphate buffered saline. AS03 is required to co-localize with antigens at the injection site to generate an enhanced immune response, and it works similarly to MF59 and can induce an enhanced immune response with co-vaccinated antigens. The critical component in AS03 is DL-α-tocopherol, an isoform of vitamin E, which is found to be antioxidant and immunogenic in human. The presence of DL-α-tocopherol can upregulate the expression of some proinflammatory cytokines and chemokines including Chemokine (C-C motif) ligand CCL2, CCL3, IL-6, and Chemokine (C-X-C motif) ligand CXCL1 (Morel et al., 2011). Moreover, AS03 induces the expression of transcription factor NF-κB and recruits innate immune cells, particularly monocytes

and granulocytes, to the site of vaccination. Specifically, monocytes recruited by AS03 at the injection site are then activated, converted to antigen-presenting cells, and migrated to draining lymph nodes. AS03 has been incorporated in the pandemic H1N1 2009 vaccine and induced increased levels of anti-influenza antibody comparing to nonadjuvanted H1N1 vaccines (Garcon, Vaughn, & Didierlaurent, 2012). AS03 as an emulsion similar to MF59 also has side effects like soreness at site of injection, granuloma formulation, and sometimes inflammatory reactions.

1.4.3 Triple adjuvants

Aside from the adjuvants used in commercial influenza vaccines, innate immune receptor ligands and host defense peptides can also be used individually as adjuvants in vaccine development, and a combination adjuvant consisting of these two adjuvants is also under development (Duthie, Windish, Fox, & Reed, 2011; Goff et al., 2015; Levast et al., 2014; Mendez-Samperio, 2014; Polewicz et al., 2013). Vaccine and Infectious Disease Organization - International Vaccine Centre (VIDO-InterVac) patented triple adjuvant (TriAdj), which is comprised of three components: polyriboinosinic acid-polyribocytidylic acid (poly（I:C)), a Toll-like receptor (TLR) agonist; Innate defense regulator (IDR)-1002, a host defense peptide; and polydi(p-oxyphenylpropionate) phosphazene (PCEP, polyphosphazene), a synthetic polymer (Garg, Latimer, Gerdts, Potter, & van Drunen Littel-van den Hurk, 2014; Mutwiri et al., 2007; Niyonsaba et al., 2013). Poly(I:C) as an adjuvant is used to mimic viral double-stranded RNAs and stimulate immune responses against intracellular pathogens. The signaling of Poly(I:C) adjuvant is dependent on TLR3 and melanoma differentiation-associated gene-5 (MDA-5), and Poly(I:C) can induce a cell-mediated immune response and type I interferon response (Hafner, Corthesy, & Merkle, 2013). The IDR-1002 could mediate immune responses through phosphoinositide 3-kinase (PI3K), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and mitogen-activated protein kinase (MAPK) pathways, and it is also associated with chemokine induction and the recruitment of monocytes and neutrophils to the injection site (Madera & Hancock, 2012; Nijnik et al., 2010). Another component of the TriAdj, PCEP, is able to induce an early innate immune response and trigger the production of pro-inflammatory cytokines and chemokines including CCL2 at the site of injection (Awate, Wilson, Lai, Babiuk, & Mutwiri, 2012).

The TriAdj proved to be highly effective in combination with various human viral and bacterial antigens for infectious diseases in different animal models. TriAdj formulated with viral

antigen from the human respiratory syncytial virus (hRSV) induced a long-term immune response in mice by producing neutralizing antibodies, memory B cells and $CD8^+$ T cells (Garg et al., 2014). Similarly, when co-formulated with *Bordetella pertussis* antigen, TriAdj induced a greater Th-1 immune response with a significant increase in levels of IgG2a antibody in mice (Gracia et al., 2011).

The mechanisms of how the TriAdj works as a vaccine adjuvant were identified in previous studies. As a mucosal vaccine adjuvant, the TriAdj could increase the antigen uptake process by dendritic cells (DCs), accelerate DC maturation, and facilitate the antigen presenting process to T cells (Garg et al., 2014). In combination with RSV antigen, the TriAdj is able to induce the expression of pro-inflammatory cytokine and chemokines in both upper and lower respiratory tracts, and the upregulation later leads to the recruitment of DCs, macrophages, and neutrophils and provides a long-term protective immune response in mice (Sarkar, Garg, & van Drunen Littel-van den Hurk, 2016).

The TriAdj as an adjuvant has the potential to stimulate tailored immune responses that is beneficial to specific viral or bacterial antigens co-formulated in the vaccine through the stimulation of multiple pathways and the induction of various immune cells.

1.5 Objectives and Aims

The HPAI H5N1 virus has a high potential to acquire inter-human transmission ability; along with its high mortality in the human population, the HPAI H5N1 virus is considered to be a candidate that could cause potential pandemics. However, the current vaccines stock against potential pandemic explosions are mostly inactivated vaccines and live attenuated vaccines, which all have relatively longer production time and higher production cost in comparison to the subunit vaccine. The novel subunit vaccine based on the production of recombinant antigenic protein is scalable, reproducible, timely, and cost effective in the case of a pandemic outbreak. Meanwhile, regarding the weak antigenicity of H5 HA antigens, adjuvants are essential elements to enhance antigenicity in subunit anti-H5 vaccines. Aside from the commercial adjuvants, the TriAdj has been shown to induce strong immune responses when formulated with various viral and bacterial antigens in multiple animal models. Thus, the **overarching objective** of my thesis project is to explore whether the recombinant subunit HA with TriAdj would be a promising vaccine to protect against H5N1 influenza infection.

I hypothesize that the H5 HA protein expressed in a mammalian system is glycosylated properly for its antigenicity compared to the HA protein expressed in a bacterial expression system. Furthermore, the TriAdj could enhance the H5 HA protein antigenicity, inducing better immune responses.

I propose three **specific aims** to reach my overarching objective.

Aim 1. Establishment of a mouse HPAI H5N1 virus model for evaluation of the potential vaccines.

Aim 2. Design, expression and purification of the HA immunogens in both mammalian and bacterial expression systems.

Aim 3. Characterization of HA immunogens *in vitro* and evaluation of the immune responses and protection efficacy of the HA immunogens *in vivo*

CHAPTER 2 ANIMAL MODEL ESTABLISHMENT FOR THE HPAI H5N1 VIRUS IN MICE

The use of animal models in disease study is important. It allows us to precisely characterize viral replication and viral pathogenicity *in vivo*; it is also essential for developing countermeasures against the viral infections. To test a vaccine candidate against HPAI H5N1 virus, I needed to first establish the disease model in mice. While human HPAI H5N1 infections have typically been reported in Asian countries, Canada reported a case of fatal human infection by the HPAI H5N1 virus in 2014. The full-genome analysis of the causative virus A/Alberta/01/2014 (H5N1) (AB14 (H5N1)) indicated that it showed over 99% identity with the avian originated H5N1 virus in nucleotide and protein levels in all its segments, except the PB2 segment. In addition to its high resemblance to the HPAI avian influenza virus, it caused a fatal infection in humans with unique neurological symptoms and encephalitis (Pabbaraju et al., 2014). The human infection case indicated that the AB14 (H5N1) virus has inter-species transmission potential and is capable of inducing neurological disorders. When I started my project there was only genome analysis available for the AB14 (H5N1) virus; the pathogenesis and the mechanisms underlying the virulence of AB14 (H5N1) *in vivo* remained elusive.

In this chapter, I investigated the pathogenicity as well as the cytokine and chemokine gene transcription profiles of the AB14 (H5N1) virus in mice and established a mouse model for this strain of the virus, which allows the evaluation of potential vaccines *in vivo*. This study is presented in the following publication.

IN VIVO CHARACTERIZATION OF AVIAN INFLUENZA A (H5N1) AND (H7N9) VIRUSES ISOLATED FROM CANADIAN TRAVELERS

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My contribution to this publication:

Performed all experiments and data analyses associated with the HPAI H5N1 virus, participated in writing the draft of the manuscript, and involved in editing the manuscript. My estimated contribution to this paper is about 40%.

2.1 Abstract

Highly pathogenic avian influenza (HPAI) H5N1 and low pathogenic avian influenza (LPAI) H7N9 viruses pose a severe threat to public health through zoonotic infection, causing severe respiratory disease in humans. While HPAI H5N1 human infections have typically been reported in Asian countries, avian H7N9 human infections have been reported mainly in China. However, Canada reported a case of fatal human infection by the HPAI H5N1 virus in 2014, and two cases of human illness associated with avian H7N9 virus infection in 2015. While the genomes of the causative viruses A/Alberta/01/2014 (H5N1) (AB14 (H5N1)) and A/British Columbia/1/2015 (H7N9) (BC15 (H7N9)) are reported, the isolates had not been evaluated for their pathogenicity in animal models. In this study, we characterized the pathogenicity of AB14 (H5N1) and BC15 (H7N9) and found that both strain isolates are highly lethal in mice. AB14 (H5N1) caused systemic viral infection and proinflammatory cytokine gene expression in different organs. In contrast, BC15 (H7N9) replicated efficiently only in the respiratory tract and was a potent inducer for proinflammatory cytokine genes in the lungs. Our study provides experimental evidence to complement the specific human case reports and animal models for evaluating vaccine and antiviral candidates against potential influenza pandemics.

2.2 Introduction

Influenza A virus (IAV) is a segmented RNA virus that infects a wide variety of species including human, avian, swine, equine, and sea mammals. The segmented genome and wide host range enable IAV to undergo antigenic variations through gene reassortment, a mechanism called antigenic shift, which is responsible for the emergence of pandemic influenza viruses. The lack of proofreading by the viral RNA-dependent RNA polymerase frequently results in point mutations throughout the viral genome (especially in HA and NA genes), which can lead to antigenic drift, a causative mechanism of annual influenza epidemics (Krammer et al., 2018; Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). Additionally, IAV also poses a severe threat to public health through zoonotic infection, because avian IAVs can directly cross the species barrier and infect humans, with avian H5N1 and H7N9 viruses being such examples. The first report of human infection by the highly pathogenic avian influenza (HPAI) H5N1 virus was in 1997 in Hong Kong; after a short period of disappearance, the virus re-emerged in 2003 in China (Claas et al., 1998). Since then, sporadic human H5N1 viral infections have been reported in several countries, causing over 400 fatalities (WHO, 2019b). The low pathogenic avian influenza (LPAI) H7N9 virus circulated exclusively among poultry in China until 2013 when the first human infection was reported (Gao et al., 2013). Similar to the HPAI H5N1 virus, human infection by the H7N9 virus is also associated with a severe and fatal respiratory disease. To date, the H7N9 virus has caused a total of six epidemic waves, having infected over 1600 humans with 623 fatalities (WHO).

In comparison to the human seasonal H1N1 and H3N2 viruses, the HPAI H5N1 virus induces more proinflammatory cytokines in the human alveolar and bronchial epithelial cells (Chan et al., 2005). H5N1 viral infection also results in the early and excessive infiltration of macrophages and neutrophils in the lungs of infected mice (Perrone, Plowden, Garcia-Sastre, Katz, & Tumpey, 2008). Animal and human studies have shown that the combinatorial effect of the unrestrained high-level virus infection together with hypercytokinemia is attributable to the increased pathogenesis of H5N1 disease (Abdel-Ghafar et al., 2008; de Jong et al., 2006). Although both H5N1 and H7N9 viral infections lead to fatality, the underlying mechanisms might be different (Meliopoulos et al., 2014). An ex vivo study showed that the human H7N9 virus replicated efficiently in human bronchial epithelial cells, alveolar epithelial cells, and alveolar macrophages, with high titres similar to that of the H5N1 virus (Chan et al., 2013). However, in animal studies, it has been reported that the H7N9 virus possesses a greater tropism for the

respiratory epithelium than the H5N1 virus, and is not capable of inducing hypercytokinemia, which is characteristic of H5N1 viral infection (Meliopoulos et al., 2014). Recently, a risk assessment of the fifth wave of H7N9 viral infection revealed that both the LPAI and HPAI H7N9 viruses were isolated from humans. Compared to the LPAI H7N9, the HPAI H7N9 virus was found to possess enhanced virulence, tropism for the brain tissue, as well as the capability to transmit by air droplets, concluding that the HPAI H7N9 virus has gained the ability to cause an H7N9 pandemic (X. Sun et al., 2019). Thus, both H5N1 and H7N9 viruses are of significant pandemic concerns.

Both HPAI H5N1 and LPAI H7N9 human infections in North America were reported in Canada, in 2014 and 2015 respectively, shortly after the patients returned from China (Maurer-Stroh et al., 2014; Skowronski et al., 2016). The A/Alberta/01/2014 (H5N1) (AB14 (H5N1)) strain isolate caused a fatal infection, whereas the A/British Columbia/1/2015 (H7N9) (BC15 (H7N9)) strain isolate caused an influenza illness from which the patients recovered. Phylogenetic analysis revealed mutations on the receptor binding site of the HA gene in AB14 (H5N1), which facilitated the direct jump from avian species to humans (Pabbaraju et al., 2014). However, for BC15 (H7N9), the analysis showed the genome to be similar to those of previous human H7N9 isolates, carrying clinically relevant markers on HA, PB2, and NA genes (Skowronski et al., 2016). Although H5N1 and H7N9 viruses have been intensively studied, the virulence of these avian-origin influenza strains isolated from these Canadian travelers has been overlooked. The purpose of this study was to experimentally investigate the pathogenicity as well as the cytokine and chemokine gene transcription profile of the AB14 (H5N1) and BC15 (H7N9) strain isolates. We report that both AB14 (H5N1) and BC15 (H7N9) strains are highly lethal in mice. AB14 (H5N1) caused a systemic viral infection as well as the proinflammatory cytokine and chemokine gene response in different organs. In contrast, the BC15 (H7N9) strain replicated efficiently only in the respiratory tract and was a potent inducer of proinflammatory cytokines in the lungs. Overall, our study not only provides experimental evidence to complement the human case report, but also offers valuable animal models for evaluating vaccine and antiviral candidates against the H5 and H7 potential pandemic influenza viruses.

2.3 Materials and methods

2.3.1 Cells and viruses

Madin-Darby canine kidney (MDCK, ATCC, #CRL-2936) cells were maintained in minimal essential medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and gentamycin (50 µg/mL, Bio Basic, Markham, ON, Canada). MDCK cells were maintained at 37ºC in a humidified 5% CO_2 incubator. Influenza A/Alberta/01/2014 (H5N1) (AB14 (H5N1)) and A/British Columbia/01/2015 (H7N9) (BC15(H7N9)) were kind gifts from Dr. Yan Li at the National Microbiology Laboratory, Public Health Agency of Canada. The viruses were propagated in MDCK cells and titrated by plaque assay. Propagated viruses were sequenced, and no mutations were found according to the reference sequences. All infectious experiments were conducted in Biosafety Containment Level 3 at the International Vaccine Centre at the University of Saskatchewan, Canada, under the guidelines of the Public Health Agency of Canada (PHAC) and the Canadian Food Inspection Agency (CFIA).

2.3.2 Ethics statement

All animal procedures were approved by the University Animal Care Committee (UACC) and Animal Research Ethics Board (AREB) of the University of Saskatchewan on 31 August 2017 (Animal Use Protocol #20170087) in accordance with the standards stipulated by the Canadian Council on Animal Care.

2.3.3 Mouse experiments

For this study, 84 six-week-old female BALB/c mice (Charles River Laboratories, Saint-Constant, QC, Canada) were randomly divided into seven groups with 12 mice in each group. These groups were housed in separate cages in Biosafety Containment Level 3 seven days prior to infection. At seven weeks of age, each mouse was intranasally infected with 50 µL of 10^3 plaque forming unit (PFU), 10^4 PFU, and 10^5 PFU of either the AB14 (H5N1) or BC15 (H7N9) strain isolates. A group of mice was mock infected with PBS. The mice were monitored daily for body weight, and on days 2 and 5 post-infection (d.p.i), three mice from each group were euthanized, to which the lung and spleen tissues were collected for viral titration as well as cytokine and chemokine profiling. Brain tissues were collected from euthanized mice on either 6 or 7 d.p.i. The rest of the mice were humanely euthanized when they dropped below 20% of their initial body weight.

2.3.4 Virus isolation and titration

Infectious lung, spleen, and brain tissues were processed immediately after collection as previously described (Pyo & Zhou, 2014). Briefly, the tissues were homogenized in MEM supplemented with Penicillin-Streptomycin (Gibco, Thermo Fisher, ON, Canada) in the TissueLyser II (Qiagen, Hilden, Germany) at 25 Hz for 5 min, followed by centrifugation at 5000× g for 10 min at 4 ºC. The supernatant was collected in screw-cap tubes and stored at −80 ºC for further titration.

For virus titration by 50% tissue culture infective dose ($TCID_{50}$) assay, MDCK cells were plated in 96-well plates, and the supernatants of homogenized tissues were serially diluted in MEM and incubated with cells for 1 h. The inoculum was removed and supplemented with MEM containing 0.2% BSA and 1 µg/mL TPCK-trypsin (Sigma-Aldrich). The development of cytopathic effects (CPE) was observed and recorded every 24 h until 96 h post-infection. The $TCID_{50}$ titre of each infectious sample was calculated by the Spearman–Kärber algorithm (Kärber, 1931; Spearman, 1908).

2.3.5 RNA extraction and quantitative RT-PCR (qRT-PCR)

Tissue samples of mice collected on 2 or 5 d.p.i. or upon necropsy were submerged into RNA later (Qiagen) and stored overnight at 4 ºC. The following day, the tissue samples were transferred to screw-cap tubes containing one 5 mm stainless steel bead (Qiagen) and 1 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The samples were homogenized using the TissueLyser II (Qiagen) at 25 Hz for 5 min, followed by centrifugation at 5000× g for 10 min at 4 ºC. The supernatant was then transferred to new tubes for RNA extraction by the TRIzol method (Invitrogen).

To determine mRNA levels of various cytokines and chemokines induced by AB14 (H5N1) and BC15 (H7N9) viral infection, qRT-PCR was performed on total RNA of the samples collected from mice infected with 10^3 PFU of the respective virus, as previously described with the following modifications (G. Liu et al., 2018). Briefly, a 500 µg portion of RNA was reverse transcribed with oligo (dT) and SuperScript III Transcriptase (Invitrogen) to obtain total mRNA. qPCR was performed on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, CA, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystems). Cytokine mRNA levels were normalized to that of the housekeeping gene HPRT and expressed using the ΔΔCT method relative to the PBS group. All sequences of qPCR primers are listed in Table 1.1.

Table 2.1 List of primers used in RT-qPCR studies in mice.

Name	Sequence (5'-3')
IFN-γ-F	TCAAGTGGCATAGATGTGGAAGAA
IFN-γ-R	TGGCTCTGCAGGATTTTCATG
IFN-α-F	CCTGTGTGATGCAACAGGTC
IFN-α-R	TCACTCCTCCTTGCTCAATC
IFN-β-F	ATCATGAACAACAGGTGGATCCTCC
IFN-β-R	TTCAAGTGGAGAGCAGTTGAG
IP-10-F	ATGACGGGCCAGTGAGAATG
IP-10-R	GAGGCTCTCTGCTGTCCATC
TNFα-F	AGGCACTCCCCCAAAAGATG
TNFα-R	CTGCCACAAGCAGGAATGAG
IL-1β-F	GTGTGGATCCCAAGCAATAC
IL-1β-R	GTCCTGACCACTGTTGTTTC
IL-18-F	TGGTTCCATGCTTTCTGGACTCCT
IL-18-R	TTCCTGGGCCAAGAGGAAGTGATT
IL-6-F	GTGGCTAAGGACCAAGACCA
IL-6-R	TAACGCACTAGGTTTGCCGA
IL-10-F	GCTGCCTGCTCTTACTGACT
IL-10-R	CTGGGAAGTGGGTGCAGTTA
RIG-I-F	CCTCCCATCTCCTTCATGACA
RIG-I-R	CCACCTACATCCTCAGCTACATGA
HPRT-F	GATTAGCGATGATGAACCAGGTT
HPRT-R	CCTCCCATCTCCTTCATGACA

All primers have been validated to have greater than 95% of amplification efficiency. The expression levels of cytokine mRNA were normalized to the expression of the housekeeping gene HPRT. F: forward primer; R: reverse primer.

2.3.6 Histopathology

The left side of the lung was fixed with 10% natural buffered formalin, processed for hematoxylin and eosin (H&E) staining, and assessed in a blind manner by a board-certified veterinary pathologist as previously described (Pyo, Hlasny, & Zhou, 2015).

2.4 Results

2.4.1 Survival rate and body weight loss of mice infected with the HPAI H5N1 and LPAI H7N9 strain isolates

To determine the pathogenicity of the AB14 (H5N1) and BC15 (H7N9), BALB/c mice were intranasally inoculated with PBS or either AB14 (H5N1) or BC15 (H7N9) at three different doses (10^3 PFU, 10^4 PFU, and 10^5 PFU). Survival and body weight loss were monitored for 10 days (Figure 2.1). Mice infected with PBS survived the duration of the trial and gained weight as the days progressed. In contrast, mice infected with the different doses of either AB14 (H5N1) or BC15 (H7N9) showed rapid body weight loss and severe mortality rates. Mice infected with 10^4 PFU or 10^5 PFU of AB14 (H5N1) exhibited rapid weight loss greater than 20% of their initial body weight within four days post-infection (d.p.i.). Furthermore, over 60% of mice infected with 10^5 PFU of AB14 (H5N1) had succumbed by 4 d.p.i. (Figure 2.1A and B). By 5 d.p.i., 100% of mice infected with 10^4 PFU and 10^5 PFU were humanely euthanized due to severe body weight loss. Albeit mice infected with 10^3 PFU displayed the slowest rate of body weight loss, they all reached a humane endpoint by 6 d.p.i. With regard to BC15 (H7N9), mice infected with 10^3 PFU displayed the slowest decline of body weight, losing over 20% of their initial body weight by 8 d.p.i. Furthermore, 50% of mice infected with 10^3 PFU reached a humane endpoint by 7 d.p.i., while the other 50% reached this endpoint by 8 d.p.i. (Figure 2.1C and D). In contrast, mice infected with 10^4 PFU and 10^5 PFU had steeper body weight losses and higher mortality rates. In the 10^4 PFU group, 100% of mice reached a humane endpoint by 6 d.p.i., while in the10^5 PFU group, 50% of the mice by 4 d.p.i. and the other 50% by 5 d.p.i. reached this endpoint. These results demonstrate that both the AB14 (H5N1) and BC15 (H7N9) strain isolates are highly virulent in mice without the need of prior adaptation even at the lowest dose of 10^3 PFU.

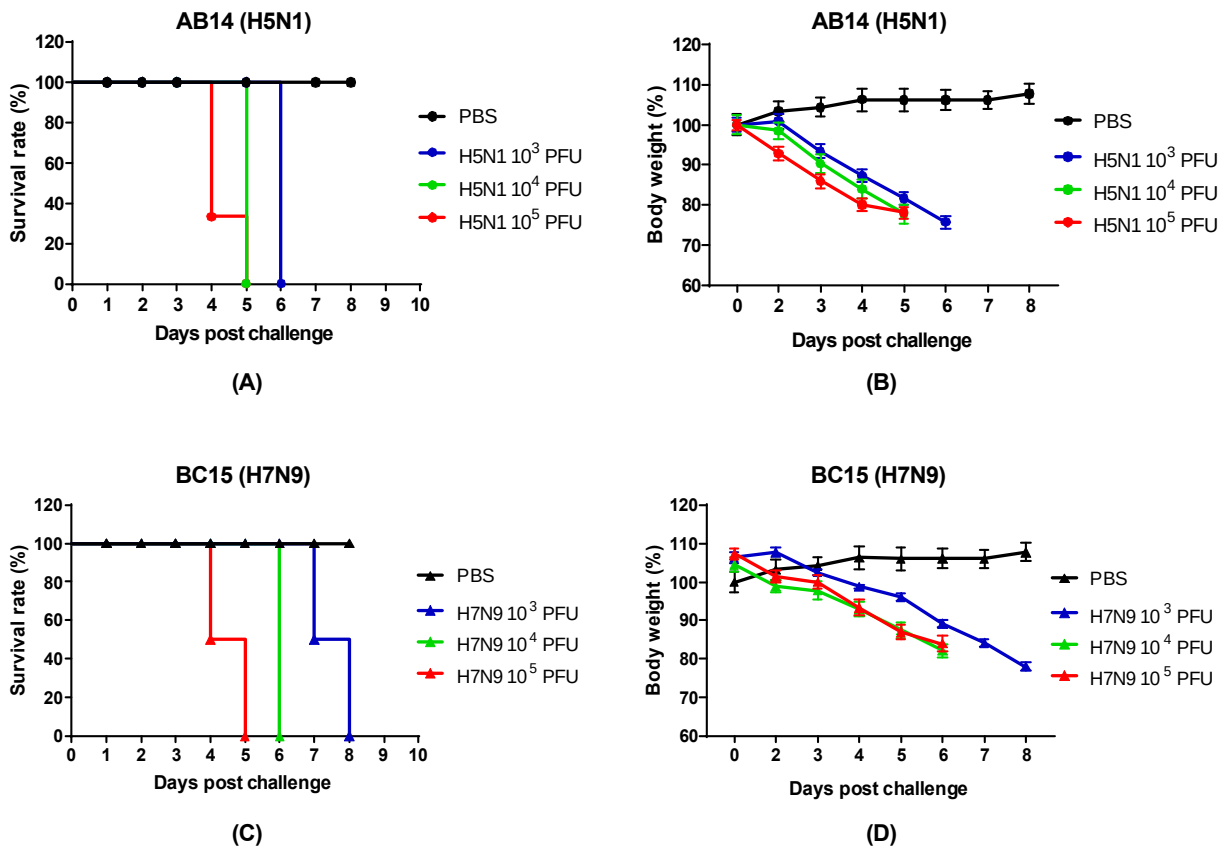


Figure 2.1 Survival rate and body weight loss for the AB14 (H5N1) and BC15 (H7N9) strain isolates.

The survival rates for (A) AB14(H5N1) and (C) BC15(H7N9) as well as body weight changes for (B) AB14(H5N1) and (D) BC15(H7N9) were determined in BALB/c mice (n=6 per group) infected with 10_3 PFU, 10_4 PFU, and 10_5 PFU of the two different strain isolates.

2.4.2 Histopathology of the Mouse Lung

To examine the levels of pulmonary pathology from AB14 (H5N1) and BC15 (H7N9) infection, we performed a histopathology study on the lungs of mice infected with 10_3 PFU that reached a humane endpoint on 6 or 7 d.p.i. Mice infected with both AB14 (H5N1) and BC15 (H7N9) strain isolates showed bronchointerstitial pneumonia, with vasculitis (Figure 2.2). Specifically, the walls of the arterioles in infected mice were found to be infiltrated with

inflammatory cells and contained some necrotic debris (Figure 2.2D and G). In addition, viral infection also led to moderate damage to the mice alveoli and bronchioles. In the alveoli of infected mice, we found moderate thickening of the alveolar walls due to congestion as well as some inflammatory infiltrate (Figure 2.2E and H). The alveolar space was filled with edema and contained small to moderate numbers of mixed neutrophils and macrophages. Occasionally, hyaline membranes lining the alveoli were observed (Figure 2.2E). Multifocally, the bronchiolar epithelium was necrotic, and the lumen was filled with some necrotic debris and inflammatory cells as seen in the alveolar space (Figure 2.2F and I).

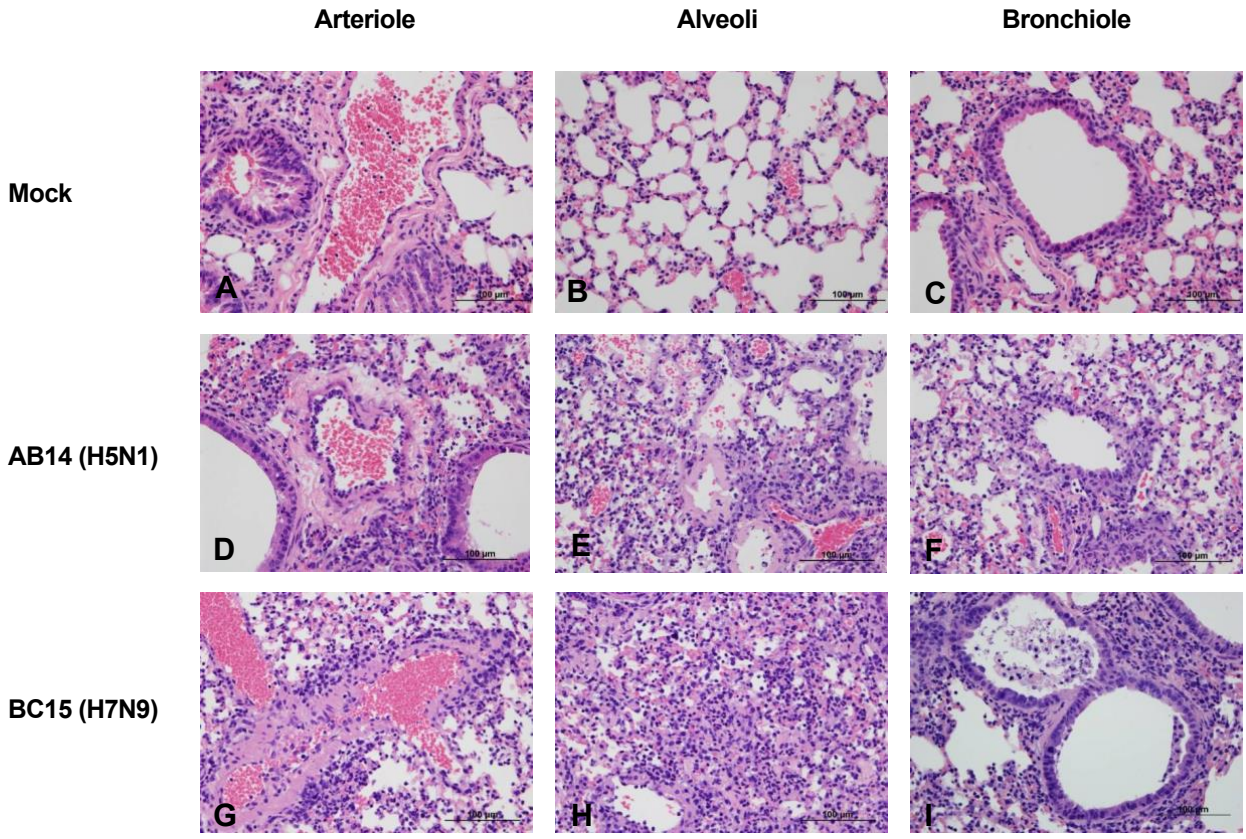


Figure 2.2 Lung histopathology of mice after infection with AB14 (H5N1) and BC15 (H7N9) strain isolates.

Lung samples were fixed, sectioned, and stained with hematoxylin and eosin. (A-C) Tissues from mock-infected lungs. (D-F) Tissues from mice infected with AB14 (H5N1) showing infiltration of inflammatory cells into the (D) wall of the arteriole, (E) alveolar walls, and (F) bronchiolar

epithelium affected by necrosis. (G-I) Tissues from mice infected with BC15 (H7N9) showing necrotic debris and inflammatory cells in the (G) wall of the arteriole, (H) collapsed alveoli, as well as degeneration and necrosis of the (I) bronchiolar epithelium. Scale bar represents 100 µm.

2.4.3 Replication efficiency of the HPAI H5N1 and LPAI H7N9 strain isolates in different organs of mice

To investigate the replication efficiency and tissue tropism of both strain isolates, mouse lung, spleen, and brain tissues were collected at predetermined days, as well as when the mice reached a critical endpoint (Figure 2.3). In all groups of mice infected with AB14 (H5N1), the peak lung viral titres were reached by 2 d.p.i., which remained at high levels throughout the trial. The mean titres on 5 d.p.i. were $10^{7.2}$, $10^{6.4}$, and $10^{6.7}$ $TCID_{50}$/g for 10^3 PFU, 10^4 PFU, and 10^5 PFU, respectively (Figure 2.3A). Brain samples taken from the lowest dose group (10^3 PFU) had very high viral titres on 6 d.p.i., with a mean virus titre of 10^7 $TCID_{50}$/g (Figure 2.3C). Note that the brain samples were only harvested at the endpoint. Spleen viral titres remained at an approximate level of $10^{2.5}$ $TCID_{50}$/g from 2 d.p.i. until 5 d.p.i. (day 2: $10^{2.64}$ $TCID_{50}$/g, day 5: $10^{2.46}$ $TCID_{50}$/g) in the mice infected with 10^5 PFU of the AB14 (H5N1) strain isolate (Figure 2.3D). Interestingly, spleen viral titres were not detected for the two lower dose groups (10^3 PFU and 10^4 PFU).

In BC15 (H7N9) viral infected lung, the 10^3 PFU group reached a titer of approximately $10^{6.5}$ $TCID_{50}$/g by 2 d.p.i. and remained at this titer throughout the duration of the trial. The 104 PFU group displayed a similar trend, with high titers present at 2 d.p.i. (10^8 $TCID_{50}$/g), which decreased by 5 and 6 d.p.i. ($10^{6.5}$ $TCID_{50}$/g). The 10^5 PFU group had the highest titer at 2 d.p.i. (10^8 $TCID_{50}$/g), which remained at this level throughout the duration of the trial (Figure 3B). The BC15 (H7N9) strain isolate was not detected in the spleen and brain of mice infected by all three doses by $TCID_{50}$ assay.

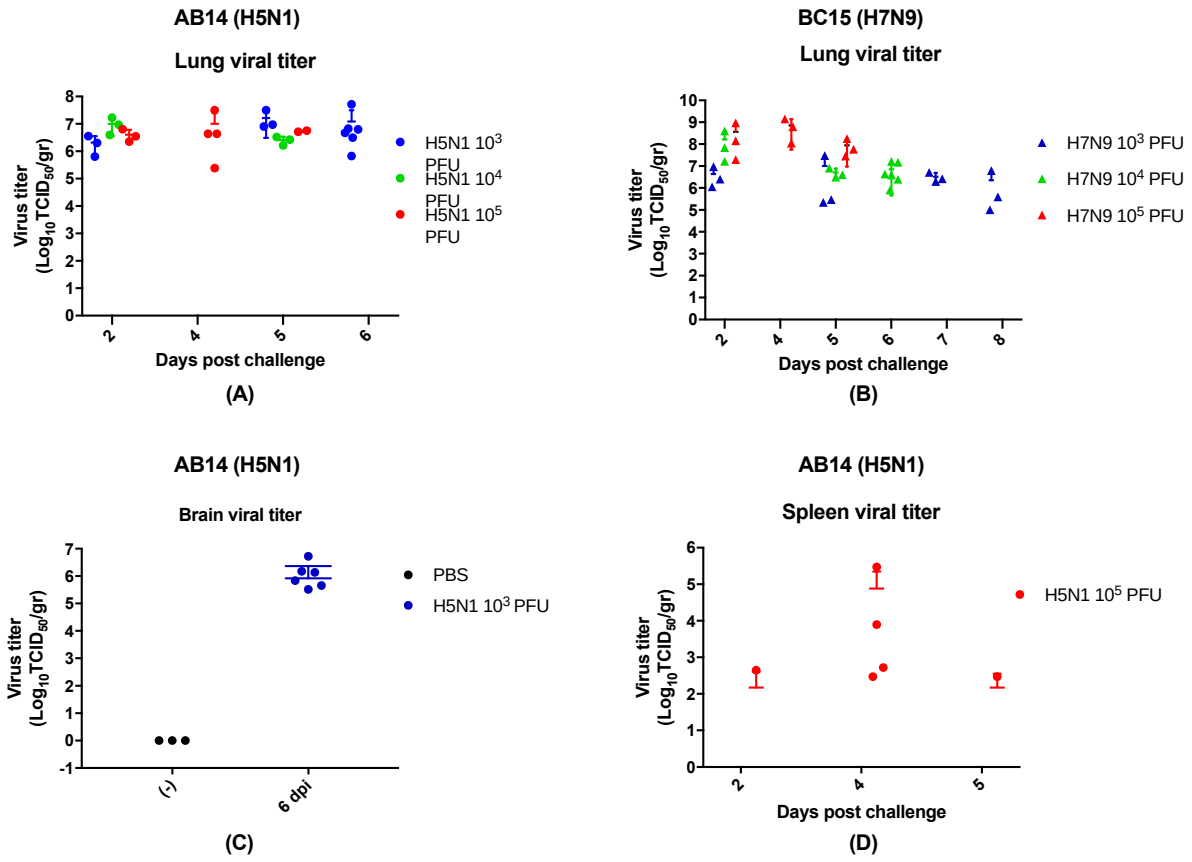
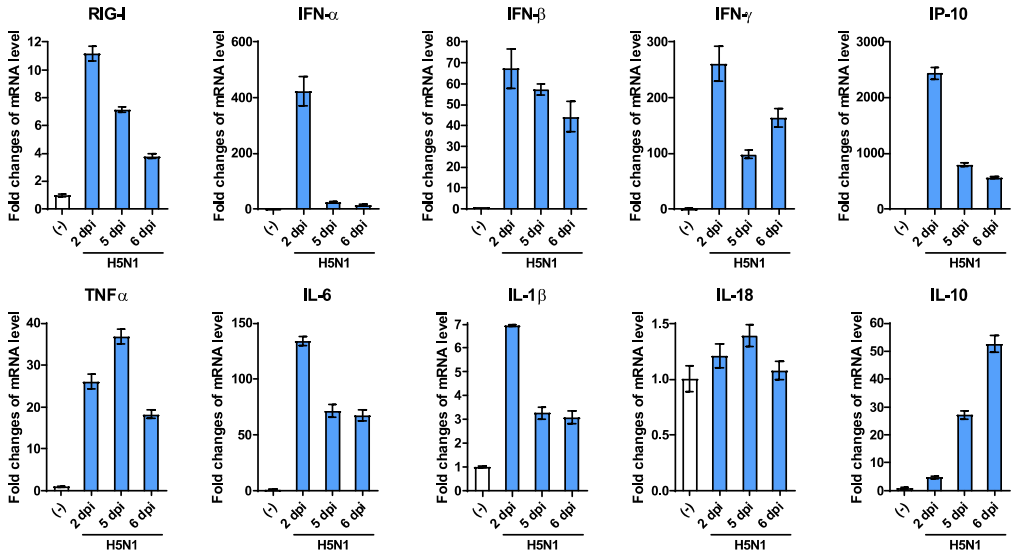


Figure 2.3 Viral titration of the mouse lung, spleen, and brain for the AB14 (H5N1) and BC15 (H7N9) strain isolates.

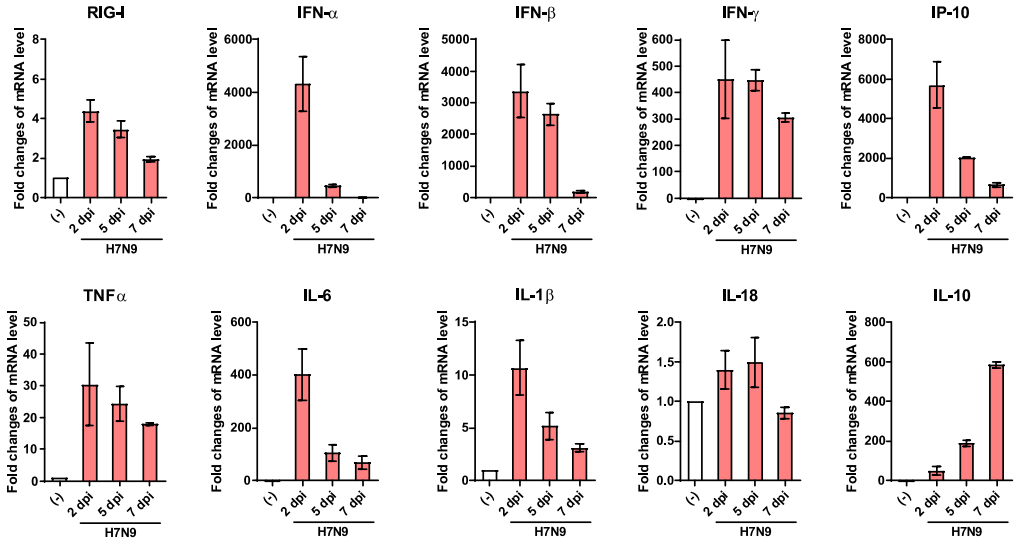
Mice were intranasally infected with 10_3 PFU, 10_4 PFU, or 10_5 PFU of AB14 (H5N1) or BC15 (H7N9). The lung, spleen, and brain tissues were collected and homogenized for virus titration by $TCID_{50}$ assay. Lung viral titration from (A) AB14 (H5N1) and (B) BC15 (H7N9) infection for all doses. Brain viral titration from (C) AB14 infection (H5N1). Spleen viral titration from (D) AB14 (H5N1) infection. Please note that BC15 (H7N9) was not detected in the spleen and brain of mice infected by all three doses.

2.4.4 Cytokine and chemokine profiling in the mouse lung

To understand the pathogenesis and immune response to AB14 (H5N1) and BC15 (H7N9) viral infection, we assessed the innate immune receptor retinoic acid-inducible gene I (RIG-I), as well as the cytokine and chemokine gene expression in the lungs and brains of mice infected with the lowest dose (10_3 PFU) of both strain isolates by qRT-PCR. To start, we investigated a group of genes that encode interferons as well as the innate immune sensor RIG-I, a major pattern recognition receptor that recognizes influenza viral infection and activates the interferon response (Opitz et al., 2007). Interferon (IFN)-α and IFN-β are both type I interferons which play essential roles in antiviral defense (Taniguchi & Takaoka, 2002). IFN-γ is a type II interferon that is the first cytokine produced in response to foreign invaders and is an important activator of macrophages and natural killer (NK) cells. IFN-γ also has antiviral activity and inhibits the proliferation of Th2 cytokines (Interleukin (IL)-4, IL-5, and IL-6) (Tau & Rothman, 1999). In the AB14 (H5N1) infected lungs (Figure 2.4A), gene expression of RIG-I peeked on 2 d.p.i., which then decreased by 5 and 7 d.p.i. IFN-α gene expression was drastically upregulated on 2 d.p.i., and then sharply dropped on 5 and 7 d.p.i. IFN-β mRNA levels increased over 60-fold on 2 d.p.i., which then slightly decreased but remained at moderately high levels on 5 and 7 d.p.i. The type I IFN gene transcription levels showed similar upregulating patterns in concordance with that of RIG-I. IFN-γ had the highest upregulation by 2 d.p.i., which dropped moderately by 5 d.p.i., and then increased slightly by 7 d.p.i. The transcription of the gene encoding interferon-gamma-inducing protein 10 (IP-10), a cytokine produced in response to IFN-γ, was significantly upregulated (over 2000-fold) on 2 d.p.i., and then remained at relatively high levels, consistent with the IFN-γ mRNA upregulation pattern. Next, we profiled the gene transcription of proinflammatory (Tumor necrosis factor (TNF) α, IL-6, IL-1β, and IL-18) and anti-inflammatory (IL-10) cytokines. TNFα is a proinflammatory cytokine produced in response to the foreign invasion and is often the main cytokine produced by macrophages following infection (Idriss, Naismith, & technique, 2000). IL-6 and IL-1β are the main contributors to severe lung inflammation in both humans and poultry infected by influenza virus (Saito et al., 2018). Both TNFα and IL-6 mRNA were markedly upregulated upon AB14 (H5N1) infection and remained at these elevated levels. IL-1β mRNA was upregulated 7-fold on 2 d.p.i., which then returned to slightly higher levels than that of the uninfected control. IL-18 mRNA remained at mock levels upon infection. On the contrary, the mRNA level of the anti-inflammatory cytokine IL-10 was gradually upregulated after infection, and peaked on 7 d.p.i.



(A)



(B)

Figure 2.4 Innate immune receptor RIG-I, as well as cytokine and chemokine gene transcription levels in the lungs of mice infected with the AB14 (H5N1) and BC15 (H7N9) strain isolates.

Cytokine and chemokine mRNA levels of RIG-I, IFN-α, IFN-β, IFN-γ, IP-10, TNFα, IL-6, IL-1β, IL-18, and IL-10 from virus-infected lungs with (A) AB14 (H5N1) and (B) BC15 (H7N9)

(n=3 mice per virus group) were measured by qRT-PCR. Samples were harvested on the indicated d.p.i.; each sample was tested in triplicate.

In the lungs of BC15 (H7N9) infected mice, substantially upregulated mRNA levels of interferon genes (IFN-α, IFN-β, and IFN-γ), interferon responsive cytokine gene (IP-10), and proinflammatory cytokine genes (TNFα and IL-6) were obtained on 2 d.p.i. compared to the uninfected control (Figure 2.4B). IFN-α, and IFN-β mRNA levels substantially dropped by 7 d.p.i., whereas mRNA levels of RIG-I, IFN-γ, and TNFα remained relatively unchanged throughout the duration of the trial. Similar to what we observed for AB14 (H5N1), the IL-18 gene transcript was not upregulated in response to viral infection, whereas the gene transcription of the anti-inflammatory cytokine IL-10 dramatically increased from 2 to 7 d.p.i.

Besides cytokine and chemokine gene transcription analysis in the lungs of viral infected mice, we also analyzed select cytokine transcription levels in the brains of mice harvested at the endpoint. In the brains of mice infected with AB14 (H5N1), the transcription of IFN genes (IFN-α, and IFN-γ) as well as proinflammatory cytokine genes (TNFα, IL-6, and IL-1β) was dramatically upregulated compared to the uninfected control (Figure 2.5A). Among all genes activated in the brain tissue upon infection, IFN-γ and TNFα were the most highly transcribed cytokines associated with HPAI AB14 (H5N1) infection. Similar to the cytokine regulation observed in the mouse lung, IL-18 transcripts did not change upon AB14 (H5N1) infection when compared to the uninfected control (Figure 2.5A). In contrast, the brains of mice infected with BC15 (H7N9) had only moderate upregulation of IFN-α, IFN-γ, and IL-6 gene transcription, which was marginal compared to the degree of upregulation observed in the AB14 (H5N1) viral infected brain. The transcripts of the other inflammatory cytokine genes, TNFα, IL-1β, and IL-18, did not change when compared to the uninfected control (Figure 2.5B).

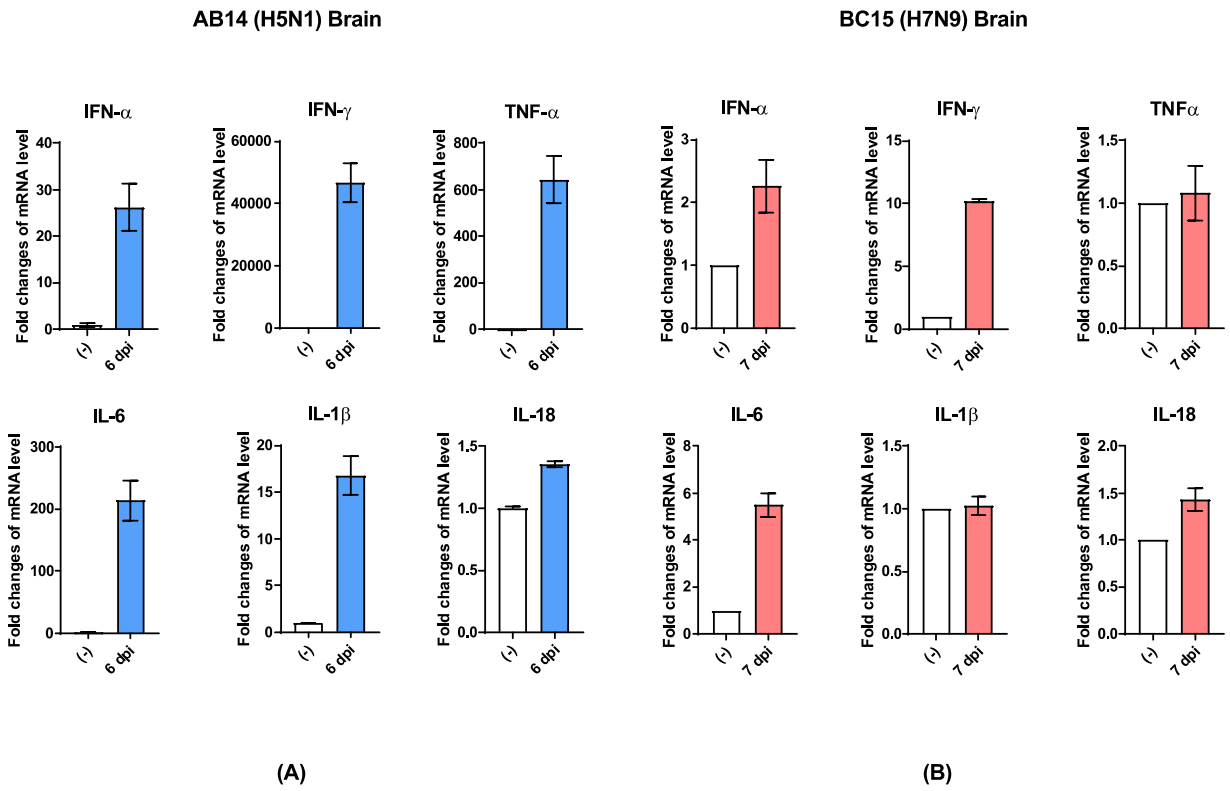


Figure 2.5 Cytokine gene transcription profile in the brains of mice infected with the AB14 (H5N1) and BC15 (H7N9) strain isolates.

Cytokine transcription levels of IFN-α, IFN-γ, TNF-α, IL-6, IL-1β, and IL-18 from virus-infected brains with (A) AB14 (H5N1) and (B) BC15 (H7N9) (n=3 mice per virus group). Samples were harvested on the indicated d.p.i; each sample was tested in triplicate.

2.5 Discussion and Conclusions

Following the reporting of the human HPAI (H5N1) and LPAI (H7N9) infection in Canada, the characterization on the causative strain isolates associated with these human infection cases, AB14 (H5N1) and BC15 (H7N9), was reported. Pabbaraju et al. analyzed the full genome of AB14 (H5N1) and assessed its molecular markers of pandemic risk (Pabbaraju et al., 2014). Skowronski et al. conducted the serological and phylogenetic analysis of BC15 (H7N9), reporting that this virus belongs to the clade W2-C, which clusters with both the 2014–2015 H7N9 human isolates

from the Jiangsu, Zhejiang, and Fujian Provinces of China as well as the 2014 chicken isolated from Jiangsu Province (Skowronski et al., 2016). Despite the analysis on these two Canadian isolates, the infectious and immunological properties as well as the histopathological correlation to disease remained elusive. In this study, we aimed to fill this knowledge gap, and to provide insightful information on the pathogenesis of the AB14 (H5N1) and BC15 (H7N9) strain isolates in mice.

To ensure that the AB14 (H5N1) and BC15 (H7N9) strain isolates would induce disease in mice, we chose to infect them with three different doses (10^3 PFU, 10^4 PFU, and 10^5 PFU). We found that even at the lowest dose (10^3 PFU), both isolates were lethal to mice. AB14 (H5N1) caused all mice to reach a humane endpoint by 6 d.p.i., whereas BC15 (H7N9) infected mice survived one to two days longer than the mice infected with AB14 (H5N1). This observation is in contrast to the various seasonal or the 2009 pandemic influenza viruses (Groves et al., 2018; Rowe et al., 2010). Groves et al. reported that mice infected with 5×10^3 PFU of influenza A/England/195/2009 (H1N1) showed mild weight loss over 7 days, whereas mice infected with 10^3 PFU of influenza A/England/691/2010 (H3N2) did not show any signs of disease (Groves et al., 2018). In the study reported by Rowe et al., infection of BALB/c mice with 10^4 EID_{50} of the 2009 pandemic virus did not cause any death when using 20% total body weight loss as an endpoint (Rowe et al., 2010). Furthermore, we could detect the AB14 (H5N1) strain in multiple organs, including the lung, spleen, and brain, with consistently high titres in the lung and brain tissues. However, we could only detect the BC15 (H7N9) strain in the mouse lung, with no detectable virus in the spleen and brain by $TCID_{50}$ assay. These results are in agreement with the previous reports that while H5N1 virus has the ability to replicate systemically and spread efficiently to non-respiratory tissues, H7N9 virus replicates well in the upper respiratory tract, covering the nasal passages, nasopharynx-associated lymphoid tissues (Meliopoulos et al., 2014), human bronchus, and the lungs (Chan et al., 2013). Research has shown that avian IAV strains need to acquire adaptation mutations in order to be infectious in mammals, with one such adaptation being in the polymerase, PB2. This adaptation resides at the amino acid at position 627 of PB2 and determines the viral replication efficiency in different hosts. It is well characterized that avian strains carry a glutamic acid (E) at this position referred to as 627E, whereas mammalian strains carry a lysine (K), namely, 627K (Hatta, Gao, Halfmann, & Kawaoka, 2001). The BC15 (H7N9) strain isolate consists of PB2 encoding 627K, which may explain its ability to replicate well in the human

respiratory tract. In contrast, the AB14 (H5N1) strain isolate consists of PB2 possessing the avian signature, namely, 627E. However, our data have shown that while AB14 (H5N1) replicates efficiently without any adaptation in mice, it replicates less efficiently in tissue culture (Lu and Zhou unpublished data). In addition to the PB2 protein, HA protein is also a key viral factor that determines whether an avian virus can replicate well in mammals. It is reported that AB14 (H5N1) HA contains two novel mutations, R189K and G221R, which are located in the immediate receptor-binding pocket (Pabbaraju et al., 2014). It was speculated that these mutations arose in an avian H5N1-infected patient, which was also associated with severe illness and spread of the virus to the brain. We observed that the AB14 (H5N1) strain isolate replicated efficiently in the mouse brain, which will provide a model for further investigation of the functional role of G221R in avian influenza HA contributing to viral pathogenesis in mammals.

Research has shown that the dysregulation of the innate immune responses that results in an unusual proinflammatory cytokine production often contributes to the pathogenicity of both the HPAI H5N1 virus and the 1918 Spanish Flu pandemic virus (Cilloniz et al., 2009; Perrone et al., 2008). Our data show that although at different magnitudes, infection by both AB14 (H5N1) and BC15 (H7N9) induced dramatically elevated levels of interferon genes and proinflammatory cytokine gene expression in the lungs. These levels peaked on 2 d.p.i., and then decreased but remained at moderate levels as the infection progressed. Concomitantly, both strain isolates induced increased gene expression of the anti-inflammatory cytokine IL-10, which peaked on 6 and 7 d.p.i., for both the AB14 (H5N1) and BC15 (H7N9) strain isolates, respectively. There has been evidence showing IL-10 to inhibit several proinflammatory cytokines and chemokines (Couper, Blount, & Riley, 2008), and that it may therefore actually hinder clearance of the virus (Frensing et al., 2016). When comparing the transcription levels of various cytokines, it was found that most cytokine genes were sharply upregulated in the tens- to hundreds-fold. Interestingly, the two inflammatory cytokines IL-1β and IL-18, whose maturation is regulated by inflammasome activity (Kaplanski, 2018), were not significantly upregulated at the mRNA level; IL-1β mRNA was only moderately upregulated, whereas IL-18 mRNA was not upregulated at all. This finding thus warrants further investigation into the mature IL-1β and IL-18 protein levels, in order to understand their roles in mediating avian IAV-induced pathogenesis. Interestingly, it has been reported that in comparison to other H5N1 strain isolates that are associated with severe human respiratory disease, H7N9 strains isolated from human infections from 2013 to 2015 are poor

proinflammatory cytokine and chemokines inducers in mammalian models (Belser et al., 2016). However, our results showed that BC15 (H7N9) is a potent inducer of proinflammatory cytokines in the mouse lung. This finding is consistent with the study of human patients infected with the H7N9 virus, where elevated cytokine and chemokine production was observed (Chi et al., 2013). With regard to the cytokine gene expression in the mouse brain, we found that AB14 (H5N1) dramatically upregulated IFN-α, IFN-γ, TNF-α, and IL-6 transcription in the brains of mice that reached a humane endpoint. This finding possibly indicates cytokine hyperinduction in the brains of mice infected with AB14 (H5N1), which may contribute to the pathogenesis. In contrast, only moderate upregulation of both IFN-γ and IL-6 transcripts was observed in the brains of mice infected with BC15 (H7N9). This finding reflects the viral titres found in the infected brains of the mice, such that while mice infected with AB14 (H5N1) had high titres, mice infected with BC15 (H7N9) had no detectable virus in the brain.

Taken together, we showed that the AB14 (H5N1) strain isolate replicates efficiently without prior adaptation not only in the mouse lung, but also in other non-respiratory tissues such as the spleen and brain. The high viral load and hypercytokinemia in multiple organs contributed to the severity of the disease associated with AB14 (H5N1) infection. Similarly, the BC15 (H7N9) strain is also highly pathogenic in mice, but its replication seemed to be more constrained to the respiratory tract. The severity of both AB14 (H5N1) and BC15 (H7N9) disease as well as the significant lung pathology observed may be a result of the unusual upregulation of proinflammatory cytokines including TNFα, IP-10, and IL-6. Our findings not only contribute to a better understanding of the pathogenesis of the strain isolates associated with Canadian human cases of avian H5N1 and H7N9 virus infection, but also provide animal models for testing vaccine and antiviral candidates for viruses that are of significant public health concerns.

2.6 Acknowledgements

We are thankful to Yan Li from the National Microbiology Laboratory, PHAC for sharing the AB14 (H5N1) and BC15 (H7N9) strain isolates with us. We are grateful to the animal care staff at VIDO-InterVac for the enormous support in housing, monitoring, infecting, and processing the mice. We also appreciate Tracey Thue in assisting the coordination with both the CFIA and PHAC for the Biosafety regulation of the Containment Level 3 facility. This manuscript was

approved for publication by the director of VIDO-InterVac and was assigned the manuscript serial number 857.

TRANSITIONS BETWEEN CHAPTER 2 AND CHAPTER 3

In chapter 2, I established a mouse disease model to test the infection of AB14 (H5N1) virus. My results showed that intranasal inoculation of 10^3 PFU of AB14 (H5N1) to mice could lead to rapid weight loss and 100% mortality rate without the need of adaptation. Furthermore, AB14 (H5N1) virus caused systematic infection in multiple organs, including lung, spleen and brains, with dysregulation of proinflammatory cytokines and chemokines in lung and brains. These results, for the first time, provide experimental data to complete the human case report of the Canadian HPAI H5N1 isolate and offer a valuable animal model for further vaccination study.

In the following chapter, I focused on design, expression and purification of HA immunogens in two expression systems; characterization of the immunogens *in vitro*; using the mouse model I established for AB14 (H5N1) to evaluate the immune responses and protection efficacy of the HA immunogens with or without the TriAdj.

These results are presented in the following manuscript.

CHAPTER 3 A NOVEL ADJUVANT FORMULATED HA BASED VACCINE PROTECTS MICE FROM LETHAL INFECTION OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 VIRUS

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This manuscript was submitted to Vaccine.

My contribution to this manuscript:

I played a leading role in performing all experiments and analyzing the data, writing the original draft of the manuscript, editing the manuscript.

My estimated contribution to this manuscript is about 80%.

3.1 Abstract

The highly pathogenic avian influenza (HPAI) H5N1 viruses and their spillover into the human population pose substantial economic burdens and public health threats. Here we developed a novel H5 influenza vaccine to improve the pandemic preparedness. The hemagglutinin (HA) of HPAI H5N1 virus A/Alberta/01/2014 (AB14) was produced using both mammalian (m) and bacterial (b) expression systems. The purified recombinant proteins were formulated with a proprietary adjuvant (TriAdj) and their efficacy as vaccine candidates was evaluated in mice. Intramuscular delivery of two doses of TriAdj formulated mammalian expressed HA (m-HA/TriAdj) provided full protection against a lethal challenge of AB14 in mice; in contrast bacterially expressed HA with TriAdj (b-HA/TriAdj), b-HA without adjuvant or m-HA without adjuvant resulted in no protection in immunized mice. Furthermore, m-HA/TriAdj elicited significantly higher levels of balanced Th1 and Th2 responses and neutralizing antibody titres. All of the mice in this group survived a lethal AB14 H5N1 challenge and showed no signs of disease or infection as demonstrated by no loss of body weight or detectable virus in the lungs. Our results suggest that m-HA formulated with TriAdj has potential to protect against pandemic H5N1 in the event of its cross over to the human host.

3.2 Introduction

Influenza A virus (IAV) is a significant health threat to the human population. It infects a wide host range, including waterfowl, poultry, swine, horse, dog, cat, human and several other mammal species (Short et al., 2015). Aquatic birds are natural reservoirs for IAVs, with 16 HA and 9 NA subtypes of IAVs being identified thus far (Fouchier et al., 2005; Sato et al., 2019). Increasing risk of zoonotic disease outbreaks exists due to the interspecies spillover of the highly pathogenic avian influenza viruses (HPAI) from avian species to human hosts. To date, 860 HPAI H5N1 human infection cases have been reported in 16 countries, of which around 52% were fatal (WHO, 2019c). The transmission of HPAI H5N1 virus from birds to humans occurs through either direct contact with infected poultry or indirect contact by exposure to the contaminated environment (Van Kerkhove et al., 2011). The adaptive mutations acquired by HPAI H5N1 viruses may lead to increased transmission efficiency from birds to humans; alternatively, the acquisition of human-to-human transmission ability of the virus might result in a devastating influenza pandemic. Although antiviral drugs have some effect in treating influenza infection, vaccination is still the most effective intervention to prevent possible pandemic outbreaks. However, conventional egg-based vaccine production strategy takes up to six months for vaccine development, during which the antigenically-distinct pandemic viruses are allowed to spread in the naïve population leading to a rapid disease progression (Krammer & Palese, 2015). Traditional inactivated vaccines against zoonotic avian influenza viruses often suffer from low immunogenicity due to the intrinsic poor antigenicity of avian influenza viruses, or they are poor inducers of cellular immunity (R. D. de Vries, Herfst, & Richard, 2018).

Currently, several human H5N1 subunit pre-pandemic stockpiling vaccines are under development (WHO, 2019a). Clinical studies and animal studies have shown that HA of influenza H5 subtype is a weak antigen and is less immunogenic; co-formulation with adjuvant or higher antigen dose in the vaccine is necessary to enhance its immunogenicity (R. B. Belshe et al., 2014; Frey et al., 2019; W. Keitel et al., 2010; Sun et al., 2017; S. H. Wang et al., 2019). Adjuvants have been used in vaccines for more than 90 years and significantly enhance immune responses towards vaccine antigens, particularly for viral antigens with poor immunogenicity (Cohet et al., 2019; Di Pasquale, Preiss, Tavares Da Silva, & Garcon, 2015). In FDA-approved vaccines, limited adjuvants are employed in vaccines for human, which include aluminum salts, MF59, AS01, AS03, AS04, and CpG ODN (Shi et al., 2019). We developed Triple Adjuvant (TriAdj) which consists

of three components: poly (I:C), a ligand of the toll-like receptor-3 (TLR3); IDR-1002, an innate defense regulatory peptide; and poly[di(sodium carboxylatoethylphenoxy)] phosphazene (PCEP), a synthetic polymer (Andrianov, Marin, & Chen, 2006; Niyonsaba et al., 2013; Sadat, Snider, Garg, Brownlie, & van Drunen Littel-van den Hurk, 2017). Formulation of TriAdj with human viral and bacterial antigens including human respiratory syncytial virus (RSV) and *Bordetella pertussis* induced efficient and long-lasting immune responses in animal models such as mice, sheep, and pigs (Garg et al., 2014; Garg, Latimer, Gerdts, Potter, & van Drunen Littel-van den Hurk, 2015; Polewicz et al., 2011). However, whether TriAdj can enhance the immunogenicity of influenza antigens has not yet been determined.

The glycosylation status of HA, particularly glycan quantity or quality, is closely associated with the induction of virus-specific antibody responses. It has been reported that large glycans can mask conserved epitopes on HA resulting in reduced immunogenicity compared to HA with a single glycan or without glycosylation (C. C. Wang et al., 2009). However, glycosylation may be required for the correct conformation of the protein and essential for the expression of protective epitopes which elicit neutralizing antibodies. Compared to N-glycosylated HA antigen, non-glycosylated HA antigen showed relatively low induction of antigen-specific antibodies *in vivo* (Hutter et al., 2013). Thus, glycosylation is an important parameter to be considered in the design of HA-based subunit vaccines.

In this study, we aimed to develop a new subunit H5 influenza vaccine to achieve better immune protection against HPAI H5N1 virus infection. Given the roles of glycosylation and adjuvant in determining the immunogenicity of antigens, we compared H5 HA antigens expressed in either mammalian or bacterial system and formulated with or without the TriAdj, with respect to their immunogenicity and protective capacity against lethal viral challenge in mice. Our results showed that the bacterially expressed HA (b-HA) did not provide protection against an H5N1 lethal challenge, regardless of the presence of TriAdj. In contrast, vaccination with the mammalian-expressed recombinant HA antigen (m-HA) formulated with TriAdj elicited significantly higher levels of IgG1 and IgG2a, as well as neutralizing antibodies in the serum compared to all the other vaccine combinations, and protected mice from a lethal challenge with the H5N1 virus.

3.3 Materials and methods

3.3.1 Cells and Viruses

Human embryonic kidney (HEK293T, ATCC, #CRL-3216) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS, Sigma) and 50µg/ml gentamycin (Bio Basic Canada, Toronto, ON, Canada). The 293T cell line stably expressing the recombinant HA protein was cultured in DMEM supplemented with 10% FBS, antibiotic-antimycotic (Gibco, Grand Island, NY, USA) and 1 µg/ml puromycin (Invivogen, San Diego, CA, USA) for maintaining.

Influenza A/Alberta/01/2014 (H5N1) [AB14 (H5N1)] (GISAID EpiFlu database ID: EPI_ISL_154130) was kindly provided by Dr. Yan Li at the National Microbiology Laboratory, Public Health Agency of Canada. The virus was propagated in MDCK cells and the titre was measured by plaque assay. All the H5N1 virus infection experiments were conducted in Biosafety Containment Level 3 at the International Vaccine Centre at the University of Saskatchewan, Canada under the guidelines of Public Health of Canada and the Canadian Food Inspection Agency.

3.3.2 Protein production

The HA amino acid sequence is derived from AB14 (H5N1). The HA gene was codon-optimized for mammalian expression and was synthesized based on the HA amino acid sequence on EpiFlu databases (EPI500771) by GenScript (Piscataway, NJ, USA). The polybasic cleavage site on the HA gene was mutated form PQRERRRKRG to PQTRG to minimize unnecessary cleavages in producing the HA proteins. The last 10 amino acids at the C terminus of the HA ectodomain was deleted as this was observed to substantially increase the yield of secreted recombinant protein. For mammalian expression, the HA coding sequence with N-terminal tissue plasminogen activator (TPA) signal peptide and C-terminal his_{12}-tag, was cloned into an episomal expression vector pEB4.3 (accession number: MG182339). The coding sequence was proceeded by a Kozak sequence and was cloned downstream of a human CMV promoter and intron IA contained within the vector; the resulting plasmid construct was named pEB-HA. For Bacterial expression, the HA sequence encoding AA 16-521 of AB14 HA fused with an N terminal His_6 tag was cloned downstream of a T7 promoter contained within a pET vector (pET30a.1); the resulting construct was named pET-HA.

I have cloned various constructs to optimize the HA expression in mammalian system.

Table 3.1 List of expression plasmids used in Chapter

	Plasmid construct	Description	Database #	Expression location	Expression level
pEB 4.6	Mini HA	HA (1-52, 320-531 aa)	1150	cell lysate	No secretion
	Mini HA mut	HA (1-52, 320-531 aa)	1151	cell lysate	No secretion
	HA ectodomain	HA (1-531 aa)	1146	cell lysate	No secretion
pEB 5.2	Mini HA	HA (17-52, 320-531 aa)	1180	cell lysate	No secretion
	Mini HA mut	HA (17-52, 320-531 aa)	1181	cell lysate	No secretion
	HA ectodomain	HA (17-531 aa)	1147	cell lysate	No secretion
	HA1	HA (17-338 aa)	1293	supernatant, cell lysate	Low supernatant expression
	HA with C-term Δ10 aa (Δpb)	HA (17-521 aa Δ339-344 aa)	1285	supernatant, cell lysate	High supernatant expression
	HA with C-term Δ20 aa (Δpb)	HA (17-511 aa Δ339-344 aa)	1286	cell lysate	No secretion
	HA with C-term Δ40 aa (Δpb)	HA (17-491 aa Δ339-344 aa)	1287	cell lysate	No secretion
	HA with C-term Δ60 aa (Δpb)	HA (17-471 aa Δ339-344 aa)	1288	cell lysate	No secretion
pEB 5.2 Foldon	HA ectodomain	HA (17-531 aa)	1232	cell lysate	No secretion
	HA ectodomain Δpb	HA (17-531 aa Δ339-344 aa)	1233	cell lysate	No secretion
	Mini HA	HA (17-52, 320-531 aa)	1234	cell lysate	No secretion
	Mini HA Δpb	HA (17-52, 320-531 aa Δ339-344 aa)	1235	cell lysate	No secretion
	HA1	HA (17-338 aa)	1294	cell lysate	No secretion
	HA with C-term Δ10 aa (Δpb)	HA (17-521aa Δ339-344 aa)	1289	supernatant, cell lysate	Low supernatant expression
	HA with C-term Δ20 aa (Δpb)	HA (17-511 aa Δ339-344 aa)	1290	cell lysate	No secretion
	HA with C-term Δ40 aa (Δpb)	HA (17-491 aa Δ339-344 aa)	1291	cell lysate	No secretion
	HA with C-term Δ60 aa (Δpb)	HA (17-471 aa Δ339-344 aa)	1292	cell lysate	No secretion

Their expression level and location are summarized in Table 3.1. The construct has the most abundant supernatant expression is bolded in the table and used in the study. The original signal

peptide of HA constructs with the N terminus 17aa deletion are replaced with TPA signal peptide on the vector.

3.3.3 Protein production

HEK293T cells (ATCC) were maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma) and 50µg/ml gentamycin (Bio Basic Canada). HEK293T cells were transfected with pEB-HA using TransIT-LT1 (Mirus, Madison, WI, USA) according to the manufacturer's protocol. Stable maintenance of the episomal vector was selected by addition of puromycin (1 µg/ml) 48 hours post transfection. Cells were grown in Hyclone CDM4HEK293 supplemented with GlutaMAX (Gibco), antibiotic-antimycotic, 1 µM sodium pyruvate, and 1 µg/ml puromycin 1 µg/ml (Invivogen) for 48 hours to produce H5 HA protein. Protein was purified from the supernatant with NI-NTA agarose affinity chromatography (Qiagen, Toronto, ON, Canada) and dialyzed against phosphate-buffered saline (PBS buffer).

For bacterial protein expression, the pET-HA construct was transformed into BL21 (DE3). Cells were grown to mid-log phase in LB medium and expression of recombinant HA was induced by the addition of 0.5 mM IPTG (Sigma-Aldrich, St. Louis, MO, USA) followed by growth with vigorous aeration at 26˚C for 5 hours. Bacteria were collected by centrifugation, resuspended in lysis buffer (150 mM NaCl, 25 mM Tris-HCl, 0.5% Triton X-100, protease inhibitor (Cell Signaling, #5871S, Whitby, ON, Canada), pH 8.0) and broken using an emulsiflex C3 homogenizer (Avestin, Ottawa, ON, Canada). After disruption, the lysate was centrifuged at 13,000 rpm for 40 min, and the pellet was collected and stored at −20°C; all recombinant HA was determined to be expressed as inclusion bodies within the pellet fraction. The recombinant HA was solubilized by resuspension in denaturation buffer (500 mM NaCl, 25 mM Tris–HCl, 5 mM imidazole, 8 M urea, pH 7.9). After centrifugation at 13,000 rpm for 40 min to remove insoluble material, b-HA was purified using NI-NTA agarose according to the manufacturer's instructions. Recombinant b-HA was eluted with elution buffer (500 mM NaCl, 25 mM Tris-HCl, 500mM imidazole, 8 M Urea, pH 7.9). The 10 ml eluted b-HA was loaded into a syringe with a 27-gauge needle and allowed to drop by gravity into 2L of refolding buffer (50 mM glycine, 5 mM EDTA, 5 mM DTT, pH 9.0) with stirring at 4°C for 48 hours. The diluted protein was concentrated using

an Amicon® Ultra-15 centrifugal filter (10 kDa) (Millipore, Burlington, MA, USA) and dialyzed for 48h at 4°C against PBS buffer.

3.3.4 Circular Dichroism (CD) Spectroscopy

The secondary structures of the m-HA and the b-HA protein were investigated by CD spectroscopy with Chirascan Plus CD Spectrometer (Applied Photophysics, Leatherhead, UK). Each of the purified protein was diluted into PBS buffer and subjected to CD analysis. CD spectrum was measured between 280-170 nm at 20˚C. The secondary structure content of each protein was analyzed with Chirascan software based on its CD spectrum.

3.3.5 Animal vaccination and challenge

Fifty-five four-week-old female BALB/c mice (Charles River Laboratories, Saint-Constant, QC, Canada) were divided into 5 groups with 11 mice per group. Acclimatization was held in Biosafety Level 2 containment 5 days prior to vaccination. At day 0, mice were vaccinated with either PBS or the different H5N1 HA antigens (1μg/mice) with or without a proprietary adjuvant (TriAdj) and boosted 21 days after the first immunization. The TriAdj consists of 10 μg poly (I: C) (Gibco Life Technologies, Burlington, ON, Canada), 20 μg IDR-1002 (VQRWLIVWRIRK) (Genscript), and 10 μg PCEP (Idaho National Laboratory, Idaho Falls, ID, USA). Nine days post boost, 3 animals per group were euthanized, and the spleens were collected, while the remaining 8 mice per group were transferred into the Biosafety Level 3 containment and challenged intranasally with 10^3 pfu of AB14 (H5N1) virus per mouse. Mice were monitored for body weight and clinical scores daily after the viral challenge. On day 5 post challenge, 4 mice from each group were euthanized, and lungs, spleens were collected. The remaining mice were either humanely euthanized when they lost over 20% of their initial weight or euthanized when the animal experiment was terminated 14 days post viral challenge.

3.3.6 Enzyme-linked immunospot (ELISPOT)

Murine IFN γ or IL-5 specific monoclonal antibodies (Southern Biotech, Birmingham, AL, USA) were coated on the ELISPOT plates (Millipore) overnight at 4˚C. Splenocytes collected after boost were added at a concentration of 10^7 cells/ ml along with the stimuli of medium alone, purified mammalian H5N1 HA protein (1 μg/μl), purified bacterial H5N1 HA protein (1 μg/μl) or Concanavalin A (Con A), and incubated overnight at 37 ˚C. Spots were developed using biotinylated rat anti-mouse IFN γ or IL-5 antibodies (BD Pharmingen, San Jose, CA, USA), followed by visualizing with Alkaline Phosphatase (AP) Streptavidin (Jackson ImmunoResearch,

West Grove, PA, USA) and SIGMAFAST BCIP/NBT (Sigma). The developed spots were counted with the ELISPOT reader (AID, Strassberg, Germany).

3.3.7 Virus isolation and titration

The mouse lung was homogenized in MEM with Penicillin-Streptomycin (Gibco) by TissueLyser II (Qiagen) at 25 Hz for 5 minutes immediately. After homogenization, tissues were subjected to centrifugation, the supernatant was collected and stored at -80°C for viral titration.

Virus titre was quantified by 50% tissue culture infectious dose (TCID50) assay. In 96-well plates, the supernatant of homogenized tissue samples was incubated with the monolayer of MDCK cells for 1 hour. After 1 hour of incubation, the inoculum was replaced with MEM containing 0.2% BSA and 1 µg/ml TPCK-trypsin (Sigma). The cytopathogenic effect (CPE) development was observed with microscopy every 24 hours after infection. The TCID50 titre of each sample was calculated by the Spearman & Kärber algorithm (Kärber, 1931; Spearman, 1908).

3.3.8 RNA extraction and quantitative RT-PCR (qRT-PCR)

Tissue samples were immediately preserved in RNA later (Qiagen) following tissue collection. RNA was extracted by homogenizing the tissues in TRIzol (Invitrogen) using a TissueLyser II (Qiagen). The downstream processing was conducted according to the manufacturer's instruction.

qRT-PCR was performed to determine the cytokine and chemokine gene expression levels in tissues of vaccinated and virus challenged mice as previously described with modification (Y. Lu et al., 2019). Briefly, 500 µl of RNA was reverse transcribed to cDNA with oligo (dT) and SuperScript III Transcriptase (Invitrogen, Carlsbad, CA, USA); qPCR was conducted with gene-specific primers on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystems). GAPDH was used as the housekeeping gene to normalize the mRNA levels of cytokines or chemokines. All sequences of qPCR primers used in this study are described previously (Y. Lu et al., 2019).

3.3.9 Enzyme-linked immunosorbent assay (ELISA)

To detect influenza-specific lgG levels induced by vaccination, ELISA was performed with serum samples taken before vaccination, after the first vaccination, and after the boost. Purified H5N1 m-HA protein (2.5µg/ml) or b-HA protein (2.5µg/ml) were used to coat the 96-well plates by incubation overnight at 4˚C. Serially diluted serum was added on to the plate and influenza-specific IgG1 and IgG2a were detected by biotinylated anti-IgG1 and IgG2a antibodies (Southern

Biotech). The reaction was developed by adding AP Streptavidin (Jackson ImmunoResearch) and p-nitrophenyl phosphate substrate (PNPP) (Sigma), followed by plate reading in a microplate absorbance spectrophotometer (Bio-Rad, Hercules, CA, USA) at 405 nm with the reference wavelength of 490 nm.

3.3.10 Hemagglutination inhibition assay (HAI)

Serum from vaccinated animals was pretreated with receptor destroying enzyme (RDE) (Sigma, #C8772) according to the manufacturer's instruction. The next day 1.5% sodium citrate (pH7.2) was added to the serum and the mixture was incubated at 56°C for 30 minutes to inactivate the RDE. Next, the serum was treated for 1 hour at 4°C with 50% red blood cells (RBCs) from chicken to reduce nonspecific agglutinations. The treated serum was then ready to be used for the assay. The serum was serially diluted by two-fold and incubated with 4 HA units of the H5N1 virus for 30 min at room temperature. After incubation, an equal volume of 0.5% chicken RBCs was added to the wells and incubated for 30 min. The HAI titre of the serum sample was determined as the minimum reciprocal dilution of serum to inhibit hemagglutination.

3.3.11 Ethics statement

All animal procedures were approved by the University Animal Care Committee (UACC) and Animal Research Ethics Board (AREB) of the University of Saskatchewan on 15 July 2018 (Animal Use Protocol #20170002) in accordance with the standards stipulated by the Canadian Council on Animal Care.

3.3.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. The difference within all groups was examined by one-way ANOVA using the Tukey test. When the difference was compared between groups, two-way ANOVA followed by the Tukey post-test was used to obtain the P-value. Data are shown as the mean plus standard deviation (SD) of each sample performed in triplicates. Significant differences are denoted by *($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

3.4 Results

3.4.1 Expression and characterization of recombinant HA in mammalian and bacterial systems

To generate the secreted form of AB14 HA (m-HA) from mammalian HEK293T cell culture, we deleted the transmembrane domain and the cytoplasmic tail of HA, along with the last ten amino acids at the C-terminus of HA. We further replaced the native HA signal peptide with that of the tissue plasminogen activator (TPA) to facilitate protein secretion from stably transfected cells. In addition, the polybasic amino acids between HA1 and HA2 (RERRRK) were replaced with a threonine (T) to avoid unnecessary HA cleavage during the protein production process (Fig. 1A). For bacterial expression, the same HA codon sequence was used except that the signal peptide was removed (b-HA). To facilitate purification, proteins were expressed with a histidine tag at the C-terminus (m-HA) or the N-terminus (b-HA) (Figure 3.1A).

The recombinant HA proteins were purified and characterized by immunoblotting and Coomassie Blue staining (Figure 3.1B). The two recombinant HA proteins showed significant variations in molecular weight, which can be accounted for by the glycosylation that occurs during mammalian expression. The recombinant HA proteins were then subjected to circular dichroism spectroscopy (CD) to estimate and distinguish their secondary structure. The CD spectra of m-HA under far-UV showed that it consists of 16.5% of α-helix and 22.6% of β-strand, while that of b-HA indicated the contents of α-helix (16.9%) and β-strand (21%) (Figure 3.1C). These are in agreement with the secondary structure composition predicted by PSIPRED programs based on their amino acid sequences, which suggests m-HA comprised of 16.6% of α-helix and 20.4% of β-strand, whereas b-HA contains18.8% of α-helix and 20% of β-strand. These data showed that m-HA and b-HA contained comparable α-helix and β-strand contents, suggesting they have similar secondary structures.

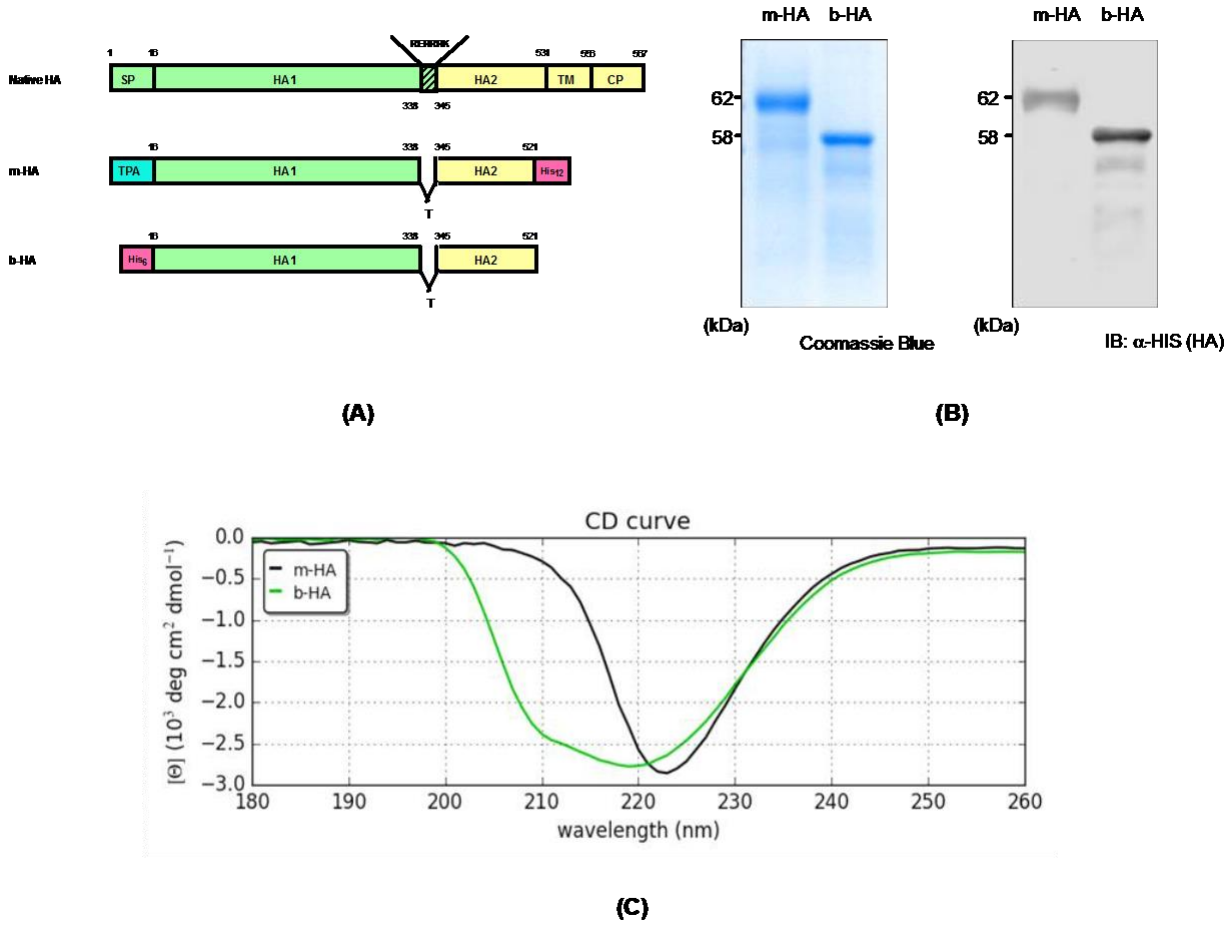


Figure 3.1 Expression and characterization of recombinant HA in two expression systems. (A) Schematic representation of the recombinant H5N1 HA. The polybasic cleavage site on the HA gene was mutated by replacing RERRRK with T, and the last 10 amino acids at the C-terminus of the HA ectodomain were deleted. SP: signal peptide; TM: transmembrane domain; CP: cytoplasmic tail; TPA: tissue plasminogen activator; His12: 12 X Histidine; His6: 6 X Histidine. (B) Purified recombinant H5N1 HA proteins from mammalian and bacterial systems. The expression of recombinant HA proteins was confirmed by Coomassie Blue staining (left) and immunoblotting (right) with the anti-His antibody. (C) Circular dichroism (CD) analysis of the recombinant HA protein's secondary structure. Far-UV CD analysis of the structure of recombinant HA proteins, the representative graph displaying the secondary structure composition.

3.4.2 Mammalian expressed H5N1 HA protein formulated with TriAdj induces high levels of neutralizing antibody titres

To investigate the immunogenicity and the protective efficacy of the recombinant HA vaccines, BALB/c mice were intramuscularly immunized with PBS or 1 μg of the recombinant m-HA or b-HA antigens formulated with or without TriAdj. Mice were boosted 21 days after the first immunization with the respective vaccines. Eleven days after the boost, mice were challenged with a lethal dose (LD100) of HPAI H5N1 virus (10_3 PFU/mice) (Y. Lu et al., 2019) (Figure 3.2A). Serum of all mice was collected on day 0, day 21 and day 30 and subjected to HA-specific antibody detection by ELISA. After the first vaccination, m-HA/TriAdj tended to induce a higher titre of IgG1 compared to pre-vaccination levels (not statistically significant). After the boost, m-HA, m-HA/TriAdj, and b-HA/TriAdj stimulated a higher level of HA-specific IgG1 in comparison to the corresponding levels induced after the first vaccination; m-HA/TriAdj elicited the highest level of IgG1 (Figure 3.2B). Mice immunized with TriAdj-formulated m-HA or b-HA produced increased levels of IgG2a after the first vaccination, which further increased following the boost with significantly higher levels of IgG2a in the m-HA/TriAdj group (Figure 3.2C) compared to the control group. At the two time points tested, b-HA alone did not induce any antibody response; In contrast, m-HA/TriAdj elicited the highest and comparable levels of IgG1 and IgG2a antibodies (Figure 3.2B and C).

Serum HAI titre is widely used in the evaluation of anti-influenza antibody response and vaccine immunogenicity (R. J. Cox, 2013; Ting Hui et al., 2019; Y. Wang, Wu, et al., 2017). Mice vaccinated with a single dose of any of the HA vaccines did not result in significantly higher HAI titres over pre-vaccination (Figure 3.2D). However, after the boost, m-HA/TriAdj induced a HAI titre of 320, which is significantly higher than that elicited by the other vaccines tested (Figure 3.2D).

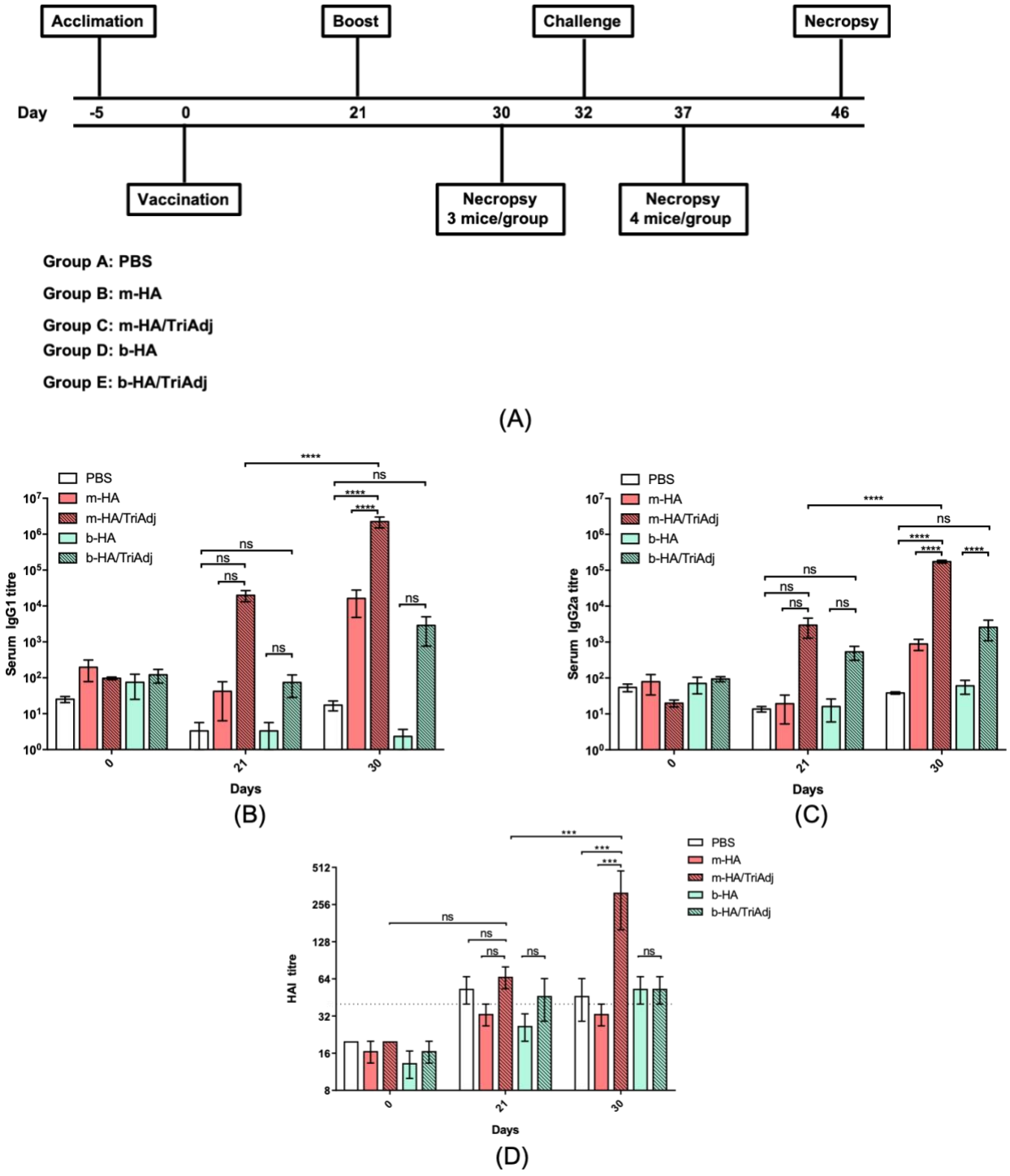


Figure 3.2 Humoral immune responses induced by recombinant HA vaccines.

(A) The timeline and grouping of the vaccination and challenge study in mice. Mouse serum was collected before the first vaccination (day 0), before the boost (day 21) and 9 days after boost (day

30). The HA-specific serum IgG1 (B) and IgG2a (C) titres were determined by ELISA. Serum HAI titre was determined by using the AB14 virus (D). Data are shown as the mean ± standard deviation (SD). Significant differences are denoted by two-way ANOVA using the Turkey test. *($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

3.4.3 Mammalian expressed H5N1 HA protein formulated with an adjuvant produces high numbers of IFN-γ and IL-5 secreting cells

To assess the ability of the HA vaccines to induce cell-mediated immune responses after vaccination, splenocytes were isolated from vaccinated and control mice 9 days after the boost, and antigen-specific responses were measured by IFN γ and IL-5 ELISPOT assays. Both m-HA and b-HA antigens formulated with TriAdj were able to induce significantly more numbers of antigen-specific IFN γ-secreting cells than the control group (Figure 3.3A). In contrast, the numbers of IL-5 producing T cells increased drastically only in the mice vaccinated with m-HA/TriAdj (Figure 3.3B).

Collectively, the results demonstrated that TriAdj formulated m-HA vaccine consistently elicited significantly higher titres of antigen-specific IgG1, IgG2a, and HAI; in addition, m-HA/TriAdj generated a balanced Th1 and Th2 immune response.

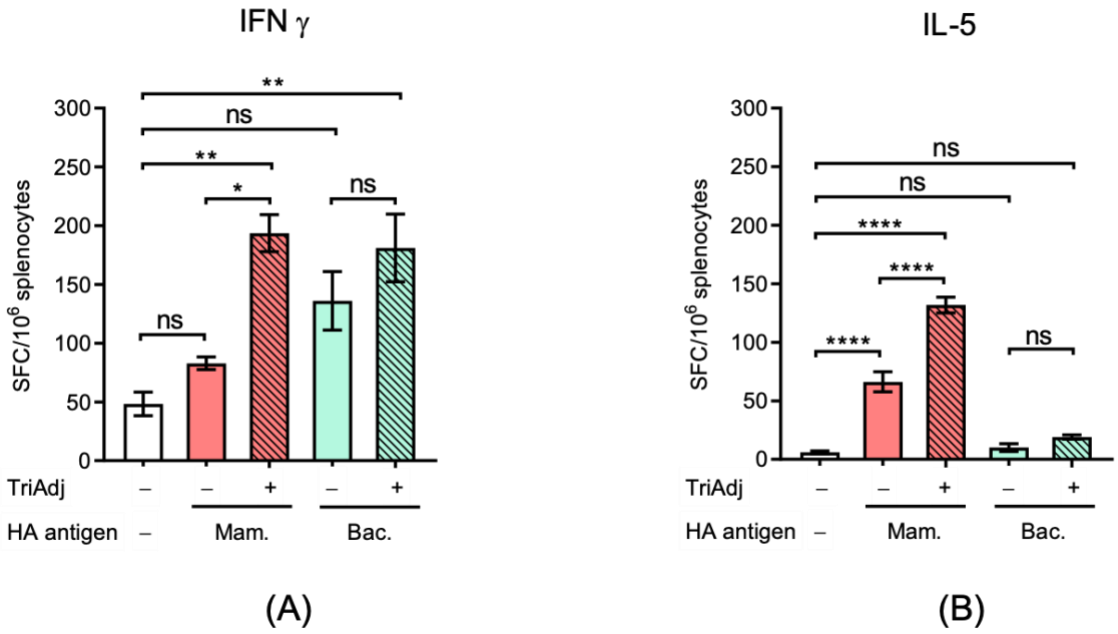


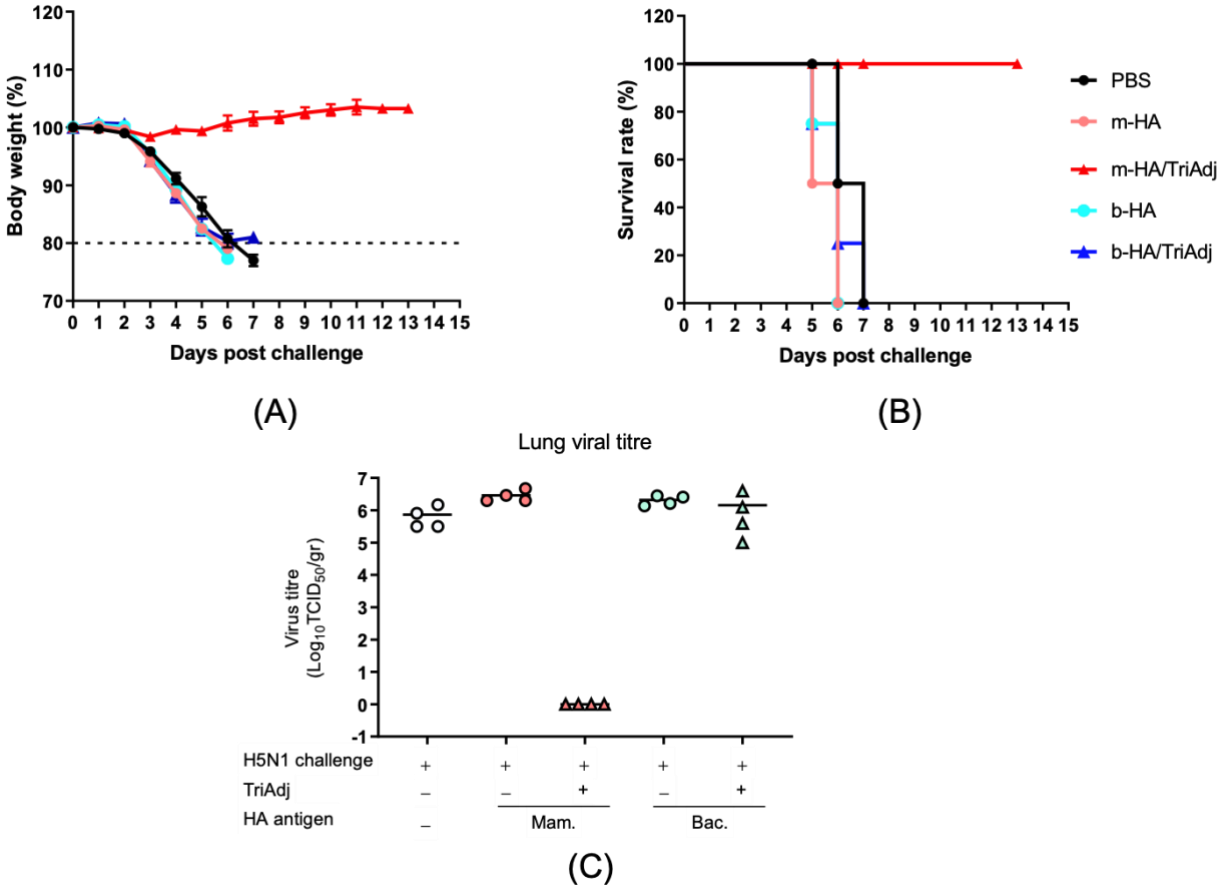
Figure 3.3 Cell-mediated immune responses elicited by recombinant HA vaccines.

Spleens were collected 9 days after boost. The number of IFN γ (A) and IL-5 (B) secreting T cells per 10_6 splenocytes was assessed by ELISPOT. The number of spots observed in wells stimulated only with medium was counted and subtracted as background. Results are reported as the number of IFN γ- or IL-5- secreting cells per 10_6 splenocytes when stimulated with either m-HA or b-HA antigens. SFC: spot-forming cells. Data are shown as the mean ± standard deviation (SD). Significant differences are denoted by one-way ANOVA using the Turkey test. *($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

3.4.4 Mammalian expressed H5N1 HA protein with the TriAdj completely protects mice from the lethal challenge with the HPAI H5N1 virus

To evaluate the protective effect induced by the recombinant HA antigens, mice that received two vaccine doses were challenged with an LD100 of HPAI H5N1 virus (10_3 PFU/mice) (Figure 3.2A). All mice that received m-HA/TriAdj survived the lethal viral challenge. During the first 3 days after virus challenge, mice in this group only showed marginal body weight loss with no disease signs and started gaining weight on day 4 post challenge. In contrast, mice immunized

with the other vaccines and challenged with H5N1 virus experienced rapid weight loss greater than 20% of their initial body weight within 7 days; they all reached the humane endpoint by exhibiting severe illness signs such as rough coat, hunched posture and were no longer mobile. These mice did not survive the duration of the trial (Figure 3.4A and B). Consistent with these results, infectious virus could not be detected in the lung of the mice that received m-HA/TriAdj, whereas mice immunized with either PBS, m-HA, b-HA or b-HA/TriAdj had a lung virus titre of 2.87x10$_6$



TCID$_{50}$/g, 2.08x10$_6$ TCID$_{50}$/g, and 1.42x10$_6$ TCID$_{50}$/g, respectively on day 5 post challenge (Figure 3.4C).

Figure 3.4 Body weight, survival rate and lung viral titre of mice.

Mice received different vaccines and were challenged intranasally with AB14 (H5N1) virus (10$_3$ PFU/mice). The body weight changes (A) and the survival rates (B) were monitored. The virus titre in the lung on day 5 post challenge was determined by TCID$_{50}$ assay (C).

To access the pathological immune responses induced by H5N1 virus infection after vaccination, we determined RIG-I receptor and cytokine gene expression levels in lung tissue of mice on day 5 post challenge by qPCR (Figure 3.5). RIG-I is a major pattern recognition receptor that recognizes the influenza virus infection and activates the interferon responses (G. Liu et al., 2018; Loo & Gale, 2011; Opitz et al., 2007). RIG-I mRNA was significantly upregulated in PBS vaccinated and H5N1 virus challenged mice; in contrast, the m-HA/TriAdj vaccinated and virus challenged mice had the lowest RIG-I gene expression compared to all other groups. The gene expression of both type I interferons (IFN α and IFN β), type II interferon IFN γ, and interferon-gamma-inducing protein 10 (IP-10) was significantly up-regulated in PBS vaccinated and H5N1 virus challenged mice; however, vaccination with m-HA/TriAdj mostly abrogated the up-regulation of IFNs that occurred following challenge with virus. Similarly, the gene expression of proinflammatory (TNF α, IL-6) and anti-inflammatory (IL-10) cytokines was significantly lower in the m-HA/TriAdj vaccinated group than in all the other vaccinated groups after virus challenge, further confirming that vaccination by m-HA/TriAdj completely protected mice from HPAI infection.

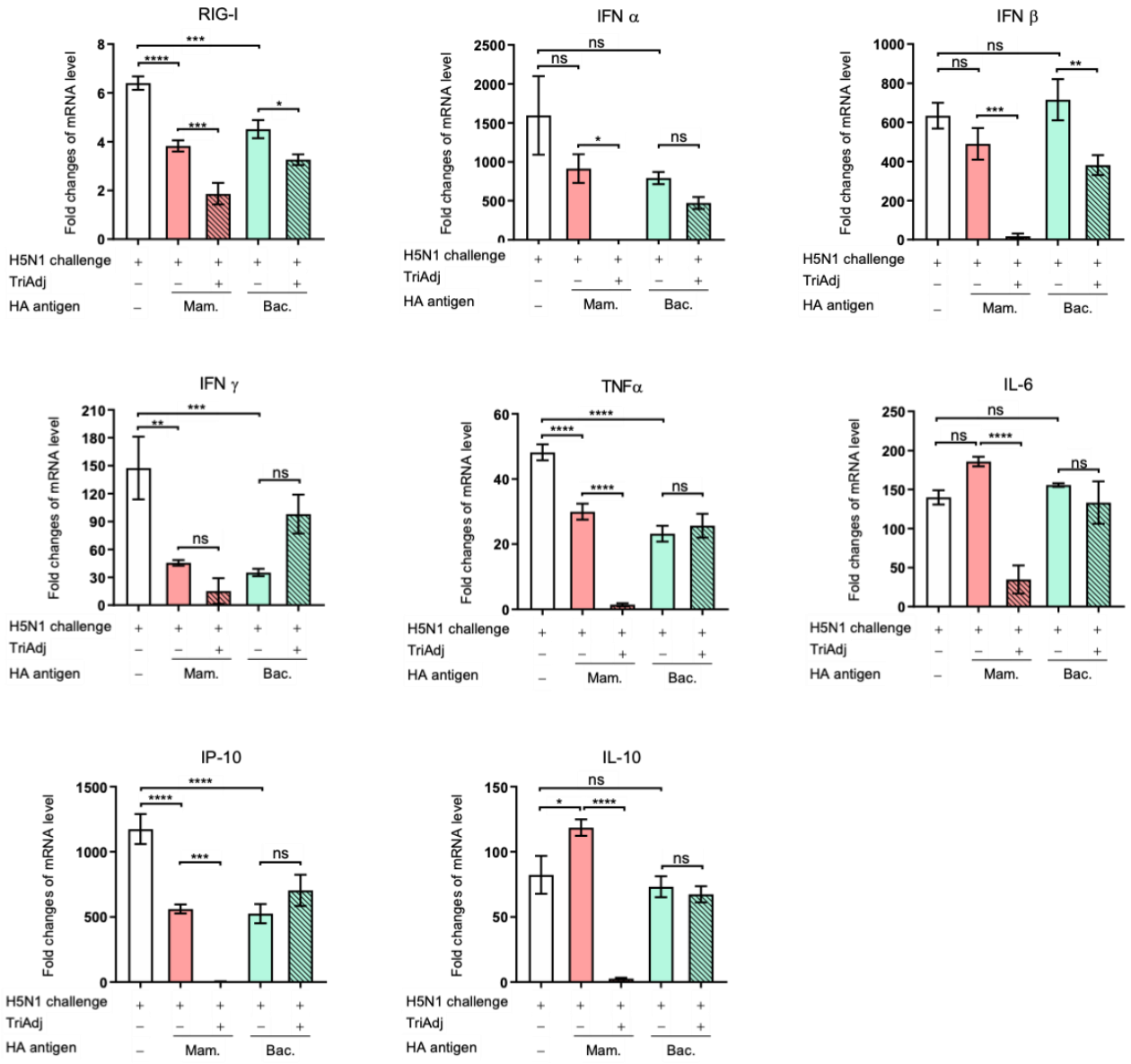


Figure 3.5 Innate immune receptor, cytokine and chemokine gene expression profiling induced by AB14 challenge after mice received two doses of vaccines.

mRNA levels of the indicated genes in mouse lung on day 5 post challenge (n = 4 mice per virus group) were measured by qRT-PCR. Each sample was tested in triplicates. Data are shown as the mean ± standard deviation (SD). Significant differences are denoted by one-way ANOVA using the Turkey test. *($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

3.5 Discussion and conclusion

In the current study, we evaluated the immunogenicity and protection of mice from H5N1 virus lethal challenge by immunization with the recombinant HA proteins expressed in a bacterial system and a mammalian system, with or without an adjuvant. Although bacterial expressed HA protein showed a similar secondary structure to that of mammalian expressed HA as determined by CD spectroscopy, bacterial expressed HA alone did not induce any antibody response. Addition of TriAdj to bacterial expressed HA led to increased production of serum IgG; however, it had no HAI activity. Vaccination with m-HA without adjuvant induced elevated levels of both antibody and cellular immune responses after the boost. However, this was insufficient to provide protection from a lethal dose infection of the H5N1 virus. These results suggested that H5 HA alone is a poor immunogen and that proper glycosylation is required to induce neutralizing antibodies. This finding is in agreement with the previous report that MDCK and Vero cell-derived glycovariants could affect T-cell activation and cytokine production; removal of glycosylation dramatically decreased HA-specific antibody titre (Hutter et al., 2013).

Formulation of TriAdj with mammalian expressed HA led to increased levels of both IgG1 and IgG2a antibodies after the first vaccination; these levels further rose after the boost. Additionally, induction of virus-specific neutralizing serum antibodies measured by HAI, and an increased number of Th1-associated IFN γ secreting- as well as Th2-associated IL-5 secreting- T cells only occurred in mice vaccinated with m-HA/TriAdj (R. M. Talaat, Mohamed, Bassyouni, & Raouf, 2015; Viallard et al., 1999). These data suggested that the m-HA/TriAdj vaccine induced balanced Th1 and Th2 immune responses with effective humoral immunity. These immune responses were clearly attributable to the TriAdj, and our challenge study showed that these immune responses provided complete protection to the lethal H5N1 virus challenge in mice.

TriAdj is comprised of three components: poly(I:C), host defense peptide, and polyphosphazene. Poly(I:C) is recognized by TLR3 and other intracellular innate immune receptors. Activation of these receptors triggers the production of pro-inflammatory cytokines, which will activate the immune cells, such as monocytes/macrophages, DCs, natural killer cells, B cells and T cells (Stahl-Hennig et al., 2009; Trumpfheller et al., 2008; Trumpfheller et al., 2012). The host defense peptide IDR-1002 is immunomodulatory and promotes the induction of multiple cytokines and chemokines, including CCL1, CCL2, CCL5, and IFN γ, and the recruitment of neutrophils and monocytes, resulting in the enhancement of the immune responses against

pathogens (Nijnik et al., 2010; Prame Kumar, Nicholls, & Wong, 2018). The third component, PCEP, is a synthetic water-soluble polymer. It allows multimeric presentation by forming a complex with the other two components and antigen through non-covalent interaction, thus enhancing the stability, immunogenicity of the antigen (Awate et al., 2012; Garg, Babiuk, van Drunen Littel-van den Hurk, & Gerdts, 2017; Mutwiri et al., 2007). Previous studies from our group have shown that large and small animals vaccinated with viral subunits formulated in TriAdj demonstrate effective protection against challenge with a balanced response and that the immunity is long-lasting (Garg et al., 2018).

Formulation of TriAdj with subunit vaccine stimulated the induction of T follicular helper cells, plasma cells, and memory B cells after intranasal immunization (Garg, Babiuk, et al., 2017). Mucosal immunization with TriAdj formulated subunit RSV vaccine elicited potent antigen-specific mucosal and systemic immune responses, which provided protection of RSV infection in cotton rats (Garg et al., 2018). Given the advantages of intranasal vaccination, future studies will look at the effectiveness of immunizing animals intranasally with m-HA/TriAdj. In addition, with the induction of a balanced Th1 and Th2 response and the high neutralizing antibodies levels observed in our study, m-HA/TriAdj vaccine holds the promise to provide broader protection against heterologous subtypic H5N1 influenza virus infection.

In summary, recombinant HA expressed and purified from mammalian cells, and formulated with novel adjuvant TriAdj induced robust and balanced immune responses, and it has the potential to protect humans against a possible pandemic HPAI H5N1 outbreak.

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CHAPTER 4 DISCUSSION AND CONCLUSIONS

Currently, avian influenza viruses have been continuously circulating in birds and occasionally caused human infections. The spread of zoonotic HPAI H5N1 virus from poultry to humans highlighted its threat to public health. The HPAI H5N1 viruses caused over 50% mortality rate in human infection cases. Fortunately, there is no evidence of interhuman transmission of the H5N1 virus to date. (H. Imai et al., 2018; WHO, 2019c). Avian influenza viruses preferably bind to α-2, 3-linked sialic acid receptors mostly located on the intestinal epithelial cells of waterfowls (Gambaryan et al., 2003; Paulson & de Vries, 2013). However, the highly pathogenic avian H5N1 viruses adapt the ability to bind to α-2, 6-linked sialic acid receptors on the human upper respiratory tract to be efficiently transmitted from poultry to humans (M. Imai & Kawaoka, 2012). Human infection cases of avian influenza H5N1 viruses are characterized by multi-organ failure, neurological disorders and fulminant pneumonia with acute respiratory distress syndrome (ARDS), which is the main reason for H5N1-associated human mortality (de Jong et al., 2006; Li et al., 2018). Fatal H5N1 human cases are also associated with high nasal virus loads, frequent detection of viral RNA in serum, and hyperinduction of proinflammatory chemokines and cytokines in the lung (de Jong et al., 2006; Westenius, Makela, Julkunen, & Osterlund, 2018).

Ever since the first human infection cases of HPAI H5N1 virus in 1997 in Hong Kong, the HPAI H5N1 viruses have spread to multiple countries. In 2014, A/Alberta/01/2014 (H5N1) (AB14 (H5N1)) virus was isolated from a Canadian traveler shortly after returning from China (Maurer-Stroh et al., 2014; Pabbaraju et al., 2014). This particular strain jumped from avian to human due to several mutations on the receptor binding site of the HA gene in the AB14 (H5N1) virus (Pabbaraju et al., 2014). We identified the pathogenicity of this particular Canadian isolate of HPAI H5N1 virus in a mouse model. We found that the AB14 (H5N1) virus could result in high mortality in mice with the induction of systemic infections as well as dysregulation of pro-inflammatory cytokines and chemokines in multiple organs without further adaptation (Y. Lu et al., 2019). These data not only provide a more thorough understanding of this human case report of HPAI H5N1 virus, but also provide a valuable animal model for vaccination studies against this AB14 (H5N1) virus.

In this study, after HPAI AB14 (H5N1) challenge, virus particles were detected in mouse lung, spleen and brain tissue. The mechanism of influenza associated spleen infection is not known. One possible explanation is that there is a period of viremia upon severe lung inflammation (Tse

et al., 2011; X. Wang, Tan, Zhao, Ye, & Hewlett, 2014). Alternatively, viral particles or antigens may migrate to the spleen with phagocytes or antigen-presenting cells. In the context of HPAI H5N1 virus-induced central nervous system infection in the brian, it has been reported that some laboratories confirmed that human HPAI-infected cases had H5N1 virus or viral RNA isolated from the brain tissues, serum and cerebrospinal fluid (de Jong et al., 2005; Gao et al., 2010; Mak et al., 2018). Moreover, administration of 10_6 50% egg infectious doses in ferrets via the upper respiratory tract induced high viral loads in the cerebrum, suggesting that HPAI H5N1 virus has the ability to cause systemic infection in animal models (Edenborough et al., 2016). My research also indicated that this particular HPAI H5N1 human isolate (AB14) is capable of replicating systematically and reaching high viral loads in the brain after challenge with 10_3 PFU of the virus (Y. Lu et al., 2019).

Animal models of influenza play an essential role in viral pathogenicity studies and in pre-clinical vaccination studies (Margine & Krammer, 2014; Oh & Hurt, 2016). We established the model in mice due to the following factors: low experimental cost, large animal numbers per experiment, and easy handling process (Marjuki et al., 2014; Zarogiannis et al., 2012). We've evaluated the viral pathogenicity through the observation of the virus-induced weight loss, mortality in mice and through the measurement of viral titers and proinflammatory cytokine upregulations in different murine organs post challenge. However, the biggest limitation of using a mouse influenza model is the lack of clinical symptoms in mice after infection (Oh & Hurt, 2016).

In contrast to the mouse influenza model, ferrets could display comparable clinical symptoms, such as elevated body temperatures, sneezing, and nasal discharges, to that of humans after influenza infection (Gooch et al., 2019; Maines et al., 2006). Furthermore, the ferret influenza model also serves as an ideal approach to display the transmission ability of influenza viruses, because influenza viruses could efficiently infect ferrets without further adaptations (Belser et al., 2018). The superior advantages of the ferret influenza model are that ferrets and humans share similar lung physiology, including similar distribution of sialic acid receptors for influenza viruses in the respiratory tract (van Riel et al., 2007; Zeng et al., 2013). However, there are some limitations about using the ferret model; these include relatively small animal group sizes due to the high experimental cost and facility requirement and lack of available immunological reagents (Enkirch & von Messling, 2015). Currently, ferret models have been used in viral susceptibility and transmission studies, and as a pre-clinical model to study influenza infection in particular age

groups including young children, the elderly or immunocompromised individuals (Huang et al., 2012; Paquette et al., 2014; Q. Zhang et al., 2013).

After the establishment of a mouse HPAI H5N1 disease model, we aimed to develop of a pre-pandemic subunit H5N1 vaccine. The preparation for pandemic vaccine stock could not only counteract a potential pandemic, but are also recommended by CDC for all laboratory workers working on developing influenza vaccines against the HPAI H5N1 viruses (Centers for Disease, Prevention, Gangadharan, Smith, & Weyant, 2013). Due to the weak immunogenicity of the HA antigen of the H5N1 virus, the subunit vaccine with HA antigen alone did not elicit strong immune response (Pitisuttithum et al., 2017). To strengthen the immunogenicity of the subunit influenza vaccine, increased antigen doses, the use of adjuvants, or the application of prime and boost strategy are used in H5 vaccines (Karron et al., 2009; K. R. Talaat et al., 2014; Treanor, Campbell, Zangwill, Rowe, & Wolff, 2006; Y. Wang, Wu, et al., 2017). Research showed that adjuvanted plant-derived recombinant HA of H5N1 induced high levels of antigen-specific IgG titres in the serum and IgA titres in bronchial secretions in mice (Ting Hui et al., 2019). Besides that, the recombinant HA H5N1 vaccine also elicited cell-mediated immunity and provide full protection against challenge with a lethal dose of H5N1 virus in ferrets (S. H. Wang et al., 2019). The application of a subunit influenza vaccine is an effective and economical option comparing to conventional vaccines.

The subunit influenza vaccine we developed in this project contained recombinant HA antigens expressed in both mammalian and bacterial systems to obtain the distinct protein glycosylation statuses. A mammalian expression system allows both N-linked and O-linked glycosylation of the secreted protein, especially cell line 293-based platforms, and is capable of providing closer post-translational modification processes and functions to protein naturally produced in human cells (P. J. Kim, Lee, & Jeong, 2009; Walsh, 2010). This system also allows easy transfection and abundant protein secretion, which will reduce the production course, simplify the harvesting process, and increase the production amount, whereas the bacterial protein expression system only provides incomplete glycosylation to expressed proteins but produces a large quantity of recombinant protein (R. Chen, 2012). These studies correlated with the data we obtained. The recombinant HA protein expressed in mammalian 293T cells but not in *E. coli* has a substantially larger size than the calculated mass of the unmodified protein when identified by

Coomassie blue staining, demonstrating the distinct post-modification modification process in a mammalian expression system.

The use of adjuvants in subunit vaccines could significantly enhance and broaden the vaccine immunogenicity, especially in subunit influenza vaccines that showed weaker immunogenicity (Awate et al., 2012; Couch et al., 2012). Previous research demonstrated that the MF59-adjuvanted influenza subunit vaccine (Fluad®) and the inactivated split virion vaccine (Vaxigrip®) had comparable immunogenicity against the H1N1 and the H3N2 virus strains (Beyer, Palache, & Osterhaus, 1998; Squarcione, Sgricia, Biasio, & Perinetti, 2003). The adjuvant we used in this research is a VIDO patented adjuvant, TriAdj, comprised of three major components, poly(I:C), a host defense peptide, and PCEP (Garg et al., 2014; Mutwiri et al., 2007; Niyonsaba et al., 2013). combination of the three components was tested in vivo in different animal models with various viral antigens (Garg, Brownlie, et al., 2017; Garg et al., 2018; Garg et al., 2019; Sadat et al., 2017). The TriAdj has been shown to significantly enhance the immune responses when co-formulated with viral or bacterial antigens of infectious diseases including some respiratory viral antigens, such as RSV (Garg et al., 2014). Similarly, our results demonstrated that the adjuvanted recombinant HA subunit vaccine (m-HA/TriAdj) could induce high levels of antigen-specific antibodies and provide complete protection against H5N1 viral infection.

One of the major components of the TriAdj, poly(I:C) have been shown to induce cell-mediated immune responses with H9 antigen against heterologous infection in ducks (A. Zhang et al., 2017). Similar adjuvant effects were observed with varies subtypes of HA antigen in pigs (Thomas et al., 2015). In mice, poly(I:C) was reported to enhance both mucosal responses and humoral responses in mice when co-formulated with inactivated H5N1 virus and administrated intranasally. These data provide insights of poly(I:C) as an adjuvant is capable of inducing both humoral and cell-mediated immune responses and generating heterologous protection in ducks and pigs. The other two components of the TriAdj are both able to upregulate the production of pro-inflammatory cytokines and chemokines in injection site (Awate, Eng, Gerdts, Babiuk, & Mutwiri, 2014; Prame Kumar et al., 2018). Moreover, PCEP activates naïve B cell directly and generates antigen-specific T cell responses (Awate et al., 2014; Awate et al., 2012). Previous study showed that the combination of TLR agonist, IDR, and PCEP is capable of developing balanced immune responses after intramuscular vaccination and enhancing mucosal immunity when administrated intranasally (Kindrachuk et al., 2009; Kovacs-Nolan, Latimer, et al., 2009; Kovacs-Nolan,

Mapletoft, Latimer, Babiuk, & Hurk, 2009). Poly(I:C), IDR, and PCEP alone as vaccine adjuvant induces moderate immune responses, while the combination of the displayed strong immune responses by enhancing the DC maturation followed by cytokines and chemokines expression (Garg et al., 2014; Snider, Garg, Brownlie, van den Hurk, & van Drunen Littel-van den Hurk, 2014). The relative low cost and significant efficacy of the TriAdj make it an ideal adjuvant candidate to administrate with multiple viral antigens.

Besides the vaccine adjuvants, the distinct glycosylation profiles of HA antigens in the vaccine also results in the varied antigenicity. It was reported that specific N-glycosylation sites on HA masked the antigenic epitopes and affected the antigenicity of the virus (W. Wang et al., 2010). Moreover, the distinct influenza HA N-glycosylation caused by the production of recombinant viruses in different cells lines resulted in remarkably different immunogenicity *in vivo*. T cell activation was significantly diminished after the stimulation of deglycosylated variant compared with fully glycosylated protein, which indicates the essential role of N-glycosylation on HA for immunogenicity. Another study using recombinant HA expressed in mammalian or insect cells showed that HA carrying complex glycan structures or single N-acetylglucosamine residues induces higher neutralizing antibodies titres than HA carrying high-mannose glycan structures (R. P. de Vries et al., 2012). Our results demonstrated that the HA produced in mammalian cells, which carries complex glycan structures, exhibited higher levels of neutralizing antibody and more balanced Th1 and Th2 immunity compared to non-glycosylated HA. In contrast, the non-glycosylated HA induced a Th1-biased immune response with low induction of neutralizing antibodies. Furthermore, fully glycosylated HA with TriAdj but not non-glycosylated HA completely protected mice from lethal infection of the homologous virus, indicating the enhanced protection may be provided by glycosylation of the HA antigen.

In this study, we used the intramuscular delivery route for the recombinant subunit H5N1 influenza vaccine. Besides the intramuscular (IM) injection, the most comment routes are intradermal (ID) injection and intranasal spray. Due to the varied distribution of immune cells in the tissue, blood, mucosal membrane, and skin, different vaccine delivery routes could result in distinct vaccine efficacy (Chaplin, 2006).

ID delivery of 9 μg of a trivalent inactivated HA vaccine showed similar humoral and cellular immune responses to IM delivery of 15 μg of a trivalent inactivated HA vaccine in healthy adults; it reached similar seroconversion rate three months after vaccination in HIV-1-infected

adults. This indicates that ID delivery of influenza vaccine is superior to that of IM delivery (Ansaldi et al., 2012; Nougarede et al., 2014). The possible reason for this may be that the DCs are predominantly in the dermis (Belyakov, Hammond, Ahlers, Glenn, & Berzofsky, 2004; D. Chen et al., 2001). The DCs in the skin could facilitate the capture and presentation of antigens, the induction of DC maturation, and the migration of matured DC into draining lymph nodes (Bonnotte et al., 2003).

Apart from IM and ID delivery of influenza vaccine, needle-free intranasal influenza vaccines have been used in the market since 2013 for 2-49 years old recipients, which is a smaller range of recipients than that targeted by intramuscularly administrated annual influenza vaccines (R. J. Cox, 2013). The IN delivery route of influenza vaccine induces both systemic and local immunity, which could prevent influenza infection and transmission (L. Zhang, Wang, & Wang, 2015). Although the FluMist®, a live-attenuated intranasal trivalent influenza vaccine, induced significantly lower levels of HAI titres, which is incomparable to the injectable influenza vaccine, it stimulated greater levels of local IgA antibody and had similar efficacy against culture-positive influenza infections (Beyer, Palache, de Jong, & Osterhaus, 2002).

In summary, the two parts of this thesis established a mouse disease model for HPAI AB14 (H5N1) virus and developed a vaccine candidate with high efficacy against HPAI H5N1 virus. Understanding the disease progression of HPAI H5N1 virus in mice and identification of a potential anti-H5 vaccine candidate would provide insights into the development of a pandemic influenza vaccine and eventually mitigate the threat of pandemic outbreaks.

This project has proved that the TriAdj-formulated mammalian HA is capable of inducing robust antigen-specific immunity in mice and protecting mice from homologous H5N1 challenge. Based on the current data, our next step would be to identify whether TriAdj-formulated mammalian HA vaccination could provide protection against heterologous virus challenge in mice. Besides that, we would also broaden the coverage of the vaccine by establishing a ferret model to explore the efficacy of the vaccine *in vivo*, and its potential to prevent viral transmission and infection in a ferret model. In the content of vaccine administration route, aside from the current IM delivery of the subunit influenza vaccine, the development of the needle-free IN vaccination route will be taken into consideration in our future project. In this way, we will better understand the immunogenicity, the safety profile, and the protection of TriAdj formulated mammalian HA

vaccine, which will all determine whether this novel adjuvanted subunit vaccine could become an efficient and safe influenza vaccine.

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APPENDIX. PUBLICATIONS, AUTHOR CONTRIBUTIONS, CONFERENCE PROCEEDINGS AND AWARDS

Publications:

A. **Lu Y**, Landreth S, Liu G, Brownlie R, Gaba A, van Drunen Littel-van den Hurk S, Gerdts V, Zhou Y. (2019). A novel adjuvant formulated HA based vaccine protects mice from lethal infection of highly pathogenic avian influenza H5N1 virus. ***Submitted to Vaccine***. JVAC-D-19-01017

B. **Lu, Y**., Landreth, S., Gaba, A., Hlasny, M., Liu, G., Huang, Y., & Zhou, Y. (2019). In Vivo Characterization of Avian Influenza A (H5N1) and (H7N9) Viruses Isolated from Canadian Travelers. ***Viruses***, 11(2), 193.

C. Liu, G., **Lu, Y**., Liu, Q., Zhou, Y. (2019). Inhibition of Ongoing Influenza A Virus Replication Reveals Different Mechanisms of RIG-I Activation. ***J Virol***. JVI-02066.

D. Gaba, A., Xu, F., **Lu, Y**., Park, H. S., Liu, G., Zhou, Y. (2018) The NS1 Protein of Influenza A Virus Participates in Necroptosis by Interacting with MLKL and Increasing Its Oligomer cation and Membrane Translocation. ***J Virol***. JVI-01835.

E. Liu, G., **Lu, Y**., Raman, S. N. T., Xu, F., Wu, Q., Li, Z., Zhou, Y. (2018). Nuclear-resident RIG-I senses viral replication inducing antiviral immunity. ***Nature communications***, 9(1), 3199.

Conference proceedings:

A. **Lu Y**, Landreth S, Liu G, Brownlie R, Gaba A, van Drunen Littel-van den Hurk S, Gerdts V, Zhou Y**.** 2019. A novel adjuvant formulated HA based vaccine protects mice from lethal infection of highly pathogenic avian influenza H5N1 virus. Oral presentation. The American Society for Virology 38th Annual Meeting. Jul 20-24, 2019. University of Minnesota, Minneapolis, US.

B. **Lu Y.** 2018. Pre-pandemic influenza vaccines preparedness. Oral presentation. 2018 Saskatchewan Veterinary Medical Association. Sep 8-10, 2018. Saskatoon, Canada

C. **Lu Y**, Liu G, Landreth S, Gaba A, Brownlie R, Zhou Y**.** 2018. Development of pre-pandemic influenza vaccines against highly pathogenic H5 strains. 73rd International Conference one Diseases in Nature Communicable to Man (INCSNCM). June 27-28, 2018. Saskatoon, Canada

D. **Lu Y**, Liu G, Landreth S, Gaba A, Brownlie R, Zhou Y. 2018. Expression of influenza hemagglutinin as a novel immunogen for highly pathogenic H5N1 vaccine development. Poster presentation (#235). The 5th International One Health Congress (IOHC). June 23-25, 2018. Saskatoon, Canada.

E. **Lu Y**, Liu G, Landreth S, Gaba A, Brownlie R, Zhou Y. 2018. Development of pre-

pandemic Influenza vaccines against highly pathogenic H5 strains. Poster presentation (#3). The 2nd Symposium of the Canadian Society for Virology (CSV). June 13-15, 2018. Halifax, Canada.

F. **Lu Y**, Liu G, Landreth S, Gaba A, Brownlie R, Zhou Y. 2018. Development of pre-pandemic influenza vaccine against highly pathogenic H5 strains. Poster presentation (Basic science 4: #92). The 25th Annual Life & Health Sciences Research Expo. May 3, 2018. Saskatoon, Canada.

G. Liu G, **Lu Y**, Liu Q, Zhou Y. 2018. Nuclear resident RIG-I senses influenza A virus replication mounting an antiviral response. Poster presentation (NSV2018-1236). The 17th Negative Strand RNA Virus meeting (NSV2018). June 17-22, 2018. Veneto, Italy.

H. **Lu Y**, Liu G, Thulasi Raman SN, Hlasny M, Brownlie R, Zhou Y. 2017. Development of influenza HA mini-stem as a universal vaccine candidate. Poster presentation (#15). The 5th Annual Protein Structure Function and Malfunction Symposium (PSFaM). June 15-17, 2017. Saskatoon, Canada.

I. **Lu Y**, Liu G, Thulasi Raman SN, Hlasny M, Brownlie R, Zhou Y. 2017. Development of influenza HA mini-stem as a universal vaccine candidate. Poster presentation (#VIR-112). The 24th Annual Life & Health Sciences Research Expo. May 5, 2017. Saskatoon, Canada.

Awards and scholarships:

2019 Student travel award. University of Saskatchewan Student Travel Award (C$ 450)

2019 Student travel award. The American Society for Virology 38th Annual Meeting (US$ 500)

2018 2nd Place, Basic Science Category-4, 2018 Life and Health Sciences Research Exposition (C$100)

2018 The Cecil E. Doige Fund Western College of Veterinary Medicine Graduate Student Travel Award (C$350)

2018 John Allen Veterinary Microbiology Graduate Student Travel Allowance (C$300)

2017-2018 WCVM Graduate Student Scholarship/Fellowship Award (C$ 8000)