The impact of influenza A hemagglutinin glycosylations on the breadth of the humoral immune response

M.Sc. Thesis

Hanni Uusi-Kerttula
Institute of Biomedical Technology
University of Tampere, Finland

May 2013

MASTER'S THESIS

Place: UNIVERSITY OF TAMPERE
Institute of Biomedical Technology (IBT)
Author: UUSI-KERTTULA, HANNI KATARIINA

Title: The impact of influenza A hemagglutinin glycosylations on the breadth of the humoral immune response

Pages: 82 pages, 8 index and appendix pages
Supervisor: Professor Rafael A. Medina Silva
Reviewers: Professors Markku Kulomaa and Rafael A. Medina Silva
Date: May 2013

Abstract

Influenza A is a highly contagious respiratory virus whose surface protein hemagglutinin (HA) constantly obtains mutations at its five antigenic sites. These mutations occasionally lead to acquisition of new N-linked glycosylation sites that may alter the antigenicity and virulence of influenza virus by shielding the major antigenic epitopes. Glycosylation sites 71, 142, 144, 172, 177 and 179 have occurred in the HA globular head of the natural seasonal H1N1 influenza A strains and become fixed over time. The immunomodulatory role of these single and multiple additional N-linked glycosylation sites in the recombinant pandemic A/Netherlands/602/09 H1N1 (rWT) background was assessed. Hemagglutination inhibition (HI) assays were performed with mouse and ferret antisera to evaluate the effect of these additional glycosylations on the breadth of humoral immune response. Poor HI activity was observed with antisera from mice infected with the rWT and the recombinant virus glycosylated at site 144 against 14 natural seasonal H1N1 isolates that occupy variable glycosylations and are antigenically distant from the pandemic strain. It was shown that mouse antisera elicited by viruses containing an additional glycosylation at site 144 or 144-172 were capable of cross-reacting with the rWT and a panel of recombinant mutant viruses with single or multiple additional glycosylations. Conversely, antiserum from mice infected with the rWT had a considerable loss in cross-reactivity against the viruses glycosylated at sites 144 or 144-172. However, antisera from ferrets infected with the 144-glycosylated or 144-172-glycosylated viruses showed narrowed cross-reactivity against a panel of glycosylation mutant viruses and were not capable of neutralising the rWT, as measured by microneutralisation assays. Based on the observations with mouse antisera, most of the polyclonal activity seems to be directed against the antigenic site Sa, efficiently masked by glycosylation at site 144. This alters the antigenicity of the 2009 pandemic H1N1 virus by redirecting the focus of polyclonal response to multiple sites around the site Sa. The broad cross-reactivity was shown to be unique to site 144, since the breadth of polyclonal response elicited by other mutant viruses with single or multiple glycosylation sites was narrowed below homologous titres, having no activity against the viruses containing the site 144. Broadening the humoral immune response by incorporation of additional glycosylation sites provides an approach for universal, broadly reactive influenza vaccines. However, further validation is warranted in both animal models.

Key words: 2009 pandemic H1N1 influenza, antigenicity, antigenic site Sa, broadly reactive influenza vaccine, cross-reactivity, hemagglutinin, humoral immune response, influenza A, neutralising antibodies, N-linked glycosylation

PRO GRADU –TUTKIELMA

Paikka: TAMPEREEN YLIOPISTO
Biolääketieteellisen teknologian instituutti (IBT)
Tekijä: UUSI-KERTTULA, HANNI KATARIINA

Otsikko: Influenssa A −viruksen hemagglutiniiniglykosylaatioiden vaikutus humoraalisen immuunivasteen laajuuteen

Sivumäärä: 82 sivua, 8 hakemisto- ja liitesivua

Ohjaaja: Prof. Rafael A. Medina Silva
Tarkastajat: Professorit Markku Kulomaa ja Rafael A. Medina Silva

Aika: Toukokuu 2013

Tiivistelmä

Influenssa A on erittäin helposti tarttuva hengitystievirus, jonka pintaproteiini hemagglutiniinin (HA) viiteen antigeeniseen kohtaan syntyy jatkuvasti mutaatioita. Nämä johtavat ajoittain uusien N-liitettyjen glykosylaatiokohtien syntymiseen HA:n pallomaiseen päähän. Kookkaat oligosakkaridiketjut saattavat peittää viruksen tärkeät antigeeniset epitoopit ja täten muuttaa sen antigeenisyyttä ja taudinaiheutuskykyä. HA:n pallomaisessa päässä sijaitsevat aminohapot 71, 142, 144, 172, 177 ja 179 ovat esiintyneet glykosyloituneina kausittaisissa H1N1 influenssa A −viruksissa ajan myötä. Tässä tutkimuksessa rekombinanttivirusten avulla selvitettiin, miten A/Netherlands/602/09 H1N1 (rWT) −pandemiavirukseen lisätyt ylimääräiset N-glykosylaatiokohdat muokkaavat humoraalista immuunivastetta. Hiirten ja frettien antiseerumeista tutkittiin näiden rekombinanttivirusten aiheuttaman immuunivasteen laajuutta hemagglutiniini-inhibitio (HI)- ja mikroneutralisaatiotesteillä. Tutkimuksessa rWT-virusta ja 144-glykosyloitua virusta vastaan tuotettu hiirten sekä frettien antiseerumi reagoi huonosti kausittaisiin H1N1 influenssa A −viruksiin, joilla on vaihteleva glykosylaatiostatus ja jotka ovat antigeenisyydeltään hyvin erilaisia kun vuoden 2009 H1N1 pandemiavirus. 144- ja 144–172 –glykosyloituja viruksia vastaan tuotetuilla hiirten antiseerumeilla todettiin olevan laajentunut ristireaktiivisuus sekä rWT-virusta että kaikkia mono- ja polyglykosyloituja viruksia vastaan. Sitä vastoin muita glykosyloituja viruksia vastaan tuotetuilla hiiren antiseerumeilla oli kaventunut reaktiivisuus 144-glykosyloitua virusta vastaan. Yllättäen frettien 144-virusta vastaan tuotetun antiseerumin ei todettu reagoivan muihin glykosyloituihin viruksiin, eikä sen myöskään todettu neutralisoivan rWT-virusta mikroneutralisaatiotesteissä. Hiirten antiseerumeilla saatujen tulosten perusteella suurin osa polyklonaalisesta immuunivasteesta näyttäisi olevan kohdistunut antigeeniseen kohtaan Sa, jonka glykosylaatio kohdassa 144 tehokkaasti naamioi. Tämä mahdollisesti muokkaa kyseisen H1N1 pandemiaviruksen antigeenisyyttä ja kohdentaa immuunitunnistuksen todennäköisesti Sa-alueen ympärille. Laajentuneen immuunivasteen todettiin olevan 144-glykosylaatiokohtaspesifinen, sillä muiden lisättyjen glykosylaatiokohtien − sekä yksittäisten että monen glykosylaatiokohdan yhdistelmien – todettiin kaventavan humoraalista immuunivastetta. Näiden virusten ei myöskään havaittu reagoivan 144-glykosyloituihin viruksiin. Immuunivasteen laajentaminen HA-glykosylaatioiden avulla tarjoaa kiinnostavan lähestymistavan uudenlaisen, laajasti reaktiivisen influenssarokotteen kehittämiseen. Lisätutkimuksia kuitenkin tarvitaan molemmilla eläinmalleilla tulosten varmistamiseksi.

Avainsanat: antigeenisyys, antigeeninen kohta Sa, hemagglutiniini, humoraalinen immuunivaste, influenssa A, laajasti reaktiivinen influenssarokote, neutralisoivat vasta-aineet, N-liitetty glykosylaatio, ristireaktiivisuus, vuoden 2009 H1N1 influenssapandemia

Acknowledgements

All practical work for this project was done in the Laboratory of Molecular Virology at Centro de Investigaciones Médicas y División de Pediatría, Pontificia Universidad Católica de Chile in Santiago (Chile) and funded by Millennium Institute on Immunology and Immunotherapy (MIII). During this project a research paper[1] was completed and recently accepted by Science Translational Medicine. The leader of the Influenza group is Professor Rafael A. Medina Silva, who was a huge personal support during my work in the laboratory. In addition to Rafael's great expertise on influenza viruses he encouraged me all the way through my thesis and shared his immense knowledge and wisdom both in my professional and personal life. I hugely respect that great personality and admit that it will be very difficult to find as adaptive, motivating and heartfelt boss in my future jobs. That person has made me trust the future of virology and vaccine development, no matter how blurry it may some days seem. This work experience has already opened doors to vaccine development in Finland, of which I'm hugely grateful to Professor Rafael.

The second most important group are my co-workers who taught me patience and gave lessons on my way to practical Spanish (although Chilean surely is a whole different language). Special love goes to Aldo Vincent Barrera Vásques who shared his energy and smiles with me every single day in the lab, except on the dark days when he was the one needing encouragement. Aldo, I know many things did not work but many did, and those moments are to be remembered. Special thanks to Constanza Martinez Valdebenito, who generously borrowed her reagents, bacteria, wisdom and happiness, and to Marco Saavedra Maass and Hansol Kim Kweon whose personality always made me joyful. Thanks also to sweet and dear Ana-Maria Contreras Toledo who was always ready to help me, no matter how busy she was with her own tasks. I am also extremely grateful to Doctor Marcela Ferres Garrido for opening the door in the first place and for accepting me for the 5-week practical training at her wonderful laboratory. Without that experience I would never have met Rafael and worked on influenza for the exciting 6 months. Special thanks also to Professor Marcelo Lopez Lastra for the perceptive comments and for making me more critical about my project. I miss you all a lot and wish you very welcome to my home in the far far land Finland. See you someday!

Dear Sebastián, whose wife I became during this research project. Without you I would never have decided to spend a year in Santiago de Chile and would never have met all these great people. Integrating into the interesting South American life style took its toll and was not always purely pleasant. However, at the end of the year I knew the streets of Santiago as my own pockets and learned to avoid the jam-packed metro at the rush hours. At times we were both very exhausted with the polluted air and the crowds but with patience we strolled through it. Thank you for motivating me at hard times and understanding what it requires to make science. I also highly appreciate you keeping me relaxed with the luscious micheladas when my stress level was getting ridiculously high. Nothing makes me happier than you.

Hoping to visit Chile soon − I truly miss all the delicious seafood and cheap wines!

[1] Medina RA, Stertz S, Manicassamy B, Zimmermann P, Sun X, Albrecht RA, Uusi-Kerttula H, Zagordi O, Belshe RB, Frey SE, Eggink D, Tumpey TM, García-Sastre A. Glycosylations in the globular head of hemagglutinin protein modulate the virulence and antigenic properties of the H1N1 viruses. *Science Translational Medicine*, accepted.

Abbreviations

ASC	antibody-secreting cell
CDC	Centers for Disease Control and Prevention
CMV	cytomegalovirus
CPE	cytopathic effect
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
GFP	green fluorescent protein
HA	hemagglutinin
HAU	hemagglutination unit
HDV	hepatitis delta virus
HEK/293T	human embryonic kidney cell
HI	hemagglutination inhibition
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
HTBE	human tracheobroncheal epithelial cell
IF	interferon
IL	interleukin
LAIV	live attenuated influenza vaccine
LRT	lower respiratory tract
MBC	memory B cell
MDCK	Madin-Darby canine kidney cell
MOI	multiplicity of infection
MSSM	Mount-Sinai School of Medicine (New York, USA)
NA	neuraminidase
nAbs	neutralising antibodies
Neth/09	2009 pandemic A/Netherlands/602/09 influenza A (H1N1) isolate
NK	natural killer cell
NLS	nuclear localisation signal
PBS	phosphate buffered saline
PFU	plaque forming unit
pH1N1/18	1918 pandemic H1N1 influenza A strain, "Spanish flu"
pH1N1/09	2009 pandemic H1N1 influenza A strain, "swine flu"
p.i.	post-infection
RBC	red blood cell
RNP	ribonucleoprotein complex
RT	room temperature (19−22˚C)
rWT	recombinant wild type
SA	sialic acid
SIV	simian immunodeficiency virus
SOIV	swine-origin influenza virus
Sf9	*Spodoptera frugiperda* insect cell
$TCID_{50}$	tissue culture infectious dose 50
TIV	trivalent influenza vaccine
UTR	upper respiratory tract
Vero	African green monkey cell
VGM	viral growth medium
VLP	viral-like particle
WHO	World Health Organization

Contents

1 Introduction

Influenza A viruses belong to the family *Orthomyxoviridae* that is divided into three genera: influenza A, B and C viruses, *Thogotovirus* and *Isavirus*. Influenza A and B viruses are responsible for the seasonal epidemic outbreaks that occur annually during the winter months. Influenza A is a highly contagious respiratory pathogen that is transmitted in close contact via aerosol droplets. It is capable of infecting the upper respiratory tract (URT) and lower respiratory tract (LRT) of humans and various animal species by binding on specific sialic acid (SA) receptors on epithelial cells via its surface glycoprotein hemagglutinin (HA). The classification of influenza viruses is based on the sequence variation in these main surface glycoproteins (Air 1981) that are the virion surface molecules containing the main antigenic epitopes, receptor binding sites and crucial enzymatic functions.

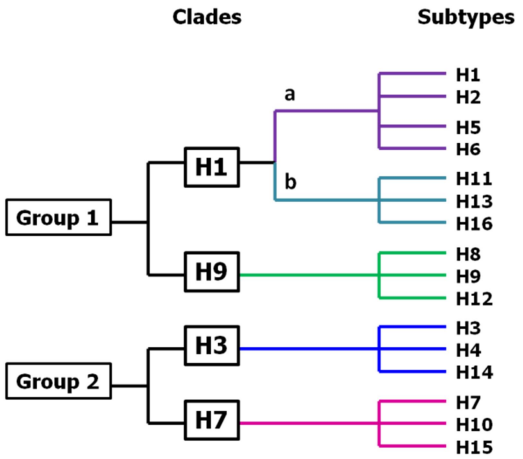


Figure 1. The two influenza A groups are divided into clades and subtypes. There are 16 antigenically characterised influenza A subtypes, all of which have been found circulating in birds. Modified from (Medina & García-Sastre 2011).

The HA subtypes are classified into two lineages and further into four clades, based on their antigenic and structural properties (Figure 1). There are 16 antigenically characterised influenza A HA subtypes and 9 NA subtypes, although only three HA subtypes (H1−H3) and two NA subtypes (N1 and N2) have been found circulating in humans. However, a unique bat influenza virus H17N10 of a distinct lineage has been very recently described (Tong *et al*. 2012) and seems to differ substantially from other HA and NA subtypes in its structural and functional characteristics (Zhu *et al*. 2013). Since the beginning of the documented medical history, rapid evolution of influenza viruses has been on-going in humans, birds, pigs, horses and other animal species. Interspecies transmission is responsible for the genetic diversity of the influenza

viruses. The changes that alter the antigenic properties of the seasonally circulating viruses are known as antigenic drift that involves minor, gradual amino acid changes within the antigenic regions of the HA and NA proteins. Some of these mutations alter the glycosylation profile of these proteins, which affects the immunogenicity and virulence of influenza viruses. At times the human strains undergo reassortment events with zoonotic subtypes. This major exchange of the gene segments between different species is known as antigenic shift that creates new variants with acquired new antigenic properties and can result in pandemics. Due to the continuous antigenic evolution, control of influenza virus transmission is challenging.

Currently two influenza A subtypes, H1N1 and H3N2, are circulating in humans[2] and causing annual epidemics. Due to cross-reactive pre-existing neutralising antibodies (nAbs) present in the population, the arising antigenically drifted variants commonly cause solely a mild self-limited respiratory disease due to their antigenic similarity with the previously circulating strains. Influenza A and B viruses cause tens of millions of human infections every year, leading to an estimated 250,000−500,000 annual deaths worldwide (World Health Organization 2009). The highest mortality rate of the seasonal influenza is seen in the elderly (> 65 years of age) that is the most susceptible portion of the population due to underlying morbidities (Nichol 2005, Fireman *et al*. 2009). Influenza remains as the main infectious disease of the developed world with significant economic losses.

This thesis gives a compact review on influenza A (H1N1) viruses based on their extensive antigenic variation and pandemic potential, although the importance of influenza B viruses on the health of the population is not to be dismissed. For more detail on the structural and functional properties of influenza viruses, the reader is kindly requested to take a look at an excellent review by Medina and García-Sastre (2011) and the Chapters 47 and 48 of Fields Virology (Palese & Shaw 2007, Wright *et al*. 2007). The natural influenza isolates are universally abbreviated as for instance A/California/7/2009 (H1N1), which means that this particular virus was an isolate number 7 of a human influenza A taken in California in year 2009 and it has an HA subtype of 1 and an NA subtype 1. This nomenclature is used throughout the thesis whose focus is on subtype H1N1 influenza A viruses.

[2] http://www.who.int/influenza/surveillance_monitoring/updates/2013_03_15_surveillance_update_181.pdf (accessed 2.4.2013)

2 Review of the literature

2.1 Structure of the influenza A virion

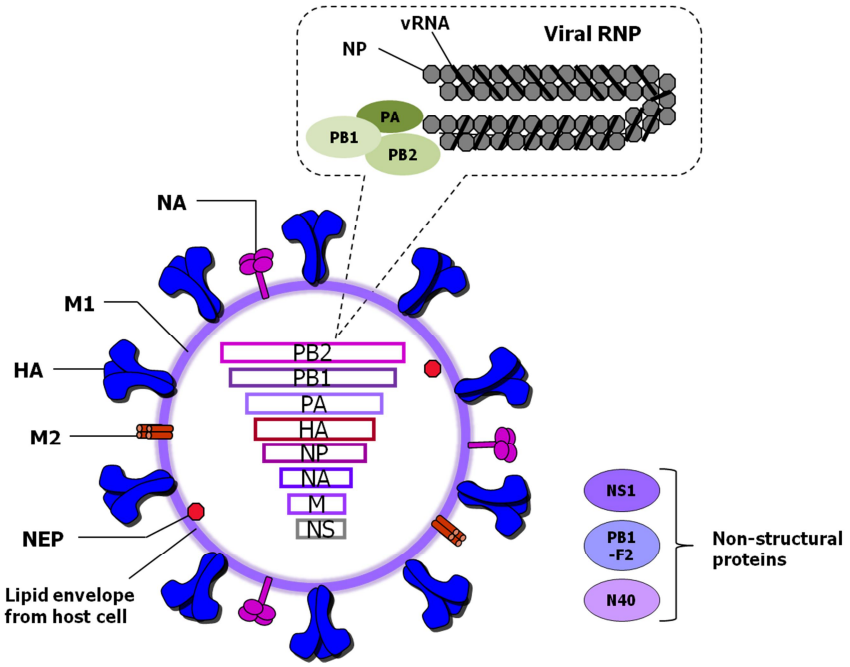


Figure 2. The influenza A viral particle with the segmented genome and the contributing proteins. HA = hemagglutinin, NA = neuraminidase, M2 = ion channel protein, NEP/NS2 = nuclear export protein, M1 = matrix protein layer, NP = nuclear protein around which the viral RNA (vRNA) is wrapped. NP and vRNA together form the viral ribonucleoprotein (RNP) complex that is bound to the viral polymerase complex PA/PB1/PB2. Modified from (Medina & García-Sastre 2011).

Influenza A viruses are spherical or filamentous viruses with a diameter of 100 nm or 300 nm, respectively. The virus is surrounded by a host-cell derived lipid envelope with five distinct proteins protruding from the lipid layer: the receptor-binding protein HA, the SA-destroying enzyme NA, the matrix protein M1, the ion channel M2 and the nuclear export protein (NEP) that is located right beneath the envelope (Figure 2). HA and NA proteins are the predominant surface proteins that form 10−14 nm spikes on the virion exterior and are present with an approximate ratio of 4:1. The eight (−) ssRNA segments of influenza A genome each code for one or two proteins (Table 1) (Palese 1977). Each segment contains a non-coding region in the 5′ and 3′ ends that is partly segment-specific and has 13 conserved nucleotides in the 5′ end and 12 conserved nucleotides in the 3′ end. The segments are transcribed into mRNAs and synthesised into (+) strand RNA copies (cRNA) that function as templates for (−) viral genomic RNA (vRNA) synthesis. In addition to cRNA and mRNA synthesis, influenza A synthesises RNA from the M gene that gives rise to spliced mRNA and produces M2 protein and RNA from the NS gene that produces NEP from a spliced mRNA.

Additionally, PA-X protein has been recently described to be expressed from an open reading frame (X-ORF) of segment 3 by +1 ribosomal frameshifting (Jagger *et al*. 2012, Lee *et al*. 2012, Shi *et al*. 2012). This results in production of 12 viral proteins in total.

Table 1. The genomic segments of an influenza A/Puerto Rico/8/1934 (H1N1) virus and their encoded proteins. The eight (−) ssRNA genomic segments have variable lengths and code for proteins with multiple functions in the complete virus particle. Table modified from (Bouvier & Palese 2008).

Segment number	Segment length (nt)	Encoded proteins	Protein length (aa)	Protein function
1	2341	PB2	759	Polymerase subunit; mRNA cap recognition
2	2341	PB1	757	Polymerase subunit; RNA elongation, endonuclease activity
		PB1-F2	87	Pro-apoptotic activity
3	2233	PA	716	Polymerase subunit; protease activity
		PA-X	252 (Taubenberger *et al*. 2005)	Cellular signalling, endonuclease activity, viral pathogenicity (Jagger *et al*. 2012, Lee *et al*. 2012)
4	1778	HA	550	Surface glycoprotein; major antigen, receptor binding and fusion activities
5	1565	NP	498	RNA binding protein; nuclear import regulation
6	1413	NA	454	Surface glycoprotein; sialidase activity, virus release
7	1027	M1	252	Matrix protein; vRNP interaction, RNA nuclear export regulation, viral budding
		M2	97	Ion channel; virus uncoating and assembly
8	890	NS1	230	Interferon antagonist protein; regulation of host gene expression
		NEP/NS2	121	Nuclear export of RNA

2.2 Influenza life cycle

The viral cycle begins with the attachment of the virus HA on the SA α2,6-galactose β1,4-N-acetyl glucosamine (SA α-2,6Gal) or on the SA α2,3-galactose β1,3-N-acetyl galactosamine (SA α-2,3Gal) receptors on the host epithelial cell surface (Figure 3). Acidification of the inside of the virion occurs through the ion exchange channel M2, which initiates viral internalisation by endocytosis via clathrin-coated pits, caveolae, macropinocytosis or alternative internalisation methods [reviewed in (Conner & Schmid 2003, Lakadamyali *et al*. 2004)] in pH 5.0−5.5 [reviewed in (White *et al*. 1983)]. This results in a simultaneous conformational change in the transmembrane domains of multiple HAs within the viral envelope and the fusion peptide domains located in the endosomal membrane, which enables the fusion of these membranes. The viral contents can then enter the cytoplasm where the RNPs become trafficked into the nucleus, a process driven by their nuclear localisation signals (NLSs). The low pH within the

endosome triggers the host cell driven proteolytic cleavage of the disulfide bonds between the HA1 and HA2 subunits and results in generation of a fusion peptide.

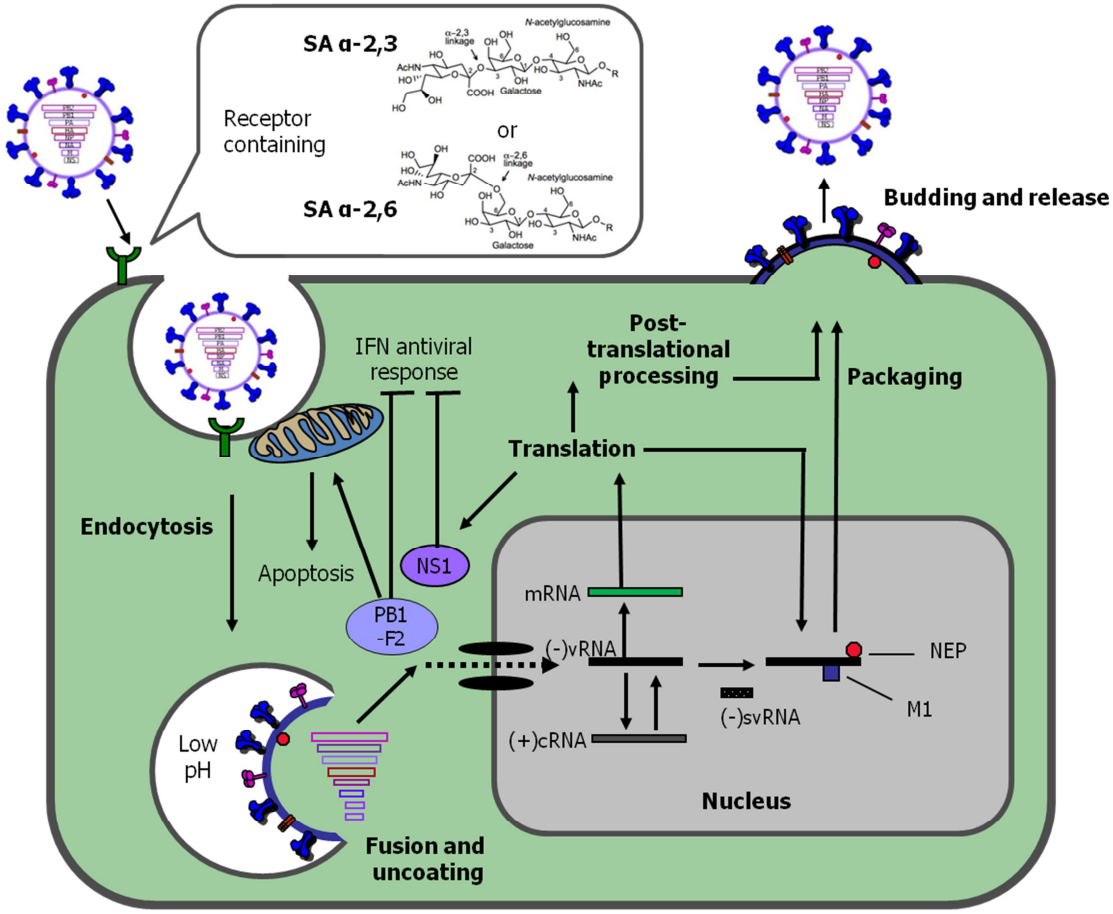


Figure 3. The replication cycle of influenza A viruses. The cycle begins with viral attachment on SA receptors (SA α2,3 or SA α2,6) on the cell surface via HA. After the virus has entered the cell, the low pH in the endosome causes fusion and uncoating of the lipid layer –surrounded virus. Viral RNA (vRNA) is trafficked into the nucleus as part of the RNP complex, where it is copied into (+) copy RNA (cRNA) and amplified to be assembled into new virions. In a parallel process the cRNA is transcribed into mRNA and the viral proteins are being translated to be packaged into new virions. Non-structural proteins NS1 and PB1-F2 are involved in apoptotic and antiviral processes. Figure modified from (Medina & García-Sastre 2011).

All RNA synthesis occurs in the host cell nucleus where the eight (−) vRNA segments are transcribed into mRNAs and a (+) cRNA is synthesised for replication of the progeny viral RNA by the viral RNA-dependent RNA polymerase. The virus uses a "cap-snatching" mechanism of stealing the host pre-mRNA 5´capped primers to initiate its own mRNA synthesis. The viral RNA-dependent RNA polymerase adds a poly (A) tail after it has accomplished the mRNA synthesis from the vRNA template. The cellular splicing machinery is used for alternative splicing of the primary transcripts and is highly controlled to enable production of both unspliced and spliced mRNAs. The initiation of replication requires a switch in the polymerase and may involve the presence of free NP. In the second step the (−) vRNA is first synthesised into full

sequence (+) cRNA by the viral RNA-dependent RNA polymerase via a primer-independent mechanism. The cRNA is then used as a template when an encapsidated (−) genomic vRNA copy is produced by the same viral polymerase. The newly produced vRNA segments are then assembled into RNP complexes and transported into the cytoplasm by the M1 and NEP proteins through nuclear pores. The mRNAs are transported into the cytoplasm, where they are translated on the membrane-bound ribosomes by the cellular machinery. In ER the integral membrane proteins HA and NA become folded and glycosylated; HA is assembled into a trimer and NA into a tetramer. Later they are palmitoylated and glycosylated in the Golgi apparatus and directed along with the RNPs to the apical membrane of the polar epithelial cells where the assembly, budding and release of the mature viruses occurs. The release requires active enzymatic degradation of the cellular receptor SAs and viral glycoproteins by the neuraminidase activity of the NA.

2.2.1 Influenza tropism & transmission route

Influenza virus has to fight its way through physical barriers such as mucus, macrophages and epithelial cells, and successfully attach, enter and replicate. Interspecies transmission of influenza viruses has been largely facilitated by common habitat use, animal trade, international travel and bird migration but is further puzzled by genetic reassortment and recombination that may provide sudden fitness advantages to the virus. More about host species barriers to influenza virus is covered in an excellent review (Kuiken *et al*. 2006). The influenza virus A can potentially transmit from human-to-human in direct or indirect contact, via large droplets or airborne aerosolisation of secretions or fomites. Direct or indirect contact involves physical contact with the infected individual or contaminated instruments/objects, which is facilitated by the ability of influenza virus to remain infectious on dry surfaces for up to 48 hours (Bean *et al*. 1982). The pathogens can also spread by large droplets that are generated from the respiratory tract of an infected individual by sneezing, coughing, talking or during a respiratory operation. Smaller aerosols can be generated from the large droplets that can remain in the air for long periods attached to dust particles or skin cells/debris. Aerosol transmission seems to be an important mode of transmission since the particles remain airborne for prolonged periods, which enables long-range infections. Transmission of influenza A is reviewed in (Tellier 2006, Brankston *et al*. 2007).

The cell tropism of influenza A viruses is determined by the receptor recognition specificity and affinity of the HA anchored on the virion surface. Bronchial epithelial cells, nasal mucosa, goblet (mucous) cells, paranasal sinuses and pharynx of the human URT carry SA α-2,6Gal surface receptors (Ibricevic *et al*. 2006, Shinya *et al*. 2006) that function as primary receptors for the H1 and H3 human influenza A viruses (Rogers & Paulson 1983, Rogers & D'Souza 1989). In contrast, the avian H5 influenza viruses use the SA α-2,3Gal as their primary receptor (Connor *et al*. 1994) that can be found in the bird gut epithelium as well as on the alveolar type-II cells and non-ciliated cuboidal bronchiolar cells of the human LRT (Shinya *et al*. 2006). Pigs have both of these SA receptor types in their trachea, which enables simultaneous infection of swine, human and avian viruses and thus makes them a possible mixing vessel for genetic reassortment events between viruses of different zoonotic origin. The SA receptors recognised by influenza A viruses and the distribution of SA receptors in various hosts is reviewed in (Hidari & Suzuki 2010).

Influenza A viruses are also known to infect natural killer (NK) cells, antigen-presenting cells, macrophages, monocytes, neutrophils, respiratory dendritic cells (DCs), B cells, CD4+ cells and CD8+ cells (Manicassamy *et al*. 2010a). In this mouse study a fully replicating reporter virus carrying a green fluorescent protein (GFP) tag in its NS1 segment was produced by reverse genetics methods. This attenuated but pathogenic virus was shown to begin spreading from UTR and to then continue deeper into the lungs, eventually infecting high levels of immune cells. Mannose receptors have been shown to be involved in macrophage infection by influenza viruses (Reading *et al*. 2000). Two seasonal H3N2 viruses were shown to have improved infectivity of murine macrophages compared to a H1N1 virus. The mannose-binding lectins and lung surfactant protein D were shown to be involved in endocytic entry and the virus sensitivity to these collectins was parallel to their relative infectivity. These C-type lectins are known to be involved in influenza virus opsonisation (Hartshorn *et al*. 1994, Hartshorn *et al*. 1996), complement-dependent neutralisation (Anders *et al*. 1994) and aggregation (Hartshorn *et al*. 1994, Hartshorn *et al*. 1997) by binding to the oligosaccharide side chains present on the glycoproteins HA and NA. Interestingly, collectin-sensitivity has been shown to correlate with the number of N-linked glycosylations of the HA globular head and to have inverse relationship with viral replication in the lungs (Reading *et al*. 1997). The authors suggest that glycosylation

pattern can act as a virulence factor that determines the character of innate immune defences in the respiratory tract.

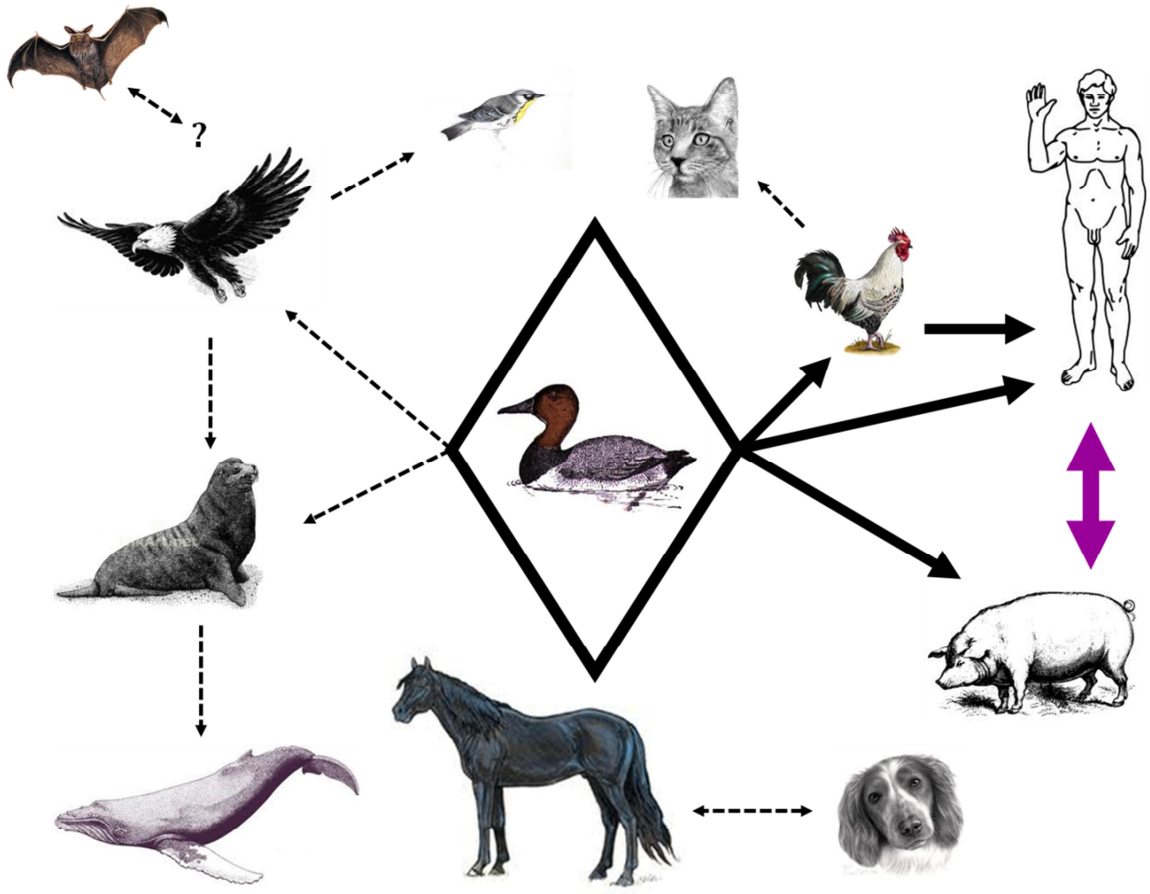


Figure 4. Influenza A host tropism. Influenza viruses infect humans and animals from various species, wild aquatic birds serving as a ubiquitous virus pool. Figure modified from (Wright *et al.* 2007).

Influenza A viruses have been extensively studied in various animal species and geographical regions and therefore a vast amount of information has been obtained on the evolution and ecology of RNA viruses during the last century. The first bird influenza virus was isolated as early as 1902, followed by isolation from pigs in 1930, from humans in 1933, from horses and domestic ducks in the 1950's, from terns 1961 and from waterfowl and shorebirds in 1974. A new influenza A subtype H17N10 was initially found from two bat populations in Guatemala and seems to be highly divergent from all known influenza A viruses, according to the preliminary characterisation studies (Tong *et al*. 2012). Bird surveillance studies have shown that aquatic birds provide a complete genetic reservoir and a ubiquitous source of all mammalian influenza virus subtypes. With this diversity they are able to infect various mammalian and avian species (Figure 4) at high concentrations by direct transmission via faeces to

different water sources. Ducks are thought to play an important role in influenza virus evolution and to function as a highly adapted asymptomatic reservoir for viral transmission to seals, whales, pigs and domestic poultry (Webster *et al*. 1978). More about the ecology of influenza can be found in an extensive review by Webster and colleagues (1992).

2.3 Evolution of influenza A viruses

2.3.1 Antigenic drift creates seasonal variation

One of the most important characteristics of influenza A viruses is their ability to undergo rapid antigenic variation in high immune selection pressure, which accounts for their high infectivity and leads to annual human epidemics. Variation is brought up by substitution point mutations that are a result of the error-prone RNA polymerase lacking proofreading activity. This polymerase introduces an erroneous base once in every 10^4 nucleotides while for DNA polymerases the error rate is as low as 1 in 10^9 (Holland *et al*. 1982). The viruses have undergone multiple amino acid changes over time, probably due to immune pressure of the pre-existing immunity and escape from the immune pressure of the host (Sun *et al*. 2011). The influenza virus undergoes unpredictable seasonal antigenic variation in its major surface proteins HA and NA. The most immunologically relevant changes occur in the five HA globular head antigenic regions that possess the main antigenic epitopes for humoral immune recognition.

An accelerated antigenic drift model was demonstrated under immune selection pressure by propagating a seasonal H1N1 strain (A/Puerto Rico/8/34) in inbred vaccinated mice (Hensley *et al*. 2009). It was shown that during nine passages in immunized mice, three single-amino acid substitutions occurred within the four antigenic regions of the HA globular head. Those mutations that lead to enhanced receptor binding affinity also enhanced the virus escape from polyclonal antibodies. In contrast, when these high-avidity mutants were introduced into naïve individuals, they acquired additional mutations and the viruses with decreased receptor binding affinity got selected. Based on these results it was proposed that those amino acid changes that lead to increased receptor binding avidity and often to altered antigenicity alike, get selected in immune individuals and provide the virus a means of escaping the pressure of pre-existing nAbs. Rapid antigenic drift in the antigenic and receptor binding regions of influenza A HA impairs the efficacy of the current influenza vaccines and therefore it is of great importance to assess the mutations that lead to seasonal variation.

2.3.2 The role of N-linked glycosylations in antigenic drift

Arginine (N)-linked glycosylation is a naturally occurring post-translational process in various different proteins and also forms a significant part of seasonal antigenic drift of influenza A viruses. The level of N-linked glycosylations has been shown to modulate the receptor binding affinity (Ohuchi *et al*. 1997, Wang *et al*. 2009, Das *et al*. 2011) and specificity (de Vries *et al*. 2010) of influenza viruses and to regulate virus growth in cell culture (Wagner R et al. 2000), probably due to steric hindrance or electrostatic repulsion by the bulky oligosaccharide moieties. The impact of additional HA glycosylations (subtype H5) on the receptor binding affinity was determined by a synthetic SA glycan array by comparing the difference of free energy change between receptor sialosides (Wang *et al*. 2009). This study showed enhanced receptor binding affinity of monoglycosylated HA molecules to sialocides compared to their fully glycosylated counterparts, which is likely due to crucial 3D structure alterations and steric hindrance in the receptor binding pocket of the fully glycosylated viruses. Additionally, improved cross-reactivity of an antisera from monoglycosylated HA protein immunizations against H5 and H1 viruses was reported, as compared to their fully glycosylated counterparts.

Glycosylations that occur in close proximity to the antigenic regions of the HA may alter the antigenicity (Aytay & Schulze 1991, Munk *et al*. 1992, Tsuchiya *et al*. 2002) and immunogenicity of the virus (Wanzeck *et al*. 2011). The attached host cell −derived oligosaccharides provide the virus an intelligent means of escape from the immune system since they are recognized as “self” by the host immune machinery (Schulze 1997). HA glycosylations are also known to mask or unmask the antigenic epitopes from antibody (Wiley & Wilson 1981, Wiley & Skehel 1987, Schulze 1997, Das *et al*. 2010) and the major histocompatibility complex recognition (Jackson *et al*. 1994) and by this means to alter the virulence and host humoral immune responses. The acquisition of these large oligosaccharides adjacent to the five antigenic sites and the receptor binding pocket helps the virus to escape antibody neutralisation by blocking their access to the antigenic epitopes, a method well described for the human immunodeficiency virus (HIV) glycoproteins (Zhang *et al*. 2004, Vigerust & Shepherd 2007, Stansell & Desrosiers 2010). It has been shown that as short as maltobiose carbohydrate side chains on synthetic peptides can inhibit the T cell recognition, even outside a T cell determinant (Jackson *et al*. 1994). However, in that study they did not

seem to prevent antibody binding despite of potential steric hindrance at the peptide epitope.

Glycosylations can also have detrimental effects on the viral pathogenicity by preventing the cleavage of HA0 into HA1 and HA2 if they occur in close proximity to the fusion peptide cleavage site of the stem region (Kawaoka & Webster 1988). It has also been observed that in comparison with the H3 HA globular head that has acquired an increasing number of glycosylation sites since the appearance of H3N2 in 1968, the H1 HA has acquired only a few glycosylations during its circulation in the human population (Das *et al.* 2010). This suggests a high selection cost for the heavily glycosylated viruses, probably due to diminished cell attachment, entry and/or replication. HA glycosylation has been shown to abolish the receptor avidity of the viruses and therefore the utilisation of hyperglycosylation as an immune evasion strategy seems to be limited (Das *et al.* 2011). In this study a seasonal H1N1 virus was able to escape from the H28-A2 monoclonal antibody neutralisation by introducing a substitution mutation N133T or K144N that resulted in N-linked glycosylation at site 131 or 144, respectively. Additional compensatory mutations in HA or NA were observed for all the escape mutants and the receptor binding affinity of the 131 or 144 glycosylation mutant viruses was shown to decrease by 71%, to lower than WT levels. These results suggest that escape from nAbs can be adjusted by receptor binding affinity and that accumulated glycosylations do not provide an escape mechanism for H1 type HA due to steric hindrance at the antigenic/receptor binding sites. It seems likely that the fitness cost caused by the additional glycosylations has to be compensated by additional mutations to retain full viral functionality.

2.3.3 Emergence of strains with pandemic potential

Occasionally major genetic changes lead to virus strains that are no longer recognised by the overall pre-existing immunity present in the human population. Major genetic reassortments between the gene segments from different animal and human influenza A viruses have resulted in pandemic outbreaks that have been capable of infecting a large proportion of the human population and causing millions of deaths worldwide (Johnson & Mueller 2002). Gene reassortment can occur in case two viruses from different subgroups infect an individual simultaneously, which enables assembly of the segments into novel virions. These events can result in antigenic shift when virus with an antigenically novel HA emerges. Pre-existing neutralising immunity against this novel reassortant virus is likely to be very low in the human population, which correlates

directly with the degree of the overall disease severity. In theory, novel emerging strains must have the HA from a non-human virus to have the ability of evading the pre-existing anti-HA immunity and causing a successful pandemic among the human population.

2.3.4 Alterations in receptor specificity

Tissue tropism is one of the most important features of influenza virus virulence and transmissibility between animal and human hosts. Small-scale genomic mutations that lead to changes in receptor specificity have been shown to modulate the host tropism and transmissibility of influenza viruses. An important characteristic of a novel pandemic variant is its ability to spread from human-to-human, which greatly increases the pathogenicity of the virus due to its broadened host tropism. Pigs are known to function as effective mixing vessels for influenza viruses with different zoonotic origins due to the fact that both types of SA receptors − α-2,6Gal and α-2,3Gal − are represented in their respiratory tract (Rogers & D'Souza 1989).

The mutations responsible for altered receptor specificity from SA α-2,6Gal to SA α-2,3Gal specificity have been mapped to the residues 190 and 225 (H3 numbering) for the swine-origin H1 influenza viruses (Matrosovich *et al*. 2000, Gambaryan *et al*. 2005, Glaser *et al*. 2005). It has been demonstrated that only one change in these two amino acids within the HA receptor-binding pocket of the 1918 pandemic H1N1 influenza A strain (pH1N1/18) is enough to switch the binding preference from human to avian SA receptors (Tumpey *et al*. 2007). Severe or fatal cases of the 2009 pandemic H1N1 influenza A strain (pH1N1/09) have been show to commonly carry a substitution mutation Asp222Gly in the HA receptor-binding site, which altered their preference from non-ciliated cells to ciliated bronchial epithelial cells and increased their binding to SA α-2,3 receptors (Chutinimitkul *et al*. 2010, Kilander *et al*. 2010, Liu *et al*. 2010) without affecting their antigenicity (Chutinimitkul *et al*. 2010). This was thought to be directly connected to the enhanced virulence and severity of the infection, although it is unsure whether the substitution presents a cause or a consequence of a fatal underlying pathology in the LRT. The HA amino acid residues related with SA receptor recognition is reviewed in (Hidari & Suzuki 2010).

The highly pathogenic avian H5N1 virus has been raising concerns of a future devastating human pandemic [H5N1 outbreaks are reviewed in (Webster *et al*. 2006)]. Although the H5N1 virus has caused global outbreaks in Asia since 2003, its spread is

limited by the apparent lack of human-to-human transmission due to restricted receptor-specificity and other factors essential for replication [reviewed in (Webster & Govorkova 2006)]. In order to ensure efficient human-to-human transmission, these viruses must gain mutations that enable SA α-2,6Gal receptor recognition and allow infection of the URT and efficient spread by sneezing and coughing (Shinya *et al*. 2006). It is also known that avian influenza viruses H5N1, H9N2 and H7N7 infect ciliated cells, whereas human influenza viruses prefer non-ciliated cells for infection (Matrosovich *et al.* 2004a). However, since all influenza A viruses are constantly evolving, the emergence of highly contagious avian virus capable of wide human transmission may only be a matter of time. A recently emerged novel avian virus H7N9 has been laboratory-confirmed in 109 Chinese citizens[3], of which 22 people have died since the 1st of April 2013. The source of this strain is currently unidentified and no human-to-human transmission has been reported. For more information on the evolution, ecology and the origins of emerging human influenza viruses, see reviews by Webster and colleagues (1992) and Medina and García-Sastre (2011).

2.3.5 Influenza A pandemics of the past century

Phylogenetic studies have revealed that the human pandemic influenza viruses have arisen either by reassortment of avian influenza viruses with current human strains or by direct transmission of avian or mammalian viruses that have acquired infectivity and novel pathogenicity in humans [reviewed in (Webster *et al*. 1992)]. The reassortment events within the influenza A genome have contributed to antigenic escape of the novel viruses from the pre-existing immunity among the human population, which has led to a five major pandemics: the Spanish flu in 1918 (H1N1), the Asian flu in 1957 (H2N2), the Hong Kong flu in 1968 (H3N2), the Russian flu in 1977 (H1N1) and the most recent swine flu in 2009 (H1N1). In addition to the pandemic outbreaks, seasonal epidemics continue devastating the human population.

The first recorded human influenza A pandemic in 1918 was caused by a highly virulent H1N1 virus that infected about 20−40% of the world's population and caused an estimated of 50 million deaths[4] worldwide. The H1N1 caused human influenza epidemic in 1950 after which it was replaced by the 1957 pandemic H2N2 and disappeared, until it again reappeared into human population in 1977. The pH1N1/18 virus and the human H1N1 viruses that circulated until 1920 subsequently recombined

[3] http://www.who.int/csr/don/2013_04_25/en/index.html (accessed 26.4.2013)
[4] http://www.flu.gov/pandemic/history/index.html (accessed 21.3.2013)

with the North American avian viruses and the human H3N2 viruses, creating a triple-reassortant North American swine H3N2 virus that was introduced into the human population in 1968. These viruses further underwent a recombination with a classical swine virus H1N1, which resulted in a triple-reassortant North American swine H1N2. Between 1968 and 1977 the dominant influenza strain was H3N2, after which it has continued its circulation in the human population (Couch & Kasel 1983). Since the 1977 pandemic the H1 HA globular head has acquired additional mutations and has been responsible for seasonal influenza epidemics until the recent outbreak in 2009 that most likely resulted from a recent reassortment event of the Eurasian ´avian-like´ swine H1N1 with the North American swine-origin influenza virus (SOIV) H1N2.

Evolutionary analysis has shown the novel triple-reassortant pH1N1/09 SOIV virus to have been circulating and possibly undergoing further reassortments in the swine population for approximately one decade before its pandemic emergence in humans (Smith *et al*. 2009). The emergence of a novel pandemic H1N1 in humans was originally announced by US CDC on the 15th of April in 2009 in California (USA) as the virus was isolated from two epidemiologically unlinked paediatric patients (Dawood *et al*. 2009). The original report revealed that the genes of this pH1N1/09 were derived from the previously circulating human, swine and avian-like swine viruses (i.e. PB2 and PA from North American avian viruses, PB1 from the human H3N2 viruses, NA and M from the Eurasian ´avian-like´ swine viruses and HA, NP and NS from the classical swine H1N1 viruses). The genetic analyses have revealed the human pH1N1/09 to have low genetic diversity, which suggests that it was introduced into the human population in a single event or in multiple events of similar viruses (Garten *et al*. 2009).

Comprehensive *in vitro* and *in vivo* studies have aimed at describing the pathogenicity, drug sensitivity and human antibody cross-reactivity of a pH1N1/09 isolate A/California/04/09 (Itoh *et al*. 2009). The authors report this virus to transmit among ferrets, to replicate efficiently in mice, ferrets and non-human primates and to cause severe pathological lung lesions that resemble infection with the highly pathogenic H5N1 viruses. However, in a pathogen-free miniature pig model the infection was non-symptomatic although the virus replicated efficiently in the respiratory tract. In this study the virus was also shown to be susceptible to current approved antiviral drugs but to be resistant to ion channel inhibitors. It showed high cross-reactivity with the antigenically similar pH1N1/18 virus antisera from individuals born before the 1950's,

which was consistent with a previous observation that 33%[5] of the population over 60 years of age have nAbs to pH1N1/09 due to exposure to swine-origin H1N1 viruses that circulated after the pH1N1/18. It is proposed that the HA of this virus remained in antigenic stasis in the pig population until it successfully hit the serologically susceptible human population and was able to cause a new pandemic.

The symptoms of this febrile illness varied from self-limited to severe lethal infection involving respiratory failure and even death due to secondary infections and underlying chronic conditions (Dawood *et al*. 2009). The first estimate of global deaths due to the pH1N1/09 influenza virus is that it lead to more than 200,000 respiratory deaths with an additional 83,000 cardiovascular deaths over a period of the first 12 months (Dawood *et al*. 2012). The range of total deaths in this modelling study was estimated to be 151,700−575,400 with 80% of the deaths within the population of < 65 years of age, which was the global age distribution trend during the 2009 pandemic. However, the real mortality rates may be far from these crude estimates since it is challenging to determine the impact of underlying co-morbidities on influenza-associated deaths. Emergence of the pH1N1/09 influenza is exclusively reviewed in (Medina & García-Sastre 2011).

2.3.6 Future outbreaks – preparing for the unknown

The glycosylation profile of the pH1N1/09 is equal to the one of the temporarily distant pH1N1/18, which indicates that due to immune selection pressure in the human population the pH1N1/09 HA may acquire similar glycosylations as those that occurred during the past decades (Wei *et al.* 2010). The N-glycosylation status seems to be important for influenza A virus evolution and therefore detailed assessment of the post-translational modifications may improve preparedness to upcoming outbreaks. Due to rapidly growing knowledge on the impact of glycosylations for influenza virulence and antigenicity, the question of N-linked glycosylation remains a hot topic in the influenza research field. However, the future of influenza A viruses is highly unpredictable since it is well known that various factors influence on the emergence of novel variants. An outbreak of a new human pandemic virus would require genetic reassortments between viruses of different zoonotic origin, receptor adaptation and acquirement of high transmissibility and virulence. The highly pathogenic avian viruses have been a potential health threat for the human population for decades but fortunately the

[5] http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5819a1.htm (accessed 21.3.2013)

adaptation of the viruses hasn't been successful enough to enable human-to-human transmission.

The chances are that a new reassortment event has already occurred in some isolated animal population and it is only a matter of time when the virus acquires the ability to cross the species barrier and spreads to the world via migrating birds, international animal transportation or other global routes. Animal and human surveillance programmes form an important part of the preparedness for novel influenza variants and are coordinated globally by the National Influenza Centres (NIC)[6] of the Global Influenza Surveillance and Response System (GISRS)[7] and the NIAID Centers of Excellence for Influenza Research and Surveillance (CEIRS)[8]. Surveillance of wild and domestic animals, especially avian species, forms an important part of preparedness for new animal outbreaks and helps to evaluate the potential risk of a new human disease (Fouchier *et al*. 2003, Peiris *et al*. 2012). Additionally, the most important method of preventing influenza transmission is by creating herd immunity with annually updated, heterosubtypic influenza vaccines.

2.4 Immune responses to influenza virus

2.4.1 Hemagglutinin (HA) – the main immunogenic protein of the virion

HA is the major envelope glycoprotein with a shape of trimeric rod at its C-terminus and a globular head structure at its N-terminus. It protrudes from the lipid envelope of the viral surface as a type I integral membrane protein, with its hydrophilic carboxy terminus within the lipid layer. The full functionality of HA requires post-translational glycosylations, palmitoylations and maturation cleavage of the precursor HA0 into two subunits, HA1 (globular head) and HA2 (stem) (Klenk *et al.* 1975). The globular head of a mature HA molecule is a trimer, derived from three HA1 monomers. Epitope mapping has revealed the type H1 HA globular head to possess the SA receptor −binding site surrounded by the five antigenic regions Ca1, Ca2, Sa, Sb and Cb, present in each HA monomer (Figure 5) (Caton *et al.* 1982). The antigenic sites are distributed over four conformational epitopes, of which sites Sa and Sb lay adjacent to the receptor-binding pocket (Xu *et al.* 2010). The globular head trimer contains the main immunodominant regions and antigenic epitopes for induction of immune responses (Wiley & Wilson 1981), which makes HA the main target of nAbs (Han & Marasco

[6] http://www.who.int/influenza/gisrs_laboratory/national_influenza_centres/national_influenza_centres.pdf
[7] http://www.who.int/influenza/gisrs_laboratory/en/
[8] http://www.niaid.nih.gov/labsandresources/resources/ceirs/Pages/centers.aspx

2011) that are induced by infection and vaccination. HA contains the major antigenic regions targeted by the current commercial influenza vaccines, which creates high selection pressure and drives rapid escape of the virus from the pre-existing nAbs (Webster *et al*. 1992).

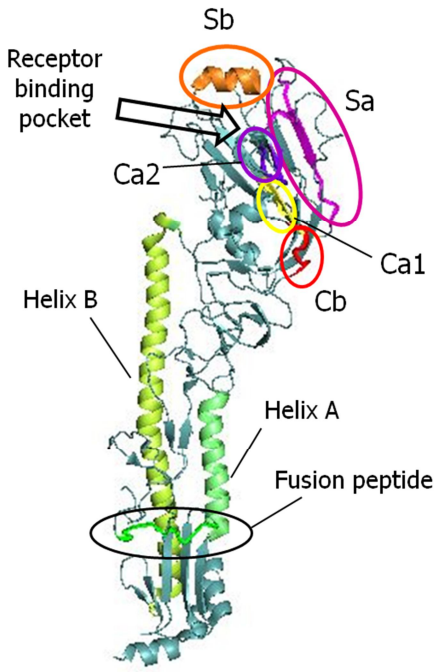


Figure 5. Structure of a hemagglutinin monomer. Ribbon diagram of an influenza A (H1N1) virus with the sialic acid receptor-binding site surrounded by the five antigenic sites. Protein Data Bank ID: 3LZG; image created with PyMol Molecular Graphics System, version 1.1eval. Figure modified from (Bouvier & Palese 2008).

The other main biological function of the HA globular head is to recognise and bind to the neuraminic acid receptors on host epithelial cell surface that express SA α-2,6Gal or SA α-2,3Gal (Weis *et al*. 1988). The receptor specificity of influenza viruses is species- and subtype-specific, as discussed in Chapter 2.2.1. The SA receptor binding site is located in the shallow pocket of the HA globular head within a close proximity to the hypervariable antigenic regions and contains the highly conserved amino acids Tyr98, Trp153, His183, Gln190, Leu194 and Tyr195 (Wilson *et al.* 1981). The HA stem is a coiled coil structure of three α-helix HA2 monomers and has a fusion peptide cleavage site at its C-terminus. It has been proposed that the stem bears a highly conserved region that might be involved in cross-neutralisation of diverse viruses and may also block membrane fusion during virus entry (Okuno *et al.* 1993, Ekiert *et al.* 2009, Sui *et al.* 2009).

N-linked glycosylation is one of the most common post-translational protein modifications in mammalian cells that occur in approximately 50% of all proteins

(Apweiler *et al.* 1999). In general, early in the protein synthesis a high mannose core is added in the amide nitrogen of asparagine at the universal Asn-X-Ser/Thr (N-X-S/T) glycosylation sequon, where X can be any amino acid except proline (Bause 1983). The oligosaccharides become further trimmed during transport through ER and Golgi, acquiring variable sugar moieties. Post-translational modifications of glycan chains are organism-, tissue- and cell type-specific and often depend on secretory context, enzymatic exposure and protein structure (An & Cipollo 2011). Viruses utilise N-glycosylation modifications to ensure correct folding, interactions, stability and functionality of their surface proteins HA and NA. Both the HA HA1 and the HA2 of seasonal H1N1 viruses pose a variable number of N-glycosylation sites in close proximity to the antigenic sites that can be potentially post-translationally modified.

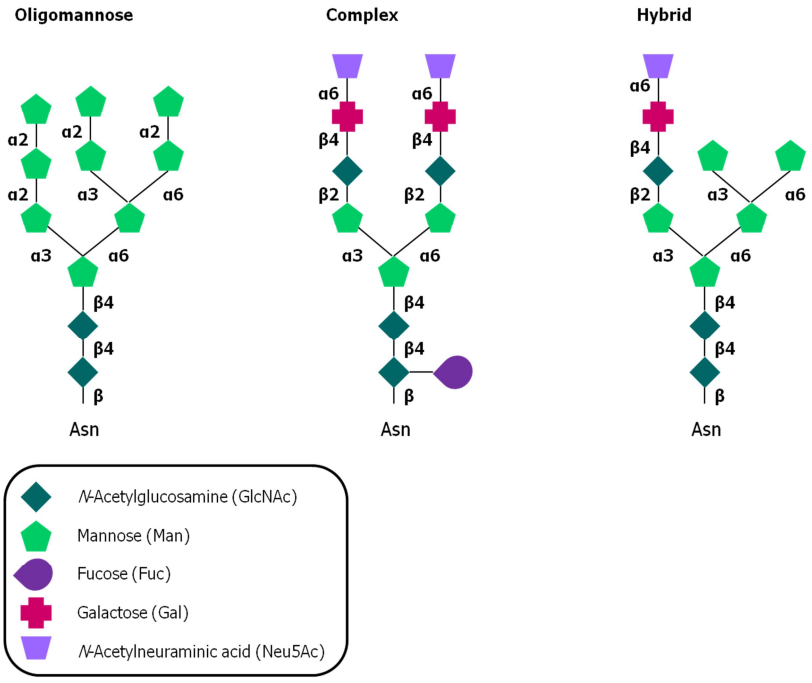


Figure 6. Types of asparagine (N)-linked glycans. The possible combinations of oligosaccharide side chains that can occur on the influenza virus surface proteins HA and NA. Figure modified from (Stanley *et al.* 2009).

The oligosaccharide side chains found in influenza virus HA can be commonly divided into high-mannose type, complex type and hybrids of these two (Figure 6) (Stanley *et al.* 2009). Multiple outer branches and additional sugar residues characterize the complex type oligosaccharides, whereas the high-mannose type contains two to six extra mannose units attached into the pentasaccharide core. The complex N-linked oligosaccharides of HA have several glycoforms and may be neutral, sialylated and/or sulfated depending on the host cell (Yagi *et al.* 2012). Sulfation occurs in the *medial*/*trans*-Golgi and *trans*-Golgi network by specific sulfotransferases (Spiro &

Spiro 2000). This structural processing may be essential for the addition of terminal anionic groups after the SA residues have been removed by the viral NA. For more information on N-linked oligosaccharides, see a review by Kornfeld & Kornfeld (1985) and for discussion of the molecular basis of N-glycosylation of influenza HA and glycoproteins of other important human pathogens, see a review by Vigerust and Shepherd (2007).

O-linked glycosylation is another common covalent modification of mammalian glycoproteins but has also been discovered from certain viral glycoproteins, such as the HIV-1 envelope glycoprotein (Env) (Bernstein *et al.* 1994). O-glycans are covalently α-linked via an *N*-acetylgalactosamine (GalNAc) moiety to the -OH of serine (S) or threonine (T) by an O-glycosidic bond, can be sialylated and occur as highly complex branched structures. More information on the general structure and functions of O-linked glycans can be retrieved from the reviews (Van Den Steen *et al.* 1998) and (Brockhausen *et al.* 2009). Herpes simplex virus type 1 glycoprotein C has numerous clustered O-linked glycans that seem to be part of the complex viral strategy but to have yet unspecified functions (Biller *et al.* 2000). Additional variable region 1 (V1)-associated N- and O-linked glycosylations in the surface component of the simian immunodeficiency virus (SIV) Env protein were shown to alter the antigenicity of the virus, likely by masking its major neutralising epitope (Chackerian *et al.* 1997). The authors suggest that these changes in S and T residues within the V1 region of the viral variants contributed to immune escape from nAb recognition during progression from clinical latency to AIDS in macaques. The functional and structural roles of O-linked glycosylation are less known for influenza A. O-glycan associated Lewis X oligosaccharides have been found solely on the HA of embryonated egg –propagated viruses but not on the HA of MDCK cell-derived viruses (Yagi *et al.* 2012).

2.4.2 Immunogenic role of the enzyme neuraminidase (NA)

NA glycoprotein is a receptor-destroying sialidase enzyme and the second most abundant protein on the influenza virion surface. It is a mushroom-shaped, tetrameric type II integral membrane protein with its N-terminus (stem) towards the virion core and the bulky head outside of the virus particle. The NA head consists of four homotetramers of six β-sheets in a propeller formation and harbours five potential N-glycosylation sites, four of which are known to be glycosylated (Varghese *et al.* 1983). The enzyme catalytic sites are located in the box-shaped head domain and are surrounded by the antigenic determinants (Colman *et al.* 1983). In a mouse study highly

purified NA was shown to elicit equal amounts of NA-specific antibodies compared to HA-specific antibodies elicited by purified HA (Johansson *et al.* 1989). However, the immunity induced by these two immunogenic proteins differed significantly − NA allowed influenza infection while the HA-specific immune responses were able to inhibit infection. It is now well known that NA antigen is capable of eliciting humoral immune responses in the host, although the infection-blocking nAbs have been shown to be mainly directed against HA. Constant mutations occur in the antigenic regions, thus affecting in the overall antigenic drift of the virus.

NA has an important role in viral replication and in the release of mature virus particles from the infected cell (Palese & Compans 1976). With its enzymatic activity the receptor SAs, viruses and mucus are removed from the cell surface, which enables virus spread. An infected cell is simultaneously being protected from further recognition of new viruses and the formation of progeny virus aggregates is prevented on the cell surface (Palese *et al.* 1974). SA-containing oligosaccharides adjacent to the receptor binding site have been shown to have inhibitory effect on the hemagglutinating activity of the influenza A viruses by possible steric hindrance or filling of the receptor binding pocket, which confirms the important role of the NA in the removal of the SA moieties (Ohuchi *et al*. 1995). The functions of NA in the final stage of the infection are well recognised but its role in the earlier stages is less known. Studies on the human airway epithelial cells *in vitro* suggest that NA may contribute to the infection by facilitating the virus entry into the cell, most likely by removal of decoy receptors on mucin, cilia and cellular glykocalix (Matrosovich *et al.* 2004b). However, further studies are required to evaluate the possible additional roles of NA in other stages of viral invasion.

2.4.3 The nucleoprotein (NP) – a structural protein with immunogenic properties

The structural protein NP has its primary role in encapsidating the viral genome into RNPs and is therefore essential for transcription, replication and packaging processes. Additionally, it is known to function as an adapter between virus and host cell processes and to contribute to viral adaptation and pathogenicity [reviewed in (Portela & Digard 2002)]. A substitution mutation D375N in the NP protein of a pH1N1/09 strain was shown to independently contribute to increased virulence and thus adaptation in mice, as compared to seven other identified mutations (Sakabe *et al.* 2011). In this study, this mutation was shown to be responsible for increased pathogenicity in mice along with four other mutations in PA and HA proteins. NP-375D is known to be highly conserved

among the avian, most classical swine viruses and most pandemic H1N1 viruses (Dawood *et al.* 2009, Garten *et al*. 2009, Smith *et al.* 2009), whereas seasonal strains consistently have V or G at this position.

Additionally, NP is the third immunogenic protein of influenza A virion, capable of eliciting antibodies in the host organism. It has been shown that inter-subunit interactions between monomeric NPs and NP-NP interactions contribute to the antigenic structure of NP polymers and the formation of the N5D3 epitope (Prokudina *et al.* 2008). NP has been shown to elicit cellular T lymphocyte responses and humoral responses that both have a role in cross-protection against new viral serotypes in mice (LaMere *et al.* 2011). In this study, anti-NP IgG was shown to promote influenza clearance by mechanisms involving Fc receptors and $CD8^+$ cells in mice. It was also capable of enhancing NP-specific CD8 T cell responses in B cell deficient mice. Additionally, all antiviral activity was lost when mice were vaccinated with an interferon antagonist protein NS1. Consequently, the authors suggest that the highly conserved NP antigens could provide additional heterosubtypic cross-protection for current influenza vaccines that are poorly effective against the constantly drifting external HA and NA antigens.

2.4.4 Influenza proteins as antagonist of the innate and cellular immune responses

A mitochondrial protein PB1-F2 has been shown to have apoptotic characteristics and to be associated with cell-type specific enhancement of cell death (Chen *et al.* 2001). This protein is now known to be expressed at differing levels in individual cells, to be degraded rapidly in a proteasome-dependent manner and to be completely absent from some influenza virus isolates. PB1-F2 carries a short positively charged amphipathic α-helix mitochondrial targeting sequence similar to other viral proteins close to its C-terminus that is responsible for mitotoxicity (Gibbs *et al.* 2003). This protein is also known to have a role in enhancing inflammation during primary viral infection in mice and in increasing frequency and severity of secondary bacterial pneumonia (McAuley *et al.* 2007). This study suggests that PB1-F2 has an important role in lung pathology, especially observed during the pH1N1/18, but its mode of pro-inflammatory activity is to be further studied. According to the study, PB1-F2 clearly affects the virulence and facilitates secondary bacterial infections by a currently unclear mechanism.

Evading immune recognition has been shown to be utilised by an influenza dsRNA inhibitory molecule NS1 that inhibits intracellular signalling and specifically type I

interferon (IFN) production in infected cells, including epithelial cells and DCs (López *et al.* 2003). This important virulence factor was shown to suppress DC maturation, migration and T cell stimulatory activity by modulating multiple cytokine genes *in vitro* (Fernandez-Sesma *et al.* 2006). The study suggests the NS1 to have an important dual role involving modulation of both innate and adaptive immune responses. The interferon antagonist mechanism of this protein is to inhibit RIG-I ubiquitination via a TRIM25 ligase (Gack *et al.* 2009). This suppresses RIG-I signal transduction, which thereby modulates antiviral immunity by inhibiting type I IFN production and thus gives a huge advantage for innate immune evasion. In a recent study the NS1 protein was shown to further suppress IFN response when expressed together with PB1-F2 (Varga *et al.* 2011). It is probable that during future influenza virus research new virulence markers arise or novel properties are found for the currently known viral factors. However, the proteins presented here are known to be the main actors at inducing influenza-mediated innate and adaptive immune responses in the host organisms.

2.4.5 Cellular and humoral responses to influenza

Adaptive immunity elicited by influenza virus infection in humans consists of antibody-mediated and cell-mediated immune responses. The cellular responses against HA viral protein involve the effector T lymphocytes – CD4+ T helper cells and cytotoxic CD8+ T cells. Optimal T cell response consists of IFN-γ –secreting CD4+ cells and CD8+ cells that lyse virus-infected cells (Zinkernagel 1996) and anti-inflammatory cytokine IL-10-producing effector CD4+ and CD8+ T cells that have been shown to regulate the extent of pulmonary inflammation (Sun *et al.* 2009). Long-lasting virus-specific effector memory CD8+ T cells have been shown to proliferate and to migrate into lung airways after recovery from viral clearance, which promotes mucosal immunity (Hogan *et al.* 2002) and can be activated against heterosubtypic challenge and response to secondary viral infection. Life-long HA-specific B cell memory seems to be operated either by antibody-secreting cells (ASC) or resting memory B cells (MBC). During a re-exposure to influenza antigens, cross-reactive helper T cells induce the production of B cells. Circulating MBCs affinity mature and differentiate into plasma cells that are able to produce protective nAbs, which gives them an ability to rapidly neutralise close variants that are introduced via subsequent vaccination or natural infection [vaccine-induced immunity is reviewed in (Dormitzer *et al.* 2011)]. Cellular immunity is essential for effective virus clearance and recovery (Graham & Braciale 1997) while nAbs seem to

provide the primary protection against re-infection (Murphy & Clements 1989, Skehel & Wiley 2000).

2.5 Influenza pathogenesis in the human host

Influenza A viruses produce a characteristic respiratory disease in each of their host organisms. In birds the disease is mostly asymptomatic but occasionally systematic infection involving the central nervous system occurs and can lead to death. The symptoms of swine influenza resemble the symptoms of human infections, including nasal discharge and congestion, coughing, high fever, laboured breathing and conjunctivitis. In humans the acute disease additionally includes body aches, severe headache, chills and sore throat. In children nausea and vomiting are possible outcomes and occasionally the disease progresses into fatal primary (viral) or secondary (bacterial) pneumonia that are a major cause of severe morbidity and mortality following influenza infection in paediatric and geriatric patients, even in previously healthy individuals (Brundage 2006, Finelli *et al.* 2008). The viral and host pathogenicity factors that affect the disease severity are known to function in a complex manner and much remains to be uncovered about the impact of different factors on the clinical manifestation of this commonly self-limited illness.

2.5.1 Viral pathogenicity factors

The virulence of influenza A is a consequence of complex and multigenic co-operation of viral determinants [reviewed in (Medina & García-Sastre 2011)]. The major factors are the host tissue tropism, adaptations in receptor binding specificity (discussed in Chapter 2.3.4) and the sequence of the HA peptide cleavage site, in addition to the immunogenic proteins HA, NA and NP discussed in the previous chapters. The sequence of HA cleavage site is variable among low-pathogenic (H1, H2, H3) and high-pathogenic (H5, H7) strains and functions as a major determinant of disease severity. Low-pathogenic strains have a single arginine amino acid at the cleavage site that is recognised by extracellular and human airway epithelium trypsin-like proteases present only in the respiratory mucosal and intestinal surfaces, whereas high-pathogenic viruses possess a multibasic cleavage site that is cleaved by intracellular proteases that can be found in multiple organs. This characteristic is believed to allow increased virulence and rapid spread of the H5N1 virus into the central nervous system and has been shown to cause severe neurological defects such as neuroinflammation and neurodegeneration in mice (Jang *et al.* 2009).

2.5.2 Host pathogenicity factors

A variety of host factors that affect the disease outcome and the virulence of influenza virus have been determined by recent RNAi-based genome-wide screenings and are discussed in reviews by Medina & García-Sastre (2011) and Watanabe and colleagues (2010). In addition to various cellular markers that interfere with different stages of viral replication, obesity (CDC 2009, Webb *et al.* 2009), diabetes (Carcione *et al.* 2010, Jain *et al*. 2012), cardiovascular diseases (Hanshaoworakul *et al.* 2009), asthma (Jain *et al.* 2012), pregnancy (Webb *et al.* 2009, Creanga *et al.* 2010, Louie *et al.* 2010, Jain *et al.* 2012), chronic pulmonary disease (CDC 2009, Hanshaoworakul *et al.* 2009, Webb *et al.* 2009), First Nations ethnicity (Canadian Aboriginals) (Zarychanski *et al.* 2010), indigenous origin (Australia and New Zealand) (Webb *et al*. 2009) and generally increased inflammatory responses have been shown to be considerable underlying risk factors of susceptibility for severe morbidity and mortality caused by seasonal and pandemic influenza A viruses.

Generally during seasonal influenza A outbreaks the portion of the population requiring most hospitalisations consists of the youngest and the oldest age groups of the population. High susceptibility has been observed within the elderly over 65-years with chronic co-morbidities (Thompson *et al.* 2004, Li *et al.* 2009). The high risk has been postulated to be due to immunological senescence and generally weakened immune responses that can cause as high as 90% excess mortality rate in the hospitalised patients [reviewed in (Webster 2000)]. Naïve immune system and frequent contacts with other children have been shown to be the factors that promote the high frequency of influenza infections in infants and school-aged children (Wallinga *et al.* 2006, Mossong *et al.* 2008). However, during the pH1N1/09 the disease severity patterns were distinctly different from the previous outbreaks. Adults at 25−64-years of age were the group with increased disease severity, highest hospitalisation rates and most visits in the intensive care units (Steens *et al.* 2011). In a case study in the USA during this pandemic, almost half of the persons who required hospitalisation were younger than 18 years old, while only 5% were senior patients of 65 years of age or older (Jain *et al*. 2012). However, risk of death from the pH1N1/09 virus increased along with increasing age (Webb *et al.* 2009). A distinct pattern was observed as the one seen during the pH1N1/18 since the mortality was highest among the young population (Simonsen *et al.* 1998).

The phenomenon known as cytokine storm involving high chemokine and cytokine levels, especially IL-15-dependent CD8+ cells, has been shown to have a critical role in acute pneumonia and influenza-induced lung injuries caused by an infection with highly pathogenic influenza viruses (Nakamura *et al.* 2010). Other host determinants of pathogenesis include the IFNγ-induced protein (IP10), monocyte chemoattractant protein 1 (MCP1), interleukins 6 (IL-6), 8 (IL-8), 10 (IL-10) (Sun *et al*. 2009) and 12 (IL-12) and other pro-inflammatory cytokines/chemokines and cell death genes whose up-regulation were shown to correlate with sustained pathology and severe tissue damage in pH1N1/18 influenza infection (Cillóniz *et al.* 2009). An earlier study has implicated high pharyngeal viral load, cytokine storm and viral presence in the blood to be the factors correlating with fatality due to viral pneumonia caused by systemic H5N1 influenza virus infection (De Jong *et al.* 2006). In addition to intense inflammatory responses, in this study mutations in the viral polymerase complex were found that were thought to indicate mammalian adaptation and virulence. Since the virus uses a complex network of cellular host functions during all steps of its life cycle, it is important to obtain detailed information on their significance on the viral replication. Improved knowledge on the impact of host factors and adaptive mutations on disease severity is an important tool for therapeutic improvements.

Protection from live influenza challenge in the respiratory tract is known to occur by co-operation of local nAbs, secretory IgA and serum subtype-specific anti-HA antibodies (Clements *et al.* 1986, Johnson *et al.* 1986). Immunoglobulins IgG, IgA, IgM and secretory IgA are involved in humoral protection against influenza infections and can also have neutralising activity (Couch & Kasel 1983). The neutralisation effect of anti-HA monoclonal IgG antibodies were initially shown to be due to their ability to inhibit the transcriptase and to prevent primary transcription (Possee *et al.* 1982, Rigg *et al*. 1989), while attachment, entry and uncoating of the virus was suggested to be successfully completed. In contrast to these results, more recent studies on H5N1 influenza showed that the nAbs are targeted against the highly conserved epitope in the stem region that contains the fusion peptide and therefore the nAbs neutralise the infection by blocking membrane fusion (Sui *et al.* 2009). While nAbs seem to be responsible for inhibition of viral entry, anti-NA antibodies are thought to provide secondary protection against influenza infections by inhibiting the release of newly assembled virions.

Unusual severity was observed from pH1N1/09 influenza infection in previously healthy middle-aged individuals (Monsalvo *et al.* 2011). The authors report influenza-induced disease severity to be due to immune complex-mediated disease involving pre-existing cross-reactive but non-neutralising low-avidity antibodies, in addition to pulmonary immune complex-mediated complement activation. Influenza immunology is further complicated by the phenomenon of "original antigenic sin" (Davenport *et al.* 1953, Francis 1953) that opposes the general Burnet's rule of B cell engagement and the existence of which has been questioned during the past 60 years of influenza research. According to the original sin theory, upon an influenza re-infection with a novel strain protective antibodies are produced extensively against the original antigens of the primary virus, even at the expense of the novel antigens. As a result, significantly decreased protective immunity is raised against the newly introduced strain that does not provide sufficient protection from the new pathogen. Recent studies on this somewhat debated topic suggest that original antigenic sin may actually exist and possibly functions as a viral bypass strategy from the immune recognition (Kim *et al.* 2009).

2.5.3 Pre-existing humoral immunity

Previous exposure to influenza viruses determines the level of pre-existing subtype-specific and cross-reactive nAbs in an individual. The level and versatility of the cross-reactive antibodies against different influenza A viruses is known to have an important role in protecting against new emerging viruses and correlates with the pandemic potential of the viruses in the overall human population. Cross-reactivity can be found among strains that contain antigenically similar regions in the HA globular head, in addition to the highly conserved main immunogenic regions in the HA stem region. Since the emergence of the pH1N1/09, the molecular mechanisms of nAb cross-reactivity has been studied extensively due to the significant antigenic resemblance of pH1N1/09 to the previous pandemic strain pH1N1/18 (Figure 7).

Recent studies have revealed neutralisation targets within the globular head domain of the HA that provide high cross-neutralisation activity among the pH1N1/18 and the pH1N1/09 strains (Manicassamy *et al.* 2010b). Cross-reactivity is based on the antigenic similarity between these pandemic strains, a finding that resulted from comparison of their sequences and N-glycosylation profiles. The seasonal H1N1 strains contain two highly conserved glycosylation sites at positions 129 and 163 within the

antigenic site Sa, whereas both pH1N1/18 and pH1N1/09 strains have non-glycosylated HA globular heads (Xu *et al*. 2010). Additionally, the amino acid identity between the HA globular head of the p/H1N1/18 and the p/H1N1/09 is as high as 80%, which is a common divergence among seasonal influenza viruses and provides resistance for antibody neutralisation, while the amino acid identity is only 58−67% between the seasonal and pandemic strains (Wei *et al.* 2010). Furthermore, the amino acid differences between pH1N1/18 and pH1N1/09 virus are localised mainly in the Ca region, while Sa, Sb and Cb epitopes show high level of conservation (Xu *et al.* 2010).

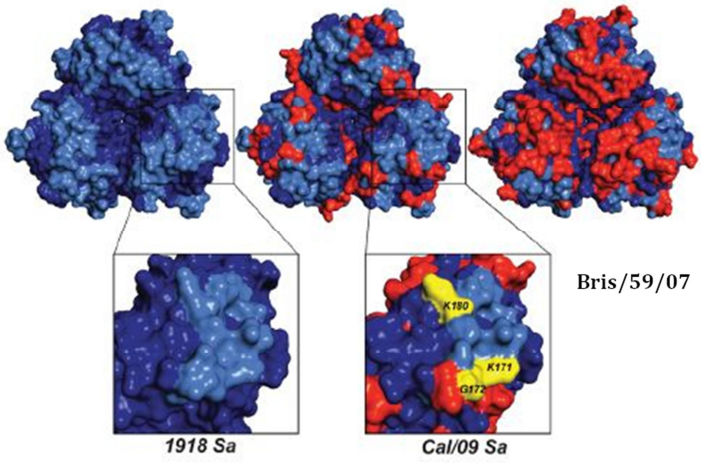


Figure 7. Comparison of antigenic differences in the HA of pH1N1/18, pH1N1/09 and a recent seasonal H1N1 strain. Surface top view of a trimeric HA showing the antigenic sites and their differences among the A/South Carolina/1/18, A/California/04/09 and a seasonal strain A/Brisbane/59/07. Blue colour corresponds to conserved amino acids, whereas red colour shows differing amino acids from pH1N1/18 HA. Antigenic sites are coloured in light blue; the close-up shows antigenic site Sa with yellow residues responsible for mAb escape mutations. Figure modified from (Manicassamy *et al.* 2010b).

An IgG antibody 2D1 from a survivor of the pH1N1/18 influenza was shown to be directed against a conserved epitope at the immunodominant antigenic site Sa and to be able to neutralise both pH1N1/18 and pH1N1/09 viruses (Xu *et al.* 2010). This finding suggests the antigenic site Sa to have a major role in antigenicity of these pandemic strains. The level of cross-reactivity between pH1N1/18 and pH1N1/09 has been addressed in various recent studies. Exposure to one pandemic strain has been shown to protect against a challenge by the other strain and to lead to significantly reduced morbidity and/or mortality. Moreover, titres nearly equal to homologous neutralisation titres were obtained between pH1N1/18 and pH1N1/09, while immunization with contemporary seasonal strains showed no cross-neutralisation activity against neither of these strains (Manicassamy *et al.* 2010b, Medina *et al*. 2010, Wei *et al.* 2010). This phenomenon has also been demonstrated in human vaccination studies. Medina and colleagues confirmed that antisera from individuals immunised with the pH1N1/09

vaccine had high cross-protection against a pH1N1/18 virus, at close to homologous titres (Medina *et al.* 2010). On the contrary to the trend observed with the seasonal strains, during the 2009 pandemic individuals older than 65 years were the most protected age group of the population by having considerably high seroprevalence of cross-reactive antibodies against the pH1N1/09 virus, which indicates previous exposure to an antigenically similar pH1N1/18 virus and/or its post-pandemic variants that continued circulating in the human population (Hancock *et al*. 2009, Xu *et al.* 2010).

This observed high cross-protection between the pandemic strains has opened new insights into studying the molecular basis of H1N1 virus antigenicity and the immunomodulatory impact of additional glycosylations in the HA globular head in the non-glycosylated pH1N1/09 virus background. In a study from 2010 by Wei and colleagues (2010), two HA globular head glycosylation sites, 142 and 177, were introduced into the pandemic A/South Carolina/1/1918 and A/California/04/2009 viruses by site-directed mutagenesis. These mutant viruses showed significantly increased resistance against neutralisation by the WT p/H1N1/18 or WT p/H1N1/09 antisera when compared to their non-glycosylated counterparts. In the same study antisera from mice immunised with the glycosylation mutant p/H1N1/18 virus was able to neutralise both the WT p/H1N1/18 and the mutant p/H1N1/18 viruses, which indicates that immunisation with a virus with additional HA globular head glycosylations could provide protection against glycosylated seasonal variants that have undergone antigenic drift.

2.6 Influenza vaccine design

2.6.1 Reverse genetics methods

Reverse genetics methods were first developed for influenza virus in the beginning of the 1990's and have ever since provided invaluable information of the genetics and replication of the (−) RNA viruses (Luytjes *et al.* 1989, Enami *et al.* 1990). Reverse genetics provides tools for constructing recombinant virus particles from cloned cDNAs and thus allows to perform functional studies at the molecular level by mutagenesis of the viral genes [see introduction to reverse genetics in (Palese & Shaw 2007)]. Influenza A genome is segmented, which has complicated the reverse genetics methods compared to viruses with a single genomic RNA molecule. This means that the segments are cloned into separate plasmids and have to be all taken up by the host cell to allow the generation of infectious particles into the growth medium. A finding that expanded our

knowledge on utilisation of these techniques was that the replication of (−) ssRNA viruses in the cell requires the formation of RNP complexes *in vitro* from synthetic cDNA (Honda *et al.* 1987, Parvin *et al.* 1989) and transfection of functional RNPs into cells, unlike the transfection of (+) ssRNA that readily leads to formation of infectious viruses. In the first systems, the constructed RNA was transfected into eukaryotic cells along with the purified NP, polymerase proteins (PB1, PB2, PA) and a helper influenza virus as a source of the other proteins required for replication (Luytjes *et al.* 1989), followed by selection of the novel virus from the helper virus (Enami *et al.* 1990).

A decade after the birth of reverse genetics, the methods had undergone huge improvements that allowed rescue of infectious viruses by a non-helper virus system (Fodor *et al.* 1999, Neumann *et al.* 1999). In these studies the genome segments were cloned between a truncated human RNA polymerase I promoter and the hepatitis delta virus (HDV) ribozyme in negative orientation and transfected into cells along with two helper plasmids expressing NP and the polymerase complex. Today the rescue of influenza viruses from cDNA can be done efficiently by eight-plasmid systems that utilise an ambisense (bidirectional) plasmid for synthesis of mRNA and vRNA from the same template (Hoffmann *et al.* 2000a, Hoffmann *et al.* 2000b). In this method the sequences are cloned in positive-sense orientation between the human pol-I promoter and mouse terminator and the pol-I transcription unit is flanked by the cytomegalovirus (CMV) pol-II promoter and a polyadenylation site. This simplified technique replaced the previous laborious systems and allowed efficient recovery of recombinant and reassortant influenza A viruses without additional plasmids. Human embryonic kidney/Madin-Darby canine kidney cell (293T/MDCK) co-culture has turned out to be the most efficient for influenza rescue [reviewed in (Neumann & Kawaoka 2004)], although African green monkey (Vero) cells (Fodor *et al.* 1999) and 293T cells alone (Neumann *et al.* 1999) propagate influenza viruses.

The drawback of the widely used systems that utilise RNA polymerase I promoter is that they only allow stringent, host-specific transcription that requires constructing a vector for each target cell type. In 2007 a novel minigenome method was developed that involves transcription of vRNA under the control of a T7 RNA polymerase (simian virus 40) promoter and allows cell type-independent transcription of influenza genes (de Wit *et al.* 2007). The vRNA was cloned into a unidirectional vector in antisense orientation between the T7 RNA polymerase in the 5’ end and a HDV genomic ribozyme and a T7 RNA polymerase terminator in the 3’ end. Although in this assay

gene expression was successfully enhanced by NLSs, the rescue efficiency was lower than for the RNA polymerase I dependent systems, which may be improved by developing a bidirectional system in the future.

By generating mutations into the coding or non-coding regions of the viral genome, the role of different proteins or RNA domains in the replication cycle or pathogenicity can be studied. Reverse genetics has allowed virus production for biotechnology applications such as protein expression vectors, live attenuated vaccines, gene therapy and immunotherapy to deliver genes into specific target cells. The biomedical applications of reverse genetics techniques are exclusively reviewed in (Palese *et al.* 1996, García-Sastre 1998). Viral-like particles (VLPs) are empty particles that structurally resemble virions but do not carry genetic material and are thus non-infectious and safe. They can be engineered by reverse genetics methods to express various viral surface proteins and their assembly requires the expression of envelope of capsid proteins. VLPs can be produced in various mammalian, insect, yeast and plant cells and can be used for comprehensive antigenicity or mutagenesis studies of influenza viruses. There have been attempts to integrate VLPs into vaccine production by engineering highly immunogenic particles with HA and NA surface glycoproteins (discussed in Chapter 2.6.3).

Reverse genetics has also enabled the rescue of the "extinct" pH1N1/18 virus from plasmid DNA, which has allowed extensive evaluation of the virulence and antigenicity of this highly virulent strain. Genomic RNA of the pH1N1/18 was initially recovered from formalin-fixed lung tissue of an Alaskan influenza victim that had died from the 1918 Spanish flu (Taubenberger *et al.* 1997, Reid *et al.* 1999). This finding opened doors to complete analysis of the antigenicity and exceptional virulence of this virus. The virus was first reconstructed in 2005 from eight DNA plasmids (Tumpey *et al.* 2005) with the available knowledge on the pH1N1/18 virus coding sequences of the eight genome segments (Taubenberger *et al.* 1997, Reid *et al*. 1999, Reid *et al.* 2000, Basler *et al.* 2001, Reid *et al.* 2002, Reid *et al.* 2004, Taubenberger *et al.* 2005). The 1918 VLPs were created by reverse genetics methods described before (Fodor *et al.* 1999, Basler *et al.* 2001) with the 5' and 3' non-coding regions from a closely related seasonal H1N1 strain (A/WSN/33).

2.6.2 Current influenza vaccines and their limitations

Since early 1940's, virus propagation in embryonated chicken eggs has remained the gold-standard method for influenza vaccine production. The present vaccine applications recommended by WHO[9] include intramuscularly administered inactivated vaccines. The seasonal trivalent inactivated vaccines (TIV) contain three selected strains − one influenza B strain and two influenza A strains − that had been the main viruses circulating in the human population during the previous season. The circulating seasonal strains are reviewed in February in the Northern Hemisphere, whereas in the Southern Hemisphere the review is carried out in September to predict which strains are likely to circulate in the population and cause human illness in the following winter. These strains are chosen for the next vaccine composition to ensure as good cross match as possible. In the absence of antigenic drift the pre-existing nAbs are likely to protect from the new circulating viruses and no alterations in the vaccine composition are required. The vaccine production circle is reviewed in (Gerdil 2003).

Influenza A viruses are known to elicit long-lasting and strong mucosal and systemic immune responses in their host and therefore engineering of live attenuated recombinant virus vaccines (LAIV) by reverse genetics methods provides an appealing approach for vaccine applications. These viruses could potentially elicit longer-lasting, more specific CD8+ T cell responses in humans than the killed viruses used in the conventional inactivated vaccines. However, the recombinant approaches require further optimisation before entering the commercial markets. In order to design safe but highly immunogenic attenuated vaccines, the virus has to be non-pathogenic in all hosts and its phenotype has to be stabilised by non-revertible mutations. The first attempt to make an attenuated influenza virus by reverse genetics methods was by mutating the promoter region of the NA protein segment of a chimeric influenza A/B virus (Muster *et al.* 1991). Attenuated viruses can also be made by introducing insertions, deletions or multiple mutations within the coding regions of the genes to produce a virus that successfully retains its attenuated temperature-sensitive phenotype (*att* and *ts*) during replication *in vivo*. Temperature-sensitive live attenuated influenza viruses have been made by introducing sequential missense mutations in the PB2 gene (Subbarao *et al.* 1995).

In addition to reverse genetics techniques, LAIVs can be produced by selecting the *att* mutations in one or more genes other than in the antigenic HA and NA. The *att* genes

[9] http://www.who.int/influenza/vaccines/virus/en/

are then transferred by genetic reassortment from the donor virus into a natural seasonal or pandemic influenza variant that carries the WT HA and NA to create a vaccine candidate. Studies on LAIVs that are cold-adapted at 25˚C, and whose growth is thus limited to the nasal passages, were initially started as early as in the 1960's (Smorodintsev 1962, Maassab 1967). A trivalent intranasal influenza vaccine FluMist™ was developed as an alternative for conventional inactivated TIV vaccines and entered commercial markets in the USA in 2003 (Mossad 2003). The cold-adapted FluMist™ vaccine strain consists of HA and NA from currently circulating WT strains and PB1, PB2, PA, NP, M and NS gene segments of A/Ann Arbor/6/60 master donor virus (6:2 reassortant) that confer *ts* and *att* phenotype by multiple amino acid residues in multiple genetic loci (Jin *et al.* 2003). The vaccine is administered as a nasal spray to induce mucosal immunity and has been shown to be immunogenic, effective and safe in humans including children (Belshe *et al.* 1998), adults (Nichol *et al*. 1999) and seniors > 65 years of age (Jackson *et al.* 1999). However, more recent studies have shown low effectivity in adults (Belshe *et al.* 2008, Block *et al*. 2008) and currently this vaccine is only approved for use in persons aged 2 to 49 years[10].

Recent studies have shown FluMist™ to promote resistance to both seasonal circulating strains and pH1N1/09 swine-origin virus via common cross-protective T cell epitopes (Sun *et al.* 2011). Additionally, in mice immunised with this vaccine the IFN-γ responses were completely suppressed, which was shown to significantly decrease the susceptibility to secondary bacterial infection. They are a common cause of severe morbidity and mortality following influenza infection (Brundage 2006, Finelli *et al.* 2008), which underlines the importance of this finding for the knowledge on influenza-bacterial interactions during epidemics and pandemics. A cold-adapted live virus challenge is proposed to induce long-lived CD4 and CD8 T cell −mediated immunity in mice and to target common epitopes that are conserved between heterosubtypic influenza viruses (Powell *et al.* 2007). The authors reported very low-dose priming with live cold-adapted virus to lead to early viral clearance, greatly improved survival after lethal virus challenge and strong heterosubtypic protection for > 70 days. This strategy presents a potential emergency prophylactic measure during a lethal influenza pandemic in case the production of TIVs is delayed.

The production of seasonal TIV vaccines follows a slow (6 months) annual process and has to take into account the accurate timing and production capacity to ensure the

[10] http://www.medimmune.com/pdf/products/flumist_pi.pdf

availability of new updated vaccines prior to the beginning of each influenza season. Since the viruses are currently propagated in the allantoic cavity of slowly reproducible embryonated hen eggs, the availability of eggs determines the production efficiency. Although current egg production meets the needs, it is likely to become the limiting factor during influenza pandemics, which highlights the urgent need for alternative rapid production systems. It is also important to note that if a novel avian pandemic strain crosses the avian-human species barrier, the egg production becomes a serious problem. The other driving forces towards new, rapid methods is the fact that the widely used TIVs are relatively poor antigens in the elderly over 65 years of age, which is the fastest growing segment of the population, and that they are not suitable for egg-allergic individuals. The limitations of the vaccine production circle are reviewed in (Gerdil 2003).

The level of cross-protection induced by current subunit or split-influenza vaccines is dependent on broadly neutralising nAbs directed against HA and NA (Palese 2006). Different chemical and physical methods can be used to inactivate the influenza vaccine viruses, the most common being formaldehyde-inactivation. The impact of γ-irradiation, formalin- and UV-inactivation protocols on the cross-protective immunity has been compared with the commercial TIV vaccine. It was shown that only γ-irradiation of all these methods was able to conserve both homologous and heterosubtypic protection and lead to cytotoxic T cell responses that most likely are a key to cross-protective and long-lasting immunity (Furuya *et al.* 2010). The current vaccines are only effective against antigenically similar, non-drifted strains and therefore have to be updated and administered each year, which involves big economical investments and annual vaccination programmes for the risk groups such as children, elderly and the health care workers. The major drawback of cold-adapted live viruses seems to be their significantly weaker immunogenicity measured by serum antibody levels, compared to their WT parental viruses with similar surface antigens (Tannock *et al.* 1984). Additionally, these vaccines still need to be administered annually to overcome the pre-existing antibodies and therefore they do not provide a solution to the great demand for broadly protecting influenza vaccines.

2.6.3 Novel vaccine approaches

There have been numerous attempts to apply novel broadly effective and convenient approaches and cell lines to the vaccine field. The new technologies include the use of mammalian cell cultures, adjuvant vaccines, recombinant DNA vaccines, VLPs and

HA-based vaccines made in insect, yeast or plant cells, universal vaccines based on conserved proteins and antigens such as the HA stem, modified live viruses and prophylactic vaccines that elicit T cell responses. The primary immunogenic target of influenza vaccines is the variable antigen HA that constantly evolves so that current vaccines may not be effective against novel pandemic of highly virulent viruses. The influenza M2 ion channel protein and the HA stem domains provide potential conserved vaccine targets to improve the level of cross-protection against different subtypes and drifted variants. An excellent summary of new technologies for new influenza vaccines can be found in reviews (Chen *et al.* 2011, Kang *et al.* 2011, Shaw 2012).

MDCKs are the standard cell line for influenza propagation for research and diagnostic applications but could also provide an alternative platform for large-scale vaccine production. The efficiency of virus replication in MDCK cells was shown to be better than in Vero cells, whereas the ratio of genome copies versus infectious units was better in Vero cells for cold-adapted and WT influenza A viruses (Youil *et al.* 2004). The immunogenicity and tolerability of an MDCK-produced inactivated HA subunit vaccine was shown to be equal to an egg-produced HA subunit vaccine (Palache *et al.* 1997). This was the first study in humans with a bivalent vaccine containing the HA antigen of an influenza A and an influenza B seasonal strain. A phase III vaccine study in adults showed a novel cell culture −derived seasonal trivalent influenza vaccine (CCIV) to be comparable to a TIV vaccine in its immunogenicity, efficacy, safety and reactogenicity (Szymczakiewicz-Multanowska *et al*. 2009). In a recent phase II/III randomised vaccine study these properties of a CCIV (Optaflu®, Novartis) were shown to be comparable to an egg-derived subunit TIV vaccine in a paediatric population (Vesikari *et al.* 2012). Both of the studied CCIV vaccines contained purified NA and HA antigens from two current seasonal influenza A and one influenza B strains. The results suggest that mammalian cell-based systems could provide a production platform for egg-protein free, non-allergenic HA subunit vaccines with better antigenic match to WT human-derived viruses compared to the avian-derived vaccines. Other advances include improved controllability of contamination for cell-culture systems, in addition to improved vaccine production yields.

Consistent results of immunogenicity were obtained with *Nicotiana benthamiana* plant –derived recombinant HA from two H5N1 strains that successfully elicited hemagglutination inhibition and nAb production in mice (Shoji *et al.* 2009). Protein Sciences produces trivalent recombinant influenza HA protein under the name of

FluBlok® that is expressed in *Spodoptera frugiperda* (Sf9) insect cells infected with a baculovirus vector (Cox & Karl Anderson 2007, Cox *et al.* 2008). This expression system has enabled the production of a highly pure vaccine free of egg-proteins, preservatives, endotoxins, adjuvants and antibiotics that contains three times more antigenic HA than TIVs and has been proved to elicit long-lasting and strong immunity against drifted influenza variants (Cox & Hollister 2009). FluBlok® has finished phase III studies and is currently waiting for final approval from the USFDA (Shaw 2012).

Glycoprotein-based vaccines and therapeutics forms an emerging field in the current drug market and efforts have been made to evaluate the possible use of highly immunogenic HA or NA proteins that would elicit broadly neutralising nAbs capable of efficiently blocking the entry to airway epithelium and eliminating the novel drifted influenza virus variants and viruses from various subgroups over an extended period of time. The extremely high reproducibility of *E. coli* cells makes it an interesting platform for recombinant HA production on a large scale. The HA proteins produced in these bacteria do not contain glycans and could thus in theory be more immunogenic. Properly folded, trimeric globular HA2 peptides from *E. coli* were shown to elicit a potential nAb response against the pH1N1/09 in ferrets (Khurana *et al.* 2010). However, the proteins secreted from *E. coli* form insoluble, misfolded inclusion bodies that have to be solubilised with detergents and purified by dialysis in addition to monitoring the level of residual endotoxins (Marston 1986).

Due to obstacles encountered with HA production in prokaryotic cells, yeast cells have been studied as a platform for fast and cost-efficient vaccine production. They have glycosylation pathways resembling the eukaryotic pathways and thus are an appealing method for large-scale vaccine production. Yeast *Pichia pastoris* −expressed recombinant H1N1 HA has been recently shown to elicit nAb responses *in vivo* (Athmaram *et al.* 2011). In this study successful HA trimers were produced in yeast that were capable of eliciting functional HA-specific antibodies in mouse and rabbit immunisations, measured by standard hemagglutination inhibition (HI) assays. The mice and rabbit antisera also lead to significant neutralisation of a pH1N1/09 infection *in vitro*, measured by plaque reduction tests. HA was produced with an excellent ratio compared to host proteins, which makes the processing of the recombinant product easy and economical on a large scale. Based on these characteristics, *P. pastoris* is an appealing vaccine production platform that warrants further evaluation.

The possibility of using insect cells as a production platform has been evaluated in various studies for the production of VLPs and recombinant DNA vaccines. VLPs produced in Sf9 cells infected with baculovirus vectors containing an expression cassette with genes of HA, NA and M1 from an H3N2 isolate have been shown to elicit broader immune responses in mice and ferrets, compared to whole inactivated influenza virions and recombinant HA (Bright *et al.* 2007). The same expression system has also been utilised by Novavax in means of creating particles that resemble a seasonal H9N2 influenza virion in their size and morphology (Pushko *et al.* 2005). These particles had preserved functional HA and NA properties and the capability of eliciting serum antibodies that inhibited virus replication following mouse challenge. An interesting advantage of VLPs is that they seem to be recognised by the particle-recognising mechanism of DCs, which has been shown to lead to virus uptake and activation of these cells (Song *et al.* 2010). In this *in vitro* DC/T cell co-culture system DCs in turn stimulated CD8+ T cells via antigen presentation, which suggests that VLPs share immunological advantages with live virus vaccines but with an improved safety profile. VLPs could potentially be more immunogenic than the HA alone, due their resemblance of complete influenza virions.

Design of chimeric VLPs containing proteins from different influenza strains could potentially serve as a mechanism for creating vaccines capable of inducing broader immune responses (Pushko *et al.* 2005). The authors highlight the fact that purification of the VLPs from the recombinant baculoviruses possesses a challenge to be overcome prior to proceeding into commercial applications. A *N. benthamiana* plant-derived H5N1 influenza vaccine was developed that involves transient expression of H5 HA on the VLP surface by an *Agrobacterium* expression cassette (Landry *et al*. 2010). This vaccine has been shown to induce cross-reactive nAbs in *in vitro* neutralisation studies and in a preclinical ferret study and to be well tolerated and immunogenic in phase I clinical studies. The authors describe the first administration of a plant-derived VLP vaccine into humans with minor adverse effects and no observed hypersensitivity reactions to plant N-glycans. With the described system VLP vaccines can be produced within a month from sequencing of a novel pandemic strain, which is a vast advantage compared to manufacture of conventional egg-based vaccines.

The HA globular head is constantly mutating, which complicates the design of broadly neutralising universal influenza vaccines. With response to that, vaccine candidates whose immunogenic focus is on conserved regions of ion channel protein M2 and HA

stem domains have been developed [reviewed in (Kang *et al.* 2011)]. Influenza A viruses are non-integrating and non-oncogenic viruses, which makes them potentially safe vectors. However, the pre-existing immunity against influenza viruses may possess a problem for use of influenza virus vectors in humans but could be circumvented by the use of antigenically different virus strains. Influenza viruses have been engineered by insertion of foreign epitopes into the HA antigenic sites and expression of polyproteins in order to create recombinant vectors for gene delivery. Chimeric influenza viruses have been created that express foreign epitopes and therefore induce humoral immune responses against the foreign pathogen or antigen. The addition of an epitope from H2 or H3 HA into the HA antigenic site B of an H1 virus created a chimeric virus capable of eliciting nAbs against both H3 and H1 subtype viruses (Li *et al.* 1992). The methods described present a very interesting approach for broadening the humoral cross-protection of influenza virus vaccines within the human population. Applications of influenza viruses as vectors are reviewed in (Garcia-Sastre & Palese 1995).

2.6.4 Prophylactic treatment of influenza-related illness

The most effective strategy for prevention of influenza-related illness is by live or inactivated influenza virus vaccines. However, during a novel influenza pandemic the conventional vaccine production will most likely be severely delayed, which is why prophylactic drugs may come in handy in means of pandemic preparedness. Two types of antiviral drugs have entered the commercial markets: adamantanes and newer neuraminidase inhibitors zanamivir (Relenza; GlaxoSmithKline) and oseltamivir (Tamiflu; Roche). While adamantanes interfere with intracellular viral uncoating by inhibiting viral ion channel protein M2, neuraminidase inhibitors block the budding of mature influenza virions from the host cell surface. Licensed neuraminidase inhibitors have been shown to shorten influenza-related symptoms by one day when administered within 48h post-exposure in an initial study in healthy adults (Hayden *et al.* 1997) and in a paediatric population (Shun-Shin *et al.* 2009). For more information on neuraminidase inhibitors for influenza, see a review (Moscona 2005). In a recent study it was shown that a synthetic FLU-v vaccine containing conserved immunogenic regions of M1, M2, NPA and NPB was able to elicit IFN-γ responses in a randomised, double-blind, placebo-controlled phase I trial (Pleguezuelos *et al.* 2012). This approach is uncommon amongst influenza vaccines, since nAb responses were absent and anti-viral effect was rather mediated by cellular mechanisms. This therapeutical agent could

offer a prophylactic approach to be used in line with novel HA/NA-based seasonal vaccines to control the influenza-related illness.

2.6.5 Future challenges

A common goal is to establish production of vaccines carrying HA proteins that occupy uniform mammalian-like glycans and are highly immunogenic and non-allergenic to humans. The glycosylation pathways are likely to depend on the enzymes naturally present in each host-cell type (Yagi *et al.* 2012), which underlines the importance of validating the glycosylation profile of novel virus or subunit vaccine candidates to ensure their antigenicity, efficiency and safety. A recent study addressed the question of N-glycosylation profiles of a H3N2 virus isolate in embryonated eggs and MDCK cells by high performance liquid chromatography (HPLC) mapping (Yagi *et al.* 2012). The HA of viruses propagated in MDCK cells showed common trend of galabiose structures, whereas these structures were absent in viruses from embryonated eggs. In contrast, the O-linked oligosaccharides were absent in the viruses from MDCKs but present in the viruses propagated in embryonated eggs. The HA of viruses grown in MDCKs exhibited considerably more (10.2%) high-mannose type sugars than viruses from embryonated eggs (2.3%). In both virus groups the proportion of neutral oligosaccharides was 90% and 10% of the anionic oligosaccharides, the latter of which were sulfated but non-sialylated. A recent mass-spectrometry –based glycoform study showed an influenza B strain to contain high-mannose hybrid complex glycans with or without sulfation (An & Cipollo 2011).

Vero cells are known to predominantly express α-2,3 galactose-linked SAs (Govorkova *et al.* 1996) which may constitute a major problem due to the variable viral N-glycosylation profile of the produced viruses compared to the current egg-propagated viruses. H3N2 isolates obtained from Vero cells have also been shown to lack the ability to hemagglutinate chicken RBCs and to grow in chicken eggs, most probably due to their altered glycosylation profile that contains an elevated share of high-mannose type oligosaccharides compared to MDCK cells (Romanova *et al.* 2003). In a recent study the glycoform composition of recombinant HAs produced in Sf9 insect cells and *N. benthamiana* plant cells was determined (Zhang *et al.* 2012). All glycosylation sites of the recombinant HAs produced in plants were shown to constantly contain high-mannose type glycans. In contrast, the HAs from insect cells showed high-mannose type glycosylation mixed with the complex type glycans. Glycosylation pathways of a yeast *P. pastoris* have been successfully modified by genetic engineering

methods to mimic human and other mammalian cell N-glycosylation patterns (Bretthauer 2003, Choi *et al.* 2003, Hamilton *et al*. 2003, Gerngross 2004), which has opened new possibilities into glycoprotein production in yeast cells that challenge mammalian cell platforms.

With a mass-spectrometry −based glycoform approach allergenic glycans, such as those containing fucosyl or xylosyl substitutions, of new vaccine candidates can be detected and the impact of new cell lines on the glycosylation profile can be evaluated (An & Cipollo 2011). A drawback of subunit vaccines can be their inability to elicit broadly neutralising immune responses directed against multiple immunogenic regions if only either HA stem or globular head is used for vaccine composition. Other factors to be considered are the stability, reproducibility and safe administration (minimal adverse effects) of the new vaccine candidates. The new vaccines should be cost-effective, easily manufactured in relatively short time-scale and their shelf life should be long enough to enable their sufficiency for the next few years to be able to cut out the costly annual vaccination programmes. There is a strong urge to design universal vaccines that would protect against multiple influenza A and B strains, be active at the respiratory mucosal surfaces and to cover various subtypes by eliciting broad, long-lasting (5−8 years) humoral immune response in the vaccinated individuals (Couch & Kasel 1983).

3 Research aims & hypothesis

The research section of this thesis concentrates on assessment of the molecular role of HA glycosylations on the induction of humoral immune responses. Recent reverse genetics and site-directed mutagenesis –based studies have revealed important clues about the significance of these post-translational modifications on the virus antigenicity, immunogenicity, receptor binding affinity and specificity and immune escape from the host immune machinery. During the past decades the amino acid residues 71, 142, 144, 172, 177 and 179 within the Sa antigenic site have acquired N-linked oligosaccharides within close proximity to the receptor-binding pocket of H1N1 HA (Figure 13). Based on the fact that these glycosylations occurred spontaneously in the nature, we were interested in assessing the impact of each single glycosylation on the breadth of the induced immune response and their possible role in masking of the antigenic site Sa. This thesis also aims at determining the level of cross-reactive antibodies elicited against the seasonally occurring H1N1 strains that have undergone antigenic drift, which would allow us to establish novel antigenic regions in the HA molecule that modulate H1N1 virus antigenicity.

The level of cross-reactivity of mice and ferret sera induced by an infection with the recombinant wild type (rWT) A/Netherlands/602/09 (Neth/09) virus glycosylated at site 144 (Neth/09-HA144) is evaluated against 14 natural seasonal H1N1 strains spanning years 1930−2009. With respect to previous studies (Medina *et al.* accepted), the Neth/09-HA144 is expected to elicit broad cross-reactivity against multiple natural seasonal H1N1 strains due to masking of Sa site. If this is true, it might suggest that this recombinant virus elicits polyclonal antibodies against other antigenic regions of HA that are likely to be outside of the antigenic site Sa. This project also aims at evaluating whether an infection with the Neth/09 virus containing additional HA glycosylations is able to induce broad polyclonal response in mice and ferrets. We aimed at engineering mutant viruses with single additional HA glycosylations at sites 71, 142, 172, 177 and 179, reflecting their temporal appearance in previous seasonal H1N1 viruses. It was speculated that some of the monoglycosylated Neth/09 viruses might exhibit broad polyclonal response against the naturally occurring H1N1 strains and the previously produced polyglycosylated Neth/09 recombinants, while some of the glycosylations might narrow the humoral response due to the diminished antigenicity of these viruses, induced by possible steric hindrance or altered accessibility of the antigenic site.

4 Materials & methods

4.1 Natural H1N1 virus sequences & alignment

The HA sequences of all the H1N1 human viruses were downloaded from the Influenza Research Database[11], aligned with ClustalW[12] (Thompson *et al*. 1994) and edited manually using BioEdit Sequence Alignment Editor software[13] (Hall 1999). The sequences containing representative glycosylation patterns for each year were selected for the alignment, whereas missing years correspond to lack of an isolate sequence for that year or unclear prototype sequence due to few sequences available. N-glycosylation sites were predicted based on the general amino acid motif N-X-S/T. Influenza Research Database accession numbers of the HA proteins in temporal order from 1918 to 2012: AF117241, CY010788, NC_002017, CY009324, CY020445, CY013271, HQ008263, CY020285, CY021709, CY009596, U02085, CY019947, CY019971, CY009332, CY022093, AB043482, CY077748, CY077725, CY008988, CY010372, CY011296, CY021909, CY020181, CY021029, CY010364, CY020437, CY021725, Z54286, DQ508873, L33485, L19023, CY036943, L20111, CY033655, CY033614, CY017011, DQ415317, AF386775, CY031336, CY025026, CY010332, CY006675, CY019883, CY007467, CY016675, CY027875, CY058487, CY040114, CY050198, CY039527, CY060454, JN790366 and CY110745.

4.2 Viruses, cells & animals

Table 2. Influenza A viruses and mammalian cells that were used for this project. The recombinant pandemic A/Netherlands/602/09 strain was used as a parent strain for all the HA glycosylation mutant viruses. The respective 1–4 additional glycosylation sites of each mutant virus are indicated in bold.

Natural H1N1 isolates	Glycosylation mutant viruses	Mammalian cells
A/Swine/Iowa/30	**Parent strain**:	**Virus propagation:**
A/PR/8/34	recombinant WT A/Netherlands/602/09 (rWT)	
A/Weiss/43		Madin-Darby canine kidney (MDCK) cells
A/FLW/52	**Recombinant mutant viruses:**	
A/Denver/57		**Virus rescue:**
A/USSR/92/77	Neth/09-HA**142**	
A/USSR/79	Neth/09-HA**144**	Human embryonic kidney (293T) cells
A/Finland/13/80	Neth/09-HA**172**	
A/Houston/84	Neth/09-HA**177**	**Animal models**
A/Texas/36/91	Neth/09-HA**179**	
A/New Caledonia/20/99	Neth/09-HA**71-142**	7 to 9-weeks-old C57BL/6 female mice
A/Brisbane/59/07	Neth/09-HA**144-172**	
A/California/07/09	Neth/09-HA**71-142-177**	8 to 12-months-old male Fitch ferrets
A/Netherlands/602/09	Neth/09-HA**71-142-172-177**	

[11] http://www.fludb.org/brc/home.do?decorator=influenza (accessed 24.-25.4.2012)
[12] version 1.4.
[13] version 7.1.3.0

MDCK cells were used for all virus propagation (Table 2) and maintained in DMEM (#SH30081.FS / high glucose, Thermo Scientific) with 15% fetal bovine serum (FBS) and 2 μg/ml penicillin streptomycin at 37˚C in humidified atmosphere with 5% CO_2. One of the available serum-free viral growth mediums (VGM) − Optimem® (#31985-047, Gibco® Life Technologies), DME/F-12 (#SH30004.03, HyClone®, Thermo Scientific) with supplementary $NaHCO_3$ and dextran or 1X MEM (#BW12-684F, 10X EMEM BioWhittaker®, Lonza) with supplementary penicillin/streptomycin, 0.21% bovine albumin, 0.12% $NaHCO_3$ and 1% HEPES − was used for viral infections with supplementary 1 μg/ml of TPCK trypsin to allow mature virus particle budding from the infected cells. Cell culture growth was monitored daily by using an Olympus CKX41 inverted microscope at 20 x magnification. All viruses (Table 2) had been previously produced by the Medina laboratory but were propagated and titrated during this project. The HA1 domains of the natural seasonal H1N1 isolates were sequenced (Macrogen, USA) to exclude the possibility of any additional mutations during passages in MDCKs. Sera collected from mice or ferrets (Table 2) were used for all serological assays. All animal research was conducted under the guidance of the Centers for Disease Control and Prevention's (CDC) IACUC in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility at Mount Sinai School of Medicine (MSSM) in New York, USA.

4.3 Site-directed mutagenesis

Table 3. Primers used for site-directed mutagenesis. The universal primers were used for producing the ambisense pDZ-HA plasmid. The glycosylation mutant primers were used for site directed mutagenesis to engineer each corresponding mutant pPol-HA plasmid with the additional HA glycosylation site 142 or 179.

Universal Primers	Glycosylation Mutant Primers
Forward primer: *SapI*-F-5′- GGCCGGCTCTTCTATTAGTAGAAACAAGG-3′	**Neth/09-HA142**: F-5′-GTTCATGGCCCAATCATACCTCGAACAAAGGTGTAACG R-5′-CGTTACACCTTTGTTCGAGGTATGATTGGGCCATGAAC
Reverse primer: *SapI*-R-5′- CATCGCTCTTCTATTAGCGAAAGCAGG-3′	**Neth/09-HA179**: F-5′-TACCCAAAGCTCAACAAATCCTAC R-5′-GTAGGATTTGTTGAGCTTTGGG

Reverse genetics techniques were used for the rescue of complete glycosylation mutant viruses from plasmid DNA (Palese *et al.* 1996, Pleschka *et al.* 1996). A rWT Neth/09 pPol-HA plasmid (kindly provided by Prof. Adolfo García-Sastre) was used for the expression (Neumann *et al.* 1994) of the glycosylation mutant HA proteins. The plasmid contains the viral RNA coding region, flanked by a RNA polymerase I

promoter at its 5' end and a HDV genomic ribozyme at the 3' end (Figure 16). Two individual glycosylation mutant plasmids pPol-HA142 and pPol-HA179 were generated by incorporating a single glycosylation site into the rWT Neth/09 pPol-HA plasmid by site-directed mutagenesis (Enami *et al.* 1990) according to the Quikchange site-directed mutagenesis kit protocol (Stratagene)[14] with a few modifications. The other proposed glycosylation mutations were not attempted due to the limited time frame. Single amino acid mutations were introduced into the plasmid in a single PCR cycle by using the high fidelity Platinum *Pfx* polymerase (#11708-013, Invitrogen) and the mutant primer pair that was designed individually for both glycosylation site mutations (Table 3) and ordered from Sigma-Aldrich (New York, USA). The amino acid changes that were made in each case to introduce a glycosylation site were D144T for site 142 and S179N for site 179.

4.4 Cloning

4.4.1 The pDZ-HA plasmid

An expression plasmid pDZ-HA was engineered by subcloning the HA sequence from the pPol-HA plasmid (Figure 16) into the pDZ plasmid (Figure 17; kindly provided by Adolfo García-Sastre). The HA sequence was inserted between the RNA polymerase promoter and terminator sequences of the ambisense pDZ plasmid that allows the bidirectional synthesis of both (−) vRNA and (+) mRNA from one viral cDNA template essential for viral assembly. The HA sequence was first amplified by a standard PCR [92˚C for 2 min, 35 cycles of (94˚C for 30 s, 55˚C for 30 s and 72˚C for 90 s) and 72˚C for 7 min] by using universal primers with *SapI* restriction sites (Table 3). The HA segment and the pDZ plasmid were then digested with *SapI* (#R0569S, New England Biolabs) for 1 h at 37˚C and their size was verified on a 1% agarose gel. The HA segment was then ligated into the pDZ plasmid with T4 ligase (#M0202S, New England Biolabs) using a protocol that involved 2 µl of 10X T4 ligase buffer, ~50 ng of the pDZ plasmid, ~100 ng of the HA insert DNA, 2 µl of T4 ligase and H_20 up to total volume of 20 µl with a 30 min incubation at room temperature (RT).

Chemically competent *E. coli* cells (prepared by Constanza Martinez Valdebenito) were transformed with 5 µl of the ligated plasmid by a general heat shock method, which in short involved incubating the plasmid with the cells on ice for 10 min, heat-shocking at 42˚C for 45 s and cooling on ice for 2 min. The bacteria were then incubated in 500 µl of LB broth (#L3022, Sigma-Aldrich) with no antibiotics on a shaker for 1 h at 37˚C,

[14] http://www.chem.agilent.com/Library/usermanuals/Public/200518.pdf

spread on ampicillin LB plates and grown overnight. One colony was picked on the following day and the plasmid was amplified in 200 ml of LB broth with 1 μg/μl ampicillin overnight, after which the plasmid DNA was extracted by using the Midiprep kit[15] (Promega). The pDZ plasmid was engineered for VLP production and other studies of Medina laboratory that were not feasible within the time range of this project.

4.4.2 The glycosylation mutant pPol-HA plasmids

The produced glycosylation mutant plasmids pPol-HA142 and pPol-HA179 were enzyme-digested with *DpnI* (#R0176S, New England Biolabs) for 1 h at 37˚C according to the Quikchange site-directed mutagenesis kit protocol to digest and eliminate the non-mutated, methylated parent pPol-HA plasmids. Chemically competent *E. coli* cells were then transformed with 3 μl of each mutant pPol-HA plasmid DNA by the heat-shock method described in Chapter 4.4.1. Three representative clones of each of the plasmids pPol-HA142 and pPol-HA179 were picked and amplified in 5 ml of LB broth (+ 1 μg/ml ampicillin) in a 15-ml culture tube overnight (> 16 h) and the plasmid DNA was then extracted by using a Miniprep kit (Promega)[16]. All the engineered mutant plasmids were sequenced (Macrogen, USA) to confirm the correct addition of the single glycosylation site and to exclude the possibility of any additional mutations.

4.5 Rescue of the glycosylation mutant viruses

Rescue of Neth/09-HA142 and Neth/09-HA179 recombinant viruses was attempted during this project by using the previously described ambisense system (Hoffmann *et al.* 2000a). The engineered pPol-HA142 and pPol-HA179 plasmids were transfected together with the Neth/09 pPol-NA plasmid and 6 pDZ ambisense plasmids encoding the viral genes and proteins of the PB2, PB1, PA, NP, M and NS segments into 293T cells by Lipofectamine® 2000 (#11668-030, Invitrogen) treatment according to manufacturer's instructions[17]. Additionally, two A/WSN/33 helper pCAGGS protein expression plasmids encoding the wild type HA and NA proteins were transfected to aid the assembly of the viral particles in the first cycle of viral replication. The pPolI plasmid encoding the rWT NA gene, the pDZ expression plasmids and the two helper plasmids had been previously generated by the Medina laboratory.

---

15 http://fi.promega.com/~/media/Files/Resources/ProtCards/PureYield%20Plasmid%20Midiprep%20System%20Quick%20Protocol.pdf

16 http://fi.promega.com/~/media/Files/Resources/ProtCards/PureYield%20Plasmid%20Miniprep%20System%20Quick%20Protocol.pdf

17 http://tools.invitrogen.com/content/sfs/manuals/lipofectamine2000_man.pdf

The transfection protocol involved first preparing the plasmid master mix in Optimem® that contained standardised concentrations (0.33 μg/transfection reaction) of each of the helper and standard plasmids. 250 μl/reaction of this master mix was aliquoted into separate tubes (in duplicates) and 0.33 μg of each mutant pPol-HA plasmid was added into these master mix aliquots. The plasmid encoding the rWT HA (pPol-HA) was used as a positive control for rescuing the glycosylation mutant viruses. Lipofectamine®-Optimem® mixture was prepared by using 2.5 μl of Lipofectamine® / 1 μg of plasmid and was incubated for 5 min in RT. Then each plasmid-Optimem® mix was mixed with 250 μl/reaction of the Lipofectamine®-Optimem® mix (in duplicates) and incubated for 20 min at RT. Meanwhile, the 293T cells were trypsinised and a cell suspension containing 10^6 cells/each well was prepared in Optimem®. 1.5 ml of this suspension was added into 6-well cell culture plates and the Lipofectamine®-plasmid-Optimem® mixtures were then added drop wise into the 293T cell suspension. 22 h post-transfection MDCK cells were seeded on top of the 293T cells and allowed to grow for additional 24 h in DMEM with penicillin/streptomycin, 0.3% bovine albumin and 1 μg/ml TPCK-treated trypsin (Sigma-Aldrich). Subsequently following this project, the supernatants were harvested at 24−36 h post-addition of the MDCKs, then once blind-passaged to MDCK cells and a plaque assay was simultaneously performed to detect virus growth.

4.6 Virus propagation

All viruses were propagated in MDCK cells in T75 tissue culture flasks (Orange Scientific). On the previous day prior to infections, the cells were washed twice with PBS and detached from the bottom of the flask with 3 ml of 0.05% trypsin (Thermo Scientific) at 37˚C for 10−15 min. Cells were then resuspended into complete DMEM, seeded into 6-well plates and grown until confluent (~24 h). On the day of infection, the cells were first washed twice with PBS to remove the residual serum in the medium that inhibits trypsin that is required for progeny virus release from the surface of the infected cells. The washed cells were then infected with 200 μl of each virus stock (1:1,000), incubated at 37˚C for 1 h and then provided with 2 ml of VGM with additional trypsin (1 μg/ml). The cultures were incubated for 48−72 h until the cytopathic effect (CPE) was visible and the cell monolayer was detached. The presence of viruses was verified by a semi-quantitative hemagglutination assay (Chapter 4.9) and virus-containing supernatants were stored in −80˚C for further use.

4.7 Virus stock titration by plaque assay

On the previous day of the infections, the MDCK cells were seeded into 6-well cell culture plates as described in the previous chapter. On the day of infection, confluent cells were washed twice with PBS and then inoculated with 200 µl of the virus stocks in 10-fold serial dilutions (10^{-2}–10^{-7}). The cells were infected at 37˚C for 1 h with frequent rotation to ensure even infection. The cells were then covered with 2 ml of an optional VGM supplemented with 2% of purified agar (#LP0028, Oxoid Ltd.) and 1 µg/ml of trypsin and incubated for ≥ 48 h until plaques were visible. Positive plaques were then counted and the number of plaque forming units (PFU/ml) was calculated considering the dilution at which the plaques were counted and the initial inoculation volume of each virus stock.

4.8 SDS-PAGE & Western blotting

The viral proteins were verified from whole-cell lysates with polyclonal antibodies by Western blot analysis. MDCK cells had been infected at an multiplicity of infection (MOI) of 5 for 12 h with the indicated viruses and lysed in NP-40 lysis buffer (50 nM Tris, 150 nM NaCl, 5 mM EDTA, 30 mM NaF, 40 mM β-glycerophosphate, 10% glycerol and 0.25% NP-40) containing Complete Mini protease inhibitor (Roche). Clarified lysates were boiled for 5 min and proteins were separated on two parallel SDS-PAGE electrophoresis gradient gels with a 4% stacking gel and a 10% resolving gel in the Mini-PROTEAN® Tetra Cell (Bio-Rad)[18]. The virus preparations were mixed with non-reducing SDS-loading buffer and run on the gel with Precision Plus Protein Dual Colour ladder (#161-0374, Bio-Rad) for a few hours.

One of the gels was stained with Coomassie stain (0.25% in 40% methanol and 7% acetic acid in dH_20) by heating in the microwave for 1 min and incubating for 10 min on a rocking plate. The gel was then destained (40% methanol and 7% acetic acid in dH_20) overnight. The other gel was transferred into a PVDF membrane overnight in the chamber provided by the Mini-PROTEAN® Tetra Cell (Bio-Rad). The membrane was then blocked with 5% skim milk (#232100, Difco™, BD), 0.5% Tween-20 in 1X PBS for 1 h and incubated with 5 ml of a rabbit anti-PR8 headless virus primary antibody 3951 (1:5,000 dilution) for 1 hour at RT. The membrane was washed three times with PBS and incubated for 1 hour in a dilution of 1:10,000 of anti-rabbit IgG-HRP conjugate. After washing five times with PBS the membrane was developed with a

[18] http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10007296.PDF

solution of 10 ml 100 mM Tris, 22 µl of 90 mM coumaric acid, 50 µl of 250 mM luminol and 3 µl of 30% H_2O_2 in a dark room at RT. The membrane was placed under an X-ray film in a closed container for 10 min and rinsed with the developing solution and H_2O.

4.9 Hemagglutination (HA) assays

Materials:

H1N1 virus stocks
1X PBS (#BM-1340, Winkler Ltda.)
0.5% turkey RBCs in 1X PBS
96-well microplates, V-shape (Nunc)

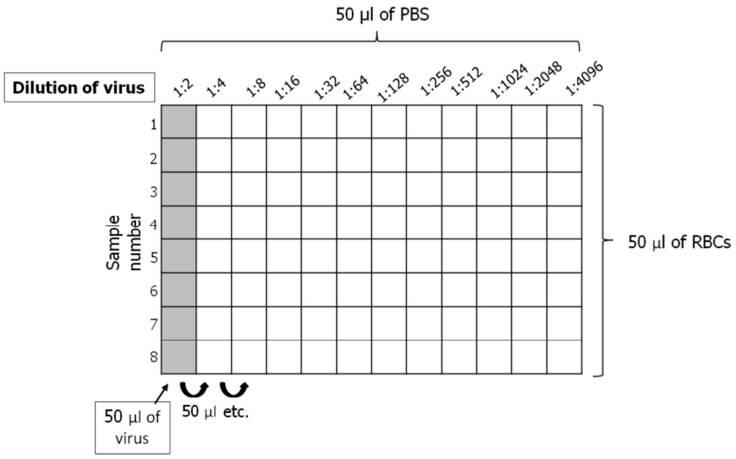


Figure 8. HA-assay settings. 50 µl of PBS was added into each well and 50 µl of initial virus stock was added into column 1. Serial 2-fold dilutions of the virus (1:2 up to 1:4,096) were done by mixing ten times and by transferring 50 µl of the virus/PBS mix to the next well across the row.

Influenza A viruses are known to agglutinate α-2,3 SA and α-2,6 SA −expressing red blood cells (RBCs) from different species, which leads to the formation of the characteristic virus-cell network (Hirst 1942). This property has been used as a detection method to confirm the presence of influenza virus in human and animal specimens for decades and functions as an excellent preliminary diagnostic method in epidemic surveillance and basic research. H1N1 viruses show agglutination of chicken RBCs, with the exception of the pH1N1/09 viruses that preferably agglutinate turkey RBCs. The hematocrit (% of erythrocytes in whole blood) of turkey blood was determined by capillary centrifugation (10,000 rpm for 5 min) and 0.5% RBC solutions were prepared in 1X PBS. A V-shaped 96-well plate was prepared for each virus to be tested (Figure 8), leaving column 12 as a negative control (PBS only). 50 µl of turkey RBCs was then added into all wells and incubated for 30−45 min at 4˚C. The hemagglutination units (HAU) of each virus were determined by the last virus dilution that showed complete hemagglutination. In the absence of virus the RBCs form a dense pellet on the bottom of the well, whereas in the presence of virus a floating virus-RBC network is formed and no pellet is observed. Following propagation in MDCK cells, the presence of all viruses used in this study was evaluated by this semi-quantitative assay prior to further assays.

4.10 Hemagglutination inhibition (HI) assays

Materials:

H1N1 virus stocks
1X PBS (#BM-1340, Winkler Ltda.)
05% turkey RBCs in 1X PBS
96-well microplates, V-shape (Nunc)
trypsin-heat-periodate treated mouse and ferret sera

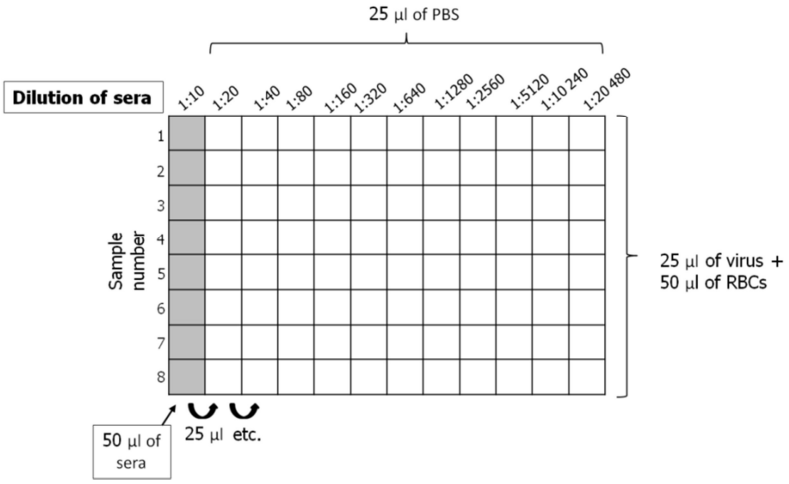


Figure 9. HI-assay settings. 25 µl of PBS was added into each well except column 1. 25 µl of trypsin-heat-periodate inactivated sera were added into column 1 and serial 2-fold dilutions of the virus (1:10 up to 1:20,480) were prepared by mixing ten times and then transferring 25 µl of the serum/PBS mix to the next well across the row.

HI assay has been used for decades to detect the presence of serotype-specific antibodies directed against the HA globular head in animal and human serum (Hirst 1942). With this simple and quick assay the cross-reactivity of any serotype-specific antisera can be evaluated. Mouse sera that had been obtained at 28 days post-infection (p.i.) and ferret sera that had been obtained at 21 days p.i. were first trypsin-heat-periodate inactivated to eliminate the activity of complement and other interfering proteins. The inactivation in short involved addition of ½ volume of trypsin into 1 volume of serum, incubation at 56˚C for 30 min, addition of 3 volumes of 0.011 M KIO_4, incubation at RT for 15 min, addition of 3 volumes of 1% glycerol saline, incubation in RT for 15 min and addition of 2.5 volumes of 85% saline. This treatment gave an initial serum concentration of 1:10, from which 2-fold dilutions were prepared up to 1:20,480 (Figure 9). The HI assays were performed as described earlier (Salk 1944). Briefly, the stock viruses were adjusted at titres of HAU 8 by the standard HA assay (Chapter 4.9) and mixed with the serial serum dilutions on the plate. The viruses were incubated with the sera for 30 min to allow serotype-specific antibody binding, after which 50 µl of turkey RBCs were added. After 30−60 min incubation in RT the HI titres were determined by visual examination as the last serum dilution that completely inhibited hemagglutination of RBCs.

4.11 Serology ELISA

Serology ELISA was utilised to measure the level of IgG antibodies in antisera from mice infected with the recombinant glycosylation mutant viruses. Immulon® 4 HBX ELISA plates (Nunc) were covered with 100 µl of each serial 2-fold dilution (1:2,000−1:64,000) in quadruplicates of trypsin-heat-periodate inactivated mouse sera that had been raised against rWT Neth/09, Neth/09-HA144, Neth/09-HA144-172, Neth/09-HA71-142-177 or Neth/09-HA71-142-172-177 mutant viruses. Following overnight incubation at 4˚C the wells were washed three times with 200 µl of 1X PBS and then blocked for 1 h in RT with 100 µl of the blocking medium that contained 5% skim milk (#232100, Difco™, BD), 0.05% Tween-20 and 5% sheep serum in 1X PBS. They were then incubated with 50 µl of a 1:5,000 dilution of Gt X Ms IgG γ chain-specific antibody (#AP503P, Millipore) in the blocking medium for 1 h in RT. 100 µl of a 1:1 mixture of HRP substrate solutions A and B (Kirkegaard & Perry Laboratories KPL) was added into each well and the absorbance at each serum dilution was measured at λ 405 on a microplate reader (PHOMO, Autobio Labtec Instruments).

4.12 Microneutralisation assays

In this assay the presence of serotype-specific nAbs in antisera collected from mice at 28 days p.i. and from ferrets at 21 days p.i. infected with the rWT or the Neth/09-HA144 glycosylation mutant viruses was determined. The microneutralisation assay was modified from WHO Influenza Manual (WHO Global Influenza Surveillance Network 2011) and consists of three sections: 1) virus-antibody reaction step, 2) inoculation step and 3) serology ELISA.

4.12.1 Determination of tissue culture infectious dose 50

The virus stocks were first titrated for the tissue culture infectious dose ($TCID_{50}$) according to the 2011 WHO Influenza Manual (WHO Global Influenza Surveillance Network 2011). Briefly, MDCK cells were seeded on 96-well cell culture plates (Orange Scientific) in VGM at 2 x 10^5 cells/ml and grown until the cell monolayer was 75% confluent (~24 h). Cells were then inoculated with ½log10 serial virus dilutions from 10^{-2} to 10^{-7} (Figure 10). $TCID_{50}$ of each glycosylation mutant virus was calculated by the Reed & Muench method (Figure 11).

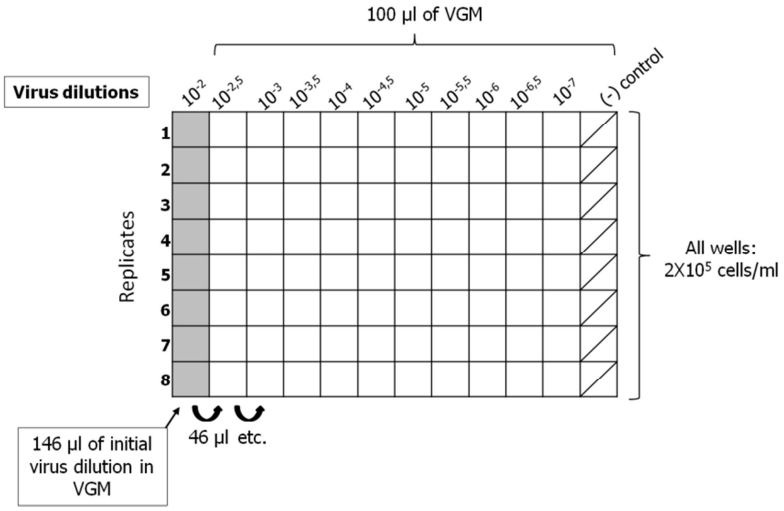


Figure 10. Virus stock titration for the microneutralisation assay. 100 µl of VGM was added into each well except column 1. 146 µl of the initial 10^{-2} dilution was added into the wells of the first column and serial ½log10 dilutions were prepared across the plate by transferring 46 µl into the next column. This virus preparation was inoculated into cells that had been seeded on 96-well cell culture plate at 2 x 10^5 cells/ml on the previous day.

Proportional distance:

$$\frac{\%\text{ positive value above 50\%} - 50 \text{ x } 0.5 \text{ (correction factor for ½log10 dilutions)}}{\%\text{ positive value above 50\%} - \%\text{ positive value below 50\%}}$$

$TCID_{50}$: dilution that gives $> 50\%$ positive wells + proportional distance

In the microneutralisation assay 100 x $TCID_{50}$ was used, for which the required volume of the stock virus was calculated by using the following equation:

100 x $TCID_{50}$: $\frac{1000\ \mu l \text{ x } 100}{\text{TCID } 50/\text{ml}} = \text{X } \mu l \text{ of virus stock/well}$

Figure 11. Determination of the $TCID_{50}$ of the virus stocks for the microneutralisation assay. The equations for calculating the tissue culture infectious dose 50 ($TCID_{50}$) of influenza stocks, modified from (WHO Global Influenza Surveillance Network 2011).

The titration of Neth/09-HA144 is presented here as an example:

$$\text{Proportional distance} = \frac{100\% - 50 \text{ x } 0.5}{100\% - 20\%} = 0.3125$$

$TCID_{50} = 10^{4+0,3} = 10^{4,3}$ $TCID_{50}$ / 100 µl (initial inoculation volume) = $10^{5,3}$ $TCID_{50}$ / ml = 1,99 x 10^5 $TCID_{50}$ / ml. 100 x $TCID_{50}$ for this virus is 0.50 µl of the virus stock / well, which equals 48 µl / 96-well plate (a 1:100 dilution).

4.12.2 Virus-serum inoculation & serology ELISA

Microneutralisation assay involved seeding the MDCK cells on sterile 96-well cell culture plates (Orange Scientific) at 2 x 10^5 cells/ml. The cells were incubated until the cell monolayer was > 75% confluent (~24 h). Mouse and ferret antisera was inactivated at 56˚C for 30 min and 2-fold serial dilutions were prepared of each serum in quadruplicates on a separate sterile 96-well cell culture plate (Figure 12). 100 x $TCID_{50}$ of the virus stocks were added into each well and was incubated with the antisera for 1 h at 37˚C to allow serotype-specific antibody binding. The cells were then inoculated with the virus-serum mixture by direct transfer from the mixing plate and incubated for 18−20 h at 37˚C, after which a serology ELISA was performed.

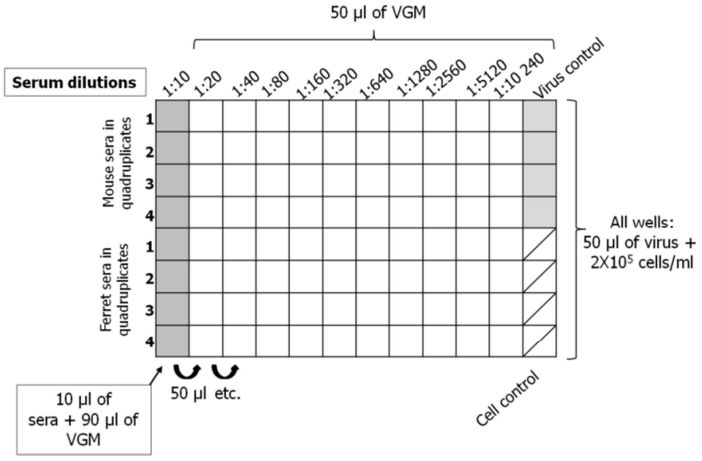


Figure 12. Microneutralisation assay test settings. 2 x 10^5 cells/ml were seeded on a sterile 96-well plate and incubated for ~24 h. On a separate sterile mixing plate, 50 µl of VGM was added into each well except 90 µl into column 1. 10 µl of heat-inactivated mouse and ferret sera were added in column 1 in quadruplicates and serial 2-fold dilutions (1:10−1:10,240) were prepared by mixing ten times and then transferring 50 µl of the serum/VGM mix to the next well across the row. 100 x $TCID_{50}$ was added into each well except the cell control wells and this serum-virus mixture was incubated at 37˚C for 1 h prior to inoculation into the cells.

The cells were fixed with ice-cold 80% acetone in PBS for 30 min, air-dried and washed with 200 µl of PBS, after which they were incubated with an α-NP protein primary antibody (1:5,000) in PBS / 0.3% Tween-20 / 5% skim milk (#232100, Difco™, BD) diluent for 1 h at RT. After three washes (PBS + 0.3% Tween-20) the cells were incubated with a secondary antibody α-rabbit HRP conjugate (1:10,000) in the diluent for 1 h in RT, washed five times with the wash buffer and developed with 100 µl of a 1:1 HRP substrate mixture A and B (Kirkegaard & Perry Laboratories KPL) for ≥ 1 h. Absorbance was measured at λ 450 on a microplate reader (PHOMO, Autobio Labtec Instruments). Neutralisation titres are represented as the geometrical mean of the highest serum dilution that was able to neutralise infection.

5 Results

5.1 Seasonal H1N1 human viruses acquired HA globular head glycosylation sites over time

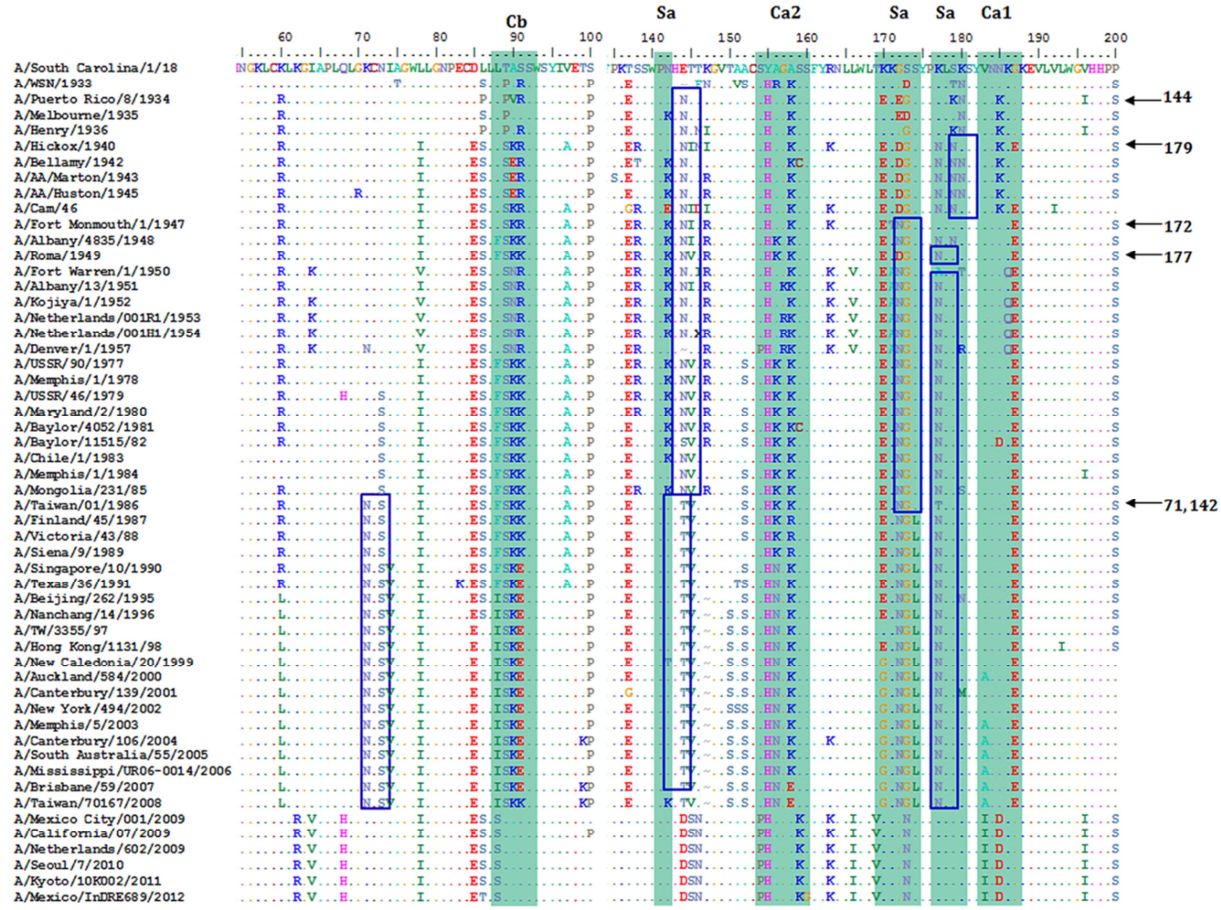
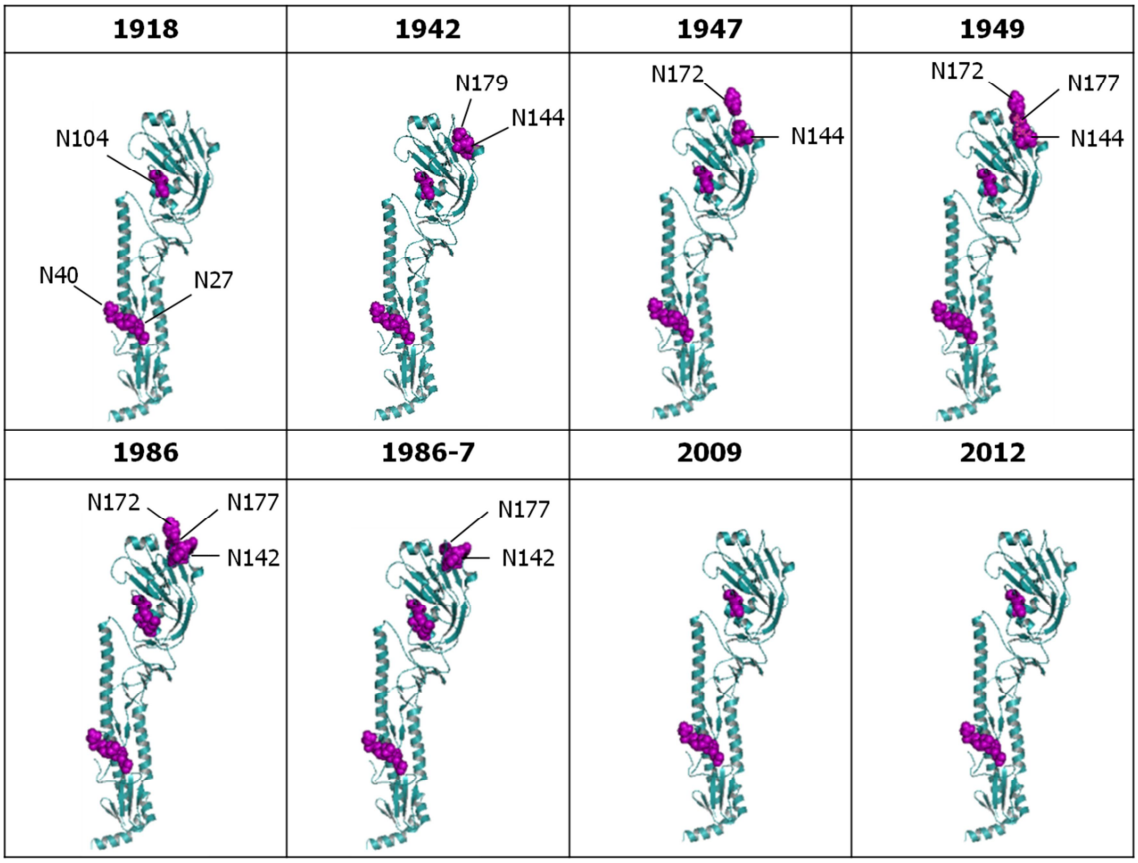


Figure 13. HA1 domain alignment of the natural seasonal H1N1 isolates between 1918 and 2009. Light blue-coloured shading depicts the known antigenic sites listed on the top and dark blue boxes represent glycosylations that appeared and disappeared over time, predicted by the N-X-S/T motif. Antigenic sites corresponding H3 numbering: Cb (78–83), Sa (128–129, 156–160, 162–167), Sb (187–198), Ca1 (169–173, 206–208, 238–240) and Ca2 (140–145, 224–226) (Manicassamy *et al.* 2010b). Arrows on the right point the year glycosylations appeared at the indicated amino acid residues. Sequences were obtained from Influenza Research Database, alignment was done with ClustalW and manual editing was done with BioEdit software.

Among other mutations, the H1 HA has acquired site-specific N-linked glycosylations at amino acid residues 71, 142, 144, 172, 177 and 179 over the past decades. These glycosylations have appeared and disappeared gradually in the HA globular head antigenic sites and have become fixed in time in the naturally occurring seasonal H1N1 strains (Figure 13), which may indicate their selective advantage on the viral antigenic profile (Medina *et al.* accepted). The analysis concentrates around the HA1 antigenic sites of seasonal H1N1 isolates, which were sequenced to confirm that no additional mutations arouse after passage in MDCK cells. Notably, there are no human influenza sequences available between 1918 and 1933 and most of the sequences are from strains that have been passaged extensively in eggs and/or nonhuman mammalian laboratory

cell cultures. The alignment shows that during the years 1935 to 1957, three different HA glycosylations were acquired sequentially at residues 144, 172 and 177. The emergence of the H2N2 pandemic influenza virus in 1957 displaced the H1N1 viruses, which disappeared from human circulation. The H1N1 virus strain re-emerged in humans in 1977 and contained the same HA glycosylation sites as the H1N1 viruses circulating in the 1950's. In 1986, glycosylation site 144 was replaced by glycosylations on positions 71 and 142. However, in 1987 glycosylation site 172 was lost, and since then the circulating H1N1 viruses contained glycosylation sites 71, 142 and 177 in the HA globular head. Since the glycosylation status of the pH1N1/09 is the same as that of the pH1N1/18, the pH1N1/09 virus has the potential to acquire the same or similar glycosylations over time as a result of immune selection pressure.

Table 4. Glycosylation models of HA monomers of natural H1N1 isolates between 1918 and 2012. Potential glycosylation sites (N-X-S/T motif) indicated with arrows and the number of the amino acid residue. The conserved glycosylation sites N27, N40 and N104 are present in all viruses. A/California/04/2009 was used as a model (PDB ID: 3LZG), the sequence was obtained from Influenza Research Database and images were created with PyMol Molecular Graphics System, version 1.1eval.



The antigenic HA1 globular head has acquired multiple different glycosylation sites (Table 4) along with antigenic drift, while the glycosylation status of the antigenically less prominent stem HA2 has remained highly conserved (Sun *et al.* 2011). These

specific HA globular head glycosylation sites seem to have been important for the evolution of influenza A viruses, which justifies further evaluation of their immunomodulatory role within the experimental part of this thesis.

5.2 Hemagglutinin expression levels of glycosylation mutant viruses are similar

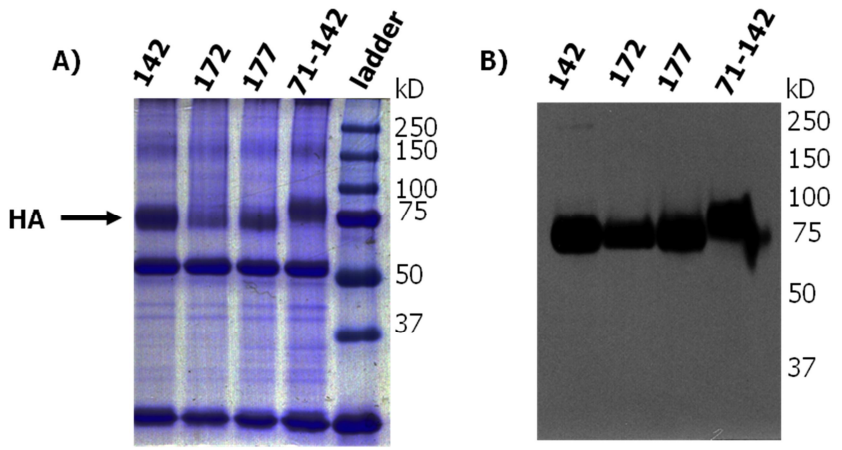


Figure 14. Hemagglutinin expression levels of recombinant viruses with single or double additional glycosylation sites. a) A Coomassie-stained SDS-PAGE (4% stacking gel and 10% resolving gel) of the glycosylation mutant viruses with the indicated HA glycosylation sites. b) Western blot of the glycosylation mutant viruses with the indicated HA glycosylation sites. Primary Ab: α-PR8 headless virus, secondary Ab: α-rabbit IgG-HRP conjugate, exposure time: 10 min.

Virus preparations had been made by propagating the rWT and glycosylation mutant viruses into high titres in MDCK cells. The supernatants were harvested and pelleted by 20% sucrose cushion ultracentrifugation (at Universidad de Santiago de Chile). The resulting virus pellets were resuspended in NP-40 containing Complete Mini protease inhibitor. The virus preparations were boiled for 5 min and analysed on two parallel SDS-PAGE electrophoresis gels, one of which was stained with Coomassie stain and the other of which was transferred into a Western blot membrane and developed on an X-ray film. Similar HA bands can be seen both on the Coomassie-stained (Figure 14: A) and the Western blot (Figure 14: B) for the studied glycosylation mutant viruses, although the band for the Neth/09-HA172 seems to be slightly weaker than for the other viruses. The results suggest that these glycosylations are utilised by the virus and that roughly similar amounts of HA are assembled into the virus particles.

5.3 Neth/09 glycosylation mutant viruses elicit equal IgG responses in mice

IgG antibody responses induced in mice infected with recombinant mutant viruses with 1−4 glycosylations were tested in an ELISA serology assay by using a Gt X Ms IgG γ chain-specific antibody. A 1:1 mixture of peroxidase substrate solutions A and B was

used for colorimetric visualisation of IgG presence in the antisera and the absorbance was measured at λ 405. The dilution factor represents the reciprocal of each mouse serum dilution used. It can be seen that the observed IgG response is very similar for all rWT, Neth/09-HA144, Neth/09-HA71-142-177 and Neth/09-HA71-142-172-177 viruses assessed in this study, although the Neth/09-HA71-142-177 seems to have slightly lower antigenicity in mice, as compared to the rWT (Figure 15).

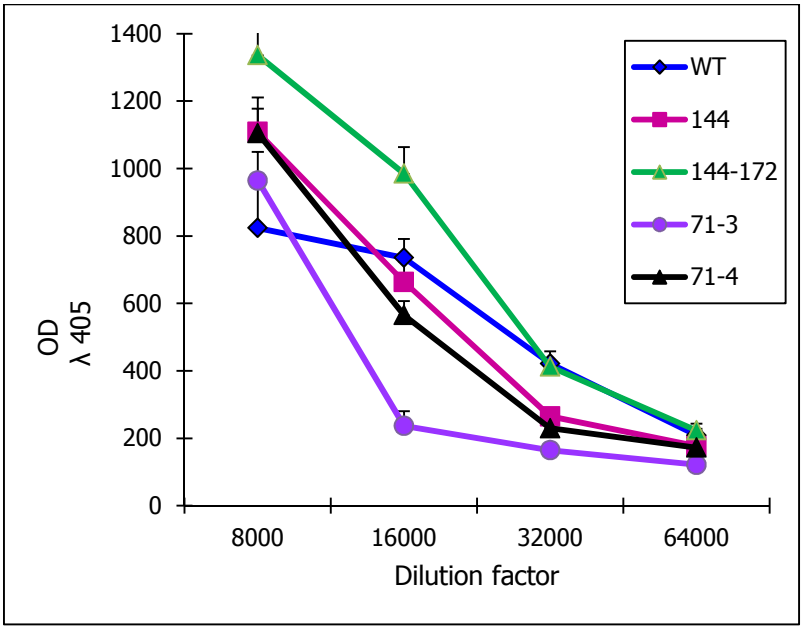


Figure 15. IgG antibody response against the glycosylation mutant viruses with 1–4 additional glycosylations. Mice had been infected with the recombinant Neth/09 viruses containing the indicated HA mutations; 71-3 = Neth/09-HA71-142-177 and 71-4 = Neth/09-HA71-142-172-177. Mouse antisera were tested at dilutions 1:2,000–1:64,000 with a 1:5,000 dilution of a Gt X Ms IgG γ chain-specific antibody.

5.4 Glycosylation site 144 in the pandemic H1N1 virus background broadens the humoral immune response in mice

The sera from mice and ferrets infected with rWT and recombinant mutant viruses with variable glycosylation levels were tested in HI assays (Chapter 4.10) against the 14 previous natural seasonal H1N1 isolates (Table 2) and each other for their hemagglutination inhibitory activity (HI titre). All viruses were propagated in MDCK cells to high titres (HAU ≥ 16), titrated by plaque assays and tested against trypsin-heat-periodate −treated mouse and ferret sera. Mouse antisera were pooled from multiple animals that had a homologous HI titre of 160, except for the highly immunogenic rWT virus that had constantly higher homologous titres (HI = 320) than the mutant viruses. The glycosylation mutant virus ferret antisera that were used for the assays were from a single selected animal that had a homologous serum HI titre of 320. All viruses were standardised by an HA assay (Chapter 4.9) at HAU = 8 prior to the HI assay. HI titre

was considered as the last serum dilution that completely inhibited hemagglutination of 0.5% turkey RBCs.

Table 5. HI titres of mouse and ferret sera after infection with rpH1N1/09 glycosylation mutant viruses against natural seasonal H1N1 isolates from 1930–2009. The HA1 glycosylation sites of the natural seasonal H1N1 isolates were predicted from the HA sequences (Influenza Research Database) based on the N-X-S/T motif. Mice and ferrets had been infected with the recombinant Neth/09 viruses containing the indicated HA mutation. HI titres represent the reciprocal of the highest dilution that completely inhibited hemagglutination of 0.5% turkey RBCs.

SEASONAL H1N1 VIRUSES		MOUSE SERUM		FERRET SERUM		
Isolate	HA1 glycosylation sites	rWT	144	rWT	144	144-172
A/Swine/Iowa/30	–	< 10	20	< 10	< 10	< 10
A/PR/8/34	144	< 10	< 10	< 10	< 10	< 10
A/Weiss/43	144, 179	< 10	10	< 10	< 10	< 10
A/FLW/52	144, 172, 177	< 10	< 10	< 10	< 10	< 10
A/Denver/57	172, 177	< 10	20	< 10	< 10	< 10
A/USSR/92/77	144, 172, 177	< 10	20	< 10	< 10	< 10
A/USSR/46/79	144, 172, 177	< 10	10	< 10	< 10	< 10
A/Finland/13/80	144, 172, 177	< 10	< 10	< 10	< 10	< 10
A/Houston/84	144, 172, 177	< 10	< 10	< 10	< 10	< 10
A/Texas/36/91	71, 142, 177	< 10	10	< 10	< 10	< 10
A/NewCal/20/99	71, 177	< 10	10	< 10	< 10	< 10
A/Brisbane/59/07	71, 142, 177	< 10	< 10	< 10	< 10	< 10
A/California/07/09	–	320	< 10	1,280	320	320
A/Netherlands/602/09	–	320	160	1,280	320	320

None of the previously produced mouse or ferret antisera showed cross-reactivity among the natural seasonal H1N1 isolates (Table 5) when assessed by two or more individual HI assays. When antisera from mice that had been infected with mutant viruses with a single additional glycosylation site were evaluated, only the Neth/09-HA144 was shown to have elicited broadly reactive antibodies that were capable of cross-reacting with all the rWT and glycosylation mutant viruses above homologous titres (Table 6). Conversely, all the rWT and glycosylation mutant virus antisera consistently lost their reactivity against the Neth/09-HA144 virus (Table 6). Infection with other recombinant mutant viruses with a single additional HA glycosylation site lead to narrowed cross-reactive responses in mice, as compared to their homologous titres (Table 6). The breadth of polyclonal response elicited in mice by recombinant mutant viruses with multiple (1−4) additional HA glycosylations was also evaluated. The Neth/09-HA144-172 was the only virus of the polyglycosylated mutant viruses that was shown to elicit polyclonal antibodies in mice, capable of cross-reacting with all the rWT and glycosylation mutation viruses (Table 7). Conversely, all rWT and

glycosylation mutant virus antisera consistently lost their reactivity against this virus. Other polyglycosylated viruses were not shown to broaden this response above homologous titres in this study (Table 7).

Table 6. HI titres of mouse sera after infection with rpH1N1/09 mutant viruses with a single additional HA glycosylation. Mice had been infected with recombinant Neth/09 viruses containing the indicated HA mutation. HI titres represent the reciprocal of the highest dilution that completely inhibited hemagglutination; homologous titres are in bold and shaded. The titres that are presented in ***italics*** were obtained in the previous study of Medina laboratory (Medina *et al.* accepted) but were all re-evaluated and verified during this project.

VIRUS	MOUSE SERUM				
	rWT	142	144	172	177
rWT	**320**	80	***320***	80	80
142	160	**160**	640	160	160
144	10	< 10	***160***	< 10	< 10
172	320	160	320	**320**	320
177	640	80	320	80	**80**
71-142	160	80	320	40	80
144-172	***20***	< 10	***80***	10	< 10
71-142-177	***80***	160	***160***	40	80
71-142-172-177	***160***	80	***160***	160	40

Table 7. HI titres of mouse sera after infection with rpH1N1/09 mutant viruses with multiple additional HA glycosylations. Mice had been infected with recombinant Neth/09 viruses containing the indicated HA mutations. HI titres represent the reciprocal of the highest dilution that completely inhibited hemagglutination; homologous titres are in bold and shaded. The titres that are presented in ***italics*** were obtained in the previous study of Medina laboratory (Medina *et al.* accepted) but were all re-evaluated and verified during this project.

VIRUS	MOUSE SERUM			
	71-142	144-172	71-142-177	71-142-172-177
rWT	80	***160***	***40***	***20***
142	160	640	320	320
144	< 10	***80***	***< 10***	***< 10***
172	160	1,280	160	80
177	80	320	20	40
71-142	**320**	640	160	320
144-172	< 10	***160***	***< 10***	***40***
71-142-177	320	***160***	***160***	***160***
71-142-172-177	80	***320***	***160***	***160***

Due to the broadened cross-reactivity observed in mice for the Neth/09-HA144 mutant virus, HI assays were extended into ferrets to further study the effect of incorporating additional glycosylation sites 144 and 144-172 into the pandemic Neth/09 virus background. Contrary to our expectations, antisera from ferrets infected with the Neth/09-HA144 or Neth/09-HA144-172 were shown to be incapable of cross-reacting with the rWT and a panel of recombinant mutant viruses with variable glycosylation levels (Table 8). In effect, these additional HA glycosylations were shown to narrow the

humoral immune response ≥ 2-fold lower than homologous levels for both assessed recombinant mutant viruses against a panel of mutant viruses with variable glycosylation levels (Table 8).

Table 8. HI titres of ferret sera after infection with pH1N1/09 glycosylation mutant viruses. Ferrets infected with recombinant pandemic Neth/09 viruses containing the indicated additional HA mutation. HI titres represent the reciprocal of the highest dilution that completely inhibited hemagglutination; homologous titres are in bold and shaded.

VIRUS	FERRET SERUM		
	rWT	144	144-172
rWT	**1280**	320	320
142	320	320	160
144	160	**640**	320
172	80	80	160
177	1280	320	160
71-142	80	80	80
144-172	20	20	**320**
71-142-177	320	160	320
71-142-172-177	40	20	160

5.5 rNeth/09 virus with 144 glycosylation elicits antibodies capable of neutralising the rWT virus in mice

Microneutralisation assays were performed on MDCK cells with mouse and ferret sera against the rWT and Neth/09-HA144 mutant viruses. $TCID_{50}$ of both viruses was determined prior to the microneutralisation assay by Reed-Muench method according to the WHO Manual (WHO Global Influenza Surveillance Network 2011). 100 x $TCID_{50}$ of both virus was tested against mouse and ferret sera at 2-fold serial serum dilutions (1:10−1:10,240). Infections were detected by incubation with a rabbit polyclonal α-NP antibody (1:5,000) and a secondary α-rabbit-HRP conjugate antibody (1:10,000). Positive wells were visualized with a HRP substrate at λ 450 nm. Neutralisation titres are represented as the reciprocal of the geometrical mean of the highest serum dilution that displayed neutralisation activity. Infection with the Neth/09-HA144 was shown to neutralise both rWT and Neth/09-HA144 viruses and the rWT antiserum was shown to have remarkably lost its reactivity against the Neth/09-HA144 virus (Table 9). However, in ferrets the infection with Neth/09-HA144 lead to 4-fold loss in neutralisation activity against the rWT virus and the rWT antiserum retained its reactivity against both rWT and Neth/09-HA144 viruses (Table 9).

Table 9. Microneutralisation assay of rWT Neth/09 and Neth/09-HA144 viruses. Titres are represented as geometrical mean of the highest serum dilution that was able to neutralise infection. Neutralisation titres of the ferret sera were obtained within this project and titres of the mouse sera were obtained subsequently by the Medina laboratory (Medina *et al*. accepted).

	MOUSE SERUM		**FERRET SERUM**	
VIRUS	**rWT**	**144**	**rWT**	**144**
rWT	**320**	320	**1280**	640
144	80	480	1280	**2560**

6 Discussion

N-linked glycosylations of the HA surface glycoprotein are believed to have an important role in altering the antigenicity, virulence and receptor-binding properties of influenza A viruses. The significance of these oligosaccharides has been studied in various recent studies but the detailed molecular determinants of the antigenicity remain unrevealed. Within this Master's thesis project the HA1 protein domains of the natural seasonal H1N1 isolates from years 1930−2012 were analysed by commonly used sequence alignment tools. This revealed that N-linked glycosylations 71, 142, 144, 172, 177 and 179 in the HA globular head are naturally occurring in the H1N1 isolates (Figure 13). Based on their constant appearance near or within the major HA antigenic regions − Sa, Sb, Cb, Ca1 and Ca2 − it was suggested that these post-translational modifications are likely to have an important role in antigenic evolution of H1N1 influenza A viruses. Their significance for influenza A antigenicity and impact on the breadth of humoral immune response was assessed by virological and serological *in vitro* methods.

Mice, cotton rats, hamsters, macaques, ferrets and guinea pigs have been widely used as model animals to study influenza virus immunogenicity, virulence and therapeutical applications [reviewed in (Bouvier & Lowen 2010)]. Mice and ferrets are the most common models and are also utilised by the Medina laboratory. The seasonal glycosylated H1N1 viruses need to be laboratory-adapted and do not generally produce human-like disease in mice, which is in strong contrast with the pH1N1/18 and pH1N1/09 strains, certain subtype H7 viruses and the highly pathogenic avian influenza H5N1 viruses that do not require adaptation and are lethal at low doses. In mice influenza viruses replicate to high titres, causing tissue damage in the LRT and lethargy that can lead to hypothermia. Ferrets are highly susceptible to the seasonal human H1N1 viruses that primarily replicate to high titres in the URT but not in the LRT. Ferrets get human-like illness that involves sneezing, nasal discharge, coughing, lethargy, anorexia and fever and are therefore considered to represent human illness most accurately. Since they are bigger animals than rodents, larger volumes of post-infection serum can be obtained without the need to sacrifice the animal. These characteristics justify the use of these model animals in this study. The use of animal models to study influenza pathogenicity and transmission is reviewed in (Bouvier & Lowen 2010).

A recent study by Medina and colleagues demonstrated that HA globular head glycosylations play an important role in modulating the pathogenesis and the antigenic properties of the pH1N1/09 virus (Medina *et al.* accepted). Recombinant pandemic H1N1 A/Netherlands/602/09 (rWT Neth/09) viruses were generated by incorporation of multiple additional HA globular head glycosylations to mimic their temporal appearance in the natural seasonal H1N1 isolates. The addition of multiple glycosylations on the non-glycosylated HA globular head of the rWT Neth/09 was shown to attenuate the virulence of this virus *in vivo* but not *in vitro*. It was shown that each virus had a reduced amount of HA incorporated into the virions compared to the rWT. However, additional glycosylations did not seem to affect infectivity since all viruses replicated at titres equal to the original WT Neth/09 isolate (> 10^7 pfu/ml) in human tracheobronchal epithelial (HTBE) cells. Glycosylation-dependent decrease in pathogenicity and severe attenuation was observed in mice and ferrets for all viruses with two or more additional glycosylations, as observed by weight loss. However, the Neth/09-HA144 virus was shown to have weight loss patterns similar to the rWT in mice, which indicates that this glycosylation does not seem to impair the virulence of the pH1N1/09 virus.

In the above mentioned study (Medina *et al.* accepted) by the Medina laboratory, incorporation of an additional glycosylation site at residue 144 alone (Table 6 of this thesis) or together with glycosylation at site 172 (Table 7 of this thesis) in the non-glycosylated rWT pH1N1/09 virus background was shown to broaden the humoral immune response in mice and showed improved cross-reactivity against the rWT and a panel of recombinant mutant Neth/09 viruses with multiple glycosylations. Conversely, the rWT mouse antiserum remarkably lost its reactivity against both viruses containing the 144 glycosylation as compared to its homologous titre, while cross-protection was retained against other glycosylation mutant viruses. The impact of this glycosylation on the antigenicity of the rWT virus had been further assessed in microneutralisation assays. Glycosylation site 144 in the pandemic H1N1 background was shown to induce polyclonal responses capable of neutralising both the rWT and 144-glycosylated virus at similar levels, whereas the rWT antiserum lost its reactivity against the 144-glycosylated virus to 4-fold lower than homologous titres. These findings suggest that the site 144 masks an important antigenic epitope, which redirects the polyclonal response to other sites within or around the antigenic site Sa.

In the same study by Medina and colleagues the significance of HA globular head glycosylations was demonstrated by modifying a recent seasonal H1N1 strain A/Texas/36/91 (Tx/91) that has three naturally occurring HA1 glycosylation sites − 71, 142 and 177. The deletion of all these glycosylation sites from this strain lead to production of antibodies that protected from the antigenically distant rWT Neth/09 and resulted in increased pathogenicity (as measured by weight loss) in mice, compared to the rWT Tx/91. The highest morbidity was observed for the Δ71-142-177 virus that resembles the rWT Neth/09 in that both have a non-glycosylated HA head, which demonstrates that HA glycosylations negatively affect the pathogenicity of human H1N1 viruses. The potential of these glycosylation deletion viruses to elicit cross-reactive antibodies against the rWT Neth/09 was also assessed in mice. Mice were first infected with the rWT Tx/91 or one of the glycosylation deletion Tx/91 viruses (Δ71, Δ71-177 or Δ71-142-177) and then challenged at 27 days p.i. with 100 x LD_{50} of rWT Neth/09 virus. Mice initially infected with the Tx/91 Δ71 had a survival rate of 20%, whereas mice infected with Tx/91 Δ71-177 or Δ71-142-177 all survived but suffered a weight loss of up to ~20%. The mice initially infected with the Δ71-142-177 showed overall lowest morbidity level after rWT Neth/09 challenge. These observations suggest that HA glycosylations mask the antigenic sites and that the equal glycosylation status is responsible for the cross-reactive antibodies elicited by these antigenically distinct viruses.

The cross-reactivity of antisera from humans vaccinated with the monovalent inactivated pH1N1/09 vaccine against a panel of glycosylation viruses was assessed in a vaccine study by the same research group described above (Medina *et al.* accepted). The authors report that most of the paediatric (< 18 years of age) and adult individuals had detectable antibody levels against the mutant viruses but the lowest HI titre was observed against the viruses with 144 and 144-172 glycosylations. This observation confirms the results obtained from the mouse cross-reactivity studies and verifies the immunomodulatory masking effect of the additional glycosylation site 144. It was also seen that the antisera from patients vaccinated with the trivalent seasonal H1N1/09 vaccine did not have cross-reactivity against the glycosylated mutant viruses, independent on their glycosylation status. This was expected since that vaccine composition contained a previously circulating seasonal H1N1 strain.

Previous studies have demonstrated the glycosylated viruses to be more sensitive to innate immune activity of collectins present in lung fluid but to retain their ability to

replicate efficiently in mouse airway macrophages and in MDCKs *in vitro* (Vigerust & Shepherd 2007, Tate *et al.* 2011). The number and position of glycosylation sites plays an important role in attenuation (Hillaire *et al.* 2012), which was also demonstrated in the study by Medina and colleagues since some mutations resulted in attenuated phenotype (eg. sites 142 & 172) while others did not (site 177) (Medina *et al*. accepted). When naïve ferrets were infected with rWT and 144-172 viruses, differences in viral load were observed in lungs and trachea of the 144-172 infected animals but not in nasal turbinates. The glycosylated H1N1 viruses were able to replicate in URT (nose) at similar levels as the rWT but low viral titres were observed in LRT (trachea and lungs). These results suggest that selection of glycosylated influenza viruses may be affected by both pre-existing nAbs and the activity of collectins. However, the outcome of influenza infection seems to be further complicated by the presence of numerous other factors, whose role is to be verified by further *in vivo* assessments in both influenza animal models. The interactions between these factors and N-linked glycans on the surface of HA protein are likely to function in complex, yet fully uncovered co-operation.

This Master's thesis project continues from the results obtained in the Medina laboratory (summarised ahead in this chapter) and aims at further assessing the impact of the naturally occurring glycosylations by virological and serological *in vitro* methods. Along with this project, Medina laboratory was relocated from New York (USA) to Santiago (Chile) and therefore all methods, cell culture and assays were simultaneously set up, revised and optimised. Initially, we proposed to engineer five glycosylation mutant viruses Neth/09-HA71, Neth/09-HA142, Neth/09-HA172, Neth/09-HA177 and Neth/09-HA179 by reverse genetics methods and to infect mice with these viruses during this project to obtain glycosylation mutant virus-specific antisera to be used in further cross-reactivity studies. However, due to challenges with cell contamination and method optimisation, these aims could not be completed within the timeline of this project. However, rescue of two glycosylation mutant viruses, Neth/09-HA142 and Neth/09-HA179, was attempted and preliminary analyses performed outside of this project suggest these rescues to have been successful. Fortunately, all animal sera, the rWT virus and glycosylation mutant viruses had already been previously produced by the Medina laboratory and were very kindly provided for the assays of this thesis.

Cross-reactivity assays with the rWT mouse and ferret sera, Neth/09-HA144 mouse and ferret sera and Neth/09-HA144-172 ferret serum were conducted against the 14 natural seasonal H1N1 isolates by HI assays. Each glycosylation mutant serum showed poor cross-reactivity against the tested strains with no major differences among the isolates (Table 5). The results suggest that the poor cross-reactivity is likely due to the major amino acid changes that occurred during the evolution of H1N1 viruses around and outside the antigenic regions, rather than due to the glycosylation status of the HA globular head. Therefore the level of cross-reactivity observed between any two strains is most likely to be influenced by the overall similarity of their HA amino acid sequence and not only the level of HA glycosylations. However, these titres were obtained from single experiments and HI assay only reveals the presence of serotype-specific nAbs in a serum but fails to give information on their ability to prevent an infection *in vitro*. Further microneutralisation assays will be needed to retrieve information on the neutralisation ability of the anti-rWT, anti-Neth/09-HA144 and anti-Neth/09-HA144-172 antisera against the natural seasonal H1N1 isolates.

Due to the close proximity of site 142 to site 144, we were also interested in assessing whether an additional glycosylation at this site in the non-glycosylated rpH1N1/09 background has a similar effect on cross-reactivity as does glycosylation at residue 144. Interestingly, incorporation of an additional glycosylation at site 142 did not lead to similar broadened cross-reactivity that has been observed for site 144 with mouse antiserum (Table 6), despite of its nearby location within the antigenic site Sa. Surprisingly, the Neth/09-HA144-172 had 4-fold increased cross-reactivity against the Neth/09-HA172 virus (Table 7), which suggests that additional glycosylation site 172 might be responsible for the broadened reactivity. However, addition of glycosylation site 172 alone (Table 6) or in the context of the Neth/09-HA71-142-172-177 virus (Table 7) did not increase cross-reactivity above homologous titres. This confirms that glycosylation site 172 alone is not likely to be responsible for the broadened cross-reactivity observed for the Neth/09-HA144-172 virus but seems to require co-operation with glycosylation site 144 to acquire this feature.

Interestingly, both mouse (Table 6) and ferret rWT antisera (Table 8) gave a very high HI titre against the Neth/09-HA177 virus, which indicates strong antigenic similarity between rWT and Neth/09-HA177. Consequently, this glycosylation site does not seem to shield any major antigenic epitopes from antibody recognition but may actually

improve the accessibility of the epitopes by unmasking the antigenic site Sa. In the future it would be interesting to further evaluate the impact of glycosylation site 177 and the neighbouring site 179 on the breadth of humoral response in mice and ferrets. Based on the observations with the Neth/09 glycosylation mutant virus antisera, the single additional HA glycosylations, with the exception of site 144, all seem to narrow the polyclonal response (Table 6) by slightly altering the focus of antigen recognition possibly around site Sa. Of the potential glycosylation sites examined, amino acid residue 144 seems to be the only site where additional N-linked glycosylation broadens the cross-reactivity of the antibodies elicited against the HA globular head.

Surprisingly, assays with the ferret antisera failed to confirm the results obtained with the mouse antisera. Glycosylation at site 144 alone in the non-glycosylated Neth/09 background did not seem to increase the cross-reactivity above homologous titres against any of the glycosylation mutant viruses in ferrets (Table 8). However, the HI titre of the 144-172 glycosylated virus antiserum was equal to its homologous titre against rWT, Neth/09-HA144 and Neth/09-HA71-142-177. Both rWT and Neth/09-HA144 ferret antisera remarkably lost their reactivity against the polyglycosylated viruses Neth/09-HA71-142, Neth/09-HA144-172 and Neth/09-HA71-142-172-177 viruses. This indicates that in ferrets the antibodies elicited against rWT and 144 viruses are possibly directed more broadly around the antigenic determinants of the HA globular head and that these multiple glycosylations remarkably distract epitope recognition by HA-specific antibodies. However, cross-reactivity was sustained against the Neth/09-HA71-142-177, which excludes the possibility of positive correlation between the increasing number of glycosylation sites and the observed loss in cross-reactivity. The homologous titres of ferret antisera were constantly higher (HI = 320−1,280) than the titres of mouse antisera (HI = 80−320), which indicates improved immunogenicity of these viruses in ferrets compared to mice. Again, these results were largely obtained from few cross-reactivity experiments and therefore more studies are to be performed with ferret antisera to verify the observations.

We were also interested in evaluating the level of IgG antibodies in mice against mutant viruses with multiple additional glycosylations. IgG levels were measured from mouse antisera by serology ELISA to assess the level of humoral response induced by the recombinant glycosylation mutant viruses. The IgG antibody levels were observed to be similar to rWT levels within the panel of viruses with 1−4 additional HA glycosylations, the Neth/09-HA71-142-177 virus being the least antigenic of the tested

viruses (Figure 15). It was confirmed that the concentration of IgG antibodies was independent on the variable cross-reactivity levels observed in the HI assays, which excludes the possibility of the broadened cross-reactivity observed for the Neth/09-HA144 virus being due to its improved ability to induce IgG antibodies in mice. Evaluation of the HA expression levels in recombinant mutant viruses with 1−2 additional HA glycosylations also confirmed that this protein is expressed at equal levels in mice, as determined from whole cell lysates by SDS-PAGE and Western blot analyses (Figure 14).

It has been shown recently that ELISA-based microneutralisation assay is preferable to the standard HI assay for evaluation of influenza vaccine efficiency. It gives more relative information on the neutralising ability of an antiserum, whereas HI titres may arise from cross-reactive antibodies and therefore detect higher but non-specific antibody titres (Cheng *et al.* 2012). Within this thesis project, the inhibitory role of serotype-specific polyclonal serum antibodies in HA binding to its SA receptor on turkey RBCs was assessed by HI assays. Additionally, microneutralisation assays were utilised to obtain further information on the inhibitory activity of mouse and ferret serum nAbs against MDCK infection by Neth/09 glycosylation mutant viruses.

Due to the limited timeline allowed for this project, microneutralisation assays were successfully performed solely for ferret rWT and Neth/09-HA144 antisera against their homologous viruses and each other. These tests showed that rWT antisera was capable of neutralising Neth/09-HA144 infection at equal to its homologous titre, while the Neth/09-HA144 antisera very surprisingly showed 4-fold decreased neutralisation of the rWT infection compared to its homologous titre (Table 9). However, these ferret results are in strong contradiction with the results obtained subsequently with mouse antisera. In the microneutralisation assays subsequently performed by the Medina laboratory following this Master's thesis project, Neth/09-HA144 was shown to neutralise both the rWT and the Neth/09-HA144 at similar levels, whereas the rWT antiserum lost its reactivity against the Neth/09-HA144 to 4-fold lower than homologous titres (Table 9). No valid conclusions can be drawn from the ferret data, since the results were obtained from single experiments. Further re-evaluations are warranted to exclude the possibility of species-specific antigenicity and to standardise the ferret data with the mouse data.

Much remains to be resolved about the 3D structure and orientation of the N-linked oligosaccharides. It is yet unknown how a glycan at site 144 changes the spatial

conformation of the antigenic site Sa and succeeds in modifying antibody recognition and/or binding. This may be due to cooperative action of steric hindrance and electrostatic repulsion or even more complex actions that involve additional changes in the structure of HA. The results obtained in this and previous studies suggest that glycosylation at site 144 is more effective at blocking the antigenic epitopes than the other single HA glycosylations, which could explain the observed alterations in the breadth of humoral response in mice. In a previous study the rescue of a mutant Neth/09 virus carrying three additional glycosylation sites 144, 172 and 177 was not successful despite of several attempts, which suggests that this combination requires additional compensatory mutations (Medina *et al.* accepted). All other viruses were successfully rescued, although the virus with four additional glycosylations (Neth/09-HA71-142-172-177) was shown to have a smaller plaque phenotype in MDCKs. Additionally, it is possible that the addition of glycosylation sites induces accessory mutations in other regions apart from the modified site that contribute to the overall viral antigenicity during multiple passages in mammalian cells.

7 Conclusions

7.1 Findings of the project

It was demonstrated that the HA globular head of the natural seasonal H1N1 isolates acquired additional N-linked glycosylations (71, 142, 144, 172 and 179) over time, until the emergence of the non-glycosylated 2009 pandemic H1N1 strain. This suggests that the HA glycosylations take part in antigenic drift and thus have an essential role in influenza antigenic evolution. Based on previous evidence, it was proposed that glycosylation at site 144 masks the antigenic site Sa from antibody recognition and therefore re-directs the focus of immune response to compensatory sites in the HA molecule. This seems to be true for the mouse sera since all the tested WT and mutant sera lost their reactivity against the Neth/09-HA144 mutant virus in the cross-reactivity assays. Bulky oligosaccharide moieties at site 144 presumably block an essential antigenic epitope, which leads to deficient recognition of this virus by antibodies that were raised against HA molecules lacking the glycosylation site 144. Consistent with the previous observations from the HI assays, microneutralisation assays and human vaccination studies (Medina *et al.* accepted), the importance of site Sa modifications, especially the role of 144 glycosylation, for viral antigenicity was confirmed in mice.

However, the cross-protective potential of the Neth/09-HA144 ferret antiserum was not verified. Further studies are warranted to draw better conclusions towards the impact of this glycosylation site on viral antigenicity in this animal model and to exclude the possibility of species-specificity. It is also important to emphasize that most of the HI titres that were obtained in this study with ferret antisera were from single experiments and therefore re-evaluation is required to confirm these preliminary data. This study is solely focused on assessing the breadth of polyclonal response elicited by the recombinant mutant Neth/09 viruses in mice and ferrets. Assays on neither the detailed 3D molecular structure nor the effect of additional N-linked glycosylations on viral functionality were performed within this project. Therefore further evaluation is recommended to establish the overall significance of additional glycosylations in these animal models and eventually in humans.

7.2 Future prospects

Influenza viruses infect millions of people worldwide each year and lead to huge economic losses due to frequent sick leaves from work, school and day care. Infection can be fatal in paediatric patients and the elderly and therefore prevention by vaccination is the first line defence mechanism. In addition to the seasonal outbreaks,

influenza viruses develop novel variants with pandemic potential with an interval of a few decades. The timing of the new pandemics cannot be predicted, which emphasises the need for constant alertness and preparedness for a new worldwide influenza epidemic. There is thrive for new vaccine applications that would allow fast, easy, safe and economical manufacture of broadly reactive vaccines that would provide effective protection against multiple influenza subtypes from various clades. In the future mammalian, insect and bacterial cell –based platforms will most likely take a stable place in the vaccine production industry and start to replace conventional embryonated egg –based vaccines.

The role of each single HA globular head glycosylation on the level and specificity of the humoral immune responses remains to be specifically determined. Knowledge on the induction of polyclonal response mechanisms at the molecular level would provide important clues to future vaccine design in means of a broadly neutralising universal vaccine. The drawback of the current seasonal influenza vaccines is that they only provide protection against known seasonal isolates but fail to neutralise the arising variants with an altered antigenic profile due to delay in the production circle. Engineering HA-glycosylated recombinant viruses with an ability to elicit broad polyclonal response in humans provides a novel therapeutic approach for further development, which is also the holistic motive behind this Master's thesis project. Current hopes and expectations are on new generation subunit and DNA vaccines as well as VLP-based vaccines that would elicit broadly cross-reactive specific humoral immune response in the vaccinated individuals. nAbs directed against the more conserved regions of the influenza virus would potentially provide protection from the yearly drifted variants, which would eliminate the need of vaccinating the population prior to each influenza season and to provide longer lasting, strong cellular and humoral immunity in all age groups.

8 References

Air GM. Sequence relationships among the hemagglutinin genes of 12 subtypes of influenza A virus. *Proc Natl Acad Sci* U S A 1981;78: 7639-7643.

An Y, Cipollo JF. An unbiased approach for analysis of protein glycosylation and application to influenza vaccine hemagglutinin. *Anal Biochem* 2011;415: 67-80.

Anders EM, Hartley CA, Reading PC, Ezekowitz RAB. Complement-dependent neutralization of influenza virus by a serum mannose-binding lectin. *J Gen Virol* 1994;75: 615-622.

Apweiler R, Hermjakob H, Sharon N. On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochimica et Biophysica Acta - General Subjects* 1999;1473: 4-8.

Athmaram TN, Saraswat S, Santhosh SR, Singh A, Suryanarayana V, Priya R, et al. Yeast expressed recombinant Hemagglutinin protein of Novel H1N1 elicits neutralising antibodies in rabbits and mice. *Virology Journal* 2011;8.

Aytay S, Schulze IT. Single amino acid substitutions in the hemagglutinin can alter the host range and receptor binding properties of H1 strains of influenza A virus. *J Virol* 1991;65: 3022-3028.

Basler CF, Reid AH, Dybing JK, Janczewski TA, Fanning TG, Zheng H, et al. Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. *Proc Natl Acad Sci* U S A 2001;98: 2746-2751.

Bause E. Structural requirements of N-glycosylation of proteins. Studieswith proline peptides as conformational probes. *Biochem J* 1983;209: 331-336.

Bean B, Moore BM, Sterner B. Survival of influenza viruses on environmental surfaces. *J Infect Dis* 1982;146: 47-51.

Belshe RB, Ambrose CS, Yi T. Safety and efficacy of live attenuated influenza vaccine in children 2-7 years of age. *Vaccine* 2008;26: D10-D16.

Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, Piedra P, et al. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N Engl J Med* 1998;338: 1405-1412.

Bernstein HB, Tucker SP, Hunter E, Schutzbach JS, Compans RW. Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. *J Virol* 1994;68: 463-468.

Biller M, Mårdberg K, Hassan H, Clausen H, Bolmstedt A, Bergström T, et al. Early steps in O-linked glycosylation and clustered O-linked glycans of herpes simplex virus type 1 glycoprotein C: Effects on glycoprotein properties. *Glycobiology* 2000;10: 1259-1269.

Block SL, Yogev R, Hayden FG, Ambrose CS, Zeng W, Walker RE. Shedding and immunogenicity of live attenuated influenza vaccine virus in subjects 5-49 years of age. *Vaccine* 2008;26: 4940-4946.

Bouvier NM, Lowen AC. Animal models for influenza virus pathogenesis and transmission. *Viruses* 2010;2: 1530-1563.

Bouvier NM, Palese P. The biology of influenza viruses. *Vaccine* 2008;26: D49-D53.

Brankston G, Gitterman L, Hirji Z, Lemieux C, Gardam M. Transmission of influenza A in human beings. *Lancet Infectious Diseases* 2007;7: 257-265.

Bretthauer RK. Genetic engineering of Pichia pastoris to humanize N-glycosylation of proteins. *Trends Biotechnol* 2003;21: 459-462.

Bright RA, Carter DM, Daniluk S, Toapanta FR, Ahmad A, Gavrilov V, et al. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine* 2007;25: 3871-3878.

Brockhausen I, Schachter H, Stanley P. O-GalNAc Glycans. In: Varki A, Cummings RD, Esko JD, et al., editor. *Essentials of Glycobiology*. 2nd Edition ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. p. Chapter 9.

Brundage JF. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. *Lancet Infectious Diseases* 2006;6: 303-312.

Carcione D, Giele C, Dowse GK, Mak DB, Goggin L, Kwan K, et al. Comparison of pandemic (H1N1) 2009 and seasonal influenza, Western Australia, 2009. *Emerging Infectious Diseases* 2010;16: 1388-1395.

Caton AJ, Brownlee GG, Yewdell JW, Gerhard W. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* 1982;31: 417-427.

CDC. Intensive-care patients with severe novel influenza a (H1N1) virus infection - Michigan, June 2009. *Morb Mortal Weekly Rep* 2009;58: 749-752.

Chackerian B, Rudensey LM, Overbaugh J. Specific N-linked and O-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. *J Virol* 1997;71: 7719-7727.

Chen J-, Ma C, Wong C-. Vaccine design of hemagglutinin glycoprotein against influenza. *Trends Biotechnol* 2011;29: 426-434.

Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, et al. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 2001;7: 1306-1312.

Cheng L-, Huang S-, Huang L-, Chang L-, Shao P-, Kiang D, et al. Comparison of neutralizing and hemagglutination-inhibiting antibody responses for evaluating the seasonal influenza vaccine. *J Virol Methods* 2012;182: 43-49.

Choi B-, Bobrowicz P, Davidson RC, Hamilton SR, Kung DH, Li H, et al. Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast Pichia pastoris. *Proc Natl Acad Sci U S A* 2003;100: 5022-5027.

Chutinimitkul S, Herfst S, Steel J, Lowen AC, Ye J, Van Riel D, et al. Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. *J Virol* 2010;84: 11802-11813.

Cillóniz C, Shinya K, Peng X, Korth MJ, Proll SC, Aicher LD, et al. Lethal influenza virus infection in macaques is associated with early dysregulation of inflammatory related genes. *PLoS pathogens* 2009;5.

Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. *J Clin Microbiol* 1986;24: 157-160.

Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic site in influenza virus neuraminidase. *Nature* 1983;303: 41-44.

Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature* 2003;422: 37-44.

Connor RJ, Kawaoka Y, Webster RG, Paulson JC. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology* 1994;205: 17-23.

Couch RB, Kasel JA. Immunity to influenza in man. *Annu Rev Microbiol* 1983;37: 529-549.

Cox MM, Karl Anderson D. Production of a novel influenza vaccine using insect cells: protection against drifted strains. *Influenza and other respiratory viruses* 2007;1: 35-40.

Cox MMJ, Hollister JR. FluBlok, a next generation influenza vaccine manufactured in insect cells. *Biologicals* 2009;37: 182-189.

Cox MMJ, Patriarca PA, Treanor J. FluBlok, a recombinant hemagglutinin influenza vaccine. *Influenza and other Respiratory* Viruses 2008;2: 201-209.

Creanga AA, Johnson TF, Graitcer SB, Hartman LK, Al-Samarrai T, Schwarz AG, et al. Severity of 2009 pandemic influenza a (H1N1) virus infection in pregnant women. *Obstet Gynecol* 2010;115: 717-726.

Das SR, Hensley SE, David A, Schmidt L, Gibbs JS, Puigbò P, et al. Fitness costs limit influenza A virus hemagglutinin glycosylation as an immune evasion strategy. *Proc Natl Acad Sci U S A* 2011;108: E1417-E1422.

Das SR, Puigbo P, Hensley SE, Hurt DE, Bennink JR, Yewdell JW. Glycosylation focuses sequence variation in the influenza a virus H1 hemagglutinin globular domain. *PLoS Pathogens* 2010;6.

Davenport FM, Hennessy AV, Francis Jr. T. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. *J Exp Med* 1953;98: 641-656.

Dawood FS, Iuliano AD, Reed C, Meltzer MI, Shay DK, Cheng P-, et al. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: A modelling study. *The Lancet Infectious Diseases* 2012;12: 687-695.

Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009;360: 2605-2615.

De Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJD, Chau TNB, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med* 2006;12: 1203-1207.

de Vries RP, de Vries E, Bosch BJ, de Groot RJ, Rottier PJM, de Haan CAM. The influenza A virus hemagglutinin glycosylation state affects receptor-binding specificity. *Virology* 2010;403: 17-25.

de Wit E, Spronken MIJ, Vervaet G, Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM. A reverse-genetics system for Influenza A virus using T7 RNA polymerase. *J Gen Virol* 2007;88: 1281-1287.

Dormitzer PR, Galli G, Castellino F, Golding H, Khurana S, Del Giudice G, et al. Influenza vaccine immunology. *Immunol Rev* 2011;239: 167-177.

Ekiert DC, Bhabha G, Elsliger M-, Friesen RHE, Jongeneelen M, Throsby M, et al. Antibody recognition of a highly conserved influenza virus epitope. *Science* 2009;324: 246-251.

Enami M, Luytjes W, Krystal M, Palese P. Introduction of site-specific mutations into the genome of influenza virus. *Proc Natl Acad Sci U S A* 1990;87: 3802-3805.

Fernandez-Sesma A, Marukian S, Ebersole BJ, Kaminski D, Park M-, Yuen T, et al. Influenza virus evades innate and adaptive immunity via the NS1 protein. *J Virol* 2006;80: 6295-6304.

Finelli L, Fiore A, Dhara R, Brammer L, Shay DK, Kamimoto L, et al. Influenza-associated pediatric mortality in the united states: Increase of Staphylococcus aureus coinfection. *Pediatrics* 2008;122: 805-811.

Fireman B, Lee J, Lewis N, Bembom O, Van Der Laan M, Baxter R. Influenza vaccination and mortality: Differentiating vaccine effects from bias. *Am J Epidemiol* 2009;170: 650-656.

Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, García-Sastre A. Rescue of influenza A virus from recombinant DNA. *J Virol* 1999;73: 9679-9682.

Fouchier RAM, Osterhaus ADME, Brown IH. Animal influenza virus surveillance. *Vaccine* 2003;21: 1754-1757.

Francis T, Jr. Influenza: the new acquaintance. *Ann Intern Med* 1953;39: 203-221.

Furuya Y, Regner M, Lobigs M, Koskinen A, Müllbacher A, Alsharifi M. Effect of inactivation method on the cross-protective immunity induced by whole 'killed' influenza A viruses and commercial vaccine preparations. *J Gen Virol* 2010;91: 1450-1460.

Gack MU, Albrecht RA, Urano T, Inn K-, Huang I-, Carnero E, et al. Influenza A Virus NS1 Targets the Ubiquitin Ligase TRIM25 to Evade Recognition by the Host Viral RNA Sensor RIG-I. *Cell Host and Microbe* 2009;5: 439-449.

Gambaryan AS, Karasin AI, Tuzikov AB, Chinarev AA, Pazynina GV, Bovin NV, et al. Receptor-binding properties of swine influenza viruses isolated and propagated in MDCK cells. *Virus Res* 2005;114: 15-22.

García-Sastre A. Negative-strand RNA viruses: Applications to biotechnology. *Trends Biotechnol* 1998;16: 230-235.

Garcia-Sastre A, Palese P. Influenza virus vectors. *Biologicals* 1995;23: 171-178.

Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 2009;325: 197-201.

Gerdil C. The annual production cycle for influenza vaccine. *Vaccine* 2003;21: 1776-1779.

Gerngross TU. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat Biotechnol* 2004;22: 1409-1414.

Gibbs JS, Malide D, Hornung F, Bennink JR, Yewdell JW. The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. *J Virol* 2003;77: 7214-7224.

Glaser L, Stevens J, Zamarin D, Wilson IA, García-Sastre A, Tumpey TM, et al. A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. *J Virol* 2005;79: 11533-11536.

Govorkova EA, Murti G, Meignier B, De Taisne C, Webster RG. African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. *J Virol* 1996;70: 5519-5524.

Graham MB, Braciale TJ. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J Exp Med* 1997;186: 2063-2068.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 1999;41: 95-98.

Hamilton SR, Bobrowicz P, Bobrowicz B, Davidson RC, Li H, Mitchell T, et al. Production of complex human glycoproteins in yeast. *Science* 2003;301: 1244-1246.

Han T, Marasco WA. Structural basis of influenza virus neutralization. *Annals of the New York Academy of Sciences* 2011;1217: 178-190.

Hancock K, Veguilla V, Lu X, Zhong W, Butler EN, Sun H, et al. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. *N Engl J Med* 2009;361: 1945-1952.

Hanshaoworakul W, Simmerman JM, Narueponjirakul U, Sanasuttipun W, Shinde V, Kaewchana S, et al. Severe human influenza infections in Thailand: Oseltamivir treatment and risk factors for fatal outcome. *PLoS ONE* 2009;4.

Hartshorn KL, Crouch EC, White MR, Eggleton P, Tauber AI, Chang D, et al. Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. *J Clin Invest* 1994;94: 311-319.

Hartshorn KL, Reid KBM, White MR, Jensenius JC, Morris SM, Tauber AI, et al. Neutrophil deactivation by influenza A viruses: Mechanisms of protection after viral opsonization with collectins and hemagglutination-inhibiting antibodies. *Blood* 1996;87: 3450-3461.

Hartshorn KL, White MR, Shepherd V, Reid K, Jensenius JG, Crouch EC. Mechanisms of anti-influenza activity of surfactant proteins A and D: Comparison with serum collectins. *American Journal of Physiology - Lung Cellular and Molecular Physiology* 1997;273: L1156-L1166.

Hayden FG, Osterhaus ADME, Treanor JJ, Fleming DM, Aoki FY, Nicholson KG, et al. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenzavirus infections. *N Engl J Med* 1997;337: 874-880.

Hensley SE, Das SR, Bailey AL, Schmidt LM, Hickman HD, Jayaraman A, et al. Hemagglutinin receptor binding avidity drives influenza a virus antigenic drift. *Science* 2009;326: 734-736.

Hidari K, Suzuki T. Glycan Receptor for Influenza Virus. *The Open Antimicrobial Agents Journal* 2010;2: 26-33.

Hillaire MLB, van Eijk M, Nieuwkoop NJ, Vogelzang-van Trierum SE, Fouchier RAM, Osterhaus ADME, et al. The number and position of N-linked glycosylation sites in the hemagglutinin determine differential recognition of seasonal and 2009 pandemic H1N1 influenza virus by porcine surfactant protein D. *Virus Res* 2012;169: 301-305.

Hirst GK. Adsorption of influenza virus hemagglutinins and virus by red blood cells. *J Exp Med* 1942;76, 2: 195-209.

Hirst G. The quantitative determination of influenza virus and antibodies by means of red cell agglutination. *J Exp Med* 1942;1;75(1): 49-64.

Hoffmann E, Neumann G, Hobom G, Webster RG, Kawaoka Y. 'Ambisense' approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. *Virology* 2000a;267: 310-317.

Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci U S A* 2000b;97: 6108-6113.

Hogan RJ, Cauley LS, Ely KH, Cookenham T, Roberts AD, Brennan JW, et al. Long-term maintenance of virus-specific effector memory CD8+ T cells in the lung airways depends on proliferation. *Journal of Immunology* 2002;169: 4976-4981.

Holland J, Spindler K, Horodyski F. Rapid evolution of RNA genomes. *Science* 1982;215: 1577-1585.

Honda A, Uéda K, Nagata K, Ishihama A. Identification of the RNA polymerase-binding site on genome RNA of influenza virus. *J Biochem* 1987;102: 1241-1249.

Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, et al. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol* 2006;80: 7469-7480.

Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature* 2009;460: 1021-1025.

Jackson DC, Drummer HE, Urge L, Otvos Jr. L, Brown LE. Glycosylation of a synthetic peptide representing a T-cell determinant of influenza virus hemagglutinin results in loss of recognition by CD4+ T-cell clones. *Virology* 1994;199: 422-430.

Jackson LA, Holmes SJ, Mendelman PM, Huggins L, Cho I, Rhorer J. Safety of a trivalent live attenuated intranasal influenza vaccine, FluMist(TM), administered in addition to parenteral trivalent inactivated influenza vaccine to seniors with chronic medical conditions. *Vaccine* 1999;17: 1905-1909.

Jagger BW, Wise HM, Kash JC, Walters K-, Wills NM, Xiao Y-, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efstathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* 2012;337: 199-204.

Jain S, Benoit SR, Skarbinski J, Bramley AM, Finelli L. Influenza-associated pneumonia among hospitalized patients with 2009 pandemic influenza A (H1N1) virus-United States, 2009. *Clinical Infectious Diseases* 2012;54: 1221-1229.

Jang H, Boltz D, Sturm-Ramirez K, Shepherd KR, Jiao Y, Webster R, et al. Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration. *Proc Natl Acad Sci U S A* 2009;106: 14063-14068.

Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang C-, et al. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology* 2003;306: 18-24.

Johansson BE, Bucher DJ, Kilbourne ED. Purified influenza virus hemagglutinin and neuraminidase are equivalent in stimulation of antibody response but induce contrasting types of immunity to infection. *J Virol* 1989;63: 1239-1246.

Johnson NP, Mueller J. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull Hist Med* 2002;76: 105-115.

Johnson PR, Feldman S, Thompson JM. Immunity to influenza A virus infection in young children: A comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine. *J Infect Dis* 1986;154: 121-127.

Kang SM, Song JM, Compans RW. Novel vaccines against influenza viruses. *Virus Res* 2011;162: 31-38.

Kawaoka Y, Webster RG. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. *Proc Natl Acad Sci U S A* 1988;85: 324-328.

Khurana S, Verma S, Verma N, Crevar CJ, Carter DM, Manischewitz J, et al. Properly folded bacterially expressed H1N1 hemagglutinin globular head and ectodomain vaccines protect ferrets against H1N1 pandemic influenza virus. *PLoS ONE* 2010;5.

Kilander A, Rykkvin R, Dudman SG, Hungnes O. Observed association between the HA1 mutation D222G in the 2009 pandemic influenza A(H1N1) virus and severe clinical outcome, Norway 2009-2010. *Euro surveillance: bulletin européen sur les maladies transmissibles = European communicable disease bulletin* 2010;15.

Kim JH, Skountzou I, Compans R, Jacob J. Original antigenic sin responses to influenza viruses. *Journal of Immunology* 2009;183: 3294-3301.

Klenk HD, Rott R, Orlich M, Bloedorn J. Activation of influenza A viruses by trypsin treatment. *Virology* 1975;68: 426-439.

Kornfeld R, Kornfeld S. Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* 1985;VOL. 54: 631-664.

Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS, Grenfell BT. Host species barriers to influenza virus infections. *Science* 2006;312: 394-397.

Lakadamyali M, Rust MJ, Zhuang X. Endocytosis of influenza viruses. *Microbes and Infection* 2004;6: 929-936.

LaMere MW, Lam H-, Moquin A, Haynes L, Lund FE, Randall TD, Kaminski DA. Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. *Journal of Immunology* 2011;186: 4331-4339.

Landry N, Ward BJ, Trépanier S, Montomoli E, Le Dargis M, Lapini G, et al. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS ONE* 2010;5.

Lee I, Kim JI, Park M-. A novel PA-X protein translated from influenza A virus segment 3. *Journal of Bacteriology and Virology* 2012;42: 368-371.

Li G, Yilmaz M, Kojicic M, Fernández-Pérez E, Wahab R, Huskins WC, et al. Outcome of critically ill patients with influenza virus infection. *Journal of Clinical Virology* 2009;46: 275-278.

Li S, Schulman JL, Moran T, Bona C, Palese P. Influenza A virus transfectants with chimeric hemagglutinins containing epitopes from different subtypes. *J Virol* 1992;66: 399-404.

Liu Y, Childs RA, Matrosovich T, Wharton S, Palma AS, Chai W, et al. Altered receptor specificity and cell tropism of D222G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. *J Virol* 2010;84: 12069-12074.

López CB, García-Sastre A, Williams BRG, Moran TM. Type I interferon induction pathway, but not released interferon, participates in the maturation of dendritic cells induced by negative-strand RNA viruses. *J Infect Dis* 2003;187: 1126-1136.

Louie JK, Acosta M, Jamieson DJ, Honein MA. Severe 2009 H1N1 influenza in pregnant and postpartum women in California. *N Engl J Med* 2010;362: 27-35.

Luytjes W, Krystal M, Enami M, Parvin JD, Palese P. Amplification, expression, and packaging of a foreign gene by influenza virus. *Cell* 1989;59: 1107-1113.

Maassab HF. Adaptation and growth characteristics of influenza virus at 25°C [31]. *Nature* 1967;213: 612-614.

Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, García-Sastre A. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. *Proc Natl Acad Sci U S A* 2010a;107: 11531-11536.

Manicassamy B, Medina RA, Hai R, Tsibane T, Stertz S, Nistal-Villán E, et al. Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines. *PLoS Pathogens* 2010b;6.

Marston FA. The purification of eukaryotic polypeptides synthesized in Escherichia coli. *Biochem J* 1986;240: 1-12.

Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol* 2000;74: 8502-8512.

Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk H-. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 2004a;101: 4620-4624.

Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk H-. Neuraminidase is important for the initiation of influenza virus infection in human airway epithelium. *J Virol* 2004b;78: 12665-12667.

McAuley JL, Hornung F, Boyd KL, Smith AM, McKeon R, Bennink J, et al. Expression of the 1918 Influenza A Virus PB1-F2 Enhances the Pathogenesis of Viral and Secondary Bacterial Pneumonia. *Cell Host and Microbe* 2007;2: 240-249.

Medina RA, Stertz S, Manicassamy B, Zimmermann P, Sun X, Albrecht RA, et al. Glycosylations in the globular head of the hemagglutinin protein modulate the virulence and antigenic properties of the H1N1 influenza viruses. *Science Translational Medicine,* accepted.

Medina RA, García-Sastre A. Influenza A viruses: new research developments. *Nat Rev Microbiol* 2011.

Medina RA, Manicassamy B, Stertz S, Seibert CW, Hai R, Belshe RB, et al. Pandemic 2009 H1N1 vaccine protects against 1918 Spanish influenza virus. *Nature Communications* 2010;1.

Monsalvo AC, Batalle JP, Lopez MF, Krause JC, Klemenc J, Hernandez JZ, et al. Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes. *Nat Med* 2011;17: 195-200.

Moscona A. Neuraminidase inhibitors for influenza. *N Engl J Med* 2005;353: 1363-1373.

Mossad SB. Demystifying FluMist, a new intranasal, live influenza vaccine. *Cleve Clin J Med* 2003;70: 801-806.

Mossong J, Hens N, Jit M, Beutels P, Auranen K, Mikolajczyk R, et al. Social contacts and mixing patterns relevant to the spread of infectious diseases. *PLoS Medicine* 2008;5: 0381-0391.

Munk K, Pritzer E, Kretzschmar E, Gutte B, Garten W, Klenk H-. Carbohydrate masking of an antigenic epitope of influenza virus haemagglutinin independent of oligosaccharide size. *Glycobiology* 1992;2: 233-240.

Murphy BR, Clements ML. The systemic and mucosal immune response of humans to influenza A virus. *Curr Top Microbiol Immunol* 1989;146: 107-116.

Muster T, Subbarao EK, Enami M, Murphy BR, Palese P. An influenza A virus containing influenza B virus 5' and 3' noncoding regions on the neuraminidase gene is attenuated in mice. *Proc Natl Acad Sci U S A* 1991;88: 5177-5181.

Nakamura R, Maeda N, Shibata K, Yamada H, Kase T, Yoshikai Y. Interleukin-15 is critical in the pathogenesis of influenza a virus-induced acute lung injury. *J Virol* 2010;84: 5574-5582.

Neumann G, Kawaoka Y. Reverse genetics systems for the generation of segmented negative-sense RNA viruses entirely from cloned cDNA. *Current Topics in Microbiology and Immunology* 2004;283: 43-60.

Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci U S A* 1999;96: 9345-9350.

Neumann G, Zobel A, Hobom G. RNA polymerase I-mediated expression of influenza viral RNA molecules. *Virology* 1994;202: 477-479.

Nichol KL. Influenza vaccination in the elderly: Impact on hospitalisation and mortality. *Drugs and Aging* 2005;22: 495-515.

Nichol KL, Mendelman PM, Mallon KP, Jackson LA, Gorse GJ, Belshe RB, et al. Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: A randomized controlled trial. *J Am Med Assoc* 1999;282: 137-144.

Ohuchi M, Feldmann A, Ohuchi R, Klenk H-. Neuraminidase is essential for fowl plague virus hemagglutinin to show hemagglutinating activity. *Virology* 1995;212: 77-83.

Ohuchi M, Ohuchi R, Feldmann A, Klenk H-. Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. *J Virol* 1997;71: 8377-8384.

Okuno Y, Isegawa Y, Sasao F, Ueda S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J Virol* 1993;67: 2552-2558.

Palache AM, Brands R, Van Scharrenburg GJM. Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells or fertilized chicken eggs. *J Infect Dis* 1997;176: S20-S23.

Palese P. The genes of influenza virus. *Cell* 1977;10: 1-10.

Palese P. Making better influenza virus vaccines? *Emerging Infectious Diseases* 2006;12: 61-65.

Palese P, Compans RW. Inhibition of influenza virus replication in tissue culture by 2 deoxy 2,3 dehydro N trifluoroacetylneuraminic acid (FANA): mechanism of action. *J Gen Virol* 1976;33: 159-163.

Palese P, Shaw M. Orthomyxoviridae: The Viruses and Their Replication. In: Knipe D.M., Howley P.M., editors. *Fields Virology*. 5th Edition ed.: Lippincott Williams & Wilkins; 2007.

Palese P, Tobita K, Ueda M, Compans RW. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* 1974;61: 397-410.

Palese P, Zheng H, Engelhardt OG, Pleschka S, García-Sastre A. Negative-strand RNA viruses: Genetic engineering and applications. *Proc Natl Acad Sci U S A* 1996;93: 11354-11358.

Parvin JD, Palese P, Honda A, Ishihama A, Krystal M. Promoter analysis of influenza virus RNA polymerase. *J Virol* 1989;63: 5142-5152.

Peiris JSM, Poon LLM, Guan Y. Surveillance of animal influenza for pandemic preparedness. *Science* 2012;335: 1173-1174.

Pleguezuelos O, Robinson S, Stoloff GA, Caparrós-Wanderley W. Synthetic Influenza vaccine (FLU-v) stimulates cell mediated immunity in a double-blind, randomised, placebo-controlled Phase I trial. *Vaccine* 2012;30: 4655-4660.

Pleschka S, Richard Jaskunas S, Engelhardt OG, Zürcher T, Palese P, Garcia-Sastre A. A plasmid-based reverse genetics system for influenza A virus. *J Virol* 1996;70: 4188-4192.

Portela A, Digard P. The influenza virus nucleoprotein: A multifunctional RNA-binding protein pivotal to virus replication. *Journal of General Virology* 2002;83: 723-734.

Possee RD, Schild GC, Dimmock NJ. Studies on the mechanism of neutralization of influenza virus by antibody: Evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus in vivo by inhibiting virion transcriptase activity. *J Gen Virol* 1982;58: 373-386.

Powell TJ, Strutt T, Reome J, Hollenbaugh JA, Roberts AD, Woodland DL, et al. Priming with cold-adapted influenza A does not prevent infection but elicits long-lived protection against supralethal challenge with heterosubtypic virus. *Journal of Immunology* 2007;178: 1030-1038.

Prokudina EN, Semenova N, Chumakov V, Stitz L. An antigenic epitope of influenza virus nucleoprotein (NP) associated with polymeric forms of NP. *Virology Journal* 2008;5.

Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. *Vaccine* 2005;23: 5751-5759.

Reading PC, Miller JL, Anders EM. Involvement of the mannose receptor in infection of macrophages by influenza virus. *J Virol* 2000;74: 5190-5197.

Reading PC, Morey LS, Crouch EC, Anders EM. Collectin-mediated antiviral host defense of the lung: Evidence from influenza virus infection of mice. *J Virol* 1997;71: 8204-8212.

Reid AH, Fanning TG, Hultin JV, Taubenberger JK. Origin and evolution of the 1918 "Spanish" influenza virus hemagglutinin gene. *Proc Natl Acad Sci U S A* 1999;96: 1651-1656.

Reid AH, Fanning TG, Janczewski TA, McCall S, Taubenberger JK. Characterization of the 1918 "Spanish" influenza virus matrix gene segment. *J Virol* 2002;76: 10717-10723.

Reid AH, Fanning TG, Janczewski TA, Taubenberger JK. Characterization of the 1918 'Spanish' influenza virus neuraminidase gene. *Proc Natl Acad Sci U S A* 2000;97: 6785-6790.

Reid AH, Taubenberger JK, Fanning TG. Evidence of an absence: The genetic origins of the 1918 pandemic influenza virus. *Nature Reviews Microbiology* 2004;2: 909-914.

Rigg RJ, Carver AS, Dimmock NJ. IgG-neutralized influenza virus undergoes primary, but not secondary uncoating in vivo. *J Gen Virol* 1989;70: 2097-2109.

Rogers GN, D'Souza BL. Receptor binding properties of human and animal H1 influenza virus isolates. *Virology* 1989;173: 317-322.

Rogers GN, Paulson JC. Receptor determinants of human and animal influenza virus isolates: Differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* 1983;127: 361-373.

Romanova J, Katinger D, Ferko B, Voglauer R, Mochalova L, Bovin N, et al. Distinct host range of influenza H3N2 virus isolates in Vero and MDCK cells is determined by cell specific glycosylation pattern. *Virology* 2003;307: 90-97.

Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawaoka Y. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. *Virus Research* 2011;158: 124-129.

Salk JE. A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. *J Immunol* 1944;49: 87-98.

Schulze IT. Effects of glycosylation on the properties and functions of influenza virus hemagglutinin. *J Infect Dis* 1997;176: S24-S28.

Shaw A. New technologies for new influenza vaccines. *Vaccine* 2012;30: 4927-4933.

Shi M, Jagger BW, Wise HM, Digard P, Holmes EC, Taubenberger JK. Evolutionary conservation of the PA-X open reading frame in segment 3 of influenza a virus. *Journal of Virology* 2012;86: 12411-12413.

Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Influenza virus receptors in the human airway. *Nature* 2006;440: 435-436.

Shoji Y, Farrance CE, Bi H, Shamloul M, Green B, Manceva S, et al. Immunogenicity of hemagglutinin from A/Bar-headed Goose/Qinghai/1A/05 and A/Anhui/1/05 strains of H5N1 influenza viruses produced in Nicotiana benthamiana plants. *Vaccine* 2009;27: 3467-3470.

Shun-Shin M, Thompson M, Heneghan C, Perera R, Harnden A, Mant D. Neuraminidase inhibitors for treatment and prophylaxis of influenza in children: systematic review and meta-analysis of randomised controlled trials. *BMJ* 2009;339.

Simonsen L, Clarke MJ, Schonberger LB, Arden NH, Cox NJ, Fukuda K. Pandemic versus epidemic influenza mortality: A pattern of changing age distribution. *J Infect Dis* 1998;178: 53-60.

Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. *Annual Review of Biochemistry* 2000;69: 531-569.

Smith GJD, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza a epidemic. *Nature* 2009;459: 1122-1125.

Smorodintsev AA. Basic aspects of the study of live influenza vaccine. *Klin Med* 1962;40: 33-39.

Song H, Wittman V, Byers A, Tapia T, Zhou B, Warren W, et al. In vitro stimulation of human influenza-specific CD8 + T cells by dendritic cells pulsed with an influenza virus-like particle (VLP) vaccine. *Vaccine* 2010;28: 5524-5532.

Spiro MJ, Spiro RG. Sulfation of the N-linked oligosaccharides of influenza virus hemagglutinin: Temporal relationships and localization of sulfotransferases. *Glycobiology* 2000;10: 1235-1242.

Stanley P, Schachter H, Taniguchi N. N-Glycans In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, et al, editors. *Essentials of Glycobiology*. 2nd Edition ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. p. Chapter 8.

Stansell E, Desrosiers RC. Functional contributions of carbohydrate on AIDS virus glycoprotein. *Yale J Biol* Med 2010;83: 201-208.

Steens A, Waaijenborg S, Teunis PFM, Reimerink JHJ, Meijer A, Van Der Lubben M, et al. Age-dependent patterns of infection and severity explaining the low impact of 2009 influenza A (H1N1): Evidence from serial serologic surveys in the Netherlands. *Am J Epidemiol* 2011;174: 1307-1315.

Subbarao EK, Eun Ju Park, Lawson CM, Chen AY, Murphy BR. Sequential addition of temperature-sensitive missense mutations into the PB2 gene of influenza A transfectant viruses can effect an increase in temperature sensitivity and attenuation and permits the rational design of a genetically engineered live influenza A virus vaccine. *J Virol* 1995;69: 5969-5977.

Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen L-, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nature Structural and Molecular Biology* 2009;16: 265-273.

Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat Med* 2009;15: 277-284.

Sun K, Ye J, Perez DR, Metzger DW. Seasonal FluMist vaccination induces cross-reactive T cell immunity against H1N1 (2009) influenza and secondary bacterial infections. *Journal of Immunology* 2011;186: 987-993.

Sun S, Wang Q, Zhao F, Chen W, Li Z. Glycosylation Site Alteration in the Evolution of Influenza A (H1N1) Viruses. *PLoS One* 2011;6: e22844.

Szymczakiewicz-Multanowska A, Groth N, Bugarini R, Lattanzi M, Casula D, Hilbert A, et al. Safety and immunogenicity of a novel influenza subunit vaccine produced in mammalian cell culture. *J Infect Dis* 2009;200: 841-848.

Tannock GA, Paul JA, Barry RD. Relative immunogenicity of the cold-adapted influenza virus A/Ann Arbor/6/60 (A/AA/6/60-ca), recombinants of A/AA/6/60-ca, and parental strains with similar surface antigens. *Infect Immun* 1984;43: 457-462.

Tate MD, Job ER, Brooks AG, Reading PC. Glycosylation of the hemagglutinin modulates the sensitivity of H3N2 influenza viruses to innate proteins in airway secretions and virulence in mice. *Virology* 2011;413: 84-92.

Taubenberger JK, Reid AH, Krafft AE, Bijwaard KE, Fanning TG. Initial genetic characterization of the 1918 'Spanish' influenza virus. *Science* 1997;275: 1793-1796.

Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG. Characterization of the 1918 influenza virus polymerase genes. *Nature* 2005;437: 889-893.

Tellier R. Review of aerosol transmission of influenza A virus. *Emerging Infectious Diseases* 2006;12: 1657-1662.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22: 4673-4680.

Thompson WW, Shay DK, Weintraub E, Brammer L, Bridges CB, Cox NJ, et al. Influenza-associated hospitalizations in the United States. *J Am Med Assoc* 2004;292: 1333-1340.

Tong S, Li Y, Rivailler P, Conrardy C, Alvarez Castillo DA, Chen L-, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. A distinct lineage of influenza A virus from bats. *Proceedings of the National Academy of Sciences of the United States of America* 2012;109: 4269-4274.

Tsuchiya E, Sugawara K, Hongo S, Matsuzaki Y, Muraki Y, Li Z-, et al. Effect of addition of new oligosaccharide chains to the globular head of influenza A/H2N2 virus haemagglutinin on the intracellular transport and biological activities of the molecule. *J Gen Virol* 2002;83: 1137-1146.

Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solórzano A, Swayne DE, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. *Science* 2005;310: 77-80.

Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solórzano A, Pappas C, et al. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. *Science* 2007;315: 655-659.

Van Den Steen P, Rudd PM, Dwek RA, Opdenakker G. Concepts and principles of O-linked glycosylation. *Critical Reviews in Biochemistry and Molecular Biology* 1998;33: 151-208.

Varga ZT, Ramos I, Hai R, Schmolke M, García-Sastre A, Fernandez-Sesma A, et al. The influenza virus protein PB1-F2 inhibits the induction of type i interferon at the level of the MAVS adaptor protein. *PLoS Pathogens* 2011;7.

Varghese JN, Laver WG, Colman PM. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. *Nature* 1983;303: 35-40.

Vesikari T, Block SL, Guerra F, Lattanzi M, Holmes S, Izu A, et al. Immunogenicity, safety and reactogenicity of a mammalian cell-culture-derived influenza vaccine in healthy children and adolescents three to seventeen years of age. *Pediatr Infect Dis J* 2012;31: 494-500.

Vigerust DJ, Shepherd VL. Virus glycosylation: role in virulence and immune interactions. *Trends Microbiol* 2007;15: 211-218.

Wagner R, Wolff T, Herwig A, Pleschka S, Klenk H-. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: A study by reverse genetics. *J Virol* 2000;74: 6316-6323.

Wallinga J, Teunis P, Kretzschmar M. Using data on social contacts to estimate age-specific transmission parameters for respiratory-spread infectious agents. *Am J Epidemiol* 2006;164: 936-944.

Wang C-, Chen J-, Tseng Y-, Hsu C-, Hung Y-, Chen S-, et al. Glycans on influenza hemagglutinin affect receptor binding and immune response. *Proc Natl Acad Sci U S A* 2009;106: 18327-18332.

Wanzeck K, Boyd KL, McCullers JA. Glycan shielding of the influenza virus hemagglutinin contributes to immunopathology in mice. *American Journal of Respiratory and Critical Care Medicine* 2011;183: 767-773.

Watanabe T, Watanabe S, Kawaoka Y. Cellular networks involved in the influenza virus life cycle. *Cell Host and Microbe* 2010;7: 427-439.

Webb SAR, Pettilä V, Seppelt I, Bellomo R, Bailey M, Cooper DJ, et al. Critical care services and 2009 H1N1 influenza in Australia and New Zealand. *N Engl J Med* 2009;361: 1925-1934.

Webster RG, Peiris M, Chen H, Guan Y. H5N1 outbreaks and enzootic influenza. *Emerging Infectious Diseases* 2006;12: 3-8.

Webster RG. Immunity to influenza in the elderly. *Vaccine* 2000;18: 1686-1689.

Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev* 1992;56: 152-179.

Webster RG, Govorkova EA. H5N1 influenza - Continuing evolution and spread. *N Engl J Med* 2006;355: 2174-2177.

Webster RG, Yakhno M, Hinshaw VS. Intestinal influenza: Replication and characterization of influenza viruses in ducks. *Virology* 1978;84: 268-278.

Wei C, Boyington JC, Dai K, Houser KV, Pearce MB, Kong W, et al. Cross-Neutralization of 1918 and 2009 Influenza Viruses: Role of Glycans in Viral Evolution and Vaccine Design. *Sci Transl Med* 2010;2: 24ra21.

Weis W, Brown JH, Cusack S, Paulson JC, Skehel JJ, Wiley DC. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. *Nature* 1988;333: 426-431.

White J, Kielian M, Helenius A. Membrane fusion proteins of enveloped animal viruses. *Q Rev Biophys* 1983;16: 151-195.

WHO Global Influenza Surveillance Network. Manual for the laboratory diagnosis and virological surveillance of influenza. Switzerland: *WHO Press*; 2011.

Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem* 1987;56: 365-394.

Wiley DC, Wilson IA. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. *Nature* 1981;289: 373-378.

Wilson IA, Skehel JJ, Wiley DC. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 1981;289: 366-373.

World Health Organization. Influenza (seasonal) factsheet N211. 2009; Available at: http://www.who.int/mediacentre/factsheets/fs211/en/index.html. Accessed 05.09.12.

Wright P, Neumann G, Kawaoka Y. Orthomyxoviruses. In: Knipe D, Howley P, editors. *Fields Virology*. 5th Edition ed.: Lippincott Williams & Wilkins; 2007.

Xu R, Ekiert DC, Krause JC, Hai R, Crowe Jr. JE, Wilson IA. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science* 2010;328: 357-360.

Yagi H, Watanabe S, Suzuki T, Takahashi T, Suzuki Y, Kato K. Comparative analyses of N-Glycosylation profiles of influenza a viruses grown in different host cells. *Open Glycoscience* 2012;5: 2-12.

Youil R, Su Q, Toner TJ, Szymkowiak C, Kwan W-, Rubin B, et al. Comparative study of influenza virus replication in Vero and MDCK cell lines. *J Virol Methods* 2004;120: 23-31.

Zarychanski R, Stuart TL, Kumar A, Doucette S, Elliott L, Kettner J, et al. Correlates of severe disease in patients with 2009 pandemic influenza (H1N1) virus infection. *CMAJ* 2010;182: 257-264.

Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, et al. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology* 2004;14: 1229-1246.

Zhang S, Sherwood RW, Yang Y, Fish T, Chen W, Mccardle JA, et al. Comparative characterization of the glycosylation profiles of an influenza hemagglutinin produced in plant and insect hosts. *Proteomics* 2012;12: 1269-1288.

Zhu X, Yu W, McBride R, Li Y, Chen L-, Donis RO, Tong S, Paulson JC, Wilson IA. Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. *Proceedings of the National Academy of Sciences of the United States of America* 2013;110: 1458-1463.

Zinkernagel RM. Immunology taught by viruses. *Science* 1996;271: 173-178.

Appendix I

The expression plasmid pPolI (Figure 16; described in Chapter 4.3) and the ambisense plasmid pDZ (Figure 17; described in Chapters 4.4.1 & 4.5) had been previously generated by Professor Adolfo García-Sastre (MSSM, USA). Infinite thanks for kindly borrowing the plasmids for the assays of this thesis.

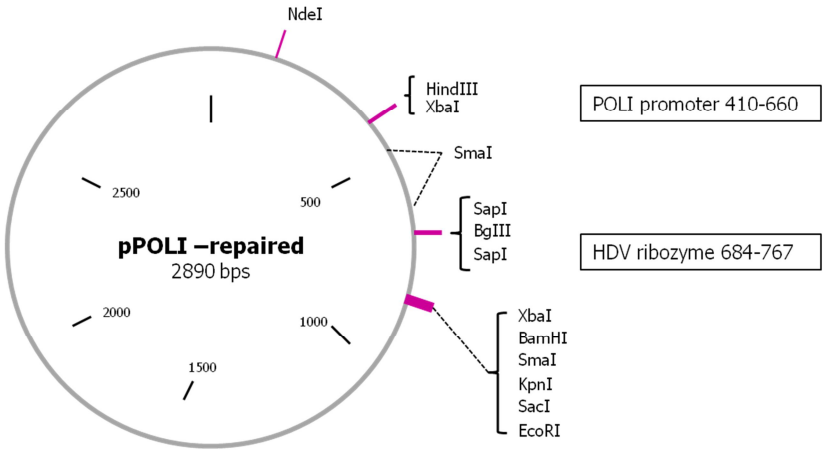


Figure 16. Map of the pPolI plasmid that was used to create the mutant pPol-HA plasmids. The plasmid was provided by Professor Adolfo García-Sastre (MSSM, USA).

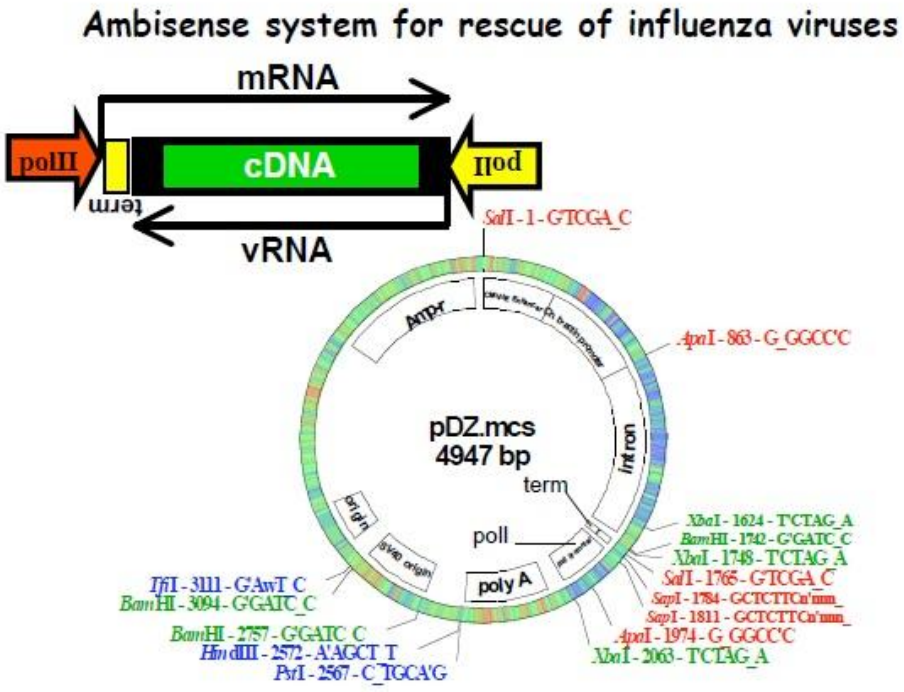


Figure 17. Map of the pDZ expression plasmid that was used for the recombinant mutant virus rescue and for generating the pPol-HA plasmid. The plasmid was provided by Professor Adolfo García-Sastre (MSSM, USA).