Chapter 1

General introduction and scope of the thesis
Epidemiology

Influenza is one of the major respiratory diseases. It has a very high disease burden globally, leading to considerable morbidity and mortality. Annual influenza epidemics result in about 3 to 5 million cases of severe illness, and about 290,000 - 650,000 deaths worldwide. Influenza appears in the community in two forms, annual epidemics or seasonal influenza, and occasional pandemics. Seasonal influenza occurs in the winter in both temperate climates of the northern and southern hemisphere. In the tropical regions, influenza occurs throughout the year and outbreaks are irregular. The spread of seasonal influenza is quite rapid, especially in the crowded areas. Influenza is caused by a respiratory virus, so if an infected person coughs or sneezes, the infectious droplets are dispersed into the air and people in the vicinity are infected if they inhale these infectious droplets or even shaking hands with an infected person. Recently, it has been shown that sneezing or coughing were not necessary for generating aerosols, only breathing was sufficient for aerosol generation and in turn transmission of influenza. Influenza virus has been detected from the air in settings like patients' rooms, health centers and emergency rooms. Recently, influenza viruses have also been detected in air especially in poultry farms.

Influenza shows the highest attack rate in young people. Children display the highest morbidity associated with influenza. However, most mortality is reported in the older adult population. Along with the elderly population, the other groups in which disease symptoms are particularly severe are pregnant women, immune-compromised individuals and people with underlying chronic conditions like chronic lung diseases, cardiovascular diseases, metabolic disorders, cancer etc. Hospitalizations and deaths especially occur in these high risk groups.

Disease symptoms and progression

Seasonal influenza can range from asymptomatic disease to severe illness leading to complications. The severity of the infection is dependent on many factors like pathogenicity of the virus, age and genetic makeup of a person and also, if a person has some underlying immune-compromising conditions. Influenza disease can range from asymptomatic to severe with secondary complications. The disease symptoms of the seasonal influenza are evident after 1-2 days incubation of the virus in the host. They include sudden onset of fever, cough, headache, muscle and joint pain, sore throat and a runny nose. Fever peaks in the first 24 hours of onset, is usually between 38-41°C, and lasts for 1-5 days. Other physiological symptoms include flushed face, severe malaise, hot and moist skin, infected eyes. Severe influenza related complications include pneumonia, Guillain–Barre syndrome, aseptic meningitis, cardiac complications, and Reye's syndrome.

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Influenza virus

Influenza is caused by the influenza virus which belongs to the family of Orthomyxoviridae, which is further subdivided into influenza A (IAV), B and C and now also D. Seasonal epidemics are caused by IAV and IBVs. Influenza C viruses cause mild infections, IAV’s are further classified depending on the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are 18 HA and 11 NA subtypes. Influenza B has 2 distinct lineages, Victoria and Yamagata. Influenza viruses are enveloped with a lipid bilayer and have a single stranded, negative sense RNA genome. An important characteristic of the influenza virus is that it has a segmented genome. It contains 8 genome segments (HA, NA, M, PA, PB1, PB2, NP, NS) coding for 10 proteins (HA, NA, M1 and M2, PA, PB1, PB2, NP, NS1 and NS2) (Figure 1). Some influenza viruses have up to 7 additional proteins along with the ones mentioned before. These are PB1-F2, N-40, PA-X, PA-N155, PA-N182, M42, NS3.

Influenza virions have a roughly spherical shape and the diameter is 80-120 nm. Influenza virions are enveloped and contain glycoproteins HA, NA and M2 on the surface. HA (80%) is the most abundant protein on the envelope followed by NA (17%). M2 is present in very small quantity, 16-20 molecules per virion. Under the envelope there is the M1 protein which covers the viral ribonucleoproteins (vRNPs) or the viral core. Each vRNP comprises three polymerase proteins: polymerase acidic (PA), polymerase basic 1 (PB1), polymerase basic 2 (PB2). The nucleoprotein (NP)-covered RNA forms a pan-handle structure which carries the polymerase complex at the end.

Figure 1. Structure of the influenza virus. Figure on the left depicts the structure of influenza virus with 8 genome segments and also surface glycoproteins HA, NA and M2. On the right are the non-structural proteins and the newly fund proteins of influenza virus.
RNA covered by NP forms a pan-handle structure which carries polymerase complex at its end 24.

**Evolution of the influenza viruses**

There are mainly two types of mutations, antigenic drift and antigenic shift, that affect HA and NA which are important proteins for the induction of neutralizing antibodies (Figure 2).

**Figure 2.** Antigenic drift and shift of influenza viruses. It depicts antigenic drift and shift within hemagglutinin. Antigenic drift is shown by change in the colour of HA head from blue to green, while antigenic shift has been shown by change in the colour of complete HA from blue to orange.

**Antigenic drift**

RNA-polymerase of influenza lacks proof-reading activity which leads to addition of faulty nucleotides during replication of IAV’s. This occurs at the rate of $10^{-3}$ to $10^{-4}$ substitutions/site, which results in high mutation rate 21,25. Accumulation of point mutations leads to a gradual change of the antigenic site of HA leading to antigenic drift (depicted in figure 2) 26. These changes impair and often prevent recognition and neutralization of this drifted virus by pre-existing neutralizing antibodies. The host immune system becomes no longer effective in protecting against this newly formed virus 25, which results in annual epidemics. Hence, to counter this, annually the flu vaccine is reformulated based on the circulating strains of that season in the northern and southern hemisphere, respectively.
Antigenic shift

The segmented genome of influenza viruses facilitates exchange of genetic material (RNA) between the genotypically different influenza viruses, leading to formation of new or re-assorted IAV strain. This phenomenon or type of mutation is termed ‘antigenic shift’ and it can lead to occasional pandemics causing unusually high morbidity and mortality. Until now the four major pandemics, 1918 H1N1 Spanish flu, 1957 H2N2, Asian flu, 1968 H3N2 Hong Kong flu and 2009 H1N1 Mexican flu pandemic, have been recorded. A pandemic virus can be produced by genetic re-assortment between avian and human IAVs. Usually, avian influenza viruses do not infect humans and vice versa. However, swine can act as a virus mixing vessel, leading to the generation of new influenza viruses, which can infect both humans and poultry. The classic example of such a virus is the H1N1 pandemic virus of 2009, which caused quite some havoc back then and is still in circulation now as a seasonal strain. These modifications in IAVs can be attributed to the factors like mechanistic errors during the replication of viral RNA polymerase, evolutionary pressure, novel environment of the host, immune pressure, or antiviral drug pressure.

Vaccination

Vaccination is the cornerstone for influenza control. It is the best strategy for prevention so far and is recommended by the WHO for elderly and populations at risk. The two types of influenza vaccine available are inactivated influenza vaccines (IIV) and live attenuated influenza vaccines (LAIV).

Historical overview

The first influenza vaccine was produced in 1940’s and it was an inactivated or “killed” virus vaccine which was monovalent containing only one influenza A strain, H1N1. With the discovery of influenza B strains, an inactivated bivalent vaccine was formulated, tested in humans and was licensed in the USA for use. With the emergence of a new strain, H3N2, in 1970, the vaccine was further adapted to consist of H1N1, H3N2 and one influenza B strain thereafter. This vaccine is termed as a trivalent influenza vaccine or a TIV. In 2009, FDA 1st considered including one more B strain to minimize the impact of IBV mismatch on vaccine effectiveness. In 2012 the WHO recommended the production of QIV’s for the seasonal vaccination. Development of influenza vaccines is summarized in figure 3.
Live attenuated influenza vaccines
Live attenuated influenza vaccine (LAIV) has been in use in Russia from the 1970's. In 2003, an LAIV was licensed in the USA (not same one as in Russia) for the age-group 2-49 years and more recently this vaccine was approved in Europe, where it is recommended for children between 2-17 years of age. It is produced by reverse genetics using HA and NA of the circulating strains, while the backbone is an attenuated, temperature sensitive and cold-adapted influenza A or B virus strain. Because of the backbone, LAIV can replicate only at 33°C essentially restricting replication to the upper respiratory tract and is administered intranasally. Based on low vaccine effectiveness particularly against H1N1pdm09 in the 2014-15 and 2015-16 seasons, LAIV was not recommended in the USA for the season of 2016-17 and the 2017-2018 season but the ban was lifted for the 2018-19 season.

Inactivated influenza vaccines (IIV)
Inactivated influenza vaccines consist of whole inactivated virus influenza vaccines (WIV), split vaccines, subunit vaccines and virosomal or virus-like particle (VLP) vaccines. The components of these vaccines are depicted in Figure 3. WIV was the first applied influenza vaccine and has shown to have superior immunogenicity compared to corresponding split and subunit vaccines. However, due to local and systemic adverse reactions associated with WIV (like generalized aching and myalgia), WIV was replaced by much safer split and subunit vaccines. However, it has been thought that altered methods of formulating WIV could conquer these problems. WIV has regained attention again in context of universal influenza vaccines. Also, when WIV was given intranasally it has shown to induce cross-protection.

Figure 3. Influenza vaccine development from 1930's.
Current influenza vaccines

Current or seasonal trivalent influenza vaccines (TIVs) comprise of either inactivated or live attenuated antigens of influenza strains (H1N1, H3N2 and influenza B strain) that are antigenically similar to the circulating strains. Co-circulation of B strains from 2 lineages (Victoria and Yamagata) has led to formulating quadrivalent influenza vaccines which contain viruses of both influenza B lineages. Identification of the circulating strains is coordinated by the World Health Organization (WHO) international surveillance system and is used to predict virus strains putatively circulating in the coming season and thus to be included in the vaccine.

Figure 4. Components of different inactivated influenza vaccines (IIV). WIV is prepared by inactivating the virus with chemicals like β-propiolactone or formalin followed by purification. Virosomes are reconstructed influenza virus envelopes that contain of HA, NA and viral phospholipids. Virosomes retain viral membrane fusion and cell-binding capabilities due to their particulate nature, which in turn make them more immunogenic compared to subunit and split vaccines. Split vaccines are prepared by treatment with diethyl ether or detergent like ammonium deoxycholate which disassociates the viral envelope exposing all virus proteins. To make subunit vaccine, which consists of HA and NA proteins, further purification is done after diethyl ether treatment.
Influenza vaccine production is mainly done using embryonated chicken eggs and is a laborious and time consuming process taking about 6 months. There are also cell culture based vaccines available in the market. However with problems associated with egg based influenza production, in 2012, the U.S. FDA approved cell culture based trivalent inactivated Flucerix® vaccine to be used in people of age 18 and above (CDC website). In 2016, quadrivalent inactivated Flucerix® was approved for the people of age 4 and older. In Europe, one of the 1st commercialized seasonal cell culture based vaccines was Optaflu® \(^53,54\). Flublok® is an insect cell based influenza vaccine, now available in the market in the USA. It is a trivalent recombinant hemagglutinin based VLP vaccine which is produced in insect cell culture using the baculovirus expression system \(^53,54\).

**Limitations of current influenza vaccines**

Current seasonal vaccines could be associated with the problem of low immunogenicity, especially in children, elderly and immune-compromised populations. Accordingly, vaccine effectiveness is modest when there is an antigenic mismatch between the vaccine strains and the circulating strains.

Moreover, inactivated vaccines administered via parenteral route induce mainly systemic immune responses and very low or no mucosal immune responses \(^53,54\). As influenza virus is mainly transmitted via airways and replicates in the airway epithelium, restricting the infection at the portal of entry would be advantageous \(^55,56\). Additionally, IgA antibodies induced by mucosal vaccines have been shown to be more cross-reactive than IgG antibodies and provide cross-protection against antigenically drifted influenza viruses \(^57,58\). There are a lot of studies done on intranasal administration of influenza vaccines; however LAIV is the only intranasal vaccine that made it to the market. Alternatively, lungs would be an excellent mucosal area for targeting influenza vaccines as lungs are large and contain a large number of antigen presenting cells \(^59,60\).

Apart from immunogenicity problems associated with current influenza vaccines, stability is also an issue \(^61\). Current vaccines are mainly administered in aqueous form and are prone to degradation if the cold chain is disturbed \(^62\). If a vaccine that has undergone conformational changes is administered, it can induce lower immune responses. Hence improving stability of current influenza vaccines is a must. One of the ways is to make dry vaccine formulations by sugar-glass technology. Dry powder vaccines are less prone to degradation by temperature changes than liquid formulations \(^63,64\). Some dry powder influenza vaccine formulations have been shown to be suitable for pulmonary administration in pre-clinical studies in mice, cotton rats and chickens \(^65-68\) and the results are quite promising.

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Apart from immunogenicity problems associated with current influenza vaccines, stability is also an issue \(^71\). Current vaccines are mainly administered in aqueous form and are prone to degradation if the cold chain is disturbed \(^72\). If a vaccine that has undergone conformational changes is administered, it can induce lower immune responses. Hence improving stability of current influenza vaccines is a must. One of the ways is to make dry vaccine formulations by sugar-glass technology. Dry powder vaccines are less prone to degradation by temperature changes than liquid formulations \(^73,74\). Some dry powder influenza vaccine formulations have been shown to be suitable for pulmonary administration in pre-clinical studies in mice, cotton rats and chickens \(^75-78\) and the results are quite promising.
The main problem associated with the currently available influenza vaccines is that they can provide only strain specific immunity. The seasonal vaccines depend on the induction of neutralizing antibodies against the globular head of HA of the virus strains present in vaccine. These antibodies fail to neutralize any mismatched HA that is not part of the flu strains in the vaccine. This makes it necessary to reformulate the vaccine every year depending on the circulating strains. Also, these seasonal vaccines fail to provide protection against newly emerging influenza strains with pandemic potential. Hence, universal or broadly protective influenza vaccines are needed.

**Universal influenza vaccine candidates**

There are many vaccine candidates that are in the pipeline for the generation of broadly protective vaccines. Conserved influenza proteins like nucleoprotein (NP), matrix protein 1 (M1) or the stem domain of HA are ideal targets for inducing broadly protective immunity. Initially, it was believed that T cells that are induced against these proteins are the main correlates of cross-reactive immune response, in particular tissue resident CD8+ T cells. But along with cross-reactive T cells, currently non-neutralizing antibodies are also getting attention for being cross-reactive. They possibly mediate cross-protection via pathways like antibody dependent cellular cytotoxicity or antibody dependent cellular phagocytosis, which currently are hot topics in the field of universal influenza vaccines.

**Inducing cross-reactive T cells**

Influenza infection can generate T cells that target epitopes derived from highly conserved internal proteins of influenza virus in mice and humans. These cross-reactive T cells have been shown to mediate heterosubtypic protection in mice. Studies have shown that the targets of T cells are mainly NP, M1 and PB1. CD8+ cytotoxic T cells can clear influenza virus infected cells and curb the virus from spreading. Even though cell mediated immunity declines by ~6 months after primary infection, a large number of memory cells is still present and has been found in animals for about 2 years. There are a number of studies which describe the importance of cross-reactive T cells in context of broad-protection from influenza in humans. Hence, targeting T cell memory with vaccines would be a promising approach to elicit broad-protection against influenza viruses. Although the protective role of CD8 T cells has been studied more in context of universal influenza vaccines, the protective role of CD4 T cells is gaining as much attention. It has been shown that without B cells or CD8 T cells, HA-specific CD4 T cells can protect a naïve host from influenza infection. CD4 T cells can target conserved M2e of influenza to induce heterosubtypic protection. There are several T cell based universal influenza vaccine candidates evaluated in clinical trials.
Inducing cross-reactive antibodies

Current influenza vaccines depend on neutralizing antibodies which provide strain specific protection. These antibodies have very limited cross-reactive potential and fail to elicit heterosubtypic protection. However, broadly neutralizing antibodies have been identified from influenza infected or vaccinated individuals during last decade, although they seem to be present in low amounts. The stem of HA contains a conserved region and antibodies targeting this region are often capable of neutralizing one or both influenza A subgroups. Recombinant vaccines can induce these antibodies directed against HA stalk. Studies with influenza vaccine consisting of chimeric HA or recombinant headless HA2 protein expressed on VLP’s have shown to induce these antibodies \(^{98}\). Apart from HA, NA and M2 have also been shown to be targets for the broadly neutralizing antibodies \(^{99,100}\). The highly conserved extracellular domain of M2, M2e, has been the most attractive target for universal influenza vaccine design \(^{91,101}\). Strategies to elicit these antibodies include sequential vaccination with antigenically distinct vaccines, use of adjuvant, different prime-boost strategies, multivalent approaches and vaccination with glycan-modified antigens, vaccination with minimal antigens and \(^{102,103}\). Alternative to the mechanism of direct neutralization, there are other non-neutralizing Fc receptor dependent mechanisms such as antibody dependent cellular cytotoxicity (ADCC), antibody dependent cellular phagocytosis (ADCP) and also complement dependent cytotoxicity (CDC). These effector mechanisms are recruited by anti-HA, anti-NA and anti-M2e antibodies \(^{72,104-107}\). ADCC is important in a sense that it can induce protection in vivo mediated by stalk specific antibodies. Protection conferred by M2e based vaccines has also been shown to be mediated by ADCC \(^{100,101}\). Passive transfer of these antibodies in humans have shown to reduce clinical symptoms \(^{112}\).
Scope of this thesis

There are several strategies that can lead us towards broadly protective and improved influenza vaccines. In the 1st part of this thesis we focus on different vaccination strategies or delivery systems to achieve broad-protection. Further we tried to elucidate which immune mechanisms these vaccines target. In the second part of this thesis we describe the cotton rat model in context of influenza vaccine evaluation and its suitability for pulmonary administration of dry powder and liquid vaccine formulations. Whole inactivated virus influenza vaccines (WIV) are capable of inducing cross-protection to some extent however it’s limited. Adjuvants and different routes of vaccination would be an interesting way to enhance cross-protection induced by WIV. In Chapter 2, we compared liposome and protein based adjuvants along with whole inactivated virus (WIV) influenza vaccines in a head to head comparison setting to assess if one or more adjuvanted vaccine candidates could confer broadly protective immunity. Also, mechanisms of cross-protection were assessed for those adjuvanted vaccines which performed the best in the head-to-head comparison.

Sequential infection with antigenically distinct influenza viruses can provide cross-protection. However not a lot of research is done on sequential vaccination for the induction of cross-protection. In Chapter 3, we assessed whether sequential immunization with antigenically distinct influenza vaccines could also provide cross-protection, in how far the form of the vaccine, whole inactivated virus vaccine (WIV) or subunit vaccine (SU), impacts on this capacity, and which immune mechanisms are involved.

Cytotoxic T cells directed against conserved proteins of influenza like NP have been shown to induce cross-protection. However, NP on its own inadequately activates antigen presenting cells leading to low induction of CTLs. This problem could be solved using influenza virosomes. In Chapter 6, we developed a novel influenza vaccine consisting of virosomes with the Toll-like receptor 4 ligand, monophosphoryl lipid A, and the metal-ion-chelating lipid DOGS-NTA-Ni incorporated in the membrane. It contains nucleoprotein of influenza which is a conserved internal influenza antigen that can induce cross-reactive cytotoxic T cells.

Mice and ferrets are used for pre-clinical influenza vaccine evaluation. However both have some advantages and disadvantages. Hence there is a need of a small animal model for influenza vaccine evaluation. In Chapter 4, we describe the introduction of the cotton rat model in our laboratory and what are the pros and cons of using cotton rat model for influenza vaccine evaluation.

Vaccine administration to the lungs is an attractive alternative to parenteral vaccine administration, as it can induce mucosal immune responses along with systemic immune responses. In Chapter 5, we studied pulmonary delivery of WIV.
first time in cotton rat model. We assessed if the site of deposition can influence the immunogenicity and protective efficacy of pulmonary administered liquid and powder influenza vaccine formulations.

In Chapter 7, I present a summary of this thesis with perspectives about different influenza vaccination strategies and delivery systems, mechanisms of cross protection and harnessing pre-existing immunity, use of suitable animal models for preclinical influenza vaccine efficacy studies, mucosal vaccination and mucosal adjuvants followed by concluding remarks.
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