Cross-protection by conventional influenza vaccines
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General Introduction
and thesis scope
THE INFLUENZA VIRUS AND DISEASE BURDEN

The influenza virus is a negative strand RNA virus belonging to the Orthomyxovirus family. Influenza viruses have been divided into 3 types, namely; Influenza A, B and C viruses. In humans seasonal influenza epidemics are caused mainly by influenza A and B viruses (1). The influenza virus has two major envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (see structure of influenza virus in Figure 1). Within the type A influenza viruses 18 different subtypes of HA and 11 NA subtypes have been identified (2). The type A influenza viruses have therefore been further divided into subclasses, based on their combination of surface proteins (e.g. H1N1, H7N9 etc.). Dependent on their HA subtype usage the influenza A viruses have additionally been divided into two groups: group 1 and group 2 influenza A viruses. The influenza B viruses are not divided into subclasses but rather into two lineages. To date two influenza A viruses (H1N1 and H3N2) and two influenza B viruses, one from each lineage co-circulate among humans and cause seasonal epidemics (3, 4).

Every year influenza viruses cause epidemics leading to substantial morbidity and mortality. The World Health Organization (WHO) estimates about 3-5 million cases of severe illness and 250,000 -500,000 deaths per year are caused by influenza epidemics worldwide(6). While influenza can cause severe illness in any age group, those at highest risk are young children below the age of two and the elderly aged above 65 (6). Next to seasonal epidemics, influenza viruses

Figure 1. Structure of the Influenza virus. Illustration from Kaiser et al, Science 2006 (5). Reprinted with permission from AAAS.
from animal reservoirs pose a global threat for causing pandemic outbreaks. Pandemics are caused by viruses to which against there is no pre-existing immunity in the infected population (herd immunity). In recent years avian influenza viruses, such as H5N1 and H7N9, have crossed the species barrier and infected humans, leading to outbreaks with significant mortality (7, 8). Since the human-to-human transmission has fortunately been limited the emergence of these viruses did not cause pandemics. However, recent findings suggest that only a few mutations are needed for the virus to acquire good transmissibility from human-to-human (9).

INFLUENZA VACCINES AND THEIR CORRELATE OF PROTECTION

To date, vaccination is the most effective way to prevent against severe illness caused by influenza. Every year the WHO predicts which circulating H1N1, H3N2 and B strains are likely to be the most prevalent in the upcoming influenza season. Seasonal influenza vaccines aim to cover the circulating strains by including components of the recommended H1N1, H3N2 and 1 or 2 influenza B viruses. The approximate efficacy of seasonal influenza vaccine in adults is 75% but drops significantly in the elderly population (10-12). Due to occasional mismatch between the circulating strain and the predicted vaccine strain the vaccine efficacy can however be substantially lower (13). The majority of seasonal influenza vaccines contain both the HA and NA component of the circulating strain (3). Depending on the type of influenza vaccine; Live attenuated influenza viruses, inactivated influenza viruses, subunit or split vaccines the vaccine also contain other influenza proteins. The dose of seasonal influenza vaccines administrated is however solely based on the HA content, and their protective efficacy is estimated by their antibody response binding to the globular head of the HA protein (14-18).

The globular head of HA is involved in viral attachment to host cell, via sialic acids on cell-surface proteins (19-21). Antibodies directed against the viral receptor binding site, located on the globular head of HA, block attachment of the influenza virus to the host cell and consequently prevent infection (19-22). In the hemagglutination inhibition (HI) assay, viral attachment to host cells is mimicked by viral agglutination of red blood cells. Antibodies that block the interaction between virus and sialic acids inhibit the agglutination of red blood cells (hemagglutination inhibition, HI) (16, 23). The induction of antibodies that are able to inhibit hemagglutination is to date the only accepted in vitro correlate of vaccine mediated protection in humans (15). However, the globular head of
HA is susceptible to a high rate of genetic drift. This is caused by the selection pressure imposed by herd immunity and an error-prone replication cycle of the influenza virus (24-26). Due to the high variability in the globular head of HA the seasonal influenza vaccine strain composition needs to be updated almost annually. Once there is a larger than 4-fold drop in cross-reactive HI titer between the dominant circulating influenza strain and last year’s vaccine strain, the WHO recommends a change in vaccine composition (27).

It is generally believed that seasonal influenza vaccines do not protect against pandemic influenza viruses as there is a large phylogenetic distance between the HA head domain of the vaccine strains and the pandemic strain, resulting in the absence of cross-reactive HI titers. Instead, strain specific pandemic vaccines are thought to be required (28). However, the production of pandemic influenza vaccines takes considerable time, resulting in a critical lag-phase between the start of the pandemic spread and vaccine availability (29). As the human population grows and our cities expand the distance between animal reservoirs and the human population is likely to decrease, increasing the risk for zoonotic influenza viruses to cross the species barrier. To more effectively prevent both influenza epidemics and pandemics caused by zoonotic viruses the development of an influenza vaccine which can protect against a broad range of influenza viruses is of great importance. Ultimately such a vaccine should be universal, i.e. preferably confer protection against all influenza viruses.

BROADLY PROTECTIVE INFLUENZA VACCINES BY CROSS-REACTIVE IMMUNITY

Cross-reactive immune responses, humoral as well as cellular, are immune responses that are considered to cover more than one strain. Thus, cross-reactive antibodies and T-cells target influenza epitopes which are conserved between two or more influenza viruses. Cross-reactive immune responses have been demonstrated to mediate protection against divergent influenza viruses, i.e. conferring cross-protection.

Cross-reactive HA antibodies
The HA protein can be divided into a “head” domain and a “stem” domain. After the virus has attached to the host cell and entered via the endosomes, the HA stem undergoes a pH dependent conformational change which enables the virus to fuse its membrane with the host endosomal membrane (30). While the head domain contains the receptor binding site of the virus and is subjected to antigen
drift the stem domain of the HA protein is highly conserved, likely to maintain functionality of this fusion mechanism, see Figure 2A for HA conservation.

In the last decade, several antibodies that target the conserved HA stem domain have been identified by using phage library technologies or cloning of human memory B–cells and plasmablasts (31-34). Due to the high degree of conservation in the HA stem, these antibodies are generally able to bind a broad range of hemagglutinins. In vivo, broadly binding HA stem antibodies have demonstrated protective efficacy against a broad range of influenza viruses (30-32, 35). While HA stem binding antibodies cannot be detected in the HI assay as they do not interfere with the receptor binding of the virus, they can inhibit influenza replication by other mechanisms. Their breadth of cross-reactivity has generally been measured by their ability to directly neutralize the influenza virus. HA stem binding antibodies can directly neutralize the influenza virus via at least 3 mechanisms. Next to blocking the conformational change of the HA2 subunit, thereby preventing fusion of the viral and endosomal membrane (30, 34, 36), HA stem directed antibodies can also block the cleavage of the precursor HA protein (HA0) by steric hindrance (30, 37). For the influenza virus to be infectious the HA protein needs to be cleaved into two subunits, HA1 and HA2. HA0 protein is cleaved by serine proteases from the host, which generally takes place in the mucosal tract (38). As the cleavage site is located on the HA stem, antibodies directed against this region of the HA protein can block cleavage of HA0 thereby preventing the virus to be infectious. The third mechanism by which the HA stem directed antibodies can directly neutralize the influenza virus is by binding to the newly produced HA protein on the host cell surface and thereby block viral budding and egress (39). Last but not least, HA stem binding antibodies can interfere with the infection and replication of influenza viruses via Fc mediated effector mechanisms. In vivo, Fc mediated effector mechanisms such as ADCC have been demonstrated to enhance the cross-protective efficacy of HA stem directed antibodies (35, 37).

While the dominant antibody response directed against the HA head is usually strain specific, a few broadly binding, neutralizing and non-neutralizing, antibodies targeting the HA head domain have also been identified (40-42). Although the broadly neutralizing antibodies can directly neutralize the virus, it has recently been shown that their in vivo protective efficacy is partly dependent on Fc mediated effector mechanisms. In fact, also the broadly binding, non-neutralizing, HA head antibodies interfere with the viral life cycle via Fc mediated mechanisms(43). In Figure 2B a schematic of a trimeric HA protein and the binding site of the various anti-HA antibodies is presented.
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Cross-reactive antibodies against NA and M2

The second most prevalent surface protein on the influenza virus is the NA protein. Although NA is also genetically variable, conserved NA epitopes have been identified (44, 45). The main mechanism by which NA specific antibodies protect against influenza is by inhibition of viral budding and egress (45). However, recently, studies have demonstrated that protection by broadly binding anti-NA antibodies in mice is dependent of Fc mediated effector mechanisms (43). The cross-protective efficacy of NA based vaccines has shown to be typically restricted to heterologous viruses of the same subtype (46, 47). A universal influenza vaccine is therefore likely not to be based solely on NA but could benefit from its incorporation.

**Figure 2. The HA protein.** A) An H1 HA trimer is colored coded according to an amino-acid conservation index (red color corresponds to most conserved and blue color to least conserved) corresponding to all H1 HA strains B) Illustration of target domains for anti-HA antibody binding. Illustration modified from Brandenburg et al. PloS One, 2013(30).
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The third surface protein on the influenza virus is M2. Antibodies directed against the ectodomain of M2 (M2e) are generally non-neutralizing and consequently do not prevent infection. Instead protection induced by M2e is suggested to be mediated via Fc mediated mechanisms (48). M2e is highly conserved making it an interesting target for broadly protective vaccines (48-50). A few M2e based vaccines have been tested in phase 1 clinical trials for safety and immunogenicity however with variable results (51-53). A cartoon illustrating the mechanisms of action by anti- HA, -NA and -M2 antibodies is presented in Figure 3.

Cross-reactive cellular immune responses

While cellular immune responses cannot prevent infection and thus cannot mediate sterile protection they can enhance the viral clearance and decrease the severity of disease. Next to providing help to B cells, enabling high production of antibodies, CD4 and CD8 T cells can function as effector cells and kill infected cells. The internal influenza proteins NP and M1 are highly conserved (54, 55) and CD8 T-cell responses against these proteins are broadly reactive and have demonstrated to mediate heterosubtypic protection in pre-clinical animal models (56-58). In humans the presence of cross-reactive CD8 T-cell responses has been suggested to correlate with protection against severe illness after infection with pandemic H1N1 and H7N9 viruses (59).

As discussed, cross-protection against influenza viruses can be mediated by a large number of mechanisms targeting various influenza proteins. For the development of novel, universal or broadly protective, influenza vaccines various approaches targeting the different influenza antigens and distinct stages of the viral life cycle are being researched.
**Figure 3. Mechanism of action by HA, NA and M2 specific antibodies.** Antibodies directed against the HA head (red), HA stem (green), NA (blue) and M2 (yellow) can mediate protection via a number of mechanisms. When the influenza virus enter the host antibodies directed against the HA head can prevent viral attachment to the host cell by blocking the interaction between HA and the sialic acids (step 1). Antibodies directed against the HA stem can further prevent fusion of the viral and endosomal membranes (step 2). Antibodies directed against the HA head, HA stem and NA can prevent budding and viral egress (step 3). HA-stem antibodies can prevent maturation of newly formed viruses by blocking cleavage of precursor HA0 (step 4). HA head, HA stem, NA and M2 specific antibodies can also indirectly neutralize the influenza virus via Fc mediated effector mechanisms (step 5). Illustration has been modified from F. Krammer et al(28). Illustration has been reprinted by permission from Macmillan Publishers Ltd: Nature reviews, Krammer et al(28), copyright 2015.
THEESIS SCOPE: CROSS-PROTECTION BY SEASONAL INFLUENZA VACCINES

As the majority of the broadly protective vaccine approaches are in the early stages of development, it is fair to assume that it will take a number of years before any of these vaccines will be in a position to impact future pandemics. In this thesis, we explore whether the protective efficacy of a trivalent virosomal seasonal influenza vaccine (TVV) can be broadened and thereby increase pandemic preparedness until more broadly protective influenza vaccines may become available.

Despite the general belief that seasonal influenza vaccines only elicit strain specific antibodies, broadly neutralizing antibodies directed against conserved regions of the HA stem have been isolated from human serum after vaccination with seasonal influenza vaccines(32, 34, 37). Furthermore, a systematic meta-analysis demonstrated that seasonal vaccines can in fact provide a degree of cross-protection also against non-matching circulating strains (10). Together, these findings suggest that the current seasonal influenza vaccines may confer a broader range of protection than previously anticipated. Next to HA many of current seasonal influenza vaccines contain also other influenza proteins such as NA, NP and M2 (60, 61). As earlier described, immune responses against these proteins have demonstrated the ability to mediate cross-protection. It is therefore plausible that immunity against these proteins could contribute to cross-protection mediated by seasonal influenza vaccines. The research described in this thesis focuses on further exploring whether adjusting the vaccination regimen can improve the cross-protective efficacy of TVV and the potential mechanisms behind such protection.

As the current correlate of protection, HI titer, only predict protection mediated by rather strain specific antibodies directed against the HA head, this assay will not accurately predict cross-protection mediated by antibodies against the HA stem or other conserved influenza proteins. Therefore, the HI assay will underestimate the cross-protective efficacy of seasonal influenza vaccines. Since there is no established correlate of protection for cross-protection, we elected not to estimate vaccine mediated protection from cross-reactive immunogenicity but rather from protective efficacy, as defined by survival in lethal challenge models. We subsequently determined which immunogenicity parameters were correlated to the protection observed in animal models.

In chapter 2-4 we explore the ability of 3 different vaccination regimens to broaden and improve the protective efficacy induced by a TVV using pre-clinical animal models:

Chapter 2 examines the ability of a vaccination regimen comprising multiple immunizations to improve the cross-protective efficacy of TVV in mice.
**Chapter 3** explores whether priming a TVV with vaccine homologous HA DNA can improve its efficacy of inducing heterologous H1N1 and heterosubtypic H5N1 protection in mice. The cross-protective efficacy of the heterologous prime/boost regimen is evaluated in parallel with the multiple vaccination regimen identified in chapter 2.

**Chapter 4** investigates the ability of improving the cross-protective efficacy of TVV by adjuvating with Matrix M. The adjuvated vaccination regimen is evaluated for cross-protection against avian H5N1, H7N7 and H7N9 influenza viruses in both mice and ferrets.

Leveraging two phase 1 clinical trials we in chapter 5-6 further evaluate the cross-reactivity and cross-protective efficacy of the humoral immune response elicited by seasonal and pandemic influenza vaccines in humans:

**Chapter 5** assesses the cross-protective efficacy of TVV in healthy adults. By making use of a novel human-to-mouse serum transfer and challenge model we evaluated the cross-protective efficacy of the humoral immune response induced by 1, 2 and 3x TVV in healthy adults.

**Chapter 6** further explores the role of ADCC in HA specific cross-reactivity and cross-protection induced by TVV and pandemic influenza vaccine in healthy adults.

**Chapter 7** comprises a summary and discussion of the findings presented in this thesis. The results are discussed in the context of the influenza vaccine field and the future perspective of broadly protective influenza vaccines.
REFERENCES


