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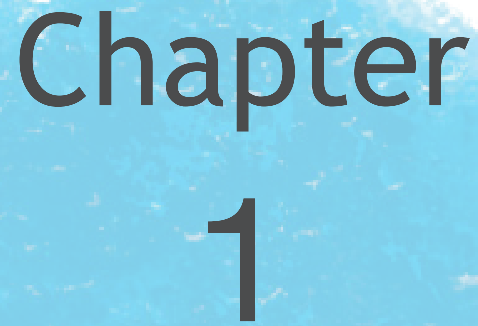

General Introduction

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Influenza infection and disease

Influenza virus is the causative agent of the flu, a mild to severe respiratory disease. It is an infectious disease, which is transmitted through the air by means of droplets, aerosols or via direct contact. Each year, influenza virus infections cause approximately 3-5 million cases of severe illness and 250,000-300,000 influenza-related deaths worldwide, resulting in an enormous social and economic impact on society (1). In addition to these seasonal epidemics, influenza virus can cause pandemics, infecting millions of individuals worldwide (2, 3). A mild influenza virus infection causes fever (38-40°C), headache, chills, dry cough and malaise lasting for 7-10 days (4). A more severe infection can cause viral pneumonia and acute respiratory distress syndrome (ARDS), which might even have a fatal outcome. Secondary complications are often caused by a superinfection with bacteria, leading to a bacterial pneumonia, and are a major cause of influenza-related deaths (5). Elderly and immunocompromised are exceptionally prone to such influenza-related illness due to an ineffective immune response, and are therefore considered for annual seasonal vaccination (6). In contrast to seasonal influenza virus, individuals of all age groups are affected during pandemic outbreaks. In the past century, at least four pandemics have occurred. These pandemics have differed in their severity, the most recent pandemic in 2009, for example, resulted in an estimate of 300,000 influenza-related deaths (7). On the other hand, the most severe pandemic in history, which occurred during 1918-1919, infected one third of the world's population and led to an estimated 50 million influenza-related deaths (8).

Influenza virus proteins and functions

Influenza virus is a single-stranded segmented RNA virus that belongs to the family of the Orthomyxoviridae. Influenza virus can be categorized into three virus types: Influenza A, B and C. In this thesis, we will focus on influenza A, since this subtype is expected to pose the greatest threat to the human population. The viral genome of influenza A consists of eight RNA segments, encoding eleven different proteins (**Figure 1**). The envelope of the viral particle is covered by hemagglutinin (HA) and neuraminidase (NA) and inserted in the lipid bilayer is the transmembrane protein matrix protein 2 (M2). HA is responsible for binding the virus to host cells by interacting with sialic acid, which is present on most cells in the host including cells in the upper respiratory tract. Then, the virus particle is taken up by a host cell via receptor-mediated endocytosis. The acidic environment in the endosome triggers a conformational change in the HA protein, upon which a fusion peptide in the HA2 subunit is inserted in the target endosomal membrane. This conformational change finally results in a hairpin formation, which induces such close contact between the viral and endosomal membrane that a fusion pore is created. The release of the virion contents is further mediated by the ion channel activity of integral membrane protein M2, which disrupts protein-protein interactions resulting in the release of free ribonculeoprotein (RNP) complex. The enzymatic protein NA is involved in virus budding and mediating release of newly formed virus particles

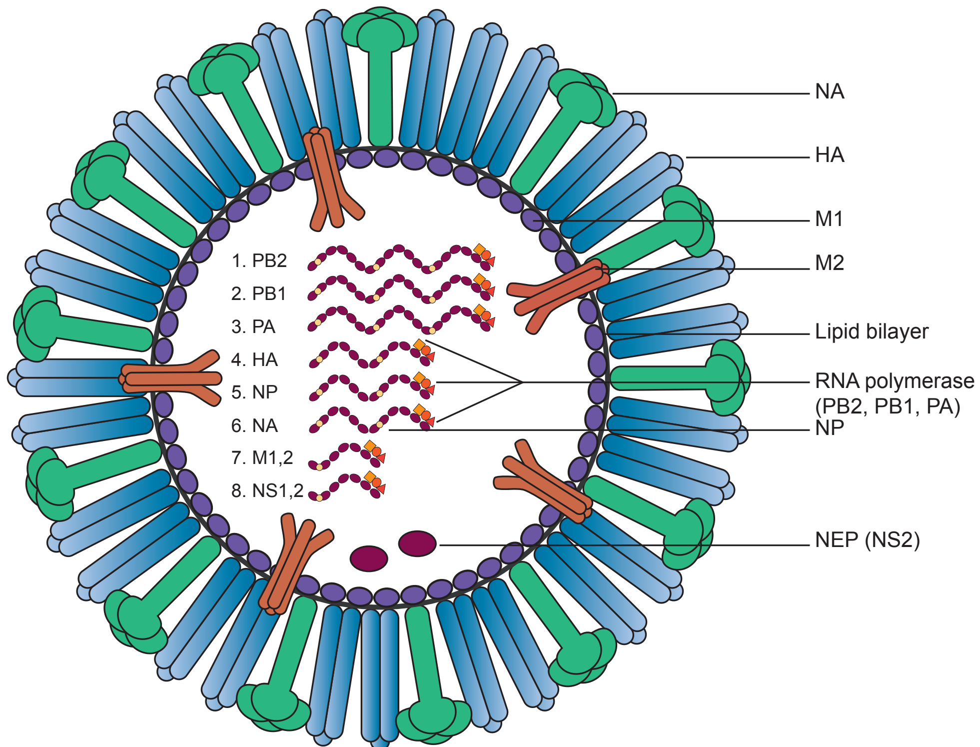

Figure 1: Structure of influenza virus particle
The viral genome of influenza A consists of eight single stranded RNA segments, as depicted by numbers 1 to 8. The envelope of the viral particle is covered by hemagglutinin (HA) and neuraminidase (NA). Transmembrane protein matrix protein 2 (M2) is inserted in the lipid bilayer. The viral envelope is lined by matrix protein 1 (M1). The viral core consists of the ribonucleoprotein (RNP) complex, of which the main viral protein is nucleocapsid protein (NP). Other proteins in the RNP complex are polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) together forming the RNA polymerase. Finally, the NS gene segment encodes for the last two proteins: Non-structural protein 1 (NS1) and nuclear export protein (NEP or NS2) (12).

from the surface of infected cells, by removing sialic acid receptor from the surface of the cell and from the HA viral protein (9).

The viral envelope is lined by matrix protein 1 (M1), a structurally important protein. The viral core consists of the ribonucleoprotein (RNP) complex, which is released from the virion upon fusion and is subsequently transported into the nucleus of the host cell, where the viral RNA serves as a template for replication and transcription. The main viral protein in the RNP complex is nucleocapsid protein (NP), which condensates viral RNA. Other proteins in the RNP complex are polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA) together forming the RNA polymerase. PB1-F2 is a protein encoded by an alternative ORF of the PB1 RNA segment, PB1-F2 is a determinant for the

pathogenesis and is involved in evasion of the innate immune response. Finally, the NS gene segment encodes for the last two proteins: Non-structural protein 1 (NS1), which plays an important role in immune evasion, among others by inhibiting the action of interferon produced by the host, and nuclear export protein (NEP), also named non-structural protein 2 (NS2), which is involved in splicing and nuclear export of viral mRNAs (10, 11).

When viral mRNAs have been translated, membrane surface proteins undergo further post translational modifications such as glycosylation. Importantly, HA is cleaved into subunits HA1 and HA2 linked via a disulfide bridge, which is required for HA to obtain the fusion active state. Proteases, responsible for this cleavage, are restricted to the tissue of the respiratory tract, which explains the limitation of influenza infection to this compartment (12). Some avian viruses, e.g. H5N1, have a multibasic cleavage site, which can also be cleaved by proteases outside of the respiratory tract and therefore allows them to spread systemically (13).

Antigenic drifts and shifts

There are 18 different HAs and nine NAs that can be distinguished serologically, based on which influenza A viruses can be further subtyped. Due to lack of a proofreading mechanism, errors during RNA replication are not corrected and point mutations can accumulate. These can lead to gradual, minor changes in the proteins encoded by the influenza genome. Since the immune pressure on the surface proteins HA and NA is very strong, favorable changes in these proteins are maintained which enables influenza virus to escape existing humoral immunity against these proteins. This phenomenon is called antigenic drift and is responsible for the annual influenza epidemics. In addition to the gradual changes caused by antigenic drifts, influenza virus can also undergo antigenic shifts. Since influenza virus is a segmented RNA virus, reassortment of genes can occur between two different influenza viruses resulting in such a shift. When, for example a single cell is infected with a currently circulating human virus of the subtype H1N1 or H3N2 and an avian virus of the subtype H5N1 or H7N9, genes of these viruses can reassort, resulting in a new virus containing genes of human and avian origin. This may result in a virus that contains the surface proteins of avian influenza and also has the ability to efficiently replicate in humans. Since individuals will not have pre-existing immunity against the surface proteins of such a virus, this poses a great threat to the human population.

Especially pigs are pointed out as so-called mixing vessels, since pig cells express both α2,3-linked sialic acid (SA), the receptor for avian influenza viruses and α2,6-linked SA, the receptor for human influenza viruses and can therefore be infected by viruses of both origins (14). In 2009, a virus containing genes from swine, avian and human origin was introduced into the human population through pigs, resulting in a pandemic (15). Another threat is that influenza viruses occasionally cross the species barrier. In the past two decades, several viruses from avian origin, of H5N1, H7N7 and H7N9 subtypes, have been shown to infect humans (16-18). Since 2013, outbreaks of H7N9 have been reported in China which resulted in the death of

approximately one third of infected individuals. To date, these avian viruses have not been shown to be transmitted from human to human (19). However, a few mutations may result in changes in the virus enabling human to human transmission and due to the absence of pre-existing immunity in the human population such a virus may cause a pandemic (20).

Treatment and prevention of influenza virus infection

There are several anti-viral treatments available, such as NA and M2 inhibitors, which also relieve symptoms of influenza virus infection (21). Neuraminidase inhibitors are generally effective in adults, but not in asthmatic children (22). Furthermore, one class of NA inhibitors have been shown to induce toxic effects, while M2 inhibitors are ineffective against 90% of H1N1 and H3N2 viruses (11, 23). More recently, the use of antibodies directed to the conserved stalk domain of the HA protein have been discussed as a therapeutic treatment (24). However, most countries reduce influenza disease burden by preventing disease instead of treatment of symptoms and therefore provide influenza vaccination. In the Netherlands, influenza vaccination is recommended for risk groups including individuals over 60 years of age, individuals with cardiac, vascular or lung diseases and individuals with other medical conditions rendering them more vulnerable to influenza infection (25). There are several types of seasonal vaccines available, which can generally be divided into live attenuated influenza virus (LAIV) vaccines and inactivated vaccines, such as whole inactivated virus (WIV), subunit, and split vaccines (26). These vaccines are mostly designed to induce neutralizing antibodies to HA. However, $CD4^+$ and $CD8^+$ T cells do play an important role in assisting the induction of an antibody response and clearing virally infected cells, respectively.

Most seasonal vaccines are administered intramuscularly, an exception is LAIV, which is administered intranasally. LAIV is adapted to grow at lower temperatures than 37°C, thereby limiting replication and preventing spread in humans. Since it establishes a limited infection via the natural route of entry it is capable of inducing mucosal and cellular immunity (27). WIV is manufactured by inactivation of viral particles by treatment with formalin or betaproteolactone (BPL). Both methods keep the viral particles structurally intact, but only treatment with BPL maintains the fusion activity of the particles. This has been shown to be an advantage for the induction of $CD8^+$ responses, since internal viral antigens are still delivered to the cytosol to be processed by the APC and subsequently presented to $CD8^+$ T cells (28). Split vaccines are produced by treating influenza virus particles with diethyl ether or detergent, causing the viral particles to lose their integrity, resulting in a mix of proteins. Split vaccines contain all viral proteins, although virus ssRNA is mostly lost. During the production process of subunit vaccines, the nucleocapsid is removed leaving only surface proteins HA and NA.

LAIV and WIV have the advantage of containing all influenza proteins. However, LAIV is not that suitable for use as a pre-pandemic vaccine since it can theoretically undergo reassortment with circulating seasonal viruses, resulting in a new virus. In addition, LAIV is less effective in

adults than in children, likely due to capture of the vaccine antigens by pre-existing immunity. The major problem with WIV vaccines occurs during production, causing lack of purity leading to adverse reactions. Although new production processes may overcome these issues, WIV vaccines are currently only used for pre-pandemic vaccines (29). Nonetheless, the most commonly used seasonal vaccines are inactivated subunit or split vaccines. Since various seasonal strains circulate in the human population, each year a selection is made of two influenza A strains and one influenza B strain (trivalent vaccine) or two influenza B strains (quadrivalent vaccine). Each year, the WHO recommends the constituents based on predictions of what strains will circulate in the Northern and Southern Hemisphere during the next influenza season, resulting in two vaccine compositions each year. When the strains in the vaccine match those circulating among the human population, influenza vaccination is quite effective. However, a suboptimal match already decreases vaccine efficacy dramatically (30). In addition, in young children and elderly, vaccines designed to induce antibodies are less effective than in (young) adults. In elderly, both qualitative and quantitative changes in the humoral response lead to ineffective antibody responses (31). In children, annual influenza vaccination with subunit and split vaccines induces a response towards a limited set of antigens. Upon natural infection, vaccination-induced antibodies will neutralize influenza virus particles, thereby hampering the development of a broad cross-reactive response to influenza virus. Therefore, subunit and split vaccines are suboptimal inducers of the immune response to influenza, especially in young children and elderly (32-34). Since the main mechanism of protection induced by subunit and split vaccines is by induction of antibodies directed to the highly variable HA protein, vaccine-induced antibodies are less effective against drift variants and are ineffective against newly emerging influenza A subtypes. Vaccines directed to more conserved parts of the virus could be more effective, since they would induce cross-reactive immunity.

Role of Toll-like receptors (TLRs) in infection and vaccination

Upon infection with influenza virus, host cells detect the virus through pattern recognition receptors (PRRs). PRRs recognize pathogen associated molecular patterns (PAMPs) of influenza virus, which trigger the initiation of antiviral signaling cascades. These signaling cascades result in an antiviral response, such as the production of interferons (35). In addition, they instruct the adaptive immune system regarding the type of response required. The main PAMP of influenza virus is ssRNA, which is recognized by TLR7 (36). TLR-ligands can also be exploited for vaccine development by using them to stimulate the immune system. The use of TLR-ligands can aid in reducing the amount of antigen required, broadening of the immune response or steering the adaptive immune response in the desired direction. The TLR2/1 ligand Pam3CSK4, for example, is a lipopeptide often used in experimental vaccine formats. Administration of Pam3CSK4 has been shown to aid in stimulation of B and T cell responses. Another often used ligand is Monophosphoryl lipid A, a derivative of LPS, which specifically stimulates TLR4. Due to its promising potency this ligand is now also produced as a clinical grade version. TLR9 ligands are another class of ligands now being developed for

clinical use. TLR9 normally recognizes unmethylated CpG rich sequences in DNA molecules, which are often located in bacterial genomes and viral DNA. By developing a CpG motif-containing oligodeoxynucleotide (ODN), which interacts with TLR9, a Th1 type of response can be induced which is especially helpful for preventive vaccination strategies for infectious diseases (37).

Humoral immune response to influenza

In addition to innate immune responses, which are the first line of defense against influenza virus infection, adaptive immune responses are able to neutralize and clear influenza virus. One of the most important correlates of protection for influenza infection are antibodies (38). When influenza virus particles enter the body, B cells can recognize the antigen and become activated. Activation of B cells leads to the development of short-lived plasma cells, responsible for the production and secretion of primary antibodies. In secondary lymphoid organs, B cells differentiate into long-lived plasma cells or memory B cells. In a process called affinity maturation, somatic hypermutation and clonal selection will lead to the presence of antibodies with greater affinity to antigen. Repeated exposure of a host to the same antigen will therefore lead to the production of antibodies with increasing affinity. Memory B cells will form plasma cells which continuously secrete antibodies, resulting in a fast response upon a new encounter with the antigen. Hereby, antibody-mediated immunity can provide protection for many years following the first encounter (39).

Antibodies are subdivided into several immunoglobulin isotypes, i.e. IgA, IgD, IgE, IgG, and IgM. IgM is expressed on immature B lymphocytes and is therefore the first isotype that is expressed during B cell development. During the maturation process, B cells also begin to express IgD, which is only secreted in small amounts. Following further activation of B cells, mature B cells undergo isotype switching, which causes the production of the other isotypes IgE, IgA, and IgG. IgE is mainly involved in allergic reactions and parasitic infections and does not play an important role in influenza infection. IgA, on the other hand, is found in mucosal sites such as the respiratory tract and is consequently an important isotype in the humoral immune response to influenza. However, the systemic antibody IgG and its four subforms IgG1, IgG2a, IgG2b and IgG3 provide the majority of humoral immunity. Which immunoglobulin subtype is secreted is largely dependent on the cytokines produced by T helper cells. IL-4, for example, induces class switching to IgG1 and IgE, while the production of IFN-γ leads to the expression of IgG3 and IgG2a (40).

For influenza virus immunity, only HA-specific antibodies are capable of preventing infection by neutralization of the virus particles (**Figure 2**), antibodies directed to NA prevent release of new virions and thereby reduce spread of the virus. However, the globular head of HA and NA protein are highly variable, allowing the virus to escape from previously induced antibodies. Antibodies that target more conserved parts of the virus, such as antibodies directed to the fusion peptide or stalk domain of HA and to the ectodomain of M2 (M2e) (41-46) may provide

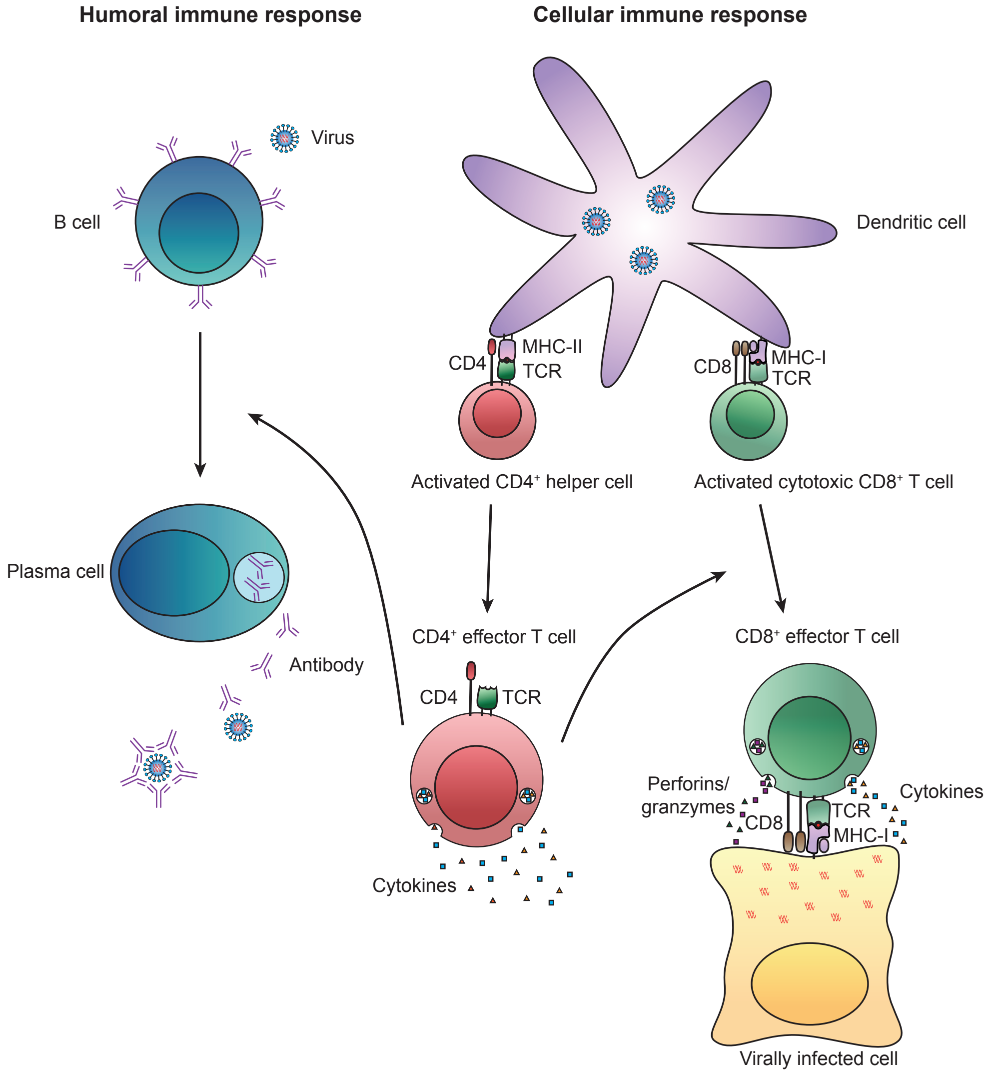

Figure 2: Immune response to influenza
The humoral response is the first line of defense of the adapted immune response against influenza virus infection. Antibodies directed to the surface proteins of influenza hemagglutinin are capable of neutralizing virus particles. When, despite the humoral response host cells are infected, a cellular immune response is needed to clear virus from the host. APCs, such as DCs present virus-specific peptides in the context of MHC class I or MHC class II. Influenza-specific $CD4^+$ T cells can recognize the peptide in combination with MHC class II, leading to its activation. The activated $CD4^+$ T cell can then provide help to B cells and $CD8^+$ T cells in the form of cytokine production. $CD8^+$ T cells recognize peptides in the context of MHC class I, after which they are activated. Activated $CD8^+$ T cells can then kill virally infected cells by the release of perforins and granzymes.

broader protection. However, thus far no vaccines are available that induce neutralizing antibodies directed to more conserved parts of the protein.

Cellular immune response to influenza

Cellular immune responses compose the second arm of the adaptive immune response (**Figure 2**). The cellular arm of the adaptive immune response consists of $CD4^+$ and $CD8^+$ T cell responses. The main task of $CD4^+$ T cells is to provide help to B and T cells by the production of cytokines, while $CD8^+$ T cells are crucial for clearance of influenza virus from the host. T cells can be activated by antigen presenting cells (APCs). During an infection, APCs such as dendritic cells and macrophages can take up extracellular proteins, which are degraded in endosomal compartments. Concurrently, the antigen presenting molecule major histocompatibility complex (MHC) class II is synthesized in the ER of APCs and transported to the Golgi apparatus where the compartment containing MHC class II can fuse with an endosomal compartment containing the degraded protein fragments. Then the antigenic protein fragments are loaded on MHC class II and the complex is then trafficked to the cell surface and presented to T cells (47). Humans have three pairs of MHC class II genes, HLA-DR, HLA-DQ, and HLA-DP. Each of these proteins can bind a different range of peptides, resulting in a broad range of peptides that can be presented on MHC class II. The MHC class II-peptide complex can interact with the T cell receptor (TCR) on $CD4^+$ T cells. $CD4^+$ T cells that recognize the peptide in combination with MHC class II, are activated and can then provide co-stimulation through CD40-CD40 ligand interaction with the APC presenting the viral peptide. This interaction can activate APCs such as DCs, but can also induce class switching of B cells enabling them to produce IgG, IgA, or IgE antibodies. Furthermore, $CD4^+$ T cells produce cytokines such as IL-4 and IFN-γ, which aid B cells and $CD8^+$ T cells. Recently, cytotoxic $CD4^+$ T cells have even been described that are capable of exerting cytotoxic functions themselves, such as the production of IFN-γ and the ability to kill cells via a perforin/granzyme mediated pathway. Pre-existing $CD4^+$ T cells were shown to result in lower virus shedding and reduced disease severity (48).

For clearance of influenza virus from the host, however, $CD8^+$ T cell responses are crucial. Viruses can infect cells directly, leading to viral replication inside the cells. Endogenous proteins in the cytosol will be degraded by the proteasome and peptide fragments will be transported into the ER via Transporter Associated Protein (TAP). Here, the peptide epitopes will be loaded onto the MHC class I antigen presenting molecule. MHC I contains two binding pockets, restricting the length of the peptide to 8-10 amino acids. Humans have three major classes of MHC class I molecules, named HLA-A, HLA-B and HLA-C. Each individual presents a different set of HLA-molecules that have their own peptide-binding requirements. When peptides fulfill the criteria of the MHC class I molecule, they can associate and a peptide-MHC class I (pMHCI) complex is formed. This complex is then transported to the cell surface of APCs via the Golgi network (47). APCs present the pMHCI complex to $CD8^+$ T cells and the

TCR of these cells can then recognize the pMHCI complex. Together with co-stimulation via CD80/86 and CD28, this will result in activation of the $CD8^+$ T cells.

Activated $CD8^+$ T cells produce cytokines such as IFN-γ, which further induce their cytotoxic function. In addition, $CD8^+$ T cells are capable of recognizing virally infected cells presenting viral peptides in the context of MHC class I and can then kill these cells by secreting granzymes and perforins (49). When the virus has been cleared from the body, a small part of the $CD8^+$ T cell population will develop into a memory pool. These memory $CD8^+$ T cells are long-lived and will constantly circulate through the body so that they can react quickly in the event of a secondary infection. Cross-reactive $CD8^+$ T cells have been shown to limit disease severity and improve recovery from disease in a pandemic situation (50, 51). Furthermore, a study in elderly described that T cell responses were a prediction for the development of influenza related illness, while antibody responses were not (34). In addition, T cell responses are often directed to more conserved parts of the virus (52-54). These findings warrant the development of a new type of influenza vaccine, which is designed to induce not only antibody- but also T cell responses.

One such strategy is peptide based-vaccines, which are mostly designed to induce cellular responses, but are also capable of inducing antibodies. Peptides can be selected that are directed to conserved sequences of influenza virus internal proteins, thereby eliciting cross-reactive immunity. Short linear peptides can be presented directly on MCH class I molecules and presentation of the pMHCI complex in combination with co-stimulatory molecules can then activate $CD8^+$ T cells. Longer peptides need processing by professional APCs, but are then capable of inducing both peptide-specific $CD4^+$ and $CD8^+$ T cells. Peptide-based vaccines can also be applied to induce antibody responses. Although B cells are mostly activated by the recognition of folded antigens, a few linear B cell epitopes are described in literature (55, 56). Since peptide-based vaccines are well-defined and easy to produce under GMP they are an attractive candidate for application in human vaccines.

Thesis outline

In this thesis, immune responses towards the highly variable influenza virus are described in the scope of a vaccination setting. We focus on conserved epitopes that mainly induce cellular responses and on the development of peptide vaccination strategies targeting these conserved epitopes. In **Chapter 2**, a general overview is provided of T cell responses to acute and chronic virus infections and how this influences the development of peptide vaccination strategies. In **Chapter 3**, we describe a clinical trial in which vaccine-specific humoral and cellular immune responses are measured in a cohort of healthy subjects during two consecutive influenza seasons, including the season in which the 2009 pandemic virus emerged. Antibody and T cell levels were determined following vaccination with an adjuvanted and unadjuvanted influenza subunit vaccine. Furthermore, analysis of booster effect of vaccination and duration of the responses were analyzed. Although these subunit vaccines were shown to induce both antibody and T cell responses, these are unlikely to be cross-protective against newly emerging subtypes, since the high variability of the different HA and NA subtypes leads to a lack of cross-reactivity of vaccine-induced antibodies and even if conserved T cells are induced, they will not provide cross-protection. Therefore, the other chapters in this thesis describe concepts within peptide-based vaccination strategies, which is one method to induce responses to highly conserved sequences of influenza virus (57).

In **Chapter 4**, we evaluated a peptide vaccine concept based on long peptides directed to highly conserved B and T cell epitopes. Such long peptides require processing by professional APCs, thereby activating both $CD4^+$ and $CD8^+$ T cells. The B epitopes were coupled to CD4 helper peptides to enhance their immunogenicity. Challenge studies were performed in C57BL/6 mice, BALB/c mice, and ferrets to evaluate immunogenicity and efficacy of these vaccine constructs. Immunogenicity was determined by analyzing vaccine-specific antibody and T cell responses, while efficacy was assessed by viral load in the lungs and parameters for morbidity of influenza infection, such as decrease in bodyweight . In **Chapter 5**, a strategy is described in which minimal influenza peptides were modified to enhance binding affinity to MHC class I. Non-natural modifications were inserted in WT influenza epitopes, leading to chemically enhanced altered peptide ligands (CPLs). Then, the effect of enhanced binding affinity on T cell responses was evaluated by vaccination of HLA-A2 transgenic mice with these CPLs and analyzing responses to these peptides ex vivo. **Chapters 6 and 7** describe formulations that improve delivery of minimal peptides. In **Chapter 6**, an influenza-specific epitope is formulated with virosomes and the immunogenicity of this formulation is evaluated in an HLA-A2 transgenic mouse model. In **Chapter 7**, the potential of using WIV as an adjuvant to enhance the immunogenicity of WT epitopes and CPLs is described.

References

1. WHO. Influenza Factsheet. 2014; 211:(Available from: http://www.who.int/mediacentre/factsheets/fs211/en/.
2. Iskander, J., et al., Pandemic influenza planning, United States, 1978-2008. Emerg Infect Dis, 2013. 19(6): p. 879-85.
3. Kilbourne, E.D., Influenza pandemics of the 20th century. Emerg Infect Dis, 2006. 12(1): p. 9-14.
4. Fiore, A.E., et al., Prevention and control of influenza: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2008. MMWR Recomm Rep, 2008. 57(RR-7): p. 1-60.
5. Nicoll, A., et al., Influenza-related deaths--available methods for estimating numbers and detecting patterns for seasonal and pandemic influenza in Europe. Euro Surveill, 2012. 17(18).
6. Nicoll, A., et al., The scientific basis for offering seasonal influenza immunisation to risk groups in Europe. Euro Surveill, 2008. 13(43).
7. Dawood, F.S., et al., Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. Lancet Infect Dis, 2012. 12(9): p. 687-95.
8. Taubenberger, J.K. and D.M. Morens, 1918 Influenza: the mother of all pandemics. Emerg Infect Dis, 2006. 12(1): p. 15-22.
9. Sugrue, R.J. and A.J. Hay, Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. Virology, 1991. 180(2): p. 617-24.
10. Wilschut, J.M., J.E.; Palache, A.M., Influenza. Second ed. 2006, Edinburgh: Elsevier. 240.
11. Knipe, D.M., Howley, P.M., Fields Virology. 2013: Lippincott Williams & Wilkins.
12. Wilschut, J.M., J.E.; Palache, A.M., Influenza. second ed. 2006: Elsevier.
13. Garten, W., et al., Influenza virus activating host proteases: Identification, localization and inhibitors as potential therapeutics. Eur J Cell Biol, 2015.
14. Ito, T., et al., Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol, 1998. 72(9): p. 7367-73.
15. Neumann, G., T. Noda, and Y. Kawaoka, Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature, 2009. 459(7249): p. 931-9.
16. Shortridge, K.F., et al., Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology, 1998. 252(2): p. 331-42.
17. Gao, R., et al., Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med, 2013. 368(20): p. 1888-97.
18. Du Ry van Beest Holle, M., et al., Human-to-human transmission of avian influenza A/H7N7, The Netherlands, 2003. Euro Surveill, 2005. 10(12): p. 264-8.
19. Li, Q., et al., Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med, 2014. 370(6): p. 520-32.
20. Horimoto, T. and Y. Kawaoka, Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev, 2001. 14(1): p. 129-49.
21. Stiver, G., The treatment of influenza with antiviral drugs. CMAJ, 2003. 168(1): p. 49-56.
22. Jefferson, T., et al., Neuraminidase inhibitors for preventing and treating influenza in healthy adults and children. Cochrane Database Syst Rev, 2014. 4: p. CD008965.
23. Moscona, A., Neuraminidase inhibitors for influenza. N Engl J Med, 2005. 353(13): p. 1363-73.

24. Krammer, F. and P. Palese, Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr Opin Virol, 2013. 3(5): p. 521-30.
25. Netherlands, H.C.o.t., Vaccinatie tegen seizoensgriep. 2011: Den Haag.
26. Amorij, J.P., et al., Development of stable influenza vaccine powder formulations: challenges and possibilities. Pharm Res, 2008. 25(6): p. 1256-73.
27. Barria, M.I., et al., Localized mucosal response to intranasal live attenuated influenza vaccine in adults. J Infect Dis, 2013. 207(1): p. 115-24.
28. Budimir, N., et al., Induction of heterosubtypic cross-protection against influenza by a whole inactivated virus vaccine: the role of viral membrane fusion activity. PLoS One, 2012. 7(1): p. e30898.
29. van Boxtel, R.A., et al., Safety and immunogenicity of influenza whole inactivated virus vaccines: A phase I randomized clinical trial. Hum Vaccin Immunother, 2015. 11(4): p. 983-90.
30. Flannery, B., et al., Early estimates of seasonal influenza vaccine effectiveness - United States, January 2015. MMWR Morb Mortal Wkly Rep, 2015. 64(1): p. 10-5.
31. Frasca, D. and B.B. Blomberg, Aging affects human B cell responses. J Clin Immunol, 2011. 31(3): p. 430-5.
32. Bodewes, R., et al., Annual vaccination against influenza virus hampers development of virus-specific CD8(+) T cell immunity in children. J Virol, 2011. 85(22): p. 11995-2000.
33. Michiels, B., et al., A systematic review of the evidence on the effectiveness and risks of inactivated influenza vaccines in different target groups. Vaccine, 2011. 29(49): p. 9159-70.
34. McElhaney, J.E., et al., T cell responses are better correlates of vaccine protection in the elderly. J Immunol, 2006. 176(10): p. 6333-9.
35. Randall, R.E. and S. Goodbourn, Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol, 2008. 89(Pt 1): p. 1-47.
36. Diebold, S.S., et al., Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science, 2004. 303(5663): p. 1529-31.
37. Duthie, M.S., et al., Use of defined TLR ligands as adjuvants within human vaccines. Immunol Rev, 2011. 239(1): p. 178-96.
38. Subbarao, K. and T. Joseph, Scientific barriers to developing vaccines against avian influenza viruses. Nat Rev Immunol, 2007. 7(4): p. 267-78.
39. Kaufmann, S.H., The contribution of immunology to the rational design of novel antibacterial vaccines. Nat Rev Microbiol, 2007. 5(7): p. 491-504.
40. Janeway, C.A., Jr., Travers, P., Walport, M., Shlomchik, M.J., Immunobiology. 6th ed. 2005, New York, USA: Garland Science Publishing.
41. Chun, S., et al., Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. Vaccine, 2008. 26(48): p. 6068-76.
42. Du, L., Y. Zhou, and S. Jiang, Research and development of universal influenza vaccines. Microbes Infect, 2010. 12(4): p. 280-6.
43. Cross, K.J., et al., Composition and functions of the influenza fusion peptide. Protein Pept Lett, 2009. 16(7): p. 766-78.
44. Fiers, W., et al., A "universal" human influenza A vaccine. Virus Res, 2004. 103(1-2): p. 173-6.
45. Magadan, J.G., et al., Biogenesis of influenza a virus hemagglutinin cross-protective stem epitopes. PLoS Pathog, 2014. 10(6): p. e1004204.

46. Throsby, M., et al., Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One, 2008. 3(12): p. e3942.
47. Neefjes, J., et al., Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol, 2011. 11(12): p. 823-36.
48. Wilkinson, T.M., et al., Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nat Med, 2012. 18(2): p. 274-80.
49. Murphy, K.T., P; Walport, M, Janeway's immunobiology. Vol. 7th edition. 2008, New York: Garland Science.
50. Sridhar, S., et al., Cellular immune correlates of protection against symptomatic pandemic influenza. Nat Med, 2013. 19(10): p. 1305-12.
51. Wang, Z., et al., Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T cells. Nat Commun, 2015. 6: p. 6833.
52. Liu, J., et al., Conserved epitopes dominate cross-CD8+ T-cell responses against influenza A H1N1 virus among Asian populations. Eur J Immunol, 2013. 43(8): p. 2055-69.
53. Grant, E., et al., Nucleoprotein of influenza A virus is a major target of immunodominant CD8+ T-cell responses. Immunol Cell Biol, 2013. 91(2): p. 184-94.
54. Chen, L., et al., Immunodominant CD4+ T-cell responses to influenza A virus in healthy individuals focus on matrix 1 and nucleoprotein. J Virol, 2014. 88(20): p. 11760-73.
55. Singh, H., H.R. Ansari, and G.P. Raghava, Improved method for linear B-cell epitope prediction using antigen's primary sequence. PLoS One, 2013. 8(5): p. e62216.
56. Sun, J., et al., Large-Scale Analysis of B-Cell Epitopes on Influenza Virus Hemagglutinin – Implications for Cross-Reactivity of Neutralizing Antibodies. Frontiers in Immunology, 2014. 5: p. 38.
57. Pica, N. and P. Palese, Toward a universal influenza virus vaccine: prospects and challenges. Annu Rev Med, 2013. 64: p. 189-202.