Immune responses against conserved influenza epitopes: Lessons for peptide vaccination strategies

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Chapter 1

General Introduction

General Introduction

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

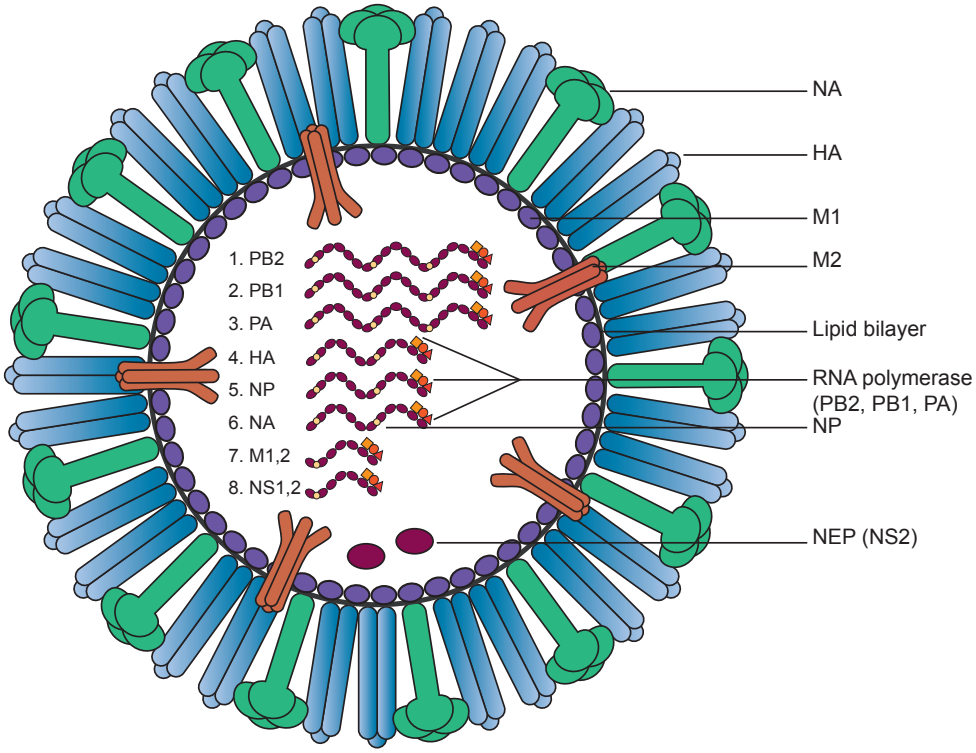

Figure 1: Structure of influenza virus particle
The viral genome of influenza A consists of eight single stranded RNA segments, as depicted by numbers 1 to 8. The envelope of the viral particle is covered by hemagglutinin (HA) and neuraminidase (NA). Transmembrane protein matrix protein 2 (M2) is inserted 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

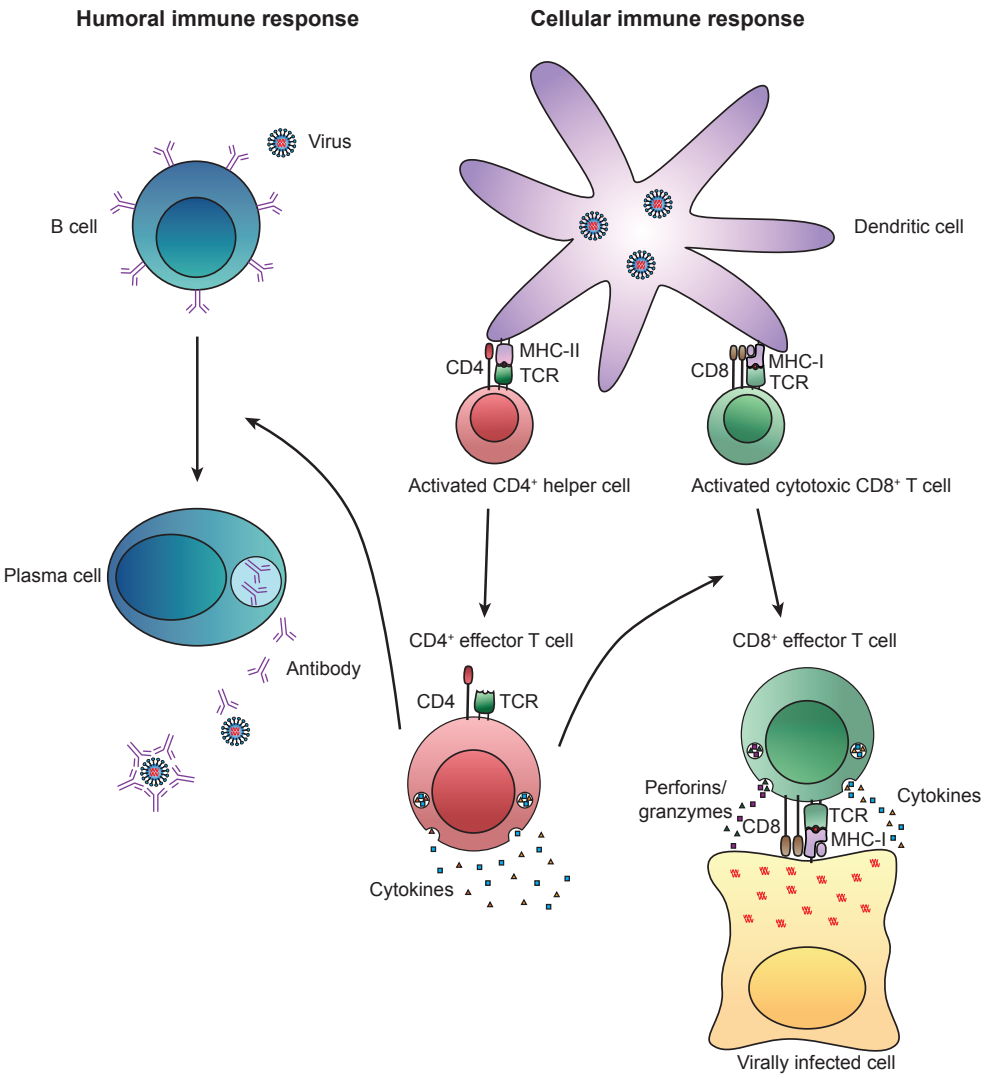

Figure 2: Immune response to influenza
The humoral response is the first line of defense of the adapted immune response against influenza virus infection. Antibodies directed to the surface proteins of influenza hemagglutinin are capable of neutralizing virus particles. When, despite the humoral response 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.

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Chapter 2

T cell responses to viral infections – opportunities for peptide vaccination

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Abstract

An effective immune response against viral infections depends on the activation of cytotoxic T cells that can clear infection by killing virus-infected cells. Proper activation of these T cells depends on professional antigen presenting cells, such as dendritic cells (DCs). In this review, we will discuss the potential of peptide-based vaccines for prevention and treatment of viral diseases. We will describe features of an effective response against both acute and chronic infections, such as an appropriate magnitude, breadth, and quality and discuss requirements for inducing such an effective antiviral immune response. We will address modifications that affect presentation of vaccine components by DCs, including choice of antigen, adjuvants, and formulation. Furthermore, we will describe differences in design between preventive and therapeutic peptide-based vaccines. The ultimate goal in the design of preventive vaccines, is to develop a universal vaccine that cross-protects against multiple strains of the virus. For therapeutic vaccines, cross-protection is of less importance, but enhancing existing T cell responses is essential. Although peptide vaccination is successful in inducing responses in Human Papilloma Virus (HPV) infected patients, there are still several challenges such as selecting the right target epitopes, choosing safe adjuvants that improve immunogenicity of these epitopes, and steering the immune response in the desired direction. We will conclude with an overview of the current status of peptide vaccination, hurdles to overcome, and prospects for the future.

Introduction

Viruses are small infectious agents that consist of nucleic acid that is coated in a simple protein shell or a cell-membrane-like protein casing, and need to infect host cells to replicate (1). Viruses can cause acute and chronic infections. In acute virus infections, such as a common cold, the virus is typically cleared from the body within a week. However, in some cases, an acute infection is followed by persistence of the virus in the host. Herpes Simplex Virus is an example of a virus causing a persistent infection, due to ability of the virus to hide in neurons. Often, these types of persistent infections do not cause any symptoms in healthy hosts (2). Chronic infections are a type of persistent infection often caused by an inefficient immune response of the host, leading to long-lasting symptoms. Especially acute and chronic virus infections have a major general health impact. Annual influenza epidemics, for instance, result in about 3-5 million cases of severe illness and approximately 250,000-500,000 deaths worldwide (3). An example of a chronic infection causing major health impact is Human Immunodeficiency Virus (HIV). In 2012, more than 35 million people were living with an HIV infection and 1.6 million people died from an AIDS-related illness (4). Some persistent virus infections, such as Epstein-Barr virus (EBV) and Human Papilloma Virus (HPV) can lead, under certain conditions, to the development of tumors (5, 6). Because viruses have such a major impact on health, strategies to limit or prevent virus infections are of major importance.

Mammals have developed a refined immune system to cope with all kinds of infections. Especially the adaptive arm of the immune response is important in limiting and clearing viral infections. The humoral immune response consists of antibodies specific for the virus that can capture and neutralize virus particles before they enter the cell. However, if these antibodies are ineffective, viruses are able to infect host cells and can only be cleared by the cellular arm of the immune response. Once a virus infects a cell, the virus will use the protein-synthesis machinery of the host cell to synthesize its own proteins. During this process, some of the newly synthesized proteins will be degraded into peptide fragments and, if they have sufficient binding affinity, bind to MHC class I molecules. These MHC class I-peptide complexes will then be presented on the cell surface of an infected cell and activated $CD8^+$ T cells, specific for the peptide, can recognize the MHC class I-peptide complex and induce apoptosis of the infected cell by releasing cytotoxic granules. Activation of these $CD8^+$ T cells occurs in the draining lymph nodes, where antigen-presenting cells (APCs), such as dendritic cells (DCs), and naïve T cells encounter each other. In these lymph nodes, DCs and $CD4^+$ T cells provide the co-stimulation necessary for proper activation of $CD8^+$ T cells. This process is summarized in **Figure 1** and will be further discussed in the next paragraphs.

During the initial phase of a viral infection, there is a significant increase in the number of $CD8^+$ T cells. Priming of these naïve T cells will not only occur through the classical pathway via infection of a cell, directly leading to presentation of peptides on MHC class I molecules, but also through cross-presentation. Cross-presentation enables the presentation of viral

peptides, taken up from extracellular sources, on MHC class I molecules. Several different cell types have been demonstrated to cross-present antigens in vivo, including professional APCs such as macrophages and DCs (7). $CD8^+$ T cells, activated either through the classical or cross-presentation pathway, induce apoptosis of virus-infected cells by the release of cytotoxic granules and the production of TNF-α and IFN-γ as depicted in **Figure 1**. The cytotoxic granules contain perforins, granzymes, and granulysin. Perforins aid in delivering contents of granules into the cytoplasm of the target cell. Granzymes, such as granzyme B and granulysin, activate apoptosis of the target cell. TNF-α can interact with the TNFR-I receptor, which induces apoptosis of infected cells. IFN-γ is an important cytokine in the immune response to various viral infections, since it can induce an antiviral state in uninfected cells and enhance the cytotoxic function of $CD8^+$ T cells. By the classical antigen presentation pathway or by the cross-presentation pathway, any form of virus can be presented on MHC class I and MHC class II and thereby stimulate antiviral responses by both $CD8^+$ T cells and $CD4^+$ T cells, respectively, leading to a broad cellular response to infection (8). After infection, some of these activated T cells will develop into memory T cells. In the event that a secondary infection occurs, these cells can rapidly mature into effector cells and respond to infection.

APCs that reside at the site of infection, can take up viral particles or remnants of virally infected cells from extracellular sources, and present them on MHC class II molecules. Subsequently, $CD4^+$ T cells, recognizing peptides in the context of MHC class II, will be activated. These activated $CD4^+$ T cells are capable of producing a wide range of cytokines and chemokines and can even exert cytotoxic functions themselves. Based on cytokine production, $CD4^+$ T cells can be divided into several subsets, the most classical being Th1, Th2, and Tregs. Th1 cells are generally characterized by the production of IFN-γ. Th2 cells, on the other hand, produce mainly IL-4, IL-5, and IL-13 and are important for providing an immune response against helminths by activating eosinophils, basophils, mast cells, and B cells. The third classical subset are the Treg cells, which are characterized by the production of IL-10 and TGF-β, and have mainly regulatory tasks such as dampening effector functions and limiting immunopathology (8, 9). In addition to their effector functions, activated $CD4^+$ T cells can provide help to $CD8^+$ T cells by CD40-CD40L interaction, which induces upregulation of ligands, such as CD80 and CD86, on DCs. These ligands interact with CD28 on naïve T cells, providing a co-stimulatory signal to activate $CD8^+$ T cells (10). The mechanism by which $CD4^+$ T cells can provide help to $CD8^+$ T cells is shown in **Figure 1**.

In this review, we will discuss the value of T cell responses in both acute and chronic viral infections and how knowledge of these responses can help in designing effective vaccines. Currently, antiviral drugs are the main treatment option to combat viral diseases. However, antiviral treatment is associated with side effects and resistance through viral escape. Making use of the hosts own immune defense system by vaccination would be another powerful approach to combat viral diseases. However, many vaccination strategies are based on antibody-mediated protection and are only partially successful. Antibodies can be very

efficient in preventing virus infection, but due to the variability of many virus surface proteins, the virus can escape and infect host cells. Once a virus has entered a cell, infection can only be cleared by a cellular response. We will highlight the history of synthetic T cell based vaccines as an important strategy to induce T cell responses and discuss current developments in this field. Then, we will discuss how the design of these vaccines, such as choice of antigen and adjuvant, influences their efficacy. Finally, we will conclude with potential pitfalls and recommendations for the design of effective peptide vaccines against virus infections.

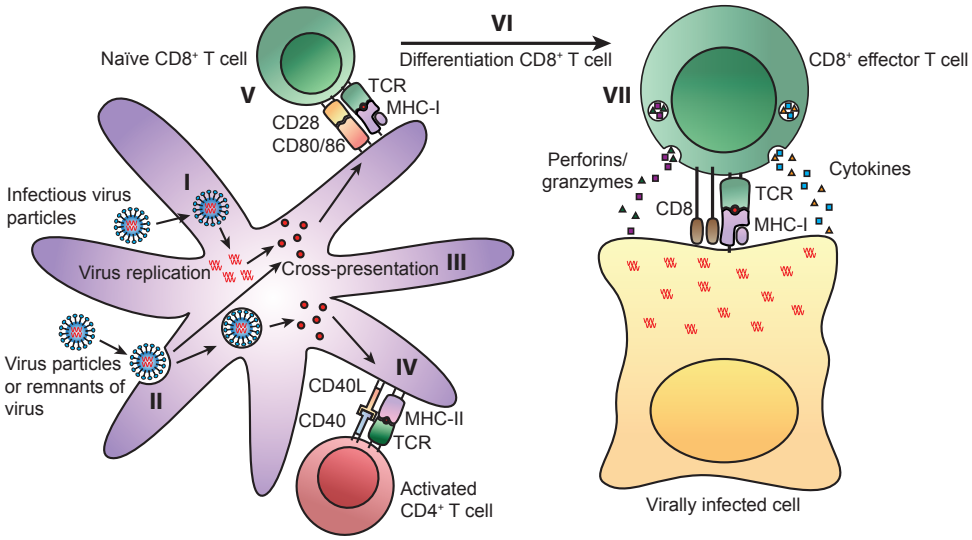

Figure 1: Routes of presentation of viral peptides on DCs
Viruses can enter cells by two ways: Some viruses can infect cells directly, leading to replication of virus inside the cells. During this process, some of the viral proteins will be degraded into peptide fragments, which will be presented on MHC class I molecules to $CD8^+$ T cells (I). APCs, such as DCs can also take up viral particles or remnants of virally infected cells (II). During processing by professional APCs, viral peptides can be presented on MHC class I molecules via the cross-presentation pathway (III). In parallel, these extracellular-derived peptides will be presented on MHC class II molecules. The TCR of virus-specific $CD4^+$ T can recognize MHC class II-peptide complexes on professional APCs. In addition to the interaction of the MHC class II-peptide complex with the TCR, $CD4^+$ T cells can activate DCs by interaction of CD40 with CD40 ligand on the DC (IV). This interaction activates DCs and results in upregulation of maturation markers CD80/CD86. CD80 and CD86 interact with CD28 on naïve $CD8^+$ T cells (V). Together with the recognition of the MHC class I-peptide complex by the TCR, CD28 signaling will result in the activation of the $CD8^+$ T cell (VI). These activated $CD8^+$ T cells will differentiate into effector T cells that can recognize the MHC class I-peptide complex on virally infected cells. Binding of the TCR to the MHC class I-peptide complex leads to activation of the $CD8^+$ T cell and the release of cytotoxic granules containing perforins and granzymes, and the production of cytokines such as TNF-α and IFN-γ (VII).

T cell responses in viral infections

There are many viruses for which T cells, both $CD8^+$ and $CD4^+$, have been shown to play a role in protection, such as measles virus, cytomegalovirus (CMV), hepatitis C virus (HCV), and HIV (11-14). In general, an efficient antiviral adaptive response is thought to be of the Th1 type (15). However, many viruses can inhibit this Th1 response by downregulating the production of interferons (16, 17). This type of manipulation of the immune response can greatly influence the outcome of the infection. In infections caused by hepatitis viruses, manipulation of the immune response by the virus can lead to a persistent infection, in which the host is incapable of clearing the virus from the body. In mice, lymphocytic choriomeningitis virus (LCMV) is used as a model to study the role of $CD8^+$ and $CD4^+$ T cells in both acute and chronic infections. $CD8^+$ T cell-deficient mice that were infected with a LCMV-strain that normally causes acute virus infection, were not able to control infection and developed a persistent infection. In mice depleted of $CD4^+$ T cells, infection with murine LCMV led to chronic infection, even in the presence of $CD8^+$ T cells. This model shows that in acute infection, $CD8^+$ T cells are sufficient to clear infection, but the help of $CD4^+$ T cells is required (18).

The importance of T cell responses during acute viral infections in humans can be illustrated by research from Sridhar et al. describing that individuals with higher numbers of pre-existing $CD8^+$ T cells specific for conserved CD8 epitopes, developed less severe illness after infection with pandemic H1N1 influenza virus (19). That not only $CD8^+$ T cells mediate protection to influenza challenge, has been shown in a unique human challenge study by Wilkinson et al.. In this study, healthy volunteers were challenged with influenza A virus, and antibody and T cell responses against influenza before and during infection were monitored. They showed that, in the absence of antibody responses, pre-existing $CD4^+$ T cells responding to influenza internal proteins were associated with less severe illness and lower virus shedding. Further characterization of these $CD4^+$ T cells showed that these cells had a cytotoxic function (20). These studies describe the importance of both $CD8^+$ and $CD4^+$ T cells in the immune response against influenza virus.

During chronic viral infections, when the host is not able to clear the virus, the main role of cytotoxic T cells is to limit disease severity and delay disease progression. This is exemplified by studies on HIV infection. Early during infection with HIV, there is a decline in viral replication as measured by the number of HIV RNA copies in plasma samples (21). In the first stages of HIV infection, it has been shown that patients with higher numbers of memory cytotoxic T cells show a much lower viral load in plasma than patients with a lower number of memory cytotoxic T cells, indicating that this decline is mediated by cytotoxic $CD4^+$ T cells (22). In addition, cytotoxic $CD4^+$ T cells are an immunological predictor of disease outcome. Patients that controlled HIV replication without antiretroviral therapy showed an increased number of $CD4^+$ T cells specific for HIV proteins (23). The importance of $CD8^+$ T cells in delaying HIV disease progression, is shown in studies where a loss of $CD8^+$ T cells coincides with disease progression (24, 25). Findings that HIV escape mutations often occur at HLA-binding sites

specific for CD8 epitopes, the strong association of certain HLA alleles with protection from HIV disease progression, the temporal relationship between viral load decline and increase in specific $CD8^+$ T cells, and $CD8^+$ T cell depletion studies in simian models, underline the importance of $CD8^+$ T cell responses (14, 26-29). Knowledge on the mechanism of protection of T cell responses in immunity against viruses can be helpful in designing preventive and therapeutic therapies, such as vaccination.

History of peptide vaccination

Many vaccines against virus infections are based on inducing antibody responses, consequently, these vaccines are often poor inducers of T cell responses (30). Since T cells are important in protection against many viral infections, there is a need for T cell inducing vaccines. By including small protein fragments (peptides), in a vaccine, which can be presented by MHC molecules to $CD4^+$ and $CD8^+$ T cells, specific T cell responses can be induced. In **Table I**, characteristics of two of the classical preventive vaccines for viral infections, i.e., protein vaccines and live attenuated vaccines, are compared to peptide vaccines. The main advantage of peptide vaccines over classical vaccines is that it is possible to specifically induce T cell responses and that the production process of these vaccines is relatively easy. The first synthetic peptide vaccine able to induce a T cell response in mice was published by Aichele et al.. This vaccine contained a 15-mer peptide, derived from the NP protein of LCMV, suspended in Incomplete Freund's Adjuvant (IFA) (31). Further experiments showed that these peptide vaccines were able to render a certain amount of protection against challenge with virus (32, 33). These results were promising, but in later studies where mice were vaccinated with 15-mer CTL epitopes derived from adenovirus type 5 early region (Ad5E1) oncogenes in combination with IFA, an enhanced outgrowth of tumors was observed following vaccination (34). In hindsight, this observation might not be that surprising. Only peptides of 20 amino acids or longer will need to be degraded by proteolytic enzymes and are therefore presented exclusively by professional APCs, thereby ensuring sufficient co-stimulation. Shorter peptides can be directly loaded on any MHC molecule, also on non-professional APCs, which may lead to the induction of tolerance. Additional research showed that indeed the problem with the 15-mer adenovirus peptides was induction of tolerance, since they were presented by non-professional APCs lacking appropriate co-stimulation, resulting in suboptimal presentation of the peptide. When mice were vaccinated with peptide-loaded DCs, there was an antitumor response and no tolerance induction, showing that presentation of these peptides on professional APCs can be effective without induction of tolerance (35).

Peptide length

Thus, the first advantage of peptides of 20 amino acids or longer, which are considered as long peptides, is that they require processing of these peptides by professional APCs, thereby reducing the chance of inducing tolerance by peptide vaccination (36). Furthermore, they may contain multiple epitopes specific for different MHC molecules. Thereby broadening the potential response both in the individual and at population level (37). Another advantage of

Table I: Comparison of classical protein vaccination, live attenuated vaccination and peptide vaccination.

	Classical protein vaccine	Live attenuated vaccine	Peptide vaccine
Composition	Inactivated split virion or purified subunit	Attenuated virus, capable of replication	Synthetic, small protein fragments
Humoral response	Yes, induces humoral response	Yes, mimics natural infection	Possible, depends on peptides included
CD4 response	No	Yes	Yes
CD8 response	No	Yes	Yes
Preexisting response	Not important	Important, Ab can capture vaccine	Not important
Adjuvant	Required for cellular response	Not required	Required
Production	Biological	Biological	Synthetic
Safety	Risk of contamination with extraneous agents and proteins of the production substrate	Risk of contamination with extraneous agents and proteins of the production substrate	Well controlled and highly pure production process
Flexibility to match escape variants	Not easy	Not easy	Easy
Target conserved components	No, primarily strain-specific response	To some extent, limited cross-reactivity	Yes, capable of inducing a broad response

Protein vaccines are a form of inactivated vaccines that consist of purified subunit or subvirion products. Live attenuated vaccines are attenuated viruses, derived from disease-causing virus. These attenuated viruses still replicate in the host, but do not cause disease. Peptide vaccines are completely synthetic vaccines, comprised of small protein fragments.

using long peptides is that, next to CD8 epitopes, this type of peptide often contains CD4 epitopes. These CD4 epitopes provide co-stimulation during priming of $CD8^+$ T cells and promote memory $CD8^+$ T cells (38, 39). One year after the first successful immunization of mice with a free synthetic LCMV peptide, Fayolle et al. described that this 15-mer peptide not only contained a CD8 epitope but also a CD4 epitope (40). This discovery confirms the valuable contribution of co-stimulation in a vaccine. In addition to considering the importance of the length of the peptide, other characteristics are equally or even more important. Therefore, considerations for the choice of antigen will be discussed next.

Choice of antigen

Aspects hampering the design of an effective preventive strategy for virus infections is that these viruses have, besides great genetic diversity, also developed multiple mechanisms to evade the host's immune response (41, 42). A promising approach is to direct the immune response to conserved parts of the virus, which do not allow for mutations. Virtually all viruses contain certain proteins or peptides that are highly conserved. Indeed, for HIV, the

Gag protein appears to be a good candidate for use as a T cell vaccine component. The Gag protein is highly conserved, and although it is a late structural protein, Sacha et al. showed in a simian model that $CD8^+$ T cells recognize Gag-derived epitopes as early as two hours post infection. This fast processing and presentation is thought to be necessary for early clearance of the virus (43). In an ex vivo study on PBMCs of HIV-infected individuals, vigorous $CD8^+$ T cell responses to Gag epitopes were observed and the breadth of the $CD8^+$ T cells specific for conserved Gag epitopes inversely correlated with viremia (44). A screening in patients with both acute and chronic HCV infection showed that specific T cell responses were found against conserved parts of the virus. Immunogenic regions were identified within core, NS3 and NS4 proteins (45). Influenza virus also contains good candidate proteins, such as nucleoprotein (NP), which is a major target of T cell responses (46). These studies show that there are T cells available directed towards conserved parts of the virus. Knowledge of these parts can be used in the design of a T cell inducing vaccine.

Since the introduction of sequence analysis tools, it is relatively easy to determine whether a certain peptide sequence is conserved. However, a high level of conservation is not the only requirement for a peptide vaccine to be effective. The peptide will have to be processed by the proteasome and then bind to the MHC molecule. Bio-informatic tools can be helpful to predict which sequences may be immunogenic for T cells. These tools can predict which sequences will bind to MHC, based on preferred amino acids of peptide anchor binding positions of these molecules. Furthermore, tools are available that predict which sequences will be processed by the proteasome and by TAP (transporter associated with antigen processing) transport (47). Together, these tools provide means of selecting a number of possible conserved T cell epitopes. Schellens et al. showed in PBMCs of HIV-infected individuals, that indeed these bio-informatics tools are valuable for predicting novel T cell epitopes (48).

Another important requirement for inducing T cell responses is that there are T cells available that can recognize the peptide. Tan et al. described the importance of the availability of naïve epitope-specific $CD8^+$ T cells in the host prior to infection and showed that precursor frequencies are indeed a good predictor for responses observed after infection, since a higher number of epitope-specific $CD8^+$ T cells led to an increased T cell response after infection (49). In addition to precursor frequencies, binding affinity of peptides to MHC is also a predictor of immunogenicity as has been shown in peripheral blood lymphocytes of acute HBV patients (50). Some groups have shown that it is possible to enhance peptides by increasing binding affinity of the peptide to the MHC molecule (51, 52). These enhanced peptides might induce a T cell response to conserved, but otherwise too low affinity epitopes. Another important consideration when vaccinating with short peptides, is HLA-specificity. Since peptides of 8-11 amino acids long bind directly into the MHC class I binding groove, the peptide has to match the HLA type of the vaccinated individual. To overcome the need for individualized vaccination, Tan et al. selected short epitopes with the capacity to bind to multiple HLA-alleles. HLA-A2 transgenic mice vaccinated with this multi-HLA peptide vaccine,

showed a reduction of virus in the lungs and increased survival following influenza infection, compared with mock vaccinated mice, showing that vaccination with peptides can positively influence disease outcome (53).

Presentation of peptides by APCs greatly depends on the form in which they are offered to APCs. Zhang et al. compared intact proteins and long peptides in the cross-presentation pathway and showed that long peptides traffic to both the endosomes and the cytosol, whereas whole protein was found to traffic only to the endosomal compartments. Therefore, whole proteins could not be processed through the cross-presentation pathway. This difference in processing led to a $CD4^+$ T cell restricted response after immunization with protein, while immunization with peptides also led to a $CD8^+$ T cell response (54). Rosalia et al. compared whole protein processing to processing of long peptides, both in mouse and in human DCs. Soluble protein antigen ended up mostly in the endolysosomes, while long peptides seemed to be more efficiently internalized by DCs leading to a faster intracellular routing. Therefore, long peptide vaccination ultimately leads to enhanced $CD8^+$ T cell activation compared to whole protein (55). In line with these findings, recent research on peptide vaccination is mainly directed to improving antigen presentation of the peptides of choice, by choosing the right form in which the peptides are presented. Rosario et al. used an HIV-synthetic long peptide vaccine to boost HIV-specific T cell responses in a macaque model and showed that boosting with these synthetic long peptides primarily increased the breadth of the $CD4^+$ T cell responses (56).

Features of an effective response

To induce an effective response against viral infections, there are several requirements that should be met. One important requirement is that there is a sufficient number of T cells available to kill virus infected cells. The need for an appropriate magnitude of T cells in order to clear virus was elegantly shown by Thimme et al. in a $CD8^+$ T cell depletion study in chimpanzees. Chimpanzees were depleted of $CD8^+$ T cells, and subsequently infected with HBV, complete depletion of $CD8^+$ T cells in the chimpanzees resulted in the inability to clear virus. When $CD8^+$ T cells reappeared in the animal, 98% of viral DNA was eliminated from the liver. However, while the number of $CD8^+$ T cells remained suppressed, the animal was not able to clear virus completely. Only when the number of $CD8^+$ T cells was able to expand further, the virus was completely eliminated (57). Furthermore, an increased breadth of T cell responses can be beneficial. Analysis of $CD8^+$ T cell responses in untreated HIV-infected individuals showed that an increasing breadth of Gag-specific responses is associated with decreased viremia (58). In parallel with these findings, vaccination of mice with a vaccine containing multiple epitopes, was more effective in generating a response to influenza infection than vaccination with single epitopes (49). These findings indicate that a broad response is more effective than a response dedicated to only one peptide. Another advantage of induction of a broad response is that small mutations of the virus will not lead to escape of the virus from the immune response. In addition to a broad response, T cell responses of high

avidity also contribute to an antiviral response. Ex vivo screening of T cell responses in HIV-infected patients showed that controllers reacted to lower antigen concentrations compared to non-controllers, indicating that controllers have T cell responses of higher functional avidity and that this higher avidity is advantageous (59).

A fourth requirement is that an effective antiviral response should be of proper functionality to enable control or clearance of the virus. $CD8^+$ T cells are the main cell type that is involved in clearance of viral infections. These cells are characterized by the production of Th1-cytokines such as IFN-γ and by the expression of degranulation marker CD107a (60). CD107a is an indicator of cytotoxic functions such as the production of granzymes and perforins. IFN-γ increases expression of both MHC class I and II molecules and enhances the antigen-presenting function of MHC class I by stimulating loading of peptides onto this molecule. Thereby, IFN-γ can induce the cytotoxic function of $CD8^+$ T cells and promote the production of other cytokines such as TNF-α, IL-2, and type I interferons. TNF-α induces apoptosis of virus infected cells and IL-2 is an important growth factor for T cells. Type I interferons, such as IFN-α and IFN-β, can induce resistance to viral infections in uninfected cells, increase MHC class I expression and antigen presentation and activate both DCs and macrophages (8, 61). Activated macrophages, in their turn, produce chemokines such as MIP-1β to attract more T cells. Together, these cytokines, chemokines, granzymes, and perforins enable control or clearance of the virus from the host. In HIV infection, a polyfunctional $CD8^+$ T cell response is observed in non-progressors, while progressors show a more limited response (62). As reviewed by Seder et al., a polyfunctional response, characterized by production of IFN-γ, TNF-α and IL-2, was indeed shown to induce more robust T cell proliferation and protection against several viral infections (63).

However, elevated amounts of inflammatory cytokines can also lead to immunopathology as has been shown in H5N1 influenza A virus infection (64). The immune system normally has its own regulatory mechanisms, such as the production of anti-inflammatory cytokines including IL-10 and TGF-β. IL-10 is produced by a wide range of cells, including T cells, macrophages and neutrophils. The main function of IL-10 is to act as a negative feedback loop to suppress the production of IFN-γ and other pro-inflammatory cytokines (65). TGF-β acts by inducing apoptosis of $CD8^+$ T cells, which regulates T cell homeostasis and prevents immune inflammation (66). These feedback loops are a way of the immune system to regulate itself, however viral factors can negatively impact this balance as is illustrated in HCV infection. Patients with progressive liver injury showed upregulation of Th1 cytokines IFN-γ and IL-2 and downregulation of the regulatory cytokine IL-10 (67). Another regulatory mechanism is the upregulation of inhibitory receptors such as PD-1, LAG-3, and CTLA-4, which leads to decreased activation potential of T cells and the activation of inhibitory genes in T cells (68). However, upregulation of these receptors has also been shown to be responsible for the exhaustion of T cells and thereby a diminished response in chronic viral infections (69). Summarizing, an effective antiviral response consists of a broad variety of antigen-specific T cells of sufficient

magnitude, affinity, and appropriate polyfunctionality. Furthermore, these T cells should be capable of performing cytotoxic functions, but should not induce immunopathology. Such a response greatly depends on the way antigen is presented to the T cells, emphasizing the important role APCs play in antiviral responses.

Co-stimulation and peptide vaccination

In recent years, multiple strategies were developed to increase the quality of antigen presentation of peptides. One of the strategies, already described above, is the addition of CD4 help. Long peptides often contain CD4 epitopes that can provide co-stimulation for $CD8^+$ T cells. However, more general CD4 helper peptides are available. One example is the non-natural pan HLA-DR binding peptide (PADRE), which is engineered by introducing anchor residues for different DR motifs within a polyalanine backbone. This peptide binds with high or intermediate affinity to the most common HLA-DR types, and allows it to activate a wide range of $CD4^+$ T cells (70). The addition of PADRE epitopes is used, for example, in Dengue virus and HBV virus vaccine development, showing promising results in vivo (71, 72). Another group of universal T helper epitopes are natural tetanus sequences, which are very promiscuous in their capacity to bind to MHC class II, and thereby very efficient in acting as a co-stimulus. (73). These universal T helper epitopes can be fused to $CD8^+$ T cell epitopes, eliciting good immunogenicity, as shown for CMV by La Rosa et al. (74). However, it remains under debate whether CD4 help should be antigen-specific or is otherwise not able to stimulate proper $CD8^+$ T cell responses. A study in which mice were vaccinated with either non-specific CD4 help or antigen specific CD4 help, showed that memory $CD8^+$ T cells can only be efficiently activated by antigen-specific CD4 help, while effector $CD8^+$ T cells can be activated by non-specific CD4 help (75).

An important factor in $CD4^+$ T cell help in short peptide vaccination appears to be CD40-CD40 ligand interaction (76). Ligation of CD40 to CD40 ligand can trigger the production of high levels of IL-12 by DCs. IL-12 induces Th1-mediated immune responses and inhibits Th2-mediated responses (77). Furthermore, CD40 ligand stimulates up regulation of ICAM-1, CD80, and CD86 molecules on DCs. By these mechanisms, DCs can trigger proliferative responses and IFN-γ production by T cells (78). By adding CD40 ligand as a co-stimulatory molecule, DCs can be activated through CD40 and in turn, DCs are able to activate $CD4^+$ T cells and $CD8^+$ T cells (79).

Another way to activate APCs is by targeting their Toll-like receptors (TLRs). TLRs are pathogen recognition receptors (PRR) that recognize molecules shared by pathogens, for example, double stranded RNA in certain viruses. Activation of these TLRs can then lead to the production of inflammatory cytokines. By covalently coupling TLR-activating lipids to the peptide, resulting in so-called lipopeptides, self-adjuvanting peptides are created. These lipopeptides can target the vaccine by activating the TLRs on the required APCs and the peptides can then be internalized and presented on MHC molecules. Thereby, lipopeptides can

signal through the TLRs to induce DC maturation, leading to enhanced antigen presentation. Jackson et al. designed a synthetic vaccine composed of a CD4 T helper epitope, a CD8 target epitope, and the lipid moiety Pam_2Cys that provided TLR2 targeting, which could induce DC maturation and antibody and CTL responses (80). Chua et al. used the TLR2 agonist Pam_2Cys to enhance the immunogenicity of their virus-like particles, containing HCV structural proteins. The addition of lipopeptide resulted in increased DC maturation at low doses of the vaccine (81). Indeed, lipopeptide vaccination can induce protective CTL responses, as shown by Day et al. in a mouse influenza virus challenge model. (82).

Adjuvants in peptide vaccination

To improve the effectiveness of peptide vaccines, there are several types of adjuvants available, with different effector mechanisms. Some adjuvants induce depot formation; others directly stimulate the immune response through additional signals. In earlier work on peptide vaccination, strong adjuvants were necessary for induction of immunogenicity. A commonly used adjuvant for peptide vaccination is IFA, which was applied in the first peptide vaccine, or the human equivalent Montanide. These water in oil formulations form a depot at the site of injection, leading to "leakage" of antigen into the body (37, 83). Research by den Boer et al. showed that the short Ad5E1 peptide still leaks from the IFA depot at 200 days post immunization (84). This depot of antigen and adjuvant can lead to chronic inflammation at the site of injection that may persist for a long time. Harris et al. showed that repeated vaccination can even lead to a site suggestive of a new lymphoid structure, including the association of mature DCs with proliferating T cells in perivascular dermal aggregates (85). However, the risk with such depots is that the peptide might be present for a long time after vaccination, but the adjuvant might not be, allowing presentation of the peptide without the necessary co-stimulation and with the risk of inducing tolerance (86). Furthermore, although effective in therapeutic vaccination, IFA does lead to the formation of lesions on the site of injection, making it less attractive for use in a preventive vaccine (87). Two clinical trials, one with HIV peptides and another with malaria surface proteins mixed in Montanide, have even been terminated because of these severe adverse events (88, 89).

An alternative for water in oil formulations could be the use of vesicular delivery systems. Depending on the nature of the delivery system, they provide the possibility to incorporate immune modulators to direct the immune response, protect against degradation of the peptide, directly target the antigen to the place of interest and, finally, actively transport the antigen across the target membrane. Currently, there are several delivery systems available for peptide vaccination i.e., liposomes, virosomes, virus-like particles, ISCOMs, and nanoparticles (90). Liposomes consist of a lipid bilayer, in which antigens or other substances can be entrapped in the lumen or the lipid bilayer, depending on traits of the peptide. The lipid bilayer of liposomes can fuse with other bilayers, such as a cell membrane. Thereby, liposomes can deliver antigens to the cytosol of APCs (91). Liposomes, containing a short CD8 lipopeptide in combination with CpG, were able to induce protection in a murine influenza

challenge model (92). However, liposomes cannot induce maturation of DCs without addition of an adjuvant and are therefore not sufficient to induce co-stimulation. To address this problem, several groups are developing modified liposomes to enhance targeting to DCs by adding targets for C-type lectin receptors such as glycans or mannose, which are typically expressed on DCs (93). Virosomes, or influenza derived virus like particles, have similar membrane-fusion capacities as live influenza virus, which allows them to actively fuse with cell membranes and thereby deliver antigens directly into the cytosol of APCs leading to cross-presentation of antigenic peptides (94). Furthermore, they have been shown to induce upregulation of maturation markers on bone marrow-derived DCs in mouse models (95, 96). However, as of yet, DC maturation capabilities of virosomes have not been shown in human systems. Thus, liposomes, virosomes, and other delivery systems can successfully be used to deliver antigens to the place of interest. In addition, they can provide the necessary co-stimulation for APCs, either due to their own properties or by adding other adjuvants to the formulation.

Current progress in peptide vaccination

The first, and most successful, peptide-based vaccine that is currently licensed is a therapeutic vaccine against human papilloma virus (HPV). This vaccine contains long synthetic peptides directed against viral oncoproteins, mixed in Montanide, which induces vaccine-specific $CD4^+$ and $CD8^+$ T cell responses in all patients (87). Since the success of this therapeutic cancer vaccine, many groups are exploring peptide vaccination for other viral agents. Therapeutic vaccination with synthetic peptides, of HCV patients not responding to standard treatment, resulted in a decrease in viral RNA as shown in two separate studies. Klade et al. performed a Phase II clinical study in HCV patients with their IC41 vaccine, consisting of five synthetic peptides formulated with a Th1 type adjuvant, poly-l-arginine. All patients that were vaccinated intradermally with TLR7 agonist imiquimod as adjuvant, showed a modest decline in viral titers (97). The study by El-Awady et al., in which HCV patients were vaccinated with a peptide vaccine consisting of three envelope proteins, showed that in two thirds of the patients both antibody and T cell responses were detectable resulting in decreased viral titers (98). However, although these studies provide a proof of concept for peptide vaccination for therapeutic use in HCV infection, the improvements are only minor.

For a preventive peptide vaccine, there are different necessities. First of all, it should target conserved sequences, which could lead to a universal vaccine. Possible target proteins have been identified for viruses such as HIV, HCV, and influenza (44-46). Especially in influenza vaccine development, the threat for a new pandemic to occur has boosted research on the development of such a universal vaccine. The research of Tan et al., in which they make use of lipopeptides directed to conserved components, is one of many examples of strategies that are currently developed and have proven themselves in mouse models but not yet in human systems (53). Other vaccination strategies, currently in development, include the use of virus-like particles in combination with an antibody-inducing influenza protein such as the relatively

conserved M2e protein or lipopeptide in combination with liposomes (92, 99, 100). A recent advancement is that there are some peptide-based vaccines against influenza virus infection in Phase I clinical trials, that are able to induce vaccine-specific cellular immunity (101, 102).

Considerations for peptide vaccine development

When designing peptide-based vaccines, there are several things to take into consideration, such as virus traits, side effects, location of the response, and traits of the host (see **Table II** for an overview). First, the objective of vaccination should be taken into consideration. Vaccines can be largely divided into therapeutic and preventive. Preventive peptide-based vaccines should elicit a robust memory T cell response, since vaccine-induced T cells need to respond rapidly after infection to clear the virus before it causes illness or at least to limit disease burden. In the case of therapeutic vaccination to chronic infections, the response should be vigorous and elongated and a rapid response is of less importance. Both for therapeutic and preventive vaccines, eliciting this response at the required location is of great value. Peptide-based vaccines for respiratory viruses, for example, might be more effective when administered intranasally, since lung-resident immune cells might then be primed more easily (103). However, changing the route of administration is not always sufficient and then adjuvants in the form of delivery vehicles might aid in transporting vaccine components to the right location in order to elicit an efficient T cell response.

Although inducing T cell responses is very important in protection against many pathogens, there are also indications that these T cell responses cause harm. This is illustrated for influenza infection, in which a high number of virus-specific $CD4^+$ T cells in patients infected with pandemic influenza A virus from 2009, correlated with more severe illness (104). In the case of HCV infection, a broad and specific T cell response is able to control virus infection (105). However, during chronic viral infection, liver damage occurs, which is assumed to be

Table II: Design of a peptide-based vaccine for preventive or therapeutic use

Factor	Preventive	Therapeutic
Route of immunization	Unimportant Time to develop response	Wanted Virus present on certain location
Existing response	Unimportant Inducing new response	Important Boost existing T cell response
Rapid effector response	Wanted Preventing or limiting disease	Unimportant Clearance in the end
Inducing memory	Wanted T cells available when infected	Unimportant Recall response not necessary
Side effects	Unwanted Reason to withdraw vaccine	Unimportant Accepted for certain diseases

There are several factors to take into consideration when designing peptide-based vaccines, such as location of the response, type of response to be induced, and side effects. The contribution of these factors in the design of preventive versus therapeutic vaccines are summarized in the table.

immune mediated. In a study by Maini et al., a high number of antigen-specific T cells in the blood did not correlate with the amount of liver damage as measured by alanine transaminase (ALT, indicative of liver damage). In contrast, Feuth et al. show a direct correlation between the number of differentiated $CD8^+$ T cells, which contain high perforin levels, and liver fibrosis measured by fibroscan elastography (106). Since a large number of T cells are detected in the liver of patients with liver damage, damage has been proposed to be caused by the recruitment of non-virus specific T cells (107). Although in humans the mechanism by which immunopathology develops is not clear, it is important to bear in mind that an exaggerated T cell response to infection or vaccination may lead to unwanted immune-mediated damage. Therefore, vaccine-induced T cell responses should be effective against the virus, without eliciting major side effects.

Traits of the host also influence the effectiveness of a vaccine. Therefore, it is important to consider the target group for vaccination. During a human's lifetime, the immune system will change continuously. Vaccination in early childhood can have a major impact on the immune response in later years as described by Bodewes et al. in which it was shown that annual vaccination with a seasonal inactivated subunit influenza vaccination hampers the development of influenza-specific $CD8^+$ T cells (108). To underline this finding, Hoft et al. compared a live attenuated influenza vaccine (LAIV) with a trivalent inactivated influenza vaccine (TIV) in young children, and found that only LAIV induced diverse T cell responses (109). Both studies show that the type of vaccination is of crucial importance both for the induction of T cell responses directly after vaccination and to T cell responses to the pathogen later in life. That age of the target group should be an important factor in the design of a vaccine is further exemplified by a study on influenza vaccination in elderly. In this study, antibody titers did not predict who developed influenza related illness, while T cell responses did (110). This effect is supported by evidence that T cell responses wane in elderly individuals. Several studies have shown that T cells from elderly individuals have a more differentiated phenotype characterized by the lack of CD27 expression and upregulation of CD57. The presence of CD57 on $CD8^+$ T cells is associated with decreased proliferation of $CD8^+$ T cells. Lack of markers, such as CD28, leads to an increased Th1-skewed response, which may contribute to decreased antibody titers in elderly individuals (111-113). Not only T cell responses wane, but also antibody responses diminish (114). Therefore, age of the target group should be an important consideration for the development of vaccines.

Prospects for peptide vaccination

Taken together, severity of side effects is an important factor in the consideration of vaccine application. The licensed HPV peptide-based vaccine contains Montanide, which is a strong adjuvant causing lesions at the site of infection (87). For the therapeutic HPV vaccine, these side effects were deemed acceptable; however, they were one of the reasons to abort studies with Montanide-containing vaccines for HIV and malaria (88, 89). Consequently, before this peptide-based vaccine concept can be widely implemented, Montanide has to be replaced by

another adjuvant. However, to elicit a response to these long overlapping peptides, a strong adjuvant is necessary. Therefore, the challenge is to increase immunogenicity of conserved targets for which T cells are available (43-45). A promising self-adjuvanting approach, which induces a broad response, is using multiple antigenic peptide (MAP). This approach was implemented in HCV patients by El-Awady et al. and was capable of inducing both antibody and T cell responses in two thirds of the patients. (98, 115).

The ultimate goal in protection against rapidly mutating viruses such as influenza, is to develop a universal vaccine, protecting against currently circulating influenza strains, but also able to cross-protect against newly emerging strains and thereby preventing future pandemics. These preventive peptide-based vaccines should elicit a robust memory T cell response, since vaccine-induced T cells need to respond rapidly after infection to clear the virus before it causes illness. To induce a pool of both memory $CD4^+$ T cells and memory $CD8^+$ T cells, efficient priming of naïve T cells is required. Professional APCs need to present the antigen to both $CD4^+$ and $CD8^+$ T cells. As most vaccines induce T cells via extracellular routing, cross-priming is of specific significance since it enables the presentation of extracellular-derived particles on MHC class I molecules. Targeting the more conserved parts of the virus by designing peptide-based vaccines, is a promising concept in the design of these preventive vaccines. Especially in influenza vaccine development, there are several examples of pilot vaccines directed to more conserved parts of the virus that should cross-protect to heterologous viruses. These vaccines often contain both antibody and T cell-inducing components (116, 117).

Concluding, in addition to antibody responses, T cell responses are of major importance in limiting and clearing virus infections. Effective therapeutic and preventive vaccines should therefore be able to induce both antibody and T cell responses. Peptide-based vaccines can meet these demands and induce both antibody and T cell responses. Furthermore, because peptides are synthetic, they are safe and relatively easy to produce. Currently, several peptide-based vaccines for viruses such as EBV, HBV and influenza virus, are evaluated in clinical trials (101). Hurdles to overcome are choosing the right target epitopes and choosing adjuvants that improve immunogenicity of these epitopes and steer the immune response in the desired direction. Adjuvants for peptide-based vaccines should target antigen to DCs, or other APCs capable of cross-presentation, and provide stimuli to ensure efficient presentation of the antigen. In addition, an overstimulation resulting in immunopathology should be avoided. Providing these criteria are met, the future of peptide-based vaccines is very promising.

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Chapter 3

Immunogenicity of influenza vaccines: evidence for differential effect of booster vaccination on humoral and cellular immunity

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Abstract

While influenza vaccines are designed to induce neutralizing antibodies, little is known on T cell responses induced by these vaccines. In contrast, more data becomes available on the important role of cellular immune responses in limiting influenza disease. The 2009 pandemic provided us with the opportunity to evaluate the immune response to vaccination in a unique setting. We evaluated both antibody and T cell responses during two consecutive influenza seasons from 2009-2011 and compared the MF59-adjuvanted pandemic vaccine with the unadjuvanted seasonal vaccine. Antibody responses were determined by a hemagglutination inhibition assay in serum and vaccine-specific T cell responses were evaluated by detecting IFN-γ producing peripheral blood mononuclear cells using whole influenza virus or vaccine-specific peptide pools as stimulating antigens. We show that one dose of the pandemic vaccine induced antibody responses sufficient for providing seroprotection and vaccine-specific T cell responses. A second dose further increased antibody responses but not T cell responses. Both responses could be boosted by the seasonal vaccine in the subsequent season. Furthermore, we show that the seasonal vaccine alone is capable of inducing vaccine-specific T cell responses, despite the fact that the vaccine did not contain an adjuvant. In addition, residual antibody levels remained detectable for over 15 months, while T cell levels had reduced back to baseline levels by that time. Hereby, we show that humoral and cellular immunity differ in their response to a second dose of the pandemic vaccine.

Introduction

Influenza virus causes seasonal epidemics resulting in a major social and economic burden and 250,000-500,000 deaths each year, while pandemic outbreaks affect the population to an even greater extent (1-3). These outbreaks of influenza are the result of the variable nature of the surface proteins of influenza virus, hemagglutinin (HA) and neuraminidase (NA). Typically, antibodies directed to these proteins can provide neutralizing immunity. However, antigenic drifts can cause small changes in antibody binding sites that may render these antibodies ineffective. In addition, completely new subtypes can arise due to antigenic shifts, which occur when circulating viruses reassort with other viruses circulating in the human population or that of other species. During the emergence of such a new subtype, individuals depend even more on the activation of other arms of the immune system than the humoral response to the globular head of HA and NA. Although they cannot prevent infection, cross-reactive cytotoxic $CD4^+$ and $CD8^+$ T cells have been shown to provide an immunological advantage by limiting disease, improving recovery, and eventually clearing infection (4-6).

In 2009, A(H1N1)pdm09, a subtype from swine origin, was introduced into the human population. This was the first time in over 30 years that an influenza virus originating from an animal reservoir was able to transmit from human to human (7). As humans were expected to be naïve to this new subtype, an MF59-adjuvanted inactivated monovalent vaccine directed to the pandemic strain, was offered to classical Dutch risk groups, pregnant women, and health care workers in a two dose schedule (8, 9). MF59 is an oil in water emulsion that was shown to activate $CD4^+$ T cells, which play an important role in the induction of high affinity class switched antibodies (10-12). In the pandemic setting, MF59 was included to allow for a lower antigen dose, while still capable of inducing seroprotective antibody titers (13).

In this study, we analyzed the immunogenicity of the pandemic vaccine during the H1N1 pandemic in 2009, which allowed for evaluation of both the unusual two dose schedule and the effect of the addition of MF59 on humoral and cellular immunogenicity of the pandemic vaccine (14, 15). In addition, this study entailed the subsequent 2010-2011 season in which the A(H1N1)pdm09 strain was included in the unadjuvanted seasonal influenza vaccine, together with a new H3N2 strain (A/Perth/16/2009). This allowed for analysis of the booster effect of previous vaccination with the A(H1N1)pdm09 strain and comparison of immunogenicity of an adjuvanted pandemic vaccine versus an unadjuvanted seasonal vaccine. Analysis of immunogenicity was performed by measuring the standard correlate of protection for influenza vaccines, i.e., antibody responses. Furthermore, vaccine-specific T cell responses were investigated since little is known on the induction of T cells by vaccination, while more evidence is being published on their important role during influenza infections. T cell responses directed against epitopes of the influenza virus surface proteins HA and NA may serve the development of specific antibodies or mediate cytotoxic effects on their own (16).

In this study, we evaluated the vaccine-specific antibody and T cell-mediated immune response during two consecutive influenza seasons from 2009 to 2011. During the first season, the additive value of a second dose of the pandemic vaccine was evaluated. In addition, a comparison of adjuvanted and unadjuvanted influenza vaccines is made. We show that one dose of the pandemic vaccine was sufficient to induce antibodies and T cell responses and that a second dose solely boosted antibody responses. The seasonal vaccine boosted both the humoral and cellular response and even induced T cell responses in individuals not vaccinated in the previous season. Antibody levels remained detectable until the end of the study, while T cell responses had reduced to baseline levels. Hereby, this study contributes to knowledge on the humoral and cellular immunity in response to influenza vaccination.

Materials and Methods

Experimental design

A non-randomized, multicenter, open-label controlled trial was conducted in the Utrecht area, during two consecutive influenza seasons between October 2009 and May 2011. The main objective was to evaluate whether a second dose with the pandemic adjuvanted vaccine was necessary for obtaining seroprotective antibody titers and whether the antibody response could be boosted in the second season with a seasonal unadjuvanted vaccine. In addition, humoral and cellular immune profiles after vaccination with pandemic and seasonal H1N1 containing vaccines were evaluated.

Healthy individuals aged between 18 and 52 years were recruited among workers of public health institutions in the Utrecht area. Exclusion criteria were: previous diagnosis with A(H1N1)pdm09, any history with serious allergic reaction to vaccine components, factors that might interfere with blood collection, and factors that might interfere with immunological analysis, including immune deficiencies, hematological disorders, bleeding disorders, usage of anticoagulants, corticosteroids, NSAIDs and/or statins, diabetes mellitus or having had an infectious disease with fever within the last two weeks before the start of the study. Study participants had the choice to be vaccinated or not in both seasons independently of their choice in the previous season, resulting in a vaccine and control group in the first season (2009- 2010) and vaccine-vaccine (VV), vaccine-control (VC), control-control (CC) and control-vaccine (CV) groups in the second season (2010-2011).

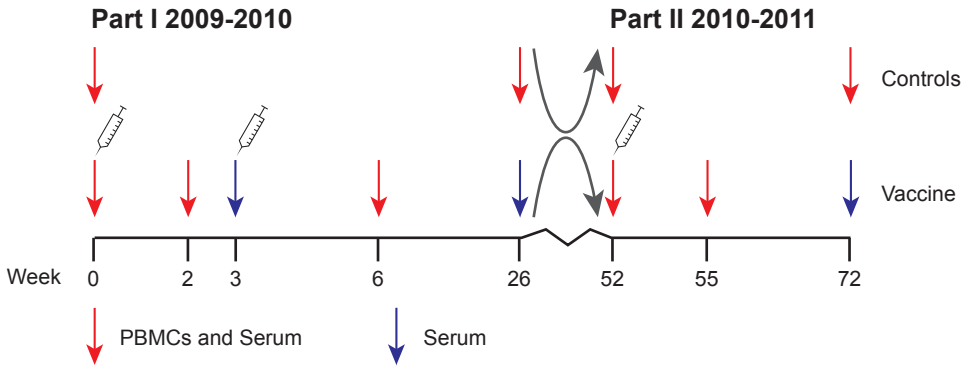

Figure 1: Design of the clinical study
The study was performed during two consecutive influenza seasons. During the first season (2009-2010) individuals were vaccinated at the start of the study and three weeks later with the MF59-adjuvanted A(H1N1)pdm09 subunit vaccine. Three weeks before the start of the study or at week six, an optional seasonal 2009-2010 vaccination was allowed. However, this was not part of the study regime. Grey arrows depict reallocation in control and vaccine groups. During the second season (2010-2011), individuals in the vaccine group received the unadjuvanted seasonal 2010-2011 subunit vaccine (including A(H1N1)pdm09) at week 52.

The protocol was approved by the medical ethical reviewing committee (Central Committee on Research Involving Human Subjects (CCMO)) of the Netherlands and the study was conducted in accordance with Good Clinical Practice and the principles of the Declaration of Helsinki. The study was registered in the Netherlands Trial Register (NTR2070) and written informed consent was obtained from each participant.

Vaccines

During the first season, individuals in the vaccine group received two pandemic influenza vaccine doses with a three-week interval (**Figure 1**). Vaccination with two doses of the A(H1N1) pdm09 vaccine was recommended by the Dutch Health Council, based on experience during outbreaks of H5N1 avian influenza, a subtype for which little to none pre-existing immunity was present in humans. The monovalent pandemic subunit vaccine (Focetria, Novartis) is a MF59-adjuvanted influenza vaccine containing A(H1N1)pdm09. MF59 already proved to be safe and immunogenic in combination with an inactivated seasonal influenza vaccine for elderly and has been registered in Europe since 1997 (17). Seasonal influenza vaccination was not part of the study regime in the first season, but was optional and had to take place at least three weeks prior to the study or at week 6 (**Table IA**), if so, individuals received the subunit vaccine Influvac 2009-2010 (Solvay, The Netherlands). This seasonal subunit vaccine contained A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and B/Brisbane/60/2008. During the second season, subjects in the vaccine group were vaccinated once with Influvac 2010-2011, containing vaccine strains A(H1N1)pdm09, A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008 (Solvay, the Netherlands).

Virus strains

The following virus strains were used for HI assays and virus ELISpots: A/California/07/09 (H1N1) was kindly provided by Institute Pasteur (Paris, France) and A/Perth/16/09 (H3N2) was obtained from the National Institute for Biological Standards and Control (NIBSC). Viruses were grown on Madin-Darby Canine Kidney (MDCK) cells. Sequences of hemagglutinin (HA) and neuraminidase (NA) proteins of these strains were obtained from GenBank and can be found under protein accession numbers ACP44189 (HA California), ACQ63272 (NA California), ACP44189 (HA Perth), and ACQ63272 (NA Perth).

Blood collection

Blood was collected before vaccination, two weeks, and three weeks after the first dose, three weeks after the second dose and at the end of the influenza season, which was approximately five months after the second dose (**Figure 1**). During the second season, blood was drawn before and three weeks after vaccination and at the end of the influenza season. At most time points, blood was collected for PBMC isolation and serum, however three weeks after the first dose of the pandemic vaccine and at the end of both seasons, only serum was collected. Blood of individuals in the control group was collected for serum and PBMC isolation at the start and the end of both seasons. Serum was stored at -20°C until

analysis. PBMCs were isolated by Ficoll (Lymphoprep, Axis-Shield, Norway) density gradient centrifugation and stored at -135°C.

Hemagglutination-inhibition (HI) assay

HI assays against MDCK cell-grown A(H1N1)pdm09 wild type virus was performed in duplicate according to standard methods of the World Health Organization (WHO) at Viroclinics (Rotterdam, the Netherlands) (Luytjes et al., 2012). In short, a dilution series of cholera filtrate-treated serum samples was incubated with four Hemagglutinin Units (HAU) influenza virus for 20 minutes and 0.25% (v/v) turkey erythrocytes for 30 minutes at 4°C and scored for agglutination.

EMA guidelines

EMA guidelines for influenza vaccines include criteria related to vaccine efficacy, which have to be met to obtain registration in the European Union (EU). First, the percentage of subjects who reach seroprotection, which is defined as an HI titer ≥40, should increase by 70%. Second, the mean geometric increase of antibodies should be >2.5. Third, the percentage of individuals who reach seroconversion, which is defined as seroprotection with at least a fourfold increase in antibody levels should be >40% (15). For a pandemic vaccine, all three criteria have to be met, while for a seasonal vaccine at least one in three is required. Antibody responses should be measured three weeks after vaccination.

Enzyme-linked immunospot (ELISpot) assays

PVDF-membrane plates (Millipore Corporation, USA) were ethanol-activated, coated with 5 µg/ml 1-D1K anti-IFN-γ antibody (Mabtech Ab, Sweden) and incubated O/N at 4°C. Plates were blocked with AIM-V medium (Thermo Scientific, The Netherlands) containing 2% human AB serum (Sigma, MO, USA). For analysis of responses to the vaccine strains, $2*10^5$ PBMCs per well were incubated in AIM-V medium (Thermo Scientific, The Netherlands) containing 2% human AB serum (Sigma, MO, USA) and stimulated with influenza virus at a multiplicity of infection (MOI) of 4, mock (cell supernatant) or 1 µg/ml Staphylococcus Enterotoxin B (SEB) (Sigma). Analysis of the vaccine-specific antigens was performed by stimulation of $4*10^5$ cells per well with 1 µg/mL of a peptide pool spanning the entire HA or neuraminidase NA protein of A(H1N1)pdm09 or A/Perth/16/2009. Per protein, 15-mer peptides with 11 overlap (JPT peptide Technologies, Germany) were pooled and dissolved in DMSO. In the negative control wells, DMSO was added to the medium. After an incubation period of 18 hours, plates were washed with phosphate buffered saline (PBS) 0.2% triton-x100 to inactivate the virus and detection IFN-γ antibody Biotin labeled antibody 7-B6-1 (Mabtech Ab, Sweden) in PBS 0.5% FCS (HyClone Thermo Scientific, USA) was added at 1 µg/ml for 2 hours at room temperature (RT). Plates were washed and incubated with streptavidin-alkaline phosphatase in PBS 0.5% FCS for 1 hour at RT. After washing the plates, 100 µl NBT/BCIP solution (Sigma, MO, USA) was added. Color reaction was stopped by washing the plates with tap water. Plates were dried O/N at RT and spots were counted with A.EL.VIS reader (A.el.vis, Germany).

Statistical analysis

Mann Whitney U and Pearson Chi Square tests were applied to analyze the characteristics of the cohort, as indicated in the Results section. Statistical significance was defined as a p-value ≤ 0.05 and statistical analysis was performed with the SPSS 19.0 statistical software program for Windows. Data from peptide ELISpots were log-transformed and tested for significance with a two-tailed student's t-test, using GraphPad Prism 6.04 software.

Results from HI assays and ELISpot assays with virus-stimulated PBMCs, were analyzed by a mixed effects Negative Binomial regression model to quantify differences in immune responses between vaccinated and unvaccinated groups (18, 19). The Negative Binomial distribution was used to describe the number of spots, while the underlying spot rates were modelled by the regression model. SEB counts were included in the regression model as denominator in the so-called offset term, i.e., if the spot rate is constant, higher SEB spot counts will automatically result in higher virus specific spot counts. Possible confounders such as sex, vaccination history, and earlier influenza infections were taken into account as categorical variables and age was entered in the model as a natural cubic spline curve. A log-link function was used to relate the response rate with these fixed effects. To account for variation between participants, a random intercept was included in the model (20). Differences between groups are therefore presented as relative rates, including 95% confidence intervals and p-values. The Holm adjustment is applied to correct for multiple testing. All statistical analyses were performed in R using the R-INLA package (21, 22).

Results

Clinical trial design

In this study, 348 individuals were included of whom 288 chose to be vaccinated (vaccine group) and 60 chose not to be vaccinated (control group) (**Figure 2**). At the start of the second season individuals again had the choice to participate and to be vaccinated or not, independent of their choice in the previous season. This resulted in four different groups: 135 individuals remaining in the vaccine group (VV), 29 individuals switching to the control group (VC), 31 individuals remaining in the control group (CC) and 7 individuals switching to the vaccine group (CV) (**Figures 1** and **2**). Baseline characteristics of the study participants are described for season one (**Table IA**) and season two (**Table IB**). Vaccination history of all participants was recorded, which shows that the number of frequent vaccinees was higher in the vaccination groups (**Table IA and IB**).

Table IA: Baseline characteristics 2009-2010, season one

		Control group (n=60)	Pandemic vaccine group (n=288)	p-value
Mean age *(range)*		39.1 *(25-52 yrs)*	39 *(19-52 yrs)*	ns
Gender (%)	Male	28.3	43.4	0.03
	Female	71.7	56.6	
Pregnant (%)		0	2.5	-
Any previous influenza vaccination (%)		20	56.6	0.001
Seasonal vaccination 2009-2010 before trial (%)		8.3	24.3	0.006
Seasonal vaccination 2009-2010 at week 6 (%)		5	35.8	ns

Table IB: Baseline characteristics 2010-2011, season two

		CC group (n= 31)	CV group (n=7)	VV group (n=135)	VC group (n=29)	p-value
Mean age *(range)*		39.71 *(27-52)*	39.57 *(25-52)*	41.01 *(19-52)*	39.14 *(23-52)*	ns
Gender (%)	Male	32.3	14.3	48.9	31	0.06
	Female	67.7	85.7	51.1	69	
Pregnant (%)		0	0	1.4	5	-
Seasonal vaccination 2009-2010 (%)		76.3	85.7	76.3	20.7	0.0001
Any previous influenza vaccination (%) 2009		3.2	100	63	27.6	0.0001

To analyze vaccine immunogenicity, antibody responses of all participants were analyzed by HI assays. Furthermore, cellular responses were determined by IFN-γ ELISpots in a subset of participants to investigate the presence of vaccine-induced T cell responses (**Figure 2**). To enable comparison of induction and duration of immune responses following vaccination, all responses were categorized. As hypothesized in **Figure 3**, baseline responses of participants are placed in category I, representing the variable background response of subjects (**Table II**). Responses of participants that are not vaccinated during the study are considered not to change considerably and therefore individuals will remain in category I, unless they do receive a vaccination during this study. Based on these rules, individuals can be placed in 11 different categories (**Figure 3** and **Table II**). To account for individual variation and other confounding factors, results were analyzed statistically using the mixed effects negative binomial regression model. Differences between groups are expressed as relative rates (RR).

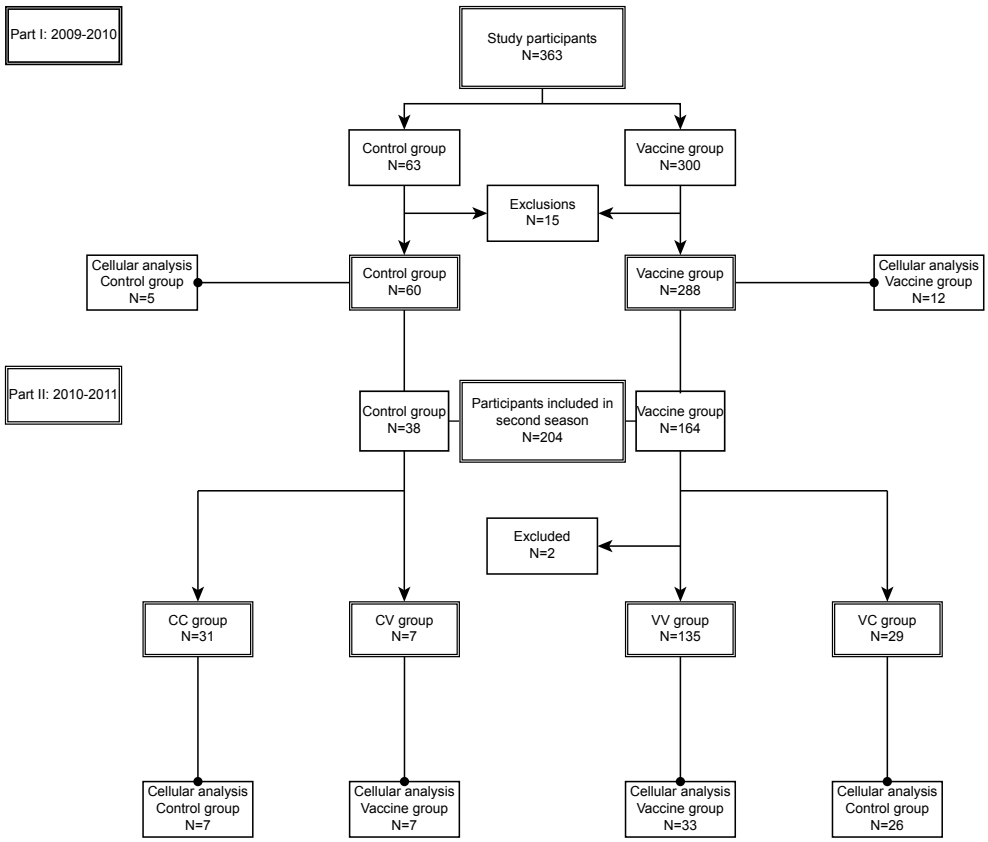

Figure 2: Study disposition:
Excluded in 2009-2010: Eight subjects were lost to follow up, two received occupational vaccination while in the control group, four only received first vaccination and, one was too old.
Excluded in 2010-2011: One withdrew consent, one due to use of corticosteroids
CC= Control group during both seasons, CV= Control group during first season, switch to vaccine group at the start of the second season, VV= Vaccine group during both seasons, VC= Vaccine group during the first season, switch to control group at the start of the second season.

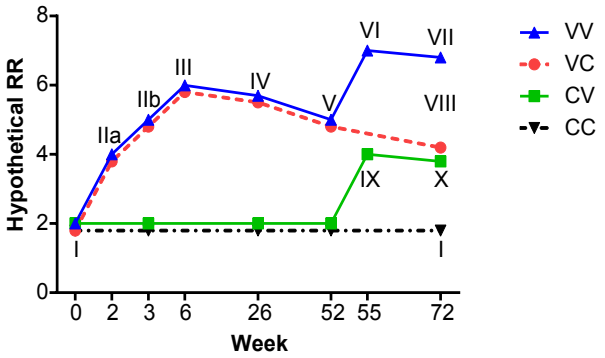

Figure 3: Hypothesis of Negative Binominal model of the immune responses
Responses of individuals can be classified by 11 different categories corresponding with both time points at which samples were collected and the vaccination status of the individual at that time point (categories I-X). For example: Category I are baseline responses of individuals that were not vaccinated at the moment of sampling. Individuals receiving their first dose were placed in category II and a booster vaccination placed individuals in category III. Categories IIa and IIb are the cellular and antibody responses, respectively, after one dose.
CC= Control group during both seasons, CV= Control group during first season, switch to vaccine group at the start of the second season, VV= Vaccine group during both seasons, VC= Vaccine group during the first season, switch to control group at the start of the second season.

Table II: Construction of categories of responses

Category	Week	Serology	ELISpot	Name
I	0-72	Yes	Yes	Baseline
IIa	*2*	*No*	*Yes*	*First dose (Cellular)*
IIb	*3*	*Yes*	*No*	*First dose (Serology)*
III	6	Yes	Yes	Second dose
IV	26	Yes	No	Contraction phase V[1]
V	52	Yes	Yes	Maintenance phase V[1]
VI	55	Yes	Yes	Secondary seasonal 2010-2011 vaccination VV[2]
VII	72	Yes	No	Contraction phase VV[2]
VIII	72	Yes	Yes	Residual level VC[3]
IX	55	Yes	Yes	Primary seasonal 2010-2011 vaccination CV[4]
X	72	Yes	No	Contraction phase CV[4]

[1]V=Vaccine group, first season
[2]VV=Vaccine vaccine group
[3]VC=Vaccine control group
[4]CV=Control vaccine group

One dose of the MF59-adjuvanted vaccine induced adequate antibody responses

In **Figure 4A**, relative antibody responses to A(H1N1)pdm09 are depicted for all groups during both seasons. The first dose of the adjuvanted pandemic vaccine increased the RR of the antibody level 17.3 fold compared to baseline (IIb versus I; p<0.001) (**Table SIA**). The second dose induced a further relative increase of 1.3 compared to primary vaccination (III versus IIb; p<0.001), showing that there is a rapid induction of antibody responses after a first dose with the pandemic vaccine and that these responses increase after a second dose. To evaluate vaccine efficacy, standard analysis of HI titers was performed according to the EMA guidelines for pandemic vaccines. One dose of vaccine induced an 18-fold increase of the GMT, seroprotection in 87.7% and seroconversion in 78% of the vaccinated, which was sufficient to meet all three EMA criteria, while the second dose induced a further increase in antibody levels (**Table III**). After vaccination, antibody levels wane quickly, however, at week 26 antibody levels were significantly higher than baseline (RR: 11.1; IV versus I; p<0.001) (**Table SIA**).

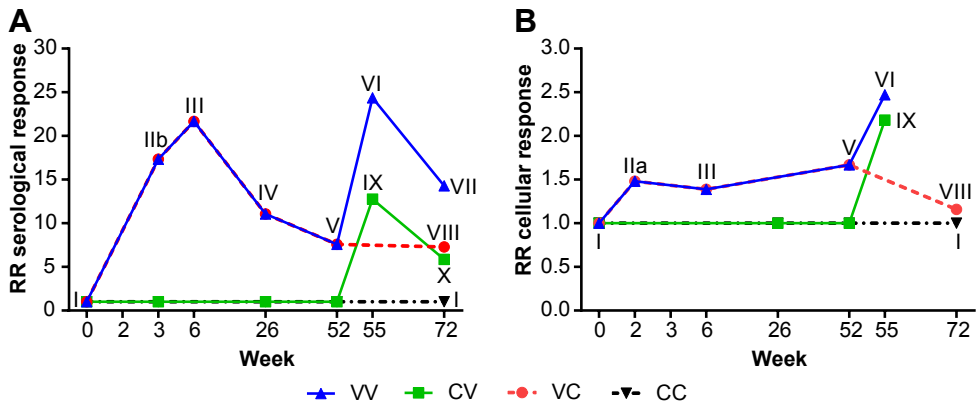

Figure 4: Relative response rates
Profile of serological (**A**) and cellular (**B**) responses. X-axis depict sampling weeks and Y-axis depicts relative response.

Residual antibody levels were boosted by seasonal vaccine

At the start of the second season the RR of antibody levels in vaccinated individuals had declined further, but still remained higher compared to control individuals (RR: 7.6; V versus I; p<0.001) (**Figure 4A**). Seasonal vaccination resulted in a significant increase in RR of individuals in the VV group with a RR of 24.4 compared to primary baseline (VI versus I; p<0.001) (**Table SIB**). Titers of individuals that were vaccinated for the first time in the second season (CV) significantly increased 12.8 fold compared to baseline (IX versus I; p<0.001). This implies a booster effect of the seasonal vaccine on the antibody levels induced by the pandemic vaccine in the previous year since the RR of VV individuals was 2-fold higher compared to CV individuals (VI versus IX; p=0.028) (**Table SIC**). However, no significant difference was found between antibody levels of individuals that had received the first dose

of the adjuvanted pandemic vaccine and individuals vaccinated only in the second year with the unadjuvanted seasonal vaccine (IIb versus IX; p=0.599). These results imply that 7.5 µg HA antigen adjuvanted with MF59 is as efficient at inducing antibodies as a regular unadjuvanted antigen dose of 15 µg HA.

One year after vaccination no further reduction in antibodies levels was observed in individuals that switched to the control group at the start of the second season, showing a duration of the antibody response for over 15 months (VIII versus V; p=0.699, RR:7.28; VIII versus I; p<0.001) (**Table SIB**). Similar as the vaccine-induced antibody response in the first season, antibody levels of individuals vaccinated in the second season (groups VV and CV) significantly reduced between week 52 and the end of the study (VI versus VII p< 0.001 and IX versus X; p=0.032) (**Table SIB**). At week 72, individuals in the VV group did end up with a larger residual antibody level compared to CV and VC individuals (VII versus X; p<0.001 and VII versus VIII; p<0.001), while no significant difference was observed between VC individuals and CV individuals at week 72 (VIII versus X; p=0.699) (**Table SIC**). These results indicate an advantage of annual vaccination with the same vaccine strain on the height of the antibody levels.

First dose of the MF59-adjuvanted vaccine induced cellular responses

Since the MF59-adjuvanted pandemic vaccine has been proposed to induce T cell responses, also cellular immune responses to the virus strains were analyzed in a subset of participants of the vaccinated and control groups. All subjects of the CV group were analyzed due to the small size of this group (n=7). **Figure 4B** depicts relative rates of T cell responses to the A(H1N1)pdm09 strain. A significant increase with a RR of 1.5 was observed two weeks after the first dose of the pandemic vaccine (IIa versus I; p<0.001) (**Table SIIA**). Three weeks after the second dose, the RR was 1.4 compared to the baseline level (III versus I; p<0.001). The difference in T cell response ratio between weeks 2 and 6 was not significant, therefore we conclude that, contrary to antibody responses, a second dose did not boost T cell responses (RR: 0.9; IIa versus III; p=0.8). Strikingly, no significant reduction in T cells, RR of 1.2, was observed between the level obtained after pandemic vaccination and the start of the second season (III versus V; p=0.11) (**Table SIIB**).

Seasonal vaccine is capable of inducing T cell responses

Similar to antibody responses, at the start of the second season, a significantly higher level of T cells with a RR of 1.7 was observed in vaccinated individuals compared to non-vaccinated individuals (**Figure 4B**; V versus I; p<0.001). Moreover, in VV individuals, a 2.5 increase in RR was observed after seasonal vaccination compared to the primary baseline (VI versus I; p<0.001). In individuals not vaccinated in the previous year (CV) a significant induction of T cells was observed with a RR of 2.2 (IX versus I; p<0.001). In addition, T cell responses to the new seasonal vaccine strain A/Perth/16/2009(H3N2) showed an increase in RR of 1.9, strengthening data on T cell induction by the seasonal vaccine (**Table SIII**). T cell levels to

A(H1N1)pdm09 obtained after singular vaccination (CV) and re-vaccination (VV) were similar (VI versus IX; p=0.819). Therefore, previous pandemic vaccination does not appear to be an advantage for VV individuals compared to T cell responses of CV individuals. By week 72, the T cell response of individuals that switched to the control group in the second year (VC) had decreased to primary baseline level (VIII versus I; RR 1.2; p=0.544), implicating a duration of the T cell response of approximately 15 months (**Table SIIC**).

Pandemic and seasonal vaccine induce HA and NA-specific responses

All cellular responses described above were analyzed by stimulation of PBMCs with live virus. As the vaccines only contained HA and NA from influenza virus, we postulate that vaccine-induced responses described after virus stimulation were mostly directed to the HA and NA proteins. To confirm this hypothesis, responses specific for the vaccine strains were further analyzed in an IFN-γ ELISpot by stimulation of PBMCs with peptide pools spanning the entire HA or NA protein of A(H1N1)pdm09. In **Figure 5**, responses to the HA- and NA-peptide pools of A(H1N1)pdm09 are depicted for the first season. After one dose, there was a significant increase in T cell responses to HA, which was not boosted by the second dose (**Figure 5A**).

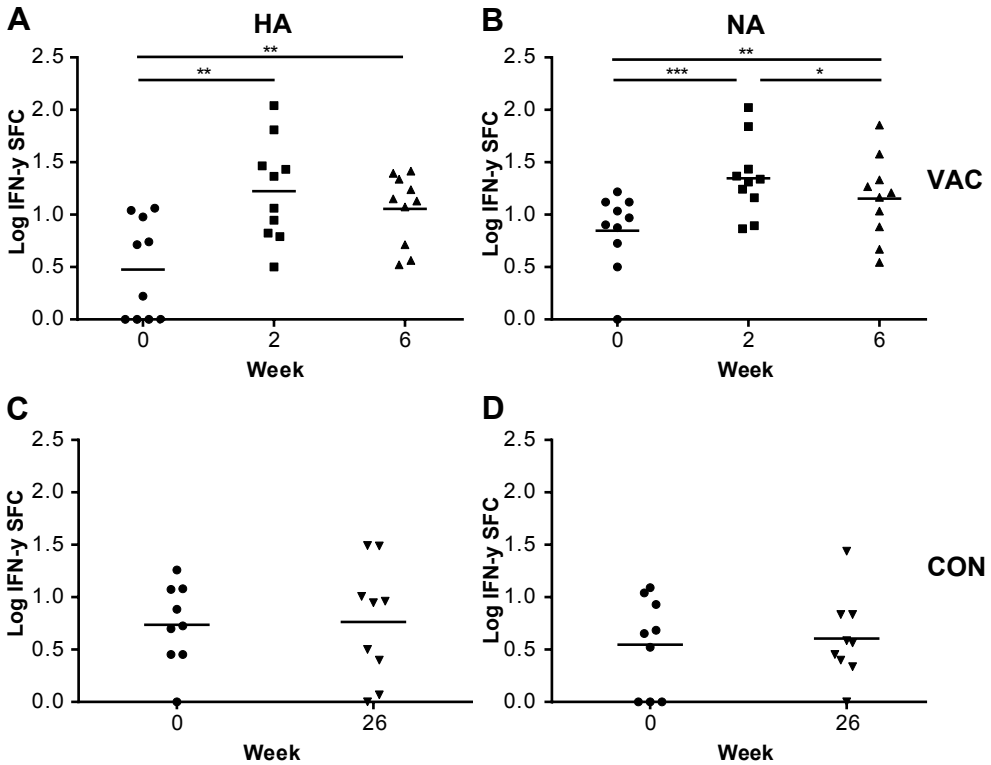

Figure 5: Vaccine-specific T cell responses in the first season

Responses against HA (**A**) and NA (**B**) peptide pools were measured with an IFN-γ ELISpot in individuals of the vaccine group on samples taken at weeks 0, 2 and 6. Responses against HA (**C**) and NA (**D**) were measured on samples drawn at weeks 0 and 26. Number of spot forming cells (SFC) are corrected for non-stimulated controls. * p<0.05, ** p<0.01, ***p<0.001

Similar observations were made for NA protein (**Figure 5B**). Responses of individuals in the control group remained similar during the first season (**Figures 5C** and **5D**). Hereby, we show that the pandemic vaccine is indeed capable of inducing HA and NA-specific T cell responses.

Likewise, vaccine-specific T cell responses were observed during the second season. Three weeks post seasonal vaccination, PBMCs of individuals in the VV and CV groups were isolated and stimulated with HA or NA of both A(H1N1)pdm09 and A/Perth/16/2009(H3N2). VV individuals showed increased T cell responses to all peptide pools (**Figure 6**). Individuals in the CV-group had a significant induction of T cell responses after stimulation with NA derived from A(H1N1)pdm09 and HA of A/Perth/16/2009(H3N2) (**Figures 6B** and **6C**). When comparing virus-stimulation and peptide-stimulation, individuals in the CV group had a significant increase in responses after only one vaccination as measured by virus stimulation which was confirmed by the NA of A(H1N1)pdm09 and HA of A/Perth/16/2009 (H3N2) peptide pool

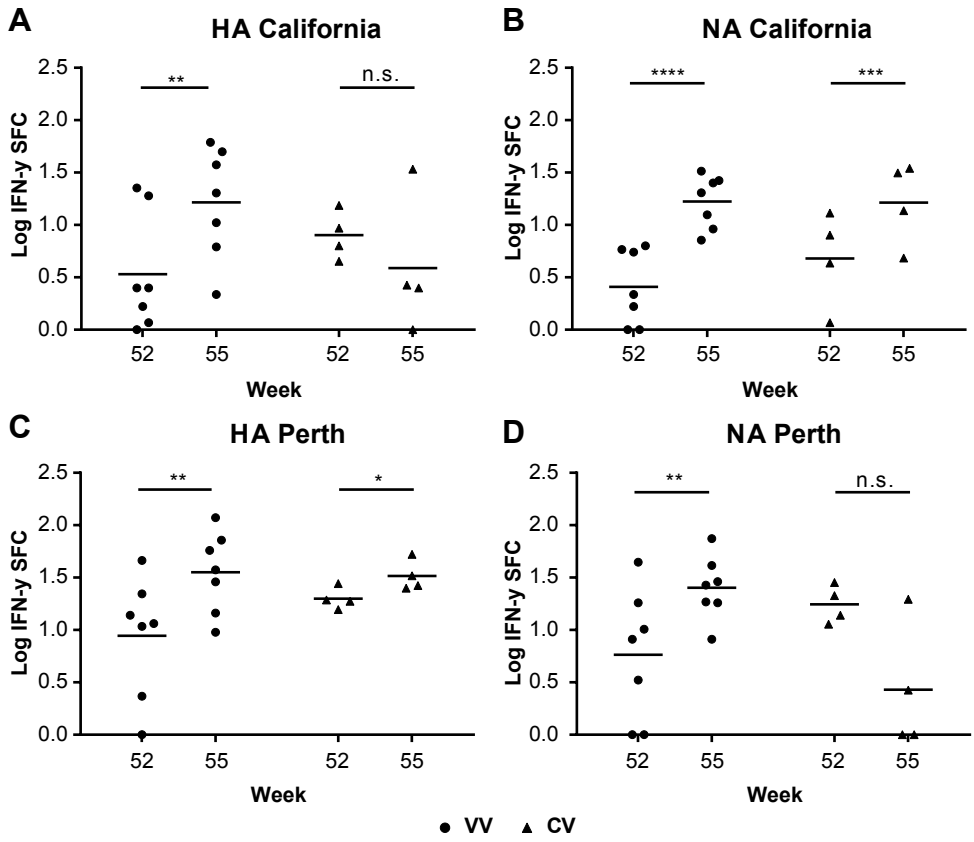

Figure 6: Vaccine-specific T cell responses in the second season
Responses against A(H1N1)pdm09 HA (**A**) and NA (**B**) peptide pools were measured with an IFN-γ ELISpot right before and three weeks after vaccination. Responses against A/Perth/16/2009(H3N2) HA (**C**) and NA (**D**) peptide pools were measured with an IFN-γ ELISpot right before and three weeks after vaccination. Number of spot forming cells (SFC) are corrected for non-stimulated controls.* p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001

stimulations (**Figures 4B**, **6B** and **C**). These results indicate an advantageous effect of 2010-2011 influenza vaccination.

Correlation of humoral and cellular immune response

Figure 4 summarizes the relative rates of antibody and T cell responses during both seasons, enabling a comparison of vaccine-specific antibody and T cell responses. The first dose of the pandemic vaccine resulted in a significant induction of both antibody and T cell responses, while a second dose only improved antibody responses. Individuals vaccinated in the first season had residual antibody and T cell responses and thus appear to have an advantage at the start of the second season. This advantage is reflected by antibody induction, but not T cell responses, as a single seasonal vaccination (CV) induces lower antibody titers but similar levels of T cell responses compared to VV individuals. At week 72, T cell responses were only measured for individuals in the control groups (CC and VC group), showing that responses of VC individuals had decreased to baseline level 15 months after their last vaccination. In contrast, antibody levels were measured for all groups and showed that residual levels of all groups that received at least one vaccination, remained significantly higher than baseline. Therefore, we can conclude that vaccine-induced antibody responses are detectable in the blood for a longer period than T cell responses measured in this study.

Discussion

In this study, the antibody and T cell mediated immune response following influenza vaccination was evaluated during two consecutive influenza seasons from 2009 to 2011. The emergence of A(H1N1)pdm09 provided us with the opportunity to evaluate influenza vaccine immunogenicity in a unique setting. The Dutch Health Council recommended vaccination with two doses of a MF59-adjuvanted monovalent A(H1N1)pdm09 vaccine, which allowed us to evaluate both the unusual two dose schedule and the effect of MF59 adjuvation on immunogenicity of the pandemic vaccine. One dose of the pandemic vaccine induced antibody responses sufficient for providing seroprotection and, in addition, induced vaccine-specific T cell responses. A second dose further increased antibody responses but not T cell responses.

Furthermore, in the subsequent influenza season, the trivalent seasonal vaccine contained the pandemic strain of the previous season, A(H1N1)pdm09, and a new H3N2 strain, A/Perth/16/2009(H3N2), allowing for analysis of booster effect of previous vaccination with the A(H1N1)pdm09 strain. Both antibody and T cell responses could be boosted by the seasonal vaccine. In addition, a comparison could be made of an adjuvanted and unadjuvanted influenza vaccine. Immunogenicity of the influenza vaccines was evaluated by measuring both vaccine-specific antibody and T cell responses during both influenza seasons. Furthermore, we show that the seasonal vaccine alone is capable of inducing vaccine-specific T cell responses, despite the fact that the vaccine did not contain an adjuvant.

In addition, residual antibody levels remained detectable for over 15 months, while T cell levels had reduced back to baseline levels by that time. We conclude that vaccine-induced antibody responses are detectable in the blood for a longer period than T cell responses measured in this study. However, this does not necessarily indicate that vaccine-specific T cells are no longer present. Memory T cells might reside in (lymphoid) tissues instead of in circulation, which is not reflected by measuring PBMC-specific T cell responses in the blood (23-25).

During the first season, immunogenicity of the MF59-adjuvanted monovalent A(H1N1)pdm09 vaccine was evaluated. Adjuvants, such as MF59, have been shown to reduce the dose of antigen needed and to induce a longer lasting antibody-mediated immune response (8). To assure seroprotection, the Dutch Health Council chose to advise a two-dose schedule as recommended by the manufacturer. The choice of administering two doses was based on studies on avian influenza vaccination where two doses were needed to obtain sufficient antibody responses (26). These studies with H5 influenza vaccines showed that two adjuvanted vaccine doses were required to obtain antibody levels that correlate with protection according to EMA criteria and furthermore they induced memory B cells (27-30). In this study, we observed in a cohort of healthy individuals that one dose induced

antibody responses sufficient to conform to EMA guidelines for the registration of pandemic vaccines. In concordance, others have shown that one dose also induced adequate levels of seroprotection in other target groups of 2009 pandemic vaccination, i.e., infants, elderly and immunocompromised individuals (31-33). In addition, data on H9N2 vaccines indicate that one dose of an adjuvanted vaccine is sufficient for protection against H9N2 subtypes (34).

Efficacy of vaccines for newly emerging subtypes appear to be affected by cross-reactive immunity. For individuals that do not have pre-existing immunity, one or even two doses might not be sufficient to provide seroprotective antibodies as shown by a study with H5 subtypes (27). In contrast, a study on H9N2 vaccines showed that individuals who had cross-reactive H2 antibodies available, responded better to one dose of an H9N2 subunit vaccine than individuals that did not have cross-reactive antibodies available. This cross-reactivity has been proposed to be due to structure similarity of H2 and H9 (35). However, there is also literature available on neutralizing antibodies that are directed to the conserved stalk domain of HA (36-38). Therefore, cross-reactive immunity may provide partial protection that can be boosted by vaccination. Thus, when an influenza subtype crosses over to the human population for the first time, the presence of cross-reactive immunity could determine whether one or two doses are needed to provide seroprotection.

Although antibodies provide primary protection against influenza virus infection, T cells are needed to clear infection when these antibodies fail to induce neutralizing protection. The importance of T cells is especially clear in situations where low cross-protective neutralizing antibodies are observed, and shows the additive value of inducing T cell responses by vaccination (39-41). The MF59-adjuvanted vaccine has been shown to induce follicular helper $CD4^+$ T cells and presence of these cells predict antibody responses (42). Furthermore, MF59 recruits immune cells, such as macrophages and monocytes, to the site of infection, and was shown to induce differentiation of monocytes to DCs, which in turn can also prime $CD8^+$ T cell responses (43). Therefore, the MF59-adjuvanted vaccine is expected to induce T cell responses in addition to antibody responses.

Analysis of vaccine-induced T cell responses was performed by stimulation of PBMCs with whole influenza virus or HA or NA-specific peptide pools using an IFN-γ ELISpot. In most individuals, we observe a background level of T cell responses before vaccination, which are more prominent in the whole virus stimulation assays. Background levels of these responses are the consequence of activation of T cells induced by natural infection or previous vaccination and will include the response to internal viral proteins. In the model, we correct for these background levels by studying an additional induction. Peptide pools solely containing vaccine antigens enabled us to make assumptions about vaccine-induced T cells alone. However, future studies are required to analyze the full cytokine profile of these responses, dissecting the nature of adjuvanted and unadjuvanted vaccine-induced T cells.

In the peptide ELISpot assay, we observed an induction of T cells already after vaccination with one dose of the adjuvanted vaccine. However, after the second dose, T cell responses remained similar to responses measured after one dose. McElhany et al. even found a negative correlation between antibody levels and cytokine ratios in elderly and proposed that a second dose might skew T cell responses to the production of IL-10, which limits CTL induction but is advantageous for antibody responses (44). We only evaluated T cell responses by IFN-γ production and are therefore currently not able to support this notion. Others reported an inverse correlation between pre-vaccination IFN-γ production and the magnitude of responses post-vaccination (45, 46). As described by Bodewes et al., annual vaccination with a seasonal vaccine hampers the development of influenza-specific $CD8^+$ T cells in children, indicating that vaccination history also affects the development of T cell responses (47). To conclude, both a second dose as well as previous vaccination and exposure to influenza might affect T cell responses induced by vaccination.

The number of doses and the quantity of antigen that are needed to induce sufficient protection during a pandemic might be related to the presence of cross-reactive antibody and T cell immunity. It is therefore important to obtain knowledge on pre-existing immunity to the virus, since this can be an indication whether a second dose is necessary. During the 2009 H1N1 pandemic, data became available that individuals had some cross-reactive T cells available that provided partial protection (48). In addition, antibodies cross-reacting to the pandemic strain were observed in older adults, which corresponds with the lower number of affected individuals in this age group (49). Although in this study individuals born during the previous H1N1 era, from 1917-1956, were excluded to limit the effects of cross-reactive immunity on the measurement of vaccine efficacy, there may still have been cross-reactive immunity present in younger individuals, which may in part explain why one dose of the pandemic vaccine already induced sufficient protection.

Therefore, it is also of significance what type of immune response is induced by regular seasonal vaccines, especially if administration of a second dose or annual vaccination might have a negative effect on the T cell response that is induced. In this study, we showed that both the adjuvanted pandemic vaccine containing 7.5 μg HA and the unadjuvanted seasonal vaccine containing 15 μg of HA were capable of inducing T cell responses. Others have shown that T cell responses can be induced by unadjuvanted split seasonal influenza vaccination in children, but focused only on internal influenza proteins (50). To date, not much data is available on the induction of T cells by the trivalent inactivated influenza vaccine containing HA and NA as viral antigens. However, we show that even an unadjuvanted subunit vaccine is capable of inducing T cell responses.

This study has some limitations. During this study, individuals were monitored for influenza-like illness (ILI) and ILI cases were laboratory confirmed for the presence of influenza within 72 hours of onset of symptoms. However, both the pandemic and consecutive year were

very mild influenza seasons in the Netherlands and only sporadic infections were observed in individuals in this study. Therefore, we could correct in our model for influenza infections during the study period. In addition, individuals with a laboratory-confirmed A(H1N1)pdm09 influenza infection before the start of the study were excluded. However, we cannot exclude the possibility that subclinical infections have occurred. Another confounding factor is the limited number of individuals that were enrolled in the CV group, which may have affected results of the HA and NA ELISpot assay, specifically. In addition, in this study, IFN-γ was used as the only read-out for T cell responses, while other cytokines or assays may provide with a more complete picture of the T cell response. For example, it would be interesting to elucidate whether the T cell responses measured in this study can be contributed to $CD4^+$ T cells, $CD8^+$ T cells or both and the additional cytokines secreted by the activated T cells.

Summarizing, we showed that one dose of the MF59-adjuvanted pandemic vaccine induced seroprotective levels of antibodies, which were boosted after administration of a second dose. This second dose did not boost the number of vaccine-induced T cell responses. At the start of the second season, a residual level of antibody and T cell levels was detectable in individuals vaccinated in the previous season. Administration of the 2010-2011 seasonal vaccine boosted both antibody and T cell levels. Comparison of the adjuvanted and unadjuvanted vaccine showed that the adjuvanted vaccine induced significantly higher antibody levels, while T cell levels induced after pandemic or seasonal vaccination were similar. Furthermore, we show that antibody levels were still detectable after 15 months, whereas T cell levels had decreased back to baseline.

These findings have key implications for influenza vaccination strategies, especially during pandemic situations. When cross-protective immunity is available, in the form of conserved antibody or T cell responses, one vaccination dose might be sufficient to provide protection. Since repeated influenza vaccination may not be favorable for the induction of T cell responses, it is important to have knowledge on cross-reactive immunity available. Therefore, studies describing the immune response following influenza vaccination should not only focus on the humoral immune response, but should also include analysis of cellular responses.

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Supplemental data

Table SIA: Serological responses 2009-2010

Vaccination response 2009-2010		RR *(2.5-97.5% CI)*	p-value
First dose *(IIb)*	Primary Baseline *(I)*	17.3 *(15.5-19.4)*	<0.001
Second dose *(III)*	Primary Baseline *(I)*	21.7 *(19.3-24.2)*	<0.001
Contraction V *(IV)*	Primary Baseline *(I)*	11.1 *(9.8-12.4)*	<0.001
Second dose *(III)*	First dose *(IIb)*	1.3 *(1.1-1.4)*	0.001
Second dose *(III)*	Contraction V *(IV)*	0.5 *(0.5-0.6)*	<0.001
Contraction V *(IV)*	Maintenance V *(V)*	1.5 *(1.3-1.7)*	<0.001

Table SIB: Serological responses 2010-2011

Vaccination response 2010-2011		RR *(2.5-97.5% CI)*	p-value
Maintenance V *(V)*	Primary Baseline *(I)*	7.6 *(6.8-8.5)*	<0.001
Secondary seasonal 2010-2011 VV *(VI)*	Primary Baseline CC *(I)*	24.4 *(21.6-27.5)*	<0.001
Contraction VV *(VII)*	Primary Baseline *(I)*	24.4 *(21.6-27.4)*	<0.001
Residual VC *(VIII)*	Primary Baseline *(I)*	7.3 *(5.8-9.0)*	<0.001
Primary seasonal 2010-2011 CV *(IX)*	Primary Baseline *(I)*	12.8 *(8.0-19.3)*	<0.001
Contraction CV *(X)*	Primary Baseline *(I)*	5.9 *(3.7-8.9)*	<0.001
Maintenance V *(V)*	Secondary seasonal 2010-2011 VV *(VI)*	3.2 *(2.8-3.6)*	<0.001
Secondary seasonal 2010-2011 VV *(VI)*	Contraction VV *(VII)*	0.6 *(0.5-0.7)*	<0.001
Maintenance V *(V)*	Residual VC *(VIII)*	1.0 *(0.8-1.2)*	0.699
Primary seasonal 2010-2011 CV *(IX)*	Contraction CV *(X)*	0.5 *(0.3-0.8)*	0.032

Table SIC: Comparison groups

Comparison groups		RR *(2.5-97.5% CI)*	p-value
Secondary seasonal 2010-2011 VV *(VI)*	Primary seasonal 2010-2011 CV *(IX)*	2.0 *(1.2-3.1)*	0.028
Contraction VV *(VII)*	Contraction CV *(X)*	0.5 *(0.4-0.6)*	<0.001
Contraction VV *(VII)*	Residual VC *(VIII)*	2.0 *(1.7-2.6)*	<0.001
Residual VC *(VIII)*	Contraction CV *(X)*	1.3 *(0.8-2.1)*	0.699

Table SID: Comparison time points

Comparison time points		RR *(2.5-97.5% CI)*	p-value
First dose *(IIb)*	Primary seasonal 2010-2011 CV *(IX)*	0.7 *(0.5-1.1)*	0.599
Second dose *(III)*	Secondary seasonal 2010-2011 VV *(VI)*	1.1 *(1.0-1.3)*	0.328
Maintenance V *(V)*	Contraction CV *(X)*	0.7 *(0.5-1.2)*	0.674

Table SIIA: Influenza A(H1N1)pdm09 specific T cell responses 2009-2010

Vaccination response 2009-2010		RR (2.5-97.5% CI)	p-value
First dose *(IIa)*	Primary Baseline *(I)*	1.5 *(1.3-1.7)*	>0.001
Second dose *(III)*	Primary Baseline *(I)*	1.4 *(1.2-1.6)*	>0.001
Maintenance V *(V)*	Primary Baseline *(I)*	1.7 *(1.4-1.4)*	>0.001
First dose *(IIa)*	Second dose *(III)*	0.9 *(0.8-1.1)*	0.819
Second dose *(III)*	Maintenance V *(V)*	1.2 *(1.0-1.4)*	0.11

Table SIIB: Influenza A(H1N1)pdm09 specific T cell responses 2010-2011

Vaccination response 2010-2011		RR (2.5-97.5% CI)	p-value
Secondary seasonal 2010-2011 VV *(VI)*	Primary Baseline *(I)*	2.5 *(2.0-3.0)*	<0.001
Primary seasonal 2010-2011 CV *(IX)*	Primary Baseline *(I)*	2.2 *(1.5-3.1)*	<0.001
Residual VC *(VIII)*	Primary Baseline *(I)*	1.2 *(0.9-1.4)*	0.544
Maintenance V *(V)*	Secondary seasonal 2010-2011 VV *(VI)*	1.5 *(1.2-1.8)*	0.001
Maintenance V *(V)*	Residual VC *(VIII)*	0.7 *(0.6-0.9)*	0.005

Table SIIC: Comparison groups influenza A(H1N1)pdm09 specific T cell responses

Comparison groups		RR (2.5-97.5% CI)	p-value
Secondary seasonal 2010-2011 VV *(VI)*	Primary seasonal 2010-2011 CV *(IX)*	1.2 *(0.8-1.7)*	0.819
Secondary seasonal 2010-2011 VV *(VI)*	Residual VC *(VIII)*	2.1 *(1.7-2.7)*	>0.001
Primary seasonal 2010-2011 CV *(IX)*	Residual VC *(VIII)*	1.9 *(1.2-2.8)*	0.021

Table SIII: Influenza A/Perth/16/2009(H3N2) specific T cell responses

		RR (2.5-97.5% CI)	p-value
Vaccination	Baseline	1.9 (1.6-2.2)	<0.001

Chapter 4

Synthetic long peptide influenza vaccine containing conserved T and B cell epitopes reduces viral load in lungs of mice and ferrets

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Abstract

Currently licensed influenza vaccines mainly induce antibodies against highly variable epitopes. Due to antigenic drift, protection is subtype or strain-specific and regular vaccine updates are required. In case of antigenic shifts, which have caused several pandemics in the past, completely new vaccines need to be developed. We set out to develop a vaccine that provides protection against a broad range of influenza viruses. Therefore, highly conserved parts of the influenza A virus (IAV) were selected of which we constructed antibody and T cell inducing peptide-based vaccines. The B epitopes vaccine consists of the highly conserved HA2 fusion peptide and M2e peptide coupled to a CD4 helper epitope. The T epitope vaccine comprises 25 overlapping synthetic long peptides of 26-34 amino acids (25 SLP), thereby avoiding restriction for a certain MHC haplotype. These peptides are derived from nucleoprotein (NP), polymerase basic protein 1 (PB1) and matrix protein 1 (M1). C57BL/6 mice, BALB/c mice, and ferrets were vaccinated with the B epitopes, 25 SLP or a combination of both. Vaccine-specific antibodies were detected in sera of mice and ferrets and vaccine-specific cellular responses were measured in mice. Following challenge, both mice and ferrets showed a reduction of virus titers in the lungs in response to vaccination. Summarizing, a peptide-based vaccine directed against conserved parts of influenza virus containing B and T cell epitopes shows promising results for further development. Such a vaccine may reduce disease burden and virus transmission during pandemic outbreaks.

Introduction

In influenza infection, both antibodies and T cell responses play an important role in viral clearance and protection against disease. In general, antibodies bind to the virus particles to prevent infection or spread of the virus, while T cells can kill virus-infected cells and provide help to other cells of the immune system. Many of the traditional influenza vaccines are only aimed at the induction of antibodies and are often poor inducers of T cell responses (1, 2). Since these vaccines mainly confer protection via antibodies directed against the highly variable surface proteins hemagglutinin (HA) and neuraminidase (NA), protection is subtype or strain-specific. Due to mutations in the antigenic site, so-called antigenic drift, influenza virus can escape vaccine-induced immunity and consequently regular vaccine updates are required. In addition, current vaccines do not provide protection against newly emerging influenza subtypes, which has multiple times led to the outbreak of a pandemic. Universal influenza vaccines could provide a solution to these issues, and are therefore an important subject in influenza vaccine research.

A universal vaccine should target conserved parts of the virus, thereby inducing cross-protection against multiple influenza subtypes. The globular head of HA is highly variable, while the N-terminal fusion peptide of the HA2 subunit is highly conserved. The fusion protein plays a crucial role in the fusion process of the viral envelope with the host membrane. Therefore, antibodies directed against the highly conserved fusion protein might be able to neutralize infectivity of influenza virus by inhibiting fusion. Chun et al. showed that it is indeed possible to induce antibodies that specifically bind to the fusion peptide, making it a promising target for vaccine development (3-5). Another highly conserved region is the extracellular domain of the M2 protein (M2e) (6). Antibodies directed against the M2e domain are not as immunogenic as antibodies directed against the globular head of HA or NA, since M2e is not directly accessible to antibodies. However, M2e-specific antibodies can limit spread of the virus by binding virus particles to the cell, thereby preventing release into the extracellular fluid (7). Furthermore, these antibodies have been shown to play a role in antibody-mediated cytotoxicity (8).

The internal proteins of influenza virus also contain several conserved regions, as reviewed by Stanekova et al.. Screening of PBMCs from healthy donors showed that NP is a major target of immunodominant cytotoxic T cell (CTL) responses (9-11). Other important targets of T cell responses to influenza are located in matrix protein 1 (M1) and polymerase basic protein 1 (PB1) (12). Recently, two studies have shown the importance of T cell mediated protection in subjects lacking strain specific pre-existing humoral immunity. Sridhar et al. described that individuals with higher numbers of pre-existing $CD8^+$ T cells specific for conserved epitopes developed less severe illness after infection with pandemic H1N1 influenza virus (13). Wilkinson et al. monitored T cell responses of healthy volunteers following influenza challenge, and observed that lower virus shedding and less severe illness correlated with the presence of

influenza-specific cytotoxic $CD4^+$ T cells (14).

Another promising strategy for universal vaccine development is therefore to induce T cell responses to conserved parts of the virus, for example by vaccination with peptides. The first, and most successful, peptide-based vaccine for virus infections is a therapeutic vaccine against human papilloma virus (HPV). This vaccine contains synthetic peptides of at least 25 amino acids long, directed against viral oncoproteins and induced vaccine specific $CD4^+$ and $CD8^+$ T cell responses in all patients (15). While peptides of 8-10 amino acids long can bind directly to MHC class I, long peptides require processing by professional APCs, which reduces the chance of inducing tolerance by peptide vaccination (16). Furthermore, long peptides often contain multiple epitopes that are not restricted to a certain MHC haplotype, thereby broadening the potential response at both the individual and population level (17). Another advantage is that, in addition to CD8 epitopes, these longer peptides often contain CD4 epitopes. Specific $CD4^+$ T helper cells support effective co-stimulation during priming of $CD8^+$ T cells and promote memory $CD8^+$ T cells (18-21). Depending on the peptides that are selected, this strategy can be applied for a wide range of viral infections.

We aimed to develop a vaccine capable of providing cross-protection against multiple influenza A subtypes, by inducing both T cell and antibody responses directed towards conserved parts of the virus. Therefore, 25 overlapping long synthetic peptides were selected based on conservation in different subtypes of influenza viruses. Furthermore, peptides containing the highly conserved HA2 fusion peptide and M2e epitope were coupled to a CD4 helper epitope to increase their immunogenicity. Mice and ferrets were vaccinated with these peptide vaccines, either alone or in combination, to evaluate immunogenicity of both the antibody and T cell components. The protective capacity of these promising vaccine candidates was evaluated in influenza A challenge models in C57BL/6, BALB/c mice and ferrets. The vaccine was shown to be capable of reducing viral load in the lungs.

Materials and methods

Ethics statement

This study was approved by the Committee on Animal Experimentation of the Netherlands Vaccine Institute (Bilthoven, the Netherlands) under permit numbers 201100334, 201200164 and 201200281. Animal handling was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation. When possible, mice were anesthesized by isoflorane in O_2 to minimize suffering. For all challenge experiments, human endpoints were set prior to the start of the study to prevent unnecessary suffering of the animals. Endpoints were based on clinical signs of influenza disease such as heavy breathing, activity, posture and fur condition and on decrease in bodyweight compared to weight prior to challenge. If animals reached these endpoints they were euthanized.

Selection of 25 synthetic long peptide (25 SLP) vaccine

H1N1 and H5N1 viruses, with a collection date from 1970 until 2010, were selected from the NCBI Influenza Virus Sequence Database. All hosts and countries/regions were included, only full-length sequences were included, and lab strains were excluded. Using the alignment tool, conserved regions were identified for each protein of the influenza virus. Then, influenza proteins NP, PB1, and M1 were selected since these contained the largest amount of conserved regions which also coded for a number of known CD8 and CD4 epitopes (**Table SI**) (12, 22-24). From these regions, 25 overlapping peptides were selected: eleven from NP, eight from PB1 and six from M1. Final length of these peptides was based on the inclusion of known epitopes as described in **Table SI**, therefore the length of these peptides ranges from 26-34 amino acids (**Table I**).

Conservancy analysis

Conservancy was analyzed using LALIGN from the Swiss Institute for Bioinformatics, based on a paper by Huang and Miller (25). Peptides used in the vaccines were aligned with proteins of vaccine strains and possible pandemic strains from different influenza A subtypes: A/California/07/2009 (H1N1), A/Victoria/361/2011 (H3N2), A/Japan/305/1957 (H2N2), high pathogenicity avian influenza virus (HPAI) A/turkey/Turkey/1/2005 (H5N1), A/Anhui/1/2013 (H7N9) and HK-X31 (a mouse adapted influenza strain containing the HA and NA proteins of A/Aichi/68 and internal genes from A/Puerto Rico/8/34). The sequence of A/Anhui/1/2013 was obtained from GISAID and provided by Lei Yang from the WHO Chinese National Influenza Center (Beijing, China).

Peptide synthesis

All peptides used in these experiments were prepared by normal Fmoc-chemistry using preloaded Tentagel resins (PyBop/NMM) for in situ activation, and 20% piperidine in NMP for Fmoc removal. Couplings were performed for 60 minutes with 6-fold acylating species. After final Fmoc removal, peptides were cleaved with TFA/H_2O 19/1 (v/v) containing additional scavengers when C (triethylsilane) or W (ethanethiol) were present in the peptide sequence.

Table I: Conservancy analysis peptides

Peptide	Sequence	H1N1	H3N2	H2N2	H5N1	H7N9	HK-X31
NP_{17-46}	GERQNATEIRASVGRMIGGIGRFYIQMCTE	96.7	90	93.3	96.2	93.3	96.7
NP_{37-65}	GRFYIQMCTELKLSDYEGRLIQNSLTIER	93.1	93.1	100	96.6	93.1	100
NP_{56-81}	LIQNSLTIERMVLSAFDERRNKYLEE	96.2	96.2	100	92.3	92.3	100
NP_{67-93}	VLSAFDERRNKYLEEHPSAGKDPKKTG	100	100	100	96.3	96.3	100
$NP_{191-220}$	ELIRMIKRGINDRNFWRGENGRKTRIAYER	93.3	93.3	100	96.7	96.7	96.7
$NP_{212-244}$	GRKTRIAYERMCNILKGKFQTAAQKAMMDQVRE	90.9	90.9	97	93.9	93.9	100
$NP_{234-263}$	AQKAMMDQVRESRNPGNAEFEDLTFLARSA	90	86.7	90	90	90	100
$NP_{254-281}$	EDLTFLARSALILRGSVAHKSCLPACVY	96.4	92.9	96.4	96.4	96.4	100
$NP_{317-349}$	RPNENPAHKSQLVWMACHSAAFEDLRVSSFIRG	100	93.9	97	100	100	90.9
$NP_{378-403}$	TLELRSRYWAIRTRSGGNTNQQRASA	96.2	96.2	100	100	100	100
$NP_{394-420}$	GNTNQQRASAGQISIQPTFSVQRNLPF	92.6	92.6	96.3	100	92.6	100
$PB1_{1-41}$	MDVNPTLLFLKVPAQNAISTTFPYTGDPPYS	96.8	100	100	96.4	96.8	100
$PB1_{21-51}$	TFPYTGDPPYSHGTGTGYTMDTVNRTHQYSE	100	100	100	100	96.8	100
$PB1_{401-434}$	ASLSPGMMMGMFNMLSTVLGVSILNLGQKRYTKT	97.1	97.1	94.1	100	97.1	100
$PB1_{474-503}$	GINMSKKKSYINRTGTFEFTSFFYRYGFVA	96.7	96.7	100	100	100	100
$PB1_{494-523}$	SFFYRYGFVANFSMELPSFGVSGINESADM	96.7	100	100	100	100	100
$PB1_{534-562}$	MINNDLGPATAQMALQLFIKDYRYTYRCH	100	100	100	100	100	100
$PB1_{588-617}$	GLLVSDGGPNLYNIRNLHIPEVCLKWELMD	100	100	100	100	100	100
$PB1_{699-727}$	FPSSSYRRPVGISSMVEAMVSRARIDAR	100	96.4	100	100	100	100
$M1_{31-60}$	VFAGKNTDLEALMEWLKTRPILSPLTKGIL	100	100	100	96.7	93.3	96.7
$M1_{51-80}$	ILSPLTKGILGFVFTLTVPSERGLQRRRFV	100	100	100	96.7	100	100
$M1_{69-98}$	PSERGLQRRRFVQNALNGNGDPNNMDKAVK	96.7	100	96.7	96.7	100	100
$M1_{87-115}$	NGDPNNMDKAVKLYRKLKREITFHGAKEI	89.7	100	96.6	89.7	89.7	100
$M1_{167-194}$	TTTNPLIRHENRMVLASTTAKAMEQMAG	100	92.9	100	96.4	100	100
$M1_{180-206}$	VLASTTAKAMEQMAGSSEQAAEAMEVA	100	96.3	100	100	100	100
M2e	SLLTEVETPIRNEWGSRSNDSSD	73.9	91.3	91.3	76.2	56.5	87
HA2	GLFGAIAGFIENGWEG	87.5	93.8	87.5	87.5	100	93.8

Numbers depict the percentage of conservancy.

Peptides were isolated by ether/pentane 1:1 (v/v) precipitation and the product was isolated by centrifugation. Following air-drying at 40°C, peptides were dissolved in acetic acid/water 1:10 (v/v) and lyophilized. Purity of the peptides was analyzed using UPLC-MS (Acquity, Waters) and integrity was confirmed by using Maldi-Tof mass spectrometry (Microflex, Bruker) (26). All peptides used in these experiments were synthesized at the LUMC (Leiden, the Netherlands).

Vaccines

The B epitopes vaccine for mice contained the following long peptides; SLLTEVETPIRNEWGSRSNDSSD deduced from M2e, and GLFGAIAGFIENGWEG deduced from the HA2 fusion peptide. For the ferret experiments, the M2e peptide and the HA2 fusion peptide were adapted for the Influenza A/turkey/Turkey/1/2005 H5N1 virus, resulting in the following sequences: SLLTEVETP**T**RNEW**E**SRS**S**DSSD and GLFGAIAGFIE**G**GW**Q**G, respectively. Adaptations are shown in bold. The HA2 and M2e peptides were coupled N-terminally to the CD4 helper peptide CPKYVKQNTLKLATG ($HA_{321-335}$) for murine experiments or to the universal helper peptide PADRE (UKXVAAWTLKAAU; U=d-Ala and X=cyclohexylalanine) for ferret experiments (27). Therefore, when there is referred to the B epitopes as a vaccine, these are always conjugated to one of the helper peptides. The 25 SLP vaccine contained 25 synthetic long overlapping peptides as shown in **Table I**. Mice received 20 nmol of each of the indicated peptides admixed in PBS, Incomplete Freund's Adjuvant (IFA, Invivogen) and 5 nmol Pam3CysSK4 (Invivogen). C57BL/6 mice also received a 2 SLP vaccine containing $HA_{321-335}$ and RGVQIASNENMETMESSTLE ($NP_{361-380}$) alone or in combination with the B epitopes. Ferrets received a combination of the 25 SLP and B epitopes vaccine, with 200 µg of each peptide, admixed with 10 nmol Pam3CysSK4 either with or without IFA, as indicated. Mice received their vaccination in a volume of 0.1 mL, and ferrets in 0.5 mL. Mice and ferrets in the positive control groups were vaccinated by means of intranasal infection (i.n.) with a low dose of live HK-X31 virus ($1*10^2$ (first infection) or $1*10^3$ (second infection) $TCID_{50}$) or Highly Pathogenic Avian Influenza virus (HPAI) H5N1 influenza A/turkey/Turkey/1/2005 virus ($1*10^4$ $TCID_{50}$), respectively. Negative control animals received the respective adjuvant mixture without peptides.

Immunization and challenge experiments in mice

Female C57BL/6 and BALB/c mice were obtained from Jackson Laboratory and used at 8-10 weeks of age. Mice were vaccinated subcutaneously (s.c.) on days 0 and 14, with respective vaccines, and prior to primary vaccination serum samples were collected. I.n. vaccinations were performed under anesthesia with isoflurane in O_2. On day 28, serum samples were collected and mice were challenged i.n. with 50 µl of a sub-lethal dose of $1*10^5$ pfu HK-X31 in PBS. Starting from challenge, bodyweight and clinical signs were recorded daily. Five days after challenge, four mice (C57BL/6) or six mice (BALB/c) per group were sacrificed by bleeding and cervical dislocation under anesthesia with isoflurane in O_2. Spleens and lymph nodes (LNs) were processed directly for use in T cell assays, and lungs were excised and

stored at <-70°C for further analysis. The remaining mice, six per group for C57BL/6 and ten for BALB/c, were sacrificed two weeks after challenge. None of the mice needed to be euthanized prior to the experimental endpoint.

Immunization and challenge experiments in ferrets

Female ferrets (*Mustela putorius furo*), aged 6-12 months, were obtained from Triple F Farms. All ferrets were confirmed to be negative for previous circulating influenza virus infection and Aleutian disease. A temperature transponder (DST micro T, Star-Oddi) was implanted in the peritoneal cavity 14 days prior to start of the experiment, which recorded the temperature every 30 minutes. Area under curve (AUC) above the baseline temperature was calculated for each group from day of viral challenge up to their sacrifice, to analyze fever development. On days 0 and 21, ferrets were vaccinated with respective vaccines. Two weeks after booster vaccination, they were challenged with $1*10^5$ $TCID_{50}$ H5N1 Influenza A/turkey/Turkey/1/2005 in 3 mL via the intratracheal route (i.t.). We decided to use the i.t. route, because vaccine-induced T cells reside mainly in the lungs and less in the nasal cavity, which is the target of i.n. challenge. Moreover, this model induces disease similar to that seen in humans after H5N1 infection (28, 29). Prior to challenge, ferrets were moved to BSL-3 isolators and from this point on, each day clinical signs were scored and every other day ferrets were weighed. Ferrets exhibiting pre-determined endpoints were euthanized by cardiac bleeding under anesthesia with ketamine (5 mg/kg) and medetomidine (0.1 mg/kg). Five days after challenge, ferrets were sacrificed and distal sections of the right lung and accessory lobes were isolated and stored at < -70°C for further virological analysis. Furthermore, serum was isolated from blood collected at day 35 and stored at -20°C until further use. Blood sampling, immunizations, and challenge were performed under anesthesia by ketamine (5 mg/kg) and medetomidine (0.1 mg/kg). After handling, the anesthesia was antagonized with atipamezole (0.25 mg/kg) with the exception of challenged ferrets when anesthesia was not antagonized to avoid a sneezing reflex.

Peptide ELISA

Streptavidin plates (Euro Diagnostica AB) were coated with HA2 or M2e peptide-biotin in a concentration of 1 nmol/ml PBS for one hour at RT. After washing the plates three times with wash buffer (PBS with 0.05% Tween-20), the plates were blocked with block buffer (5% skim milk powder (Sigma Aldrich) in PBS) for one hour at RT. Subsequently, plates were washed, sera were diluted in dilution buffer (PBS containing 1% BSA (Roche)), in two-step dilutions and incubated for two hours at RT. Next, plates were washed and goat α-mouse IgG, IgG1, IgG2a, IgG2b or IgG2c HRP (Southern Biotech) in a dilution of 1:5000 or goat α-ferret IgG (Acris antibodies) in a dilution of 1:10,000 in dilution buffer were added to the wells. After one hour incubation at RT, 50 µl TMB (Sigma) was added and the reaction was stopped with 50 µl 1M H_2SO_4. Absorbance was read at 450 nm.

Virus ELISA

Immulon II (Thermo Scientific) plates were coated with HK-X31 which had been purified by sucrose gradient centrifugation, in a concentration of 400 ng/ml HA as determined by calculating 1/3 of total protein measured by a Pierce assay. Alternatively, plates were coated with whole inactivated virus (WIV) prepared from the H5N1 vaccine strain NIBRG-23 in a concentration of 1200 ng/ml HA. Following O/N incubation, plates were washed with wash buffer (water with 5% Tween-80) and sera were diluted in two-fold steps in dilution buffer (PBS with 0.1% Tween-80). Then, plates were incubated at 37°C and after one hour, goat α-mouse IgG HRP conjugate (Southern Biotech) was added in a concentration of 1:5000 or goat α-ferret IgG HRP conjugate in a concentration of 1:10,000 (diluted in PBS with 0.5% Protifar (Nutricia)) and plates were incubated for another hour at 37°C. Next, wells were washed with wash buffer and substrate (NaAC, TMB and H_2O_2) was added to the wells. After 10 minutes, 100 µL/well of 2M H_2SO_4 was added per well to stop the reaction and OD was measured at 450 nm.

Enzyme-linked immunospot (ELISpot) assay

IFN-γ ELISpot assays were performed according to the manufacturer's protocol (U-Cytech). Spleens and inguinal lymph nodes were homogenized and passed through 70 µM filters (BD Bioscience). Then, spleen cells and LN cells were washed with RPMI (containing 10% FCS, Penicillin, Streptomycin and Glutamin) and counted using a Casy cell counter (Roche). Cells were plated in a concentration of $4*10^5$ cells/well in an IFN-γ antibody coated PVDF membrane plate (MSIP plates Milipore). Cells were stimulated in duplo with 1 µg of two NP-derived peptides selected from the 25 overlapping peptides pool (FYIQMLTEL and AYERMCNIL). After O/N incubation, spots were visualized according to the manufacturer's protocol (U-Cytech). Spots were counted using A.EL.VIS software (A.el.vis).

Virological analysis

Lungs were homogenized using FastPrep (MP Biomedicals) homogenizer and clarified by low speed centrifugation. Virus titers were determined by end-point titration on Madin-Darby canine kidney (MDCK) cells. In short, MDCK cells were seeded in 96-well plates at a density of $1\text{-}5*10^4$ cells/well and incubated at 37°C until 90-100% confluence was reached. The cells were inoculated in quadruplets/sextuplets with 200/100 µL of homogenized lungs and diluted five-fold serially. After six days of incubation at 37°C, wells were scored for cytopathic effects (CPE) and infection was confirmed by an hemagglutination assay. The $TCID_{50}$ titer was determined by the Reed and Muench method (30).

Statistical analysis

All data were log transformed and tested for normality by the d'Agostino-Pearson test. Normally distributed data were analyzed by the unpaired-t test and not normally distributed data were analyzed by the Mann-Whitney test. All statistical analysis was performed using Graph Pad Prism 6.04 (GraphPad Software Inc., San Diego, CA).

Results

Design of universal long peptide influenza vaccine

For the development of a universal influenza vaccine, we included either T cell or B cell epitopes, derived from conserved parts of influenza A virus. At time of peptide selection, H1N1 had just evolved into a pandemic and H5N1 viruses posed a threat of inducing a new pandemic. Therefore, selection of the long overlapping peptides was based on these two virus subtypes. **Table I** shows sequences of all peptides included in the vaccines and conservancy analysis of these peptides against several subtypes of influenza A viruses. A/California/07/2009 (H1N1) and A/Victoria/361/2011 (H3N2) were included in the 2013-2014 seasonal vaccine. A/Leningrad/137/1957 (H2N2), A/turkey/Turkey/1/2005 (H5N1), and A/Anhui/1/2013 (H7N9) are of subtypes that could possibly cause a new pandemic. As shown in **Table I**, HA2 fusion peptide and all T cell peptides are highly conserved in all, over 3000, strains analyzed, as illustrated by a similarity of 85% or higher. These peptides also contain a high number of known CD8 and CD4 T cell epitopes presented by a number of different MHC molecules (**Table SI**). M2e protein also has large sequence similarities, although sequence similarity is not as high as for the HA2 fusion peptide. In mice, the peptides were admixed with IFA and Pam3CysSK4 as an adjuvant combination. Although, IFA is not suitable for use in humans, it was included to guarantee an optimal adjuvant effect.

Immunogenicity of B epitopes in mice

Evaluation of the B epitopes vaccine was performed in C57BL/6 and BALB/c mice. Sera collected 14 days post booster vaccination were analyzed by ELISA for HA2-specific and M2e-specific antibody responses, to determine whether immunization with B epitopes induced vaccine-specific antibodies. No HA2 responses were detected in an HA2-specific ELISA (data not shown). M2e-specific IgG antibodies, however, were detected in sera of C57BL/6 and BALB/c mice, proving the immunogenicity of the M2e B cell epitope (**Figures 1A** and **1B**). To evaluate whether these antibodies could recognize the epitopes in their natural conformation, the sera were analyzed by ELISA for recognition of intact influenza virus. Specific antibodies in the serum of both C57BL/6 and BALB/c mice bound to the complete virus particles (**Figures 1C** and **1D**). Therefore, antibodies induced by the vaccine are not only specific for the M2e peptide, but are also capable of recognizing the epitopes in their natural conformation, which is critical for a protective immune response.

Effect of addition SLP on antibody-response

IgG antibody responses to M2e were further subtyped for IgG1, IgG2b or IgG2a/IgG2c. C57BL/6 mice have a more Th1-skewed response that is correlated with IgG2a/IgG2c antibodies (31). However, no differences were found between the different subtypes (**Figure 1E**). BALB/c mice have a more Th2-skewed response which correlates with higher levels of IgG1 antibodies. As expected, BALB/c mice showed a markedly higher IgG1 response (**Figure 1F**) (32). These types of antibody response alone are often not sufficient for complete protection against influenza

virus infection, therefore, we also evaluated the addition of T cell-inducing components to the vaccine. Primary evaluation was performed in C57BL/6 mice. Mice were vaccinated with the B epitopes to which two known T epitopes, $HA_{321-335}$ and $NP_{361-380}$, (2 SLP + B epitopes) were added. $HA_{321-335}$ is the same peptide as used for conjugation to the B epitopes, $NP_{361-380}$ contains a known CD8 peptide that is dominant in the murine response to influenza (33). Both peptides contain epitopes specific for this H-2^b mouse strain. **Figure 1E** shows that the addition of 2 SLP does not affect the antibody response. Sera of BALB/c mice vaccinated with the B epitopes and 25 SLP (25 SLP + B epitopes), screened for M2e-specific antibodies, showed no differences between the B epitopes alone or the 25 SLP + B epitopes. Thus, even the addition of 25 SLP does not negatively affect antibody responses.

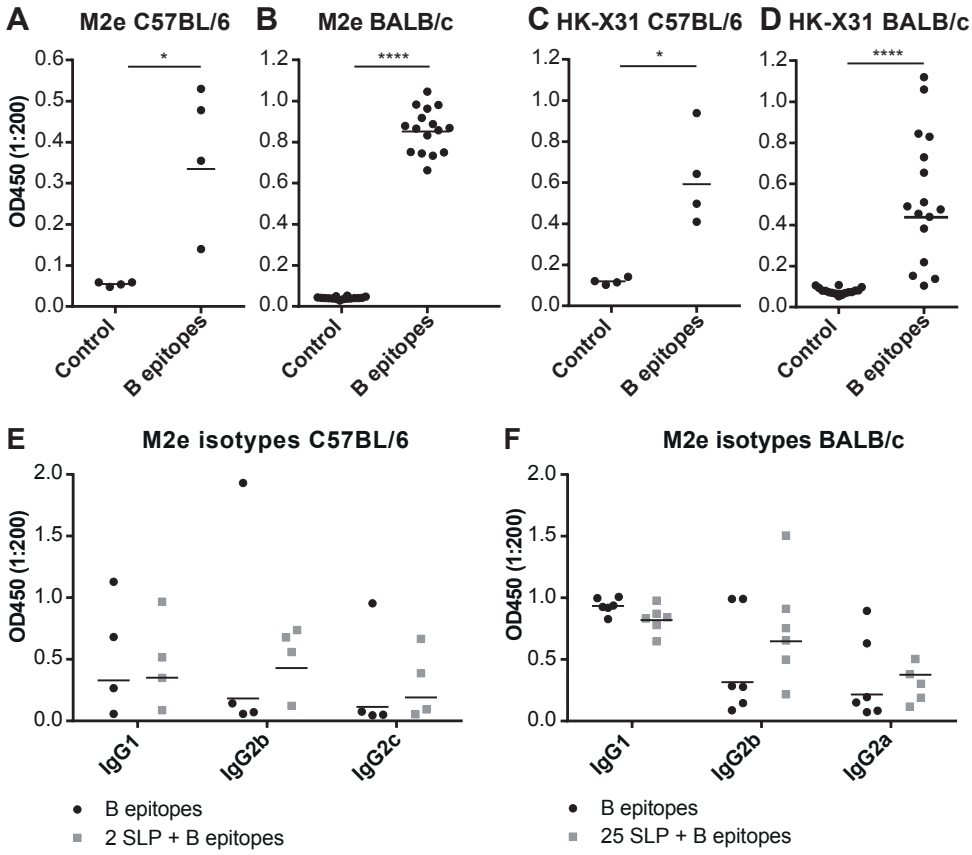

Figure 1: Immunogenicity of B epitopes.
Total IgG antibody levels as measured by an ELISA in sera of C57BL/6 and BALB/c mice vaccinated with B epitopes, two weeks after booster vaccination. M2e specific antibodies in C57BL/6 mice (**A**) and BALB/c mice (**B**) and antibodies directed to HK-X31 in C57BL/6 mice (**C**) and BALB/c mice (**D**). IgG subtyping on M2e antibody responses showed that the addition of T epitopes had no negative effect in C57BL/6 mice (**E**) and BALB/c mice (**F**). When a Gaussian distribution was found data were analyzed with an unpaired t-test, which was the case for the BALB/c experiments. For C57BL/6 experiments a Mann-Whitney test was performed *p=<0.05, ****p=<0.0001

25 SLP induces specific T cell responses

In BALB/c mice, we analyzed whether the 25 SLP vaccine induced T cell responses in an IFN-γ ELISpot assay, on spleen cells and inguinal LNs isolated five days after viral challenge. Cells were stimulated overnight with two short conserved peptides derived from the NP protein (FYIQMLTEL and AYERMCNIL). These two peptides were selected from 25 SLP based on MHC class I prediction of BALB/c epitopes by the ANN method of the Immune Epitope Database (34). **Figure 2A** shows that IFN-γ responses to FYIQMLTEL could be detected in spleen cells of the positive control group that was vaccinated i.n. with a low virus dose. Furthermore, responses were detected in 25 SLP-vaccinated mice (alone or in combination with the B epitopes), but not in B epitopes vaccinated mice. **Figure 2B** shows responses to FYIQMLTEL only in inguinal LNs of 25 SLP-vaccinated mice but not in the virus vaccinated mice. This is not surprising, since the inguinal LNs are not the draining LNs of the lung, to where the i.n. virus vaccination is targeted. To AYERMCNIL, only very low responses were observed in the LNs. These results show that the T cell vaccine is capable of inducing influenza-specific T cell responses.

Effect of vaccination on virus challenge-induced clinical manifestations in mice

Two weeks after booster vaccination, mice were challenged with a sub-lethal dose of HK-X31 virus and weighed daily for 14 days (**Figure S1**). **Figure 3** shows the average bodyweight during the challenge phase, calculated as the average percentage relative to the bodyweight at day of challenge. As a measure for recovery from disease, the average percentage bodyweight should be higher than in the negative control animals. However, all mice lost bodyweight, except the positive control group, which was completely protected. Mice vaccinated with 2 SLP lost bodyweight comparable to the mock vaccinated group, showing that such a minimal amount of peptides is not sufficient for protection from disease. C57BL/6 mice vaccinated

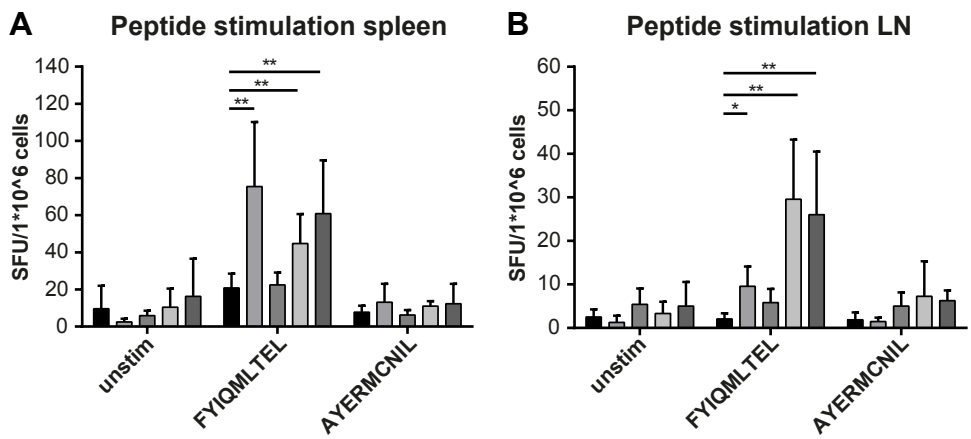

Figure 2: T epitope-specific responses.

IFN-γ ELISpot of splenocytes and LN cells of BALB/c mice five days after viral challenge. Results are shown as spot forming units (SFU) per $1*10^6$ cells. Responses in spleen cells (**A**) and LN cells (**B**) after O/N incubation with either medium, FYICMLTEL or AYERMCNIL. Data were log-transformed and analyzed by Mann-Whitney test. *p=<0.05 **p=<0.01

with B epitopes and mice vaccinated with 25 SLP did show a slightly higher average bodyweight than mock-vaccinated mice although this is not a significant difference. In the experiment with BALB/c mice, the group size was enlarged to increase power. As expected, the positive control group did not lose any weight. Furthermore, recovery was significantly increased in mice vaccinated with B epitopes (p=<0.05). The other vaccine groups did not show a significant increase in weight recovery. Based on bodyweight, the vaccines show no major protection against loss of bodyweight, but do show some improved recovery.

Effect of vaccination on virus replication in mice

As another parameter for vaccine efficacy, viral load in the lungs was analyzed five days after challenge using a $TCID_{50}$ assay. A reduction in viral titers is an important measure of protection, since lower viral titers will lead to reduced spread of the virus. The positive control group (virus vaccination) was completely protected against virus replication in both C57BL/6 and BALB/c mice, as titers were below detection level. **Figure 4** shows that vaccination with both 25 SLP and B epitopes achieved a 1.5-2 log reduction in viral load in the lungs of C57BL/6 mice and BALB/c mice. The combined vaccine achieved a comparable reduction as the B epitopes and 25 SLP alone. 2 SLP was the only vaccine that did not induce a reduction in viral load, indicating that the two long peptides alone are not sufficient for reducing viral load in the lungs. This shows that the peptide-based vaccines significantly reduced virus replication in vivo.

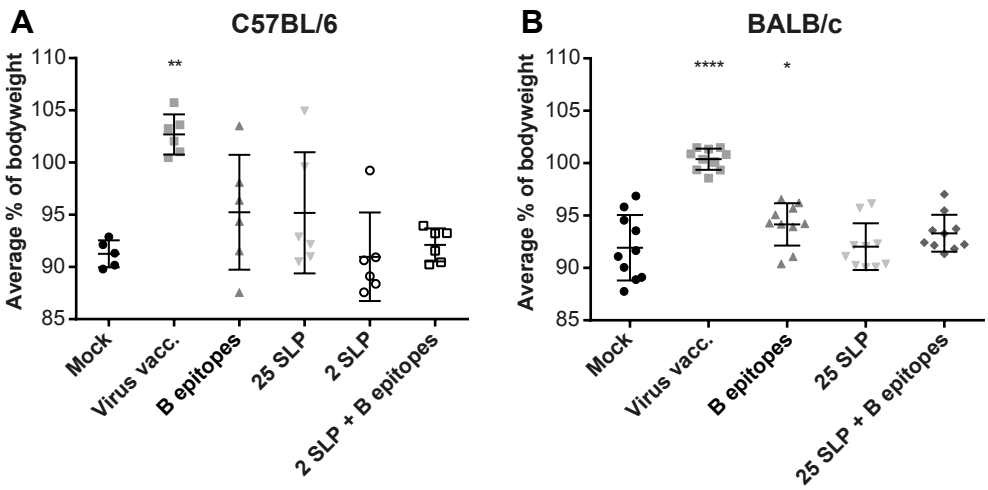

Figure 3: Bodyweight loss post challenge.
C57BL/6 mice (**A**) and BALB/c mice (**B**) were challenged i.n. with $1*10^5$ $TCID_{50}$ of HK-X31 virus and their bodyweight was recorded daily. Results are shown in average percentage of bodyweight relative to the bodyweight at the day of challenge. Data were log-transformed and analyzed for Gaussian distribution. C57BL/6 data were then analyzed with Mann-Whitney test and BALB/c data were analyzed with an unpaired t-test. *p=<0.05 **p=<0.01 ****p=<0.0001 compared to mock vaccinated animals.

Evaluation of combined vaccine in ferrets

Our studies in mice showed that the B epitopes and 25 SLP induced antibody and T cell responses, respectively, and were capable of reducing viral load in the lungs. Next, we evaluated our vaccine concept in a ferret model. Ferrets respond clinically similar to humans to influenza infection and are therefore a commonly used follow up in pre-clinical vaccine development (35). Both B and T cell responses are important in influenza infection and a universal vaccine should therefore target both the humoral and cellular immune response. For this reason, we only included the 25 SLP + B epitopes vaccine. Since route of immunization can also affect efficacy of vaccines, several routes of administration were evaluated in the ferret experiment. Pam3CysSK4 was included as an adjuvant in all vaccines and depending on the route of immunization, IFA was or was not added. IFA is a strong adjuvant for peptide vaccines that forms a depot at the site of injection and is not suitable for routes of immunization other than subcutaneously. Therefore, no IFA was included in the vaccines that were administered via i.m. and i.n. routes.

Immunogenicity of B epitopes in ferrets

Sera collected two weeks after booster vaccination were analyzed for antibodies to HA2 and M2e peptide. No antibodies directed to HA2 peptide were detected in any of the vaccine groups. M2e-specific IgG antibody responses, on the other hand, were detected in sera of all ferrets vaccinated with 25 SLP + B epitopes i.m., s.c. and s.c. with IFA and in two ferrets that received the 25 SLP + B epitopes i.n.. No antibodies were detected in the control groups (**Figure 5A**). To determine whether vaccine-induced antibodies recognize the epitopes in their natural conformation, an ELISA was performed with H5N1 WIV vaccine to enable analysis at

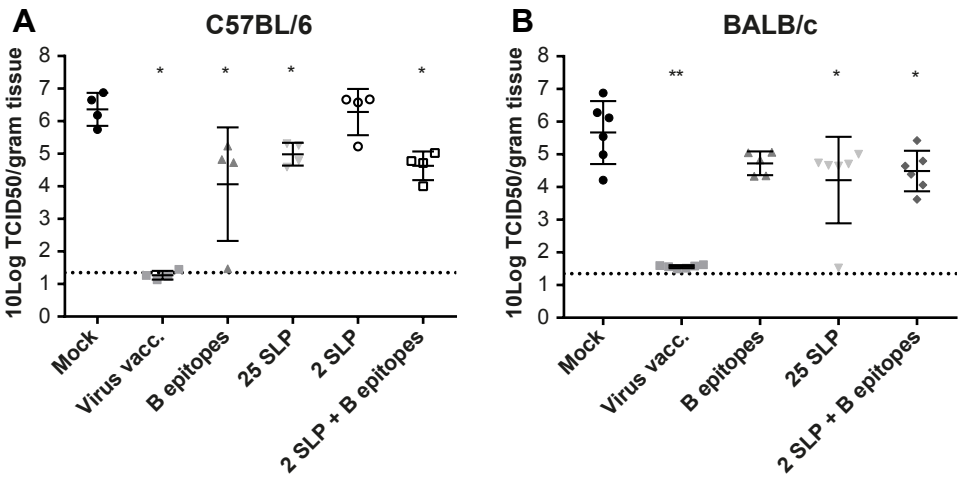

Figure 4: Reduced viral load in the lungs.
Five days after challenge four (C57BL/6) and six (BALB/c) mice were sacrificed and virus titers in the lungs were determined by end point titration. Depicted are log transformed titers in the lungs for C57BL/6 mice (**A**) and BALB/c mice (**B**). Data were analyzed by the Mann-Whitney test.*p=<0.05 **p=<0.01 compared to mock vaccinated mice.

BSL-2. The M2e-epitope of this vaccine is derived from the PR8 strain, which is 73.9% identical to the A/turkey/Turkey/1/2005 sequence. Of the groups that had detectable M2e antibodies, the ferrets that received 25 SLP + B epitopes IFA s.c. and 25 SLP + B epitopes i.m. also had detectable antibodies directed to WIV H5N1 (**Figure 5B**). This shows that the B epitopes are also immunogenic in the ferret model and that these antibodies are capable of recognizing the M2e epitopes even though sequences are not completely homologous.

Effect of vaccination on virus challenge-induced clinical manifestations in ferrets

Two weeks after booster vaccination, ferrets were i.t. challenged with H5N1 influenza virus. The administered challenge dose ($1*10^5$ $TCID_{50}$) was previously determined to be non-lethal in ferrets from a different provider, however a few ferrets in the current study succumbed or had to be sacrificed before scheduled termination. Two animals of the 25 SLP + B epitopes IFA s.c. group and one ferret of the 25 SLP + B epitopes s.c. group had to be terminated on the day prior to section, because they had reached the pre-determined clinical endpoint. One ferret of the mock i.n. group died in the night before section. However, these deviations from the original protocol only had a minor impact on the study. Four to five days after challenge, all ferrets became inactive and showed heavy breathing. No significant differences in bodyweight were observed in the peptide vaccine groups, all ferrets had lost approximately 15% of their bodyweight by day five (data not shown). Bodyweight of the positive control ferret previously vaccinated with virus, by means of a low dose i.n. infection, did remain stable over the five-day challenge period. Since ferrets were sacrificed five days after challenge, which is the optimal day to measure virus titers, recovery in bodyweight could

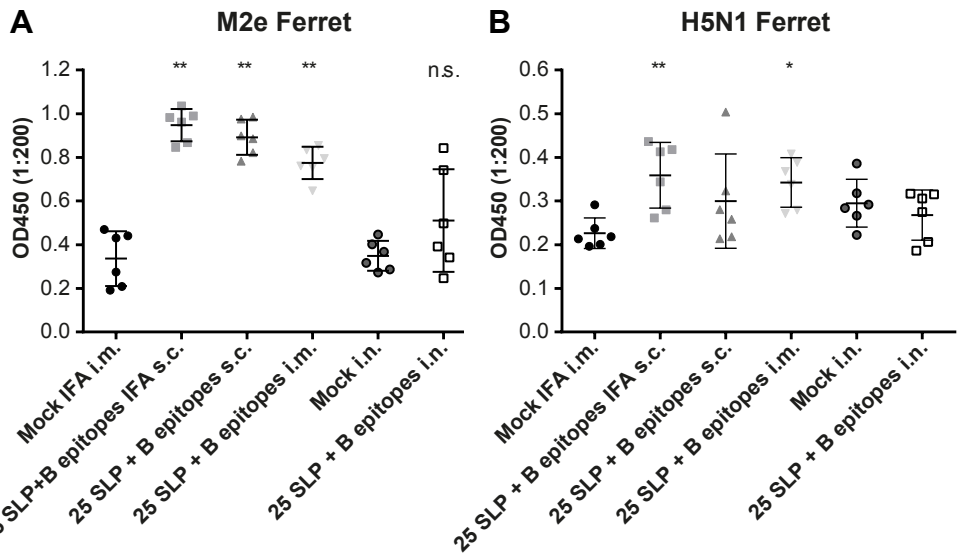

Figure 5: Immunogenicity of B epitopes in ferrets.
IgG responses to the vaccines were measured in ferret sera collected two weeks after booster vaccination, by using M2e peptide (**A**) or whole inactivated influenza H5N1 virus vaccine (**B**) as ELISA antigens.

not be measured as this normally starts seven days after infection. During the five days after viral challenge, all ferrets developed fever, except for the positive control ferret. The ferrets vaccinated i.m. and s.c. with and without IFA also had a significantly lower temperature than the corresponding control (**Table II**). Overall, the vaccine did not ease clinical symptoms of influenza virus infection, did not prevent initial body weight loss, and only had a limited effect on preventing fever.

Effect of vaccination on virus replication in ferrets

Figure 6A shows viral titers in the lungs. A significant difference is observed between the ferrets vaccinated i.m. with the 25 SLP + B epitopes and the corresponding negative control ferrets. **Figure 6B** shows viral load in the trachea, in which there is a significant difference observed between both the i.m. and i.n. 25 SLP + B epitopes vaccinated ferrets and their corresponding controls. The positive control ferret had no detectable virus titers in the lungs, but some virus remained detectable in the trachea. These results show that, depending on the route of immunization, the 25 SLP + B epitopes are capable of reducing virus load in both the lungs and the trachea of ferrets. Concluding, although vaccination of ferrets with the 25 SLP + B epitopes did not significantly ease symptoms of influenza virus challenge, the vaccine is capable of reducing virus titers in the lungs and trachea, which is especially of relevance from a transmission point of view.

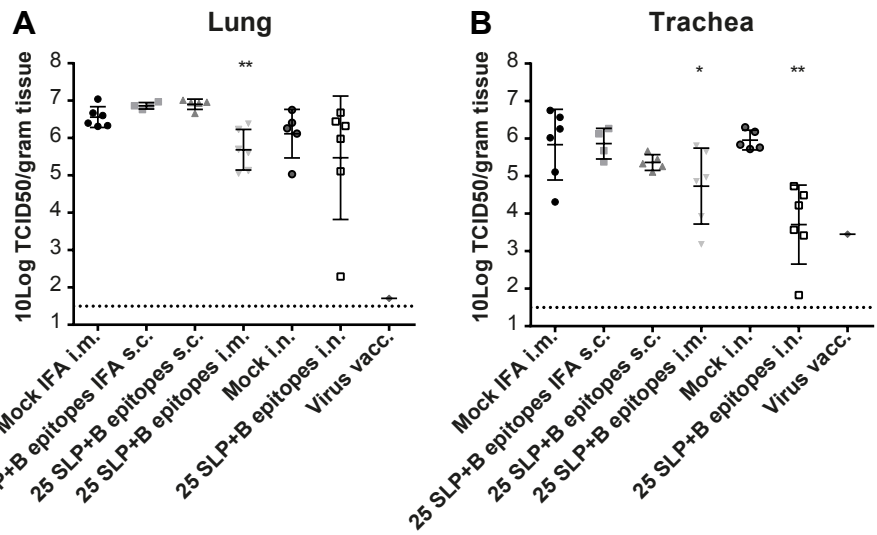

Figure 6: Virological analysis of lungs and trachea of ferrets.
Five days after challenge ferrets were sacrificed and virus titers in the lungs and trachea were determined by end point titration. Depicted are log transformed titers in the lungs (**A**) and trachea (**B**). Ferrets that had to be sacrificed or died prior to scheduled section were excluded from the analysis. Data were analyzed by the Mann-Whitney test.*p=<0.05 **p=<0.01 compared to mock vaccinated mice.

Table II: Body temperature analysis of ferrets during viral challenge

Group	AUC	SD	No. ferrets	Max ΔT (˚C) #	SD	No. ferrets
Mock IFA i.m.	9.3	1.1	6	3.9	0.2	6
25 SLP + B epitopes IFA s.c.	7.4*	2.0	4	4	0.2	6
25 SLP + B epitopes s.c.	5.9**	0.7	5	3.6*	0.1	6
25 SLP + B epitopes i.m.	7.2**	1.9	6	3.7	0.1	6
Mock i.n.	8.3	0.9	5	3.8	0.2	6
25 SLP + B epitopes i.n.	9.3	2.5	5	3.8	0.3	5
Virus vacc.	0.6	n.a.	1	1.3	n.a.	1

Max ΔT depicts the largest increase in temperature during the period after challenge.
Data were analyzed with a Mann-Whitney test. * p=<0.05 ** p=<0.01.

Discussion

Since the emergence of pH1N1 and spillover of potential pandemic H5N1 and H7N9 influenza viruses from the avian reservoir to humans, there has been a boost in research on vaccines that are capable of inducing cross-protection against multiple influenza A subtypes. In the past years, several strategies have been investigated in which conserved parts of influenza virus were targeted. In general, these strategies can be divided into antibody-inducing and T cell-inducing vaccines. Potential antibody-inducing universal vaccines often aim to induce antibodies directed to M2e or HA2 epitopes, which are two promising highly conserved antibody-inducing vaccine candidates (6, 36). T cell responses, on the other hand, are often directed towards conserved parts of the internal proteins of the virus, such as NP, M1, and PB1 proteins (11, 12). While antibodies can limit spread of the virus, T cells are responsible for elimination of the virus by killing virus-infected cells mainly by $CD8^+$ T cells, and $CD4^+$ T cells have an important helper function in several arms of the immune response to viruses. Although T cell inducing vaccines cannot prevent infection, they might be advantageous by inducing rapid clearance of infection and limiting disease severity and virus transmission (37).

In this study, an antibody-inducing vaccine, a T cell-inducing vaccine, and a combined vaccine format were evaluated in C57BL/6 mice, BALB/c mice, and ferrets. The antibody-inducing vaccine consisted of M2e and HA2 fusion peptide. Both epitopes were conjugated to a highly conserved hemagglutinin-derived CD4 helper peptide or to the universal PADRE epitope, to increase immunogenicity of the peptides. In this study, we were indeed able to detect antibodies directed to M2e in C57BL/6 mice, BALB/c mice, and ferrets. Antibodies specific for the HA2 fusion peptide were, however, detected only in a few mice and ferrets.

The antigenic site on the HA2 fusion peptide was first described by Atassi et al., who showed that antibodies directed to the HA2 fusion peptide indeed could bind to synthetic HA2 fusion peptides (38). A more recent study by Stanekova et al. showed induction of antibodies after two immunizations of mice with HA2 fusion peptide conjugated to keyhole limpet hemocyanin (KLH). Following challenge with a lethal dose of homologous or heterologous influenza virus, these antibodies even induced effective cross-protection (39). However, the HA2 fusion peptide is known to be less immunogenic than M2e. (39, 40). This might explain why, in our study, we were not able to detect antibodies directed towards HA2 fusion peptide. However, since we did find lower viral titers in the lungs of mice vaccinated with B epitopes alone, the inclusion of M2e peptide alone appears to be sufficient for reducing viral load in the lungs.

Because not only antibodies, but also T cells can play an important role in influenza infection, in our studies, also a T cell-inducing vaccine was included. The 25 SLP vaccine alone induced a 1.5-2 log reduction in viral titers in the lungs of mice, similar to that found with B epitopes alone. Furthermore, in BALB/c mice, vaccine-specific T cell responses were detected. There are several other articles describing peptide-vaccination strategies that successfully induce

T cell responses and even achieve a certain amount of protection. For example, Matsui et al. coupled $CD8^+$ T cell-specific influenza epitopes to liposomes and were able to induce partial protection in mice. Tan et al. coupled several influenza epitopes to Pam2Cys, resulting in lipopeptides. Vaccination with these constructs reduced viral titers in the lungs of mice with 1-2 log compared to controls or led to increased survival in mice (41-45). Some other strategies for inducing T cell responses to conserved peptides are reviewed by Pica et al. (46).

Although the peptide-vaccine strategies described above are promising, there are some limitations. The sequences used in these papers are indeed all highly conserved, but due to their length also restricted to a certain HLA molecule. Alexander et al. provided a solution by identifying several highly conserved influenza epitopes that can bind to more HLA types. These supertype epitopes could provide broad population coverage, and might therefore be interesting vaccine candidates (22). We chose a different strategy by selecting longer peptides of minimally 26 amino acids to avoid the necessity for HLA typing. In addition to CD8 epitopes, these long peptides also include possible CD4 epitopes. This strategy turned out to be effective in patients with HPV-induced vulvar lesions (15). In the past years, two Phase I clinical trials with influenza peptide-based vaccines were published that implemented a similar strategy. In these studies, respectively four and six long peptides derived from highly conserved sequences, were selected and tested for their safety and immunogenicity. Both vaccines proved to be safe and immunogenic (47, 48). Since our vaccine contains a larger amount of peptides, it has the potency to raise broader responses and cover even more HLA types.

By combining the B epitope and 25 SLP vaccine, we designed a vaccine capable of inducing both antibody and T cell responses against conserved regions and thus potentially providing broad protection. We evaluated this universal peptide influenza vaccine in C57BL/6 mice, BALB/c mice and ferrets. After viral challenge, the protective effects of the peptide vaccines based on clinical symptoms, such as fever and weight loss, were minimal. However, we did find a reduction of viral load in the respiratory tract of both ferrets and mice and although the vaccine did not completely protect the animals, vaccination did lead to some reduction in disease severity. Recently, Laidlaw et al. described that CTLs might act cooperatively with non-neutralizing antibodies. In this antibody dependent cell cytotoxicity (ADCC), killing of virus infected cells is mediated by complement or other cells of the innate immune system (8, 49, 50). Although more research is needed, there are indications that the cooperation of $CD8^+$ T cells, non-neutralizing antibodies, and alveolar macrophages can improve protection towards heterosubtypic viruses (51). These findings promote development of a universal vaccine generating both antigen-specific CTLs and antibodies.

By optimizing the route of administration, the formulation, and the adjuvants used in combination with the 25 SLP + B epitope, efficacy of the vaccine might be enhanced. Therefore, several routes were evaluated in the ferret model. Although ferrets vaccinated

s.c. had M2e-specific antibodies in serum, these ferrets did not show a reduction in virus replication. Ferrets vaccinated i.m. and i.n., on the other hand, did show a reduction in viral titers. There are indications that the efficacy of CTL-inducing vaccines is increased by i.n. vaccination compared to s.c. vaccination, due to enhanced recruitment of CTLs to the lungs (42, 52). Similar findings were observed for M2e, where only i.n. vaccination induced IgA-producing cells in the lungs of BALB/c mice (53).

In addition to the induction of antibody- and T cell responses to conserved parts of the virus, there are other advantages to the use of peptides in a vaccine. In contrast to many of the currently implemented vaccines, peptides can be produced synthetically and are therefore often safer and easier to produce than vaccines containing biological agents. Furthermore, since there are practically no biological limitations, peptide vaccines can easily be produced in large amounts, which is convenient in case of a possible pandemic, when stock-piling is often needed (54).

At this point, a universal influenza vaccine is not expected to completely protect individuals. Reduced virus titers in the lungs will already lead to decreased spread of the virus, which might confine an outbreak to only a small area. Furthermore, disease severity in vaccinated individuals might be decreased and therefore lead to an increased survival rate. We show here that an easy-to-produce non-MHC biased peptide-based vaccine directed against conserved regions containing both B and T epitopes is able to reduce virus replication in lungs of mice and ferrets. After further development, this vaccine might potentially be used in a pandemic situation or supplementary to seasonal vaccines.

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Supplemental data

Peptide	Sequence	Specificity	Allele	Literature
$NP_{273-281}$	KSCLPACVY	CD8	A1	Alexander 2010 (1)
NP_{44-52}	CTELKLSDY	CD8	A1	Wang 2007 (2)
$NP_{329-339}$	QLVWMACHSAA	CD8	A2	Assarsson 2008 (3)
NP_{39-47}	FYIQMCTEL	CD8	A24	Alexander 2010 (1)
NP_{67-76}	RMVLSAFDER	CD8	A3	Assarsson 2008 (3)
$NP_{383-391}$	SRYWAIRTR	CD8	B27	Wang 2007 (2)
NP_{17-25}	GERQNATEI	CD8	B44	Alexander 2010 (1)
$NP_{221-230}$	YERMCNILKG	CD8	B44	Assarsson 2008 (3)
$NP_{338-346}$	FEDLRVSSF	CD8	B44	Assarsson 2008 (3)
$NP_{199-207}$	RGINDRNFW	CD8	B58	Wang 2007 (2)
$NP_{225-233}$	ILKGKFQTA	CD8	B8	Wang 2007 (2)
$NP_{192-208}$	ELIRMIKRGINDRNFWR	CD4	nd*	Lee 2008 (4)
$NP_{264-281}$	LILRGSVAHKSCLPACVY	CD4	nd*	Lee 2008 (4)
$NP_{386-403}$	WAIRTRSGGNTNQQRASA	CD4	nd*	Lee 2008 (4)
$NP_{258-273}$	FLARSALILRGSVAHK	CD8	nd*	Lee 2008 (4)
$NP_{265-273}$	ILRGSVAHK	CD8	nd*	Lee 2008 (4)
$NP_{329-346}$	VWMACHSAAFEDLRVSSF	CD8	nd*	Lee 2008 (4)
NP_{40-57}	YIQMCTELKLSDYEGRLI	CD8	nd*	Lee 2008 (4)
$PB1_{30-38}$	YSHGTGTGY	CD8	A1	Alexander 2010 (1)
$PB1_{489-497}$	TFEFTSFFY	CD8	A1	Assarsson 2008 (3)
$PB1_{590-599}$	LVSDGGPNLY	CD8	A1	Alexander 2010 (1)
$PB1_{591-599}$	VSDGGPNLY	CD8	A1	Assarsson 2008, Wang 2007 (2,3)
$PB1_{407-415}$	MMMGMFNML	CD8	A2	Assarsson 2008 (3)
$PB1_{412-421}$	FNMLSTVLGV	CD8	A2	Assarsson 2008 (3)
$PB1_{413-421}$	NMLSTVLGV	CD8	A2	Alexander 2010 (1)
$PB1_{501-509}$	FVANFSMEL	CD8	A2	Assarsson 2008 (3)
$PB1_{505-514}$	FSMELPSFGV	CD8	A2	Assarsson 2008 (3)
$PB1_{7-14}$	LLFLKVPA	CD8	A2	Assarsson 2008 (3)
$PB1_{496-505}$	FYRYGFVANF	CD8	A24	Alexander 2010 (1)
$PB1_{41-49}$	DTVNRTHQY	CD8	A26	Wang 2007 (2)
$PB1_{488-497}$	GTFEFTSFFY	CD8	A3	Alexander 2010 (1)
$PB1_{490-497}$	FEFTSFFY	CD8	B44	Assarsson 2008 (3)
$PB1_{509-517}$	LPSFGVSGI	CD8	B7	Assarsson 2008 (3)
$PB1_{540-548}$	GPATAQMAL	CD8	B7	Wang 2007, Lee 2008 (2,4)
$PB1_{402-419}$	SLSPGMMMGMFNMLSTVL	CD4	nd*	Lee 2008 (4)
$PB1_{21-38}$	TFPYTGDPPYSHGTGTGY	CD8	nd*	Lee 2008 (4)
$PB1_{1-15}$	MDVNPTLLFLKVPAQ	CD4	DR	Assarsson 2008 (3)
$PB1_{404-418}$	SPGMMMGMFNMLSTV	CD4	DR	Assarsson 2008 (3)
$PB1_{408-422}$	MMGMFNMLSTVLGVS	CD4	DR	Assarsson 2008 (3)
$M1_{52-61}$	ILSPLTKGIL	CD8	A2	Assarsson 2008 (3)
$M1_{58-66}$	GILGFVFTL	CD8	A2	Alexander 2010, Assarsson 2008 (1,3)
$M1_{60-67}$	ILGFVFTL	CD8	A2	Assarsson 2008 (3)
$M1_{100-110}$	LYRKLKREITF	CD8	A24	Assarsson 2008 (3)
$M1_{179-188}$	RMVLASTTAK	CD8	A3	Assarsson 2008 (3)
$M1_{48-58}$	KTRPILSPLTK	CD8	A3	Assarsson 2008 (3)
$M1_{173-181}$	IRHENRMVL	CD8	B39	Wang 2007 (2)
$M1_{196-205}$	SEQAAEAMEV	CD8	B44	Alexander 2010 (1)
$M1_{173-189}$	IRHENRMVLASTTAKAM	CD4	nd*	Lee 2008 (4)
$M1_{180-199}$	VLASTTAKAMEQMAGSSEQA	CD4	nd*	Lee 2008 (4)
$M1_{55-72}$	LTKGILGFVFTLTVPSER	CD4	nd*	Lee 2008 (4)
$M1_{63-80}$	VFTLTVPSERGLQRRRFV	CD4	nd*	Lee 2008 (4)
$M1_{71-88}$	ERGLQRRRFVQNALNGNG	CD4	nd*	Lee 2008 (4)
$M1_{33-49}$	AGKNTDLEALMEWLKTR	CD8	nd*	Lee 2008 (4)
$M1_{40-57}$	EALMEWLKTRPILSPLTK	CD8	nd*	Lee 2008 (4)
$M1_{179-193}$	RMVLASTTAKAMEQM	CD4	DR	Assarsson 2008 (3)
$M1_{58-72}$	KGILGFVFTLTVPSE	CD4	DR	Assarsson 2008 (3)

Table SI: List of known epitopes present in the synthetic long overlapping peptide vaccine.
*nd = not determined

1. Alexander J, Bilsel P, del Guercio MF, Marinkovic-Petrovic A, Southwood S, et al. (2010) Identification of broad binding class I HLA supertype epitopes to provide universal coverage of influenza A virus. Hum Immunol 71: 468-474.

2. Wang M, Lamberth K, Harndahl M, Roder G, Stryhn A, et al. (2007) CTL epitopes for influenza A including the H5N1 bird flu; genome-, pathogen-, and HLA-wide screening. Vaccine 25: 2823-2831.

3. Assarsson E, Bui HH, Sidney J, Zhang Q, Glenn J, et al. (2008) Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. J Virol 82: 12241-12251.

4. Lee LY, Ha do LA, Simmons C, de Jong MD, Chau NV, et al. (2008) Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J Clin Invest 118: 3478-3490.

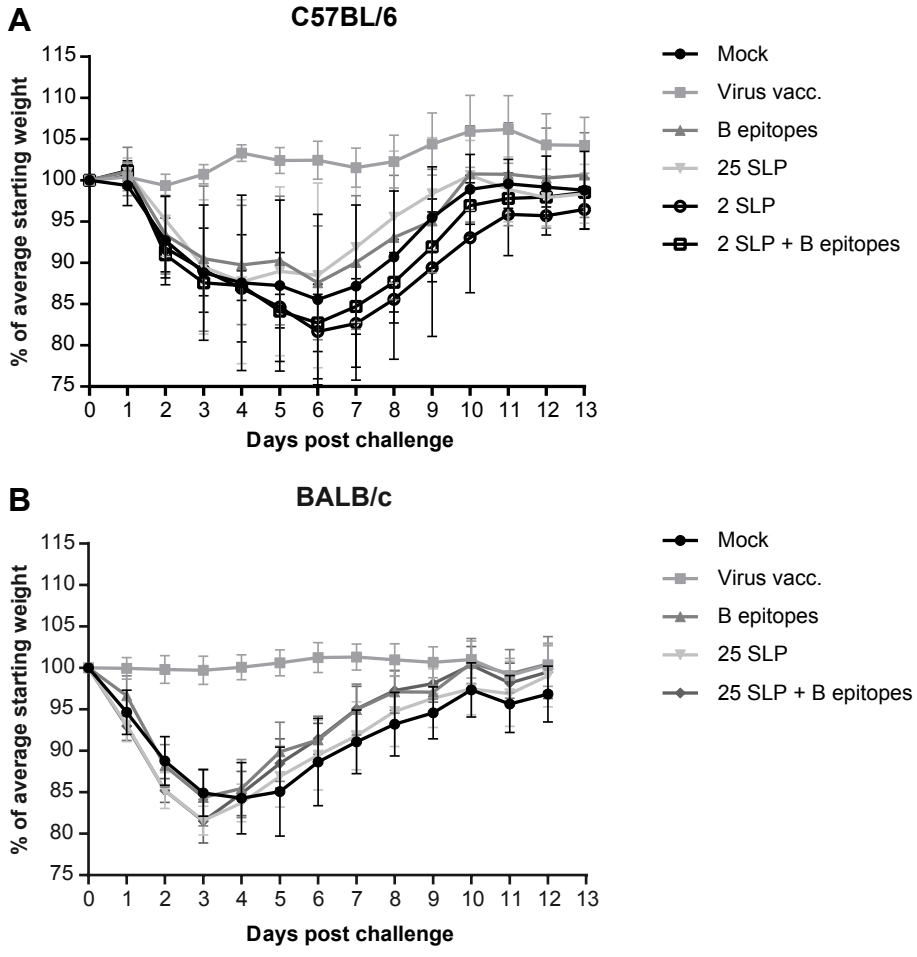

Figure S1: Bodyweight loss post challenge.
C57BL/6 mice (**A**) and BALB/c mice (**B**) were challenged i.n. with 1*10^5 $TCID_{50}$ of HK-X31 virus and their bodyweight was recorded daily. Results are shown as average per group relative to the bodyweight at the day of challenge. Error bars depict SD per group.

Chapter 5

Towards chemically altered peptide ligands as T cell-targeted influenza vaccines

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Abstract

T cells are essential players in the defense against infection. By targeting the MHC class I antigen-presenting pathway with peptide-based vaccines, antigen-specific T cells can be induced. However, low immunogenicity of peptides poses a challenge. Here, we set out to increase immunogenicity of influenza-specific $CD8^+$ T cell epitopes. By substituting amino acids in wild type sequences with non-proteogenic amino acids, affinity for MHC can be increased, which may ultimately enhance cytotoxic $CD8^+$ T cell responses. Since preventive vaccines against viruses should induce a broad immune response, we used this method to optimize influenza-specific epitopes of varying affinity and dominance. For this purpose, HLA-A*0201 epitopes GILGFVFTL, FMYSDFHFI and NMLSTVLGV were selected in order of decreasing dominance and affinity. For all epitopes, we designed chemically enhanced altered peptide ligands (CPLs) that exhibited greater binding affinity than their WT counterparts; even binding scores of the high affinity GILGFVFTL epitope could be improved. When HLA-A*0201 transgenic mice were vaccinated with selected CPLs, at least 2 out of 4 CPLs of each epitope showed an increase in IFN-γ responses of splenocytes. Moreover, modification of the low affinity epitope NMLSTVLGV led to an increase in the number of mice that responded. By optimizing three additional influenza epitopes specific for HLA-A*0301, we show that we established a general strategy that can be used to improve binding of essentially any class I epitope and to any allele. Thus, this strategy provides a valuable tool to improve the range and immunogenicity of preventive T cell-targeted peptide vaccines.

Introduction

For many infectious diseases, cellular responses are required for clearance of the pathogen from the host. One such disease that causes serious health threats worldwide is influenza (1). Preventive influenza vaccines mainly confer protection via antibodies directed against the highly variable surface proteins hemagglutinin (HA) and neuraminidase (NA). Influenza virus can escape previously induced immunity due to mutations in antigenic sites, so-called antigenic drifts. Consequently, protection is subtype or strain-specific and regular vaccine updates are required. In addition, current vaccines do not provide protection against newly emerging influenza subtypes, which has led to pandemics four times in the last century and most recently in 2009 (2, 3). Cellular responses are often directed towards more conserved parts of the virus and may therefore provide cross-protection; however, eliciting these responses by vaccination remains a challenge (4, 5). Vaccination with peptides that target antigen-specific T cells is one of the approaches that could induce these cross-protective cellular responses (6).

In general, peptide vaccines may aid in treating or preventing various types of diseases (7). Kenter et al. reported a therapeutic cancer vaccine based on long overlapping peptides that induced robust T cell responses leading to clinical effectiveness (8). Over the past years, preclinical research and two phase I clinical trials were reported, in which preventive influenza vaccines containing a set of long overlapping peptides capable of inducing T cell responses were described (9-11). Whether or not a peptide is capable of inducing such responses is dependent on characteristics such as length of the peptide and adjuvation. The latter is required, since peptides alone are often weak immunogens (12). We recently described a method to increase immunogenicity of peptides in the context of therapeutic vaccination, by substitution with amino acids that are not naturally incorporated into proteins, so-called non-proteogenic amino acids (13). By expanding the natural protein code, we aimed to generate peptides that increase peptide-MHC binding more than achieved by using substitution with proteogenic amino acids. The resulting chemically enhanced altered peptide ligands (CPLs) had increased binding affinities compared to the wild type peptides, which in turn led to enhanced T cell responses. Here, we use this approach to modify peptides encoding highly conserved influenza-specific class I epitopes of varying dominance in the context of preventive vaccination.

Individuals with pre-existing cytotoxic influenza-specific T cells were shown to have an immunological advantage upon encounter with influenza virus due to cross-reactivity of these T cells (14-16). The presence of cross-reactive cytotoxic T cells has even been shown to limit disease (17). Several preventive short (9-10 aa) peptide vaccination concepts, focusing on highly conserved $CD8^+$ T cell-specific influenza peptides, have been described (18-21). Immunogenicity of these peptide vaccines was enhanced by methods such as incorporation of peptides into virosomes or liposomes and ligation of the peptides to a lipid tail. These

methods proved promising in mouse experiments. However, these approaches were aimed at increasing immunogenicity by adding adjuvants or by using different modes of delivery, but none increased inherent immunogenicity of the peptides.

Immunogenicity of a peptide is defined by three interacting partners: peptide, MHC and TCR (22). Class I peptides are generated during degradation of a protein by the proteasome, followed by loading of the peptides on MHC class I molecules (23). Each MHC allele has a different peptide-binding groove with specific binding pockets in which amino acid side chains of a peptide's anchor residues can protrude (24, 25). Amino acid positions of peptides are referred to as P_1-P_C, P_1 being the N-terminal and P_C the C-terminal residue. By altering the anchor residues, which are usually found towards the C- and N-termini of the peptide, the number and/or quality of interactions between the peptide and MHC molecule can be altered, thereby increasing peptide affinity (26, 27). This will result in prolonged presentation of peptides on the cell surface, which may lead to enhanced T cell immunogenicity (28 ,29). Modification of the central amino acids of the peptide, on the other hand, frequently results in abrogated T cell reactivity, since this part of the epitope is directly recognized by the TCR (30-32).

In this study, we focused on improving the binding affinity of short (9-10 aa) highly conserved influenza-specific epitopes in order to enhance their immunogenicity. We chose influenza virus as a model to study the effect of immunization with CPLs that have increased binding affinity on the immune response. This strategy can eventually be used for the development of a preventive vaccine. We selected three highly conserved influenza epitopes specific for HLA-A*0201, the most abundant HLA allele in the Caucasian population, based on their varying binding affinities and dominance in influenza A virus infection: the highly dominant GILGFVFTL ($M1_{58-66}$), the less dominant FMYSDFHFI (PA_{46-54}), and the low affinity subdominant NMLSTVLGV ($PB1_{413-421}$) epitopes (33). We show that substitution with non-proteogenic amino acids can lead to improved HLA binding and T cell responses as measured by IFN-γ production in both in vitro and in vivo models. Moreover, we demonstrate that binding affinity of epitopes specific for other alleles can also be improved by optimizing binding of influenza epitopes ILRGSVAHK ($NP_{265-273}$), SFSFGGFTK ($PB2_{322-330}$), and RMVLSAFDER (NP_{67-76}) (in order of decreasing dominance) to HLA-A*0301, another frequently occurring allele in the Caucasian population (34). Thus, by enhancing binding affinity, responses to dominant and more importantly to otherwise subdominant epitopes can be improved and thereby a general tool is provided to increase and broaden T cell-mediated immune responses to a variety of antigens.

Materials and Methods

Ethics statement

This study was approved by the Committee on Animal Experimentation of the Netherlands Vaccine Institute (Bilthoven, the Netherlands) (permit numbers PO201200042, PO201200222) and the Committee on Animal Experimentation of the Antonie van Leeuwenhoek terrain (DEC-ALt) (permit numbers PO201300122, PO201400121, PO201400177 and PO201400188) (Bilthoven, the Netherlands). Animal handling was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

Peptide design and synthesis

Peptides were designed as described before and synthesized at the Netherlands Cancer Institute Peptide Facility by standard solid-phase peptide synthesis using Syro I and Syro II synthesizers (13). Amino acids were purchased from Chiralix, NovaBiochem, Chem-Impex or Creo Salus. Resins were purchased pre-loaded with proteogenic amino acids (Nova Biochem) or loaded with non-proteogenic amino acids. Typically, 2-chlorotrityl chloride resin corresponding to a loading of 0.3 mmol (Nova Biochem) was swollen in dichloromethane (DCM, Biosolve); 0.15 mmol of amino acid and 0.51 mmol di-isopropylethylamine (DIPEA, Sigma-Aldrich) were added and the mixture was shaken for 10 minutes. Another 0.99 mmol DIPEA in DCM was added and the mixture was shaken for one hour. The reaction was quenched by addition of methanol.

Fluorescence polarization-based peptide binding assay

Peptide-MHC affinity was measured using a fluorescence polarization (FP) assay based on UV-mediated ligand exchange (35-39). Since the fluorescence emission of MHC-bound tracer peptide is polarized to a greater extent than that of non-bound tracer, the total FP is a measure for the ratio of bound versus unbound tracer peptide. MHCs were refolded with conditional ligand KILGFVFJV for HLA-A*0201 and RIYRJGATR for HLA-A*0301, in which J is the photocleavable 3-amino-(2-nitrophenyl)propionic acid. Soluble MHC was dissolved in PBS containing 0.5 mg/ml bovine γ-globulin (BGG, Sigma-Aldrich) to a final concentration of 0.75 µM. The HLA-A*0201 tracer peptide FLPSDCFPSV and the HLA-A*0301 tracer peptide KVPCALINK were fluorescently labeled at the cysteine residues with 5-N-maleimide tetramethylrhodamine. Tracer peptides were diluted to a concentration of 6 nM in 1×BGG/PBS. Peptides of choice were dissolved at 125 µM in DMSO. Using a Hamilton MicroLab Liquid Handling Workstation the components were automatically transferred in triplicate into a 384-well microplate (black polystyrene, Corning). MHC, tracer and peptide were combined to reach final concentrations of 0.5 µM, 1 nM and 4.2 µM, respectively. The plate was exposed to UV light (365 nm) for 30 minutes at 4°C to exchange the UV-sensitive peptide for the desired peptides. FP values were measured using a BMG PHERAstar plate reader. To generate IC_{50} curves the FP-based peptide binding assay was performed using serial peptide dilutions ranging from 224 nM to 4 µM. Data were analyzed using GraphPad Prism 5 software.

IFN-γ induction in a GILGFVFTL specific T cell clone

TAP-deficient T2 cells, which are incapable of transporting peptides from the cytosol into the ER and thus only present exogenously loaded peptides, were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS. The GILGFVFTL-specific T cell clone was cultured in RPMI 1640 medium containing 10% FCS supplemented with 3 U/ml IL-2. Per well of a 96-well plate, 50,000 T2 cells were pulsed with 10 pM of the desired peptides at 37 °C for 1 hour. After washing away any unbound peptides, T2 cells were cultured in RPMI 1640 medium containing 10% FCS with 50,000 specific T cells for 24 hours in presence of 1 µl/ml Golgiplug (BD Biosciences). As positive control, T cells were stimulated with 0.05 µg/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich). Unstimulated cells were included as negative control. After incubation, the plate was centrifuged at 700 g for 2 minutes. The medium was discarded and cells were resuspended and stained with 20 µl/ml CD8-FITC antibody (BD Biosciences) in PBS with 0.5% BSA and 0.02% sodium azide). Cells were fixed and permeabilized using a Cytofix/CytoPerm kit (BD Biosciences) according to manufacturer's recommendations. Then, cells were stained for intracellular IFN-γ using 20 µl/ml anti-IFN-γ-APC (BD Biosciences) and analyzed using a Beckman Coulter CyAn ADP flow cytometer. The percentage of IFN-γ^+ cells was determined from the $CD8^+$ gate. Data were analyzed using FlowJo version 7.6.1. software (Tree Star Inc).

Isolation and culture of human DCs

PBMCs of HLA-A2-typed healthy human donors were isolated from fresh blood by gradient centrifugation using Lymphoprep (Nycomed). Next, monocytes, $CD8^+$ T cells, and then $CD4^+$ T cells were magnetically purified using CD14, CD8 or CD4 antibody-labeled magnetic beads, respectively, using LS columns according to manufacturer's recommendations (Miltenyi Biotec). Following elution from the columns, $CD8^+$ T cells and $CD4^+$ T cells were frozen in FCS (Hyclone) with 10% DMSO and stored at -80°C until further processing. $CD14^+$ cells were plated in a concentration of $0.4*10^6$ cells/ml in DC culture medium (IMDM (GIBCO, Invitrogen) containing 1% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml glutamine (all Sigma), supplemented with 500 U/ml human GM-CSF (PeproTech) and 800 U/ml human IL-4 (Active Bioscience) and incubated for seven days at 37°C.

Maturation and co-culture of DCs

After seven days of culture, half of the DC culture medium was replaced with DC culture medium containing GM-CSF only, and 1 nmol peptide per well was added. After an incubation period of one hour, 10 ng/ml E. coli LPS (Invivogen) was added to mature the DCs. After 48 hours, DCs were harvested and plated in a U-bottom 96-well plate in a concentration of $5*10^3$ cells/well in co-culture medium (AIM-V (GIBCO) containing 2% human AB serum (Sigma)). Samples of the DCs were collected for analysis of maturation markers by flow cytometry. Next, autologous $CD8^+$ and $CD4^+$ T cells were added to the DCs, both in a 10:1 ratio. After seven days of co-culture, cells were collected for analysis by flow cytometry.

Flow cytometry

To determine maturation status, DCs were harvested two days after addition of peptides and maturation factor LPS. Cells were stained in FACS buffer (PBS (GIBCO) containing 0.5% BSA (Sigma) and 0.5 mM EDTA (ICN Biomedicals)) for 30 minutes at 4°C with either one of two panels that contained the following maturation markers: anti-CD80-FITC, anti-CD14-PE, anti-DC-SIGN-APC, anti-HLA-DR-Pacific Blue and Live/dead-AmCyan (Invitrogen) (panel 1) or anti-CD83-FITC, anti-CD40-PE, (BD Biosciences), anti-PD-L1-APC (eBioscience), anti-CD86-Pacific Blue (BioLegend) (panel 2). Live/dead-AmCyan (Invitrogen) was included in both panels. For analysis of the co-culture, the following markers were used: anti-CD8-FITC (Sanquin), anti-CD3-PerCP, anti-TNFα-PE-Cy7, anti-IFN-γ-APC (BD Biosciences), anti-CD4-Pacific Blue (eBioscience) and Live/dead-AmCyan (Invitrogen). Four hours prior to staining, Brefeldin A (BD Biosciences) was added to the culture; then cells were stained using the Cytofix/Cytoperm kit from BD Biosciences according to manufacturer's recommendations. Cells were measured using a FACS Canto II (BD Biosciences) and results were analyzed using FlowJo version 9.7.5 software. First, lymphocytes were gated, followed by gating of live cells, then $CD3^+$ cells and finally $CD8^+$ or $CD4^+$ cells were placed in a quadrant with TNF-α^+ or IFN-γ^+ cells.

Immunization of mice

HLA-A2 transgenic mice, B6.Cg-Tg (HLA-A/H2-D)2Enge/J (Jackson Laboratory, USA), maintained in house, or C57BL/6 mice (Charles River, Germany) were vaccinated with the indicated peptides at their respective doses in a volume of 100 µl. Peptides were adjuvanted with Incomplete Freund's Adjuvant (IFA) (1/1 (V/V)) and CpG (50 µg/mouse) by vortexing the mixture for 30 minutes. In all experiments, mice were subcutaneously vaccinated at days 0 and 21 in alternating flanks. Two weeks after booster vaccination, mice were sacrificed, spleens were excised and spleen cells were restimulated for 18 hours with WT peptide or CPL. Specific IFN-γ responses were assessed using an ELISpot assay.

ELISpot assay

IFN-γ ELISpot assays were performed according to the manufacturer's protocol (U-Cytech). Spleens were homogenized and passed through 70 µm filters (BD Biosciences), washed with RPMI 1640 containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 292 µg/ml glutamine and counted using a Casy cell counter (Roche). Cells were plated in a concentration of $4*10^5$ cells/well in an IFN-γ antibody-coated PVDF membrane plate (Millipore MSIP) and stimulated with 0.1 nmol/well of either WT peptide or corresponding CPL. After 16 hours of incubation spots were visualized according to the manufacturer's protocol (U-Cytech) and counted using an A.EL.VIS reader (A.el.vis).

Results

Optimizing HLA-A*0201 binding affinity of influenza epitopes

Three influenza-specific epitopes were selected based on their varying binding affinity and dominance in the immune response. To enhance affinity for HLA-A*0201, amino acids of the WT peptide were substituted with non-proteogenic amino acids (**Figure 1**). Per epitope, approximately 200 peptides were rationally designed based on available crystal structures and on side chain similarities. Binding affinity was determined by a fluorescence polarization (FP) assay, in which CPLs compete with fluorescent tracer peptide for HLA-A*0201 binding (35, 39). From the difference in FP of MHC with tracer alone and in combination with CPL, the binding strength of the test peptide was scored as percentage inhibition of tracer peptide binding. This method allowed for high-throughput testing of multiple peptides. Per epitope, we selected 20 CPLs for their varying binding affinities in order to study the correlation between binding scores and in vitro and in vivo responses (**Table I**). After 4 hours, many of the peptides showed increased binding, but those peptides that still showed increased binding after 24 hours are likely to have a lower off-rate as a result of their higher affinity. As depicted in **Table I**, the binding score of WT GILGFVFTL was 84% after 24 hours of incubation. Insertion of the non-proteogenic amino acid D-α-methyl-phenylglycine (am-phg) on P_1, resulted in the most successful CPL with a binding score of 98% (G1; see **Table I**). Other successful substitutions on P_1 were mainly aromatic amino acids, such as DL-phenylglycine (Phg) (G7; see **Table I**), or the D (represented in lowercase) and L (represented in uppercase) amino acids of 3'- and 4'-pyridyl-alanine (3- and 4-pyra; 3- and 4-PYRA), which also resulted in increased binding scores (G8, G15, G4 and G10, **Table I**).

Since the two less immunodominant influenza epitopes FMYSDFHFI and NMLSTVLGV naturally have lower affinities compared to GILGFVFTL, we expected an even larger improvement for CPLs derived from these peptides. Substitution with the aromatic 4-fluorophenylalanine (4-FPHE) in combination with a substitution with L-2-amino-octanoic acid (2-AOC) resulted in CPLs with the highest binding score for both FMYSDFHFI and NMLSTVLGV epitopes. The binding score for FMYSDFHFI was raised from 75% to 94% after substitution of P_1 with 4-FPHE, in combination with 2-AOC on P_9 (F5; **Table I**). 4-FPHE on P_1 in combination with 2-AOC on P_2, increased the binding score of NMLSTVLGV from 55% to 92% (N95; **Table I**). Apart from these peptides, 2-AOC alone led to increased binding when substituted at or near the anchor positions P_2 and P_9 for both FMYSDFHFI and NMLSTVLGV (F143, F19, F95, N39, N41, N40; see **Table I**). Thus, using non-proteogenic amino acid substitutions, we were able to increase the binding of peptides such that they nearly inhibited 100% of the tracer peptide from binding, regardless of the affinity of the parent epitope.

In vitro and ex vivo T cell activation screening assays

Since modifications could change the T cell-exposed peptide side chains in such a way that they do not resemble those of the WT peptide anymore, we investigated whether CPLs were

still capable of activating WT-specific T cells. To determine this for modifications of GILGFVFTL, antigen-presenting T2 cells were pulsed with CPLs and co-cultured with a GILGFVFTL-specific T cell clone. Subsequently, IFN-γ production was determined by flow cytometry after 24 hours of culture. Approximately half of the 16 tested CPLs showed higher IFN-γ responses compared to the WT epitope (**Table SI**). After 24 hours, G1 and G7, the CPLs with the highest binding affinity induced high IFN-γ responses. In addition, G16 and G25 with moderately improved binding affinity also induced high IFN-γ responses; however, 4 out of 13 CPLs with similar or improved binding showed strongly reduced to no activation. Therefore, affinity is to a certain extent indicative for $CD8^+$ T cell activation, but fails as a predictor in some cases. The latter may indicate that the T cell-exposed peptide structure is altered.

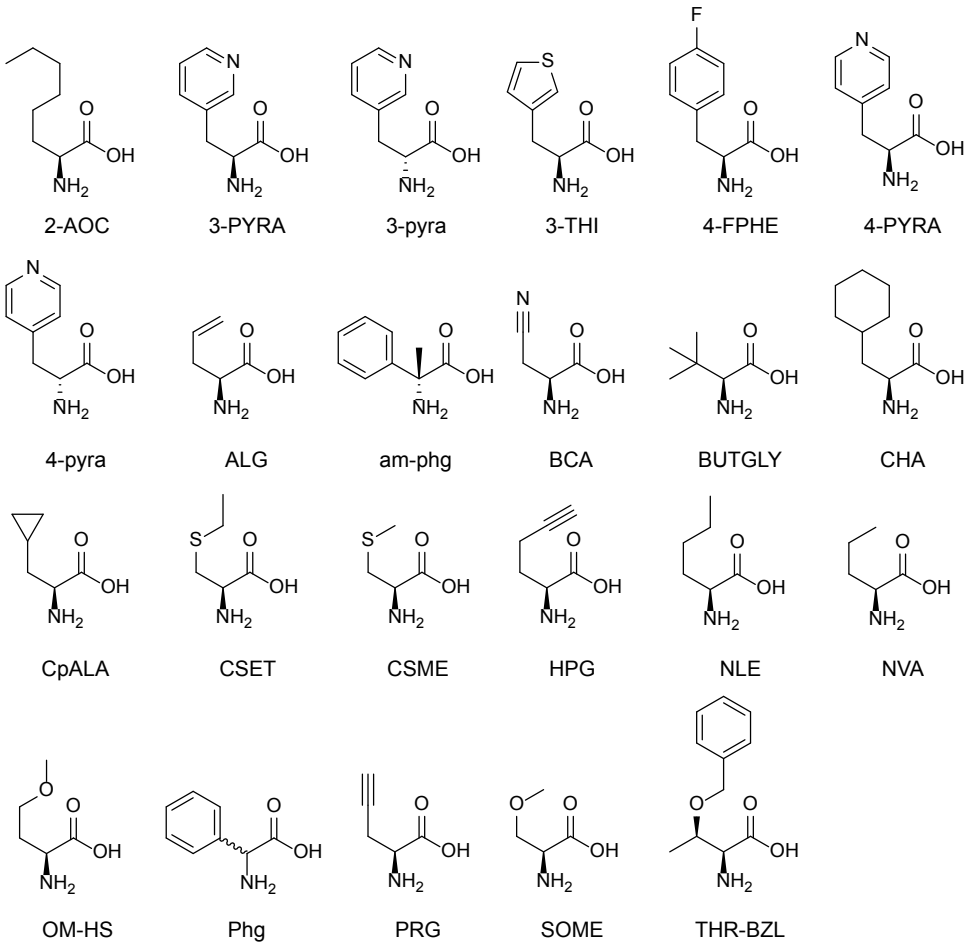


Figure 1. Structures of non-proteogenic amino acids found in the best CPLs.

L-amino acids are denoted in uppercase characters; D-amino acids in lowercase characters. Incorporation of Phg results in a racemic mixture.

Table I. FP binding scores of selected HLA-A*0201 peptides

A

#	GILGFVFTL	4h	SD	24h	SD
G1	[am-phg]ILGFVFTL	97	4	98	4
G7	[Phg]ILGFVFTL	96	0	94	2
G8	[3-PYRA]ILGFVFTL	94	3	93	1
G15	[4-PYRA]ILGFVFTL	93	3	93	3
G4	[3-pyra]ILGFVFTL	92	6	92	6
G12	GILGFV[4-FPHE]TL	92	3	92	5
G27	[am-phg][CpALA]LGFVFTL	92	1	91	2
G10	[4-pyra]ILGFVFTL	90	2	91	4
G9	yILGFVFTL	94	2	90	2
G16	G[NLE]LGFVFTL	91	3	90	5
G3	GILGFVFT[CpALA]	90	5	90	5
G25	[SOME]ILGFVFTL	89	12	89	13
G13	GILGFVFT[ALG]	88	8	88	7
G17	GILGFVFT[PRG]	88	3	88	5
G WT	**GILGFVFTL**	85	0	84	2
G24	fILGFVFTL	86	8	81	7
G20	GILGFV[BUTGLY]TL	82	10	79	12
G22	GILGFVFT[2-AOC]	79	14	77	15
G11	[CSME]ILGFVFTL	72	28	70	32
G26	G[2-AOC]LGFVFT[PRG]	60	4	54	2
G29	[3-PYRA]ILGFVFT[2-AOC]	44	7	38	5

B

#	FMYSDFHFI	4h	SD	24h	SD
F5	[4-FPHE]MYSDFHF[2-AOC]	95	2	94	2
F118	[CSET][2-AOC]YSDFHFI	95	4	93	3
F141	[THR-BZL][2-AOC]YSDFHFI	94	2	93	2
F48	F[2-AOC]YSDFHF[CHA]	93	1	92	1
F143	F[2-AOC]YSDFHFI	93	2	91	2
F102	[3-THI][2-AOC]YSDFHFI	92	0	91	0
F112	[BCA][2-AOC]YSDFHFI	92	4	91	3
F7	[am-phg]MYSDFHF[2-AOC]	91	4	90	4
F69	F[2-AOC]YSDFHF[NLE]	90	3	89	2
F49	FMYSDFHF[CHA]	88	2	86	3
F19	FMYSDFHF[2-AOC]	87	2	86	2
F193	[am-phg][NVA]YSDFHFI	85	3	81	2
F54	FMYSDFHF[CSET]	82	4	80	4
F95	[2-AOC]MYSDFHFI	82	3	80	3
F105	[4-FPHE]MYSDFHFI	86	1	78	1
F52	FMYSDFHF[CpALA]	81	3	78	4
F63	FMYSDFHF[HPG]	81	7	78	5
F142	[THR-BZL]MYSDFHFI	80	2	77	3
F WT	**FMYSDFHFI**	78	3	75	2
F100	[3-PYRA]MYSDFHFI	79	7	73	5
F111	[am-phg]MYSDFHFI	75	9	72	9

C

#	NMLSTVLGV	4h	SD	24h	SD
N95	[4-FPHE][2-AOC]LSTVLGV	94	2	92	2
N92	[SOME][2-AOC]LSTVLGV	91	4	88	2
N91	[OM-HS][2-AOC]LSTVLGV	91	1	87	2
N98	[3-THI]MLSTVLG[2-AOC]	90	3	87	1
N172	[Phg][2-AOC]LSTVLGV	89	1	87	2
N11	[am-phg]MLSTVLG[2-AOC]	88	1	85	1
N15	[NVA]MLSTVLG[2-AOC]	88	3	83	5
N8	[3-PYRA]MLSTVLG[2-AOC]	85	6	82	5
N169	[CSME][2-AOC]LSTVLGV	85	6	82	6
N46	[am-phg]MLSTVLGV	85	11	81	9
N39	[2-AOC]MLSTVLGV	81	4	78	6
N177	F[2-AOC]LSTVLGV	80	16	78	14
N41	NM[2-AOC]STVLGV	81	3	76	5
N43	[3-PYRA]MLSTVLGV	79	6	73	6
N40	N[2-AOC]LSTVLGV	75	2	72	3
N176	[THR-BZL]MLSTVLGV	75	5	71	4
N53	N[NLE]LSTVLGV	69	4	61	3
N52	[NLE]MLSTVLGV	64	31	59	30
N WT	**NMLSTVLGV**	66	9	55	9
N61	[SOME]MLSTVLGV	61	2	55	1
N122	NMLSTVLG[CpALA]	56	11	46	9

HLA-A*0201 binding of CPLs of influenza epitopes; (**A**) dominant GILGFVFTL ($M1_{58-66}$), (**B**) subdominant FMYSDFHFI (PA_{46-54}) and (**C**) low affine subdominant NMLSTVLGV ($PB1_{413-421}$), was determined using an FP-based competition assay. Binding was scored as percentage inhibition of tracer peptide binding after 4h and 24h in three independent experiments. Maintained binding after 24 hours indicates a lower off-rate, presumably due to increased stability. This table shows binding scores in a heat map for the WT epitopes (bold) and 20 CPLs that were selected for in vitro and ex vivo testing. Green indicates high binding scores, yellow medium and red low binding scores. Peptides highlighted in blue were used in vaccination experiments. SD: standard deviation.

Since T cell clones for FMYSDFHFI and NMLSTVLGV were not available, other assays were developed to allow pre-selection for in vivo experiments. To be able to compare the predictive value of these assays with that of the T cell clone-based assay, we also performed these assays with GILGFVFTL CPLs. The first alternative strategy included testing responses following CPL stimulation in a human HLA-A2$^+$ DC T cell co-culture model. For this purpose, HLA-A2$^+$ donors were selected based on the presence of CD8$^+$ T cell-specific IFN-γ responses after stimulation with WT peptide. Monocytes from these donors were isolated, differentiated into immature DCs, and subsequently pulsed with different CPLs. After pulsing, DCs were matured and co-cultured with autologous T cells for seven days. Then, IFN-γ production of CD8$^+$ T cells was measured by flow cytometry. Several CPLs appeared to induce a higher response than their corresponding WT peptides (**Table SI**); however, this assay had both a high assay variation and a high variation between donors.

To limit inter-individual variation, a third strategy was developed, in which CPLs were tested ex vivo on splenocytes of HLA-A2 tg mice vaccinated with either one of the three WT epitopes. Two weeks post booster vaccination, spleen cells were isolated and restimulated for 16 hours with selected CPLs and IFN-γ levels were measured by ELISpot. In this assay, only CPLs G13 and F100 induced similar responses compared to their corresponding WT peptide (**Table SI**). In general, the positive results of the three assays correlate poorly, as shown in **Table II** for the upper three CPLs after ranking the results based on T cell activation for each assay. However, a correlation between the three assays was found for the lower ranked CPLs derived from GILGFVFTL and NMLSTVLGV, which allowed for negative selection. We therefore used both positive and negative results from all assays to include or exclude CPLs for further investigation.

In vivo stimulation using modified peptides

Vaccination of HLA-A2 tg mice with either of the three WT epitopes confirmed their dominance in the immune response as shown by the corresponding induction of IFN-γ as measured by ELISpot (**Figure S1A**). Since the HLA-A2 tg mice had a C57BL/6 background and co-expressed H2-Kb, a control experiment in C57BL/6 mice was performed. In these mice, no responses to the selected WT HLA-A*0201 epitopes were observed, which confirmed that responses in the HLA-A2 tg mice were HLA-A*0201-specific (**Figure S1A**). Subsequently, four CPLs per epitope were selected for in vivo testing. GILGFVFTL CPLs were selected based on binding scores and T cell clone data. To analyze a broad spectrum, CPLs with varying binding scores were selected (**Table I**). Of these CPLs G1, G16 and G25 induced highest responses in the T cell clone, while G8 induced a response similar to that of the WT epitope (**Table SI**).

HLA-A2 tg mice were vaccinated with different doses of WT GILGFVFTL peptide or CPLs G1, G8, G16, or G25 on days 0 and 21. Two weeks post booster vaccination, spleen cells were isolated and stimulated for 16 hours with different peptides and analyzed using an IFN-γ ELISpot assay. First, the effect of enhanced binding affinity on a T cell response was investigated using homologous peptide as a stimulus (**Figure 2A**). Overall, responses of G1-vaccinated mice

were highest and those of G8-vaccinated mice lowest. Responses of G16- and G25-vaccinated mice, on the other hand, were highest at a vaccination dose of 25 nmol peptide and did not increase at higher doses. However, these CPL-specific T cells might not recognize the WT epitopes. Restimulation of splenocytes of CPL-vaccinated mice with WT peptide mimics a natural situation in which CPL-induced T cells respond to infection with a virus containing the WT epitope. As shown in **Figure 2B**, responses of WT-vaccinated mice were low at peptide vaccination doses of 10, 25 and 50 nmol, but increasing the dose to 100 nmol resulted in higher T cell responses. G1- and G8-vaccinated mice, on the other hand, showed a higher response compared to WT vaccinated mice at lower doses (**Figure 2B**). At a dose of 100 nmol the difference between CPLs and WT-peptide vaccinated mice was reversed, which might be due to overstimulation by CPLs at these high doses. Overall, vaccination with G1 followed by G8 resulted in the largest increase in responses after restimulation with homologous peptide and also maintained these responses after WT restimulation.

Selection of CPLs for the other two epitopes was more challenging, since data obtained using the different pre-selection strategies did not correspond well (**Table II** and **Table SI**). We therefore selected CPLs based on data from vaccination experiments with GILGFVFTL CPLs in addition to the results of the screening assays. The final selection for FMYSDFHFI comprised

Table II. Summary of pre-selection experiments

	GILGFVFTL			FMYSDFHFI		NMLSTVLGV	
	T cell clone	DC model	Mouse spleno-cytes	DC model	Mouse spleno-cytes	DC model	Mouse spleno-cytes
	G1	G26	G13	F49	F100	N172	N92*
Upper 3	G16	G7*	G3*	F5	F102*	N169	N40*
	G25	G15*	G22*	F54	F143*	N41	N172*
	G4	G24	G24	F69	F49	N11	N15
Lower 3	G9	G17	G9	F19	F5	N46	N11
	G24	G20	G4	F102	F7	N8	N8

* Lower response than WT peptide

CPLs were analyzed for their capacity to induce a response in WT-specific T cells. Therefore, three assays were developed in which IFN-γ production was used as a measure of response. The first was analysis of GILGFVFTL-CPLs on a WT-specific T cell clone (T cell clone). However, no T cell clone was available for the two other WT epitopes. Therefore, in the second assay, CPLs were loaded onto DCs of HLA-$A2^+$ human donors and co-cultured for seven days with autologous $CD8^+$ T cells (DC model). Due to high variation in the DC model, another assay was performed by 16 hours stimulation of splenocytes of WT-vaccinated HLA-A*0201 mice with CPLs (mouse splenocytes). This Table shows the upper three and lower three CPLs after ranking the results based on T cell activation for each assay separately. CPLs marked with the same color show similarities between CPLs in the assays and * indicates when a CPL induced a response lower than that of the WT peptide control. As visualized by the colored CPLs, a correlation between assays was found for the lower three CPLs derived from GILGFVFTL and NMLSTVLGV in all three assays. However, no correlation was found between assays for the upper CPLs.

F5 based on the DC co-culture model, F100 because it performed well in WT-specific mouse splenocytes and F111 and F193 based on favorable substitutions observed in pilot experiments with GILGFVFTL CPLs in mice. Mice were vaccinated with these CPLs using three doses of peptide, since in the previous experiment we observed minimal responses at the lowest dose used (10 nmol). Homologous peptide restimulation showed that vaccination with all four CPLs dramatically increased T cell responses compared to WT peptide (**Figure 2C**). When cells were restimulated with WT peptide three out of four CPL-vaccinated mice (F5, F100, F111) clearly showed higher IFN-γ responses than WT peptide-vaccinated mice (**Figure 2D**). One CPL (F193) only showed higher responses than WT peptide at a vaccination dose of 25 nmol. Thus, modification greatly enhanced T cell responses for three out of four peptides, even at low vaccination doses.

In case of both GILGFVFTL and FMYSDFHFI the best responses after homologous and WT stimulation were induced by those CPLs that also showed the highest binding scores in FP assays (G1 and F5, **Table I**). This strongly suggests a direct correlation between binding affinity and height of the T cell response. Possibly, the increased affinity results in increased residence time, i.e., lower off-rate, of the peptide in the MHC, allowing prolonged presentation to T cells. However, both F100 and F111 also induced higher IFN-γ responses in mice than the WT FMYSDFHFI, while their binding scores were similar to the WT epitope. CPL F193, on the other hand, induced responses similar to the WT peptide in mice, even though the binding score of the CPL was higher. Therefore, we could conclude that improved binding may lead to better responses, but this is not necessarily the case.

For the epitope NMLSTVLGV, CPLs N46 and N53 were selected based on modifications that were successful in previous in vivo experiments with GILGFVFTL, N92 because it was one of the few peptides that induced a response similar to that of WT peptide in WT-specific mouse splenocytes and N172 based on the DC co-culture data. Since NMLSTVLGV is a very low affinity epitope, these CPLs had, as expected, the largest improvement in binding score (**Table I**). Earlier experiments indicated that the WT peptide induced responses only in approximately one out of six mice; therefore we chose to focus on just one vaccination dose and to increase the number of mice to seven or eight per group to assure that at least 1-2 mice responded to WT peptide vaccination. **Figures 2E** and **2F** show that vaccination with CPLs N46, N53 and N172 increased the number of responding mice compared to vaccination with WT peptide, whereas N92-vaccinated mice did not respond to restimulation (**Figure 2E** and **2F**). All of the N172-vaccinated mice (n=7), half of the N53-vaccinated mice (n=4) and four of the N46-vaccinated mice responded to homologous peptide restimulation (**Figure 2E**). When spleen cells of N172-vaccinated mice were restimulated with WT peptide, half of these mice (n=3) responded (**Figure 2F**). For CPL N53 the number of responders remained stable (n=4), while there were no responders for CPL N46. By modifying NMLSTVLGV, responses could be induced in a larger proportion of mice compared to WT peptide and these responses were higher in all cases, which is a major enhancement for this very subdominant peptide. CPL N172

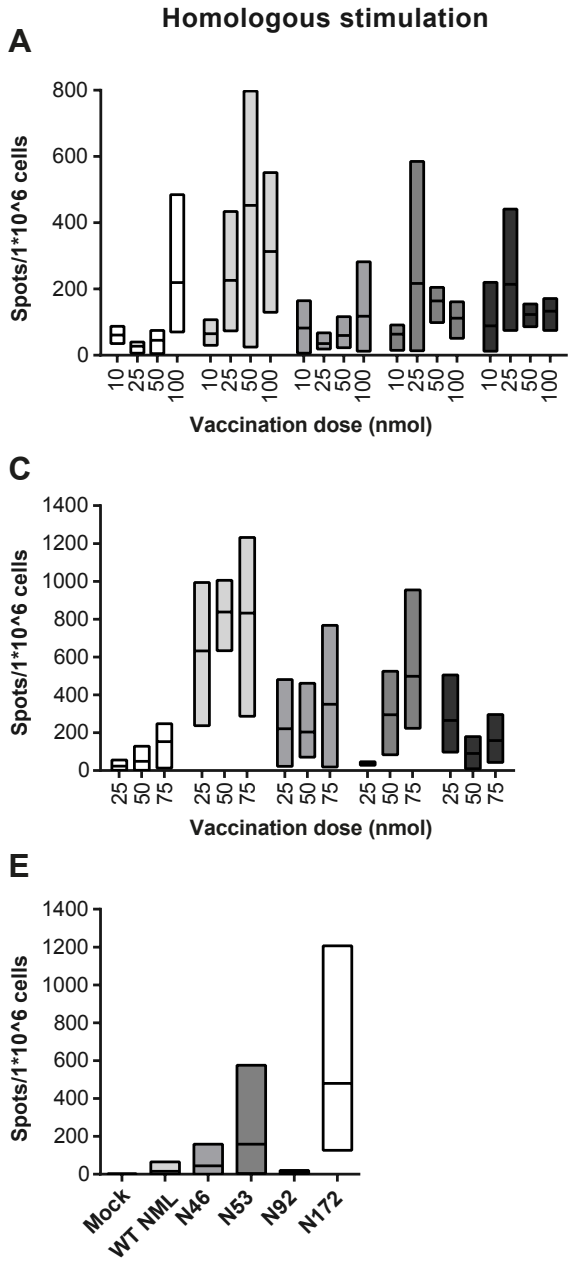
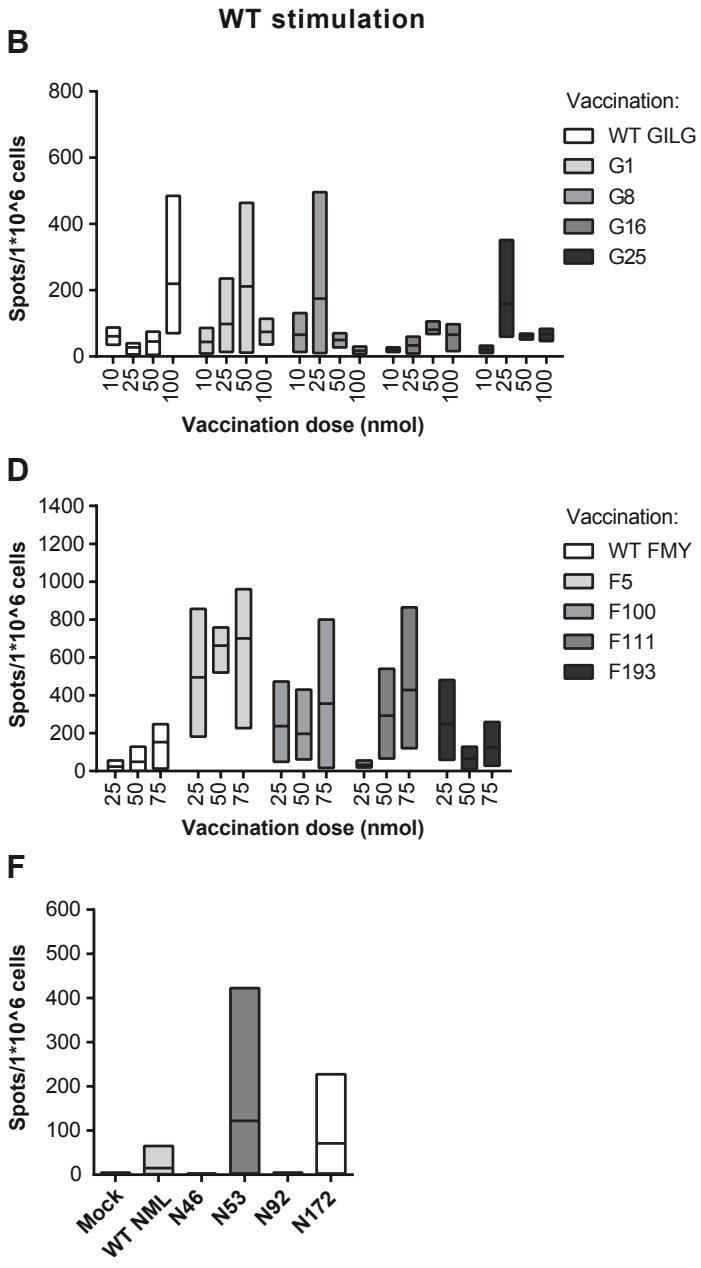


Figure 2: Vaccination with CPLs shows enhanced IFN-γ responses in vivo compared to vaccination with WT peptide.

Mice were vaccinated with different doses of WT peptide or CPLs on day 0 and day 21 and two weeks later spleen cells were isolated and restimulated with homologous peptides or WT peptide. Responses were measured by IFN-γ ELISpot after 16 hours stimulation with 0.1 nmol peptide/well. Mice were vaccinated with 10, 25, 50 or 100 nmol of WT GILGFVFTL or with the indicated CPLs. Spleen cells were restimulated with homologous (**A**) or WT (**B**) peptide. Overall, responses were highest after stimulation with CPL G1. For FMYSDFHFI mice were vaccinated with 25, 50 or 75 nmol of WT peptide or the indicated CPLs. Cells



were restimulated for 16 hours with homologous (**C**) or WT (**D**) peptide. Three out of four CPLs (F5, F100 and F111) induced higher responses compared to WT-peptide vaccination. For NMLSTVLGV mice were only vaccinated with a dose of 75 nmol of WT peptide or respective CPLs. Spleen cells were restimulated with homologous (**E**) or WT (**F**) peptide. CPL N172 induced most T cells that responded to homologous stimulation, whereas N53 induced most T cells responding to WT peptide. Figures 2A-2D depict three mice per dose. Data in Figure 2E and 2F are derived from 7-8 mice per group, with the exception of the mock, for which three mice were included. Bars are min to max, with line at mean.

was among the top binders, further showing a correlation between binding affinity and T cell reactivity.

Detailed analysis of the most immunogenic CPLs

For each of the three epitopes the most immunogenic CPLs were selected for a more detailed analysis. To this extent, G1 and F5 were selected, since these CPLs induced highest and most robust responses after homologous and WT-peptide restimulation. N53 and N172 were selected since both peptides induced higher responses in a larger number of mice than WT peptide. However, first an additional control experiment was performed in C57BL/6 mice to confirm that CPL responses were HLA-A2-specific. Unexpectedly, CPL F5 induced responses in these non-transgenic mice. From this, we can conclude that part of the extent of the responses of F5 in the HLA-A2 tg mice is due to presentation of the F5 peptide on

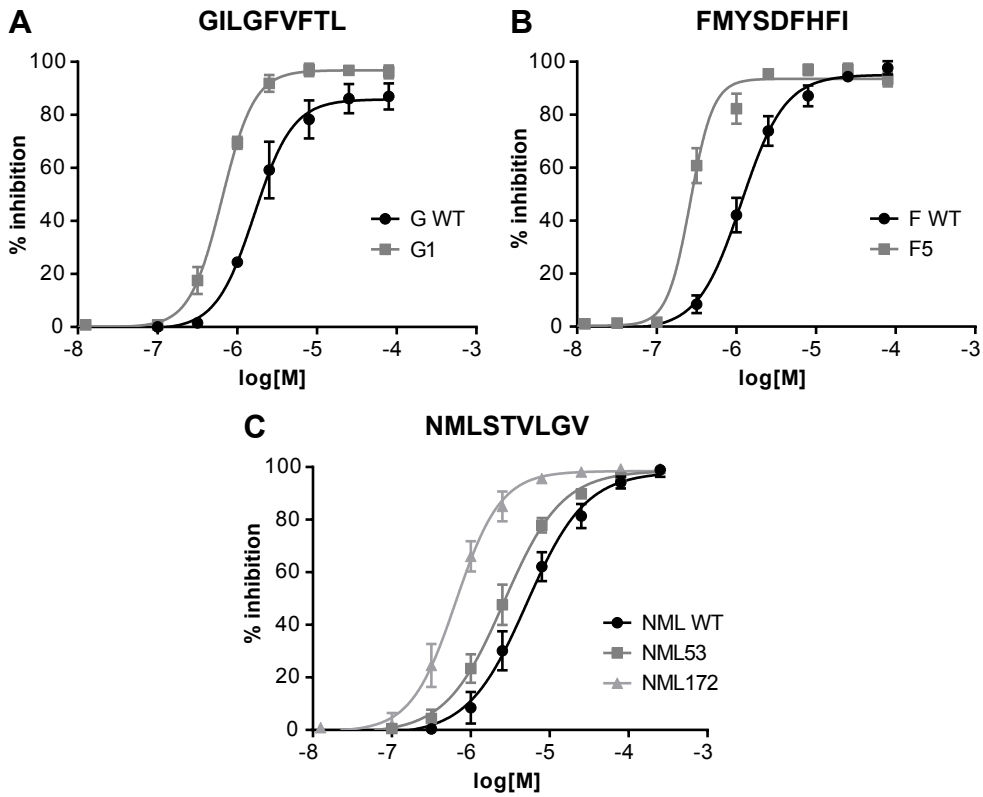


Figure 3: Binding affinity dose-response curves of CPLs and WT peptides
The IC_{50} curves of the selected CPLs show increased HLA binding affinity compared to IC_{50} curves of the corresponding WT-peptides. To generate IC_{50} curves the FP-based competition assay was performed using three-fold peptide dilutions in the presence of a standard amount of tracer peptide. Shown are averages and their standard deviation of three independent experiments for GILGFVFTL (**A**), FMYSDFHFI (**B**) and NMLSTVLGV (**C**) peptides. Curves of CPLs are shifted to the left compared to WT peptides, indicating that a lower dose of CPLs is needed to inhibit tracer binding.

H2-Kb. However, the response of HLA-A2 tg mice is still substantially higher; therefore, the improvement observed for F5 is at least in part mediated by HLA-A*0201 (**Figure S1B**).

To provide more insight into binding affinity, serial peptide dilutions were used in the FP binding assay to determine the half-maximal inhibition of tracer binding concentration (IC_{50} values). Peptide binding scores as shown in **Table I** were determined at a single concentration. Analysis of the dose-response curves shows that in all cases the CPLs have a lower IC_{50} value than their WT counterparts (**Figure 3**). These results are in line with findings in vaccination experiments with mice, in which the GILGFVFTL- and FMYSDFHFI-derived CPLs induced an IFN-γ response at lower doses than the WT epitope (**Figure 2**). The increase in binding affinity probably not only results in an increased on-rate, but more importantly also a decrease in off-rate due to increased peptide-MHC (pMHC) stability (40). This would cause a prolonged presentation to T cells and hence a higher IFN-γ response.

All responses obtained in the in vivo vaccination experiments were analyzed using an ELISpot assay with a complete pool of splenocytes. To prove that responses are indeed $CD8^+$ T cell-specific, splenocytes were analyzed by flow cytometry. In **Figure 4**, flow cytometry dot plots show that the response towards CPL G1 was similar compared to WT peptide, which might be explained by the fact that a dose of 75 nmol was used. In the dose response experiments,

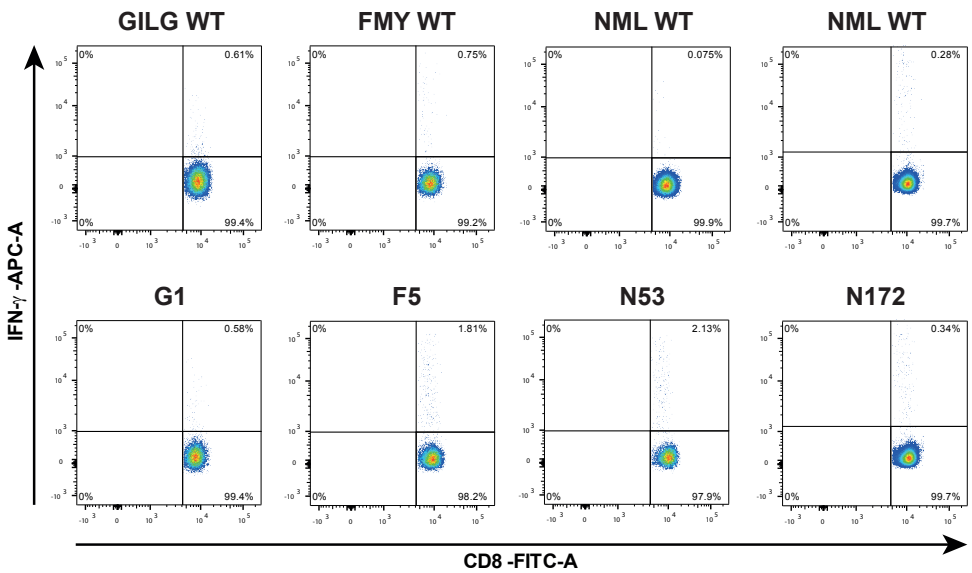
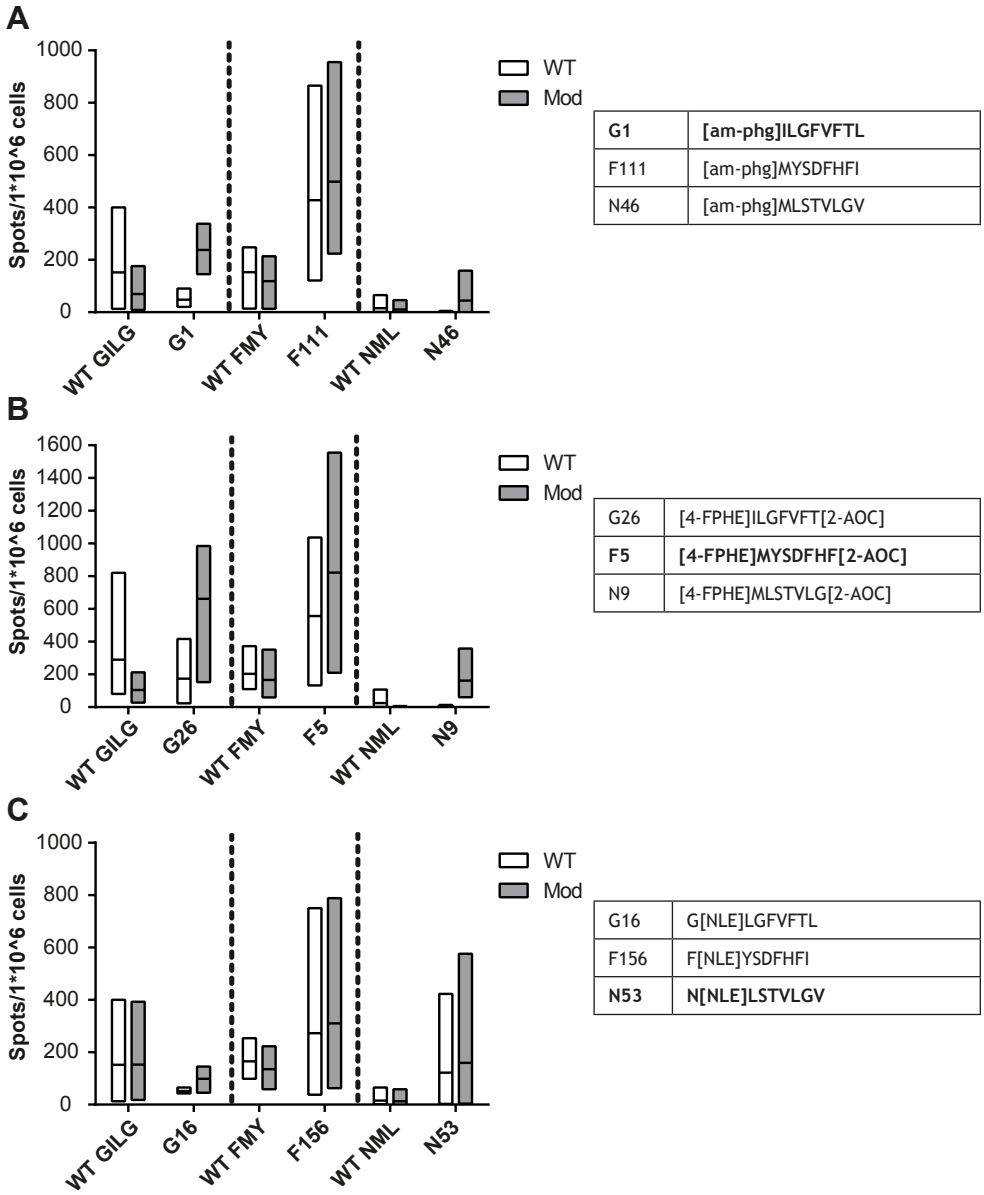


Figure 4: Flow cytometry analysis on $CD8^+$ T cell responses of CPL- and WT-vaccinated mice
Dot plots showing IFN-γ production by $CD8^+$ T cells of mice vaccinated with 75 nmol of either WT peptide or CPL (G1, F5, N53 and N172). In the upper panel, the respective WT-peptide control of that particular experiment is shown. In the lower panel, the CPL-induced IFN-γ responses are shown. Spleen cells were stimulated O/N with 0.1 nmol/well WT peptide. Highest responders of each group are shown. Vaccination with F5 and N53 induced the largest improvement in IFN-γ production compared to WT peptide-vaccinated mice.



G1	**[am-phg]ILGFVFTL**
F111	[am-phg]MYSDFHFI
N46	[am-phg]MLSTVLGV

G26	[4-FPHE]ILGFVFT[2-AOC]
F5	**[4-FPHE]MYSDFHF[2-AOC]**
N9	[4-FPHE]MLSTVLG[2-AOC]

G16	G[NLE]LGFVFTL
F156	F[NLE]YSDFHFI
N53	**N[NLE]LSTVLGV**

Figure 5: Predictive value of modifications
IFN-γ ELISpot on spleen cells of mice vaccinated with 75 nmol of either WT peptide or CPLs and stimulated for 16 hours with 0.1 nmol WT peptide or CPL per well. The three different modifications are based on final selected peptides for each epitope: (**A**) am-phg on P_1 based on G1, (**B**) 4-FPHE on P_1 and 2-AOC on P_9 based on F5 and (**C**) NLE on P_2 based on N53. X-axis depicts peptide used for vaccination. White boxes represents restimulation with WT peptide and grey boxes restimulation with CPL. Bars are min to max, with line at mean. Although it appears difficult to predict whether a modification will work in a certain epitope, an effective modification in one epitope is in some cases also effective in other epitopes. Bars represent a minimum of three mice (GILGFVFTL and FMYSDFHFI) and a maximum of eight (NMLSTVLGV).

a high dose of G1 appeared to result in suboptimal induction of IFN-γ production. Responses to CPLs F5 and N53, however, did show a major improvement as indicated by the increased production of IFN-γ by $CD8^+$ T cells. $CD4^+$ T cells did not produce IFN-γ in response to peptide restimulation, showing that the enhanced IFN-γ production measured in the ELISpot assay was produced by $CD8^+$ T cells and not by $CD4^+$ T cells (**Figure S2**).

Predictive value of modifications

Next, modifications of the CPLs described above were analyzed further to determine whether an effective substitution in one epitope is a prediction for the success of that particular substitution for other epitopes. For each epitope, CPLs were synthesized with modifications that are present in G1, F5, and N53, resulting in a set of three CPLs per type of modification. **Figure 5A** shows IFN-γ responses of mice vaccinated with either of the selected epitopes of which P_1 was substituted for the residue am-phg, the modification that was most successful for GILGFVFTL (G1). Grey bars visualize that after stimulation with homologous peptides, enhanced responses were observed in all CPL-vaccinated mice. After WT stimulation, responses remained more or less similar, except for the response to N46, which was reduced to zero. Based on CPL F5 we introduced 4-FPHE on P_1 and 2-AOC on P_9 of GILGFVFTL and NMLSTVLGV. This combination of substitutions again led to a greatly enhanced response after restimulation with homologous peptide (**Figure 5B**). However, for both epitopes, responses after WT restimulation were lower in CPL-vaccinated mice compared to responses of WT-vaccinated mice. Perhaps by changing the amino acids the structure of these CPLs differed too much from the WT, such that specificity for the WT sequence was lost. Finally, we substituted P_2 for norleucine (NLE) in GILGFVFTL and FMYSDFHFI based on CPL N53 (**Figure 5C**). This substitution showed a slightly enhanced response for CPL F156 compared to its WT counterpart, but led to decreased responses for CPL G16. Although it appears difficult to predict whether a modification will work in a given epitope, an effective modification in one epitope proves in some cases also effective in other epitopes.

Optimizing HLA-A*0301 binding affinity of influenza epitopes

The response to vaccination can be broadened by selecting more HLA-A*0201 peptides, but even more so by targeting multiple alleles. We therefore set out to optimize three influenza epitopes specific for HLA-A*0301 as an example that incorporation of non-proteogenic amino acids is a strategy that can be extended to other alleles. The main difference between HLA-A*0201 and HLA-A*0301 is the preference of HLA-A*0301 for long positively charged residues on P_9, demonstrated by the frequent occurrence of lysine and arginine on the C-terminal anchor position, whereas side chains of amino acids on P_2 still dock into a hydrophobic pocket (41). Using a HLA-A*0301-specific tracer peptide we performed the FP-based competition assay described in Materials and Methods for 96 peptides per epitope and measured binding after 4 and 24 hours (42). Similar to HLA-A*0201, the selected epitopes vary in immunodominance, with ILRGSVAHK being the most dominant and 10-mer RMVLSAFDER a low affinity epitope (33, 43, 44). SFSFGGFTK is an intermediate HLA-A*0301 binder with

unknown dominance. Substitution with non-proteogenic amino acids on or near anchor positions resulted in greatly enhanced binding, as shown in **Table III**. Since HLA-A*0301 has a hydrophobic binding pocket at P_2 just like HLA-A*0201, incorporation of norvaline (NVA) or 2-AOC on P_2 resulted in increased binding scores. Substitutions on P_9 did not enhance binding for any of the epitopes tested, probably because the lysine in the WT sequence forms strong ionic interactions that are hard to improve with the pool of amino acids tested. These data show that the technique of substituting amino acids by non-proteogenic amino acids to increase binding affinity can be applied to epitopes of other alleles, which is valuable for the development of broadly immunogenic vaccines.

Table III. FP binding scores HLA-A*0301 peptides

A

#	ILRGSVAHK	4h	SD	24h	SD
I65	ILRGSV[2-AOC]HK	87	2	94	0
I74	I[AHA]RGSV[NLE]HK	77	2	92	1
I53	[AHA][NLE]RGSVAHK	73	0	91	2
I87	I[AHA][4-FPHE]GSVAHK	73	2	91	0
I60	[2-AOC][NLE]RGSVAHK	72	4	91	1
I89	I[AHA][CHA]GSVAHK	75	1	91	1
I59	I[AHA][2-AOC]GSVAHK	71	1	91	0
I73	I[AHA]RGSV[CSET]HK	74	3	90	1
I61	[CSME][AHA]RGSVAHK	71	2	90	1
I49	I[OrnN2]RGSVAHK	71	2	90	1
I79	I[AHA]RGSV[2-FUR]HK	77	3	90	1
I86	I[AHA][CSME]GSVAHK	71	2	89	1
I84	I[AHA][PRG]GSVAHK	71	2	89	0
I77	I[AHA]RGSV[CpALA]HK	71	2	89	1
I63	ILRGSV[NLE]HK	74	2	89	1
I17	[2-AOC][AHA]RGSVAHK	61	5	89	2
I58	I[AHA][NLE]GSVAHK	68	3	89	1
I83	I[AHA][CpALA]GSVAHK	70	3	89	0
I94	IL[4-FPHE]GSVAHK	70	4	89	1
I85	I[AHA][SOME]GSVAHK	69	2	88	1
I WT	**ILRGSVAHK**	66	3	87	1

B

#	SFSFGGFTK	4h	SD	24h	SD
S70	S[NVA]SFGG[2-AOC]TK	90	0	96	0
S84	SF[4-FPHE]FGGFTK	82	1	95	0
S50	S[NVA][2-AOC]FGGFTK	83	1	94	1
S74	S[NVA]SFGG[CpALA]TK	78	1	94	1
S68	S[NVA]SFGG[NLE]TK	79	2	93	0
S95	S[NVA][4-FPHE]FGGFTK	80	2	93	0
S94	S[NVA][NLE]FGGFTK	80	1	93	1
S52	S[NVA][AHA]FGGFTK	80	1	93	1
S72	S[NVA]SFGG[4-FPHE]TK	79	0	93	0
S93	S[NVA][CSME]FGGFTK	80	0	93	1
S53	S[NVA]RFGGFTK	80	1	93	1
S54	S[AHA]RFGGFTK	80	1	92	1
S92	S[NVA]SFGGF[TOME]K	80	2	92	1
S73	S[NVA]SFGG[BPG]TK	73	0	91	1
S69	S[NVA]SFGG[NVA]TK	72	2	91	0
S96	S[NVA][CpALA]FGGFTK	76	3	91	0
S56	S[NVA]SFGGFHK	78	2	91	1
S76	S[NVA]SFGG[2-FUR]TK	75	1	91	0
S71	S[NVA]SFGG[PRG]TK	70	2	90	0
S90	S[NVA]SFGGF[AHA]K	78	0	90	1
S WT	**SFSFGGFTK**	67	2	58	3

C

#	RMVLSAFDER	4h	SD	24h	SD
R71	R[NVA]VLSAF[2-AOC]ER	71	2	82	2
R67	R[AHA]VLSAF[2-AOC]ER	74	2	78	1
R81	RMVLSAF[AHA]ER	71	2	76	2
R38	RMVLSAF[2-AOC]ER	69	3	75	2
R70	R[NVA]VLSAF[SOME]ER	64	4	74	1
R42	RMVLSAF[CpALA]ER	65	2	73	1
R36	RMVLSAF[NLE]ER	66	4	73	1
R72	R[NVA]VLSAF[4-FPHE]ER	68	1	72	2
R83	RM[OrnN2]LSAFDER	71	3	72	2
R4	RMVLSAFDEK	61	1	69	1
R59	R[NVA]VLSAFDKR	62	4	68	0
R2	R[AHA]VLSAFDEK	58	1	67	1
R50	RM[2-AOC]LSAFDER	70	3	66	1
R55	RM[4-FPHE]LSAFDER	70	4	65	1
R68	R[AHA]VLSAF[4-FPHE]ER	67	3	64	0
R66	R[AHA]VLSAF[SOME]ER	63	3	63	1
R41	RMVLSAF[ORN]ER	65	3	63	0
R39	RMVLSAF[PRG]ER	62	4	62	1
R37	RMVLSAF[CSET]ER	60	3	60	2
R40	RMVLSAF[4-FPHE]ER	63	3	57	1
R WT	**RMVLSAFDER**	18	2	6	1

HLA-A*0301 binding data for three influenza epitopes: (**A**) ILRGSVAHK, (**B**) SFSFGGFTK* AND (**C**) RMVLSAFDER. Affinity was determined as in Table I after 4h and 24h in three independent experiments. This table shows percentage inhibition in a heat map for the WT epitopes (bold) and 20 CPLs with highest binding scores. Green indicates high binding scores, yellow medium binding scores and red low binding scores. For all three epitopes binding scores could be greatly increased by substitution with non-proteogenic amino acids.

* SFSFGGFTK was incorrectly referred to in the immune epitope database; the epitope originally described as an HLA-A*0301 binder by Assarsson et al. has amino acid sequence: SFSFGGFTFK (43, 61).

Discussion

Current vaccination strategies to prevent influenza infection are mainly aimed at antibody-mediated immune responses, yet, cytotoxic responses have also been proven to contribute to protection against influenza infection (14, 16, 17, 45). One of the approaches to induce these responses is by vaccination with peptides that encode T cell epitopes. However, immunogenicity of peptides is often inadequate; therefore, additional optimization is required. Here, we designed and synthesized CPLs with enhanced affinity for class I MHCs to ultimately improve T cell responses towards these peptides. Three highly conserved HLA-A*0201-specific influenza epitopes that have varying binding affinity and dominance in the immune response were selected: GILGFVFTL, a highly immunodominant epitope; FMYSDFHFI, a less dominant epitope, and NMLSTVLGV, which is a low affinity subdominant epitope. By studying available crystal structures and by replacing amino acids at or adjacent to the anchor positions with non-proteogenic amino acids, CPLs were designed with a theoretically increased number and quality of interactions with the MHC binding groove. Using non-proteogenic amino acids, modification was no longer limited to the repertoire of naturally occurring amino acids. With this approach, we succeeded to enhance binding affinity of all three epitopes and after in vitro evaluation, the most promising CPLs were tested in mice. We showed that CPLs G1, G8, F5, F100, F111, N53, and N172 were capable of inducing improved T cell responses in HLA-A2 tg mice, as measured by IFN-γ production in splenocytes. As expected, especially the response towards the more subdominant peptides was greatly improved.

The first objective was to improve binding affinity of the peptides to MHCs by introducing non-proteogenic amino acid substitutions. Earlier, we reported improved effectivity of a melanoma-specific peptide by substitution of am-phg on P_1. This substitution led to additional interactions between the peptide and the MHC, thereby stabilizing the complex as shown in a crystal structure (13). These findings may explain increased binding scores of CPLs G1 and N46, which contain the same substitution (**Table I**). For FMYSDFHFI, introduction of am-phg on P_1 retained the binding score at a similar level as WT peptide: (F111, **Table I**). Surprisingly, G1 and F111, but not N46 showed improved immunogenicity in mice to the homologous and WT epitope (**Figure 5**).

Since the HLA-A*0201 allele prefers long hydrophobic residues on P_2 and the C-terminus of a peptide, other stabilizing interactions were created by introducing hydrophobic residues into the peptide. 2-AOC, NLE, and NVA are examples of amino acids with hydrophobic side chains that can protrude deeply into the hydrophobic binding pockets of HLA-A*0201 (46, 47). CPLs of FMYSDFHFI and NMLSTVLGV with the largest increase in binding score indeed had a substitution of 2-AOC on P_2 or P_9, often in combination with other substitutions (**Table I**). While introduction of 2-AOC did not enhance binding of GILGFVFTL-derived CPLs, introducing another hydrophobic residue, NLE, on P_2 did enhance its binding score. This NLE substitution improved homologous immunogenicity of CPLs F156 and N53 showed improved recognition

of the WT epitope (**Figure 5**).

A point of interest is that from these binding results, it becomes clear that an amino acid preferred in one epitope is not necessarily preferred in another epitope, even when they are specific for the same HLA allele. Amino acid preferences are determined by the binding pockets in the binding groove of MHC and should therefore in theory be similar for every peptide specific for that allele. As discussed before, substitution of am-phg on P_1 of the GILGFVFTL epitope resulted in the highest binding score (G1, 98% compared to 84% for the WT; Table I) and a major improvement was seen for NMLSTVLGV after the same substitution on P_1 (N46, 81% compared to 55% for the WT). The success of substitution on P_1 is not surprising, since secondary anchor residues, which for HLA-A*0201 are found on P_1, P_3 and P_7, were previously discovered to also have significant effect on binding (48, 49). However, substitution of am-phg on P_1 in the FMYSDFHFI epitope did not increase binding scores (F111, 72% compared to 75% for the WT, **Table I**). Likewise, incorporating 3-PYRA on P_1 was successful for the GILGFVFTL epitope (G8, 93%; **Table I**), but did not enhance binding as much for NMLSTVLGV and FMYSDFHFI (both 73%; **Table I**). This discrepancy could be due to conformational heterogeneity in the peptide backbones, since peptide binding strength is not only dependent on interactions of the side chains of anchor residues with the binding pockets, but also on those of the peptide backbone with the MHC binding groove (32, 50, 51). The structure of the backbone is dependent on the size and fit of the amino acid side chains in the binding groove. Modifications may change the structure of the peptide backbone in one CPL in such a way that the interaction with the binding groove is weakened, while in another CPL there is no effect of the same substitution on this interaction. Alternatively, the change in structure of the backbone may affect the positioning of the anchor residue in such a way that it does not fit smoothly into the binding pocket.

Changes in the central region of the peptide may in turn affect recognition by the TCR (52, 53). Thus, by introducing too many modifications in one peptide, T cell responses may be perturbed significantly and therefore we substituted a maximum of two amino acids. In addition, introduction of a single non-proteogenic amino acid in one peptide at a non T cell-exposed position might influence the structure of the backbone and thus the central region, while the same amino acid in another peptide might have little or no effect (54). This could be the reason that some modifications seem to always lead to higher responses after restimulation with a CPL, likely due to the improvement of affinity, but that these CPL-induced T cells do not always react to restimulation with WT peptide (N46, G26, N9; **Figure 5**). These CPLs may induce a different subset of T cells than the WT peptide, which is not necessarily problematic in a vaccination setting as long as the CPL-induced T cells still recognize the WT peptide (55).

For the selection of CPLs for in vivo experiments, we set out to exclude CPLs that were not capable of inducing a response in WT-specific T cells as we hypothesized that these CPLs would

likely not induce the correct T cells to recognize the WT epitope. Therefore, we performed three different assays in which CPLs were presented to WT-specific T cells. For the GILGFVFTL epitope a T cell clone was available, which facilitated analysis of responses of the WT-specific T cells to the CPLs. Activation of these cells by CPLs indicated that WT-specific TCRs are still capable of recognizing the CPLs (**Table SI**). For FMYSDFHFI and NMLSTVLGV, other methods needed to be developed and we therefore included a human DC-T cell co-culture method and analysis of WT-specific mouse splenocytes stimulated by CPLs (**Table SI**). The former analysis was effective in showing differences between the CPLs; however, donor variation was too large to draw definite conclusions. Analysis in splenocytes of an inbred HLA-A2 tg mouse strain allowed for little donor variation, but none of the CPLs were shown to induce better responses than the WT peptide in this model, in contrast to the other two methods. It did reveal some CPLs that induced little or no responses in the WT-specific splenocytes, allowing for negative selection. However, in these assays we were only able to mimic a reversed setting, i.e., WT-specific T cells that recognize CPLs. Such reverse immunology does not exclude the possibility that CPLs may induce T cells that are still capable of recognizing WT peptide even though this is not true for the reversed argument. For this reason, reverse immunology appears to be a suboptimal predictor for vaccine development (56). Therefore, CPLs still needed to be tested for their ability to induce T cells that recognize the WT peptide in a vaccination setting.

Thus, we evaluated whether increased binding affinity also led to enhanced T cell responses by vaccination of HLA-A2 tg mice with a selection of CPLs (**Figure 2**). Based on results from the assays described above, four CPLs per WT peptide were selected for further in vivo testing. As our data for the GILGFVFTL-derived CPLs indicate, CPLs can facilitate a dose reduction while similar responses to WT peptide are maintained. At lower doses, vaccination with CPLs G1, G8 and G25 induced higher T cell responses after restimulation with WT peptide, compared to WT-vaccinated mice. The diminished responses at higher doses could be explained by overstimulation, as described for density of pMHC interactions on an APC (57). In addition, modification of the FMYSDFHFI peptide led to the induction of higher T cell responses compared to the WT peptide in almost all doses tested. Surprisingly, F193 induced lowest homologous and heterologous responses, even though it did show improved binding affinity. In contrast, binding affinity of F100 and F111 was similar to that of the WT epitope, while these CPLs induced higher homologous and heterologous responses in mice. These observations indicate that factors other than binding affinity play a role in the increased immunogenicity. The effect of increasing binding affinity by introducing non-proteogenic amino acids on T cell responses was most remarkably shown by CPLs N53 and N172. These CPLs increased the number of responders to this subdominant epitope from approximately 1/6 to half of the mice and induced higher responses than the WT peptide, after WT restimulation.

Hence, while increased binding may result in higher responses in mice, this appears not to be a general rule, which has some implications for vaccine development. Namely, the process to

find modifications that lead to improved responses is not only affinity based, but also includes a trial and error-factor. This may lengthen the development time of a peptide-based vaccine; however, with respect to the complete development process the impact is estimated to be minor.

Preventive vaccines should most of all induce a broad immune response, in contrast to therapeutic vaccines, where high affinity peptides are needed to overcome self-tolerance. By inducing a broad range of CTLs, the chance of generation of escape mutants decreases, rendering a vaccine more effective (58, 59). Some successful phase I clinical trials describing influenza peptide vaccines capable of inducing T cell responses have been reported (9, 10). However, these vaccines consist of long peptides and are mostly based on immunodominant epitopes, which might not be the best epitopes to induce a response to since there are indications that these epitopes overrule other T cell responses (56, 60).

We have shown for six influenza epitopes, all with different characteristics, that it is possible to improve their MHC binding affinity and that the immunogenicity of the three HLA-A*0201 epitopes could be improved considerably. Furthermore, by improving binding of HLA-A*0301-specific peptides we have shown that it is possible to target alleles other than HLA-A*0201, which is essential for broad population coverage. In order to enhance immunogenicity and efficacy of short peptides for T cell-targeted vaccines as used in our studies it is necessary to further develop adjuvants and to include a broader range of peptides. Our results illustrate the potential of inducing responses to otherwise subdominant epitopes by modification of amino acid residues and enhancing binding affinity. Especially since there are indications that inducing a broad response is more efficacious, our approach provides a promising method to induce responses to a larger range of epitopes (60).

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Supplemental data

Table SI. GILG, FMY and NML specific CD8⁺ T cell responses after stimulation with CPLs in in vitro and ex vivo screening models.

A		T cell clone		DC model		Mouse splenocytes	
		Average	SD	Average	SD	Average	SD
G1	[am-phg]ILGFVFTL	50.0	2.2	0.11	0.03	27	24
G7	[Phg]ILGFVFTL	47.0	1.8	0.20	0.09	75	20
G8	[3-PYRA]ILGFVFTL	37.0	2.1	0.09	0.01	49	19
G15	[4-PYRA]ILGFVFTL	33.4	3.3	0.17	0.10	53	19
G4	[3-pyra]ILGFVFTL	8.6	0.1	0.11	0.06	12	7
G12	GILGFV[4-FPHE]TL	47.6	2.3	0.10	0.04	87	19
G27	[am-phg][CpALA]LGFVFTL			0.10	0.05	35	25
G10	[4-pyra]ILGFVFTL	45.4	0.7	0.14	0.07	47	16
G9	yILGFVFTL	1.1	0.1	0.11	0.03	20	11
G16	G[NLE]LGFVFTL	49.6	3.6	0.09	0.03	n.a.	n.a.
G3	GILGFVFT[CpALA]	42.0	0.5	0.11	0.05	99	10
G25	[SOME]ILGFVFTL	48.1	1.6	0.12	0.02	58	18
G13	GILGFVFT[ALG]	11.4	1.8	0.11	0.02	101	14
G17	GILGFVFT[PRG]	8.9	1.0	0.07	0.01	87	15
GILG WT	**GILGFVFTL**	36.3	0.9	0.26	0.13	100	0
G24	fILGFVFTL	0.7	0.1	0.08	0.02	24	18
G20	GILGFV[BUTGLY]TL	n.a.	n.a.	0.07	0.06	36	19
G22	GILGFVFT[2-AOC]	23.3	4.5	0.12	0.06	90	20
G11	[CSME]ILGFVFTL	40.9	1.9	0.17	0.03	67	24
G26	G[2-AOC]LGFVFT[PRG]			0.45	0.47	61	15
G29	[3-PYRA]ILGFVFT[2-AOC]			0.12	0.04	39	19
+	SEB	91.6	0.1	n.a.	n.a.	15	10
-	medium	0.7	1.5	0.12	0.07	2	2

B		DC model		Mouse splenocytes	
		Average	SD	Average	SD
F5	[4-FPHE]MYSDFHF[2-AOC]	0.16	0.15	58	16
F118	[CSET][2-AOC]YSDFHFI	n.a.	n.a.	81	9
F141	[THR-BZL][2-AOC]YSDFHFI	n.a.	n.a.	67	12
F48	F[2-AOC]YSDFHF[CHA]	0.11	0.04	61	19
F143	F[2-AOC]YSDFHFI	n.a.	n.a.	92	8
F102	[3-THI][2-AOC]YSDFHFI	0.06	0.02	96	12
F112	[BCA][2-AOC]YSDFHFI	n.a.	n.a.	86	14
F7	[am-phg]MYSDFHF[2-AOC]	0.13	0.10	54	18
F69	F[2-AOC]YSDFHF[NLE]	0.08	0.07	63	20
F49	FMYSDFHF[CHA]	0.18	0.16	60	17
F19	FMYSDFHF[2-AOC]	0.07	0.04	62	21
F193	[am-phg][NVA]YSDFHFI	n.a.	n.a.	n.a.	n.a.
F54	FMYSDFHF[CSET]	0.14	0.06	78	8
F95	[2-AOC]MYSDFHFI	0.12	0.09	90	15
F105	[4-FPHE]MYSDFHFI	0.08	0.04	62	10
F52	FMYSDFHF[CpALA]	0.13	0.07	79	15
F63	FMYSDFHF[HPG]	0.08	0.03	83	14
F142	[THR-BZL]MYSDFHFI	0.08	0.03	79	9
FMY WT	**FMYSDFHFI**	0.08	0.03	100	0
F100	[3-PYRA]MYSDFHFI	n.a.	n.a.	104	7
F111	[am-phg]MYSDFHFI	0.08	0.05	86	14
+	SEB	n.a.	n.a.	33	39
-	medium	0.12	0.09	0.17	0.41

C		DC model		Mouse splenocytes	
		Average	SD	Average	SD
N95	[4-FPHE][2-AOC]LSTVLGV	0.07	0.06	3	n.a.
N92	[SOME][2-AOC]LSTVLGV	0.04	0.03	380	n.a.
N91	[OM-HS][2-AOC]LSTVLGV	n.a.	n.a.	344	n.a.
N98	[3-THI]MLSTVLG[2-AOC]	0.04	0.02	93	n.a.
N172	[Phg][2-AOC]LSTVLGV	0.19	0.21	375	n.a.
N11	[am-phg]MLSTVLG[2-AOC]	0.03	0.06	3	n.a.
N15	[NVA]MLSTVLG[2-AOC]	0.07	0.05	3	n.a.
N8	[3-PYRA]MLSTVLG[2-AOC]	0.01	0.02	1	n.a.
N169	[CSME][2-AOC]LSTVLGV	0.09	0.05	366	n.a.
N46	[am-phg]MLSTVLGV	0.02	0.01	363	n.a.
N39	[2-AOC]MLSTVLGV	0.05	0.02	334	n.a.
N177	F[2-AOC]LSTVLGV	0.04	0.05	46	n.a.
N41	NM[2-AOC]STVLGV	0.08	0.05	5	n.a.
N43	[3-PYRA]MLSTVLGV	0.06	0.08	108	n.a.
N40	N[2-AOC]LSTVLGV	0.06	0.04	375	n.a.
N176	[THR-BZL]MLSTVLGV	0.03	0.02	355	n.a.
N53	N[NLE]LSTVLGV	n.a.	n.a.	365	n.a.
N52	[NLE]MLSTVLGV	0.03	0.03	341	n.a.
NML WT	**NMLSTVLGV**	0.04	0.03	436	n.a.
N61	[SOME]MLSTVLGV	n.a.	n.a.	348	n.a.
N122	NMLSTVLG[CpALA]	n.a.	n.a.	128	n.a.
+	SEB	0.20	0.21	135	n.a.
-	medium	0.05	0.04	1	n.a.

Three different assays were used to investigate whether CPLs derived from (**A**) GILGFVFTL (**B**) FMYSDFHFI and (**C**) NMLSTVLGV were still capable of activating WT-specific T cells. GILGFVFTL derived CPLs were analyzed for their ability to activate a GILGFVFTL-specific $CD8^+$ T cell clone. Percentage of IFN-γ producing T cells after stimulation with the indicated peptides are shown in the left column in Panel A. CPLs that induced the highest IFN-γ production are G1, G16, G25 and G7. Values represent the average of three independent experiments after 24 hours. The second assay depicts results of the DC co-culture model. IFN-γ-positive $CD8^+$ T cells of a HLA-$A2^+$ donor were analyzed in duplicate by flow cytometry after seven days of co-culture with peptide-pulsed DCs. CPLs G26, F49, F5, F54, F7, F52, N172, N169, N41, N95, N15, N40 and N43 induced a higher number of IFN-γ^+ $CD8^+$ T cells than the WT peptide, negative control or any of the other CPLs. The third assay depicts results of an ex vivo assay in mouse splenocytes. IFN-γ ELISpot was performed on spleen cells of mice vaccinated with 75 nmol WT GILGFVFTL, FMYSDFHFI or NMLSTVLGV on days 0 and 21. Two weeks post booster vaccination, spleen cells were restimulated ex vivo for 16 hours with 0.2 nmol of CPL. Only G13 and F100 induced slightly higher responses than corresponding WT peptides, all other CPLs induced lower responses than WT peptide. Results depict average spot count and SD of six mice for GILGFVFTL and FMYSDFHFI and spot count of the only one out of the six mice that responded to NMLSTVLGV and are shown in the right columns. Activation scores are presented in a heat map; Green indicates high activation scores, yellow medium and red low activation scores.

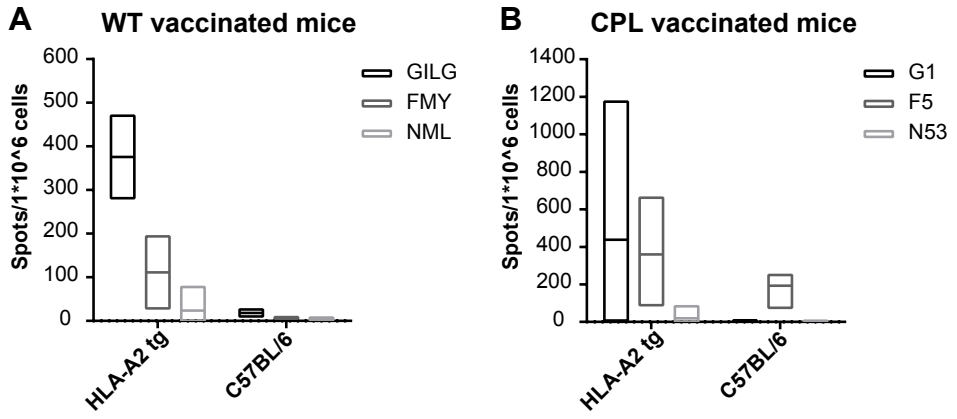

Figure S1: Epitope MHC specificity control experiment in C57BL/6 mice.
C57BL/6 and HLA-A2 tg mice were vaccinated with a dose of 75 nmol of WT peptides (A) or CPLs (B) on days 0 and 21. Two weeks later spleen cells were isolated and stimulated O/N with 0.1 nmol of WT peptides or corresponding CPLs. WT GILG and WT FMY (n=2), WT NML in HLA-A2 tg mice (n=5), WT NML in B6 (n=7), G1 and F5 (n=4), N53 HLA-A2 tg (n=6), C57BL/6 (n=7). No responses were detected in C57BL/6 mice vaccinated with WT peptides. However, C57BL/6 mice did respond to F5, although responses of HLA-A2 transgenic mice were higher. All WT and CPLs induced responses in the HLA-A2 tg mice.

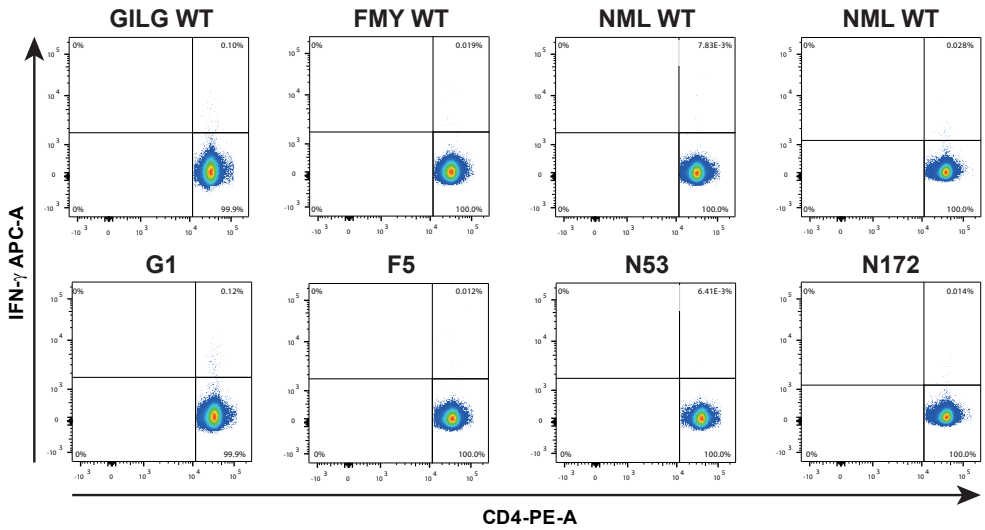

Figure S2: Flow cytometry dot plots showing IFN-γ-positive CD4⁺ T cells of HLA-A2⁺ transgenic mice.

Dot plots show IFN-γ production by CD4⁺ T cells of mice vaccinated with 75 nmol of either WT peptide or CPL (G1, F5, N53 and N172). Spleen cells were stimulated for 16 hours with 0.1 nmol/well WT peptide. Highest responders of each group are shown. Although for GILGFVFTL some background staining is visible, these dot plots show that CD4⁺ T cells did not produce IFN-γ in response to peptide restimulation, indicating that the enhanced IFN-γ production measured in the ELISpot assay was indeed produced by CD8⁺ T cells and not CD4⁺ T cells.

Chapter 6

Influenza T cell epitope-loaded virosomes adjuvanted with CpG as a potential influenza vaccine

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Abstract

Influenza $CD8^+$ T cell epitopes are conserved amongst influenza strains and can be recognized by influenza-specific cytotoxic T cells (CTLs), which can rapidly clear infected cells. An influenza peptide-based vaccine that elicits these CTLs would therefore be an alternative to current influenza vaccines, which are not cross-reactive. However, peptide antigens are poorly immunogenic due to lack of delivery to antigen presenting cells, and therefore need additional formulation with a suitable delivery system. In this study, the potential of virosomes as a delivery system for an influenza-specific T cell peptide was investigated. The conserved human HLA-A*0201 influenza T cell epitope $M1_{58-66}$ was formulated with virosomes. The immunogenicity and protective effect of the peptide-loaded virosomes was assessed in HLA-A2 transgenic mice. Delivery properties of the virosomes were studied in mice and in in vitro dendritic cell cultures. Immunization of HLA-A2 transgenic mice with peptide-loaded virosomes in the presence of the adjuvant CpG-ODN 1826 increased peptide-specific CTLs. Vaccination with adjuvanted peptide-loaded virosomes reduced weight loss in mice after heterologous influenza infection. Association with fusion-active virosomes was found to be crucial for antigen uptake by dendritic cells, and subsequent induction of CTLs in mice. These results show that influenza virosomes loaded with conserved influenza epitopes could be the basis of a novel cross-protective influenza vaccine.

Introduction

The need for cross-protective influenza A vaccines has increased in recent years after several global outbreaks of highly pathogenic influenza A strains, such as avian H5N1 (1), swine H1N1, and avian H7N9 (2, 3). The current, mainly antibody-inducing influenza A vaccines are generally ineffective against influenza A strains which underwent antigenic shifts and drifts, which leads to vaccine mismatch. Influenza-specific antibodies induced by mismatched vaccines fail to recognize the surface proteins hemagglutinin (HA) and neuraminidase (NA). As a result, the composition of the current influenza vaccines has to be adjusted frequently to cope with these antigenic changes. $CD8^+$ cytotoxic T cells (CTLs) that are specific for conserved epitopes of internal influenza A proteins, such as matrix protein and nucleoprotein, may provide cross-protection and are less affected by antigenic changes (4). Influenza-specific CTLs can efficiently clear virus-infected cells, thereby reducing viral replication and spread. Influenza-specific $CD8^+$ T cells induced by influenza infection were recently correlated with less severe illness in adults infected with pandemic H1N1 virus (5). Inducing influenza-specific CTLs by vaccination could therefore be a promising approach to achieve cross-protection against heterologous influenza A strains (6).

The influenza $M1_{58\text{-}66}$ peptide is a highly conserved human major histocompatibility complex (MHC) class I restricted epitope (7, 8), which can induce influenza-specific CTLs. However, before $CD8^+$ T cells can be induced, several critical processes have to take place (9). Delivery of the peptide antigens to antigen presenting cells (APCs), in particular dendritic cells (DCs), is crucial for antigen presentation on MHC class I molecules. Therefore, formulation of the peptide antigens with a suitable delivery system, such as virosomes, is required. Influenza virosomes were previously shown to be capable of efficient delivery of peptide antigens and subsequent CTL induction (10). However, virosomal formulations can only deliver low doses of peptide antigens and lack pathogen-associated molecular patterns (PAMPs). The inclusion of immunopotentiators such as Toll-like receptor (TLR) agonists could improve the immunogenicity of the vaccine formulation, without the need to increase the peptide-loading efficiency in virosomes, which requires complicated methods (11). While adjuvants have been used to increase neutralizing antibody levels induced by virosomal vaccines (12), their effect on influenza peptide-specific $CD8^+$ T cell responses in combination with virosomes has yet to be determined.

In this study, we investigated the immunogenicity of virosomes loaded with the human HLA-A*0201 restricted peptide $M1_{58\text{-}66}$, derived from influenza matrix protein 1. Influenza-specific $CD8^+$ T cell responses and supporting antibody titers were assessed in HLA-A2 transgenic mice after immunization. The addition of TLR9 agonist CpG was found to be an effective adjuvant for MHC class I restricted peptides in conjunction with virosomes. Furthermore, immunization with peptide-loaded virosomes adjuvanted with CpG increased the rate of recovery in mice infected with a heterologous influenza strain. Finally, delivery

properties of virosomes were extensively characterized in in vitro human DC models and in vivo in transgenic mice. Both peptide association with the virosomes and virosomal cell binding and membrane fusion capabilities were found to be crucial for peptide uptake by DCs and induction of peptide-specific CTLs in mice.

Materials and Methods

Preparation of virosomes

Virosomes were prepared from β-propiolactone inactivated egg-derived influenza A/PR/8/34 H1N1 virus as described previously (13). In brief, whole inactivated influenza virus (WIV) was disrupted by the addition of 100 mM 1,2-dihexanoyl-*sn*-phosphatidylcholine (DHPC, Avanti Polar Lipids) in HNE (5 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.3) buffer. Nucleocapsid was removed from the membrane lipids and surface proteins by ultracentrifugation. Virosomes were reconstituted by removal of the detergent by dialysis against HNE buffer. Subsequently, virosomes were purified by centrifugation on a discontinuous sucrose gradient (10%/60% w/v in HNE), and sucrose was removed by dialysis against HNE buffer. Peptide-loaded virosomes were obtained by adding 125 μg/mL $M1_{58\text{-}66}$ peptide (GILGFVFTL, kindly provided by the Netherlands Cancer Institute) to the virosomes (36:1 peptide:protein w/w ratio) prior to detergent removal to enable peptide encapsulation. As negative control, fusion-inactivated virosomes were prepared. Virosomes were fusion-inactivated after peptide-loading by lowering the buffer pH to 4.5 with a pretitrated volume of 1 M HCl, and subsequently incubated at 37°C for 15 min. Afterwards, pH was restored to pH 7.3 with a pre-titrated volume of 1 M NaOH.

Characterization of virosome formulations

Protein composition of the peptide-loaded virosomes was determined by SDS-PAGE, by using a 12% precast polyacrylamide gel (Thermo Scientific) and Coomassie Brilliant Blue (Thermo Scientific) staining. Mean particle size distribution and zeta potential were determined by dynamic light scattering (DLS) with a Malvern Nano ZS (Malvern Instruments). Samples were diluted six fold in MilliQ for zeta potential analysis.

Hemolysis assay

Virosome fusion activity was determined by using a hemolysis assay as described previously (14). Vaccine preparations were mixed with human blood erythrocytes and 0.1M 2-(N-morpholino)ethanesulfonic acid (MES) buffer with different pH ranging from 4.5 to 5.5, and incubated at 37°C. After allowing fusion for 30 min, the released hemoglobin was quantified in the supernatant after centrifugation by reading absorbance at 540 nm using a Synergy Mx reader (Biotek). Hemoglobin release of erythrocytes mixed with water was set as maximal hemolysis (100%).

Association of peptide to virosomes

The interaction between peptides and virosomes was studied using size-exclusion chromatography (SEC). $M1_{58\text{-}66}$ peptide was labeled with fluorescein for detection purposes. Peptide-loaded virosomes or peptide mixed with empty virosomes were applied on a pre-washed PD-10 column (GE Healthcare). Samples were eluted with HNE buffer, and fractions of 1 mL were collected in tubes and subsequently analyzed for peptide and protein content

by using fluorescence (excitation at 480 nm and emission at 530 nm) and Lowry protein assay, respectively.

Immunizations

Animal experiments were performed according to the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee on Animal Experimentation of the Antonie van Leeuwenhoek terrain (DEC-ALt) under registration numbers PO201200156 and PO201300046. 8-12 week old female transgenic C57BL/6-Tg(HLA-A2.1)1Enge/J mice (Jackson Laboratory, maintained in-house) were used in all studies. Mice received subcutaneous injections (100 µl/dose) in the left hind flank at days 0 and 21. Immunizations consisted of either PBS, peptide mixed with CpG, peptide in 50% Incomplete Freund's Adjuvant (IFA, Sigma-Aldrich) with CpG, peptide-loaded virosomes (mixed with and without CpG), inactivated peptide-loaded virosomes mixed with CpG, and free peptide mixed with empty virosomes and CpG. Mice received 1 µg of $M1_{58-66}$ peptide per dose and 180 µg of virosomal protein. When mentioned, 50 µg of CpG-ODN 1826 (Invivogen) per dose was used as an adjuvant. As a positive control, one group of mice was infected with $1*10^3$ PFU of influenza HK-X31 H3N2 virus in 50 µl PBS by intranasal administration under isoflurane anesthesia. On day 35 animals were sacrificed by bleeding and cervical dislocation under isoflurane anesthesia.

Challenge study

For the challenge study, six mice were immunized as described previously. Additionally, one group of mice was immunized twice with 180 µg (total protein) of influenza A/PR/8/34 WIV vaccine on the same immunization schedule as previous groups. On day 35, mice were infected with a sublethal dose of $1*10^5$ PFU of influenza HK-X31 virus in 50 µl PBS by intranasal administration. Subsequently, mice were weighed daily until day 14 after challenge, after which mice were sacrificed.

Intracellular staining

Peptide-specific cytotoxic T cells were quantified by flow cytometry analysis. Single cell suspensions of excised spleens were obtained using 70 µm cell strainers (BD Falcon). Subsequently, $2*10^6$ splenocytes were plated per well in 48-wells culture plates (Greiner) and restimulated with 50 ng $M1_{58-66}$ peptide for 18 hrs at 37°C 5% CO_2. Next, Golgi-plug (1:1000, BD) was added to the cells to inhibit cellular protein and cytokine transport, and cells were incubated for another 4 hrs. Subsequently, cells were transferred to a 96-wells plate, washed twice with FACS buffer (PBS, 0.5% BSA), and stained with anti-mouse CD4-PE (BD Biosciences), anti-mouse CD8-FITC (BD Biosciences) and Live/dead-Aqua (Invitrogen). Next, cells were washed twice with FACS buffer, and fixed with fixation-permeabilization buffer (BD Biosciences). Subsequently, cells were washed with permeabilization wash buffer (BD Biosciences), and intracellular staining was performed with IFN-γ-APC (Biolegend). Finally, cells were washed with FACS buffer and 1.5 to 2 million cells were measured on a FACS Canto II flow cytometer (BD Biosciences). Data was analyzed using FlowJo software version 9 (Tree

Star) for Mac OSX.

Enzyme linked immunosorbent spot assay (ELISpot)

Cytokines produced by spleen cells were determined by ELISpot. 96-wells Multiscreen PVDF filter plates (Millipore) were activated by adding 25 µL 70% ethanol for 2 min, and subsequently washed three times with PBS. Plates were coated overnight (O/N) with anti-mouse IFN-γ antibodies (U-Cytech) at 4°C. Next, filter plates were washed three times and blocked with 5% Hyclone fetal calf serum (FCS, Thermo Scientific) for 1 hour at 37°C. Subsequently, $4*10^5$ isolated spleen cells in IMDM medium, 5% FCS were added to each well with or without 50 ng $M1_{58-66}$ peptide, and incubated O/N at 37°C. After O/N stimulation, filter plates were washed five times and IFN-γ was detected using biotinylated anti-mouse antibodies (U-Cytech) and 100 µL BCIP/NBT reagent (Thermo Scientific) per well. Spots were allowed to develop for 15 minutes after which the plates were thoroughly washed with tap water. Spots were counted using an A.EL.VIS ELISpot reader (A.el.vis). The number of IFN-γ producing cells in antigen stimulated spleen cells was obtained after background correction (subtracting the number of spots produced by splenocytes incubated with medium).

Hemagglutination inhibition assay

Hemagglutination inhibition (HI) titers in mouse sera were determined by an HI assay. Sera were treated O/N with diluted receptor-destroying enzyme from *Vibrio cholerae* (1:5, Sigma-Aldrich) at 37°C to remove non-specific inhibitors, and were subsequently inactivated at 56°C for 30 min. Finally, PBS was added to the sera to obtain a 1:10 dilution. Diluted sera were serially diluted two-fold with PBS. Four hemagglutinating units of inactivated influenza A/PR/8/34 or influenza HK-X31 were subsequently added to each well and incubated for 20 min at room temperature after mixing. Next, an equal amount of a 0.5% (v/v) turkey erythrocyte suspension was added to the wells and incubated for 45 min at room temperature. HI titers are reported as the reciprocal of the highest serum dilution capable to completely prevent hemagglutination.

Enzyme linked immunosorbent assay (ELISA)

Influenza antigen-specific antibody titers were determined by ELISA as described previously (15). In short, Microlon 96-wells flatbottom plates (Greiner) were coated O/N with 600 ng (HA) of A/PR/8/34 subunit per well at 4°C. Serial two-fold dilutions of individual mouse sera in PBS, 0.5% BSA, 0.1% Tween80 were applied on the plate and incubated for 1 hour at 37°C. Subsequently, plates were incubated for 1 hour at 37 °C with horseradish peroxidase-conjugated goat antibodies against mouse IgG, IgG1 or IgG2c (1:5000, Southern Biotech). Detection of antibodies was performed with TMB substrate buffer (0.4 mM TMB in 0.11 M sodium acetate, 0.006% H_2O_2, pH 5.5). The reaction was stopped by adding 2 M sulfuric acid, after which the optical density (OD) was measured at a wavelength of 450 nm by using a Synergy Mx platereader (BioTek). Titers are reported as the reciprocal of the serum dilution corresponding to OD_{450}=0.2 after background correction.

Dendritic cell uptake studies

Peptide antigen uptake by DCs was determined as follows. Fresh blood was collected from healthy donors and collected in heparin-coated tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using a Lymphoprep (Axis-Shield) gradient. Subsequently, $CD14^+$ monocytes were isolated from the PBMC fraction by labeling with human CD14 MicroBeads (Miltenyi Biotec) and subsequent separation with a magnetic LS MACS column (Miltenyi Biotec). Finally, monocytes were plated at a concentration of $0.4*10^6$ cells/mL in 48-wells plates in IMDM medium (Invitrogen) containing 1% FCS, 500 U/mL GM-CSF (Peprotech) and 800 U/mL IL-4 (Sanquin). After 6 days, vaccine formulations were incubated for 4, 24, and 48 hours with the immature DCs at a concentration of 50 ng/mL $M1_{58\text{-}66}$-FITC peptide in IMDM with 500 U/mL GM-CSF. Subsequently, cells were transferred to a 96-wells plate, washed twice with FACS buffer, and stained with Live/dead-Aqua. Cells were washed twice with FACS buffer and analyzed on a FACS Canto II flow cytometer. Data was analyzed by using FlowJo 9 software for Mac OSX. Peptide uptake is reported as mean fluorescent intensity (MFI) of the FITC signal.

Statistics

Data were analyzed by using one-way ANOVA with Tukey-Kramer's method for multiple comparisons. Probability (p) values of $p \leq 0.05$ were considered statistically significant. Statistics were performed by using GraphPad Prism Software version 6.03 for Windows.

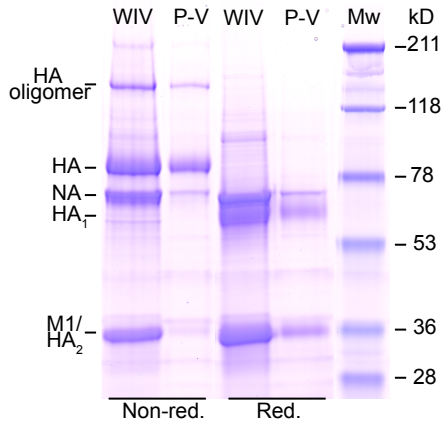

Figure 1. Protein profile of peptide-loaded virosomes
SDS-PAGE analysis of peptide-loaded virosomes (P-V) and source material whole inactivated influenza virus (WIV) under non-reduced and reduced conditions on a 12% precast gel stained with Coomassie Brilliant Blue. Protein identities were confirmed by mass spectrometry.

Results

Characteristics of peptide-loaded virosomes

To confirm that virosome production was successful, the protein composition of peptide-loaded virosomes was analyzed by SDS-PAGE. We observed that peptide-loaded virosomes (P-V) retained HA and NA proteins, whereas internal proteins, such as matrix protein 1 (M1), were removed from the virosome particles (**Figure 1**). Additionally, SDS-PAGE analysis of P-V under reducing conditions revealed that subunits HA_1 and HA_2 from HA mono- and dimers were formed, similar to WIV. Dynamic light scattering showed a particle size of 140 ± 2 nm (mean ± SD, n=3) for P-V, which was comparable to the size of source material WIV (143 ± 1 nm). The polydispersity index (PDI) of P-V was 0.121, indicating that the particle distribution was very homogeneous and comparable to the PDI of WIV (0.036). The zeta potential of virosomes was -21.2 ± 1.7 mV (mean ± SD, n=3), which was similar to that of WIV (-21.5 ± 0.3 mV). Therefore, the peptide-loaded virosomes closely resembled WIV particles in terms of particle size and surface charge, but were enriched in HA and NA.

Size-exclusion chromatography confirmed that simple mixing of peptide and virosomes did not result in substantial association between the two components (**Figure 2A**). As expected, both peptide and virosomes co-eluted when peptide-loaded virosomes were applied onto

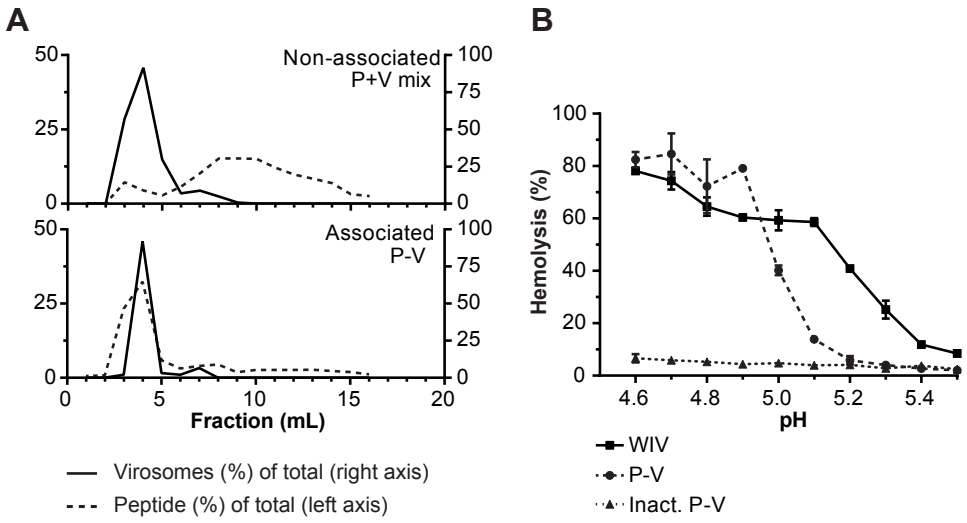

Figure 2. Characteristics of peptide-loaded virosomes
Peptide association of peptide mixed with virosomes (P+V mix) and peptide-loaded virosomes (P-V) analyzed by size exclusion chromatography (**A**). Solid lines show the virosome elution pattern (based on protein determination), whereas the broken line shows the elution of peptide (based on fluorescence of $M1_{58-66}$-FITC). The fusogenic activity of WIV (solid), P-V (dashed) and fusion-inactivated P-V (dotted) was determined between pH 4.6 and 5.5 by a hemolysis assay (**B**). Data represents mean ± SD (n = 3).

the SEC column, indicating association. The association efficiency of the peptide with the virosomes was typically 4-6% of the total amount of added peptide.

In order to confirm whether P-V still possessed fusion activity, a hemolysis assay was performed. Both peptide-loaded virosomes and WIV showed low-pH induced fusion activity in the pH range that is representative for the endosome (**Figure 2B**). Additionally, P-V were shown to be successfully fusion-inactivated by short (15 min) exposure to pH 4.5. P-V fused at slightly lower pH compared to WIV, which might be caused by small differences in HA confirmation and stability in peptide-loaded virosomes.

Immunogenicity of peptide-loaded virosomes in HLA-A2 transgenic mice

To assess whether the produced peptide-loaded virosomes were able to induce peptide-

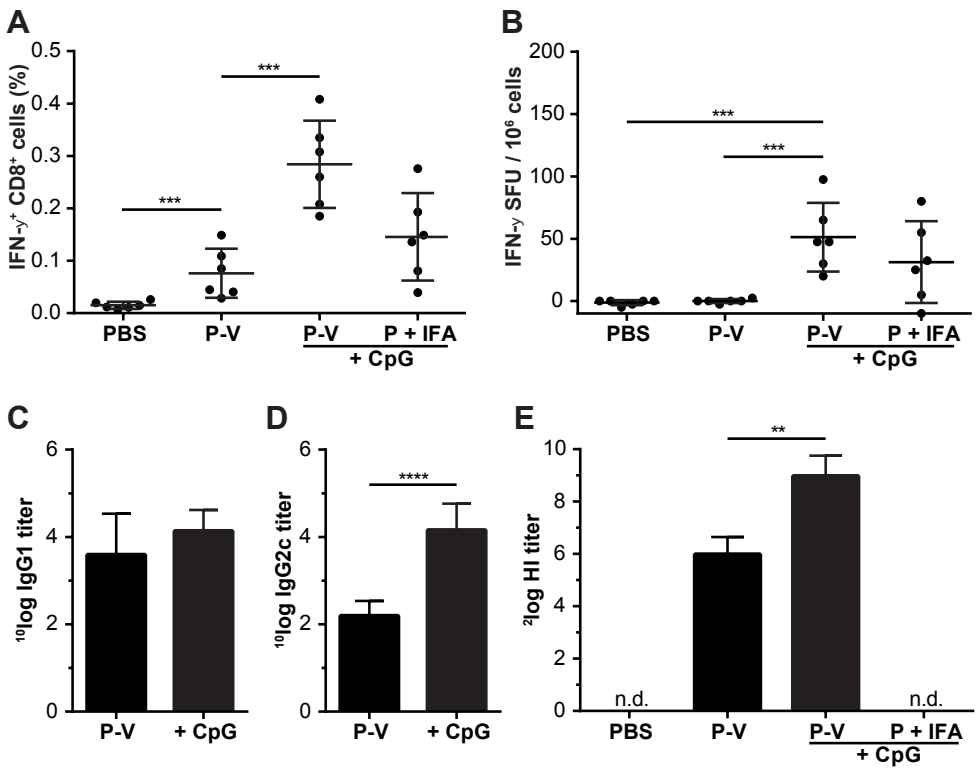

Figure 3. Immunogenicity of (non-)adjuvanted peptide-loaded virosomes

HLA-A2 transgenic mice were immunized twice with 1 µg of $M1_{58-66}$ peptide formulated in either virosomes (P-V), virosomes adjuvanted with CpG (P-V + CpG) or Incomplete Freund's Adjuvant (IFA) with CpG (P + IFA + CpG). Mice were immunized with PBS as negative control. Two weeks after the final vaccination, peptide-specific $CD8^+$ T cell responses in ex vivo stimulated splenocytes were determined using flow cytometry (**A**) and ELISpot (**B**). Antibody isotypes IgG1 (**C**) and IgG2c (**D**) titers were determined in mice sera. HI titers were also determined (**E**). Data represents mean ± SD (n = 6); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; n.d., not detectable.

specific T cell responses, HLA-A2 transgenic mice were primed and boosted three weeks after priming with either peptide-loaded virosomes or P-V adjuvanted with CpG. PBS and peptide mixed with IFA and CpG were administered as negative and positive control, respectively. Peptide-specific T cell responses in restimulated splenocytes were determined two weeks after booster vaccination. Flow cytometry analysis revealed that splenocytes from mice immunized with peptide-loaded virosomes contained peptide–specific IFN-γ$^+$ CD8$^+$ T cells after ex vivo stimulation (**Figure 3A**), as opposed to PBS injected mice. P-V were able to induce specific IFN-γ$^+$ CD8$^+$ T cell levels comparable to the levels induced by P mixed with IFA and CpG. The addition of CpG to P-V significantly ($p < 0.001$) increased the number of peptide-specific CD8$^+$ T cells, which confirmed the immunopotentiating effect of CpG. The increase of IFN-γ$^+$ CD8$^+$ T cells after adding CpG to the P-V formulation was in line with the increased frequency of IFN-γ producing cells observed utilizing an ELISpot assay (**Figure 3B**). Several other TLR ligands (poly(I:C), imiquimod and Pam3CSK4) were tested in combination with P-V, but none were as effective as CpG (data not shown).

While virosomes mainly act as an efficient vehicle to deliver the peptide antigen to the APCs, the presence of CD4$^+$ T cell epitopes in the sequence of virosomal surface proteins enables virosomes to provide helper T cell (Th) responses. Th responses are able to support the induction of CD8$^+$ T cells (16), and CD4$^+$ T cell epitopes are thus an important part of the vaccine formulation. To gain further insight into the possible T cell help that virosomes and the adjuvant provide, the Th1/Th2 balance was assessed by determination of IgG1 and IgG2c isotype titers induced by P-V with or without CpG. The results show that IgG1 titers remained unchanged (**Figure 3C**) after addition of CpG, but IgG2c titers were significantly ($p < 0.0001$) increased after vaccination with CpG adjuvanted P-V when compared to P-V alone (**Figure 3D**).

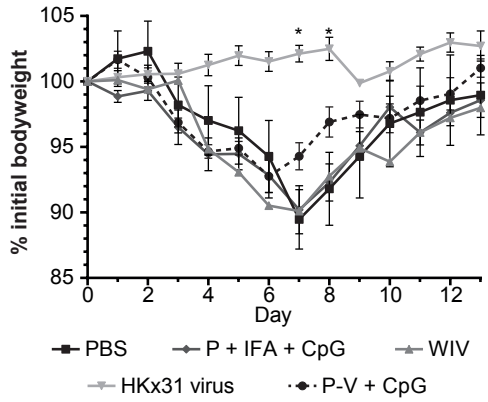

Figure 4. Efficacy of vaccine after sublethal heterologous influenza infection
HLA-A2 transgenic mice were immunized twice with either peptide-loaded virosomes with CpG, peptide mixed with incomplete Freund's adjuvant and CpG, WIV or PBS . As a positive control, mice were challenged with a sublethal dose of HK-X31 virus. Subsequently, mice were infected with heterologous HK-X31 (H3N2) influenza virus and their weight was monitored for 14 days. Data represents mean ± SEM ($n = 6$); *$p < 0.0001$ for PBS and P + IFA + CpG groups versus HK-X31.

This indicates that addition of CpG to P-V skewed influenza-specific responses towards a Th1 response, which supports the $CD8^+$ T cell response against the influenza peptide.

In addition to the T cell epitopes, the P-V formulations contain virosomal B cell epitopes (mainly on surface antigens HA and NA) that induce influenza-specific antibodies. While these antibodies are usually not cross-reactive, they do provide protection against homologous influenza strains. Thus, in case the circulating virus matches the source influenza strain of the virosomes, additional humoral responses can aid in protection. CpG adjuvanted P-V induced significantly ($p < 0.01$) higher HI titers compared to non-adjuvanted P-V (**Figure 3E**). Total IgG titers showed a similar significant ($p < 0.05$) increase after addition of CpG to the P-V

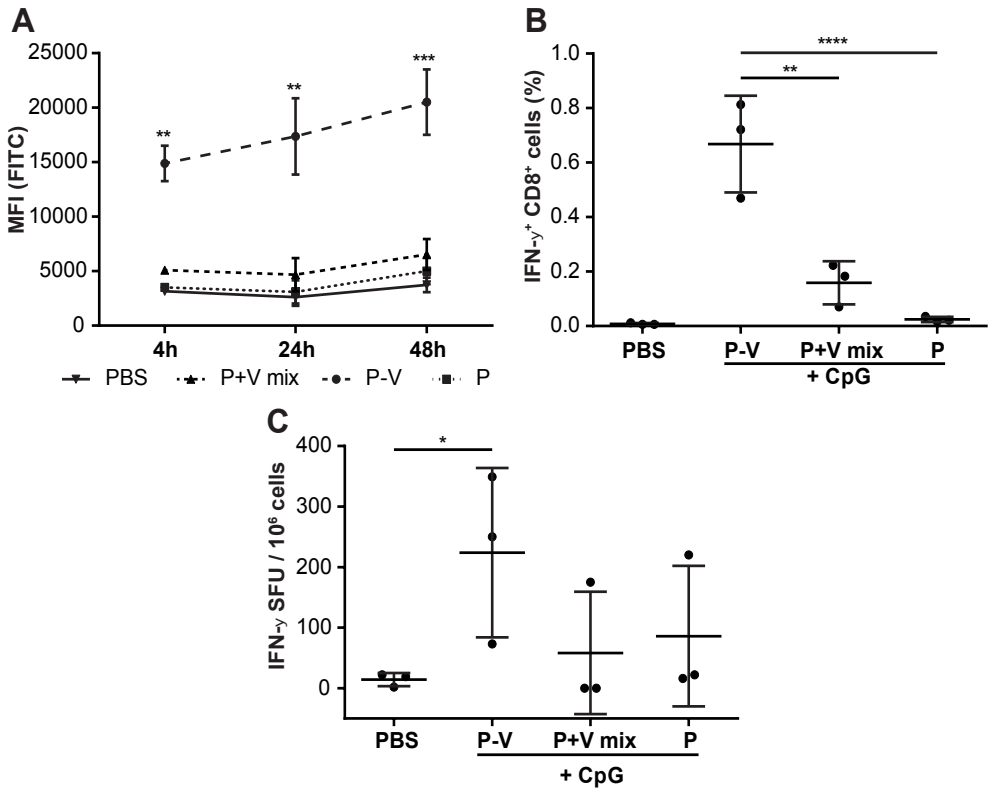

Figure 5. Influence of peptide-virosome association on in vitro dendritic cell uptake and in vivo T cell responses

$M1_{58-66}$-FITC peptide uptake by human immature DCs was determined by flow cytometry (**A**). Either PBS, peptide-loaded virosomes (P-V), peptide mixed with empty virosomes (P+V mix) or free peptide (P) were incubated at 37°C with immature DCs for 4, 24 and 48 hours. Data represents mean ± SD (n = 3) performed with DCs obtained from three different donors. HLA-A2 transgenic mice were immunized twice with aforementioned formulations adjuvanted with CpG. Peptide-specific $CD8^+$ T cell responses were determined in ex vivo stimulated splenocytes by flow cytometry (**B**) and ELISpot (**C**). Data represents mean ± SD (n = 3) and is representative of three individual experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

(**Figure S1**). This underlines that CpG is not only an effective adjuvant for T cell induction, but also improves B cell responses, as observed previously (12). As expected, there were no detectable HI and total IgG titers in sera of control mice receiving PBS or peptide mixed with IFA and CpG, due to the lack of influenza surface antigens in these formulations.

Efficacy of peptide-loaded virosomes against heterologous influenza infection in mice

In addition to the assessment of immunological responses, the efficacy of the P-V vaccine against heterologous influenza infection was examined. HLA-A2 transgenic mice were immunized with the vaccine and subsequently infected with a sublethal dose of influenza HK-X31 (H3N2) virus. The weight of the infected animals was monitored for 14 days (**Figure 4**). Mice that were previously infected with a small dose of live influenza HK-X31 did not show any weight loss after challenge. Mice that received either PBS, WIV, or peptide mixed with IFA and CpG showed a decline in weight until day 7, after which the animals recovered slowly. In contrast, mice immunized with CpG adjuvanted P-V started to recover already after day 6, and their total weight loss was less severe than that of mice immunized with PBS or peptide mixed with IFA and CpG. Moreover, at day 7 and 8, the weight of mice immunized with P-V adjuvanted with CpG was not statistically different than the weight of protected mice pre-exposed to HK-X31, whereas mice immunized with PBS, WIV, or peptide with IFA and CpG did show a significant ($p < 0.0001$) difference with protected mice. Furthermore, no HK-X31-specific HI titers were detected in sera of mice immunized with the PR8-based P-V or WIV groups (**Figure S1**). Since WIV contains all influenza antigens and does not show any effect against HK-X31 infection, it can be assumed that virosomes alone (which only contain HA and NA proteins) would not mediate any response against HK-X31. Thus, this indicates that the increased recovery was not mediated by cross-reactive neutralizing antibodies but by the increase of $CD8^+$ T cells. Vaccination with P-V mixed with CpG may therefore contribute to the recovery from influenza after heterologous influenza infection.

Influence of association between peptide and virosomes on peptide association with dendritic cells and $CD8^+$ T cell responses

In order to gain some mechanistic insight into the mode of action of our P-V vaccine, we investigated the importance of peptide association with the virosomes for the induction of peptide-specific CTLs. The delivery concept of virosomes and the importance of peptide association was first assessed in vitro. The uptake of peptide antigens by immature DCs was determined in vitro for P-V and free peptide mixed with empty virosomes (P+V, **Figure 5A**). After 4 hours of incubation, P-V showed a significantly ($p < 0.01$) higher peptide association with DCs compared to P+V or free peptide without any carrier. This trend was also observed after 24 and 48 hours of incubation, resulting in even larger ($p < 0.001$) differences between P-V and the other formulations. Incubation of P-V with DCs at a temperature of 4°C also showed a significant increase of peptide signal (data not shown), indicating that P-V readily associated with the cell membrane of DCs prior to internalization. This indicates that the

virosomes attached themselves to the DC surface most probably by sialic acid binding. Thus, association of the peptide with the virosomes positively affects the antigen association with DCs, proving that virosomes act as a delivery system for the peptide antigen.

In addition to the in vitro DC studies, in vivo studies in HLA-A2 transgenic mice revealed that association of the peptide with the virosomes is crucial for the induction of peptide-specific CTLs. $CD8^+$ T cell responses were determined in in vitro stimulated splenocytes two weeks after the boost vaccination (**Figure 5B**). P-V + CpG induced significantly ($p < 0.0001$) higher CTL numbers in mice than the peptide + CpG mixture. When CpG adjuvanted peptide was mixed with empty virosomes, the number of peptide-specific CTLs in the spleen was significantly ($p < 0.01$) lower compared to P-V + CpG. The frequency of IFN-γ producing cells showed a similar trend; only P-V + CpG showed increased IFN-γ spot-forming units compared to peptide mixed with empty virosomes or free peptide alone (**Figure 5C**). This suggests that

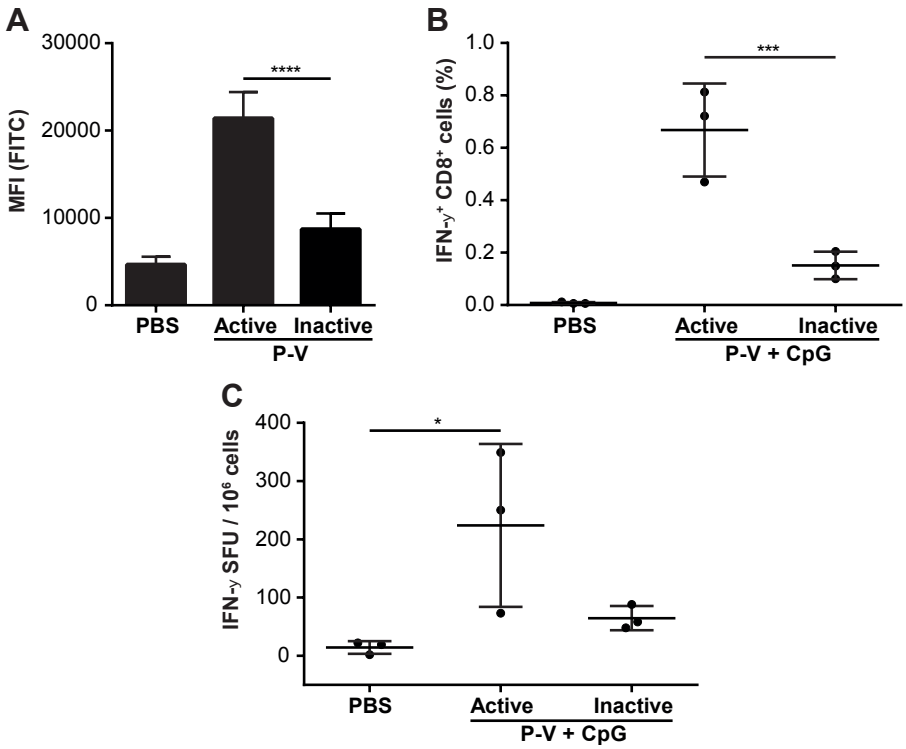

Figure 6. Impact of loss of fusogenic activity on immunogenicity of peptide-loaded virosomes
$M1_{58\text{-}66}$-FITC peptide uptake by human immature DCs was quantified for both fusion-active and inactive P-V after 16 hours of incubation at 37°C (**A**). Data represents mean ± SD (n = 3) performed with DCs obtained from three different donors. HLA-A2 transgenic mice were immunized twice with peptide-loaded virosomes with CpG (Active P-V + CpG) or fusion-inactivated peptide-loaded virosomes with CpG (Inactive P-V + CpG). Peptide-specific $CD8^+$ T cells were subsequently quantified in ex vivo stimulated splenocytes by flow cytometry (**B**) and ELISpot (**C**). Data represents mean ± SD (n = 3) and is representative of three individual experiments. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

addition of the adjuvant alone to the $M1_{58-66}$ peptide is not sufficient to induce peptide-specific T cell responses. Furthermore, association of the peptide with virosome was not critical for the humoral responses or the Th1/Th2 balance (**Figure S2**), suggesting that these particular responses are only influenced by the virosome and the presence of an adjuvant.

Influence of virosomal cell binding and membrane fusion capabilities on the immunogenicity of peptide-loaded virosomes

In addition to the role of peptide association, the role of virosomal cell binding and membrane fusion activity in the immunogenicity of peptide-loaded virosomes was studied. Content of virosomes is released into the cytosol of APCs through pH-mediated fusion with the endosomal membrane. First, peptide association by DCs was quantified by flow cytometry after 16 hours of incubation with (inactivated) vaccine formulations (**Figure 6A**). When P-V were fusion-inactivated, the association of peptide decreased significantly ($p < 0.0001$) compared to fusion-active P-V. This indicates that the inherent cell binding and membrane fusion capabilities of virosomes are needed to ensure efficient uptake by APCs.

Next, we investigated whether the limited uptake of peptide by DCs had an impact on T cell induction after administering inactivated P-V to mice. HLA-A2 transgenic mice immunized with fusion-inactivated P-V generated significantly ($p < 0.001$) less peptide-specific $CD8^+$ T cells than mice immunized with fusogenic P-V (**Figure 6B**). The frequency of IFN-γ producing cells also showed a decreasing trend after inactivation (**Figure 6C**), showing that inactivation of the influenza virosomes has a significant negative impact on the immunogenicity of $M1_{58-66}$ peptide associated with the virosome.

To assess the impact of fusion inactivation on the ability of P-V to induce humoral responses, influenza-specific antibody titers were determined in serum. The fusion-inactivated formulation induced a significantly ($p < 0.0001$) lower influenza-specific neutralizing antibody response than the fusion-active P-V (**Figure S3A**). However, the total serum IgG titers were only slightly but significantly ($p < 0.01$) lower for the group receiving fusion-inactivated P-V (**Figure S3B**). This indicates that HA-specific antibodies were unable to inhibit hemagglutination, which could be the effect of reduced antibody avidity or the generation of antibodies recognizing different epitopes. Furthermore, fusion-inactivation of P-V did not affect IgG1 titers, but did negatively affect IgG2c titers (**Figures S3C and S3D**), which indicates a shift to a Th2 response.

Discussion

Current research on universal influenza vaccines is directed at targeting conserved parts of the influenza virion. Aside from B cell epitopes that can induce broadly-protective neutralizing antibodies, a T cell component is considered to be an important component of future influenza vaccines (17). Influenza-specific CTLs can increase viral clearance and limit morbidity across multiple influenza strains; moreover, recent studies indicate that cellular responses might be a correlate of protection against pandemic influenza strains (5, 18). Inducing potent immune responses against influenza-specific T cell epitopes, however, is challenging due to the nature of the antigen. Subunit (peptide) vaccines generally possess poor immunogenicity due to the lack of any particulate structure or presence of PAMPs. Virosomes have proven to be an efficient delivery system for peptides in previous studies (10, 11, 19, 20), but generally have low peptide association or encapsulation rates. This makes proper dosage of the peptide antigen difficult; if encapsulation rates are low, a disproportionate amount of virosome material is present in the vaccine. Several alternative production methods have been developed that enhanced peptide encapsulation efficiencies. These included chimeric virosomes (11), virosome lyophilization and subsequent reconstitution (19), or covalent attachment of the peptide (20). However, these methods complicate the production process, and might not be suitable for every virosomal peptide formulation. Thus, the addition of an adjuvant to virosomal peptide formulations could be a viable alternative to raise the immunogenicity of the peptide, without increasing peptide and virosome dose or altering the formulation process.

The selected $M1_{58\text{-}66}$ peptide epitope is restricted to the human HLA-A2 haplotype. To produce a vaccine that is effective in a global population, several peptide epitopes covering all the HLA serotypes must be included to ensure acceptable coverage. Since few (animal) models currently exist to screen for the various HLA types, we selected an HLA-A*0201 epitope, which is a common serotype in the Caucasian population and can be tested in HLA-A2 transgenic mice. Thus, in contrast to other preclinical peptide-based vaccine concepts, this concept influenza vaccine could be used directly in humans without changing the peptide antigen.

We demonstrated that the addition of CpG as an adjuvant significantly increased cellular responses in mice immunized with peptide-loaded virosomes. Additionally, CpG skewed antibody responses induced by peptide-loaded virosomes towards the IgG2c isotype. The production of IgG2c antibodies is stimulated during Th1 type responses (21), which supports the induction of $CD8^+$ T cells (16), which in turn is associated with influenza virus clearance (22). Since our current peptide-loaded virosome production process was inefficient, we opted to mix CpG with our formulation, rather than incorporating it in the virosome, which was previously performed with an avian virosome vaccine (12). Incorporating both CpG and peptide antigen in a virosome potentially could increase immunogenicity due to the simultaneous signal delivery of both adjuvant and antigen. This would be a logical next step

for future research.

The interaction between the peptide antigen and the virosome particles was shown to be an important factor for the overall efficacy of the peptide-loaded virosome vaccine, which confirmed an earlier report (10). Additionally, antigen uptake studies with DCs revealed that association of the antigen with the carrier is important for antigen uptake by APCs. While SEC analysis showed that the peptide was indeed associated with the virosomes, the exact localization of the peptide, e.g., in the aqueous inner compartment or the lipid membrane, remains unknown. The localization of peptide antigens in virosomes could have an impact on presentation on MHC class I molecules by APCs (23), which in turn can affect the quality of the immune response, and is therefore a relevant topic for future studies.

Hemagglutinin conformational integrity and activity, mediating virosomal cell binding and membrane fusion, were shown to be crucial for the induction of $CD8^+$ T cell responses. In addition to an earlier report which indicated that fusion activity might affect CTL responses induced by $NP_{147\text{-}155}$ peptide-loaded virosomes (10), we demonstrated that virosome fusion inactivation had a profound impact on the capacity of virosomes to deliver peptide to DCs, and on the induction of peptide-specific T cell responses by peptide-loaded virosomes. The role of fusion activity is not only important for binding and cell entry of virosomes, but also for CTL induction by WIV vaccines (24, 25). Furthermore, fusion-inactivation impaired the induction of influenza-specific IgG2c antibodies, which could affect helper T cell responses. In addition, fusion-inactivation impaired the neutralizing ability of influenza-specific antibodies generated after vaccination with peptide-loaded virosomes significantly, while total influenza-specific IgG levels only were reduced slightly. Thus, inactivation of the fusion capacity not only reduces peptide-specific T cell responses, but also severely impairs supporting helper T cell and humoral responses induced by the virosomal vaccine.

To assess the efficacy of the vaccine, mice were immunized with CpG-adjuvanted P-V and subsequently challenged with a sublethal heterologous HK-X31 influenza infection. The mouse weight loss data shown in this study indicate that the elevated numbers of influenza-specific CTLs after vaccination contributed to the recovery of the mice after heterologous influenza infection, independent of neutralizing antibodies. An increase of circulating $CD8^+$ T cells might however not be enough to provide complete protection against influenza infections. A boost in $CD8^+$ T cells may help clear the virus and improve the rate of recovery of the mice after infection, but is arguably insufficient to prevent the early onset and spread of infection. Slütter et al. recently showed the importance of local memory $CD8^+$ T cells in the upper respiratory tract to combat influenza A infections in the early stages (26). This insight implicates that the local induction of respiratory $CD8^+$ T cells could be an important goal for further T cell based influenza vaccine development. The increased systemic T cell levels reported in clinical studies might be an indication that elevated local influenza-specific $CD8^+$ T cells in the lungs can provide accelerated recovery and decreased morbidity in influenza-

infected patients (5, 27).

Aside from $CD8^+$ T cell responses, it has been established that T cell help (in the form of $CD4^+$ T cells) and B cell responses should not be overlooked (28), and therefore a vaccine concept that utilizes both T cell and B cell responses should be pursued to obtain a universal influenza vaccine (29). Influenza virosomes could be an excellent candidate platform for a cross-protective influenza vaccine, as it is an effective peptide delivery system and a natural carrier of $CD4^+$ T cell and B cell epitopes.

In conclusion, we demonstrated that peptide-loaded virosomes are able to induce peptide-specific $CD8^+$ T cells. The addition of CpG as an adjuvant further increased the efficacy of peptide-loaded virosomes. Aside from a greater number of peptide-specific $CD8^+$ T cells, CpG adjuvanted P-V also induced T cell help and influenza-specific antibodies. Peptide-virosome association and virosome fusion activity are important factors for the effectiveness of P-V. The synergistic effect of virosome particles, fusion activity and CpG make a potent combination to increase the immunogenicity of peptide antigens. Thus, peptide-loaded virosomes are a promising approach for the development of a cross-protective influenza vaccine.

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Supplemental data

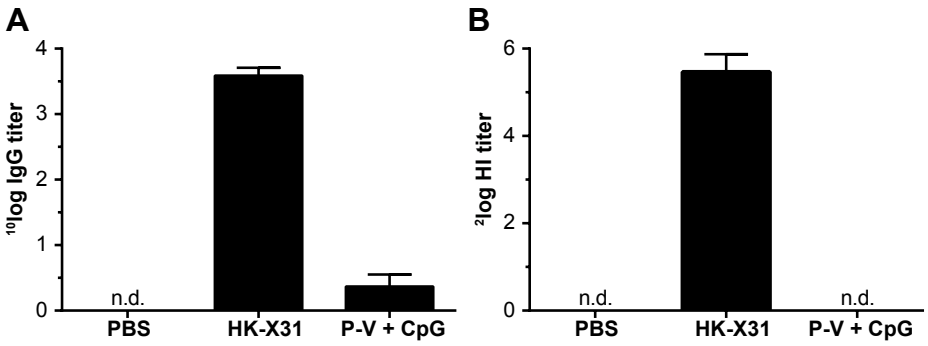

Figure S1. Total influenza-specific IgG titers after immunization
Influenza-specific total IgG titers in sera from mice. Data represents mean ± SD (n = 6). *p < 0.05; n.d., not detectable.

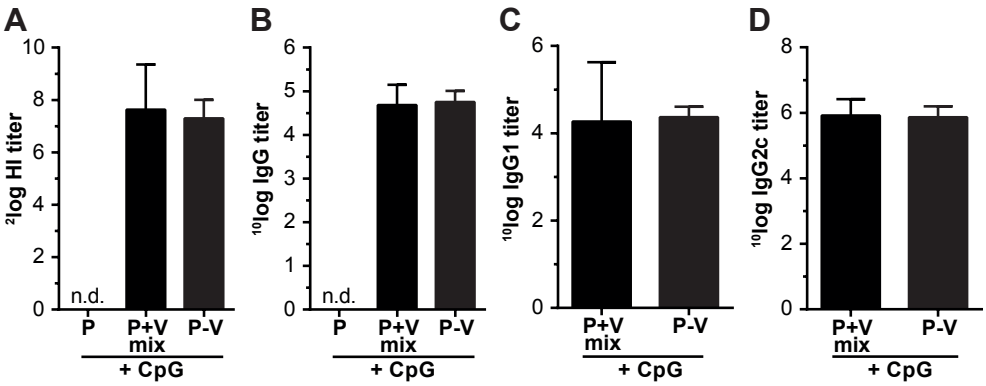

Figure S2. Effect of association between peptide and virosomes on humoral responses
Mice sera were analyzed for HI (**A**) and total IgG (**B**) titers. IgG1 (**C**) and IgG2c (**D**) isotypes were also determined from sera. Data represents mean ± SD (n = 3) and is representative of three individual experiments. n.d., not detectable.

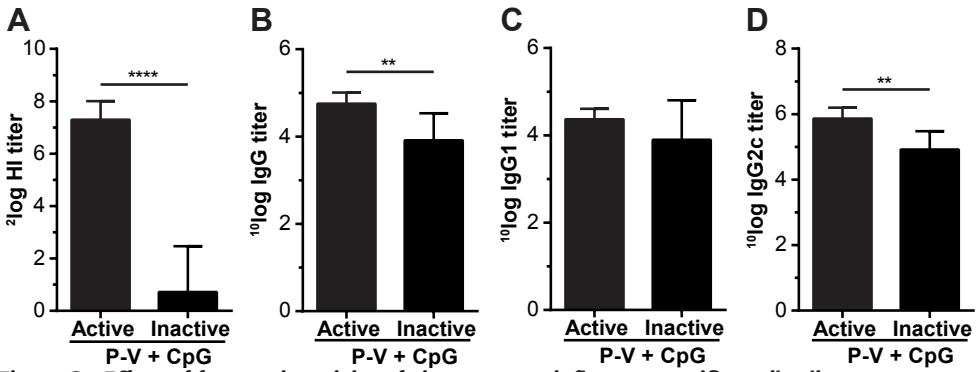

Figure S3. Effect of fusogenic activity of virosomes on influenza-specific antibodies.
Influenza-specific HI titers (**A**) and total IgG titers (**B**), and antibody isotypes IgG1 (**C**) and IgG2c (**D**) titers in sera from mice. Data represents mean ± SD (n = 3) and is representative of three individual experiments. **p < 0.01, ****p < 0.0001.

Chapter 7

Whole-inactivated influenza virus as an adjuvant for influenza peptide antigens

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Abstract

Influenza peptide antigens have the potential to induce cross-protective influenza-specific T cells. However, short peptide antigens are poorly immunogenic and therefore need to be formulated with a potent adjuvant. In this study, it was investigated whether whole inactivated influenza virus (WIV) can act as an adjuvant for influenza peptide antigens. The immunogenicity of WIV mixed with the HLA-A*0201 restricted influenza peptide GILGFVFTL (GIL) was assessed in HLA-A2 transgenic mice by quantification of peptide-specific IFN-γ^+ $CD8^+$ T cells after vaccination. Subsequently, a Design of Experiments (DoE) approach was utilized to study the synergistic effect between WIV adjuvant and peptide antigen at different doses. Moreover, the roles of WIV fusogenicity, peptide-WIV association and co-localization on the adjuvanticity of WIV were investigated. To assess whether WIV could also act as an adjuvant for other peptides, a peptide pool with three wild type (WT) influenza peptides was adjuvanted with WIV. In addition, three chemically enhanced peptide ligands (CPLs) derived from the WT peptides, which possessed a higher binding affinity to the MHC molecules, were adjuvanted with WIV and screened for their immunogenicity compared to the WT peptides. WIV was found to be a potent adjuvant for the GIL peptide. The DoE study revealed that WIV was able to act as an adjuvant at even low concentrations. Co-localization of the peptide antigen and WIV adjuvant was important for the induction of a peptide-specific immune response, whereas peptide-WIV association and WIV fusogenicity were not. WIV was also able to act as an adjuvant for both WT and CPL peptide pools. This study shows the potential of WIV as an adjuvant for influenza peptides. The simple formulation process and the existing safety track record of WIV make this an attractive adjuvant, which could also be used for non-influenza antigens.

Introduction

Seasonal influenza vaccines mediate their protective effect mainly through the induction of virus-specific neutralizing antibodies. These antibodies are directed against the influenza surface proteins hemagglutinin (HA) and neuraminidase (NA). However, these proteins can undergo changes due to antigenic shifts and drifts. These antigenic changes impair the neutralizing ability of antibodies induced by vaccines, rendering these vaccines ineffective. Therefore, additional immune responses such as cellular responses against influenza need to be induced to increase vaccine effectiveness (1).

Cellular immune responses represent a potential alternative to antibody-mediated immune responses. Recently, Sridhar et al. found that cellular immune responses correlated with reduced morbidity in patients infected with pandemic influenza (2). Similarly, Wang et al. showed that patients with early influenza-specific $CD8^+$ T cell responses recovered faster from severe H7N9-induced disease (3). These studies confirmed that cellular responses against influenza can indeed be effective. Cellular immune responses such as cytotoxic T cells (CTLs) can effectively clear virus-infected host cells, thereby inhibiting viral replication and spread. Unlike most vaccine-induced antibodies, these CTLs recognize epitopes located on internal influenza proteins, which are conserved in many influenza strains. Owing to the conserved nature of these epitopes, cellular responses directed against these epitopes are potentially cross-reactive. Short linear peptides representing these epitopes are therefore attractive antigens for the development of cross-reactive influenza vaccines.

Peptide antigens as such, however, suffer from low immunogenicity due to inefficient delivery to antigen presenting cells (APCs) and the absence of pathogen-associated molecular patterns (PAMPs) or adjuvants to activate the APCs. Delivery of the peptide antigen to the cytoplasm of APCs is considered to be crucial for proper processing and subsequent presentation on MHC-I molecules, while activation of the APCs is important for licensing of naïve effector and memory $CD8^+$ T cells (4).

Formulation of peptide antigens with an appropriate adjuvant (which can be a delivery system or an immunopotentiator (5)) is thus crucial to induce a cellular immune response against the peptide antigen. Water-in-oil emulsions such as Incomplete Freund's Adjuvant (IFA) are commonly used and effective adjuvants for peptides, but are associated with severe adverse events such as lesion formation at the site of injection (6). Thus, alternative adjuvants for peptide antigens are highly sought after. Particulate adjuvant systems such as liposomes or virosomes formulated with influenza peptides derived from internal proteins have proven to be effective for the induction of peptide-specific CTLs (7, 8), especially in combination with Toll-like receptor (TLR) agonists (9, 10). However, the formulation of peptides into these delivery systems can be complicated and may result in low encapsulation rates. Adjuvants that can be directly admixed with peptide antigens would therefore be preferable.

Whole inactivated influenza virus (WIV) possesses an innate adjuvant capability in the form of viral single-stranded RNA (ssRNA). Previously it was shown that influenza ssRNA is a potent TLR7 agonist (11), that increases antibody responses and promotes cellular immune responses. Furthermore, WIV contains, aside from $CD8^+$ epitopes, $CD4^+$ epitopes, which provide invaluable T cell help that supports the induction of functional $CD8^+$ T cells (12). We therefore hypothesize that the addition of WIV to peptide antigens could promote the induction of peptide-specific T cell responses.

In addition to proper formulation of the peptide antigen, modification of the peptide could also improve the immunogenicity of the antigen. Previously, chemically enhanced altered peptide ligands (CPLs) derived from HLA-A2*0201-restricted epitopes were shown to possess a higher binding affinity to HLA-A2*0201, and to induce higher amounts of IFN-γ compared to wild type (WT) epitopes in an in vitro system (13). However, like other peptides, these CPLs are currently adjuvanted with IFA. Thus, we investigated whether WIV can act as an adjuvant for these modified peptides.

In the current study, we first investigated the adjuvanticity of WIV for the GILGFVFTL (GIL, $M1_{58-66}$) influenza peptide, an HLA-A2*0201-restricted $CD8^+$ T cell epitope, in a proof-of-principle study. Next, we performed a dose-finding study for the optimal WIV adjuvant and peptide antigen concentration to induce peptide-specific T cells by use of a Design of Experiments (DoE) approach. Furthermore, we studied the effect of WIV-peptide co-localization and WIV membrane fusion activity on the adjuvanticity of WIV. Finally, we tested the adjuvanticity of WIV with three WT T cell peptides and three CPL variants of the WT peptides.

Materials and methods

Formulation of vaccines

Influenza A/PR/8/34 virus was propagated on fertilized eggs and inactivated with β-propiolactone on a pilot scale as described before (14), which yielded PR8 WIV bulk vaccine. To study the effect of fusion activity on the immune response, WIV was fusion-inactivated by lowering the buffer pH to 4.5 with a pretitrated volume of 1 M HCl, and subsequently incubated at 37°C for 15 min. Afterwards, the sample was brought to physiological pH by dialyzing overnight against PBS pH 7.2. Membrane fusion capacity was subsequently determined by a hemolysis assay as described previously (10).

The Netherlands Cancer Institute kindly provided the HLA-A2*0201-restricted influenza GILGFVFTL (GIL, $M1_{58-66}$), FMYSDFHFI (FMY, PA_{46-54}), and NMLSTVLGV (NML, $PB1_{413-421}$) peptides, and CPLs [am-phg]ILGFVFTL (G1), [4-FPHE]MYSDFHF[2-AOC] (F5), and N[NLE]LSTVLGV (N53). Nonproteogenic amino acids introduced in the peptide sequences are shown in **Figure S1**. Influenza PR8 WIV and peptide antigens were formulated in PBS pH 7.2 (Life Technologies) at various concentrations. When mentioned, 50 μg of CpG ODN1826 (Invivogen) or 50% (v/v) Incomplete Freund's Adjuvant (Sigma-Aldrich) was added to the formulation.

Animal studies

Animal studies were conducted according to the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation (DEC) of the National Institute for Public Health and the Environment (RIVM). Eight- to ten-week-old female HLA-A2 transgenic mice (Jackson Laboratory, maintained in-house) were used in all studies.

In the proof-of-principle study, mice (three per group) received immunizations subcutaneously (s.c.) in the flank at days 0 and 21 under isoflurane anesthesia, containing either PBS, 50 μg WIV, 1 μg GIL peptide adjuvanted with 50 μg WIV or 100 μg GIL adjuvanted with 50 μg CpG. For the dose finding study, a DoE approach was used (as described below). The selected formulations consisting of various doses of WIV and GIL peptide (shown in **Table SI**) were administered s.c. in the flank of mice (six per group) at day 0 and 21.

To study the effect of adjuvant co-localization, mice (six per group) were immunized at days 0 and 21 either s.c. in one flank with PBS or 100 μg GIL peptide adjuvanted with 25 μg WIV, or s.c. in separate flanks with 100 μg GIL peptide in one flank and 25 μg WIV adjuvant in the opposite flank. The effect of membrane fusion activity was assessed by immunizing mice (six per group) s.c. in the flank at day 0 and 21 with 100 μg GIL peptide adjuvanted with either 25 μg of fusion-active WIV or fusion-inactive WIV.

The adjuvant effect of WIV on a mix of multiple peptides was assessed with either a WT

peptide pool (GIL, FMY and NML; 100 µg each) or a modified peptide pool (G1, F5 and N53; 100 µg each). Mice (six per group) received an s.c. immunization in the flank at day 0 and 21 containing either PBS, WT peptide pool adjuvanted with 5 µg WIV or IFA, CPL peptide pool adjuvanted with 5 µg WIV or IFA, or only 5 µg WIV. In all studies, animals were sacrificed by cervical dislocation and bleeding under anesthesia at day 35.

Dose finding by design of experiments

An initial dose-response study was performed by a design of experiments approach in order to detect potential interactions and effects between the GIL peptide antigen dose and WIV adjuvant dose on the induction of GIL-specific T cell responses in vivo. A full factorial design was created using MODDE 10.0.0 (Umetrics AB). Results from both flow cytometry and ELISpot methods were selected as response parameters. The limits of the doses ranged from 1-100 µg GIL peptide and 1-25 µg WIV. This resulted in a design with seven formulations including three center points. To accommodate for the high variability in animal experiments, it was chosen to administer each formulation to six mice, resulting in a design as shown in **Table SI**. The models were fitted using partial least squares and subsequently optimized by deleting non-significant terms (15), until the model performance parameters goodness of fit (R^2), goodness of prediction (Q^2), validity, and reproducibility were at their highest.

Intracellular staining and flow cytometry

T cell populations were assessed by flow cytometry. In short, single-cell suspensions of spleens were plated at a concentration of $2*10^6$ cells in a 48-well plate in RPMI medium (Life Technologies) with 10% Hyclone fetal calf serum (FCS, Thermo Scientific), and stimulated overnight with either medium, 50 ng peptide or PR8 WIV. Cytokine transport was inhibited by incubating with Golgi-plug (BD Biosciences) for 4 hours. Cells were subsequently stained with anti-mouse CD8-FITC (BD Biosciences), anti-mouse CD4-PE (BD Biosciences) and Live-dead-Aqua (Invitrogen). Next, cells were fixated with fixation/permeabilization buffer (BD Biosciences) and washed with permeabilization wash buffer (BD Biosciences). Finally, cells were stained intracellular with anti-mouse IFN-γ-APC (BD Biosciences), and IFN-γ^+ $CD8^+$ T cells were quantified on a FACS Canto II flow cytometer (BD Biosciences). Acquired data was analyzed with FlowJo version 10 for Mac OSX (TreeStar Inc.).

Enzyme linked immunosorbent spot assay (ELISpot)

An ELISpot assay was used to determine IFN-γ spot-forming units in restimulated splenocytes. 96-wells Multiscreen PVDF filter plates (Millipore) were activated by adding 25 µL 70% ethanol for 2 min, and subsequently washed three times with PBS. Plates were coated overnight with anti-mouse IFN-γ antibodies (U-Cytech) at 4°C. Next, filter plates were washed three times and blocked with 5% Hyclone FCS (Thermo Scientific) for 1 hour at 37°C. Subsequently, $4*10^5$ isolated splenocytes resuspended in IMDM medium, 5% FCS were added to each well with or without 50 ng relevant peptide, and incubated overnight at 37°C. After overnight stimulation, filter plates were washed five times and IFN-γ was detected using biotinylated anti-mouse

antibodies (U-Cytech) and 100 µL BCIP/NBT reagent (Thermo Scientific) per well. Spots were allowed to develop for 15 min after which the plates were thoroughly washed with tap water. Spots were counted using an A.EL.VIS ELISpot reader (A.el.vis). The number of IFN-γ producing cells in antigen-stimulated splenocytes was obtained after background correction (subtracting the number of spots produced by splenocytes incubated with medium).

Determination of association between peptides and WIV

The association of peptides to WIV particles was studied by quantification of unassociated peptide in a mixture of peptides and WIV. Peptides were admixed with WIV in similar concentrations used in the animal studies. WIV particles were subsequently spun down by ultracentrifugation for 2 hours at 30,000 g. Supernatant was collected and analyzed for peptides by mass spectrometry. Percentage of unassociated peptide was calculated by comparing peptide content in supernatants of peptide mixed with WIV to peptide content in supernatants collected from solutions without WIV. No traces of WIV proteins were detected in supernatants, indicating that WIV was successfully separated from the free peptide.

Hemolysis assay

Virosome fusion activity was determined by using a hemolysis assay as described previously (16). Formulations were mixed with human blood erythrocytes and 0.1M 2-(Nmorpholino) ethanesulfonic acid (MES) buffer with pH's ranging from 4.5 to 5.5, and incubated at 37°C for 30 min. The released hemoglobin was quantified in the supernatant after centrifugation by reading absorbance at 540 nm using a Synergy Mx reader (Biotek). Hemoglobin release from erythrocytes mixed with water was set as maximal hemolysis (100%).

Statistics

Results were statistically analyzed with a one-way ANOVA followed by a Tukey-post test for multiple comparisons. All statistical analyses were performed using GraphPad Prism 6.04 for Windows (GraphPad Software Inc.).

Results and Discussion

Proof-of-principle of WIV as an adjuvant

The adjuvant effect of WIV for GIL peptide was assessed in HLA-A2 transgenic mice. Mice received two vaccinations of either peptide adjuvanted with CpG, peptide adjuvanted with WIV or WIV alone. Splenocytes restimulated with GIL peptide were analyzed for peptide-specific T cells by flow cytometry. The specificity of $CD8^+$ IFN-γ^+ T cells was determined by comparing peptide-stimulated splenocytes with mock-stimulated splenocytes (**Figure 1A**).

As expected, 100 µg GIL peptide adjuvanted solely with 50 µg CpG did not induce any peptide-specific CTL response in mice (**Figure 1B**). This can be attributed to a number of factors, such as the absence of $CD4^+$ helper epitopes, and the lack of delivery of antigen and adjuvant, both of which are crucial for the immunogenicity of short peptide antigens (6). In contrast, only 1 µg GIL peptide antigen adjuvanted with 50 µg WIV induced peptide-specific responses in mice. Mice that received only WIV also showed considerable T cell responses, which was attributed to the high dose of WIV as described earlier (17, 18). The GIL epitope is indeed present in PR8 WIV, which explains the induction of GIL-specific T cells by WIV at high concentrations. Furthermore, WIV might still act as an adjuvant for peptides at lower doses. Thus, in order to maximize peptide-specific T cell responses with minimal use of WIV, a dose-finding study was conducted.

Dose-finding and interaction study between GIL peptide and WIV using design of experiments

To investigate which concentrations of both WIV and peptide were still able to induce a peptide-specific T cell response a dose-finding study of both WIV and peptide was conducted by using a DOE approach. DOE approaches are commonly used for the optimization of (bio)

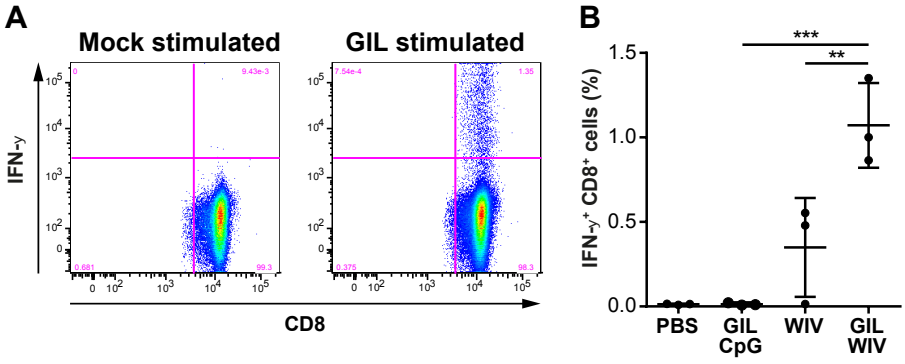

Figure 1. WIV acts as an adjuvant for peptide antigens.
Flow cytometry plot displaying specificity of IFN-γ^+ $CD8^+$ T cells in splenocytes from HLA-A2 transgenic mice immunized twice with 1 µg GIL peptide adjuvanted with 50 µg WIV (**A**). Splenocytes restimulated with GIL peptide were analyzed for IFN-γ^+ $CD8^+$ T cells with flow cytometry (**B**). Data is represented as mean ± SD, n=3; **p<0.01, ***p<0.001.

pharmaceutical formulations (19). However, there are currently no reports that utilized a DoE approach to assess the effect of formulation parameters on in vivo responses, such as cellular immune responses. A full factorial design was implemented by varying the peptide antigen dose from 1-100 µg, and the WIV adjuvant dose from 1-25 µg (**Table SI**).

The formulations, containing a variety of GIL peptide and WIV doses, were tested for their ability to induce GIL-specific T cell responses in HLA-A2 transgenic mice (**Figure 2A** and **2B**). A combination of 1 µg GIL peptide and 1 µg WIV was unable to induce CTL responses. However, when the GIL dose was increased to 100 µg, a significant increase of GIL-specific T cells was observed. This effect was also observed when the peptide dose was increased from 1 to 100 µg combined with a dose of 25 µg WIV. These results indicate that WIV is still able to boost the immune responses towards GIL peptide at concentrations as low as 1 µg WIV when combined with a high dose (100 µg) of peptide antigen.

Association between the GIL peptide and the WIV particles may be a contributing factor to the immunogenicity of peptide antigen. Thus, the association between the peptide and WIV was determined (**Table I**). Only low amounts of GIL peptide (1 µg) mixed with relatively high amounts of WIV (25 or 50 µg) showed some association. At higher peptide concentrations, association with WIV was negligible, which can be explained by the high molar abundance of GIL peptide compared to WIV or a low affinity between the two. In general, it can be concluded that association of the peptide to WIV did not have a significant influence on immunogenicity, contrarily to other delivery systems such as liposomes or virosomes (10, 20).

To assess the synergistic effect between the peptide antigen and the WIV adjuvant, a partial least squared (PLS) regression model was fitted for the T cell responses. Valid models were obtained for both flow cytometry (R^2=0.706, Q^2=0.633) and ELISpot (R^2=0.712, Q^2=0.629)

Table I. Association between GIL peptide and WIV at different concentrations.

Peptide (µg)	WIV (µg)	Unassociated peptide (%)
1	1	112 ± 10
100	1	111 ± 6
50	13	96 ± 5
1	25	77 ± 8
100	25	92 ± 20
1	50	87 ± 9

The fraction of unassociated peptide was determined in the supernatant by mass spectrometry. Data represents mean ± SD, n=3.

responses, and model prediction contour plots were generated (**Figure 2C** and **2D**). The contour plots illustrate that addition of WIV is essential for the peptide antigen to become immunogenic. Furthermore, the model indicates that theoretically the optimum of T cell responses has not been reached yet; however, the dose ranges used in this DoE model for both GIL peptide and WIV are at their maximum concerning peptide solubility and feasible WIV dose for human use, respectively.

The use of the DoE approach enabled us to illustrate the synergy between antigen and adjuvant, and to predict their effect on the cellular immune responses in vivo. The use of DoE in preclinical animal studies is difficult, due to the multiple factors, such as biological variability between animals and T cell assay variability, which can cause variability in each

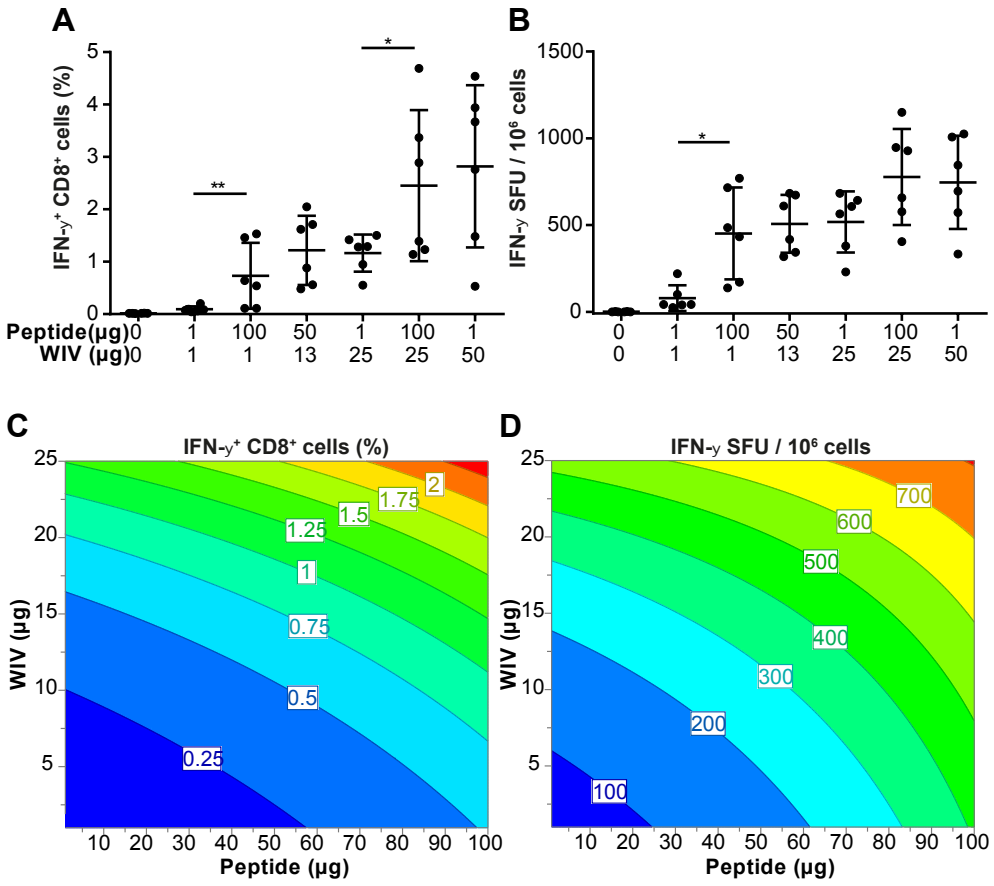

Figure 2. Dose-finding study of WIV and peptide by DoE.
HLA-A2 transgenic mice were vaccinated two times with different doses of GIL peptide and WIV. Splenocytes restimulated with GIL peptide were analyzed for peptide-specific $CD8^+$ T cells with flow cytometry (**A**) and ELISpot (**B**). Prediction contour plots obtained by DoE visualize the interaction between peptide antigen and WIV for the flow cytometry (**C**) and ELISpot (**D**) responses. The predicted responses are displayed in the white boxes. Data is presented as mean ± SD, n=6; *p<0.05, **p<0.01.

animal study. Nonetheless, the use of DoE provides valuable insight in the effect of antigen and adjuvant dose on the immune response in mice, and could be implemented in future vaccine development.

Involvement of WIV-peptide co-localization on immunogenicity

The viral ssRNA present in WIV is a TLR7 agonist, and likely contributes to the observed immunostimulating effect of WIV (11). For most adjuvants, including TLR ligands, co-localization with the antigen is necessary to provide local immunostimulatory signals.

In the model contour plots, it is predicted that a dose of 25 µg WIV combined with 100 µg GIL peptide is able to induce the highest peptide-specific T cell responses. Therefore, 25 µg WIV and 100 µg peptide was selected as the formulation to be used in mechanistic studies. To investigate the importance of co-localization, we administered 100 µg GIL peptide and 25 µg WIV s.c. at separate flanks, each draining to a different lymph node. When the peptide and WIV were administered separately at different sites, a significant decrease of the peptide-specific T cell response was observed (**Figure 3**). The observed response after separate vaccination is probably caused by the WIV only. It is likely that co-localization of WIV and GIL peptide in the endosomal compartment of APCs is required to benefit from the co-stimulatory adjuvant signal provided by the viral ssRNA (21). Moreover, a recent study suggested that particulate delivery of a TLR7 agonist can improve its immunostimulatory effect due to efficient delivery to the endosomal compartment (22), where TLR7 is located. WIV can deliver its own viral ssRNA in a similar manner, which might explain the immunostimulatory potential of WIV.

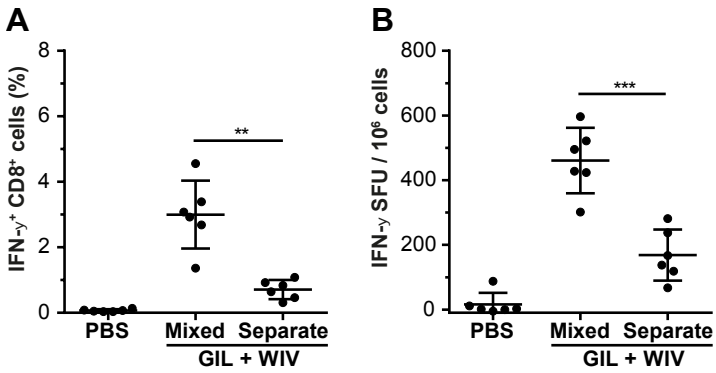

Figure 3. Effect of co-localization on WIV adjuvanticity.
HLA-A2 transgenic mice were vaccinated twice with 100 µg GIL peptide and 25 µg WIV either in one single flank (mixed) or separate flanks (separate). Splenocytes were restimulated with GIL peptide and analyzed with flow cytometry (**A**) or ELISpot (**B**). Data is presented as mean ± SD, n=6; **p<0.01, ***p<0.001.

Involvement of WIV membrane fusion activity on immunogenicity

Aside from co-localization, the role of membrane fusion activity of WIV was investigated. Fusion activity was shown to be important for the induction of cross-reactive T cell responses by WIV (23). Furthermore, other nearby molecules, such as the peptide antigen in our WIV-adjuvanted vaccine, can escape the endosomal compartment during membrane fusion of WIV with the endosomal membrane (24). Fusion activity might thus play a role in the adjuvanticity of WIV. Surprisingly, mice vaccinated with fusion-inactivated WIV mixed with GIL peptide still produced high amounts of peptide-specific T cells, comparable to those in mice receiving fusion-active WIV with peptide (**Figure 4A** and **5B**). A hemolysis assay confirmed the loss of pH-dependent fusion activity of WIV (**Figure 4C**). The current results indicate that fusion activity of WIV is not important for the induction of T cell responses against peptide antigens. The immunogenicity of antigens located inside the WIV particle itself might be compromised by fusion inactivation as shown before (23), but in the current study the admixed GIL peptide apparently was taken up and processed correctly by APCs regardless of WIV fusion activity.

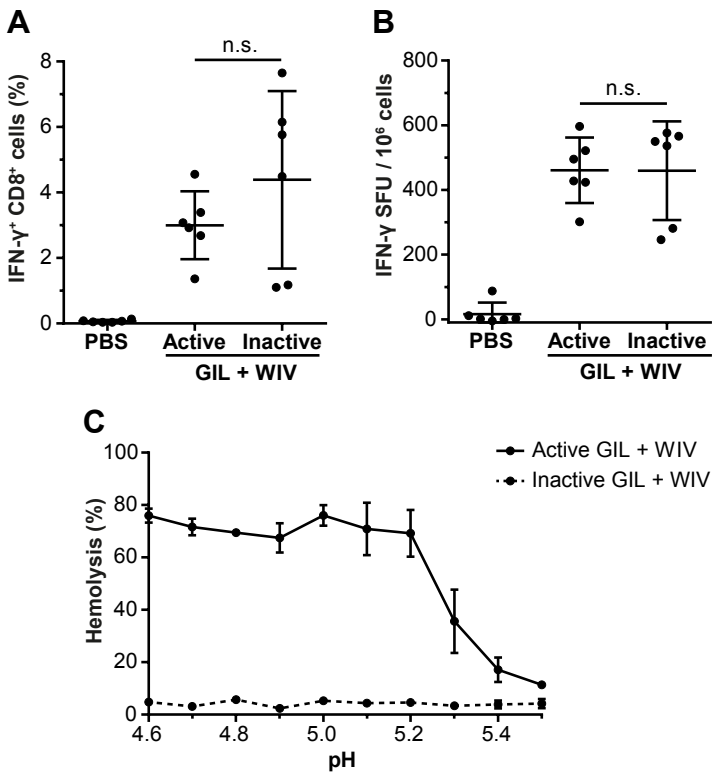

Figure 4. Effect of membrane fusion activity on WIV adjuvanticity.
HLA-A2.1 transgenic mice were vaccinated twice with 100 µg GIL peptide and 25 µg fusion-active (active) or fusion-inactive WIV (inactive). Splenocytes were restimulated with GIL peptide and analyzed by flow cytometry (**A**) or ELISpot (**B**). Fusion activity of active and inactive WIV-GIL formulations was determined by hemolysis assay (**C**). Immunogenicity data is presented as mean ± SD n=6; n.s.=not significant. Hemolysis data is presented as mean ± SD n=3.

This indicates that WIV is a robust adjuvant that retains its function even after loss of fusogenicity.

Adjuvation of multiple peptides by WIV

To investigate whether WIV also acts as an adjuvant for multiple peptides, a peptide pool of GIL and two additional human HLA-A*0201 restricted influenza epitopes, FMY and NML, was studied in combination with WIV. In addition, we selected three modified peptides to be combined with WIV, being G1, F5 and N53, which are CPLs derived from the three aforementioned WT peptide epitopes. Modification of WT peptides with non-proteogenic amino acids has previously shown to increase binding affinity with the MHC-I molecules, which might result in increased T cell responses (13). Since the selected epitopes are also present in WIV, a reduced WIV dose (5 μg) was chosen from the previously established prediction model. At this concentration, it was predicted that WIV still had an immunostimulating effect, while bringing the inherent T cell response generated by WIV itself to a minimum. Mice were vaccinated with either WT or modified peptide pools adjuvanted with WIV. As a control, peptide pools adjuvanted with IFA were included to compare the adjuvanticity of WIV to that of IFA.

The individual peptides in both WT and modified pools did not show significant association with the WIV particles, similar to the previous observations with the GIL peptide alone in this study (**Table SII**). Thus, it is unlikely that differences in induced immune responses by the peptide vaccines are caused by differences in association between peptide and WIV.

As seen previously in this study, the GIL peptide in the peptide pool was able to induce GIL-specific T cell responses after immunostimulation with WIV (**Figure 5A**). In contrast, IFA adjuvanted GIL peptide induced significantly lower T cell responses. The modified G1 peptide, however, was unable to induce potent GIL-specific responses, regardless of adjuvant. The G1 peptide adjuvanted with either WIV or IFA did induce a G1-specific T cell response, indicating that while the modified peptide was immunogenic in combination with an adjuvant, it failed to induce responses that reacted with the WT analog.

The WT FMY peptide was able to induce modest FMY-specific T cell responses in combination with either WIV or IFA (**Figure 5B**). Interestingly, the modified F5 peptide was able to induce significantly higher FMY-specific responses compared to the WT FMY peptide when adjuvanted with WIV. F5 peptide adjuvanted with IFA did not show such an increase, indicating that WIV is a more potent adjuvant than IFA for the F5 peptide. This difference was also observed with the F5-specific responses; F5 peptide induced significantly higher F5-specific T cell responses when adjuvanted with WIV than with IFA.

The subdominant NML peptide and the modified N53 were unable to induce any significant T cell responses, regardless of adjuvant (**Figure 5C**). IFA-adjuvanted peptides showed

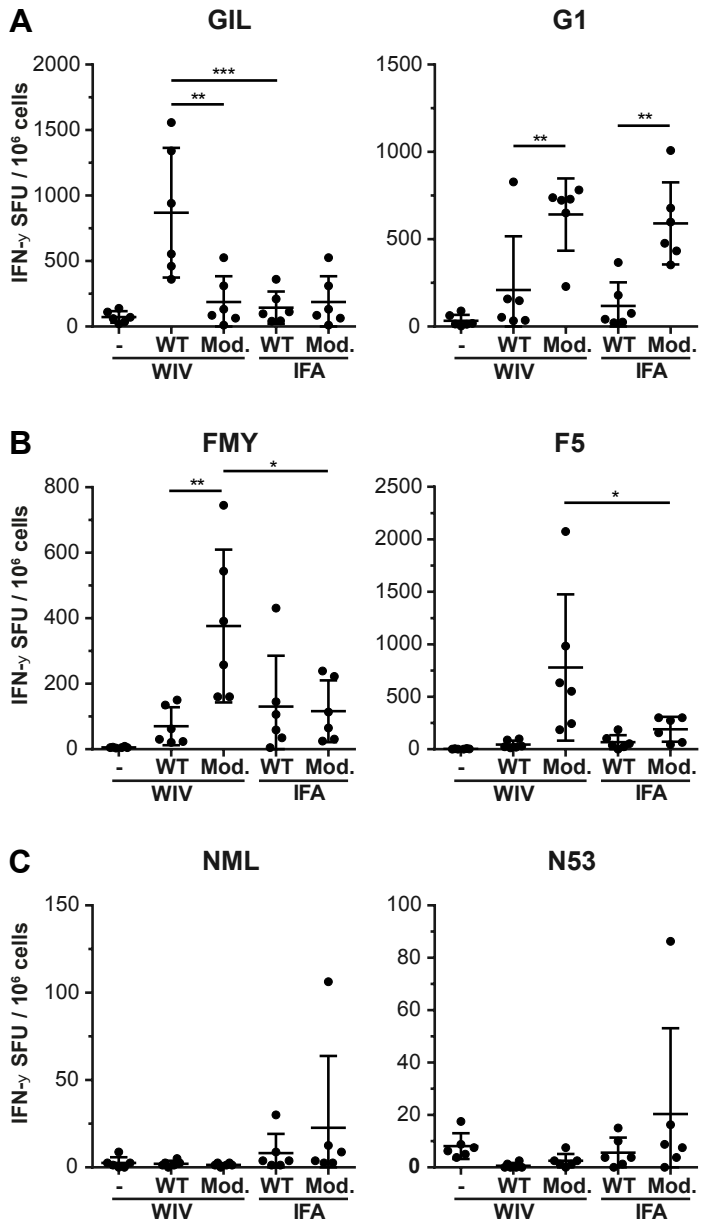

Figure 5. T cell responses against wild-type and modified peptides adjuvanted with WIV.
HLA-A2 transgenic mice were vaccinated twice with peptide pools containing 100 µg of wild type (WT) peptides (GIL, FMY and NML) or modified (mod.) peptides (G1, F5 and N53) adjuvanted with 5 µg WIV or 50% (v/v) IFA. Specific T cell responses induced by the peptide pools towards either GIL or G1 (**A**), FMY or F5 (**B**), NML or N53 (**C**) were determined for all groups. IFN-γ spot-forming units were determined with ELISpot. Data is presented as mean ± SD n=6; *p<0.05, **p<0.01, ***p<0.001.

incidental T cell responses in some animals, suggesting that IFA is a slightly better adjuvant than WIV for this specific peptide. It is unclear why WIV was not effective with NML and N53 peptides, while IFA-adjuvanted NML and N53 managed to induce a response in a few animals. It is possible that WIV contains epitopes which are more immunodominant than the NML epitope, decreasing the NML-specific T cell responses. However, since responses induced by IFA-adjuvanted NML and N53 peptides were not consistent in all animals, there was no significant difference between IFA- and WIV-adjuvanted groups.

These data indicate that WIV is a potent adjuvant for short peptides, both in WT or modified form. Other approaches such as peptide-lipid conjugates (25), liposomes (9), virosomes and nanoparticles have been used previously to increase the immunogenicity of short peptides (10, 26), but require multiple formulation steps and might not be suitable for every peptide due to differences in physicochemical attributes. In contrast, WIV can be readily mixed with peptide antigens, which is a simple process to scale up. Furthermore, WIV is already licensed and used for decades as an influenza vaccine, and recent studies show an excellent safety profile (27). With this prior knowledge on safety and tolerability, it should be feasible to include WIV in any prospective vaccine as an adjuvant.

Conclusion

While it is known that WIV possesses an innate adjuvant capacity, so far it has never been used as an adjuvant for peptide antigens. We showed that WIV is capable of effectively increasing the T cell response against GIL and FMY influenza peptides in HLA-A2 transgenic mice. Co-localization of antigen and adjuvant were necessary to induce a potent T cell response, but the membrane fusion capacity of WIV was not important for the immunogenicity of the formulation. Furthermore, we showed that WIV was also able to immunostimulate non-natural, modified peptides effectively. Due to the ease of production of WIV and its long time safety track record, it is an excellent candidate adjuvant for low-immunogenic antigens that induce cellular responses.

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Supplemental data

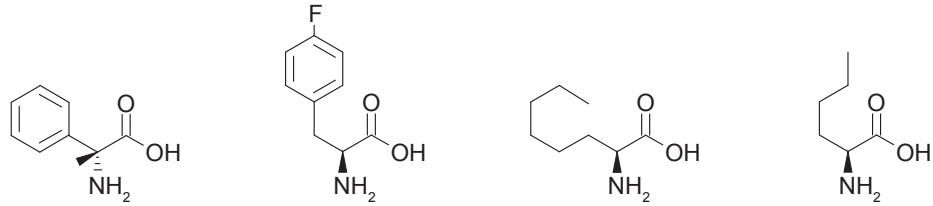

Figure S1. Nonproteogenic synthetic amino acids used for peptide modification.
The four nonproteogenic synthetic amino acids were introduced either in GILGFVFTL, FMYSDFHFI or NMLSTVLGV peptides, resulting in modified [am-phg]ILGFVFTL, [4-FPHE]MYSDFHF[2-AOC] and N[NLE] LSTVLGV peptides.

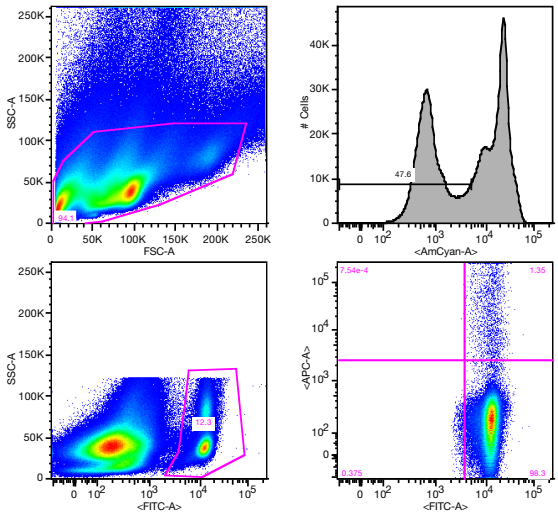

Figure S2. Gating strategy of CD8⁺ IFN-γ⁺ T cells in splenocytes.
An example of the gating strategy. The lymphocyte population was first gated (upper left). From this population, all live cells were selected (upper right). Subsequently, CD8⁺ cells were gated (lower left), after which a quadrant gate was created to select for CD8⁺ IFN-γ⁺ cells (lower right).

Table SI. Worksheet of full factorial design of dose-finding study.

All experimental points (initially seven) were duplicated and included six times to accommodate biological variation between the animals in the in vivo study.

No.	WIV	Peptide
1	1	1
2	1	1
3	1	1
4	1	1
5	1	1
6	1	1
7	25	1
8	25	1
9	25	1
10	25	1
11	25	1
12	25	1
13	13	50.5
14	13	50.5
15	13	50.5
16	13	50.5
17	13	50.5
18	13	50.5
19	1	100
20	1	100
21	1	100
22	1	100
23	1	100
24	1	100
25	25	100
26	25	100
27	25	100
28	25	100
29	25	100
30	25	100

Table SII. Association of peptides with WIV.

Peptides were admixed with WIV (in similar concentrations as used in vivo) and subsequently separated by ultracentrifugation. The fraction of unassociated peptide was determined in the supernatant by mass spectrometry. Data represents mean ± SD, n=3.

Peptide	Unassociated peptide (%)
GIL	87 ± 18
FMY	81 ± 22
NML	115 ± 21
G1	138 ± 34
F5	96 ± 8
N53	99 ± 12

Chapter 8

Summarizing discussion

Summarizing discussion

Current seasonal influenza vaccines are effective inducers of antibody responses against matching circulating strains. However, the variable nature of HA and NA renders these vaccine-induced antibodies ineffective. In contrast, T cell responses are often directed towards internal proteins of influenza, such as NP, PB1, and M1, which contain a greater amount of conserved epitopes (1, 2). The relevance of T cell responses to conserved epitopes during influenza infection has been shown by studies describing the effect of the presence of cross-reactive T cell responses on influenza disease severity. Sridhar et al. described that a higher frequency of pre-existing $CD8^+$ T cells specific for conserved epitopes correlated with less severe illness after infection with pandemic H1N1 influenza virus (3). Wang et al. observed that the presence of early robust $CD8^+$ T cell responses correlated with faster recovery from H7N9 infection (4). Wilkinson et al. monitored T cell responses of healthy volunteers following influenza challenge, and observed lower virus shedding and less severe illness in the presence of influenza-specific cytotoxic $CD4^+$ T cells (5). In this thesis, we describe the current status of influenza vaccination and the role for vaccine-induced T cell immunity to influenza. We applied this knowledge to the development of new broadly protective influenza peptide-based vaccination strategies.

Current status influenza vaccination

In **Chapter 3**, a clinical trial is described in which we evaluated humoral and cellular immune responses during two consecutive influenza seasons from 2009 to 2011. The study was initiated in 2009 at the start of the influenza pandemic, following the introduction of the A(H1N1)pdm09 strain into the human population. This strain contains genes from swine, avian and human origin and was introduced into the human population through pigs. The virus had already infected individuals in different countries, and since individuals were expected to be naïve to this virus it was expected that the virus would spread easily and would be able to affect a high proportion of the human population worldwide. Therefore, the Dutch government decided to order pandemic vaccines in addition to the seasonal vaccine. The pandemic vaccine evaluated in this study was a monovalent MF59-adjuvanted subunit vaccine. The adjuvant was included to decrease the antigen-dose needed to obtain seroprotective antibody responses by half and induce a broader immune response in the form of enhanced functional antibody titers and greater breadth of antibody cross-reactivity (6). Based on studies on avian influenza vaccination the vaccine was registered for a two dose schedule to obtain seroprotective antibodies, which was also recommended by the Dutch government (7).

In the study described in **Chapter 3**, healthy adult participants received the MF59-adjuvanted monovalent pandemic vaccine in a two-dose setting. To account for background response and to be able to compare humoral and cellular immune responses, a statistical model was developed to analyze the data. Analysis of antibody titers showed that one dose would have

been sufficient to provide seroprotection, however, a second dose did further enhance antibody levels. In contrast, T cell responses were induced after the first dose, but were not boosted by a second dose. At the start of the second season, antibody levels and T cells were still detectable in vaccinated individuals. Administration of the unadjuvanted seasonal vaccine, also containing the pandemic strain of the previous year, boostered both these responses. Antibody levels of individuals that did not receive the seasonal vaccine in 2010 remained detectable until the end of the study indicating the presence of vaccine-specific antibodies for over 15 months. T cells remained detectable until one year after vaccination, but had reduced back to baseline level at 15 months post vaccination.

The finding that one dose of the A(H1N1)pdm09 pandemic vaccine is sufficient for providing seroprotection is supported by literature (8-10). We hypothesize that it may be possible to predict whether a two-dose setting is necessary during a pandemic threat, by predicting cross-reactive immunity. Cross-reactive immunity is in the first place dependent on the presence of cross-reactive responses in individuals. Ikonen et al. described the presence of cross-reactive antibodies to A(H1N1)pdm09 in elderly, which may have contributed to the low number of elderly affected by this pandemic (11). These data indicate that when cross-reactive immunity is present, one dose is sufficient (12), while in individuals that do not have pre-existing immunity even two doses might not be sufficient to provide seroprotective antibodies as shown by a study with H5 subtypes (13). However, characteristics of the virus itself should also be taken into account. While unadjuvanted vaccines for H5N1 subtypes already required a two dose schedule to induce seroprotective antibody titers (14-16), there are some viruses that have such a low immunogenicity that the design of an effective vaccine will be very difficult. The effect of immunogenicity of the virus itself on vaccine efficacy is demonstrated by data on H7N9, a subtype that emerged in China in 2013 and has a high lethality record (17). It was predicted by de Groot et al. that H7N9 had a low number of cross-reactive $CD4^+$ T cell epitopes, which are required for antibody induction. Indeed, unadjuvanted vaccines targeting H7N9 HA had a low efficacy (18), which underlines the importance of cross-reactive T cell responses and shows that these are not only dependent on pre-existing immunity present in the human population, but also on epitopes in the virus itself.

In **Chapter 3**, we also monitored T cell responses to the vaccine during the clinical trial. Vaccine-specific T cell responses were initially demonstrated by stimulation of PBMCs with the WT virus strains used in the vaccines to show the effect of vaccination on T cell responses following infection with influenza virus. IFN-γ production in response to whole virus stimulation was measured by ELISpot. Hereby, we showed that both pandemic and seasonal influenza vaccination induced T cell responses in vaccinated individuals. However, in contrast to antibody levels, T cell levels were not boostered by a second dose of the pandemic vaccine. The finding that booster vaccination is not favorable for T cell responses has been described earlier. McElhany et al. even found a negative correlation between antibody levels and cytokine ratios in elderly and proposed that a second dose might skew T cell responses

to an IL-10 dominated cytokine profile (19). IL-10 is associated with an anti-inflammatory response, which limits T cell and antibody responses. The presence of high levels of IL-10 is therefore not desired during a response to influenza (20). Others reported an inverse correlation between pre-vaccination IFN-γ production and the magnitude of responses post-vaccination (21, 22). In the clinical trial, a booster effect on T cell responses was, however, visible at the start of the second season; residual T cell levels of individuals vaccinated with the A(H1N1)pdm09 vaccine in the previous year, were further enhanced after vaccination with the seasonal vaccine. This apparent discrepancy in the ability of vaccination to boost T cell responses could be explained by timing of vaccination. The second dose of the pandemic vaccine was administered three weeks after the first dose, while the seasonal vaccine was administered approximately one year later. After three weeks, T cells will still have been activated or the memory T cell response may not have been fully developed (23, 24), thereby limiting further activation of T cells. However, the effect of vaccine dose administered cannot be excluded since the pandemic vaccine contained 7.5 μg HA, while the seasonal vaccine contained 15 μg HA.

To confirm that T cell responses detected in individuals that received pandemic and seasonal vaccination were vaccine-specific, T cell responses were also analyzed by stimulation with vaccine-specific peptide pools. During stimulation with live virus, T cells to the more conserved internal virus proteins induced by previous natural infections may have been activated. However, by stimulation of PBMCs with a peptide pool containing 15-mer peptides with 11 amino acid overlap spanning the entire HA or NA protein of the vaccine strains, only vaccine-specific responses were analyzed. A similar profile of responses was detected as with whole virus stimulation, showing that T cell responses were indeed vaccine-specific. Residual T cell levels were still detectable at the start of the second season, which implies that T cell memory is maintained for over a year. After 15 months T cell levels had reduced back to baseline, which is in line with findings that memory $CD4^+$ and $CD8^+$ T cells have a maximum life span of approximately one year (25, 26). Thereafter, a minimal compartment of memory cells may still be present, which is the background response or baseline of our assays. Following infection or vaccination, these T cells can be re-activated and this will be measurable as an induction of T cell levels (27). In **Chapter 3**, we described general T cell levels in response to influenza vaccination, however, it remains to be clarified by which cell type the measured IFN-γ levels were produced. Since peptide pools were used as a stimulating antigen, which are specifically designed to stimulate T cells, cytokines measured are most likely produced either by $CD4^+$ T cells, $CD8^+$ T cells, or both. Thus far, both types of T cells have been shown to be important in providing cross-reactive immunity during influenza infection (3, 5). However, the impact of each type of T cell following influenza vaccination remains to be elucidated. The impact of cellular responses also depends on specific characteristics of the T cell response, such as clonality of the T cells and their cytokine profile. As described in **Chapter 2**, for viral infections, a Th1-skewed T cell response, as characterized by the production of IFN-γ is more desirable than a Th2-skewed immune response as characterized by the production of IL-4,

IL-5, and IL-13. Furthermore, the response should be of magnitude and breadth to be able to clear virus and have an effect during influenza infection.

In addition, it will be interesting to identify to which epitopes the responses were directed. Much research is focused on T cell responses to internal influenza proteins, such as NP and PB1, since these proteins are more conserved and thus interesting for the development of universal vaccines. However, as elegantly shown in a model in which mice were infected with HK-X31, HA protein is, in addition to NP, one of the major contributors of MHC class II epitopes. Approximately 51% of the MHC class II epitopes submitted to the immune epitope database is HA-specific (28, 29). However, research on $CD4^+$ T cell epitopes may be focused on responses to antigens to which also antibodies were directed, explaining the large number of $CD4^+$ epitopes identified for HA. In concordance, the immune epitope database contains a large number of conserved $CD8^+$ T cell epitopes of internal viral proteins and only a minor percentage of epitopes derived from HA, which can be explained by the fact that many groups specifically investigate conserved $CD8^+$ T cell responses directed to these proteins (29). Therefore, a potential bias in literature cannot be excluded and it remains difficult to predict whether $CD4^+$ T cells or $CD8^+$ T cells are the main contributors to responses in **Chapter 3** and whether the T cell responses are directed to relatively conserved HA and NA epitopes or to subtype or strain specific antigens.

As of yet, no literature is available describing T cell responses to subunit vaccines in humans. One explanation is that current seasonal vaccines are designed to induce antibody responses to circulating strains, since antibodies are still the correlate of protection for influenza. Therefore, evaluation of these types of vaccines generally does not include an evaluation of T cell responses induced by vaccination. For example, Lambe et al. did evaluate the induction of T cell responses by a trivalent inactivated split vaccine, but focused only on conserved epitopes present in the remaining internal viral proteins M1, NP and NS1 (30). Other registered influenza vaccines that have been shown earlier to be capable of inducing T cell responses have components facilitating the induction of T cells. LAIV and WIV, for example, both contain ssRNA, which is a ligand for TLR7 (31). In addition, LAIV still has limited replicative potential, leading to presentation of viral antigens on MHC class I, while BPL-treated WIV has maintained fusion activity, facilitating the induction of T cell responses (32). However, we have shown that both the MF59-adjuvated pandemic vaccine and the unadjuvanted subunit vaccine induce T cell responses even though these vaccines did not contain internal viral proteins and were not designed to induce T cell responses. Apparently, administration of antigen alone is sufficient to induce a certain level of T cell responses, which could be due to activation of memory T cells already present due to earlier influenza infection or vaccination. For example, although the globular head of HA and NA is highly variable, these proteins do contain some conserved epitopes and individuals may have had T cells available directed to these epitopes explaining the induction of a response to the vaccine antigens. Although it could also be possible that the vaccine may have induced naïve T cells.

T cell-inducing influenza vaccines

Even though we have shown that current vaccines are capable of inducing a certain level of T cell responses, they are designed to induce antibody responses to circulating influenza viruses and do not induce a cross-reactive immune response. Therefore, current vaccines have several limitations; they need to be updated each year to match with circulating strains and if strains do not match, the vaccine is not as effective. In addition, antibody-induced vaccine-immunity might not be optimal for target groups of influenza vaccination, i.e., elderly and young children. McElhany et al. showed that T cell responses might provide a better correlate for protection than antibody responses in elderly (33). In addition, Bodewes et al. showed that although seasonal vaccination of children is effective in preventing disease, it does hamper the development of virus-specific $CD8^+$ T cells, normally acquired through natural infection (34). By specifically inducing T cell responses, an influenza vaccine would not only be more efficient in elderly, but by targeting conserved epitopes it would also provide additional immunity to a broader variety of influenza virus subtypes. Hereby, such a vaccine could limit disease and spread of infection when a new virus subtype emerges.

In scope of the development of such a new type of influenza vaccine, there are many strategies under development that are designed to induce influenza-specific T cell responses as reviewed by Soema et al. (35). Live attenuated influenza vaccines are already licensed and in use for vaccination of individuals of 2-49 years of age. LAIV has been shown to induce both $CD4^+$ and $CD8^+$ T cell responses in unprimed children (21, 36). T cell-inducing influenza vaccines that are under development include viral vectors, DNA vaccines, protein vaccines, and peptide-based vaccines. Viral vectors are replication-defective viruses capable of expressing sustained levels of antigens. As extensively reviewed by Tripp et al., there are many types of viruses that can be used as a vector to express high levels of influenza-specific antigens. (37). Recently, a vaccinia virus Ankara expressing NP and M1 was shown to induce T cell responses (38). DNA vaccines are also under development as a method to induce influenza-specific T cell responses. At the site of immunization, cells become transfected with plasmid DNA that encodes the protein of interest. This protein can then be processed and presented to the immune system in the context of MHC class I or II. Hereby, both $CD8^+$ and $CD4^+$ T cells can be activated (39). A third novel strategy are protein vaccines, recently a successful Phase I clinical trial has been described with a vaccine containing HA, M1, and NP that was capable of inducing cellular responses in adults and elderly (40). In this thesis, we focused on peptide vaccination as a means of inducing T cell responses to conserved influenza epitopes, which have the advantage of being fully synthetic and can therefore be readily produced for human use in a GMP setting. In addition, while the production of currently used vaccines first requires the generation of vaccine seed strains, which is time consuming and may result in low yield strains. To produce the vaccine, the virus seeds need to be grown on eggs, of which may be a shortage during a pandemic (41) and has the downside of possible contamination with egg antigens and extraneous agents. On the contrary, the production of peptides only requires the sequence of a new virus and is much faster and cleaner.

Peptide-based vaccine strategies

In **Chapter 2**, we described the features of an effective cellular response against both acute and chronic infections, including an appropriate magnitude, breadth and quality. An effective immune response against viral infections largely depends on the activation of cytotoxic T cells capable of clearing infection by killing virus-infected cells. Proper activation of cytotoxic T cells depends on professional antigen presenting cells. In **Chapter 2** we discussed the potential of peptide-based vaccination as a strategy to prevent and treat viral infections. Many factors influence the efficacy of peptide vaccines, including choice of antigen, adjuvants and formulation. As already mentioned previously, it is desirable to specifically target conserved regions of influenza virus, making a vaccine efficacious against a broader range of influenza subtypes. For the selection of peptides there are databases available in which known CD4 and CD8 epitopes are listed, specific for a wide variety of pathogens including influenza virus (29). Furthermore, there are tools available to check whether selected regions are conserved, whether these regions contain T cell epitopes and whether selected sequences do not contain auto-antigens (42-44). Following successful selection, a choice needs to be made on the type of peptide used. Minimal epitopes only induce a $CD8^+$ or $CD4^+$ T cell response, while long peptides need to be processed by professional APCs and will induce both $CD8^+$ and $CD4^+$ T cells. In this thesis, we described both strategies. **Chapter 4** discusses the potential of a long overlapping peptide vaccine while **Chapters 5**, **Chapter 6** and **Chapter 7** describe the modification and formulation of minimal epitope-based vaccine concepts.

In **Chapter 4,** we set out to develop a vaccine that provides protection against a broad range of influenza virus subtypes. We selected 25 synthetic long overlapping peptides (SLP), derived from highly conserved regions of nucleoprotein (NP), polymerase basic protein 1 (PB1), and matrix protein 1 (M1). Length of the peptides ranged from 26-34 amino acids to ensure that known $CD4^+$ and $CD8^+$ T cell epitopes were included. Hereby, an immune response could be induced to a wide variety of antigens, thereby also providing a broad HLA coverage. In addition, we selected linear B cell epitopes directed to the highly conserved HA2 fusion peptide and to M2e peptide and conjugated these epitopes to a CD4 helper epitope. The CD4 epitopes were selected based on their known strong immunogenicity. By including a $CD4^+$ epitope, $CD4^+$ T cells can be activated in parallel to B cells. The activated $CD4^+$ T cells can then provide help to the B cell, resulting in enhanced immunogenicity of the B cell epitope. This will result in higher B cell epitope-specific antibody titers. C57BL/6 mice, BALB/c mice, and ferrets were vaccinated with the 25 SLP, the B epitopes, or a combination of both. We detected vaccine-specific antibodies in sera of both mice and ferrets. Vaccine-specific T cell responses were only analyzed in BALB/c mice and were shown to be directed to vaccine-specific peptides. Following challenge, a reduction of virus titers in the lungs was observed in mice vaccinated with the 25 SLP and the B epitope alone and in mice and ferrets vaccinated with the combined vaccine. Hereby, we have developed a promising universal influenza vaccine format capable of inducing both T and B cell responses. Although vaccination did not reduce disease severity, reduction of virus replication is valuable since it may reduce spread

of the virus in a pandemic situation.

In **Chapter 5**, we discuss a method in which immunogenicity of peptides itself can be enhanced. By substitution of amino acids in the wild type sequences with non-proteogenic amino acids, resulting in chemically enhanced altered peptide ligands (CPLs) the affinity for HLA can be increased. This method was applied to optimize minimal influenza-specific epitopes of varying natural affinity and dominance: the highly dominant GILGFVFTL ($M1_{58-66}$), the less dominant FMYSDFHFI (PA_{46-54}) and the low affine and subdominant NMLSTVLGV ($PB1_{413-421}$) epitopes. Based on these HLA-A*0201-specific epitopes, approximately 600 CPLs were designed and tested for binding affinity. For all three epitopes, we succeeded to design CPLs that exhibited greater binding affinity than their WT counterparts. FMYSDFHFI and NMLSTVLGV had the greatest window of improvement and therefore, the largest increase in binding score was obtained for modifications to these epitopes; however, even binding scores of the already high affinity GILGFVFTL epitope could be improved. To evaluate immunogenicity of these CPLs compared to their corresponding WT epitopes, IFN-γ responses in splenocytes of mice vaccinated with CPLs were compared to WT vaccinated mice. An increased response was measured for CPLs of GILGFVFTL and FMYSDFHFI and in addition to an increased response, modification of the low affinity epitope NMLSTVLGV also led to an increase in the number of mice that responded. Additionally, by optimizing three HLA-A*0301-specific epitopes: ILRGSVAHK ($NP_{265-273}$), SFSFGGFTK ($PB2_{322-330}$), and RMVLSAFDER (NP_{67-76}), we show that a general strategy is established that can be used to improve binding of essentially any class I epitope and to any allele.

In addition to the experiments described in **Chapter 5**, a challenge experiment was performed in HLA-A2 transgenic mice to assess the efficacy of a CPL-based vaccine and to determine whether modified epitopes have an additional value compared to WT epitopes. To this end, mice were vaccinated with a combination of the three HLA-A*0201-specific WT epitopes described above or with a combination of the three optimal CPLs derived from these epitopes (G1 ([am-phg]ILGFVFTL), F5 ([4-FPHE]MYSDFHF[2-AOC]), and N53 (N[NLE] LSTVLGV)). An identical formulation was used as in the immunogenicity experiments, i.e., adjuvation with IFA and the TLR9-adjuvant CpG. As a negative control, mice were vaccinated with adjuvants alone, while positive control mice received a low dose of the mouse adapted HK-X31 (H3N2) influenza A strain. Two weeks following booster vaccination, at the peak of the T cell response, mice were challenged with the HK-X31 strain. No significant differences were observed between the lung viral load of mock-vaccinated mice and both groups of peptide-vaccinated mice. In addition, all mice lost bodyweight after challenge, however, CPL-vaccinated mice demonstrated significantly less bodyweight loss than mock vaccinated mice and WT epitope-vaccinated mice. Pathological examination of lungs of mice showed a reduction in relative lung weight (a measure for edema formation), percentage affected tissue, and the end score of histopathological markers such as inflammation of tissues and damaged epithelium, in CPL-vaccinated mice compared to mock-vaccinated mice. WT epitope-

vaccinated mice also had a lower percentage of affected tissue, but other histopathological markers were not decreased as much. These results indicate a small advantage of CPLs over the WT epitopes, although additional experiments should be performed to strengthen these findings. Altogether, modification of epitopes provides a valuable tool to improve the range and immunogenicity of preventive T cell-targeted peptide vaccines, although optimization of these vaccines is needed to obtain a vaccine also capable of reducing viral load in the lungs.

However, the limited immunogenicity of peptides requires strong adjuvation. In the vaccines described in **Chapters 4** and **5**, peptides were formulated in Incomplete Freund's Adjuvant in combination with a TLR1/2 and TLR9 adjuvant, respectively. IFA is a depot-forming adjuvant, which is highly effective for peptide-based vaccines, however, it is not desirable for human use (45, 46). Therefore, other methods need to be developed to enhance immunogenicity of peptide-based vaccines. In **Chapter 6**, we formulated the GILGFVFTL-peptide with virosomes and assessed its immunogenicity in HLA-A2 transgenic mice. We showed that vaccination of mice with peptide-loaded virosomes (P-V) induced peptide-specific $CD8^+$ T cells. Addition of TLR9 ligand CpG to the formulation increased IFN-γ production of $CD8^+$ T cells in response to stimulation of splenocytes with the GILGFVFTL-peptide. Furthermore, IgG2c titers measured to HA of the virosome were increased in mice vaccinated with P-V in combination with CpG. The presence of IgG2c-antibodies indicates a Th1-skewed response, which is advantageous for the induction of $CD8^+$ T cell responses. Mice vaccinated with P-V in combination with CpG demonstrated less weight loss and increased recovery compared to PBS and mice vaccinated with peptide and CpG formulated with IFA after challenge with influenza. **Chapter 6** shows that virosomes are an effective peptide delivery system, however, depending on characteristics of the peptide, encapsulation of peptides can be challenging. Therefore, virosomes are not yet broadly employable and additional development is needed before it can be considered for clinical testing in humans.

Improving existing influenza-vaccine formats may also be a possibility. In **Chapter 7**, we investigated whether T cell responses can be improved by using whole inactivated influenza virus (WIV) as an adjuvant for influenza peptides. Immunogenicity of WIV mixed with minimal peptides was investigated in HLA-A*0201 transgenic mice. WIV was formulated with GILGFVFTL alone or with a peptide pool containing the three HLA-A*0201-specific WT epitopes described in **Chapter 5** and the three optimal CPLs corresponding with these epitopes. WIV was found to be a potent adjuvant for GILGFVFTL alone, even at low concentrations and for the WT and CPL peptide pools. In mice vaccinated with CPLs adjuvanted with WIV the IFN-γ response was enhanced compared to mice vaccinated with CPLs formulated in IFA. When WIV was used as an adjuvant, responses to GILGFVFTL and FMYSDFHFI were higher than with IFA. Especially the response to the more subdominant FMYSDFHFI peptide was increased. In addition, injecting peptide and WIV separately at different sites or mixed at a single site, showed that co-localization of the peptide antigen and WIV adjuvant was important for an efficient peptide-specific immune response. The relatively simple formulation and the use of a

known influenza vaccine component make WIV an attractive adjuvant. However, in contrast to the vaccination strategies described in **Chapters 4** and **5**, which describe the development of possible universal influenza vaccines, the strategy described in **Chapter 7** is meant to improve current vaccines. By adding T cell inducing components to current vaccine formats, the response to vaccines can be broadened thereby improving vaccine-specific responses.

Future perspectives

One of the major issues for T cell inducing vaccines is designing a vaccine that provides a broad HLA-coverage. The minimal epitopes described in this thesis are directed to HLA-A*0201 and HLA-A*0301. Both are alleles frequently occurring in the Caucasian population, however allele frequencies differ between different ethnicities as described in an online database (47). Minimal epitopes are described that are recognized by a broader set of alleles,

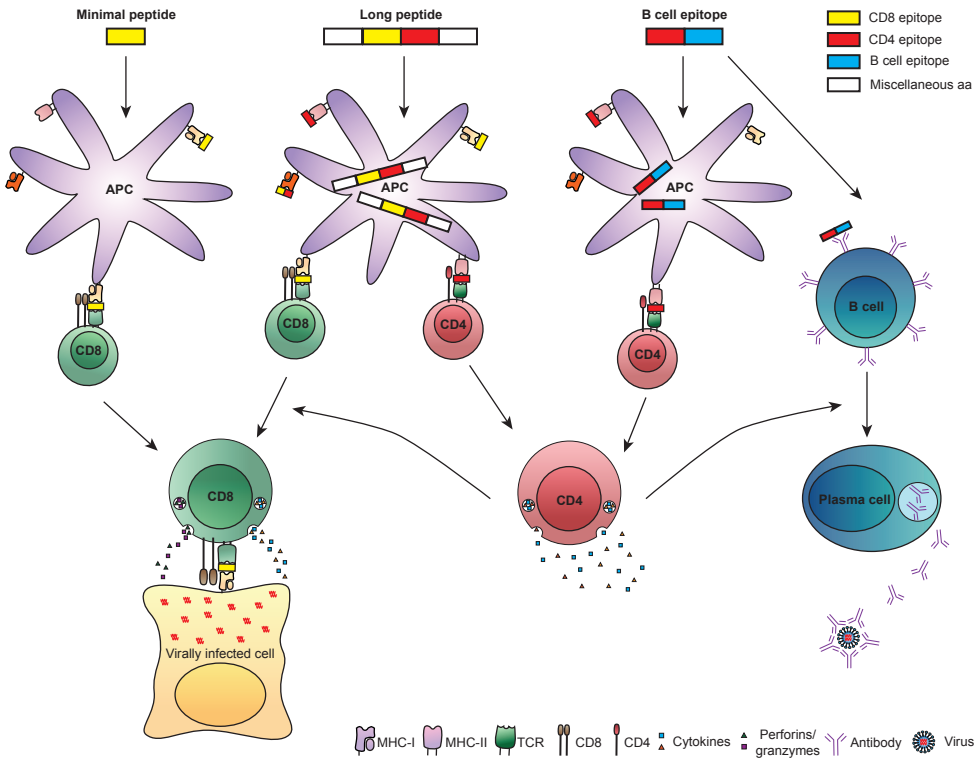

Figure 1: Overview of peptide vaccination strategies.
This Figure provides an overview of the peptide vaccination strategies described in this thesis. Minimal peptides (left) fit directly into a specific MHC class I molecule, require no processing by APCs and will therefore only induce $CD8^+$ T cells. Compared to WT peptides, CPLs will induce an enhanced $CD8^+$ T cell response compared to WT epitopes. Long peptides (middle) do need to be processed by a professional APC and are capable of inducing both $CD8^+$ and $CD4^+$ T cells. The B epitopes (right) described in this thesis contain both a $CD4^+$ T cell epitope and a B cell epitope and are therefore capable of activating $CD4^+$ T cells and inducing the production of B cell epitope-specific antibodies.

however these epitopes are not presented equally efficient by the different alleles (28). Long peptides provide part of the solution, since they need to be processed by professional APCs and are thus not specific for one allele (48). However, a large enough selection needs to be made to provide for sufficient epitopes for each allele and even if long peptides can be selected that provide a broad HLA-coverage, the question still remains how many and which type of epitopes are sufficient for providing at least a reduction of disease burden. There are publications describing potent trials with synthetic peptide vaccines containing immunodominant epitopes (49, 50). However, these trials have only shown that synthetic peptide vaccines are immunogenic in a small population. It remains to be evaluated whether this vaccine is applicable worldwide and whether it is effective against natural infection with influenza. A recent paper describes in an in vitro model of human epithelial cells that although the immunodominant GILGFVFTL epitope is present in a high number of copies per cell following infection, GILGFVFTL-specific T cells are unable to effectively recognize infected human epithelium (51). These results warrant caution when selecting epitopes for use as a vaccine, since some epitopes might induce an unwanted T cell response.

As already recognized as early as 1969, efficacy of influenza vaccines is also influenced by the route of immunization (52). With the exception of LAIV, which is administered intranasally, registered influenza vaccines are administered either intramuscularly or intradermally. As also discussed in **Chapter 2** and evaluated in **Chapter 4**, route of immunization will play an important role in efficacy of peptide-based influenza vaccines. Lung-resident T cells will naturally play an important role in clearance of influenza virus and as Rose et al. described, lung-resident immune cells will be primed more easily via intranasal vaccination (53). In **Chapter 4**, no major differences in efficacy were observed between the different routes of immunization with the SLP vaccine in ferrets. A study in mice, however, which compared pulmonary immunization to systemic immunization found that while circulating memory $CD8^+$ T cells are efficiently induced by systemic immunization these cells do not prevent viral replication and damage to the lung epithelium. In pulmonary vaccinated mice, a T cell response is observed followed by efficient clearance of virus from the lungs (54). These findings demonstrate the potential advantage of inducing T cell immunity at the site of entry of the pathogen. This can be obtained by selecting a certain route of immunization, as described above. However, other factors also contribute to the induction of an efficient T cell response in the lungs. Lung-primed DCs, for example, play an important role in recruiting T cells by inducing upregulation of the CCR4 receptor on these cells. CCR4 was shown to be an important homing marker on T cells for lung tissue, specifically (55), illustrating that additional actions are needed to obtain a localized immune response.

However, a risk of inducing T cell responses too efficiently is that overstimulation of the immune response may lead to immune pathology. To examine the influence of vaccination on pathological effects induced by a virus challenge, lungs of mice and ferrets collected five days after infection in studies described in **Chapter 4**, were fixed in formalin after which

haematoxilin and eosin (HE) stained sections were examined for general histopathology. In C57BL/6 mice, the overall pathology in the vaccine groups and the mock vaccinated group was similar. In BALB/c mice, however, lung pathology in the group vaccinated with B epitopes, but mostly in the group vaccinated with the 25 SLP alone was higher than that of mock vaccinated group. Surprisingly, the pathology in mice vaccinated with 25 SLP + B epitopes was not enhanced. In addition, in the group vaccinated with 25 SLP alone, inflammation around the bronchi, bronchiole, and blood vessels was increased. In ferrets, gross pathology, as examined by percentage affected lung tissue and relative lung weight, was increased in the vaccinated groups. An increase in the latter represents edema formation and plays a role in acute respiratory distress. On the microscopic level, an increase in pathology was also observed although there was a large lobular variation and variation between animals. Although the increased parameters were mainly of inflammatory nature, in some animals increased damage to the epithelium was also observed.

While there was a large variation in lung pathology observed in the mice and ferret studies, the observations described above indicate enhanced lung pathology after challenge. Based on the BALB/c mice study, this appears to be linked to the T cell component of the vaccine. Increased pathology, and especially the increased inflammation, may be explained by the induction of lymphocytes by this type of vaccine. In addition, the timing of influenza challenge in these studies is approximately at the peak of the T cell response and it could be that a completely developed effector T cell response in combination with an unnaturally high challenge dose induces exaggerated pathological effects. Recently, when we used the intratracheal challenge model in ferrets to investigate an H7N9 LAIV, also known to induce cellular responses, increased inflammation compared to the placebo group was observed (manuscript in preparation). In addition, when mice vaccinated with a low dose of BPL-inactivated WIV (5 µg), also known to induce T cell responses, with or without the addition of T cell epitopes as described in **Chapter 7** were challenged with influenza virus, enhanced pathology in the lungs was observed compared to the mock vaccinated group. This indicates that increased pathology is not an effect of the peptide vaccine specifically, but it might be the result of the design of the challenge model in combination with vaccines that induce T cell responses. Future research, in which we, a.o., want to identify the cells involved in pathology by histochemical examination and look into a model which addresses a different timing of the challenge and a lower challenge dose, will hopefully reveal the mechanism behind these observations. However, when developing T cell inducing vaccines for respiratory infections, the risk of the induction of immune pathology should be taken into account and proper models to examine these side effects should be developed.

Vaccine-related immunopathology has also been reported by others. Studies in mice indicate a role for $CD4^+$ T cells in influenza-related immunopathology. Mice that had memory $CD4^+$ T cells available specific for the challenge virus, exhibited more clinical signs, greater lung damage, and died sooner than mice that did not have $CD4^+$ memory T cells available. It was

hypothesized that memory $CD4^+$ T cells tend to cluster together during a secondary challenge, resulting in a concentrated secretion of antiviral cytokines. These localized clusters resulted in more extreme damage of the epithelium than during a normal immune response in the lungs (56, 57). Another group demonstrated that influenza infection in aged mice resulted in increased pathology, which was contributed to $CD8^+$ T cells. Enhanced lung pathology has also been observed for other respiratory pathogens. A mouse model to elucidate the mechanisms behind the failure of the 1960 trial with formalin-inactivated RSV, which induced enormous vaccine-related morbidity and mortality, a Th2-skewed $CD4^+$ T cell response as the cause of enhanced disease (58). In other RSV virus models, there are indications that $CD8^+$ T cells provide protection at the cost of pulmonary immune pathology (Knudson, Keystone 2014, unpublished data) (59).These results illustrate that the presence of memory T cells in the lungs may aid in efficient clearance of the virus, but may also induce immunopathology. This leaves us with an apparent paradox, in which important antiviral cytokines, such as IFN-α and IL-6 are also inducers of pathology in the lungs (60). Therefore, an efficient antiviral response requires the presence of T cells that, upon activation, will produce a balanced set of cytokines. Antiviral cytokines such as interferons are needed to aid in clearance of the virus, but should be followed by cytokines regulating this cytotoxic response, such as IL-10, to restrict pathological effects induced by the production of pro-inflammatory cytokines (61).

Summarizing conclusion

When a successful T cell inducing vaccine would be available, a constraint is that even if memory T cells induced by this vaccine are present, time is needed for a T cell response to develop. Therefore, a vaccine only focused on the induction of T cells, will not clear an influenza virus infection before the onset of symptoms. This is in contrast to the current antibody-inducing vaccines that are capable of providing neutralizing immunity. While we showed that current subunit vaccines are capable of inducing both humoral and cellular responses, they do not offer cross-protection. As long as no neutralizing cross-reactive antibodies can be induced by vaccination, it will already be an advantage to induce T cell responses towards conserved parts of influenza virus. A few studies describe the role of cross-reactive T cells in reducing disease burden during infection with a new virus subtype. However, a vaccine inducing cellular responses is not only desired in case of a possible pandemic, in which T cells can aid in limiting spread of the virus, but it may also be the key to improve vaccines for children and elderly. In this thesis, we have described several peptide vaccination strategies targeting conserved influenza epitopes (summarized in **Figure 1**) and we investigated formulation strategies to enhance immunogenicity of peptide-based vaccines.

Therefore, we propose that while a new T cell inducing format will not replace current seasonal vaccines, it is desirable to add T cell inducing components to current seasonal vaccines. This will not only enhance immunogenicity of these vaccines in elderly and young children, but may also aid in providing cross-protection when the humoral components of the seasonal vaccine do not match circulating vaccine strains. In parallel, completely new vaccine formats

should be developed that ideally induce humoral and cellular immunity to highly conserved influenza antigens to be able to protect against a wide variety of influenza virus subtypes. Optimally, such vaccines will induce memory T and B cells at the location of influenza virus entry, i.e., the respiratory tract, to ensure a quick response upon infection. One can apply a different route of immunization or make use of adjuvants to obtain a memory response at the desired location. Concluding, there is still a long road ahead, but the findings in this thesis may contribute to the development of the next generation influenza vaccines, which offer broad and cross-reactive protection in the form of both humoral and cellular responses to conserved influenza epitopes.

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Appendix

Nederlandse samenvatting

Dankwoord

Curriculum vitae

Nederlandse samenvatting

Achtergrond

Influenza virus infecteert jaarlijks 5 tot 10% van de wereldbevolking en veroorzaakt ernstige griep bij 3 tot 5 miljoen mensen. Griep karakteriseert zich door hoge koorts, koude rillingen, keelpijn, hoesten, verstopte neus, hoofdpijn en spierpijn. Iedereen wordt ongeveer 1-2 keer per tien jaar geïnfecteerd door influenza virus en het immuunsysteem bouwt dan afweer op tegen een volgende infectie. Bij een zeer ernstige infectie kunnen echter ook de lagere luchtwegen worden aangedaan en kunnen (vaak) verzwakte mensen zelfs ten gevolge van de infectie overlijden. Daarnaast kunnen additionele infecties, met bijvoorbeeld bacteriën, longontsteking veroorzaken met ernstige ziekte of dood tot gevolg, zogenoemd influenza-gerelateerd overlijden. Tijdens de jaarlijkse griepepidemie overlijden wereldwijd tussen de 250.000 en 500.000 personen aan influenza of aan een aan influenza-gerelateerde ziekte. Naast deze jaarlijkse griepepidemieën kunnen ook nieuwe influenza-varianten opduiken in de mens die zich wereldwijd kunnen verspreiden, met een **pandemie** tot gevolg. Het meest beruchte voorbeeld van een dergelijke pandemie stamt uit 1918, toen wereldwijd naar schatting 50 miljoen mensen aan influenza infectie zijn overleden. De meest recente pandemie was in 2009, toen de "Mexicaanse griep" wereldwijd vele mensen infecteerde.

Influenza virus

Zoals hierboven beschreven, wordt griep veroorzaakt door het influenza virus. Op basis van verschillende eigenschappen kan er onderscheid gemaakt worden tussen drie typen influenza virus: type A, B en C. Type A kan zowel mensen als dieren, zoals vogels en varkens, infecteren en is de voornaamste veroorzaker van griep epidemieën en pandemieën. Type B is alleen in staat om mensen te infecteren en is, naast type A, een belangrijke veroorzaker van de jaarlijkse griepepidemie. Type C infecties komen vooral voor bij kinderen en veroorzaken slechts een infectie vergelijkbaar met een verkoudheid. In dit proefschrift wordt het onderzoek beperkt tot influenza type A virussen, aangezien deze momenteel de grootste bedreiging vormen voor de bevolking.

Structuur influenza virusdeeltje

Het oppervlak van het influenza virusdeeltje bestaat uit twee eiwitten: Hemagglutinine (HA) en neuraminidase (NA), welke zeer variabel zijn en op basis waarvan het influenza A virus verder onderverdeeld wordt in subtypen. Daaronder zit een lipide-laag, het membraan, waarin ook een transmembraan eiwit, matrix eiwit 2 (M2), zit. De binnenkant van de lipide laag wordt gecoat door matrix eiwit 1 (M1). De kern van het virusdeeltje bestaat uit het ribonucleoproteine (RNP) complex, waarvan het voornaamste eiwit nucleoprotein (NP) is. Andere eiwitten in het RNP complex zijn het polymerase basische eiwit 1 en 2 (PB1 en PB2) en het polymerase zure (acidische) eiwit (PA). Deze drie eiwitten vormen samen het RNA polymerase, dat een belangrijke rol speelt in de replicatie van virusdeeltjes. Daarnaast bevat een influenza virusdeeltje nog twee andere eiwitten: non-structureel eiwit 1 (NS1) en nucleair

export eiwit (NEP of NS2). In tegenstelling tot de twee oppervlakte eiwitten HA en NA zijn de interne eiwitten relatief geconserveerd tussen verschillende influenza subtypen.

Antigenic drift

De subtypering van influenza A virussen geschiedt op basis van de twee oppervlakte-eiwitten HA en NA. In totaal zijn er 18 HA subtypen bekend en 9 NA subtypen die met een HxNx code worden benoemd. Momenteel worden mensen alleen door de subtypen H1N1 en H3N2 geïnfecteerd en deze zijn dan ook de veroorzakers van de jaarlijkse griep epidemieën. Vogels daarentegen worden geïnfecteerd door een grote verscheidenheid aan subtypen en worden ook wel als het reservoir voor influenza A beschouwd. De HA en NA eiwitten veranderen voortdurend, dit komt doordat het RNA-polymerase complex fouten maakt tijdens de replicatie die niet hersteld worden. Hierdoor verandert de **aminozuurvolgorde** van de eiwitten wat ervoor zorgt dat het immuunsysteem deze eiwitten niet meer kan herkennen (antigenic drift). En is de eerder opgebouwde afweer door vaccinatie of een eerdere infectie niet in staat een nieuwe infectie tegen te gaan. Wel kan de gevormde afweer in staat zijn om de ziektelast te verlagen of het herstel te bevorderen.

Antigenic shift

Daarnaast is de opmaak van een influenza virusdeeltje zo ingericht dat virussen van twee verschillende subtypen kunnen combineren en zo een nieuw subtype kunnen vormen. Dit kan gebeuren als een cel geïnfecteerd is door twee verschillende virussen en dat wordt dan 'antigenic shift' genoemd. Met name varkens worden genoemd als gastheren waarin deze zogenoemde recombinatie kan plaatsvinden. De nieuwe subtypen die zo ontstaan vormen een groot gevaar voor de bevolking, aangezien er geen immuniteit tegen deze virussen is. De "Mexicaanse griep" is een voorbeeld van een infectie die van varkens op mensen is overgegaan en zo vele mensen kon infecteren. Daarnaast zijn er subtypen, zoals H5N1 en H7N9, die van vogels (veelal pluimvee en eenden) kunnen overgaan op de mens. Op dit moment zijn deze infecties beperkt gebleven tot bepaalde regio's omdat mensen alleen geïnfecteerd konden raken door direct contact met vogels, maar als het virus zich ook van mens tot mens kan verspreiden, is er een kans dat ze een pandemie veroorzaken.

Adaptieve immuunrespons

Eén van de belangrijkste beschermingsmechanismen tegen influenza virus infectie bestaat uit de humorale immuunrespons. Als influenza virusdeeltjes het lichaam binnen komen worden deze herkend door B cellen. Deze cellen worden hierdoor geactiveerd, wat leidt tot de ontwikkeling van plasma cellen. Deze cellen kunnen **antilichamen** produceren die in staat zijn om influenza viruspartikels te neutraliseren. Na infectie zal een deel van de B cellen zich **differentiëren** tot geheugen (memory) B cellen. Bij een volgende infectie met een soortgelijk virus zullen deze cellen geactiveerd worden, waarna er veel sneller een immuunrespons opgestart kan worden. Hierdoor kunnen antilichamen nog vele jaren na een eerste infectie (gedeeltelijke) bescherming bieden bij een her-infectie. In het geval van influenza virus

infectie of vaccinatie zijn het met name antilichamen gericht tegen de kop van het HA eiwit die infectie kunnen voorkomen en een neutraliserende bescherming induceren. Antilichamen gericht tegen NA kunnen wel de verspreiding van virus tegengaan, maar voorkomen niet infectie van cellen. Daarnaast zijn er ook antilichamen beschreven tegen geconserveerde delen van HA en tegen het ectodomein van het M2 eiwit (M2e). Hoewel ook deze antilichamen geen neutraliserende werking hebben, kunnen ze mogelijk bredere bescherming bieden omdat deze delen van de eiwitten minder aan verandering onderhevig zijn en dus tegen meer influenza varianten bescherming kunnen bieden.

Naast de humorale immuunrespons wordt ook de cellulaire immuunrespons in werking gezet. De cellulaire immuunrespons bestaat uit geactiveerde T cellen die op basis van oppervlaktemarkers onderscheiden kunnen worden in o.a. $CD4^+$ en $CD8^+$ T cellen. In het kort werkt een cellulaire respons als volgt: Een antigeen presenterende cel (APC), zoals een dendritische cel (DC), komt een virusdeeltje of gedeeltes van een virusdeeltje tegen en neemt deze op. Via een interne route wordt vervolgens een gedeelte van een viruseiwit (**peptide**) aan het oppervlakte van de APC gepresenteerd op een zogenaamd MHC-molecuul. De combinatie van dit peptide-MHC (pMHC) complex kan vervolgens herkend worden door virus-specifieke $CD4^+$ T cellen of door $CD8^+$ T cellen. $CD4^+$ T cellen die een langer peptide (>10 amino zuren) op een MHC klasse II molecuul herkennen, worden ook wel helper cellen genoemd. Ze produceren **cytokines** die APCs aanzetten tot betere antigeenpresentatie en cytokines die $CD8^+$ T cellen verder activeren. Daarnaast spelen $CD4^+$ T cellen een belangrijke rol in de humorale immuunrespons door B cellen te activeren om antilichamen te produceren. $CD8^+$ T cellen herkennen een peptide van 8-10 aminozuren lang op een MHC klasse I molecuul en zijn over het algemeen de cellen die uiteindelijk in staat zijn om het virus op te ruimen uit het lichaam. $CD8^+$ T cellen specifiek voor influenza antigenen zullen virus-geïnfecteerde cellen herkennen en zijn vervolgens in staat om door middel van cytokines en **granzymen** een virus-geïnfecteerde cel te doden.

Behandeling en vaccinatie

Als de natuurlijke immuunrespons niet in staat is om influenza virus te klaren, zijn er behandelingen mogelijk die de symptomen van griep verminderen. De meest gangbare middelen remmen de functie van het NA eiwit, waardoor het verspreiden van virus wordt tegengegaan. Echter, de standaard strategie om kwetsbare groepen zoals ouderen en individuen die om andere redenen vatbaar zijn, te beschermen tegen influenza virus infectie is een jaarlijkse griepvaccinatie. Het merendeel van de huidige seizoensvaccins die gebruikt worden in Nederland zijn zogenaamde subunit of split vaccins. Subunit vaccins worden tijdens de productie zo gezuiverd dat ze alleen HA en NA eiwitten bevatten. Split vaccins worden ook gezuiverd, maar bevatten naast de oppervlakte-eiwitten ook nog wat interne eiwitten, zoals M1 en NP. Deze vaccins worden ontwikkeld op basis van voorspellingen welk virusstam het volgende seizoen zal gaan circuleren. Ieder jaar bevat het seizoenale vaccin twee influenza A stammen en één influenza B stam. Na toediening van dit type vaccin worden

er voornamelijk antilichamen opgewekt tegen de oppervlakte-eiwitten in het vaccin. Gezien de continue verandering van de oppervlakte-eiwitten vindt er frequent (soms jaarlijks) een stamwijziging van het vaccin plaats. Daarnaast zal het vaccin minder goed werken als de voorspelling van de circulerende virussubtypen niet juist blijkt te zijn en kunnen mensen ondanks vaccinatie toch ziek worden. In deze gevallen zorgt vaccinatie dan vaak nog wel voor verminderde ziektelast. In het geval van split vaccins is het bekend dat er ook T cellen worden geactiveerd die voornamelijk delen van de meer geconserveerde interne eiwitten herkennen. Deze respons is echter niet afdoende om bescherming af te dwingen.

Aangezien influenza virus continue verandert en er een constante dreiging is van de introductie van een geheel nieuw influenza virus dat een pandemie zou kunnen veroorzaken, is er een grote vraag ontstaan naar de ontwikkeling van een nieuw griepvaccin die een bredere immuunrespons induceert. Een dergelijk vaccin zou bescherming kunnen bieden tegen drift varianten van de seizoenale griep en zou ook in staat zijn (gedeeltelijke) bescherming te bieden tegen pandemische griepvirussen. De strategie zoals beschreven in dit proefschrift gaat uit van het opwekken van een immuunrespons tegen geconserveerde delen van het griepvirus. Hierbij kan gedacht worden aan het opwekken van antilichamen tegen meer geconserveerde delen van het HA eiwit of tegen het extracellulaire domein van het M2 eiwit (M2e). In de ontwikkeling van dit type vaccins zullen juist ook T cellen een belangrijke rol spelen, aangezien dit type immuunrespons vaak al gericht is tegen de interne eiwitten van het influenza virus welke veel geconserveerde delen bevatten. Een voorbeeld van een methode waarmee een brede immuunrespons tegen dergelijke geconserveerde delen van het influenza virus kan worden geïnduceerd is peptidevaccinatie.

Proefschrift

Doel van het onderzoek

Het onderzoek beschreven in dit proefschrift kan in twee delen uiteen gezet worden. In het eerste deel werd inzicht verkregen in de immuunrespons tegen influenza virus na natuurlijke infectie en na vaccinatie. Daarnaast werd de rol die verschillende cellen daarin spelen onderzocht. Deze kennis vormde mede een basis voor de ontwikkeling van nieuwe influenza vaccin-concepten. Het tweede deel van dit proefschrift beschrijft de ontwikkeling van vaccin-concepten die in staat zijn brede bescherming tegen influenza A virussen te bieden.

T cel respons en peptide-vaccinatie concepten

In hoofdstuk 2 van dit proefschrift staat een literatuuronderzoek beschreven waarin het belang van een cellulaire immuunrespons bij verschillende virusinfecties (influenza, hepatitis C virus en humaan immunodeficiëntie virus (HIV)) wordt uitgelegd in het kader van de ontwikkeling van peptide vaccinatiestrategieën. Deze peptide vaccinatiestrategieën kunnen grofweg worden onderverdeeld in twee concepten: Vaccinatie met korte peptiden en vaccinatie met lange peptiden. Korte peptiden kunnen direct worden gepresenteerd door MHC klasse I moleculen, die aanwezig zijn op een groot deel van de lichaamscellen. Presentatie van het pMHC-complex aan $CD8^+$ T cellen kan deze cellen activeren waarna deze in staat zijn om virus-geïnfecteerde cellen te doden. Dat zoveel cellen MHC klasse I presenteren is ook meteen het nadeel, aangezien hierdoor $CD8^+$ T cellen suboptimaal geactiveerd kunnen worden, waardoor een ongewenste immuunrespons ontstaat. Lange peptiden daarentegen, moeten worden verwerkt door professionele antigeen-presenterende cellen, waarna peptiden zowel op MHC klasse I en MHC klasse II gepresenteerd kunnen worden wat resulteert in de mogelijkheid tot activatie van zowel $CD4^+$ als $CD8^+$ T cellen. Het voordeel van peptidevaccinatie strategieën is dat peptiden in het algemeen relatief goedkoop, eenvoudig en daardoor snel en zuiver geproduceerd kunnen worden.

Immuunrespons huidige influenza vaccins

Hoofdstuk 3 omvat een klinische studie waarin de immuunrespons van gezonde individuen na vaccinatie gedurende twee influenza seizoenen gevolgd is. Huidige influenza vaccins zijn ontworpen voor het induceren van neutraliserende antilichamen. Er is echter weinig bekend over de T cel responsen die door deze vaccins worden geïnduceerd, terwijl er steeds meer data beschikbaar komt over het belang van cellulaire responsen in het verminderen van ziektelast door influenza besmetting en in het reduceren van verspreiding. Om deze reden zijn in de studie beschreven in Hoofdstuk 3 zowel de humorale als cellulaire vaccin-specifieke responsen gemeten. Het eerste seizoen startte in 2009 tijdens de pandemie, waarin mensen gevaccineerd konden worden met een pandemisch vaccin, dat een **adjuvant** bevatte waardoor de dosis van het antigeen verlaagd kon worden en wat daarnaast ook de immuunrespons verbreedt. Aan het begin van het tweede seizoen werden deelnemers gevaccineerd met het seizoenale vaccin van 2010-2011 dat, naast twee influenza stammen die

voor het eerst aan het vaccin werden toegevoegd, ook de pandemische stam van het vorige jaar bevatte. Het pandemische vaccin van het eerste studie jaar werd, in tegenstelling tot seizoensvaccinatie, in twee doses aangeboden.

Het doel tijdens het eerste deel van de studie was om te bepalen of de tweede dosis nodig was voor de inductie van een beschermende immuunrespons en om de toegevoegde waarde van het adjuvant te onderzoeken. Daarnaast is bepaald of jaarlijkse vaccinatie een voordeel oplevert in de inductie van een immuunrespons door de immuunrespons van deelnemers te bepalen die beide seizoenen gevaccineerd zijn met individuen die niet of alleen in het eerste jaar gevaccineerd zijn. De belangrijkste bevindingen zijn dat één dosis van het pandemische vaccin leidde tot de productie van een hoeveelheid antilichamen die voldoet aan de standaard criteria voor influenza vaccins. Daarnaast was het vaccin, ondanks dat het alleen HA en NA eiwitten bevatte, in staat om T cellen te induceren. Een tweede dosis van het vaccin induceerde een hogere antilichaamrespons, terwijl de T cel respons gelijk bleef. Zowel de humorale als de cellulaire immuunrespons werden **geboosterd** door seizoenale vaccinatie in het daaropvolgende seizoen, wat impliceert dat herhaalde vaccinatie een voordeel oplevert. Daarnaast hebben we aangetoond dat het seizoenale vaccin ook in staat is om vaccin-specifieke T cellen te induceren, ondanks het ontbreken van een T cel-inducerend adjuvant.

Vaccinatie met lange peptiden

Hoofdstuk 4 beschrijft een studie van een lang peptide vaccin concept waarin muizen en fretten werden geïmmuniseerd met peptide vaccin-formuleringen die zowel B als T cellen kunnen activeren. Het B **epitoop** vaccin, gericht op de inductie van antilichamen, bevatte het zeer geconserveerde HA2 fusie peptide en het M2e peptide. Aangezien deze peptiden van zichzelf niet zo **immunogeen** zijn, werden deze peptiden gekoppeld aan een universeel CD4 helper epitoop. Het T epitoop vaccin bevatte 25 lange synthetische peptiden geselecteerd uit zeer geconserveerde delen van influenza eiwitten NP, PB1 en M1 met het doel virus-specifieke T cellen op te wekken die een breed spectrum van influenza virus varianten kan herkennen. Deze vaccins zijn getest in muizen en fretten. Muizen werden gevaccineerd met het B epitoop vaccin, de 25 lange peptiden of een combinatie van beide. Fretten werden gevaccineerd met het combinatie vaccin. Hiermee lieten wij zien dat het B epitoop vaccin in staat is om antilichamen tegen een geconserveerd deel van M2e op te wekken en dat deze antilichamen in staat zijn om virus te herkennen. Daarnaast hebben wij laten zien dat het T epitoop vaccin influenza-specifieke T cellen activeert. Een aantal dagen na vaccinatie werden muizen en fretten vervolgens gechallenged met influenza A virus. In de gevaccineerde dieren vonden wij een verlaging van de hoeveelheid virus in de longen. Dit zijn hoopvolle resultaten voor de verdere ontwikkeling van peptide-gebaseerde vaccinconcepten gericht tegen geconserveerde delen van influenza virus, aangezien een dergelijk vaccinconcept in staat kan zijn zowel ziektelast als virusverspreiding te verlagen gedurende een uitbraak van een pandemisch influenza virus.

Verbeteren immunogeniciteit korte peptiden

Zoals eerder beschreven zijn T cellen een belangrijk onderdeel van de immuunrespons tegen influenza infectie. Door middel van peptide vaccinatie kunnen **antigeen**-specifieke T cellen worden geïnduceerd, echter peptiden zijn van zichzelf laag immunogeen en niet of nauwelijks in staat een goede respons te induceren. In hoofdstuk 5 is de mogelijkheid beschreven om de immunogeniciteit van korte peptiden, specifiek voor het induceren van $CD8^+$ T cellen te verbeteren door modificaties in de peptide-sequentie aan te brengen. Deze modificaties hebben als doel om de binding tussen het peptide en het MHC klasse I molecuul te versterken. De theorie is dat een versterkte binding tussen peptide en MHC klasse I voor een langer durende presentatie aan $CD8^+$ T cellen zorgt, wat mogelijk resulteert in een verbeterde respons. Peptiden afkomstig van zeer geconserveerde delen van influenza virus werden geselecteerd op basis van hun natuurlijke binding, zodat een breed scala aan eigenschappen getest kon worden. Vervolgens hebben wij laten zien dat het inderdaad mogelijk is om door het modificeren van peptiden een verbeterde binding aan het MHC molecuul te bewerkstelligen en dat dit werkt voor zowel peptiden met een natuurlijk sterke binding en peptiden die normaal gesproken zwak binden. Daarna hebben wij door middel van immunisatie van muizen onderzocht of deze gemodificeerde peptiden ook tot een verbeterde T cel respons leidden. Belangrijkste bevindingen zijn dat gemodificeerde peptiden een hoger aantal cytokine-producerende $CD8^+$ T cellen induceren dan de corresponderende wildtype peptiden, met name de gemodificeerde peptiden afkomstig van zwak bindende peptiden. Hiermee hebben wij aangetoond dat modificatie een belangrijke manier is om de immunogeniciteit van T cel-inducerende peptide vaccins te verbeteren en dat dit concept breed toegepast kan worden.

Verbeteren aflevering van peptiden

Hoofdstuk 6 beschrijft een andere methode om de immunogeniciteit van $CD8^+$ specifieke peptiden te verbeteren. De voornaamste reden dat peptiden van zichzelf niet immunogeen zijn is doordat ze niet efficiënt aangeboden worden aan APCs. In dit hoofdstuk is de mogelijkheid van virosomen als methode voor het aanbieden van peptiden beschreven. Virosomen zijn in feite lege influenza virusdeeltjes waarin theoretisch allerlei soorten peptiden geladen kunnen worden. De immunogeniciteit en beschermende effect van peptide-beladen virosomen werd bepaald in muizen. Immunisatie van muizen met peptide-beladen virosomen in combinatie met een adjuvans liet een betere peptide-specifieke respons zien dan het peptide alleen. Daarnaast hadden muizen gevaccineerd met dit geadjuveerde peptide-beladen virosoom verminderd gewichtsverlies na besmetting met influenza virus. Deze resultaten bieden een basis voor het gebruik van influenza virosomen voor een nieuw type kruis-beschermend influenza vaccin.

Toevoeging peptide aan bestaand concept

Een vroeger veelgebruikt vaccin tegen influenza virus waren geïnactiveerde influenza virussen (WIV). Dit zijn complete influenza virusdeeltjes die zo behandeld worden dat ze

niet in staat zijn te repliceren en ook niet in staat zijn om zich te verspreiden. Aangezien ze alle influenza antigenen bevatten kunnen ze zowel antilichamen als T cellen activeren. Hoofdstuk 7 beschrijft WIV als adjuvant voor verschillende peptiden. WIV in combinatie met drie natuurlijke en drie gemodificeerde peptiden zoals beschreven in Hoofdstuk 5, was in staat om de respons van een aantal van deze peptiden te versterken zelfs als een zeer lage dosis WIV werd toegevoegd. Daarnaast werd aangetoond dat co-lokalisatie van peptide en WIV erg belangrijk was voor de inductie van een peptide-specifieke immuunrespons, wat het vermogen aantoont om WIV als adjuvant voor influenza peptiden te gebruiken. Het voordeel van dit concept is dat het voortborduurt op bestaande vaccinconcepten, waarvan de veiligheid bekend is.

Perspectief

Om een effectief vaccin tegen influenza te ontwikkelen is het in eerste instantie belangrijk om de respons tegen het virus goed te begrijpen. Van influenza virus is het bekend dat de virusdeeltjes te neutraliseren zijn door antilichamen gericht tegen het oppervlakte eiwit HA te induceren. Huidige griepvaccins gebruiken deze strategie dan ook om bescherming te kunnen bieden. Wij hebben laten zien dat er door deze vaccins ook T cellen worden geïnduceerd, die een bijdrage kunnen leveren aan het verminderen van ziektelast. Echter, deze vaccins zijn onder bepaalde condities niet effectief, waardoor mensen ondanks vaccinatie toch griep krijgen. Een van de belangrijkste redenen dat vaccins soms niet effectief zijn is de variabiliteit van het influenza virus zelf, waardoor de opgewekte immuniteit niet meer effectief is. Daarnaast is er een continue dreiging van virussen die vanuit bijvoorbeeld vogels op mensen kunnen overspringen. Als het virus zich vervolgens zo aan de mens aanpast dat infectie van mens op mens kan optreden is het een reële optie dat er een pandemie zal ontstaan. Door gebruik te maken van geconserveerde delen van het virus en hier een respons tegen op te wekken, zal een vaccin bescherming kunnen bieden tegen een breder scala aan influenza subtypen. In de ontwikkeling van een dergelijk vaccin spelen T cellen een grote rol aangezien deze vaak al gericht zijn tegen meer geconserveerde delen van influenza virus. Ook is bekend dat T cellen gericht tegen geconserveerde epitopen in staat zijn ziektelast te verlagen. Om deze reden is het wenselijk om een respons tegen dergelijke geconserveerde epitopen te induceren. Zoals beschreven in dit proefschrift is peptide vaccinatie een voorbeeld van een strategie die hiertoe in staat is, aangezien hiermee niet alleen T cellen maar ook B cellen gericht geïnduceerd kunnen worden. Hoewel er nog een lange weg te gaan is, kunnen de bevindingen in dit proefschrift bijdragen aan de ontwikkeling van de volgende generatie influenza vaccins die in staat zijn een brede kruis-reactieve bescherming te bieden in de vorm van zowel humorale als cellulaire responsen gericht tegen geconserveerde influenza epitopen.

Verklarende woordenlijst

Adjuvant: Een hulpstof die aan een vaccin kan worden toegevoegd om de immuunrespons tegen het antigeen te verbeteren.

Aminozuur: Een bouwstof van eiwitten, welke gecodeerd wordt door letters. De aminozuurvolgorde geeft aan in welke volgorde aminozuren aan elkaar zitten.

Antigeen: Een stof die in een organisme een immuunrespons kan doen ontstaan.

Antilichaam: Een eiwit die door plasma B cellen wordt uitgescheden en die als reactie wordt gevormd op antigenen die door vaccinatie of infectie het lichaam zijn binnengekomen.

Booster: Het voor de tweede keer tegenkomen van een antigeen door bijvoorbeeld herhaalde infectie of vaccinatie, wat tot een verbeterde immuunrespons kan leiden.

Cytokine: Een molecuul die een rol speelt in de immuunrespons en die in staat is om bepaalde receptoren op cellen te activeren. Er zijn verschillende soorten cytokines, elke met andere eigenschappen. Cytokines worden uitgescheiden door verschillende lichaamscellen.

Differentiëren: De ontwikkeling van een celtype, bijvoorbeeld van een cel die nog nooit antigeen heeft gezien (naïeve cel) tot een cel die actief optreedt tegen een antigeen.

Epidemie: Een situatie waarin veel mensen binnen een bepaald gebied tegelijk aan dezelfde ziekte lijden.

Epitoop: De minimale moleculaire structuur die door een B of T cel herkend kan worden. In het geval van T cellen, een peptide die in staat is om aan een MHC-I of MHC-II molecuul te binden en vervolgens herkend kan worden door de T cel receptor.

Granzym: Een enzym dat cellen doodt door geprogrammeerde celdood (apoptose) te veroorzaken in bijvoorbeeld virus-geïnfecteerde lichaamscellen. Granzymen kunnen worden uitgescheiden door verschillende immuuncellen.

Immunogeen: Immunogeniciteit geeft de mate aan waarin een antigeen in staat is een immuunrespons op te wekken.

Pandemie: Een wereldwijde verspreiding van een besmettelijke ziekte.

Peptide: Een keten van aminozuren.

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Curriculum vitae

Sietske Karla Rosendahl Huber was born on the 21st of July in 1987 in Heemstede. After graduating from the Sancta Maria Lyceum in Haarlem in 2005, she started her study Bio-Medical Sciences at the Vrije Universiteit (VU) in Amsterdam. During her bachelor internship at the department of Neuropathology of the VU Medical Center she studied the effect of statins on glial cells under the supervision of dr. Hedwich Kuipers. For the master specialization 'Infectious diseases' she performed an internship at the department of Medical Microbiology and Infection Control of the VU Medical Center, where she worked on the validation of a zebrafish screening method using a macrophage cell line under supervision of Esther Stoop and dr. Astrid van der Sar. Her second internship for the specialization 'Immunology' was performed at the Netherlands Vaccine Institute under supervision of Inonge van Twillert and dr. Cécile van Els and involved investigating the role of inhibitory receptors on pertussis-specific T cell clones. In July 2010 she obtained her Master's degree from the Vrije Universiteit (VU) Amsterdam and in October 2010 she started her PhD project on peptide vaccination strategies at the Centre for Infectious Disease Control of the National Institute of Public Health and the Environment (RIVM), which resulted in this thesis. From May 2015 she started working at TNO Triskelion as a junior study director immunology.

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