Formulation of influenza T cell peptides: In search of a universal influenza vaccine

Peter Soema

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About the cover: A combined graphic of a T cell peptide and the three adjuvants central in this thesis: whole inactivated influenza virus, virosomes and liposomes.

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Chapter 1

General introduction and thesis outline

INTRODUCTION

Influenza

The influenza virus is a negative-stranded ssRNA virus of the *Orthomyxoviridae* family. Influenza viruses are able to infect numerous species, which include humans, birds, pigs, dogs and horses. In humans, influenza viruses infects via the respiratory tract. Symptoms of influenza infection include coughing, rhinitis, headache, fever, chills, muscle pain and fatigue. Severe cases of influenza infections may lead to primary viral pneumonia, secondary bacterial pneumonia and sinus infections, which are potentially lethal. Influenza epidemics occur annually in the northern (October through May) and southern (May through October) hemispheres. Estimations of the World Health Organization (WHO) indicate that annually, around 3 to 5 million influenza cases lead to severe illness and 0.5 million cases lead to influenza-associated death. Furthermore, each influenza season has considerable financial consequences, from hospitalization and treatment costs, to costs involved with sick personnel. Thus, for both health and economic reasons, it is essential that influenza infections are prevented.

Influenza vaccination

Vaccination is the only way to prevent influenza infections. Today, influenza vaccines are adapted each season to match circulating strains as predicted by worldwide epidemiological monitoring. These vaccines elicit antibodies specific to the main surface proteins of influenza, hemagglutinin and neuraminidase (Figure 1). However, these proteins are highly variable due to random genetic mutations (antigen drift) or genetic reassortment (antigenic shift). Antigenic drift is a continuous process that leads to small antigenic changes within an influenza subtype. However, antigenic shift is a complete genetic reassortment of the influenza subtypes, and vaccine-elicited immune responses are generally unable to prevent infection of influenza viruses that do not match with the vaccine strain. The greatest concern currently is the emergence of a pandemic influenza strain, which is created by recombination of influenza strains hosted by different species. Since current influenza vaccines lack cross reactivity, a pandemic can only be managed by antiviral drugs. While these antivirals are effective, increasing numbers of resistant influenza virus strains, including prepandemic H1N1, have been reported since 2007, casting serious doubt on the effectiveness of these antivirals during future influenza outbreaks. Increasing the cross reactivity of influenza vaccines is therefore an important goal in influenza vaccine development.

T cell-based influenza vaccines

To address the lack of cross reactivity of current influenza vaccines, several novel vaccine approaches are currently being pursued. One of these approaches is the induction of influenza-specific T cells that recognize conserved epitopes located on internal proteins. Such T cells have the potential to recognize any influenza virus, regardless of antigenic shift or drift. Peptide antigens derived from influenza proteins can be used to induce such T cells. However, peptides are poorly immunogenic. Multiple factors contribute to this poor immunogenicity: peptides are easily degraded, they are not adequately recognized and taken up by antigen presenting cells (APCs), and lack immunostimulatory

signals to activate APCs. Additional formulation of peptide antigens is thus needed to increase the immunogenicity of the peptides and to make an effective T cell-based influenza vaccine. In this thesis, several formulation strategies for peptide-based influenza vaccines were investigated.

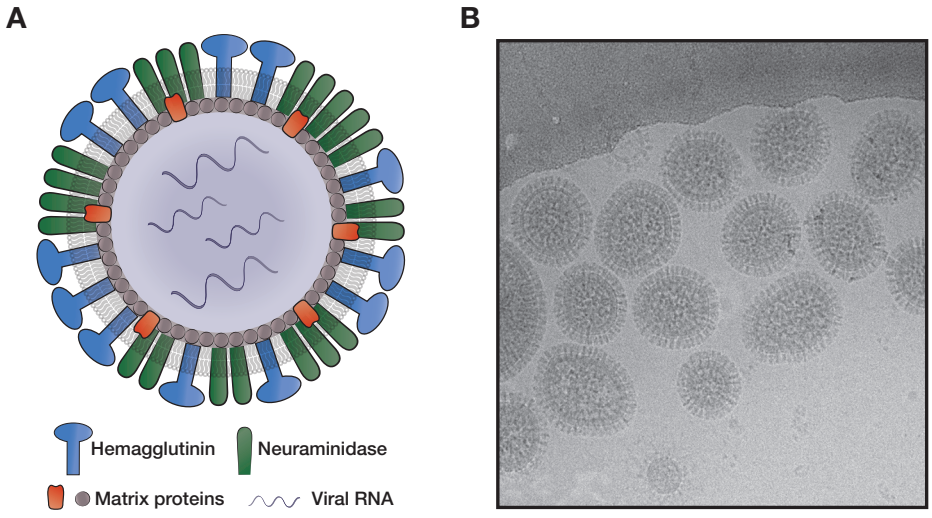

Figure 1. Schematic drawing of an influenza virus particle (A), and a cryogenic transmission electron microscopic image of inactivated influenza virus particles (B).

THESIS SCOPE AND OUTLINE

The main objective of this thesis is to investigate and develop novel formulations for peptide-based influenza vaccines that ultimately could be used as universal influenza vaccines. Several formulation approaches are evaluated in this thesis. Furthermore, the current landscape of influenza vaccine research in general is assessed and the feasibility of T cell-based influenza vaccines in particular is reviewed.

In **Chapter 2** the status of current and future influenza vaccines is reviewed. The limitations of current seasonal vaccines and possible solutions to these limitations are addressed. Immunological and formulation aspects play important roles in these solutions, and are highlighted in this review. Finally, the production of current influenza vaccines and the production feasibility of future influenza vaccines are discussed.

In **Chapter 3** the development of peptide-loaded virosomes, a T cell-based influenza vaccine, is described. Peptide-loaded virosomes were produced from whole inactivated influenza virus (WIV) and synthetic T cell peptides. Physicochemical characteristics such as peptide loading efficiency, protein content of virosomes and membrane fusion capacity of the peptide-loaded virosomes were studied. The immunogenicity and protection against virus challenge of peptide-loaded virosomes with or without adjuvant was evaluated in HLA-A2.1 transgenic mice. In addition, the ability of virosomes to deliver peptides was assessed in a human dendritic cell (DC) model and in mice.

Chapter 4 presents the use of WIV as an adjuvant for influenza T cell peptides. The adjuvant effect of WIV in combination with a T cell peptide was investigated in HLA-A2.1 transgenic mice in a proof-of-principle study. Subsequently, a dose response study was carried out by varying both WIV and peptide doses. A DoE approach was used to identify potential synergistic effects between WIV and peptide doses. Moreover, the effect of WIV and peptide co-localization and membrane fusogenicity of WIV on the immunogenicity was investigated in the same transgenic mice. Finally, WIV was used as an adjuvant for mixtures of either three wild type influenza peptides or three chemically modified influenza peptides.

A novel method to develop optimized liposomal formulations for peptide antigens is described in **Chapter 5**. Using a DoE approach, the influence of liposomal lipid composition on liposomal size, surface charge and liposome-induced DC maturation was investigated. Four different lipids were varied during the formulation of liposomes. The liposomes were tested in a human DC maturation model and the influence of the liposomal lipid composition on the expression of DC maturation factors was modeled. Finally, the accuracy of the generated prediction model was evaluated.

In **Chapter 6**, the latest developments in T cell-based influenza vaccine research are reviewed. The immunological steps of T cell activation are presented and an overview of T cell-based vaccines in both preclinical and clinical development is given. Furthermore, the need for cellular correlates of

protection for influenza vaccines and the risks and limitations associated with T cell-based influenza vaccines are discussed.

An alternative delivery system for influenza vaccines, the Bioneedle, is presented in **Chapter 7**. Bioneedles were filled with either subunit, split, virosomal or WIV influenza antigens, stabilized with trehalose. Antigen-filled Bioneedles were implanted in C57BL/6 mice and influenza-specific humoral and cellular responses were evaluated and compared to those induced by intramuscular or subcutaneous administered influenza vaccines. In addition, the thermostability of vaccine-filled Bioneedles and conventional liquid vaccines were assessed at temperatures up to 60°C.

Chapter 8 summarizes the findings and conclusions of this thesis. The prospect of T cell-based influenza vaccines and alternative delivery systems for influenza vaccines are discussed.

Chapter 2

Current and next generation influenza vaccines: Formulation and production strategies

Peter C. Soema [1,2], Ronald Kompier [1,3], Jean-Pierre Amorij [1] and Gideon F. A. Kersten [1,2]

1 Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands.
2 Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.
3 FluConsult, Noordwijk, The Netherlands.

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ABSTRACT

Vaccination is the most effective method to prevent influenza infection. However, current influenza vaccines have several limitations. Relatively long production times, limited vaccine capacity, moderate efficacy in certain populations and lack of cross-reactivity are important issues that need to be addressed. We give an overview of the current status and novel developments in the landscape of influenza vaccines from an interdisciplinary point of view. The feasibility of novel vaccine concepts not only depends on immunological or clinical outcomes, but also depends on biotechnological aspects, such as formulation and production methods, which are frequently overlooked. Furthermore, the next generation of influenza vaccines is addressed, which hopefully will bring cross-reactive influenza vaccines. These developments indicate that an exciting future lies ahead in the influenza vaccine field.

INTRODUCTION

Influenza viruses are negative stranded RNA viruses of the *Orthomyxoviridae* family. Three types of influenza viruses, influenza A, B and C, are capable of infecting humans, of which influenza A and B are the most common circulating types. Individuals infected with influenza virus generally display symptoms such as chills, fever, headache, muscle pain, fatigue, rhinitis and coughing. Progressed influenza infections can lead to severe complications including bronchitis, pneumonia, secondary bacterial infections, acute respiratory distress and cardiovascular complications, which all can lead to death if left untreated. Individuals with a weakened immune system, such as immunocompromised patients, elderly and young children [1-3], are particularly vulnerable to influenza infections and are thus classified as high-risk populations.

Global influenza epidemics emerge seasonally and typically occur during the winter seasons of the northern and southern hemispheres. The WHO estimates that there are 3-5 million cases of severe influenza infections annually, with 250.000-500.000 deaths globally. The reemergence of a pandemic H1N1 strain in 2009 [4], and the emergence of highly pathogenic avian H5N1 and H7N9 influenza viruses [5, 6], has reaffirmed that influenza remains a global threat to this day.

Vaccination against influenza is the most cost-effective method to prevent influenza infections. Fast availability of influenza vaccines to the world population is one of the key factors for effective coverage against seasonal and pandemic influenza. Despite the fact that influenza vaccines are on the market since the 1930s, several limitations still exist involving both their availability and their effectiveness, which are listed in Table 1.

Current influenza vaccines are predominantly produced by egg-based production methods. Being dependent on the supply of vaccine-quality eggs, vaccine manufacturers cannot be flexible in the amount of doses produced. This can lead to vaccine shortages, especially during pandemic situations. Alternative production platforms, such as cell culture-based vaccine production, plant-based vaccine production or synthetic vaccines, could increase the flexibility of manufacturers. It is often thought that these novel production methods decrease the time needed to develop and release an influenza vaccine. However, the availability of strain-specific reagents for vaccine potency and release tests such as the single radial immunodiffusion (SRID) assay and subsequent clinical trials are the main factors that delay the commercial release of influenza vaccines.

Directly tied to the commercial release of influenza vaccines are the regulatory approval procedures. To speed up these procedures, mock-up vaccines are developed to generate a registration dossier, which can subsequently be used for the licensing of an actual seasonal or pandemic influenza vaccine.

Limited vaccine availability is not only caused due to the inflexibility of the vaccine production capacity; especially not in developing countries. Technology transfer of production methods

to developing countries increases the worldwide vaccine production capacity. Increasing the (heat) stability and shelf life of influenza vaccines negates the need of a cold chain, which is imperfect in developing countries. This prevents unnecessary vaccine loss. Furthermore, decreasing antigen dose by the addition of adjuvants can also increase the number of influenza vaccines. Development of stabile vaccine formulations and effective adjuvants is thus important.

In several population groups, such as unprimed young children, the elderly and immunocompromised individuals, influenza vaccines have limited efficacy. Unprimed individuals have a reduced response to influenza vaccines, whereas elderly, due to immunosenescence, and immunocompromised individuals generally suffer from a declined immune function. Increasing the immunogenicity and breadth of the immune response elicited by influenza vaccines might improve vaccine efficacy in these vulnerable groups.

Current influenza vaccines induce neutralizing antibodies against the viral membrane surface proteins hemagglutinin (HA) and neuraminidase (NA). Due to antigenic shift and drift of HA and NA genes, neutralizing antibodies elicited by influenza vaccines lack cross-reactivity against non-matching influenza strains. While seasonal adjustments to the vaccine strains are made to cope with this problem, it is not as convenient and fast as a potential cross-protective influenza vaccine. Thus, the identification of alternative correlates of protection (CoPs) against influenza is an important step towards the development of cross-reactive influenza vaccines.

Table 1. Limitations of current influenza vaccines and potential solutions.

Limitations	Potential solution(s)
Dependence on egg-based production	- Cell culture-based production of virus - Recombinant antigens - Synthetic vaccines
Regulatory approval procedures	- Mock-up vaccines to generate regulatory dossier
Limited worldwide vaccine availability	- Technology transfer of vaccine production methods - Dose sparing by the addition of adjuvants or alternative administration routes - Increase stability and shelf life of vaccines to prevent vaccine loss in unfavorable conditions
Limited efficacy in elderly and unprimed populations	- Increase vaccine immunogenicity by increasing antigen dose, the addition of adjuvants or using alternative administration routes - Increase breadth of immune response by the addition of adjuvants, alternative administration routes or by inclusion of novel antigens
Lack of cross-reactivity by current vaccines	- Vaccines inducing stalk-reactive antibodies - M2e-targeted vaccines - T cell inducing vaccines - Heterologous prime-boost strategies with seasonal and cross-reactive vaccines

The aforementioned limitations of current influenza vaccines may be resolved through the implementation of new technologies in the field of influenza production and vaccine formulation. Novel antigens often require novel production methods, which carry their own advantages and disadvantages. Additionally, these novel antigens often need to be formulated with excipients and adjuvants to be sufficiently immunogenic. While important, the development of alternative administration methods and devices for influenza vaccines is not within the scope of this current review, and has been thoroughly reviewed by Amorij et al previously [7]. In this review, we will discuss advances in immunological, formulation and production aspects for current and promising novel influenza vaccine antigens, and discuss their potential to solve the limitations of influenza vaccines today.

IMMUNE RESPONSES AGAINST INFLUENZA

The efficacy of current influenza vaccines is determined by the presence of adequate hemagglutination inhibition (HI)- or virus neutralization (VN)-titers in vaccinated individuals. HI titers indicate antibody responses against HA, which are not cross-reactive, and do not protect against mismatching influenza strains. Ideally, an influenza vaccine would protect against all strains, uninfluenced by antigenic changes. VN titers indicate antibody responses that are able to neutralize influenza virus, and thus can potentially be applied for cross-reactive vaccines. Nonetheless, identification of alternative CoPs, such as cross-reactive antibodies or T cell responses would significantly aid the development of universal vaccines [8].

Induction of immune responses against novel and more conserved epitopes, other than the variable epitopes of HA, has come under the attention in recent years (Figure 1). These include vaccines that induce antibodies directed against stalk regions of HA and matrix protein 2 ectodomains (M2e), and vaccines that induce cellular responses against internal influenza proteins. These vaccine could potentially be the basis of a universal influenza vaccine.

HA-specific antibodies

Antibodies against HA can be divided into categories: those reactive against the globular head domain, and those reactive to the stalk domain. Current influenza vaccines induce mainly antibodies directed against the head domain, which is highly variable due to antigenic drifts. In contrast, the stalk domain is more conserved, which makes it an attractive target for the induction of a cross-reactive humoral response. Certain stalk-reactive antibodies, such as globular head-reactive antibodies, inhibit the virus attachment to cell membranes [9], thereby preventing infection (Figure 1A). Other stalk-reactive antibodies disrupt viral membrane fusion (Figure 1B), preventing endosomal escape of the virus. Indeed, several monoclonal antibodies directed against these stalk domains proved to be effective, and are currently under development to provide therapeutic treatment of acute influenza infections [10].

Several HA stalk-directed vaccines are currently under development, which proved effectiveness against both influenza A group 1 and 2 viruses [11], as well as influenza B. However, the potential side effects of these antibodies still need to be carefully evaluated. Khurana et al showed that HA2 stalk-reactive antibodies promoted viral fusion and respiratory disease symptoms by pH1N1 influenza in pigs [12], indicating that the induction of stalk-reactive antibodies is not without risk. Further clinical studies should determine whether stalk-reactive antibodies are suitable for protection against influenza infection.

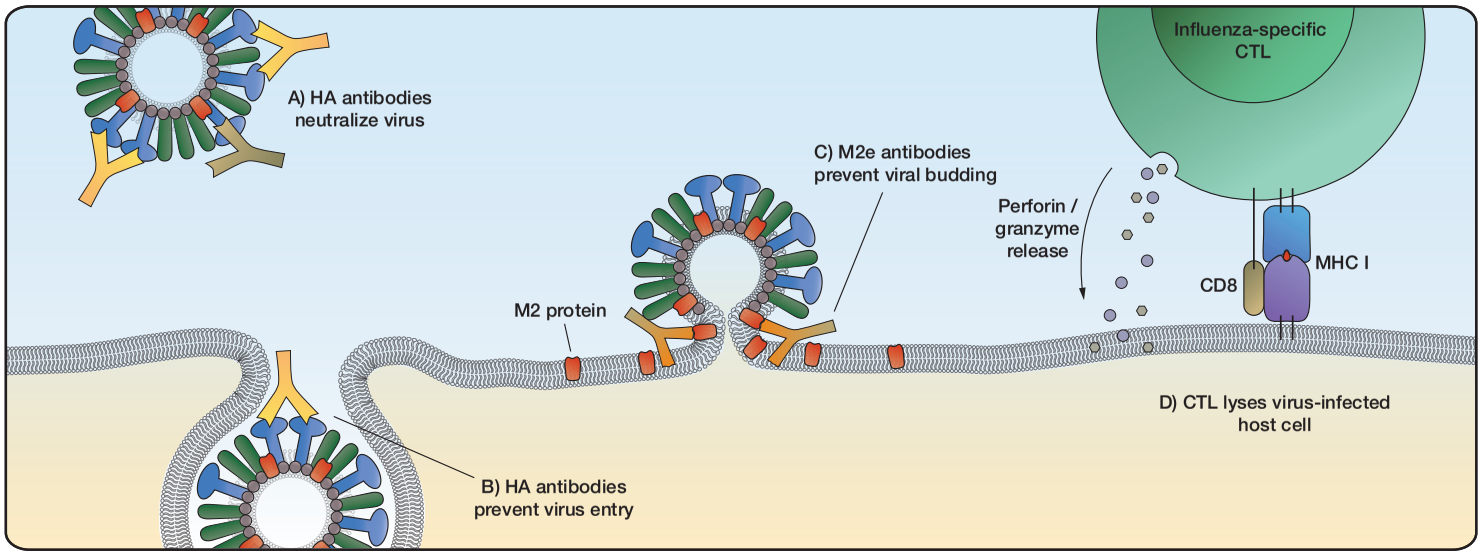

Figure 1. Immune responses against influenza virus. (A) HA head- or stalk-reactive antibodies neutralize the virus. (B) HA-specific antibodies prevent virus attachment and entry to host cells. (C) M2e-specific antibodies prevent the budding and release of virus particles produced in infected host cells. (D) Cytotoxic T cells recognize epitopes from internal influenza proteins presented on the host cell surface by MHC-I molecules, and subsequently lyse the infected host cell through perforin and granzyme release.

Matrix protein 2 ectodomain-specific antibodies

Matrix protein 2 (M2) is a tetrameric transmembrane protein that acts as a proton-selective ion channel. It plays a crucial role in the acidification and subsequent destabilization of the viral membrane, which facilitates the release of the genetic material of the virus into the host cell. The M2 protein is, except in low amounts in whole inactivated virus (WIV) and live attenuated influenza virus (LAIV) vaccines, not included in current seasonal vaccines; M2-specific antibodies are generally not detected in subjects vaccinated with seasonal influenza vaccines. Nonetheless, it possesses a sequence of amino acids that is highly conserved among influenza subtypes, located on the N-terminal ectodomain.

Since M2e is conserved among influenza subtypes, it is a potential target for cross-reactive immune responses. M2e is expressed abundantly by influenza-infected host cells [13], and M2e-specific antibodies are able to efficiently mark these cells for phagocytosis by natural killer cells or macrophages through antibody-dependent cellular cytotoxicity (ADCC) [14]. Furthermore, M2e-specific antibodies disrupt the budding process of viral particles, preventing virus release from infected host cells (Figure 1C). Thus, M2e vaccines do not prevent viral infection, but efficiently inhibit viral replication once inside the host. Several vaccine concepts utilizing M2e-derived antigens are currently being evaluated as universal influenza vaccines.

T cell responses

Cellular immune responses appear to play an important role in the cross-protective immune response against influenza virus [15]. $CD8^+$ T cells (CTLs) can actively eliminate infected cells through perforin-mediated cell lysis (Figure 1D), but also exhibit other effector activities such as Fas ligand- and TRAIL (TNF-related apoptosis-inducing ligand)-mediated cytotoxicity and cytokine secretion, which all contribute to the protective cellular immune response against influenza infections [16]. Recently, several clinical studies correlated cellular responses with a decrease of influenza-related illness, indicating that influenza-specific cellular responses might be an alternative CoP for influenza. Sridhar et al. showed that individuals which possessed preexisting $CD8^+$ T cells displayed decreased morbidity after infection with pH1N1 influenza, underlining the cross-reactivity of $CD8^+$ T cells [17]. Wang et al. found that patients infected with H7N9 required prolonged hospitalization in the absence of early $CD8^+$ T cell responses, whereas patients with early $CD8^+$ T cell responses recovered quickly [18]. Additionally, Wilkinson et al. showed that influenza specific $CD4^+$ T cells decreased viral shedding and illness in individuals infected with pH1N1 in the absence of influenza-specific antibodies [19]. A novel vaccine concept based on the induction of influenza-specific T cells, MVA-NP+M1, reduced symptoms and viral shedding of individuals infected with influenza, demonstrating that such an approach has merit [20].

Most T cell epitopes, which are highly conserved, are located on internal influenza proteins such as nucleoprotein (NP), matrix protein 1 (M1) or polymerase subunits (PA, PB1 and PB2). While these antigens are not very immunogenic, several formulation strategies have been utilized to successfully induce influenza-specific T cell responses. The induction of influenza-specific cellular responses might be a great addition to current antibody-inducing influenza vaccines.

CURRENT INFLUENZA VACCINES

Current seasonal trivalent influenza vaccine (TIV) formulations contain either inactivated influenza antigens or live attenuated influenza viruses, derived from two influenza A strains and one influenza B strain. Next to TIV formulations, quadrivalent influenza vaccine (QIV) formulations have entered the market recently, which adds an additional influenza B strain. Additionally, several pandemic vaccines have been developed in the preparation of possible future outbreaks of highly pathogenic influenza strains. These vaccines, which are all currently in the market, will be examined below.

Inactivated influenza vaccines

Inactivated influenza vaccines comprise either whole inactivated virus, split, virosomal or subunit antigen, all differing in either structural organization or viral components (see Figure 2). WIV vaccines were the first to be used in widespread annual influenza vaccination campaigns. However, these WIV formulations caused local and systemic adverse effects upon administration [21]. This was possibly due to the presence of impurities, such as egg proteins, in the vaccine. WIV vaccines were therefore mostly abandoned when split vaccines entered the market, which were considered to be less reactogenic. However, the use of current vaccine production technologies results in better defined and pure WIV vaccines than previously, which give rise to very low levels of side effects [22].

Nowadays, influenza vaccines usually consist of either split viruses or subunit influenza antigens. Split vaccines are influenza virus particles disrupted by diethyl ether or detergent treatment. While split vaccine still contains all viral proteins, the original viral particulate organization and viral ssRNA are mostly lost, losing some of the inherent immunogenicity of the virus [23]. Split viruses are currently widely used in TIV formulations, due to their adequate immunogenicity and relative ease of production. Aside from standard intramuscular (i.m.) split vaccines, an intradermal (i.d.) influenza split vaccine is currently licensed, which was proven to induce non-inferior immune responses as a dose of 9 µg HA compared to the standard 15 µg HA in adults [24]. This dose-sparing effect is likely to be mediated by the high density of antigen presenting cells (APCs) in the skin [25]. In contrast, elderly still require a normal dose of 15 µg when receiving an i.d. influenza vaccine.

Subunit antigens, that is HA and NA proteins, are also frequently used in TIV formulations. HA and NA proteins are separated from the viral nucleocapsid and lipids after diethyl ether or detergent splitting. However, the addition of adjuvants to the antigens is sometimes required to reach adequate immunogenicity in the elderly [26]. Recently, a recombinant HA (rHA) subunit vaccine has entered the market, which contains a high dose (45 µg per strain) of antigen to reach the required immunogenicity. The administration of a higher dose of rHA compared to other non-recombinant TIV formulations resulted in higher seroconversion rates in healthy adults and the elderly [27], but lower efficacy rates in children [28]. Therefore, rHA vaccines need additional formulation with adjuvants to optimize immunogenicity in children.

In addition to split and subunit vaccines, virosomal TIV formulations have been used mainly in EU countries since 1997 [29]. Virosomes are reconstituted influenza virus envelopes consisting of HA, NA and viral phospholipids. Their particulate structure enables virosomes to retain viral membrane fusion and cell-binding capabilities, which could increase their immunogenicity compared to subunit and split vaccines.

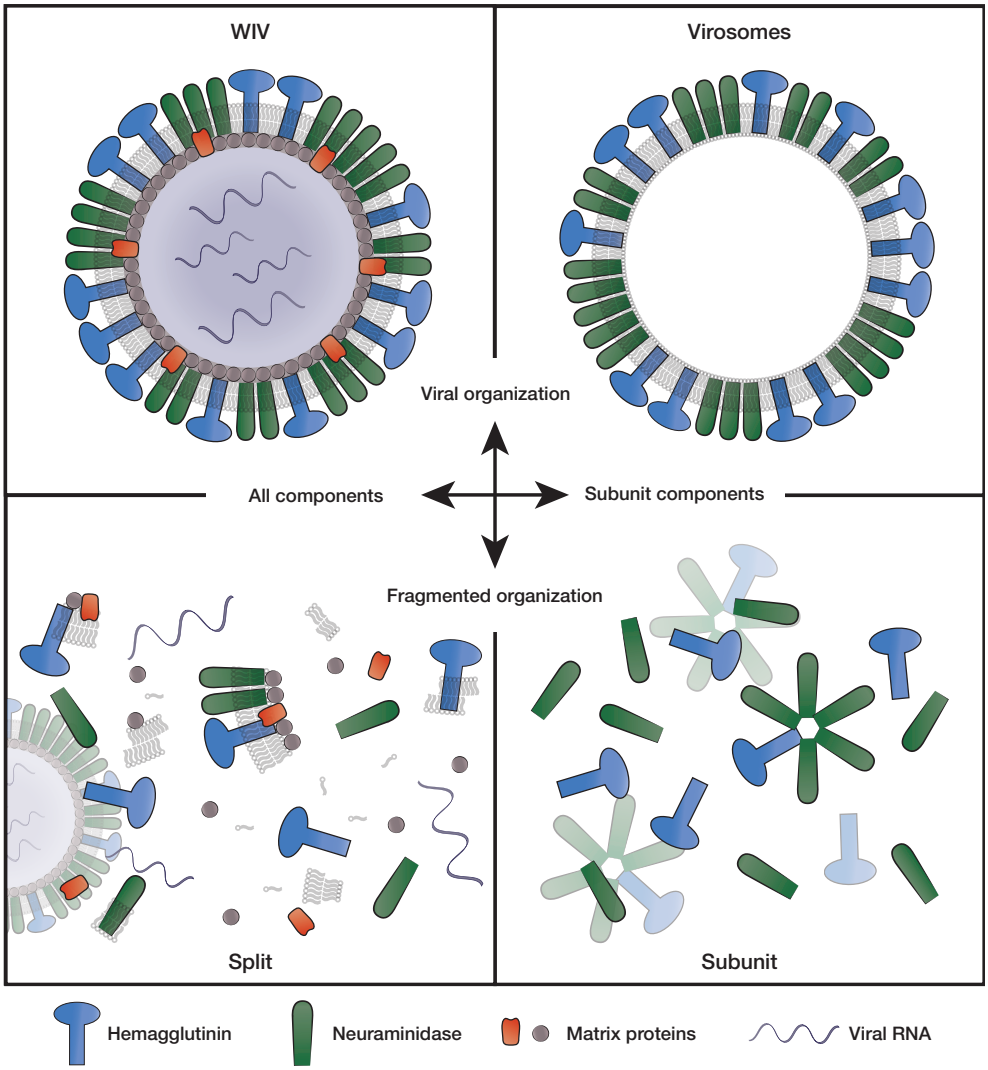

Figure 2. Composition of inactivated influenza vaccines. The four different compositions of influenza vaccine differ in antigen components and structural organization. These differences also have an impact on the immunogenicity of the vaccine.

In the literature, there are many studies which state that there are differences in immunogenicity and safety between the different inactivated influenza vaccine formulations. However, meta-analyses show that they all are similarly immunogenic and safe [30, 31]. Individual studies often compare vaccines of a single season, which might give a limited view on these formulations.

Live-attenuated influenza vaccines

Aside from inactivated influenza vaccines, there are also live-attenuated influenza vaccines. Intranasal administration of LAIV mimics the natural route of infection of influenza, resulting in a localized mucosal immune response at the site of infection [32]. In contrast to inactivated vaccines, LAIV induces strong mucosal IgA responses and cell-mediated immune responses, which are effective at preventing influenza infection [33]. While proven effective, the use of LAIV raised two major concerns. The virus in LAIV can theoretically undergo genetic reversion into a pathogenic, transmissible influenza strain. However, this event has yet to be proven, and is unlikely to ever happen [34]. The second concern is the use of LAIV in young children, which caused wheezing in infants under 2 years. Therefore, LAIV is currently approved for use in children and adults between 2 and 49 years old.

Quadrivalent influenza vaccines

In recent years, more focus has been laid on including a second influenza B strain in the seasonal influenza vaccine. Two distinct influenza B lineages have been circulating since 1985, thereby decreasing the efficacy of TIV, which only includes one influenza B strain [35]. Indeed, five strain mismatches have occurred between 2001 and 2011, indicating that inclusion of an additional influenza B strain, resulting in a quadrivalent influenza vaccine, has become necessary. The first QIV (a LAIV formulation) entered the market in 2012, and several other QIV formulations based on inactivated vaccine formulations, such as split and subunit formulations, have been licensed since. Several manufacturers continue to develop novel QIV formulations, expanding the market share of quadrivalent influenza vaccines.

Pandemic influenza vaccines

In the last decade, the global outbreaks of H5N1 and H1N1 influenza viruses have increased the demand for pandemic influenza vaccines. Both WIV and split antigens have been used (with or without adjuvants) for the development of pandemic vaccines. While WIV is infrequently used in seasonal influenza vaccines, it is used in pandemic vaccines due to its high intrinsic immunogenicity. During the H1N1 pandemic of 2009, several adjuvanted and non-adjuvanted pandemic vaccines were widely used [36, 37]. In addition to H1N1 vaccines, several pandemic H5N1 mock-up vaccines have been currently licensed. Mock-up vaccines are developed to generate a registration dossier, which can subsequently be used for the licensing of an actual pandemic vaccine after inclusion of a pandemic vaccine strain. This could speed up the regulatory approval process in case of a pandemic.

LAIV formulations are also considered as a pandemic vaccine candidate, since they elicit strong local mucosal and cellular immune responses. Chen and Subbarao summarized the preclinical development of prepandemic live-attenuated influenza vaccines against H5N1 previously [38]. While these pandemic vaccines are effective against their matched strains, they still generally lack cross-reactivity against heterosubtypic strains.

FORMULATION STRATEGIES FOR INFLUENZA VACCINES

With the advent of novel concepts for immunity against influenza, as described above, novel types of antigens such as recombinant proteins, viral vectors, peptides and DNA are under development. Many of these antigens are poorly immunogenic, and thus need advanced formulation with adjuvants (immunopotentiators and delivery systems) to become sufficiently immunogenic [39]. Most of these concepts aim to be universal influenza vaccines, and thus need to induce cross-protective immune responses. Aside from increasing and steering the immunogenicity, formulation of antigens with excipients can increase vaccine stability for unfavorable conditions such as elevated temperatures and freezing [40], thereby preventing the loss of vaccines. In the following paragraphs we will discuss potential novel antigens and adjuvants for influenza vaccines, as well as formulation methods to stabilize them.

Recombinant antigens

The use of recombinant technology enables the production of a wide array of influenza protein antigens that can induce different immune responses. These include not only conventional antibody responses against HA, but also immune responses against HA stalk regions and M2 ectodomains, which are potentially cross-reactive.

Recombinant antigens are the main type of antigen to induce HA stalk-specific antibodies. Recombinant headless HA2 protein was expressed on virus-like particles (VLPs) [41], which induced cross-reactive antibodies that showed immunogenicity against heterologous influenza strains in mice. Recombinant VLPs were also utilized to present the A-helix domain of HA2 [42], which were able to induce stalk-reactive antibodies that recognized several influenza group 1 and 2 HA subtypes. Next to recombinant VLP antigens, nanoparticles were used to increase the immunogenicity of recombinant HA ectodomains. HA ectodomains were fused to ferritin nanoparticles [43], which induced high antibody titers in mice to both the globular head and stalk domains of HA.

Recombinant proteins are being widely used to induce M2e-specific immune responses [14]. Purified recombinant M2e proteins (in a multimeric state) were also combined with several adjuvants to induce M2e-specific antibodies [44]. Vaccines with covalently bound M2e antigen to a carrier protein or adjuvant could induce potent cross-protective immune responses in mice. Some studies reported a shift to IgG2a as the predominant IgG subtype [45, 46], indicating a skewing towards T_H1 responses, which support the induction of cytotoxic lymphocytes. This additional immune response could further broaden the protection of these vaccine concepts.

Viral vectors

Recombinant technology is applied to engineer replication-deficient viral vectors, which produce influenza antigens once administered in the host. These vectors are usually immunogenic and can display multiple antigens. One of the most studied viral vectors is Modified Vaccinia virus Ankara (MVA), which has already been used to express multiple influenza antigens such as HA, M2e, M1

and NP [47]. One of the most promising influenza vector vaccines is MVA-NP+M1, which was able to elicit potent T cell responses in both healthy adults and elderly individuals in multiple phase I clinical trials [20, 48]. These studies did not find any severe adverse effects associated with the vector vaccine, other than an increased reactogenicity profile. MVA-NP+M1 could therefore be a possible universal influenza vaccine in the future.

Adenoviruses are other viral vectors used to engineer influenza vaccines. Clinical studies with adenoviruses expressing either HA or NP+M1 have been performed successfully [49, 50], indicating that adenoviruses are also a suitable vector platform for influenza vaccines.

The possibility of anti-vector immunity remains one of the risks involved with vector-based vaccines, since it could induce tolerance to the vaccine. Indeed, both humoral and cellular vector-specific immune responses negatively impacted the efficacy of a HIV vaccine based on a adenovirus vector in a clinical trial [51], indicating the importance of monitoring for anti-vector immunity in such studies.

Peptides

Peptides can be used for the induction of both influenza-specific immune B cell and T cell responses against conserved epitopes. Peptide antigens can either be minimal epitopes, which generally suffer from poor immunogenicity, or long peptides which are composed of multiple epitopes [52].

Several preclinical studies have used minimal epitope peptides as their main antigen to induce influenza-specific cellular responses. Liposomes have proven to be effective adjuvants for these peptides in numerous studies. $NP_{366-374}$ peptide encapsulated in liposomes was able to induce potent T cell responses in the presence of anti-CD40 mAbs, and reduced viral lung titers of influenza-infected mice [53]. HLA-A2.1 and HLA-A24.2 restricted peptides conjugated to liposomes were able to minimize morbidity in influenza-infected mice [54, 55]. Remarkably, these peptide-liposome conjugates were able to induce $CD8^+$ memory T cells without contribution of $CD4^+$ T cells, which are thought to be crucial for the support of effective CTL responses [56]. Conjugation of an influenza PA-derived peptide to Pam2Cys, a bacterial lipopeptide and natural PAMP, efficiently induced peptide-specific CTL responses, which reduced viral lung titers in influenza-infected mice [57]. Ichihashi et al. showed that, surprisingly, influenza peptides conjugated to phosphatidylserine were more immunogenic alone than incorporated in a liposomal formulation [58], indicating that particulate formulations not always have superior immunogenicity.

Aside from liposomes, virosomes have also been used as delivery systems for short peptide antigens. An early study showed that virosomes loaded with the $H\text{-}2K^d$ binding influenza $NP_{147–155}$ peptide induced CTLs that were able to lyse influenza-infected target cells [59]. The addition of the adjuvant CpG ODN 1826 to influenza $M1_{58-66}$ peptide-loaded virosomes increased peptide-specific

$CD8^+$ T cell responses even further [60], which resulted in an increased recovery of mice infected with heterologous influenza virus.

Long peptide antigens that include multiple epitopes are, compared to short peptides, in an advanced stage of development. Recently, FP-01.1 was tested in a phase I clinical trial [61]. FP-01.1 is composed of six 35-mer peptides, each consisting of multiple $CD4^+$ and $CD8^+$ epitopes derived from influenza A internal proteins, which have been conjugated to a fluorocarbon chain. The vaccine formulation was found to be safe and induced cross-reactive immune responses in most subjects.

Multiple antigenic peptide (MAP) constructs are also effective for enhancing the immunogenicity of peptide antigens. The MAP approach has been widely studied with M2e-derived antigens [62]. Multimeric-001, which consists of nine linear B cell and T cell epitopes of HA, NP and M1 combined in a single 50 kDa polyepitope [63]. Multimeric-001 was able to induce considerable cellular immune responses when administered twice in both adults and elderly [64]. Interestingly, Multimeric-001 was also used in a prime-boost approach with seasonal TIV in the same study. Individuals who were primed with Multimeric-001 and subsequently boosted with TIV had significantly higher HI titers than individuals who were primed and boosted with TIV. Further formulation with adjuvants might increase the immunogenicity of standalone Multimeric-001 vaccine in the future.

Aside from the induction of T cell responses, peptide antigens have also been used to induce HA stalk-specific antibodies. Polypeptide $HA2_{76\text{-}130}$ (the binding domain of stalk reactive mAb 12D1) was conjugated to the carrier protein keyhole limpet hemocyanin, which induced 12D1 antibodies that protected mice against influenza H5N1 and H1N1 infections [65].

Peptides are thus promising influenza antigens, especially for the induction of influenza-specific T cell responses. While the formulation of these antigens remains challenging, the approaches discussed above have shown promising results, specifically in prime-boost regimens with regular influenza vaccines. Furthermore, peptide antigens do not require folding or post-translational modifications, and might be more stable compared to protein antigens in unfavorable conditions.

DNA and RNA

Unlike protein or peptide-based antigens, DNA vaccines induce antigen production in the host itself. In short, a DNA copy is made of the viral RNA segment coding for the antigen of interest (i.e. an influenza protein), which is then inserted into an expression plasmid. Bacteria carrying the production plasmid are cultured and subsequently the plasmid is purified. The purified plasmid is administered, and the plasmid enables antigen production in cells of the host, which results in an immune response against the antigen.

The concept has been evaluated in a phase I efficacy and safety study with an epidermal administered influenza DNA vaccine containing an HA gene. The DNA plasmids were coated on gold particles, which were subsequently applied epidermally using a gene gun. A trivalent DNA vaccine was able to protect individuals from influenza infection, proving that the DNA vaccine concept was viable [66].

Aside from the replacement of seasonal influenza vaccines, DNA antigens are also used to induce more broadly reactive immune response. In a clinical study, Ledgerwood et al. showed that priming with an H5 encoding DNA vaccine in advance of a monovalent H5N1 subunit boost vaccine significantly improved antibody responses [67], and induced influenza-specific T cell responses. This prime-boost regimen is an example of a novel antigen supplementing existing influenza vaccines. The same group showed that vaccination with a H1N1 HA-encoding DNA vaccine and subsequent boosting with subunit vaccine induced broadly-protective stalk-directed antibodies in mice and ferrets [68, 69]. Both approaches did not require any additional formulation of the DNA vaccine, which suggests that DNA plasmids are efficiently taken up by host cells.

An influenza DNA vaccine encoding for H5N1 HA, NP and M2 proteins induced antibody and T cell responses in combination with the cationic liposome adjuvant Vaxfectin in a clinical study [70]. The vaccine was able to induce HI titers comparable to titers induced by a subunit vaccine, showing that adjuvanted DNA vaccines have the potential to be used in humans.

While influenza DNA vaccines are a promising concept, several concerns regarding safety have to be considered. Antibodies against the DNA plasmid could render the vaccine ineffective. Also, the continued production of influenza antigens in the host might alter the immune system, or induce tolerance against influenza antigens. Arguably, the largest issue is the introduction of extraneous DNA into the vaccinated subject, which could lead to unwanted genetic changes such as tumor growth. Extensive safety and efficacy studies are therefore necessary to overcome these concerns.

RNA-based influenza vaccines are recently in preclinical development. Like DNA, mRNA enables influenza antigen production in host cells. A non-amplifying mRNA encoding for HA was able to confer protective HI responses in mice and ferrets with a single immunization of 80 µg mRNA [71]. Another study incorporated a self-amplifying mRNA encoding for HA and NA in lipid nanoparticles, which were able to induce HI titers with a mRNA dose as low as 0.1 µg [72]. This concept vaccine was fully synthetic, and is thought to have limited safety concerns compared to DNA-based and protein vaccines, which are usually generated in *in vitro* platforms.

Adjuvants for influenza vaccines

Enhancing the immunogenicity of vaccine antigens by the addition of adjuvants has several advantages, such as dose sparing, increased efficacy in the elderly, unprimed individuals and

Table 2. Adjuvants for influenza vaccines.

Adjuvant category	Adjuvant	Antigen(s)	Stage of development	Ref.
Salts	Alum	Split, WIV (pandemic)	Licensed	N/A
Oil-in-water emulsions	MF59 (squalene, Span 85, polysorbate 80)	Subunit (seasonal and pandemic)	Licensed	N/A
	AS03 (squalene, DL-α-tocopherol, polysorbate 80)	Split, WIV (pandemic)	Licensed	N/A
	AF03 (squalene, Brij 76)	Split (pandemic)	Licensed	N/A
	CoVaccine HT (squalene, polysorbate 80, sucrose fatty acid sulphate esters)	WIV (pandemic)	Animal model	[73]
Saponins	Iscomatrix	WIV (seasonal)	Clinical development	[74]
	Matrix-M	Virosomes (pandemic)	Clinical development	[75]
Glycolipids	Alpha-GalCer (alpha-galactosylceramide)	LAIV (seasonal) DNA (HA-encoding)	Animal model	[76] [77]
Liposomes	CCS/c (cationic liposomes of ceramide carbamoyl-spermine/cholesterol)	Subunit (seasonal)	Animal model	[78]
	CAF01 (cationic liposomes of DDA/TDB)	Split (seasonal)	Animal model	[79]
	Vaxfectin (cationic liposomes of GAP-DMORIE/ DPyPE)	Split (seasonal, WIV (pandemic) DNA (HA-encoding)	Animal model (split, WIV) Clinical development (DNA)	[80] [70]
Bacterial components	CTA1-DD (Cholera toxin subunit A)	Peptide (M2e-based)	Animal model	[81]
	LT patch (Escherichia coli enterotoxin)	Split (pandemic)	Clinical development	[82]
	Salmonella and Escherichia coli flagellins	rHA (pandemic) rM2e	Clinical development	[83] [84]
Cytokines	IL-12, IL-23	WIV (laboratory strain)	Animal model	[85]
	GM-CSF (Granulocyte-Monocyte Colony Stimulating Factor)	DNA (HA-encoding)	Animal model	[86]
	Type 1 IFN (IFNα)	Subunit (seasonal)	Animal model	[87]
TLR agonists / immunomodulators	GLA (glucopyranosyl lipid A) (TLR4)	rHA (pandemic)	Clinical development	[88]
	Bacterial flagellins (TLR5)	rHA (pandemic) rM2e	Clinical development	[83] [84]
	CpG oligodeoxynucleotide (TLR9)	Split (seasonal)	Clinical development	[89]
	PolyI:C (TLR3)	Split (seasonal) LAIV (laboratory strain)	Animal model	[90] [91]
	IC31 oligodeoxynucleotide (TLR9)	Subunit (seasonal)	Animal model	[92]
	sLAG-3 (IMP321) (MHC-II ligand)	Split (seasonal)	Clinical development	[93]
Polymers	Chitosan	Subunit (laboratory strain) DNA (M2 and NP encoding)	Animal model	[94] [95]
	PCPP (poly[di(carboxylatophenoxy) phosphazene])	Subunit (pandemic)	Animal model	[96]
	Advax (delta inulin)	Split (pandemic) rHA (pandemic)	Clinical Development	[97] [98]

immunocompromised, and broadening of the influenza-specific immune response. Many novel antigens such as peptide and DNA antigens require the addition of adjuvants to steer the immune response towards a specific response, such as a cellular immune response. The development of suitable adjuvants for influenza vaccines is therefore imperative. A comprehensive overview of adjuvants currently on the market or in development is shown in Table 2.

There are currently several adjuvants that are approved for use in influenza vaccines. The most commonly used vaccine adjuvant, aluminum salt, is currently used in pandemic influenza vaccines. However, no beneficial effect of alum with these vaccines was observed during the 2009 H1N1 pandemic [36].

In contrast to aluminum salts, oil-in–water emulsions have proved to be suitable adjuvants for influenza vaccines. MF59 was the first of these adjuvants approved for use with influenza vaccines in 1997. MF59 is an oil-in-water emulsion, which consists of 150 nm-sized biodegradable squalene oil droplets stabilized by non-ionic surfactants. Several modes of action have been attributed to this adjuvant [99], including enhanced regulation of genes for cytokines and chemokines, local release of ATP as an endogenous danger signal, increased influx of macrophages and monocytes to the site of injection, differentiation of monocytes to active dendritic cells, and antigen transportation to draining lymph nodes. Numerous reports observed increased immunogenicity and efficacy of MF59-adjuvanted subunit vaccine in young children, healthy adults, and elderly individuals [100-103]. Additionally, MF59 has similar immunostimulatory effects in combination with prepandemic vaccine formulations [104]. Overall, MF59 has thus far proven to be a very effective adjuvant for the stimulation of humoral responses against both seasonal and prepandemic influenza vaccines.

Similar to MF59, AS03 is also an oil-in-water emulsion based on squalene droplets. However, unlike MF59, AS03 is currently only used in pandemic influenza vaccines. AS03 adjuvanted influenza vaccines were significantly more immunogenic than their unadjuvanted counterparts both in primed and unprimed individuals [105, 106]. Furthermore, AS03-adjuvanted influenza vaccines were also able to confer seroprotection in immunocompromised patients infected with HIV-1 [107]. In contrast, adjuvanted vaccines failed to increase seroprotection rates in other immunocompromised groups, such as transplant recipients or patients with lymphoid malignancies [108, 109].

Saponin-based adjuvants are currently in clinical development for use with influenza vaccines. Natural or synthetic saponin QS-21 (a fraction from soluble triterpene glycosides purified from Quillaja saponaria) was clinically tested with TIV vaccine, but failed to increase HI titers significantly compared to unadjuvanted TIV [110]. These saponins can form complexes with lipids like cholesterol resulting in particles, the so-called immune stimulating complexes (ISCOMs). These are hollow, cage-like particles of around 40 nm diameter [111]. Clinical studies with ISCOM-adjuvanted influenza split vaccines revealed accelerated antibody responses in individuals who received

ISCOM-adjuvanted influenza vaccines [74]. Furthermore, this coincided with a notable increase of influenza-specific $CD8^+$ T cell responses [112]. A third generation of saponin based adjuvants, named Matrix-M, was evaluated in a clinical study in combination with a pandemic virosomal influenza vaccine [75]. The addition of Matrix-M resulted in a significant dose sparing of the antigen, and increased vaccine-induced T cell responses. Matrix-M was successfully used as an adjuvant for a H7N9 VLP vaccine in a phase II clinical trial, in which the adjuvanted VLP vaccine showed significantly higher seroconversion rates after vaccination compared to non-adjuvanted VLP vaccine [113].

Bacterial-derived components can also serve as potent adjuvants for influenza vaccines. Flagellin was fused genetically to the globular head of a HA1 subdomain, and was able to induce protective HI titers in healthy adults with only 2 μg of antigen, and in elderly with 4 μg of antigen [83, 114]. Currently advancing to phase III trials, this fusion vaccine shows that bacterial-derived components can be very effective adjuvants. Indeed, the co-administration of heat-labile enterotoxin via a patch after immunization with an influenza split vaccine boosted HI responses to the vaccine in healthy adults [82].

Recently a novel polysaccharide adjuvant, Advax, was used as an adjuvant for pandemic influenza vaccines. Made from delta inulin, this adjuvant stimulated both humoral and cellular responses induced by split vaccines in ferrets, which protected the animals from a lethal influenza challenge [115]. However, an Advax-adjuvanted rHA H1N1 vaccine failed to induce the required EMA/FDA seroprotection rates after two immunizations, except with a high antigen dose of 45 μg [98]. While the mode of action of Advax might also be partially through the induction of cellular responses, this still needs to be assessed in well-designed clinical studies.

Besides increasing the immunogenicity of the antigens, the addition of adjuvants to influenza vaccines can induce unwanted immune responses. AS03-adjuvanted influenza vaccines have been under close attention since a sudden increase of childhood narcolepsy incidence was observed in Scandinavian countries after the pandemic influenza epidemic of 2009-2010 and subsequent administration of AS03-adjuvanted Pandemrix influenza vaccines [116]. The 2009 pandemic H1N1 influenza was associated with the incidence of narcolepsy in patients with a HLA-DQB1*06:02 allele, and it is suspected that the pH1N1 vaccine caused an autoimmune response leading to narcolepsy in individuals with this genotype [117]. A recent study identified higher amounts of structurally altered influenza NP protein in the Pandemrix vaccine than Arepanrix, another AS03-adjuvanted influenza vaccine [118]. Strikingly, they found higher levels of NP-specific antibodies in children with the HLA-DQB1*06:02 allele, which suggests a link between the antigen content of Pandemrix and narcolepsy, rather than a link between narcolepsy and the adjuvant. Another group also suggested that differences between vaccine antigens might be related to the higher incidence of narcolepsy associated with Pandemrix [119]. Nonetheless, extra care should be given to the safety profile when combining powerful adjuvant with complex protein vaccines such as WIV, split, virosomal or

subunit influenza vaccines, since the induction of broad antibody responses increases the risk of cross-reactivity with self-proteins.

Improving influenza vaccine stability

The shelf life of influenza vaccines is limited to approximately one year if stored refrigerated (2 -8°C). The potency of the HA antigen can be negatively affected by either elevated temperatures or temperatures below 0°C [40]. Consequently influenza vaccines need to be refrigerated during distribution and storage (so-called cold-chain), which is costly and can be difficult to guarantee in developing countries. Increasing the stability of influenza vaccines would therefore reduce the dependency on the cold chain, and would ensure that antigen retains its potency until administration. Additionally, improving the antigen stability can also prolong the vaccine shelf life, which would facilitate stockpiling of influenza vaccines in the preparation of a possible pandemic.

Stabilization of liquid antigens is commonly achieved through conversion to dry formulations. The solid state provides stability by decreasing the mobility of the protein antigen and the absence of water-based degradation pathways. However, drying methods are associated with their own stress factors that can affect the stability of the antigen. The addition of excipients such as sugars to influenza vaccines can stabilize the antigen during the freeze-drying process and subsequent storage [40]. During drying, sugars form a glassy matrix that protects the antigen by providing a physical barrier. Furthermore, the glass matrix of some carbohydrates such as inulin or trehalose possesses high glass transition temperatures, which increases the heat stability of the formulations due to decrease in molecular mobility.

The sugars inulin and trehalose both have been used as stabilizing excipients to facilitate influenza vaccine drying (either freeze-, spray- or spray freeze-drying). All four types of inactivated influenza antigens have been stabilized successfully by one or more drying methods with various excipients [120-123]. This proves that the addition of stabilizing excipients can greatly enhance influenza vaccine stability under extremely unfavorable conditions.

UNIVERSAL INFLUENZA VACCINES

Some of the aforementioned novel formulations are prospective universal influenza vaccines; these should be able to protect against all influenza strains regardless of any antigen shifts or drifts. In the last few years, many of such universal vaccine concepts have entered clinical trials, as listed in Table 3. Vaccines based on HA stalk-reactive antibodies have yet to enter the clinical phase, indicating that this concept still has a long way to go.

The most advanced are the M2e- and T cell-based vaccine concepts, with several vaccine concepts having completed phase II trials. Most concepts proved to be immunogenic in humans (either healthy adults or elderly) and had positive safety profiles. It is expected that some of these vaccines will enter phase III trials in the coming years, from which we will finally be able to conclude whether these concepts are able to offer increased cross-reactivity against multiple influenza strains.

Interestingly, prime-boost or simultaneous immunizations combining these novel vaccines with seasonal vaccine formulations are gaining popularity [64, 126], indicating that these concepts are more prone to supplement rather than to replace existing seasonal vaccines.

Table 3. Universal influenza vaccine concepts currently in the clinical phase of development.

Targeted response	Concept	Status	Registration number	Ref.
M2e antibodies	VAX102 (recombinant M2e fused to flagellin)	Phase I/II completed	NCT00603811, NCT00921947, NCT00921206	[84]
	VAX102 + seasonal vaccine (coadministered)	Phase I completed	NCT00921973	N/A
	ACAM FLU-A (recombinant M2e fused to hepatitis B core protein)	Phase I completed	NCT00819013	N/A
	VGX-3400X (DNA plasmid encoding for HA, NA and M2e-NP of H5N1 delivered by electroporation)	Phase I completed	NCT01184976, NCT01142362	N/A
Influenza-specific T cells	FP-01.1 (long peptides containing multiple T cell epitopes)	Phase I completed, phase II ongoing	NCT01265914, NCT01677676, NCT02071329	[61]
	FP-01.1 + undisclosed adjuvant	Phase I completed	NCT01677676	N/A
	FP-01.1 combined with seasonal vaccine (prime) + FP-01.1 (boost)	Phase I completed	NCT01701752	N/A
	Flu-v (long peptides containing multiple T cell epitopes)	Phase I completed	NCT01226758, NCT01181336	[124]
	Multimeric-001 (recombinant protein containing multiple T cell epitopes)	Phase I/II completed	NCT01010737, NCT00877448, NCT01146119	[63]
	Multimeric-001 (prime) + seasonal vaccine (boost)	Phase I/II completed	NCT01419925, NCT02293317	[64]
	MVA-NP+M1 (modified vaccinia virus Ankara vectored vaccine containing multiple T cell epitopes)	Phase I/II completed	NCT00942071, NCT00993083	[20, 48, 125]
	MVA-NP+M1 + seasonal vaccine (coadministered)	Phase I completed	NCT01465035	[126]
	ChAdOx1 NP+M1 (simian adenovirus vectored vaccine containing multiple T cell epitopes) + MVA-NP+M1 (mixed prime/boost)	Phase I ongoing	NCT01818362	[50]

PRODUCTION STRATEGIES FOR INFLUENZA VACCINES

Production of current influenza vaccines

The production of seasonal influenza vaccines is performed each year in a limited window of time between influenza strain selection and the release of the final vaccine. The steps involved are depicted in Figure 3. Several time determining steps, such as the availability of reagents for the SRID assay, are crucial for the advancement of the production process, and thus limit the speed of vaccine production each year, regardless of production platform.

Virus propagation on embryonated chicken eggs remains the most frequently used method to manufacture influenza vaccines. Furthermore, securing sufficient vaccine-quality eggs to manufacture influenza vaccines for the world population is a daunting, probably impossible task. Several other risks with egg-based vaccine production also need to be considered. An influenza outbreak among poultry is a serious possibility that would decrease the availability of vaccine-quality eggs [127]. Additionally, influenza wild type (WT) strains need to be optimized for growth in eggs, which involves recombination of these WT strains with high-yield laboratory strains such as A/PR/8/34. During this step, mutations in the egg-adapted reassortant strain can contribute to a mismatch between the vaccine strain and the circulating strain, which occurred recently during the

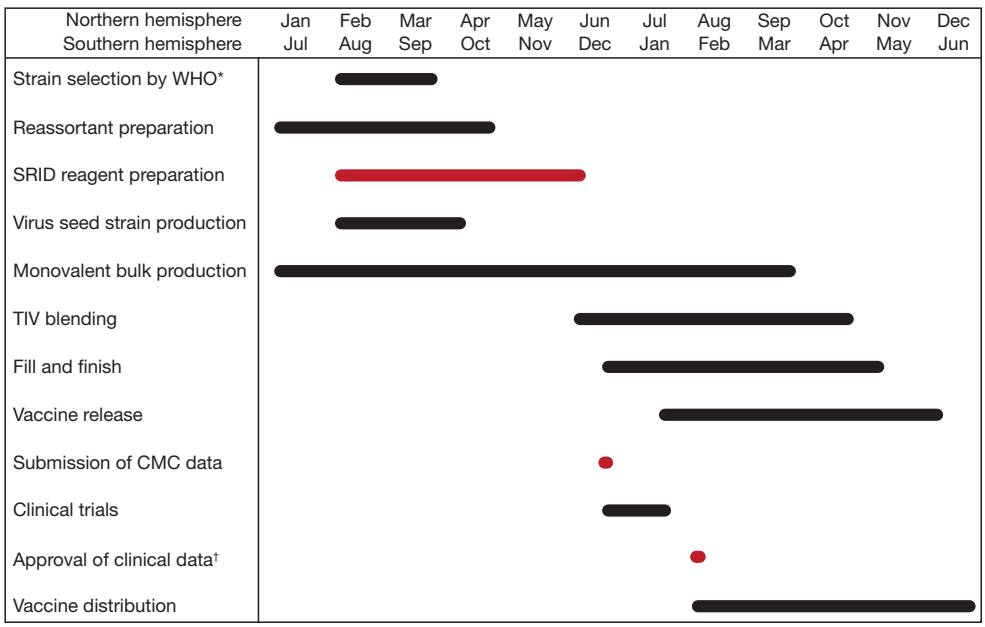

Figure 3. Timeline of seasonal influenza vaccine production. While some novel production methods can grow influenza viruses faster, the vaccine production timeline contains several steps that are time determining (red lines). This means that while the vaccine can be produced faster, the time from strain selection to vaccine release remains similar for all production platforms.

2012-2013 influenza season [128]. Decreasing dependence on egg-based influenza propagation is thus a crucial step towards the increase of influenza vaccine production capacity worldwide. Current influenza production platforms are listed in Table 4.

One alternative to egg-based production systems is cell culture-based systems. Cell culture-based influenza propagation is not dependent on the availability of vaccine-quality eggs. More importantly, cell-culture based production platforms are easy to scale up, and theoretically should be able to meet the high demand for influenza vaccine in case of a pandemic situation [129]. However, WT influenza strains still need to be adapted for growth on cells, and building costs and validation of cell-based manufacturing plants are high, which might be unattractive for manufacturing companies [127].

As of yet, only a few cell culture-based seasonal and prepandemic influenza vaccine formulations are currently approved. Madin-Darby canine kidney (MDCK) cells were the first to be used for the production of seasonal TIV vaccines [130]. Most WT human influenza viruses grow efficiently in MDCK cells, and existing egg-adapted reassortant strains can grow to similar titers [131]. These advantages make MDCK cells an acceptable substitute for egg-based influenza virus production. Vero cells have also been used as a production platform for both seasonal and prepandemic vaccines [132, 133]. Influenza virus cultivation in laboratory-scale bioreactors was compared between MDCK and Vero cells, but production yields between cell lines were not significantly different [134]. However, different virus strains showed differences in growth stability depending on culture medium and cell line. Searching other cell lines suitable for influenza production is therefore important, since influenza viruses might grow more efficiently on cell lines other than the ones that are currently used.

One of these novel cell lines is human retina-derived PER.C6, which is able to grow without the need of solid support for growth such as microcarriers [135]. The growth of suspension cell cultures is limited to the concentration of cells in the medium, rather than surface area in case of adherent cell cultures. This might allow easier scale-up of the vaccine production if necessary. A split H7N1 influenza vaccine grown on PER.C6 cells was successfully tested for safety in a phase I clinical trial [136], but failed to induce adequate immune responses. It is believed that, similar to recombinant HA, higher doses of antigen are needed to confer protective antibody titers. Other cell-based platforms

Table 4. Influenza vaccine production platforms.

Production platform	Vaccines	Status
Fertilized eggs	Seasonal and (pre)pandemic subunit and split (Pre)pandemic WIV	Licensed worldwide
MDCK cells	Seasonal and (pre)pandemic subunit and WIV	Licensed in EU and USA
Vero cells	(Pre)pandemic WIV and split	Licensed in EU and Japan
Baculovirus	Seasonal subunit	Licensed in US and Japan

for influenza production such as Human Embryonic Kidney (HEK)-293 and Amniocyte-derived (CAP) cell lines are currently still in preclinical development [137, 138]. These human-derived cell lines might be more suitable for the growth of human-adapted influenza strains compared to the currently used animal-derived cells. Indeed, there are indications that influenza viruses grow better in cell lines derived from their preferred host [139].

While these production methods are definitely an improvement, vaccines produced on cell lines have to be thoroughly screened for adventitious viruses and residual cell line DNA and cell line proteins, which might cause adverse effects [140]. Nonetheless, cell-based influenza virus production remains an improvement over egg-based production methods, due to increased vaccine purity.

Recombinant influenza antigens represent another alternative technology to traditional egg production methods. The baculovirus expression vector system (BEVS) efficiently produces recombinant HA in insect Sf9 cells, resulting in a recombinant influenza subunit vaccine [141]. By producing merely the HA antigen and not the entire influenza virus, several purification and inactivation steps can be omitted from the production process. This also results in predictable and more robust yields during production. However, the current rHA vaccine on the market requires a dose of 45 µg HA per strain to be effective, which is 3 times higher than the standard 15 µg HA dose in non-recombinant influenza vaccines. Further formulation of rHA with adjuvants might be required for considerable dose sparing.

Aside from technological improvements of the vaccine production process, it is necessary to increase the number of influenza vaccine manufacturers worldwide to meet demand. Technology transfer of influenza vaccine production methods to new manufacturers is therefore important. Incentives like the International Technology Platform for Influenza Vaccines (ITPIV) and other projects provide the transfer of influenza vaccine production knowledge to new vaccine manufacturers [142], expanding the number of influenza vaccine producers and increasing influenza vaccine production capacity in the world.

Production of future influenza vaccines

The advent of novel influenza vaccines antigens also requires production technologies that are different from classical egg- or cell-based virus propagation. This could offer several advantages, such as faster production times, increased capacity and product consistency, and less risk of adventitious agents in the final product.

Recombinant protein technology is bound to play a major role in the production of these novel antigens. Already utilized for the production of rHA, it is clear that recombinant technology is a viable option for the production of influenza antigens. The previously discussed peptide-based Multimeric-001 vaccine is produced in *E. coli*, for instance. HA and M2 ectodomain antigens, both in

peptide and in protein forms, are regularly produced by recombinant technology in various vectors, such as *E. coli*, tobacco mosaic virus, papaya mosaic virus, bacteriophage T7 and baculovirus. The ability to fuse a carrier protein or immunopotentiators to the antigen during production is a great advantage of recombinant systems, and negates the need of post-production antigen formulation with, for instance, an adjuvant. Similar strategies have also been used with T cell inducing antigens such as NP epitopes [143]. With its versatility and the recent approval of rHA vaccine, recombinant technology is bound to be used widely for the production of novel influenza antigens.

The production process of DNA vaccines has rapidly evolved since the approval of several veterinary DNA vaccines [144]. Nowadays, manufacturing kilogram-scale batches of DNA plasmids is not uncommon. However, several problems still exist, such as getting an adequate concentration of DNA in a small enough volume for vaccination. These problems are expected to be resolved in the coming years, as the realization of influenza DNA vaccines comes closer.

Peptide antigens are fundamentally different from aforementioned protein antigens in terms of manufacturing process. Short to medium length peptides that do not require specific folding can be chemically synthesized rather than biologically produced. Thus, these antigens can be produced without the inherent risks of using biological systems, such as the presence of adventitious agents or cellular components in the final product. Technological developments in the field of chemical peptide synthesis over the last two decades have enabled the industry to manufacture large quantities of peptides at competitive prices, underlining the feasibility of large scale production of peptide vaccines [145]. Additionally, chemical synthesis of peptides is relatively fast, which is required for the production of influenza vaccines. The aforementioned FP-01.1 influenza peptide vaccine shows the potential of peptide-based vaccines [61]. However, most peptide antigens will need additional formulation with adjuvants or delivery systems in order to be immunogenic, which may add complexity and time to the production process of the final vaccine formulation. Another more simple option would be combining universal vaccines with current seasonal vaccines in a prime/boost regime, in order to broaden the immune response.

The aforementioned antigen production methods are all relatively fast and flexible, certainly compared to the egg-based influenza vaccine production. While most of the novel influenza antigens are still in development, there is great potential for these antigens from a formulation and production point of view.

CONCLUSION

The field of influenza vaccine development is constantly changing. While improvements on formulation and production level are continuously being made for traditional influenza vaccines, great steps are being made in the development of universal influenza vaccines. The introduction of novel influenza antigens and accompanying novel correlates of protection will be the most crucial and revolutionary step that has to be taken. Before a universal influenza vaccine is developed, it is likely that novel more conserved antigens will supplement current day influenza vaccine formulations in order to broaden the immune response by combining strong humoral and cellular responses. Fortunately, the production methods for these novel antigens seem more flexible than production methods of current influenza vaccines. While novel production methods can produce vaccines faster, the timely availability of reagents for vaccine potency testing remains the main time-delaying factor, and should therefore be considered. Furthermore, universal vaccines could be produced continuously opposed to the current seasonal vaccines, which would greatly increase vaccine production capacity and coverage. The next decade will thus be an exciting time for the influenza vaccine field.

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Chapter 3

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

Peter C. Soema [1,2], Sietske K. Rosendahl Huber [3], Geert-Jan Willems [1], Wim Jiskoot [2], Gideon F.A. Kersten [1,2] and Jean-Pierre Amorij [1]

1 Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands.
2 Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.
3 Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

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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 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 T cell peptide was investigated. The conserved human HLA-A2.1 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-A2.1 transgenic mice. Delivery properties of the virosomes were studied in mice and in *in vitro* dendritic cell cultures. Immunization of HLA-A2.1 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 that are specific for conserved epitopes of internal influenza A proteins, such as matrix protein and nucleoprotein, may provide cross-protection and are thus unaffected 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)-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-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-A2.1-restricted peptide $M1_{58\text{-}66}$, derived from influenza matrix protein 1. Influenza-specific $CD8^+$ T cell responses and antibody titers were assessed in HLA-A2.1 transgenic mice after immunization. The addition of TLR9 agonist CpG was found to be an effective adjuvant for the peptide in conjunction with virosomes. Furthermore, immunization with peptide-loaded virosomes adjuvanted with CpG increased the rate of recovery in mice after heterologous influenza infection. Finally, delivery properties of virosomes were extensively characterized in human DC models and in mice. Both peptide-virosome association 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/PR8/34 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 $M1_{58-66}$ 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 pretitrated 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). $M1_{58-66}$ 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 of Animal Experimentation (DEC) of the National Institute of Public Health and the Environment (RIVM) 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 day 0 and 21 under isoflurane anesthesia. 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 A/HKx31 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/PR8 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 A/HKx31 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 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 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 (Aelvis). The number of IFN-γ producing cells in antigen stimulated spleen cells was obtained after background correction (subtracting number of spots produced by splenocytes incubated with medium).

Hemagglutination inhibition assay (HI assay)

Hemagglutination inhibition (HI) titers in mouse sera were determined by a HI assay. Sera were treated overnight 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 A/HKx31 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 overnight with 600 ng (HA) of A/PR8/34 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 $M1_{58-66}$-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 6.03 for Windows (GraphPad Software Inc.).

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 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.

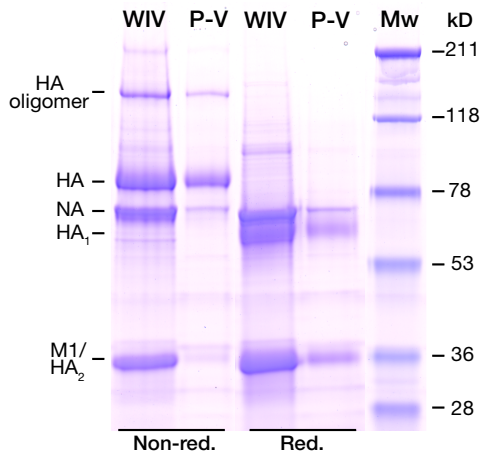

Figure 1. Protein profile of peptide-loaded virosomes. SDS-PAGE analysis of peptide-loaded virosomes (P-V) and source material whole inactivated influenza virus (WIV) under non-reduced and reduced conditions on a 12% precast gel stained with Coomassie Brilliant Blue. Protein identities were confirmed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

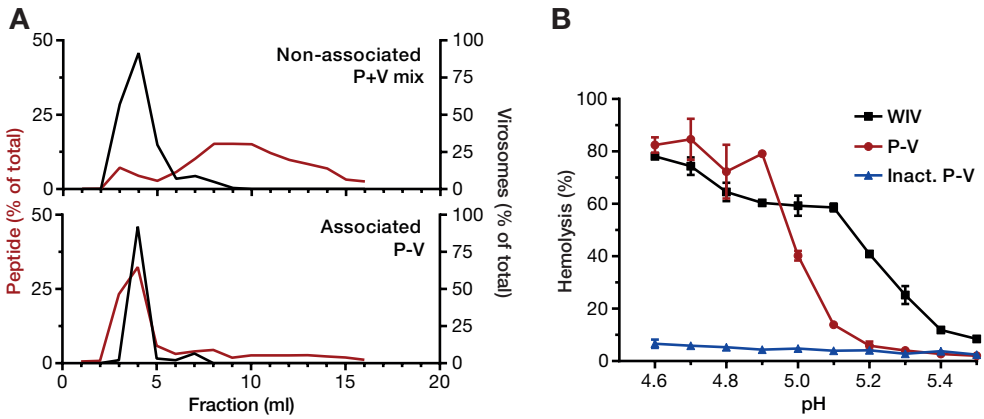

Figure 2. Characteristics of peptide-loaded virosomes. Peptide association of peptide mixed with virosomes (P+V mix) and peptide-loaded virosomes (P-V) analyzed by size exclusion chromatography (A). Black lines show the virosome elution pattern (based on protein determination), whereas the red line shows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

Immunogenicity of peptide-loaded virosomes in HLA-A2.1 transgenic mice

To assess whether the produced peptide-loaded virosomes were able to induce peptide-specific T cell responses, HLA-A2.1 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 enable 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

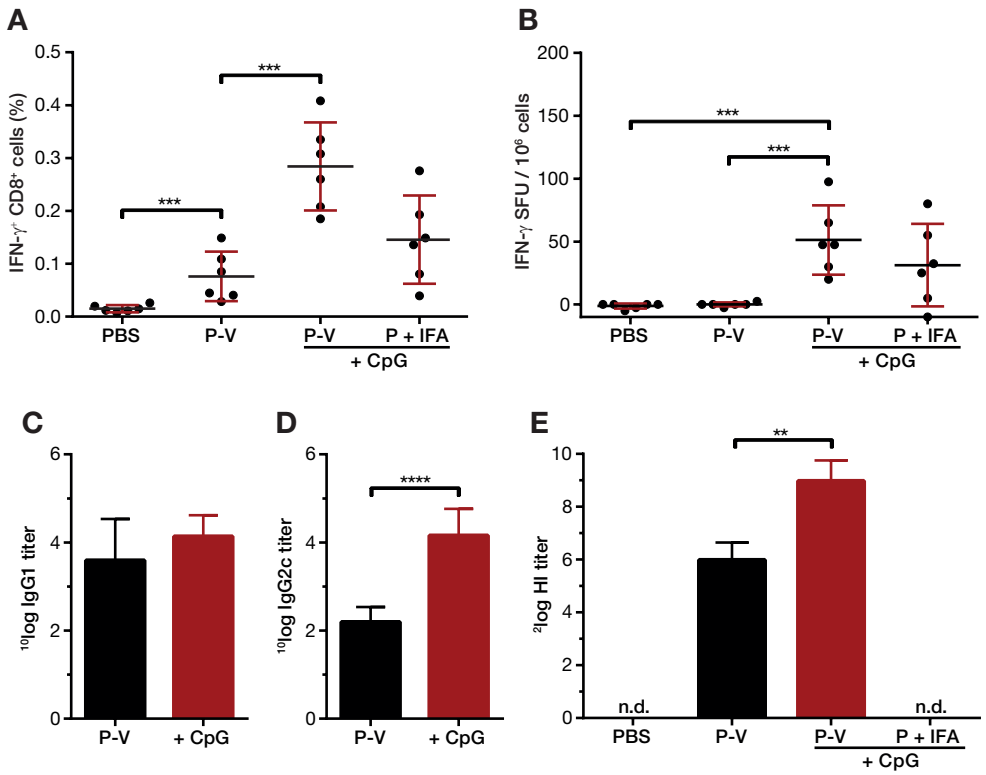

Figure 3. Immunogenicity of (non-)adjuvanted peptide-loaded virosomes. HLA-A2.1 transgenic mice were immunized twice with 1 µg of $M1_{58-66}$ peptide formulated in either virosomes (P-V), virosomes adjuvanted with CpG (P-V + CpG) or Incomplete Freund's Adjuvant (IFA) with CpG (P + IFA + CpG). Mice were 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 represent mean ± SD (n = 6); *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; n.d., not detectable.

when compared to P-V alone (Figure 3D). 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 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

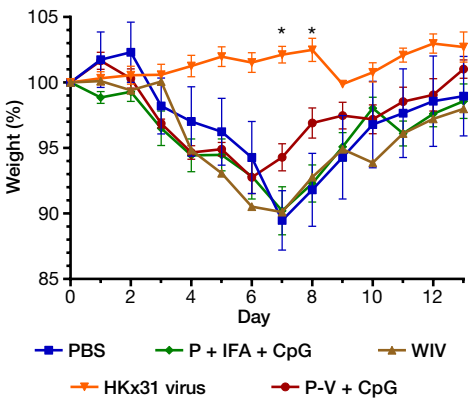

Figure 4. Efficacy of vaccine after sublethal heterologous influenza infection. HLA-A2.1 transgenic mice were immunized twice with either peptide-loaded virosomes with CpG (red), peptide mixed with incomplete Freund's adjuvant and CpG (green), WIV (brown) or PBS (blue). As a positive control, mice were challenged with a sublethal dose of HKx31 virus. Subsequently, mice were infected with heterologous HKx31 (H3N2) influenza virus and their weight was monitored for 14 days. Data represent mean ± SEM (n = 6); *p < 0.0001 for PBS and P + IFA + CpG groups versus HKx31.

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-A2.1 transgenic mice were immunized with the vaccine and subsequently infected with a sublethal dose of influenza HKx31 (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 HKx31 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 HKx31, 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 HKx31-specific HI titers were detected in sera of mice immunized with the PR8-based P-V or WIV groups (data not shown). Since WIV contains all influenza antigens and does not show any effect against HKx31 infection, it can be assumed that virosomes alone (which only contain HA and NA proteins) would not mediate any response against HKx31. 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.

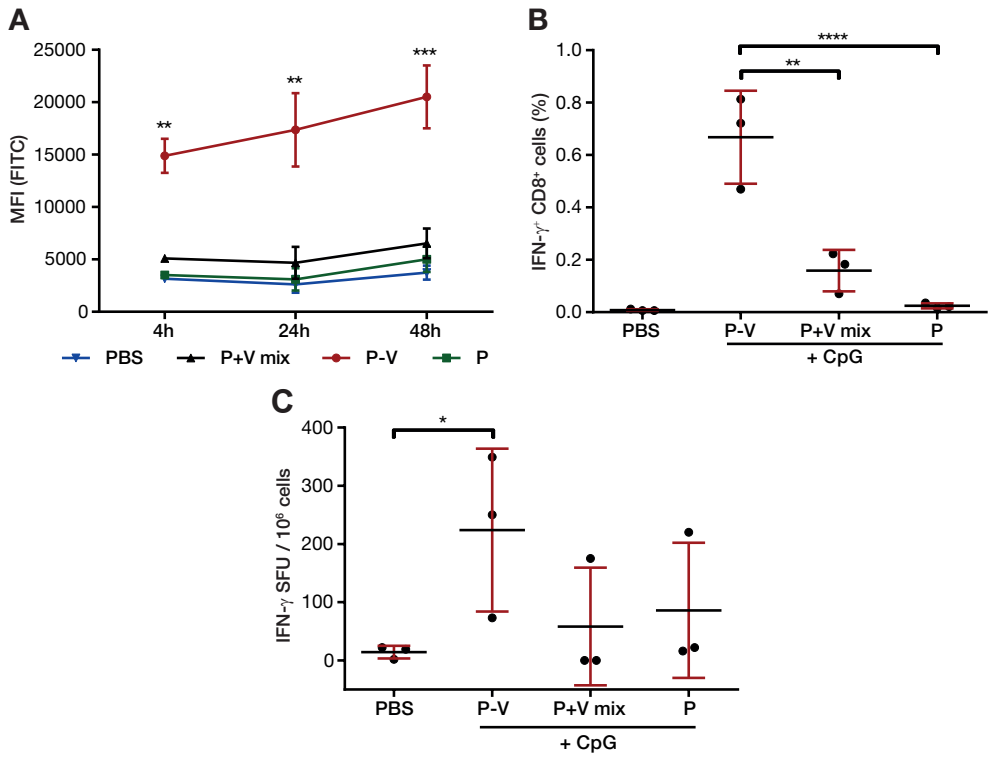

Figure 5. Influence of peptide-virosome association on *in vitro* dendritic cell uptake and *in vivo* T cell responses. $M1_{58-66}$-FITC peptide uptake by human immature DCs was determined by flow cytometry (A). Either PBS, peptide-loaded virosomes (P-V), peptide mixed with empty virosomes (P+V mix) 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-A2.1 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 represent mean ± SD (n = 3) and is representative of three individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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

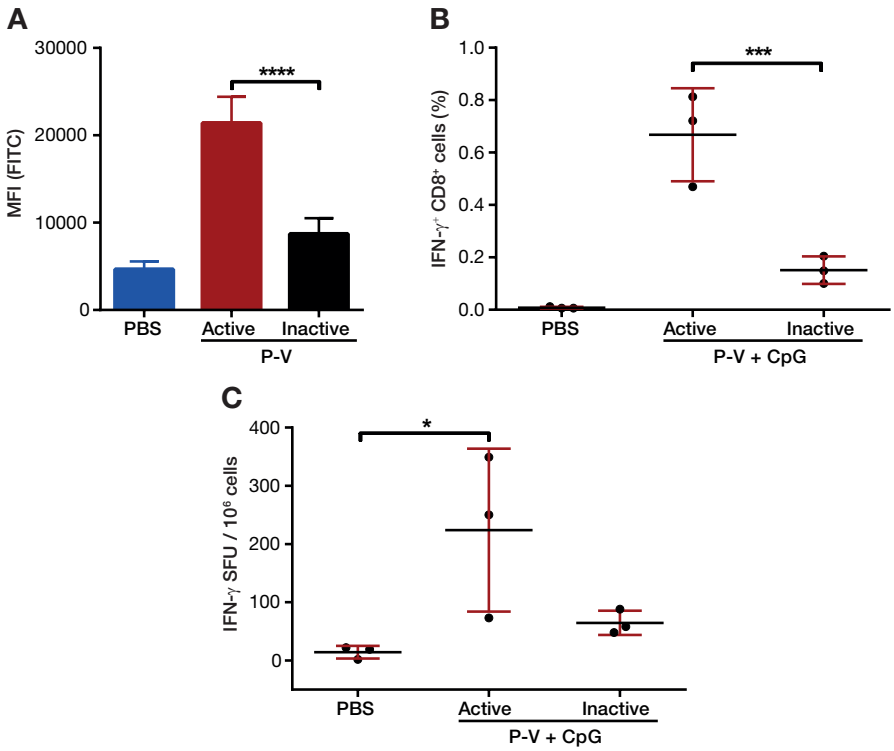

Figure 6. Impact of loss of fusogenic activity on immunogenicity of peptide-loaded virosomes. $M1_{58-66}$-FITC peptide uptake by human immature DCs was quantified for both fusion-active and inactive P-V after 16 hours of incubation at 37°C (A). Data represents mean ± SD (n = 3) performed with DCs obtained from three different donors. HLA-A2.1 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 represent mean ± SD (n = 3) and are representative of three individual experiments. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

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-A2.1 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 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 T_H1/T_H2 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-A2.1 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 $M1_{58\text{-}66}$ 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 (Figure S3C and S3D), which indicates a shift to a T_H2 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-A2.1 serotype. 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 a HLA-A2.1 epitope, which is a common serotype in the Caucasian population and can be tested in HLA-A2.1 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 T_H1 type responses [21], which support 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-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 $NP_{147-155}$ 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 HKx31 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

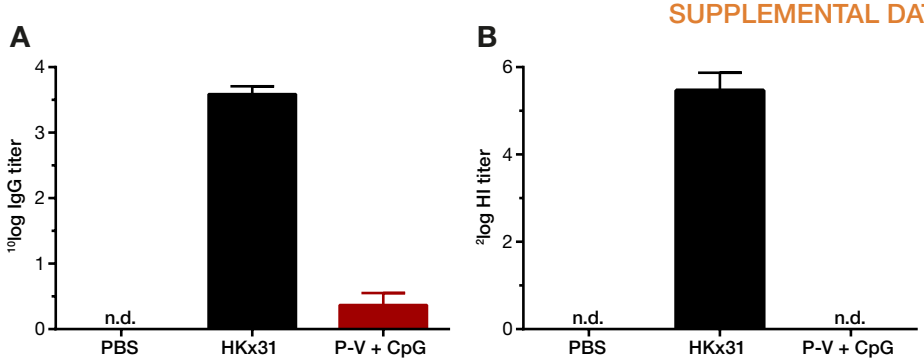

Figure S1. Total influenza-specific IgG titers after immunization. Influenza-specific total IgG titers in sera from mice. Data represent mean ± SD (n = 6). *p < 0.05; n.d., not detectable.

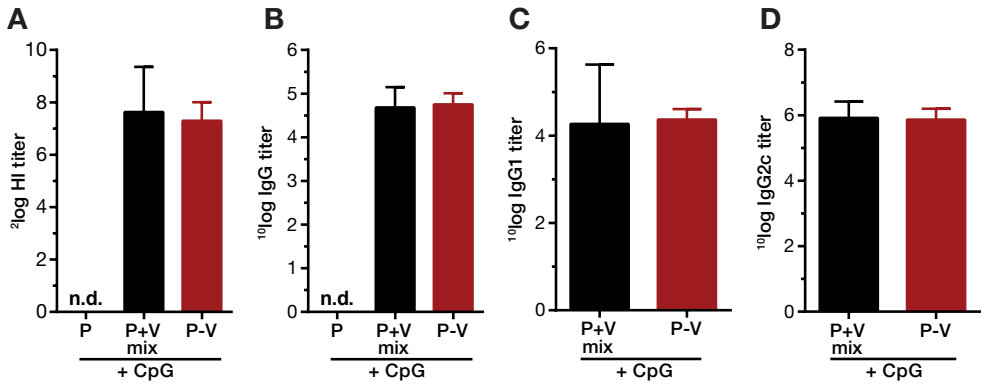

Figure S2. Effect of association between peptide and virosomes on humoral responses. Mice sera were analyzed for HI (A) and total IgG (B) titers. IgG1 (C) and IgG2c (D) isotypes were also determined from sera. Data represent mean ± SD (n = 3) and is representative of three individual experiments. n.d.,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

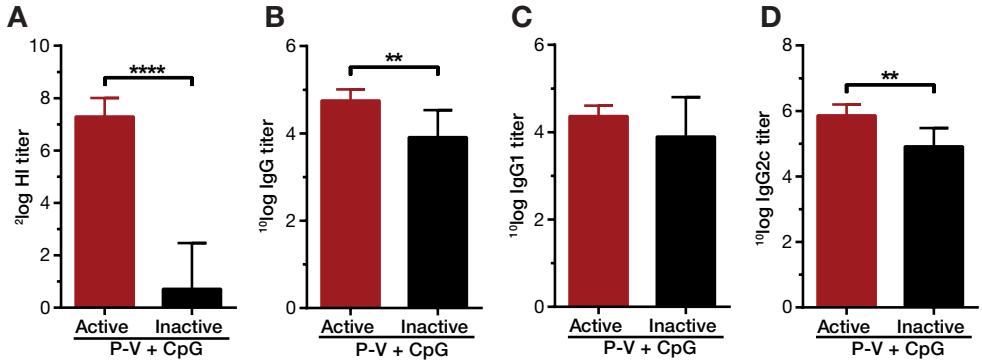

Figure S3. Effect of fusogenic activity of virosomes on influenza-specific antibodies. Influenza-specific HI titers (A) and total IgG titers (B), and antibody isotypes IgG1 (C) and IgG2c (D) titers in sera from mice. Data represent mean ± SD (n = 3) and is representative of three individual experiments.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

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Chapter 4

Whole inactvated influenza virus as an adjuvant for influenza peptide antigens

Peter C. Soema* [1,2], Sietske K. Rosendahl Huber* [3], Geert-Jan Willems [1], Wim Jiskoot [2], Jørgen de Jonge [3], Josine van Beek [3], Gideon F.A. Kersten [1,2] and Jean-Pierre Amorij [1]

* Authors contributed equally

1 Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands.
2 Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.
3 Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

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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-A2.1-restricted influenza peptide GILGFVFTL (GIL) was assessed in HLA-A2.1 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-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-A2.1-restricted epitopes were shown to possess a higher binding affinity to HLA-A2.1, 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, a HLA-A2.1-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.

Formulation of vaccines

Influenza A/PR8/34 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-A2.1-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 ODN 1826 (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 transgenic C57BL/6-Tg(HLA-A2.1)1Enge/J (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 S1) were administered s.c. in the flank of mice (six per group) under isoflurane anesthesia 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 S1. 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 fetal calf serum (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 (Aelvis). The number of IFN-γ producing cells in antigen-stimulated splenocytes was obtained after background correction (subtracting 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 [15]. Formulations were mixed with human blood erythrocytes and 0.1M 2-(*N*-morpholino) 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-A2.1 transgenic mice. They 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)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

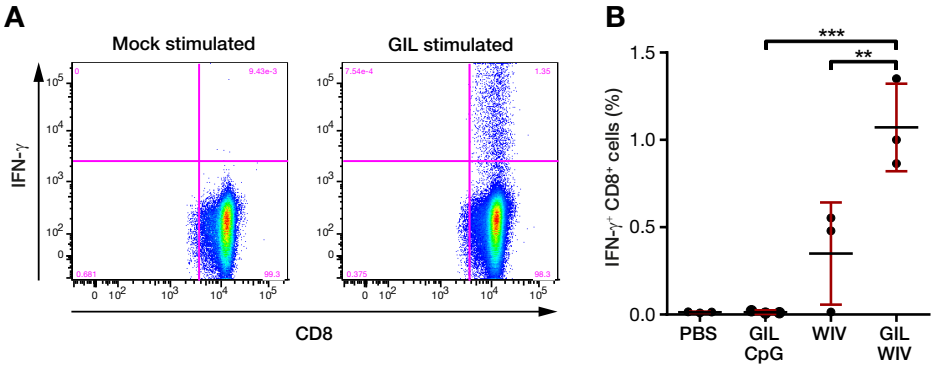

Figure 1. WIV acts as an adjuvant for peptide antigens. (A) Flow cytometry plot displaying specificity of IFN-γ^+ $CD8^+$ T cells in splenocytes from mice immunized twice with 1 µg GIL peptide adjuvanted with 50 µg WIV. (B) Splenocytes restimulated with GIL peptide were analyzed for IFN-γ^+ $CD8^+$ T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

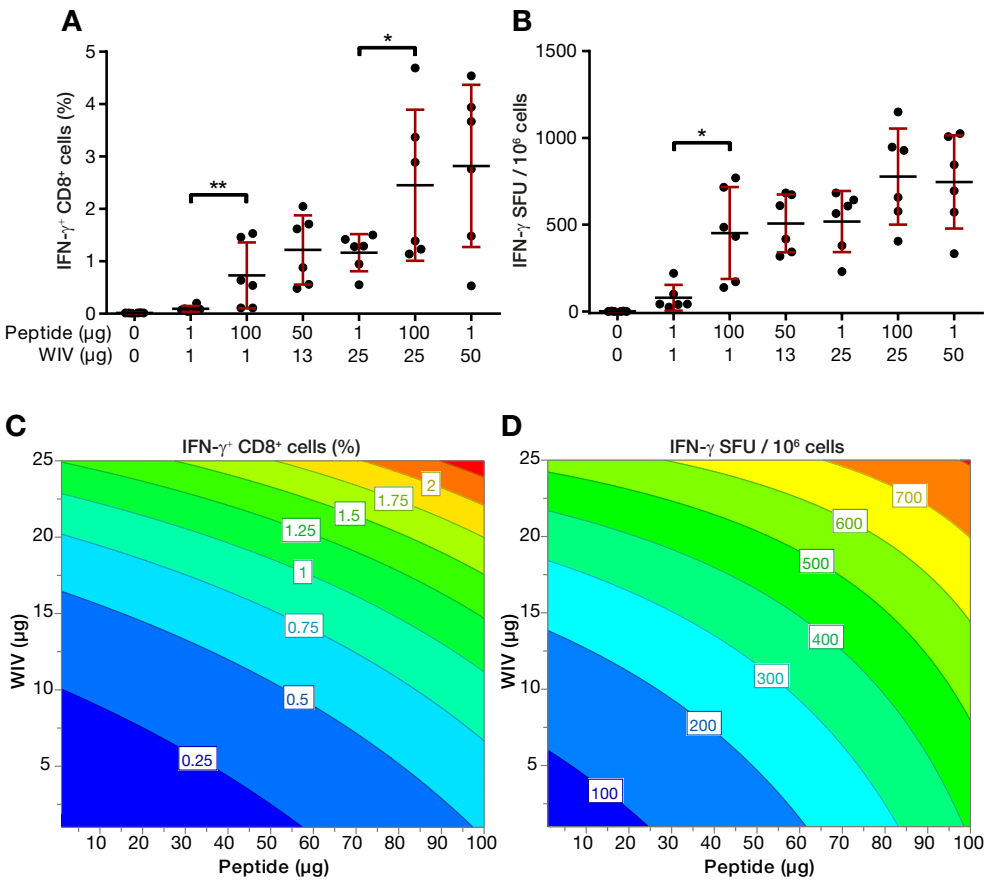

Figure 2. Dose-finding study of WIV and peptide by DoE. HLA-A2.1 transgenic mice were vaccinated two times with different doses of GIL peptide and WIV. Splenocytes restimulated with GIL peptide were analyzed for peptide-specific CD8+ T cells with flow cytometry (A) and ELISpot (B). Prediction contour 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 represent mean ± SD (n = 6); *p<0.05, **p<0.01.

adjuvant dose from 1-25 µg (Table S1).

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-A2.1 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.

Table 1. Association between GIL peptide and WIV at different concentrations. The fraction of unassociated peptide was determined in the supernatant by mass spectrometry. Data represent mean ± SD (n = 3).

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

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 1). 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 the 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) 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 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

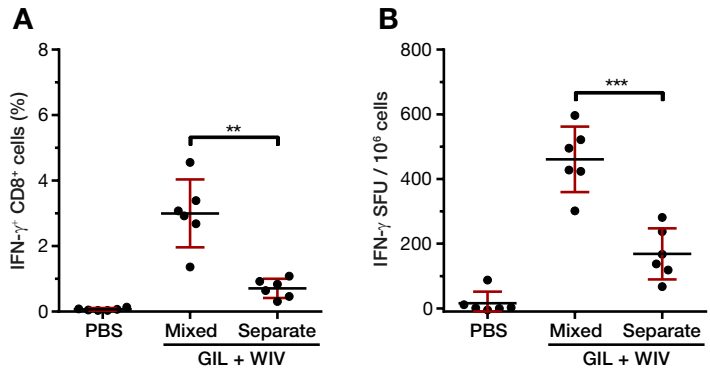

Figure 3. Effect of co-localization on WIV adjuvanticity. HLA-A2.1 transgenic mice were vaccinated twice with 100 µg GIL peptide and 25 µg WIV either in one single flank (mixed) or separate flanks (separate). Splenocytes were restimulated with GIL peptide and analyzed with flow cytometry (A) or ELISpot (B). Data represent mean ± SD (n = 6); **p<0.01, ***p<0.001.

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 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.

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 4B). 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. This indicates that WIV is a robust adjuvant that retains its function even after loss of fusogenicity.

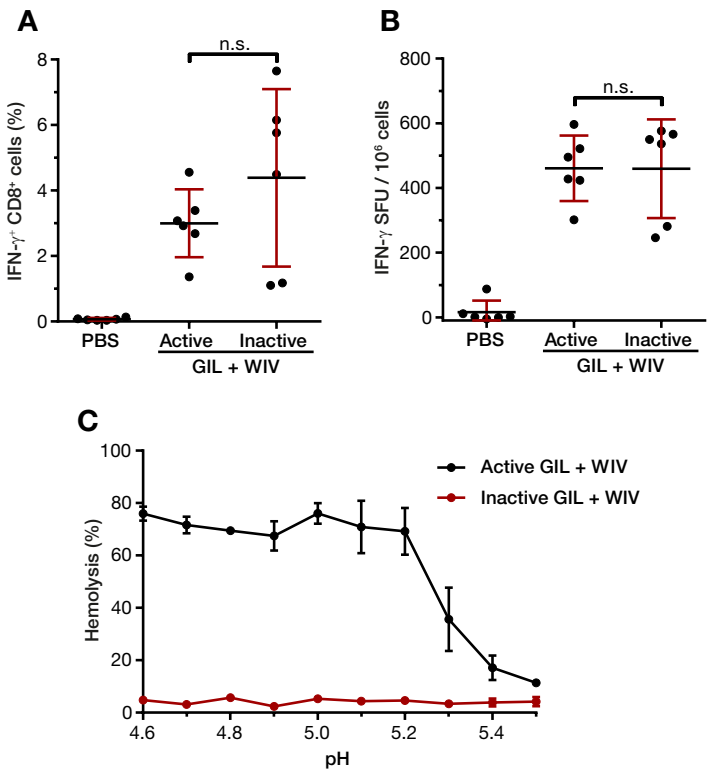

Figure 4. Effect of membrane fusion activity on WIV adjuvanticity. HLA-A2.1 transgenic mice were vaccinated twice with 100 µg GIL peptide and 25 µg fusion-active (active) or fusion-inactive WIV (inactive). Splenocytes were restimulated with GIL peptide and analyzed by flow cytometry (A) or ELISpot (B). 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 represent mean ± SD (n = 3).

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-A2.1-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 S2). 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 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.

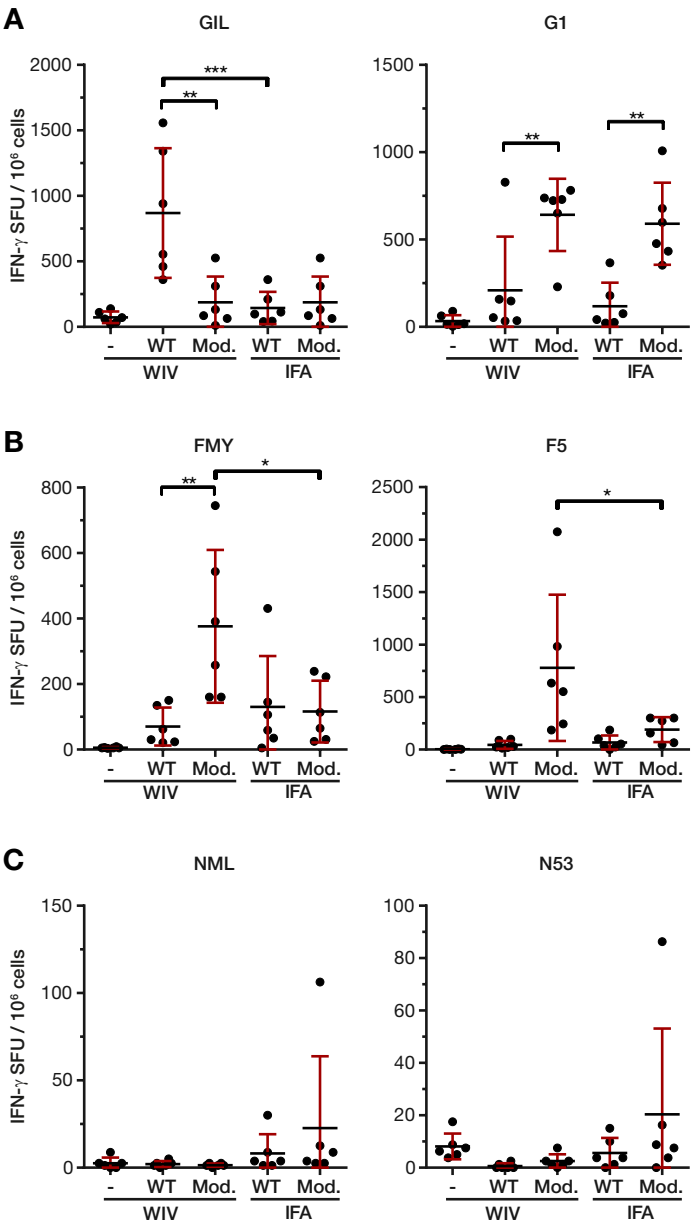

Figure 5. T cell responses against wild-type and modified peptides adjuvanted with WIV. HLA-A2.1 transgenic mice were vaccinated twice with peptide pools containing 100 µg of wild type (WT) peptides (GIL, FMY and NML) or modified (mod.) peptides (G1, F5 and N53) adjuvanted with 5 µg WIV or 50% (v/v) 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 represent mean ± SD (n = 6); *p<0.05, **p<0.01, ***p<0.001.

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-A2.1 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.

ACKNOWLEDGMENTS

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SUPPLEMENTAL DATA

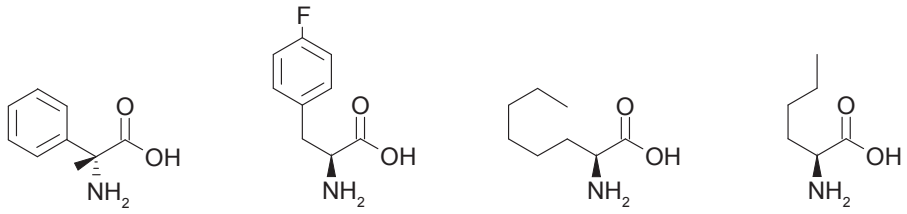

Figure S1. Nonproteogenic synthetic amino acids used for peptide modification. The four nonproteogenic synthetic amino acids were introduced either in GILGFVFTL, FMYSDFHFI or NMLSTVLGV peptides, resulting in modified [am-phg]lLGFVFTL, [4-FPHE]MYSDFHF[2-AOC] and N[NLE]LSTVLGV peptides.

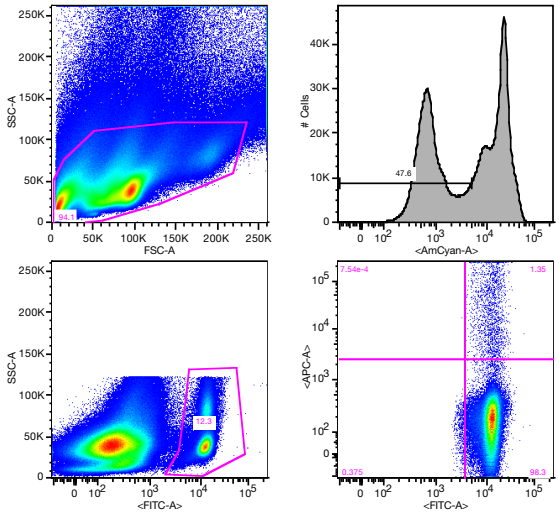

Figure S2. Gating strategy of CD8⁺ IFN-γ⁺ T cells in splenocytes. An example of the gating strategy. The lymphocyte population was first gated (upper left). From this population, all live cells were selected (upper right). Subsequently, CD8⁺ cells were gated (lower left), after which a quadrant gate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

Table S1. 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 (µg)	Peptide (µg)
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 S2. 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 represent 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

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Chapter 5

Predicting the influence of liposomal lipid composition on liposome size, zeta potential and liposome-induced dendritic cell maturation using a design of experiments approach

Peter C. Soema [1,2], Geert-Jan Willems [1], Wim Jiskoot [2], Jean-Pierre Amorij [1] and Gideon F.A. Kersten [1,2]

1 Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands.
2 Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.

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ABSTRACT

In this study, the effect of liposomal lipid composition on the physicochemical characteristics and adjuvanticity of liposomes was investigated. Using a design of experiments (DoE) approach, peptide-containing liposomes containing various lipids (EPC, DOPE, DOTAP and DC-Chol) and peptide concentrations were formulated. Liposome size and zeta potential were determined for each formulation. Moreover, the adjuvanticity of the liposomes was assessed in an *in vitro* dendritic cell (DC) model, by quantifying the expression of DC maturation markers CD40, CD80, CD83 and CD86. The acquired data of these liposome characteristics were successfully fitted with regression models, and response contour plots were generated for each response factor. These models were applied to predict a lipid composition that resulted in a liposome with a target zeta potential. Subsequently, the expression of the DC maturation factors for this lipid composition was predicted and tested *in vitro*; the acquired maturation responses corresponded well with the predicted ones. These results show that a DoE approach can be used to screen various lipids and lipid compositions, and to predict their impact on liposome size, charge and adjuvanticity. Using such an approach may accelerate the formulation development of liposomal vaccine adjuvants.

INTRODUCTION

Many vaccines are based on purified or synthetic antigens derived from their respective pathogens. These include antigens, such as peptides and proteins, which are poorly immunogenic on their own. Adjuvants, based on delivery systems and/or immunopotentiators, are used frequently to improve the immunogenicity of antigens [1]. Liposomes are important delivery systems for vaccines because of their high versatility, which enables them to be suited for many types of antigens [2].

Numerous lipid compositions and preparation methods for liposomes can be chosen, which affect several liposomal characteristics, such as size, zeta potential, bilayer fluidity and encapsulation or association of antigen or adjuvant. In turn, these characteristics can influence the adjuvant effect of liposomes [3]. The adjuvanticity of liposomes is attributed to several mechanisms, such as antigen depot formation, induction of local inflammation and increased antigen uptake by antigen presenting cells.

Antigen presenting cells, with dendritic cells (DCs) in particular, play a pivotal role in the induction of adaptive immune responses. DCs recognize, internalize and process antigens, and ultimately present them to naïve $CD4^+$ or $CD8^+$ T cells [4]. The uptake of antigens by DCs is affected by several antigen characteristics, of which size and surface charge are the most influential. Generally, the size of most subunit antigens is too small for the DC to be taken up efficiently. Incorporation of an antigen into a particulate delivery system such as a liposome, whose size is comparable to that of a virus particle, can therefore significantly increase antigen uptake by DCs through endocytosis [5].

The surface charge density of a liposome influences its zeta potential, and thereby its electrostatic interaction with the surface of a DC. Since cellular membranes are anionic, cationic liposomes are ideally suited to increase antigen uptake by DCs [6]. It is generally accepted that anionic and neutral liposomes are less suited for the induction of immune responses [7]. The cationic liposome formulation CAF01 is currently advancing through clinical trials in combination with HIV and tuberculosis antigens, indicating the potency of cationic liposomes [8, 9].

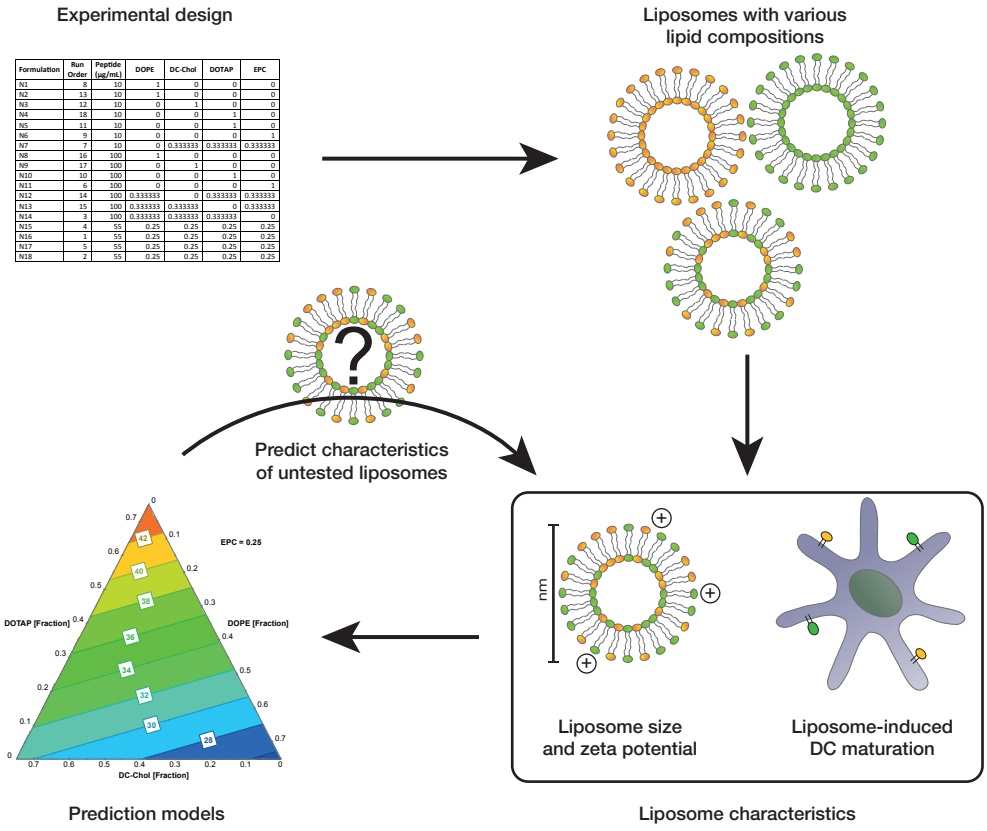
For the successful priming of naïve B- or T cells by DCs, more is needed than efficient antigen uptake and processing. During antigen presentation by the DCs to naïve lymphocytes, costimulatory signals are required. These are provided by the DCs, which can express costimulatory molecules such as CD40 (for B cells), CD80 and CD86 (for T cells) after maturation [10]. The maturation of DCs is considered to be of vital importance for the overall immunogenicity of a vaccine antigen [11]. *In vitro* DC maturation models can therefore be used as preclinical screening tools for vaccine formulations [12].

Immunostimulatory signals, which are often provided by pathogen-associated molecular patterns (PAMPs), are required for the activation of DCs. Inclusion of PAMPs such as Toll-like receptor (TLR) ligands or other molecules in liposomes is therefore a popular strategy to increase liposome

adjuvanticity [13, 14]. Cationic lipids also seem to affect DC maturation [15]. Besides the positive charge, other physical characteristics, such as lipid bilayer fluidity, may affect DC maturation [16]. Chemical differences between cationic lipids indeed have shown to affect DC maturation, underlining the significance of the lipid composition of cationic liposomes.

Design of experiments (DoE) is a statistical method to screen, identify and optimize important factors in various processes, such as pharmaceutical formulation development [17, 18]. It uses a minimal number of experiments to model the effects of each formulation parameter, which significantly accelerates the identification of optimal conditions. A DoE approach was recently employed to optimize the formulation process of itraconazole-loaded liposomes [19]. The authors were able to predict drug loading with a mathematical model obtained with DoE, and identify critical formulation parameters affecting drug loading. However, no attempts have been made yet to predict biological parameters, such as the adjuvanticity of liposomes, with DoE-like approaches.

In this study, the effects of liposomal lipid composition and peptide incorporation on the physicochemical characteristics and the adjuvanticity of liposomes were studied. To gain insight into the effects of each component with a minimal number of experiments, a DoE approach was used. The physicochemical characteristics of the liposomes were determined as the liposome size and zeta potential, while the liposome adjuvanticity was determined as liposome-induced *in vitro* expression of DC maturation factors CD40, CD80, CD83 and CD86. To this end, four lipids, i.e., egg-phosphatidylcholine (EPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), and the HLA-A2.1-restricted influenza peptide GILGFVFTL ($M1_{58-66}$), were used to generate peptide-loaded liposomes with different lipid compositions. Liposome size and zeta potential were determined for each formulation, and prediction models for these parameters were generated by using a DoE approach. Simultaneously, the ability of these liposomes to maturate DCs was evaluated by determining the expression of DC maturation markers CD40, CD80, CD83 and CD86. With DoE, the most influential lipids were identified, and prediction models were generated for each maturation marker. Finally, the prediction models were validated by selecting a liposome with a previously untested lipid composition. A complete overview of the study is depicted in Figure 1.



Formulation	Run Order	Peptide (µg/mL)	DOPE	DC-Chol	DOTAP	EPC
N1	8	10	1	0	0	0
N2	13	10	1	0	0	0
N3	12	10	0	1	0	0
N4	18	10	0	0	1	0
N5	11	10	0	0	1	0
N6	9	10	0	0	0	1
N7	7	10	0	0.333333	0.333333	0.333333
N8	16	100	1	0	0	0
N9	17	100	0	1	0	0
N10	10	100	0	0	1	0
N11	6	100	0	0	0	1
N12	14	100	0.333333	0	0.333333	0.333333
N13	15	100	0.333333	0.333333	0	0.333333
N14	3	100	0.333333	0.333333	0.333333	0
N15	4	55	0.25	0.25	0.25	0.25
N16	1	55	0.25	0.25	0.25	0.25
N17	5	55	0.25	0.25	0.25	0.25
N18	2	55	0.25	0.25	0.25	0.25

Figure 1. Overview of the study concept. An experimental design describing liposomes with various lipid compositions and peptide concentrations is generated with DoE software. Liposomes are formulated according to the design. Then, liposomes characteristics such as size, zeta potential and liposome-induced dendritic cell maturation are determined for each liposome formulation. Models are subsequently fitted to the generated data. Finally, these models can be used to predict the liposome characteristics of liposomes with an untested lipid composition.

MATERIALS AND METHODS

Reagents

The influenza peptide GILGFVFTL ($M1_{58-66}$) was synthesized at the Dutch Cancer Institute (NKI). All lipids (EPC, DOPE, DOTAP, DC-Chol) were purchased from Avanti Polar Lipids, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and sodium chloride from Sigma-Aldrich, Iscove's Modified Dulbecco's Medium (IMDM) from Invitrogen, human granulocyte macrophage colony-stimulating factor (GM-CSF) from Peprotech, human IL-4 from Sanquin, anti-human CD40-PE and CD80-FITC from BD Pharmingen, anti-human CD83-APC and CD86-Pacific Blue from Biolegend, phosphate-buffered saline (PBS; 155 mM NaCl, 1.5 mM potassium phosphate monobasic, 2.7 mM sodium phosphate dibasic, pH 7.2) and Live/dead-Aqua from Life Technologies, lipopolysaccharide (LPS) *E. coli* K12 from Invivogen and Hyclone fetal calf serum (FCS) from Thermo Scientific.

Experimental design

To investigate the effect of the liposome composition on liposome size, zeta potential and liposome-induced DC maturation, a linear mixture model was selected with MODDE 10 (Umetrics) software. Boundaries for EPC, DOPE, DOTAP and DC-Chol fractions were set at 0 and 1 (with 1 being 100% of total lipid content). GILGFVFTL peptide content was set between 10 and 100 µg/mL. A D-optimal design was selected, which was composed of 18 runs, including a quadruple center point [20]. After the runs were completed, models for liposome zeta potential and DC maturation factors CD40, CD80, CD83 and CD86 were created with a partial-least square (PLS) regression. Data were log-transformed, if needed, and non-significant factors were removed from the model until R^2 (model fit) and Q^2 (model prediction power) were optimal.

Liposome formulation

Lipids were admixed (ratios according to the experimental design) to a total amount of 7.5 µmol in 10 ml chloroform. The lipid mixture was transferred to a 50 mL round bottom flask, and the chloroform was evaporated under reduced pressure at 40°C with a rotary evaporator (Buchi Rotavapor R-3). The obtained lipid film was subsequently rehydrated for 2 hours, room temperature at 250 rpm with a shaker (Edmund Bühler Swip KS-10) after addition of GILGFVFTL peptide (concentrations according to experimental design) dissolved in 1.5 mL buffer (10 mM HEPES, 100 mM NaCl, pH 7.4). After rehydration, crude liposomes were extruded five times through a 0.2-µm Nucleopore Track-Etch membrane (Whatman) with a 10-mL Lipex extruder (Northern Lipids Inc.). Each liposome formulation from the experimental design was made in duplicate.

Characterization of liposomes

Liposome size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Nanosizer ZS (Malvern Instruments). The zeta potential of the liposomes was determined after a 5 fold dilution in MilliQ water by laser Doppler velocimetry using a Nanosizer ZS with a universal dip cell (Malvern Instruments).

Maturation of human dendritic cells

Human $CD14^+$ monocytes were isolated from fresh donor blood as described previously [21]. Monocytes were plated at a concentration of $0.4*10^6$ cells/mL in 24-wells plates in IMDM medium containing 1% FCS, 500 U/mL GM-CSF and 800 U/mL IL-4. Monocytes were differentiated to immature dendritic cells (iDCs) after 6 days. iDCs were subsequently stimulated with either medium, LPS or liposomes in duplicate. After 24 hours incubation, cells were transferred to a 96-wells plate and washed twice with FACS buffer (PBS, pH 7.2, 0.5% BSA, 0.5 mM EDTA). DCs were stained with anti-human CD40, CD80, CD83, CD86 and live/dead staining for 30 minutes, and subsequently washed twice with FACS buffer. Samples were measured on a FACS Canto II flow cytometer (BD). Data were analyzed by using FlowJo 10 software for Mac OSX (Tree Star Inc.). Surface markers are reported as % of mean fluorescent intensity (MFI) relative to that induced by LPS.

RESULTS

Liposome characteristics

A linear mixture model was selected to screen the effects of the lipids EPC, DOPE, DC-Chol and DOTAP on the size distribution and zeta potential of the liposomes and their ability to induce DC maturation. A D-optimal design was chosen, which generated a worksheet with 18 formulations (Table 1). Physical characteristics of the liposomes, i.e., size, PDI and zeta potential, were determined. Formulations N1, N2 and N8, which all contained DOPE as the only lipid, did not yield liposomes and were excluded from further experiments. Liposome sizes ranged from 150 to 194 nm, with an average of 170 nm (Figure 2A) and a low PDI (< 0.2), indicating that the liposomes were relatively monodisperse. As expected, the zeta potential of the liposomes containing a cationic lipid (DOTAP and/or DC-Chol) was positive, whereas formulations lacking a cationic lipid (N6, N11) showed a zeta potential close to zero (Figure 2B).

Based on the experimental results, PLS regression models were fitted for both liposome size and zeta potential data using MODDE software. These regression models allowed the identification and qualification of input parameters (being peptide, DOPE, DC-Chol, DOTAP and EPC) which significantly contributed to the output parameters (size and zeta potential). The model regression coefficients reflect the influence of the particular input parameter on the response of the output parameter. Valid models were obtained for both output parameters (Figure 3). Liposome size was influenced the most by EPC (Figure 3A), which increased liposome size when present in high amounts. Model validity for the zeta potential model was low (a value >0.25 indicates a good model fit), which is likely a model artifact caused by the high reproducibility [20]. DOTAP and EPC were the most significant model terms, with DOTAP increasing the zeta potential, and EPC decreasing it (Figure 3B). The incorporation of the peptide antigen had no influence on both liposome size and zeta potential, and was thus removed as a model term.

Response contour surface plots were generated for both liposome size and zeta potential after the fitting of the regression models (Figure 4). These surface plots visualize the predicted value of a response factor according to the corresponding lipid composition at that specific position in the plot (due to the two-dimensional nature of these plots and the multi-dimensional nature of the regression models, one input parameter is kept constant).As expected, both cationic lipids (DOTAP and DC-Chol) increased to zeta potential of the liposomes, whereas the zwitterionic lipids (EPC and DOPE) decreased it.

Table 1. Design of experiments worksheet. Lipids (DOPE, DC-Chol, DOTAP, EPC) are presented as fraction of total lipid content (1=100%).

Formulation	Peptide (µg/mL)	DOPE	DC-Chol	DOTAP	EPC
N1	10	1	0	0	0
N2	10	1	0	0	0
N3	10	0	1	0	0
N4	10	0	0	1	0
N5	10	0	0	1	0
N6	10	0	0	0	1
N7	10	0	0.333333	0.333333	0.333333
N8	100	1	0	0	0
N9	100	0	1	0	0
N10	100	0	0	1	0
N11	100	0	0	0	1
N12	100	0.333333	0	0.333333	0.333333
N13	100	0.333333	0.333333	0	0.333333
N14	100	0.333333	0.333333	0.333333	0
N15	55	0.25	0.25	0.25	0.25
N16	55	0.25	0.25	0.25	0.25
N17	55	0.25	0.25	0.25	0.25
N18	55	0.25	0.25	0.25	0.25

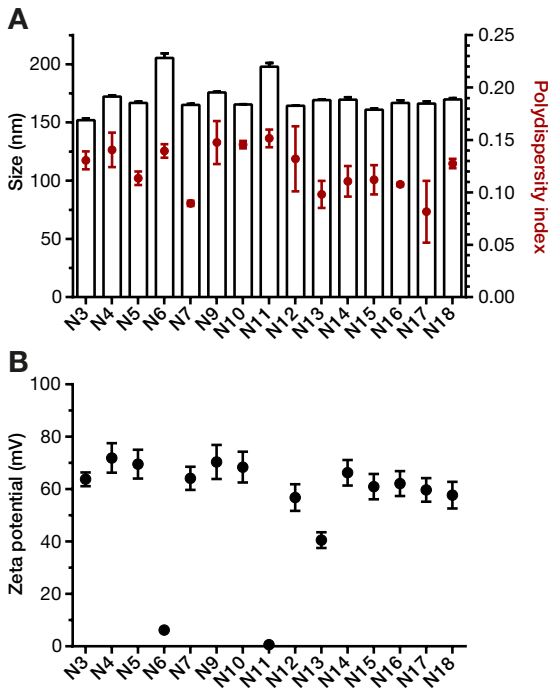


Figure 2. Liposome characteristics. (A) Size and polydispersity index (PDI) of liposomes were determined by dynamic light scattering. (B) The zeta potential of the liposomes was determined by laser Doppler velocimetry. Data represent mean ± upper/lower values (n = 2).

DC maturation by liposomes

The effect of the liposomal lipid composition on DC maturation was evaluated by measuring four DC maturation markers (CD40, CD80, CD83 and CD86) on maturated DCs 24 hours after stimulation with the liposome formulations from the experimental design. The formulations were tested in duplicate on immature DCs isolated from two different donors (donors 1 and 2). LPS was taken as a positive control and reference sample in both experiments. The expression of CD40, CD80, CD83 and CD86 by DCs (derived from donor 1) after stimulation with the liposomes is presented in Figure 5.

Datasets from both experiments were fitted with PLS regression models per individual maturation marker, and the resulting models and their coefficients are summarized in Figure 6 (maturation experiment on DCs derived from donor 1) and Figure S1 (maturation experiment on DCs derived from donor 2). While the resulting models differed between experiments (most likely due to donor variability), the models showed similar trends. Since this study concerned a proof-of-principle, we opted to investigate the models obtained with DCs derived from donor 1 in more detail, since

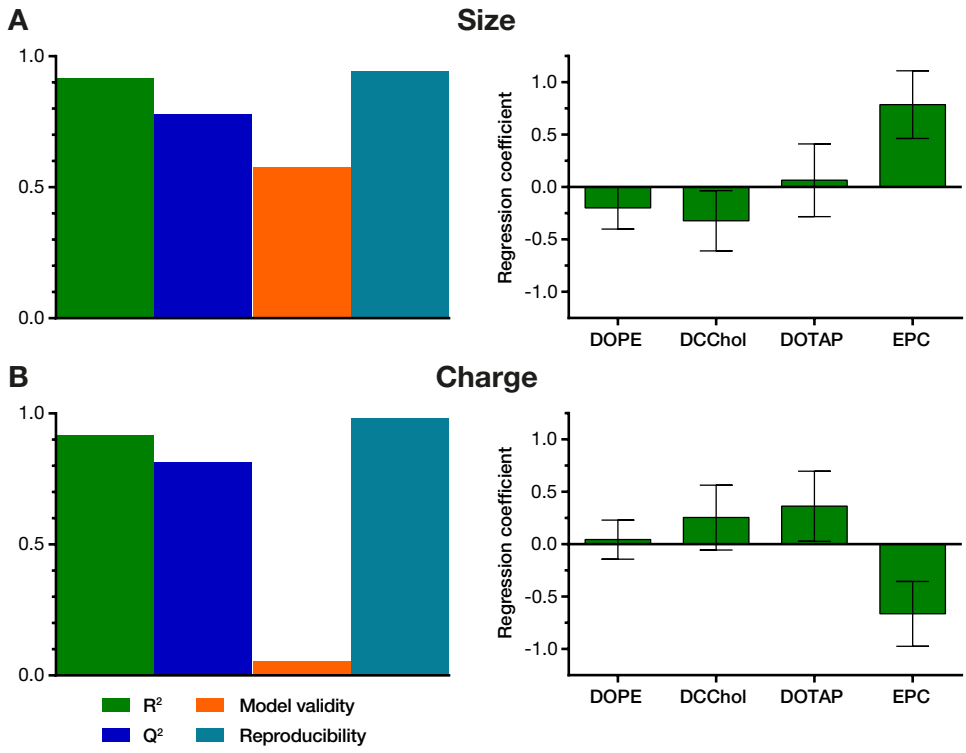


Figure 3. Regression models for liposome size (A) and zeta potential (B). On the left summaries of fit for the models are displayed. Model fit (R^2, >0.5 indicates a good model fit to the data), prediction power (Q^2, > 0.5 indicates sufficient prediction power), model validity (>0.25 indicates that the model error is smaller than the experimental error) and reproducibility (>0.5 indicates a small experimental error) are shown. On the right, normalized model regression coefficients are displayed. Coefficients with a 95% confidence interval that does not cross zero are significant terms.

overall fit of the models from donor 1 were better than those of donor 2.

For both CD40 and CD80 responses models were yielded with a high model fit, validity and reproducibility. Formulation N14 was statistically found to be an outlier, and was subsequently removed from all models. For both CD40 and CD80, the DOTAP lipid was found to be the most significant model term, indicating that the presence of DOTAP in the liposomes induces CD40 and CD80 expression by DCs. The model for CD83 had a relatively low model validity, which again might be a model artifact caused by the high reproducibility. The two cationic lipids, DC-Chol and DOTAP, were the most significant model terms for CD83. The model for CD86 was valid, but suffered overall from a relatively low model fit, predictability and reproducibility. This was confirmed with the model

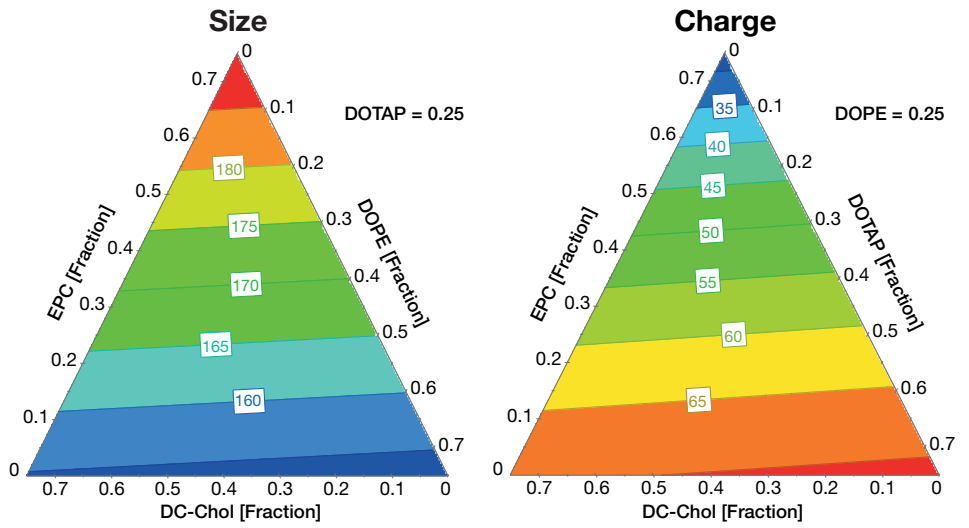


Figure 4. Response contour plots for liposome size and charge. Lipid amounts are displayed as a fraction of 100% total lipid. The fraction of the least influential lipid was set at a constant fraction of 0.25. The values in the boxes and associated color regions represent the predicted response (either size (nm) or zeta potential (mV)) for that particular lipid composition.

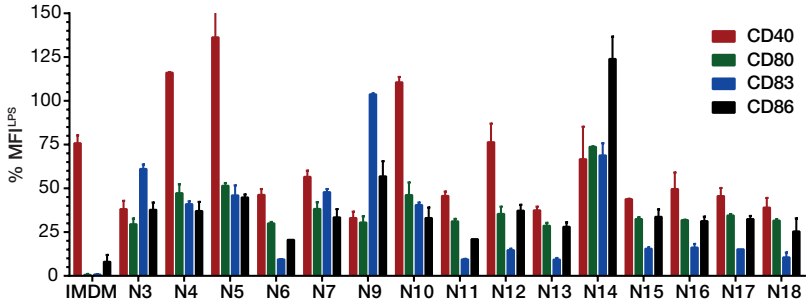


Figure 5. Dendritic cell maturation marker expression after stimulation with either medium or liposomes. Maturation markers are expressed as % mean fluorescent intensity (MFI) relative to that induced by LPS (MFI^{LPS}). Data represent mean + upper value (n = 2).

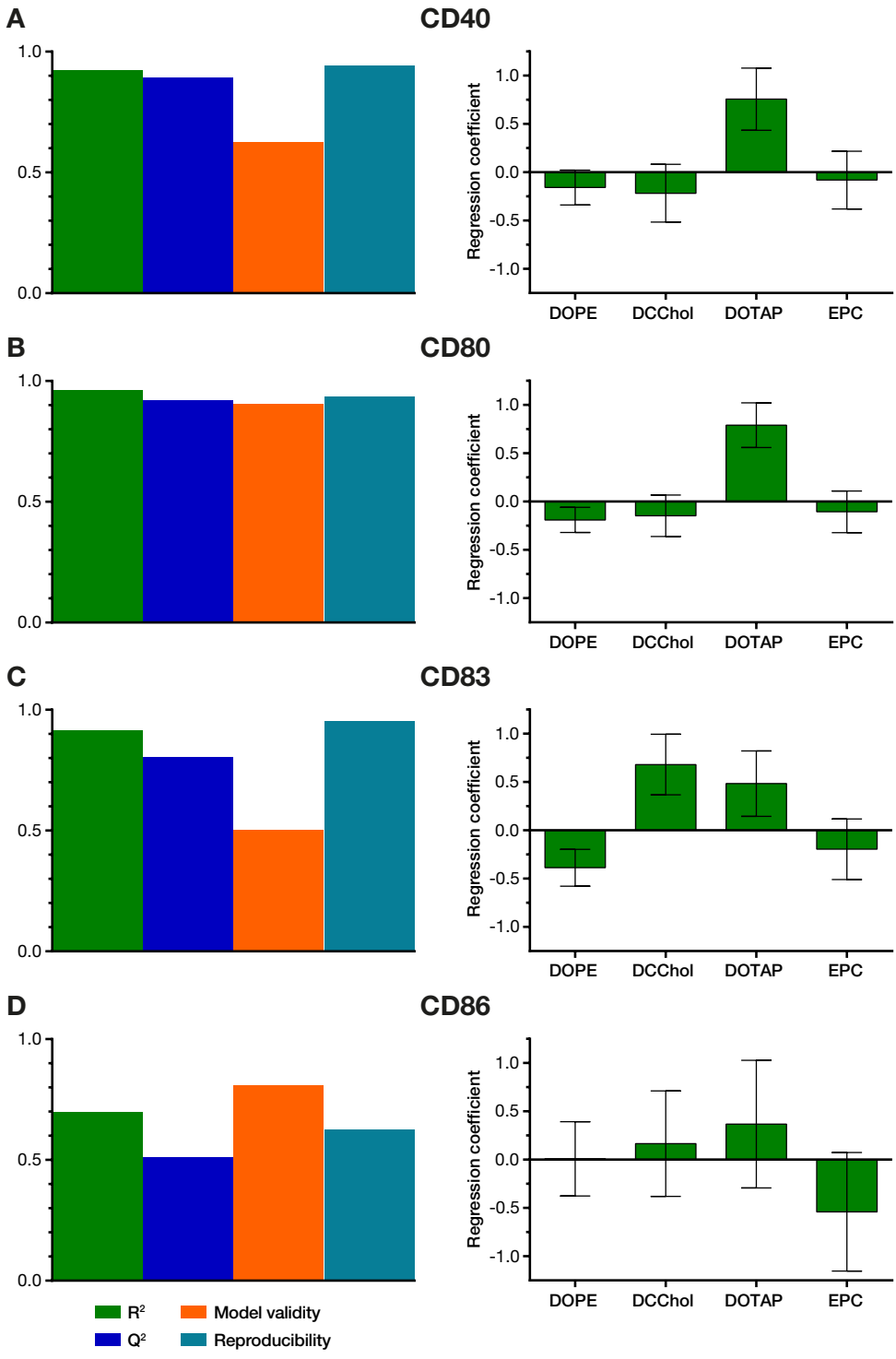

A
B
C
D
1.0
0.5
0.0
R²
Q²
Model validity
Reproducibility
CD40
CD80
CD83
CD86
Regression coefficient
1.0
0.5
0.0
-0.5
-1.0
DOPE
DCChol
DOTAP
EPC

Figure 6 (left). Regression models for dendritic cell maturation markers CD40 (A), CD80 (B), CD83 (C) and CD86 (D). On the left summaries of fit for all models are displayed. Model fit (R^2, >0.5 indicates a good model fit to the data), prediction power (Q^2, > 0.5 indicates sufficient prediction power), model validity (>0.25 indicates that the model error is smaller than the experimental error) and reproducibility (>0.5 indicates a small experimental error) are shown. The normalized model regression coefficients are displayed on the right. Coefficients with a 95% confidence interval that does not cross zero are significant terms.

coefficients, which all have a non-significant contribution to the CD86 response, indicating that no single lipid had a great effect. Similar to the models for liposome size and zeta potential, the peptide content was a non-significant model term in all the models for the maturation markers. The response contour surface plots of all four maturation markers are displayed in Figure 7. From these figures it can be clearly seen that in general, a high fraction of DOTAP and to a lesser extent DC-Chol, has a positive effect on the expression of all maturation markers. Furthermore, the inclusion of DOPE generally had a negative effect on the maturation. The lipid EPC was non-influential for most responses, and is therefore still suited as a helper lipid to produce stable liposomes.

Prediction power of obtained models

As described previously, valid prediction models were obtained for liposomal size, zeta potential and all four DC maturation markers. The prediction power of the models was tested by selecting a liposomal formulation that was not yet included in the D-optimal design. For proof-of-principle purposes, an initial target response factor was set for the liposome formulation. In this case, a target liposomal zeta potential was set. Since most formulations in the experimental design showed a zeta potential of either above 60 mV or 0 mV, a target zeta potential of 30 mV was chosen. The zeta potential prediction model subsequently gave a lipid composition (Table 2) which should yield liposomes with a zeta potential of 30 mV. The liposome formulation (N19) was made, and size and zeta potential were determined (Table 3), which indeed correlated with the predicted values. Subsequently, the selected lipid composition could now be used as an input for the previously acquired prediction models for liposomal adjuvanticity.

Next, liposome formulation N19 was added to immature DCs, and DC maturation markers were determined. The experimentally acquired data were similar to the predicted means (Table 3), indicating that the predictions made by the models were accurate.

Table 2. Lipid composition of a liposome formulation predicted to have a zeta potential of 30 mV by the zeta potential model.

Formulation	Peptide (µg/mL)	DOPE	DC-Chol	DOTAP	EPC
N19	13.4	0.03	0.07	0.13	0.77

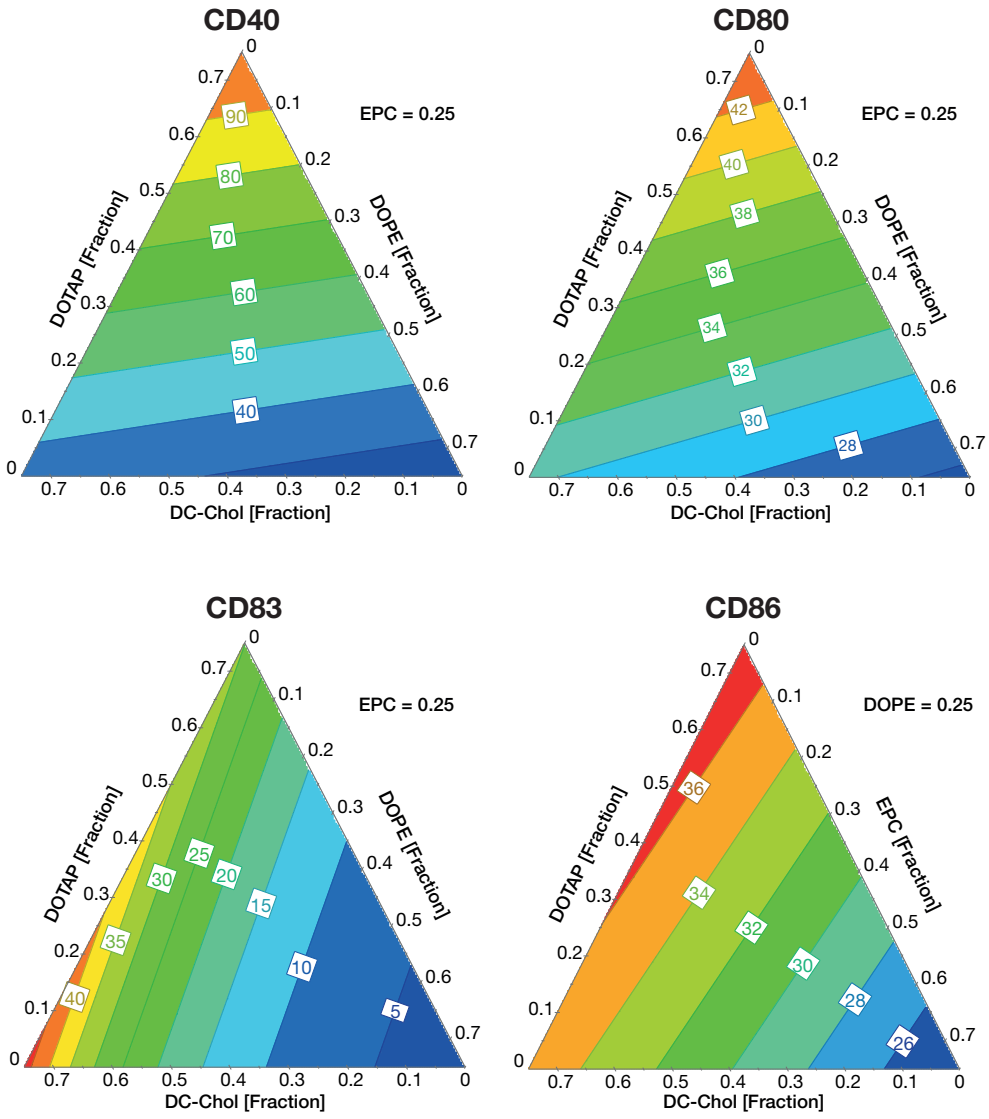


Figure 7. Response contour plots for DC maturation markers induced by liposomes. Lipid amounts are displayed as a fraction of 100% total lipid. The fraction of the least influential lipid was set at a constant fraction of 0.25. The values in the boxes and associated color regions represent the predicted response (either CD40, CD80, CD83 or CD86, all in % of LPS-induced expression) for that particular lipid composition.

Table 3. Assessment of the validity of the prediction models. The liposome size, zeta potential and liposome-induced maturation markers were predicted by the acquired models for formulation N19. Predictions are expressed as mean ± 95% confidence intervals. Measured values are given as mean ± upper/lower values (n = 2).

	Predicted mean	Lower	Upper	Measured
Size (nm)	188.5	183.3	193.7	181.1 ± 8.7
PDI	n.a.[a]	n.a.	n.a.	0.12 ± 0.01
Zeta potential (mV)	30.0	17.1	39.5	30.3 ± 6.2
CD40 (% MFI^{LPS})	52.3	40.1	64.5	46.2 ± 16.8
CD80 (% MFI^{LPS})	32.9	30.9	34.7	31.1 ± 3.9
CD83 (% MFI^{LPS})	13.1	9.6	17.7	13.0 ± 4.0
CD86 (% MFI^{LPS})	24.8	19.7	29.8	26.1 ± 6.8

[a] Not applicable; no valid prediction model for PDI was generated in this study.

DISCUSSION

From a historical perspective, most researchers are inclined to vary one factor at a time (OFAT) when systematically screening or optimizing a certain system or formulation. Such OFAT approaches however are ineffective, since the number of experiments increases exponentially when a variable is added to the design. Another drawback of OFAT is that important interactions between the parameters can be missed. Utilizing a DoE approach instead solves some of these OFAT-associated constraints, by decreasing the number of experiments needed to screen multiple variables, and to visualize interactions with the aid of statistical models. Furthermore, prediction models can be generated from the existing data, which can predict inter- or extrapolated variables that have not been tested yet.

In the current study, a DoE approach was used to investigate a five-component (one antigen and four lipids) liposomal system with respect to physicochemical properties and biological activity: size, zeta potential and liposome-induced DC maturation. While DoE approaches are increasingly used for the formulation and process development of pharmaceuticals [4], they have been rarely used in studies involving liposomes. Two previous studies investigated the role of different liposome formulation processes on the encapsulation efficiency of either a poorly soluble drug molecule [19], itraconazole, or a small peptide [22]. These studies proved that the DoE approach is applicable for the development and optimization of liposomal formulations. In this current study, it was found that the liposomal lipid composition affected liposomal characteristics such as size, zeta potential and liposome-induced DC maturation. The inclusion of a peptide antigen, however, was not of influence on any of these factors.

It is clear from our results that the liposomal lipid composition influenced the expression of DC maturation markers. However, not much is yet known on the individual effects of these lipids on expression of CD40, CD80, CD83 or CD86. Vangasseri et al. previously demonstrated that liposomes containing DOTAP effectively induced CD80 and CD86 expression by DC2.4 cells [16]. When the cationic head group of DOTAP was replaced by anionic or neutral head groups, the liposomes lost their ability to induce DC maturation. Similarly, replacement of the unsaturated fatty acid chain of DOTAP with saturated analogues was detrimental to the maturation response. Addition of counter ions to the cationic liposomes also did not affect their ability to induce DC maturation. Another study showed similar results with DOTAP:DOPC liposomes; a higher molar ratio of DOTAP correlated with increased CD83 and CD86 expression by human monocyte-derived DCs [23]. From these results, it was hypothesized that not only the zeta potential of the liposomes, but also the chemical composition of the lipids influenced the immunostimulatory properties of liposomes.

Our results confirm that liposomes containing cationic lipids, particularly DOTAP, were able to induce DC maturation. Contrarily to the expression of CD80, CD86 and CD40, the CD86 marker expression was more sensitive to DC-Chol than to DOTAP. It has been previously reported that DC-Chol liposomes also have an immunostimulatory effect on DCs [15]. The difference in expression of the

maturation markers with these two cationic lipids might be related to the chemical and structural differences between the lipids, as mentioned earlier. Further studies are needed to elucidate the underlying mechanisms for these differences, in order to support the rational design of optimal cationic liposomes for the induction of DC maturation and subsequent immune responses.

The maturation experiments in the current study were performed on immature DCs derived from human blood monocytes isolated from donors. This introduces a donor variety into the DC studies, which can have a large effect on the prediction models. Indeed, the obtained prediction models from experiments using two differed iDC donor sources showed some differences due to biological donor variety. To eliminate this biological variability from the models, future investigations could be performed on immortalized DC cell lines. Human-derived DC cell lines such as MUTZ-3 have been used to screen vaccine immunogenicity, and showed consistent maturation responses opposed to monocyte-derived DCs from fresh blood, which showed a large donor variability [24]. Using such cell lines would probably yield prediction models that can be used continually on the same cell line, which is a huge advantage for the reproduction of the experiments. When combined, the current DoE approach and established DC cell lines could form an effective platform to rapidly screen liposomal (and other) vaccine formulations without the use of animal studies [12].

Aside from the liposome-induced DC maturation responses, the effects of lipid composition on liposome size and zeta potential were investigated and modeled. While the size of the liposomes is mostly dictated by the formulation method (e.g., extrusion and sonication), the lipid composition does influence the size to some extent. This may be accredited to differences in lipid tail length, molecular shape and membrane fluidity, but also the incorporation of charged lipids. Nonetheless, the size variations observed in this study were small, and therefore most likely did not influence size-dependent mechanisms, such as uptake by DCs [25].The zeta potential of the liposomes was influenced by the lipid composition. The cationic lipids DC-Chol and DOTAP both increased the zeta potential of the liposomes, while EPC had a neutralizing effect on the zeta potential. The acquired model for zeta potential could accurately predict a suitable lipid composition of a liposome with a zeta potential of 30 mV. The ability to predict the zeta potential of a liposome according to its lipid composition could be a powerful tool, since the zeta potential of liposomes affects several factors [26], such as their colloidal stability (electrostatic repulsion), encapsulation efficiency of a drug or antigen (electrostatic attraction) and depot formation at the injection site.

In conclusion, this study shows the usefulness of a DoE approach to investigate the influence of the lipid composition and antigen content of liposomes on their physicochemical characteristics (size and zeta potential) and biological effect (maturation of DCs). The obtained models were able to accurately predict liposome size, zeta potential, and relative levels of liposome-induced DC maturation factors CD40, CD80, CD83 and CD86. This approach could be a valuable method for the development of liposome-based vaccine adjuvants.

ACKNOWLEDGMENTS

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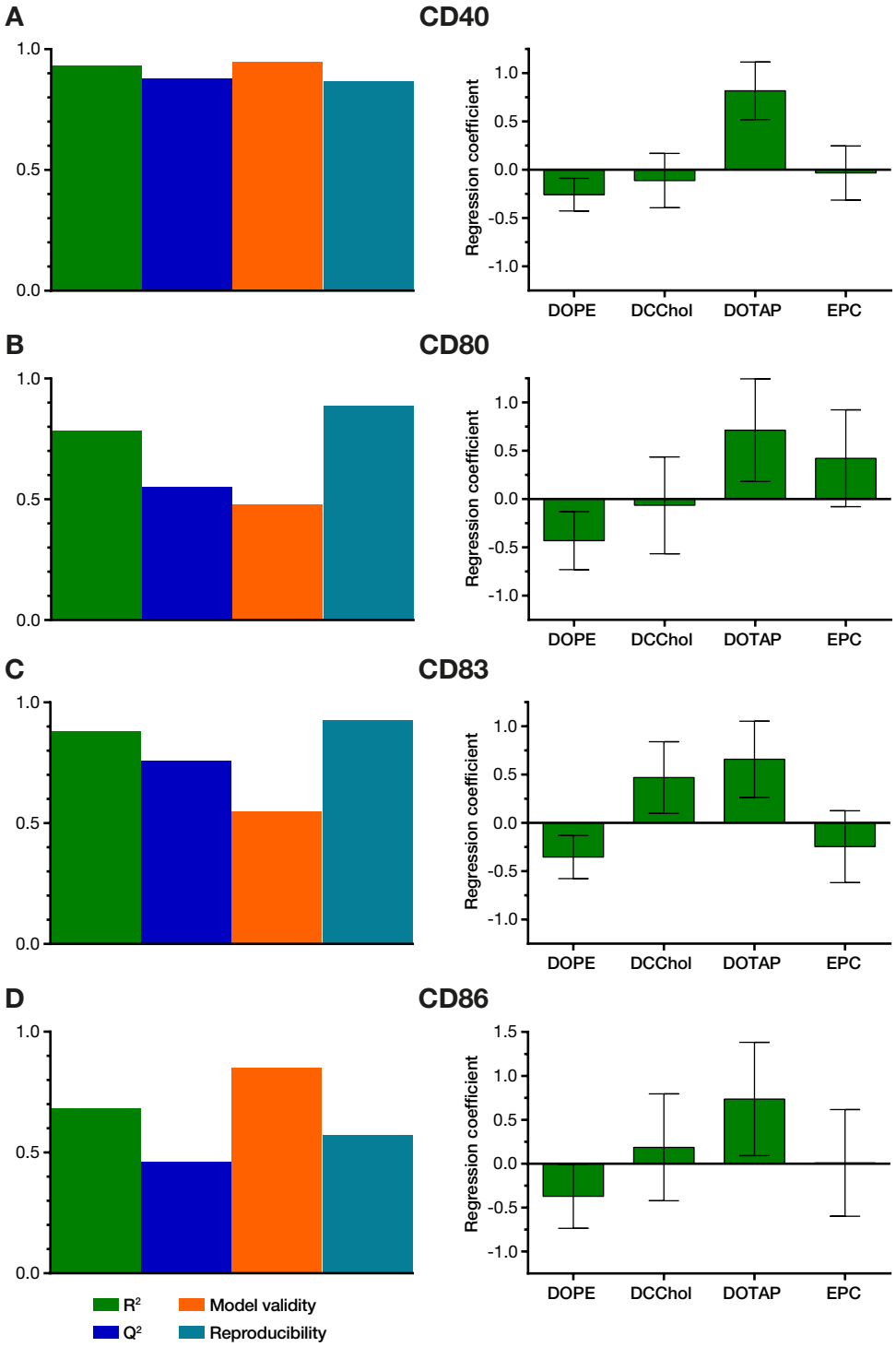
Figure S1 (right). Regression models for dendritic cell maturation markers CD40 (A), CD80 (B), CD83 (C) and CD86 (D) of the second maturation experiment. On the left summaries of fit for all models are displayed. Model fit (R^2, >0.5 indicates a good model fit to the data), prediction power (Q^2, > 0.5 indicates sufficient prediction power), model validity (>0.25 indicates that the model error is smaller than the experimental error) and reproducibility (>0.5 indicates a small experimental error) are shown. The normalized model regression coefficients are displayed on the right. Coefficients with a 95% confidence interval that does not cross zero are significant terms.

SUPPLEMENTAL DATA



Chapter 6

Development of cross-protective influenza A vaccines based on cellular responses

Peter C. Soema [1,2], Elly van Riet [1], Gideon F.A. Kersten [1,2] and Jean-Pierre Amorij [1]

1 Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands.
2 Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.

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ABSTRACT

Seasonal influenza vaccines provide protection against matching influenza A virus (IAV) strains mainly through the induction of neutralizing serum IgG antibodies. However, these antibodies fail to confer a protective effect against mismatched IAV. This lack of efficacy against heterologous influenza strains has spurred the vaccine development community to look for other influenza vaccine concepts, which have the ability to elicit cross-protective immune responses.

One of the concepts that is currently been worked on are influenza vaccines inducing influenza-specific T cell responses. T cells are able to lyse infected host cells, thereby clearing the virus. More interestingly, these T cells can recognize highly conserved epitopes of internal influenza proteins, making cellular responses less vulnerable to antigenic variability. T cells are therefore cross-reactive against many influenza strains, and thus are a promising concept for future influenza vaccines.

Despite their potential, there are currently no T cell based IAV vaccines on the market. Selection of the proper antigen, appropriate vaccine formulation and evaluation of the efficacy of T cell vaccines remains challenging, both in preclinical and clinical settings.

In this review, we will discuss the current developments in influenza T cell vaccines, focusing on existing protein-based and novel peptide-based vaccine formulations. Furthermore, we will discuss the feasibility of influenza T cell vaccines and their possible use in the future.

INTRODUCTION

Several million people worldwide are infected with influenza viruses annually, which can result in hospitalization and even death from complications in severe cases. Vaccination is the preferred method to prevent influenza virus infections. Two types of influenza, influenza A and B, currently circulate among the human population. The influenza A virus (IAV) however can be further divided in several subtypes and strains. The surface antigens of IAV, hemagglutinin (HA) and neuraminidase (NA), frequently alter due to antigenic drift and sometimes alter due to antigenic shifts. Seasonal influenza vaccines need to be updated accordingly to match the circulating IAV strains. While seasonal influenza vaccines are effective against their matched IAV strains, they are unable to cross-react with unmatched strains. The lack of cross-reactivity of vaccine-elicited immune responses, mainly antibodies, is a major limitation of current influenza vaccines.

Several novel concepts for the development of cross-reactive IAV vaccines have been pursued in recent years. One concept is a vaccine that induces mucosal IgA responses, which can induce strong cross-protective antibody responses against closely related IAV strains (Figure 1). However, the cross-reactivity of these IgA responses with respect to more divergent strains is modest [1]. Alternatively, vaccines that induce (IgG) antibody responses against conserved antigens, such as HA stalk-reactive- or M2e-specific antibodies, might be promising [2, 3]. Studies however indicate that these approaches mostly lead to cross-reactive responses within the same phylogenetic group of IAV, such as H5N1 and H1N1 [4], with some exceptions [5, 6]. Finally, vaccines inducing influenza-specific T cell responses can offer broad and long-lasting immune responses. Since T cells recognize epitopes that are mostly derived from viral proteins located in the nucleocapsid, which are conserved between IAV strains, T cell responses can be effective against a broad range of influenza strains. This averts the necessity of seasonally changing the influenza vaccine composition, and thus could be a significant improvement over the current influenza vaccines. A drawback of a purely T cell-inducing vaccine for the prevention of seasonal influenza could be that, unlike IgA antibodies, T cell responses cannot prevent infection but prevent (severe) disease. For the application as a universal vaccine, currently T cell responses are thought to have the highest potential to induce such broad heterosubtypic responses that can react to any IAV subtype.

Natural IAV infections induce, next to antibody responses, T cell responses that are potentially cross-reactive. Indeed, it is assumed that memory T cell established by previous IAV infections prevent subsequent IAV infection in some instances; most individuals experience severe IAV-induced symptoms only a few times in their life. However, there are indications that the CTL activity of T cell recall responses wanes over times in humans, suggesting that T cell responses established by IAV infections can only protect for a few years [7]. Additionally, the number of available influenza-specific memory T cells should be large enough to be able to rapidly respond to IAV infection without excess additional expansion of the T cell pool [8]. Furthermore, it is known that regulatory T cells suppress T cell responses during IAV infections, which can have a negative effect on the subsequent formation of a memory T cell pool [9]. Natural IAV infections therefore

do not mount a T cell response potent enough to provide long-lasting protection against all heterologous IAV strains. T cell-inducing influenza vaccines might overcome this shortcoming by establishing long-lasting, cross-reactive T cell responses. In this review, we will focus on the latest developments in T cell-inducing influenza vaccine research. The selection of antigen, formulation and administration strategies, as well as possible risks and limitations of T cell-inducing vaccines are evaluated.

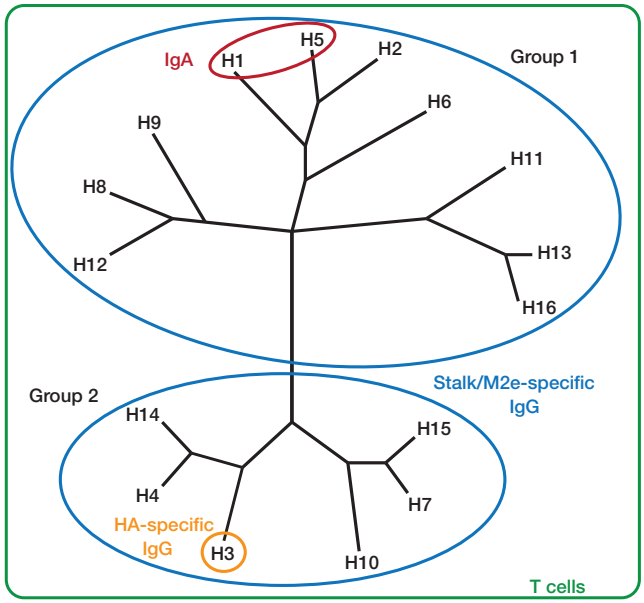

Figure 1. Reactogenicity of immune responses against influenza strains. Influenza A strains are displayed in their respective phylogenetic groups. HA-specific IgG responses (orange) react only with homologous influenza strains. Mucosal IgA responses (red) can provide heterosubtypic reactivity against related influenza strains. Stalk- or M2e-specific antibodies (blue) are cross-reactive within either group 1 or group 2 influenza strains. T cells react universally against all influenza strains, regardless of subtype or group.

ROLE OF T CELLS IN INFLUENZA

CD8$^+$ T cells

Primed CD8$^+$ T cells, otherwise known as cytotoxic T cells (CTLs), are able to lyse influenza-infected cells. Via the endogenous antigen presentation pathway, infected cells will present influenza-derived epitopes on their cell surface, which are recognized by influenza-specific CTLs. The CTLs then induce apoptosis of the target cell either through the secretion of perforins and granzymes, or through the Fas ligand pathway. Furthermore, CTLs produce proinflammatory cytokines such as TNF-α and IFN-γ that exert antiviral activity, which further aids viral clearance [10, 11].

Several recent studies have elucidated the importance of CD8$^+$ T cells during IAV infections in humans. Sridhar et al. showed that individuals that possessed a higher frequency of CD8$^+$IFN-γ$^+$IL-2$^-$ T cells experienced a decreased clinical illness during infection with pandemic 2009 H1N1 IAV [12]. CD8$^+$IFN-γ$^+$IL-2$^-$ T cells were correlated with a decreased risk of fever, an absence of viral shedding and reduced influenza-like illness (ILI) symptoms. These cells also expressed the lung-homing marker CCR5, which might explain their effectivity. CD8$^+$ T cells induced by seasonal IAV strains were shown to be cross-reactive with several influenza A strains such as 2009 H1N1, swine-origin H3N2 and the recently emerged H7N9 IAV [13-15]. Indeed, when cellular responses were studied in individuals infected with pandemic 2009 H1N1 IAV, rapid recall responses of CD8$^+$ T cells were observed, which peaked within 1 week after infection [16]. These responses were thought to originate from lymphoid memory CD8$^+$ T cells established from prior seasonal IAV infections. Memory T cells were demonstrated to last for at least several years in a study which assessed IAV-specific T cell responses in PBMC's of individuals collected from 1999 to 2012 [17]. PBMC's from several donors were stimulated with Resvir-9 (a H3N2 reassortant strain), and IAV-specificity and CTL activity was subsequently determined by intracellular staining with several labeled, highly conserved CTL peptides and IFN-γ.

Taken together, these studies indicate that CD8$^+$ T cells can play a role in the protection against IAV infections, that these T cells are long-lived and are able to cross-react with multiple IAV strains. Thus, the induction of these T cells may be the basis of broadly reactive universal influenza vaccines.

CD4$^+$ T cells

CD4$^+$ T cells also play an important role in IAV infections, but contrarily to CD8$^+$ T cells, have not been studied extensively yet in humans. In animal models, activated CD4$^+$ T cells can exert different roles in relation to IAV infections. CD4$^+$ T cells can act as T helper cells (TH), providing costimulatory signals by CD40/CD40L signaling to antigen presenting cells (APCs) during the priming of B cells and CD8$^+$ T cells [18, 19]. Interestingly, reactivation of adoptively transferred CD4$^+$ TH (from IAV challenged mice) increased the recall capacity of both memory CD4$^+$ and CD8$^+$ T cell responses in receptive mice after IAV infection [20]. While CD4$^+$ TH is not necessarily required for the induction of effector CTLs, it is crucial for the transition of CD8$^+$ T cells to the memory phase, which is essential for the maintenance of long-lived immunity [21, 22]. Surprisingly, CD4$^+$ T cells can also acquire cytotoxic

activity through the release of perforin in mice, providing direct protection against IAV infection [23].

In humans, it was found that preexisting $CD4^+$ T cells were reactive to pandemic 2009 H1N1 peptides, and were correlated with lower virus shedding and reduced illness during IAV infection [24]. Unexpectedly, $CD8^+$ T cell responses were not associated with reduced illness in this study. Nonetheless, it can be concluded that preclinical and clinical studies indicate that targeted induction of $CD4^+$ T cell responses, next to $CD8^+$ T cell responses, may be an attractive goal for novel vaccines.

T CELL-INDUCING INFLUENZA VACCINES

Immune responses and in particular the antibodies elicited by current seasonal influenza vaccinesare limited in their effectiveness against heterologous IAV infections. From the current knowledge on T cell responses during IAV infections in preclinical and clinical studies, as described above, it is believed that T cell-inducing influenza vaccines have the potential to result in broadly reactive, universal influenza A vaccines. While most vaccines are still in preclinical development, a few concepts have recently entered the clinical phase. In Table 1 the most recent developments in T cell-inducing vaccines are listed.

Recently, the potency of viral vector-based influenza vaccines has been reviewed [47]. In the following paragraphs, several other potential T cell-inducing influenza vaccines are highlighted.

Live attenuated influenza vaccines

Live attenuated influenza vaccines (LAIV) are currently on the market as intranasal (i.n.) IAV vaccines. LAIV induces next to humoral responses both $CD4^+$ and $CD8^+$ T cells in children [25, 26]. Remarkably, no cellular immune responses are detected in adults receiving LAIV; the cause of this discrepancy might be related to the naïve status of children. Furthermore, LAIV is more effective than current seasonal trivalent inactivated influenza vaccines (TIV) in children but not in adults, suggesting that the induction of cellular immune responses increases the efficacy of LAIV [48]. The encapsulation of LAIV in a biopolymer of alginate and subsequent subcutaneous (s.c.) administration induced $CD8^+$ T cell responses that protected mice from a heterologous IAV challenge [49], indicating that LAIV can induce T cell responses via immunization routes other than i.n. by use of formulation strategies. The induction of cellular responses by LAIV might be explained by the "live" state of the vaccine antigen; it can still infect after vaccination. During the viral replication, many viral proteins containing $CD8^+$ and $CD4^+$ epitopes are produced within the infected host cell, leading to efficient antigen processing via the endogenous pathway, which leads to MHC-I presentation and subsequent T cell activation.

Whole inactivated influenza virus

Like LAIV, whole inactivated influenza virus (WIV) contains internal proteins such as nucleoprotein (NP), matrix proteins 1 and 2 (M1 and M2 respectively), polymerase basic proteins 1 and 2 (PB1 and PB2 respectively) and polymerase acidic protein (PA), which possess conserved T cell epitopes. WIV vaccines were replaced by subunit and split vaccines due to incidence of adverse effects associated with WIV [50], but have been given increased attention the past few years in the search for cross-reactive vaccines [51]. Improvements on WIV production and purification methods have decreased WIV-associated side effects, making this vaccine acceptable for use again, especially for the induction of broadly reactive immune responses. At normal clinical dose, which typically does not exceed fifteen micrograms of HA protein, WIV induces adequate neutralizing antibody titers, but generally fail to induce any cellular responses regardless of administration route [52]. However, studies by Budimir et al. showed that multiple high doses of WIV, such as two times 6 micrograms, were able to induce significant amounts of IAV-specific CTLs in mice [53-55]. The critical roles of

Table 1. T cell-inducing influenza vaccines in recent development.

Class	Concept name	Antigen(s)	Adjuvant(s)	Immune response	Status	Ref.
Whole virus or protein vaccine	Live attenuated influenza vaccine	Live attenuated influenza vaccine (various strains)	None	Induces $CD4^+$ and $CD8^+$ T cell responses in unprimed children	Licensed	[25, 26]
		Single-cycle live attenuated influenza vaccine (H3N2)	None	Induced $CD8^+$ T cell responses in mice that protected against heterologous challenge	Preclinical	[27]
	Gamma-irradiated whole inactivated influenza vaccine	Whole inactivated influenza vaccine (H3N2)	None	Induces robust influenza-specific T cell responses in mice	Preclinical	[28]
	Influenza virosomes	Virosomes (H5N1)	Matrix-M	Induces good influenza-specific $CD4^+$ T cell responses in healthy adults, but $CD8^+$ T cell responses were limited	Phase I trials	[29]
	Multimeric-001	Synthetic protein containing B and T cell epitopes from HA, M1 and NP	Montanide ISA 51VG	Induces cellular responses in healthy adults and elderly that are reactive against multiple IAV strains	Phase I trials	[30, 31]
Peptide vaccine	Lipopeptides	Minimal T cell epitopes from M1, PA and NS1	Pam2Cys	Induces $CD8^+$ T cell responses that protect mice against heterologous IAV challenge	Preclinical	[32]
		Minimal T cell epitopes from HA and NP combined with seasonal influenza vaccine	Pam2Cys	Induces $CD8^+$ T cell responses that reduces lung viral load in mice after heterologous challenge	Preclinical	[33]
		Minimal T cell epitope from NP	Phosphatidylserine	Induces peptide-specific $CD4^+$ and $CD8^+$ T cell responses in mice	Preclinical	[34]
	Liposome-conjugated peptides	Minimal T cell epitopes from M1, NP, PA, PB1 or PB2	Liposomes, CpG ODN 5002	Induces T cell responses that protect mice from IAV challenge	Preclinical	[35, 36]
	Peptide-loaded virosomes	Minimal T cell epitope from M1	Virosome, CpG ODN 1826	Induces peptide-specific $CD8^+$ T cells that reduce body weight loss of mice after heterologous IAV infection	Preclinical	[37]
	FP-01.1	Long peptides containing T cell epitopes from M1, NP, PB1 and PB2	Peptides conjugated to fluorocarbon moiety	Induces $CD4^+$ and $CD8^+$ T cells in healthy adults that are cross-reactive against IAV-infected target cells	Phase I trials	[38]
	Flu-v	Long peptides containing T cell epitopes from M1, M2 and NP	Montanide ISA 51VG	Induces peptide-specific $CD8^+$ T cells in healthy adults	Phase I trials	[39]
Virus-like particle / viral vector vaccine	Peptide fused to PapMV nanoparticles	T cell epitope from NP	Papaya mosaic virus nanoparticles	Induces peptide-specific $CD8^+$ T cells in mice	Preclinical	[40]
	DdFluM1	T cell epitopes from M1	Adenoviral dodecahendron particles	Induces peptide-specific $CD4^+$ and $CD8^+$ T cells in chickens	Preclinical	[41]
	PIV5-NP	T cell epitope from NP	Parainfluenza 5	Induces $CD8^+$ T cells in mice that reduce morbidity and lethality after IAV challenge	Preclinical	[42]
	MVA-NP+M1	T cell epitopes from M1 and NP	Modified vaccinia virus Ankara vector	Induces influenza-specific cellular responses in healthy adults and elderly that reduce viral shedding and reduction of symptoms	Phase II trials	[43-45]
DNA vaccine	DNA plasmids encoding for T cell epitopes	DNA encoding for B and T cell epitopes from HA and NP	None	Induces T cell responses that reduce body weight loss of mice after IAV challenge	Preclinical	[46]

membrane fusion activity and the presence of viral ssRNA for the induction of CTLs were established [54, 55]. Intramuscular (i.m.) administration of WIV proved to be more effective at inducing CTLs than i.n. administration [53]. This was confirmed by Takada et al., who found that intranasal vaccination with WIV failed to induce T cell responses [56]. In contrast, one study utilizing gamma-irradiated WIV showed that the protective effect of WIV was mainly mediated by T cell responses [28]. It is suspected that the method of WIV inactivation can have an effect on its immunogenicity. Aside from increased dosage, WIV-induced cellular responses can also be boosted by the addition of adjuvants. For instance, a dose of 2.5 micrograms WIV adjuvanted with cationic lipid/DNA complex (CLDC) was able to induce influenza-specific $CD4^+$ and $CD8^+$ T cell responses in mice, whereas alum adjuvanted WIV only induced high antibody responses [57]. Similar to studies with WIV, the addition of alum to virosomes proved to be detrimental to cellular responses in mice [58], since it skewed the T_H to a T_H2-type response.

Virosomes

Virosomal vaccines can also induce influenza-specific CTL responses. The addition of adjuvants to virosomes is necessary to induce T cell responses, since unadjuvanted virosomes only induce humoral responses. The incorporation of LpxL1, a detoxified lipopolysaccharide, in virosomes significantly increased IFN-γ secretion in mice [59]. Madhun et al. showed that addition of the saponin-based Matrix-M adjuvant to virosomes significantly increased the production of T_H1-associated cytokines IL-2 and IFN-γ when administered i.m. to mice [60]. Strikingly, a significant induction of multifunctional $CD4^+$ T cells was also observed in a murine model after the addition of Matrix-M to the virosomal vaccine. In a similar study, Radosevic et al. screened multiple adjuvants (i.e. aluminium phosphate, aluminium hydroxide, MF59 and Matrix-M) in combination with virosomes in mice [61]. Unlike the study by Madhun et al., virosomes were readily able to induce $CD4^+$ T cells, and addition of any adjuvant, including Matrix-M, did not increase these responses. However, only MF59 and Matrix-M adjuvanted virosomal vaccines were able to induce IAV-specific $CD8^+$ T cell responses. Furthermore, addition of any aluminium salt-based adjuvants proved to be ineffective at eliciting any cellular responses, which was probably due to T_H2-skewed immune responses by aluminium salts.

The ability to induce cellular immune responses by some marketed influenza vaccines is of great value in order to offer limited cross-reactivity against non-matched influenza strains. These vaccine formulations can play a role as an intermediate solution until the next generation of cross-protective influenza vaccines is developed.

Peptide antigens

Peptides are another type of antigen that can be used in T cell-inducing influenza vaccines. However, short peptides that consist of a minimal epitope are generally not immunogenic, and thus require additional modification or formulation to be able to induce T cell responses [62].

Several preclinical studies have used minimal epitope peptides as their main antigen to induce influenza-specific cellular responses. Short influenza peptides conjugated to phosphatidylserine were able to induce $CD8^+$ T cell responses in mice [34]. The conjugation of lipids to peptides opens up several possibilities; a PA-derived peptide conjugated to Pam2Cys, a lipid and TLR2 ligand, efficiently induced peptide-specific CTL responses in mice [63]. Furthermore, peptides conjugated to liposomes were able to minimize morbidity in IAV-infected mice through the induction of $CD8^+$ T cells [35, 36]. Remarkably, these peptide-liposome conjugates were able to induce $CD8^+$ memory T cells without the contribution of $CD4^+$ T cells. Liposomes act as a delivery system for the peptides, which are then internalized more efficiently by APCs than unformulated peptides. Direct conjugation of the peptide to a lipid or liposome is however not required. $NP_{366-374}$ peptide encapsulated in liposomes was able to induce potent T cell responses when mixed with anti-CD40 mAbs, and reduced viral lung titers of influenza-infected mice [64].

Aside from liposomes, virosomes have also been used as delivery systems for short peptide antigens. These virosomes utilize the membrane fusion activity of HA proteins to deliver the loaded peptide to the cytosolic compartment of the APC. An early study showed that virosomes loaded with the $H\text{-}2K^d$ binding influenza $NP_{147–155}$ peptide induced CTLs that were able to lyse IAV-infected target cells [65]. The addition of the adjuvant CpG-ODN 1826 to influenza $M1_{58-66}$ peptide-loaded virosomes was shown to increase peptide-specific $CD8^+$ T cell responses even further [37], which resulted in a faster recovery of vaccinated mice after heterologous influenza virus infection.

Long peptide vaccines consisting of multiple epitopes are, opposed to short peptide vaccines, already in the clinical testing phase. Flu-v consists of an equimolar mixture of four synthetic polypeptides derived from M1, M2 and NP IAV proteins, formulated with the adjuvant Montanide [39]. Flu-v induced peptide-specific T cells in healthy subjects; unfortunately, reactivity against actual IAV strains were not determined. However, vaccination studies in mice showed that $CD8^+$ T cell responses induced by Flu-v did reduce mortality after IAV-infection [66].

Similar to Flu-v, FP-01.1 consists of six polypeptides derived from M1, NP, PB1 and PB2, which were conjugated to a fluorocarbon moiety. The vaccine was able to induce $CD4^+$ and $CD8^+$ T cells in healthy subjects [38]. Moreover, these T cells were cross-reactive with H1N1 and H3N2 IAV-infected target cells. This is the first study that shows a peptide vaccine capable of inducing cross-reactive T cells in humans, which is very encouraging for the development of cross-reactive T cell-inducing vaccines.

The studies described above suggest that peptide-based approaches are very promising in the development of T cell-inducing IAV vaccines. However, an important challenge is the genetic variability among the human population in relation to epitope recognition and presentation. $CD4^+$ and $CD8^+$ T cells recognize IAV epitopes displayed on MHC molecules, which are called human

leukocyte antigen (HLA) molecules in humans. Different HLA polymorphisms occur in the human genome, resulting in a host of varying HLA molecules in the human population. Each HLA can only bind specific viral epitopes, which means that multiple epitopes of the same antigen need to be in a peptide-based vaccine to cover the human population [67]. *In silico* prediction methods can be employed to determine the potential T cell immunogenicity of conserved epitopes across multiple IAV strains [68]. Furthermore, several transgenic mouse strains have been bred that express HLA molecules, which can be used in preclinical development. Nonetheless, there remains a significant challenge for peptide-based vaccines to include enough epitopes to cover each HLA type, which would be required for a vaccine to be effective in the entire population.

Other T cell influenza vaccine concepts

Aside from the vaccine strategies described above, several other concepts are currently in clinical development (Table 1). Multimeric-001 is a synthetic recombinant protein composed of 9 T cell and B cell epitopes derived from HA, NP and M1 influenza proteins [30]. The vaccine in combination with the adjuvant Montanide ISA 51VG was able to induce cellular responses in healthy subjects. The cellular responses showed limited reactivity to multiple IAV strains. In a follow up study, the Multimeric-001 vaccine showed an induction of humoral and cellular responses in elderly subjects similar to responses observed in healthy adults [31]. While the results of these studies are encouraging, the true effectiveness of the induced cellular responses against homologous and heterologous IAV infections has yet to be determined.

Another concept which has advanced to the clinical stage of development is the modified vaccinia virus Ankara vectored vaccine MVA-NP+M1 [43]. This vaccine consists of a vaccinia virus Ankara expressing the influenza proteins NP and M1. Several clinical trials, including a phase II study, were conducted with this vectored vaccine. MVA-NP+M1 was able to expand pre-existing memory $CD8^+$ T cells in both healthy adults and elderly, and also increased the IAV-specific $CD4^+$ T cell population [44, 45].

T cell-based influenza vaccine concepts in the clinical phase

The protein-based influenza vaccines such as LAIV, WIV and virosomes currently have the advantage that they are already licensed and have been widely used. Such vaccines might be excellent candidates to prime naïve populations for both cellular and humoral responses.

Peptide-based vaccine concepts have the advantage that they can be easily engineered and produced synthetically. However, as mentioned above, selection of the right epitopes remains vital. These vaccines also require additional formulation with adjuvants to increase their immunogenicity. Nonetheless, several peptide-based vaccines have entered the clinical phase.

Vectored T cell-inducing vaccines are a sophisticated concept. They include both antigen and

adjuvant in a single particle. Since they express whole proteins rather than epitopes, vectored vaccines might have a higher coverage amongst different populations compared to peptide-based vaccines. A recent study also combined a seasonal influenza vaccine with MVA-NP+M1 to increase the breadth of the immune response [69]. Such an approach is a major improvement and might be an ideal solution to induce both humoral and cellular immunity with a single vaccine. Other concepts, such as peptide-based influenza vaccines, are also eligible to be used simultaneously with seasonal influenza vaccines, as demonstrated recently [33]. This is a good step towards a universal influenza vaccine.

VACCINE PRIMING

The IAV-naïve status and age of persons may influence the immunogenicity of T cell-inducing IAV vaccines. This was already observed with LAIV vaccines, which effectively induce cellular responses in naïve children, but not induce such responses in adults, who already established an immunological memory to IAV [25, 26]. A study in mice reported that $CD8^+$ T cells primed by LAIV rapidly differentiated to IAV-specific memory T cells after short-interval boosting, and were able to protect against heterologous challenge [70]. Several T cell-inducing vaccine concepts consider the potency of the prime-boost approach; a DNA-protein prime-boost concept enhanced the T cell responses to IAV in mice [71], and in a clinical trial priming with Multimeric-001 before a seasonal influenza vaccine boost greatly increased IAV-specific cellular responses in elderly subjects [31]. Priming at an early age in naïve mice with IAV resulted in the induction of long-term memory $CD8^+$ T cells with the broadest reactivity, while priming at an older age resulted in a $CD8^+$ T cell population with a reduced diversity [72]. Thus, T cell priming at an early age, when the subject is still naïve, should be considered before immunization with an influenza vaccine that only induces humoral responses. As a result, the intended target population of a vaccine is key for vaccine design and development [73].

VACCINE PRIMING

Many T cell-inducing vaccine concepts aim for the induction of systemic IAV-specific T cell responses. However, local T cell responses at the site of IAV infection are potentially more effective. The presence of IAV-specific resident memory T cells (TRM) in the lungs was correlated with clearance of heterologous IAV infection in mice [74]. $CD4^+$ T cells mediated the formation of $CD8^+$ TRM cells, adding yet another important function for $CD4^+$ TH [75]. Current knowledge on the establishment of TRM cells has been reviewed recently [76]. While the process of TRM induction is not completely unraveled, some possible mechanisms can be exploited to induce IAV-specific TRM responses with vaccines. A recent study specifically targeted an antigen to resident lung DCs using antibodies, and were able to generate IAV-specific $CD8^+$ TRM cells in mice that provided protection against a lethal influenza challenge [77]. Furthermore, it is known that CXCR3-expressing $CD8^+$ T cells play an important role in the establishment of $CD8^+$ TRM cells in the lungs [78]. The near future may learn us whether specific targeting of certain T cell populations, e.g. by adjusting the route of administration to the lungs [79, 80], may add to the potential of T cell-inducing influenza vaccines.

PRECLINICAL CORRELATES OF PROTECTION

There is clear evidence that cellular responses correlate with a reduction of symptoms after IAV infection. However, current correlates of protection (CoP) for influenza vaccines are all based on the induction of antibodies, such as the presence of hemagglutination inhibition- or virus neutralization titers, which are inadequate CoPs for T cell-inducing vaccines. Instead, responses that indicate the presence of effector T cells such as IFN-γ and IL-10 cytokines, combined with cytotoxic effector molecules like granzyme B may be more suitable as CoP for T cell-inducing vaccines [81]. These parameters also need to be further evaluated in epidemiological studies in order to define their efficacy. For instance, it is still unclear what quantitative levels of IAV-specific $CD8^+$ or $CD4^+$ T cell responses are required for protection against an IAV challenge. Furthermore, an adequate translation from animal models to the human setting has to be made. While there is quite some experience with humoral responses against IAV in animal models and their relation to the clinic, such experience has not been established yet for cellular responses. Establishing these responses as human CoPs, and translating study findings from animal models to humans remain important tasks for the development of T cell-inducing IAV vaccines.

CONCERNS AND LIMITATIONS OF T CELL-INDUCING IAV VACCINES

There are some concerns whether IAV-specific T cells can provide the same level of protection compared to IAV-specific antibodies. While T cells have a broader reactivity, they can only recognize and lyse IAV-infected host cells. Most likely, an IAV infection is already spreading before an efficient T cell response is mounted. It can therefore be debated whether T cells responses actually provide protection (i.e. sterilizing immunity) or only shorten the length and severity of influenza symptoms (i.e. decreased morbidity). The difference between these two can be very hard to distinguish. Therefore, elucidation of T cell responses after influenza infection in humans is of critical importance to determine the efficacy of T cell-inducing influenza vaccines. Nonetheless, reduction of morbidity of IAV infections would already be a great success in situations where seasonal influenza vaccines would be ineffective, such as a mismatched influenza epidemic or an influenza pandemic. The definition of protection should therefore not only be limited to sterilizing immunity, but also to reduction of disease morbidity.

Another concern is the possibility of excessive T cell responses to IAV infections, which could cause immunopathology in the lungs [82]. There are indications that excessive T cell responses mediate severe lung inflammation and subsequent lung damage after IAV infection in mice. Only one study describes the phenomenon in humans; elevated IAV-specific $CD8^+$ and $CD4^+$ T cell responses were found in pandemic 2009 H1N1-infected children with severe pneumonia [83]. It was however unclear whether these T cell responses were the cause of pneumonia or simply present due to the infection.

It is yet unknown whether T cell-inducing influenza vaccines can mount long-lasting T cell responses after a limited number of immunizations. As already discussed above, natural IAV infections are able to induce T cell responses, but their effectivity is limited. Studies suggest that local inflammation and inflammatory cytokine production caused by IAV infection suppress $CD8^+$ T cell responses in mice. This was partly attributed to an increased expression of PD-L1 on the $CD8^+$ T cells, which cripples the functionality of these T cells [84, 85]. T cell-based vaccines however should not experience the effects of these immunosuppressive pathways, since inflammation after immunization is generally limited. It is thus likely that these vaccines can induce T cell responses which are more potent than those elicited by natural IAV infections. Nonetheless, it is important that T cell-inducing vaccines elicit balanced T cell responses, and special interest should be given to T cell-mediated immunopathology during safety studies of these vaccines.

Aside from the intensity of T cell responses, special attention should be given to the selection of target epitopes derived from IAV. A recent study described the existence of tolerizing epitopes in certain influenza strains, which are recognized by autologous regulatory T cells and may suppress protective T cell responses [86]. Another study found that T cells against certain immunodominant epitopes such as $M1_{58\text{-}66}$ have a poor functionality, and are unable to clear IAV-infected cells [87]. It was hypothesized that these immunodominant epitopes are actually a decoy of IAV to evade T cell-

mediated immunity and to prevent the generation of more potent T cells against other epitopes. It is therefore important that such epitopes, which could lead to decreased or impotent T cell responses, are identified and excluded in any prospective T cell-inducing IAV vaccines.

CONCLUSION

Humoral immune responses elicited by current IAV vaccines do not provide sufficient cross-protection against non-matched IAV infections. IAV-specific T cells recognize conserved epitopes of IAV and thus have to potential to be cross protective. Many different T cell-inducing vaccines are currently under development, and some have even reached clinical phases. Selecting suitable preclinical testing models and clinical CoPs are vital for further development of such vaccines. In addition, proper understanding the effectiveness of each T cell response and their possible pathological effects is of great importance. The current developments with T cell-inducing IAV vaccines, including novel formulations and extended immunological insight, are fast evolving and may ultimately result in universal influenza vaccines.

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Chapter 7

Solid Bioneedle-delivered influenza vaccines are highly thermostable and induce both humoral and cellular immune responses

Peter C. Soema [1,2], Geert-Jan Willems [1], Klaas van Twillert [1], Gijsbert van de Wijdeven [3], Claire J. Boog [1], Gideon F.A. Kersten [1,2] and Jean-Pierre Amorij [1]

1 Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands.
2 Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands.
3 Bioneedle Technologies Group BV, Eindhoven, The Netherlands.

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ABSTRACT

The potential of Bioneedles to deliver influenza vaccines was investigated. Four influenza vaccine formulations were screened to determine the optimal formulation for use with Bioneedles. The stability of the formulations after freeze-drying was checked to predict the stability of the influenza vaccines in the Bioneedles. Subunit, split, virosomal and whole inactivated influenza (WIV) vaccine were formulated and lyophilized in Bioneedles, and subsequently administered to C57BL/6 mice. Humoral and cellular immune responses were assessed after vaccination. The thermostability of lyophilized vaccines was determined after one-month storage at elevated temperatures. Bioneedle influenza vaccines induced HI titers that are comparable to those induced by intramuscular WIV vaccination. Delivery by Bioneedles did not alter the type of immune response induced by the influenza vaccines. Stability studies showed that lyophilized influenza vaccines have superior thermostability compared to conventional liquid vaccines, and remained stable after one-month storage at 60°C. Influenza vaccines delivered by Bioneedles are a viable alternative to conventional liquid influenza vaccines. WIV was determined to be the most potent vaccine formulation for administration by Bioneedles. Lyophilized influenza vaccines in Bioneedles are independent of a cold-chain, due to their increased thermostability, which makes distribution and stockpiling easier.

INTRODUCTION

The conventional method of influenza vaccine delivery is intramuscular injection of liquid formulations using syringes and needles. The use of needles may cause fear and stress in children and adults [1]. Needle stick injuries and reuse of needles and syringes are additional risks associated with conventional injections. To overcome these problems, extensive research is being carried out on alternative delivery methods and delivery routes for influenza vaccines [2]. Multiple delivery routes are currently being studied, including nasal, pulmonary, sublingual, oral and dermal routes. These routes usually require different delivery methods than needles; these can be sprays, dry powders or microneedles. Alternative delivery methods for the intramuscular and subcutaneous routes are limited. Examples in development are liquid jet injections and powder jet injections [3, 4].

Recent outbreaks of influenza A strains, such as the highly pathogenic avian influenza A H5N1 [5], the 2009 pandemic influenza A H1N1 and more recently avian influenza A H7N9 [6, 7], have increased the need for more effective vaccines. Novel influenza vaccines are required to be quickly available for mass vaccination in case of epidemics. Current influenza vaccines have limited stability, and thus require a cold-chain. This makes distribution and storage of these vaccines expensive and challenging, specifically in developing countries due to the limited cold-chain infrastructure.

There are four types of marketed non-adjuvanted inactivated influenza vaccines: whole inactivated virus, virosomal, split and subunit vaccine. These vaccines differ in terms of viral components and particulate organization [8]. Subunit and virosomal vaccines contain only the influenza hemagglutinin (HA) and neuraminidase (NA) surface antigens, while WIV and split vaccines also contain internal viral components such as internal proteins and, in case of WIV, viral RNA. WIV and virosomes maintain a viral particulate organization of approximately 150 nm, whereas subunit and split vaccines consist of a less organized mixture of components. These differences in characteristics have effects on vaccine immunogenicity and efficacy of the different influenza vaccines, which is important for the development of novel influenza vaccine formulations and delivery methods.

Another potential alternative delivery system for influenza vaccines are Bioneedles [9]. Bioneedles are small hollow implants made from thermoplastic starch (Figure 1). They are loaded with an antigen by filling the inner compartment (volume of 5 µl) with a liquid vaccine formulation followed by subsequent lyophilization. Vaccination with antigen-filled Bioneedles is performed by intramuscular or subcutaneous implantation under high velocity using compressed air [9]. After implantation, the Bioneedle dissolves, resulting in the release of the antigen. A phase I clinical study showed that empty Bioneedles are well tolerated by healthy volunteers during and after administration [10]. No local toxicity other than tissue damage from Bioneedle injection was observed at the site of implantation. Previous studies with tetanus toxoid and hepatitis B vaccines have shown that antigens delivered by Bioneedles induce comparable or improved immune responses in mice compared to liquid vaccines delivered by conventional injection [11, 12]. Moreover, the lyophilized vaccine antigens in these Bioneedles showed improved thermostability. This reduces the need for

a cold-chain and allows long-term storage of vaccines. Furthermore, Bioneedles are ideally suited for mass vaccinations. Vaccination with Bioneedles is relatively easy, very quick and does not have the risk of needle stick injuries. Applicators (currently under development) will be low cost devices working on compressed air. Pressurizing the device is done manually, which make it ideal for use in developing countries. Furthermore, cost assessments have indicated that Bioneedle applicator devices could be supplied free of charge for the use in public health care in developing countries.

In this current study, we compared the immunogenicity of influenza vaccine filled Bioneedles with the immunogenicity of conventional liquid influenza vaccines in mice. In order to identify the most potent influenza vaccine formulation for inclusion in Bioneedles, we included four types of non-adjuvanted influenza vaccine. Furthermore, the thermostability of the lyophilized influenza vaccine formulations was evaluated. Finally, vaccine release from Bioneedles was imaged *in vivo*, in order to determine vaccine release kinetics from the Bioneedles at the site of injection.

Figure 1. Freeze-dried Bioneedles filled with influenza vaccine.

MATERIALS AND METHODS

Preparation of influenza vaccines

Bioneedles (15 mm long and 1 mm wide, internal volume of 5 µl) were obtained from the Bioneedles Technologies Group. Influenza A/PR/8/34 whole inactivated virus was produced by Intravacc. The process was based on egg virus propagation and β-propiolactone virus inactivation [13]. Split and subunit vaccines were produced by solubilization of WIV with *n*-octyl-β-D-glucopyranoside (Sigma-Aldrich) as described previously [8]. Virosome vaccine was produced as described previously [14]. All vaccines were concentrated with Centriprep centrifugal filters (Millipore) with a molecular weight cut-off (MWCO) of 10 kDa, and formulated in HBS (20 mM HEPES, 125 mM NaCl, 9 mM $CaCl_2$, 5 mM $MgCl_2$). Vaccine formulations for Bioneedles contained 2.5% (w/w) D-trehalose dihydrate (Sigma-Aldrich) as a stabilizer. Influenza vaccine-containing Bioneedles (subunit, split, virosomal and WIV vaccine) were prepared by filling Bioneedles with 5 µL of 1 mg/mL (HA content) liquid vaccines from the hollow back of the Bioneedle using specially designed filling apparatus, and frozen on a metal plate at minus 50°C [12]. Next, Bioneedles were freeze-dried using a Zirbus Sublimator 3x4x7 (Zirbus Technology). Lyophilized Bioneedles were stored in glass vials with rubber stoppers under ambient air and relative humidity.

Liquid vaccine characterization

The protein composition and purity of the vaccine formulations was determined by SDS-PAGE. Formulations were run under non-reducing conditions on a 12% precast gel (Thermo Scientific), and stained with Coomassie Brilliant Blue (Thermo Scientific). Particle size was measured by dynamic light scattering (DLS) using a Malvern Nano ZS (Malvern Instruments). The results are given as the average particle size diameter and the polydispersity index (PDI).

Lyophilized vaccine characterization

Samples for recovery and stability studies were prepared by filling 3 ml glass vials (Müller + Müller) with 25 µl vaccine formulated with 2.5% trehalose and subsequently freeze-dried using the same drying procedure as the vaccine-filled Bioneedles. Vials were closed with rubber stoppers under ambient air and relative humidity. In order to assess the initial recovery, the vaccines were reconstituted in 100 µl MilliQ water immediately after lyophilization. In order to determine their heat stability, lyophilized and liquid vaccines were stored for 1 month at 4°C, 24°C, 37°C or 60°C; lyophilized vaccines were subsequently reconstituted in 100 µl MilliQ water. Recovery data of heat-stressed samples were compared to data of unstressed samples acquired immediately after lyophilization. Each condition was performed in triplicate, and individual samples were measured three times for each method.

Relative moisture content (RMC) was determined by Karl-Fischer titration. In brief, lyophilized vaccines were dissolved in Hydronal-Coulomat A solution (Sigma-Aldrich) and titrated using a Mitsubishi CA-06 coulometric moisture meter (Mitsubishi).

HA structural quantification by RP-HPLC

The HA1 subunit of HA was quantified by RP-HPLC according to the method of Kapteyn et al [15]. In short, influenza vaccines were dissolved in 0.15 M Tris-HCl, pH 8.0 and solubilized by incubation with 1% (w/v) Zwittergent 3-14 (Millipore). Trypsin agarose beads (Sigma-Aldrich) were subsequently added in order to cleave HA into HA1 and HA2 subunits. After removal of the beads, samples were reduced with 25 mM dithiothreitol (DTT, Sigma-Aldrich) and subsequently alkylated with 50 mM iodoacetamide (IAA, Sigma-Aldrich). IAA was neutralized by addition of 25 mM DTT. Prepared samples were analyzed on an Agilent 1100C system (Agilent Technologies) using a polystyrene POROS R1/10 2.1 mm x 100 mm column (Applied Biosystems) equipped with a 2 µm precolumn filter and frit (Upchurch Scientific). The autosampler and column heater were set at 6°C and 60°C respectively. Mobile phases used were 0.1% trifluoroacetic acid (TFA), 5% acetonitrile in water (solvent A), and 0.1% TFA in acetonitrile (solvent B). The solvent gradient from A to B was 0-32% in 2 min, 32-64% in 3.5 min, 64-100% in 1 min and 100% for 1 min, with a flow of 0.8 mL/min. HA_1 protein was detected by an Agilent 1046A fluorescence detector (Agilent Technologies) with excitation and emission wavelengths set at 280 nm and 335 nm respectively. After each sample, the system was rinsed with 100 µL of 1% (w/v) Zwittergent 3-14 with a gradient elution of solvent B from 100% to 0% in 6 min.

HA antigenic quantification by surface plasmon resonance (SPR)

Antigenicity of the influenza formulations was quantified by a SPR method modified from Estmer Nilsson et al [16]. Samples were analyzed on a Sensor Chip CM5 with a Biacore T200 biosensor system (GE Healthcare). HBS-EP+ (GE Healthcare) was used as analysis buffer. Recombinant HA protein from influenza A/PR/8/34 (Protein Sciences) was immobilized to 7000-10000 response units using an Amine coupling kit (GE Healthcare) with ~65 µL rHA (10 µg/mL) in 10 mM phosphate buffer, 0.05% Surfactant P20 (GE Healthcare), pH 6.0. Dilutions series of the vaccine samples were made, and anti-influenza A/PR/8/34 sheep serum (1:150, NIBSC) was added to each dilution. The sample-serum mixture was subsequently injected during 400 seconds during which sensorgrams were acquired. In between each sample the sensor chip surface was regenerated using 50 mM HCl, 0.05% Surfactant P20. Acquired sensorgrams were analyzed using Biacore T200 evaluation software (GE Healthcare). Antigenicity was calculated relative to a known concentration of rHA A/PR/8/34.

Immunizations

Animal experiments 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 of Public Health and the Environment (RIVM). For all experiments, 7-week-old female C57BL/6 mice (Harlan) were used. Prime and boost immunizations were performed at day 0 and 21 respectively under isoflurane anesthesia. Animals were sacrificed by bleeding under anesthesia at day 28. Mice received 5 µg HA of WIV, split, virosome or subunit influenza in either liquid or Bioneedle form; the placebo group received HBS. Liquid formulations (50 µL) were administered

subcutaneously (s.c.) in the neck between the ears. Bioneedles were implanted subcutaneously in the neck between the ears using a sterilized trocar with mandrin under anesthesia as reported previously [12]. To compare the s.c. route with the classical intramuscular (i.m.) route, fluid WIV and subunit vaccine were also applied i.m. in the hind-left leg.

Hemagglutination inhibition assay (HI assay)

Hemagglutination-inhibiting titers in mouse sera were determined by an HI assay. Individual sera were treated overnight 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 transferred to a 96-wells V-bottom plate (Greiner) and serially diluted two-fold with PBS. Four hemagglutinating units of inactivated influenza A/PR/8/34 was subsequently added to each well and incubated for 20 min at room temperature after mixing. Next, an equal amount of 0.5% (v/v) turkey erythrocyte suspension (Harlan) was added to the wells and incubated for 45 min at room temperature. HI titers are given as the reciprocal of the highest serum dilution capable of preventing hemagglutination. Sera without detectable titers were scored 2, 1/5th of the detection limit.

Enzyme linked immunosorbent assay (ELISA)

Influenza antigen specific antibody titers were determined by ELISA. Microlon 96-wells flatbottom plates (Greiner) were coated overnight with 600 ng of A/PR8/34 subunit HA (as determined by SPR) per well at 4°C. After washing twice with 0.05% Tween80, 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. Plates were washed three times and subsequently 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 3,3'5,5'-tetramethylbenzine (TMB) substrate buffer (0.4 mM TMB in 0.11 M sodium acetate, 0.006% H_2O_2, pH 5.5) after washing three times and incubated for 10 min at room temperature. Enzymatic reaction was stopped by adding 2 M sulfuric acid, after which the optical density (OD) was measured at a wavelength of 450 nm using a Synergy Mx platereader (BioTek). Titers are given as the reciprocal of the serum dilution corresponding to OD_{450}=0.1 after background correction.

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 incubating with 25 µL 70% ethanol for 2 min, and subsequently coated overnight with anti-mouse IFN-γ or IL-4 antibodies (U-Cytech) at 4°C after washing with three times PBS. 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, 5% FCS were added to each well with or without 50 ng influenza A/PR/8/34 subunit antigen, and incubated overnight at 37°C. After overnight stimulation, filter plates were washed five times

and IFN-γ and IL-4 were detected using biotinylated anti-mouse antibodies (U-Cytech) and 100 µL BCIP/NBT reagent (Thermo Scientific) per well. Spot were allowed to develop for 15 min after which the plates were thoroughly washed with water. Spots were counted using an A.EL.VIS ELISpot reader (Aelvis). The number of IFN-γ or IL-4 producing cells in antigen stimulated spleen cells was obtained after background correction (subtracting number of spots produced by splenocytes incubated with buffer lacking antigen). Subsequently this number was corrected for the number of spots found in splenocytes from mice immunized with HBS.

***In vivo* imaging**

WIV vaccine was labeled with infrared dye IRdye 800CW (LI-COR) using the manufacturer's instructions for imaging purposes. Antigenicity of labeled WIV was checked by HA quantification by SPR. Hairless, immune-competent 7-week-old female SKH1-Elite mice (Charles Rivers) were immunized with IRdye or labeled WIV (5 µg) administered either s.c. or by Bioneedle between the ears in the neck. Mice were subsequently scanned several times with an IVIS Spectrum imaging system (PerkinElmer) during one week. The excitation wavelength was set at 710 nm and emitted light was measured at 760, 780, 800 and 820 nm, after which spectral unmixing was performed to distinguish auto-fluorescence from label-specific fluorescence. The fluorescent signal at the site of injection was quantified over time. After the experiment, animals were sacrificed by bleeding under anesthesia and cervical dislocation. To determine antigenicity of labeled WIV, HI titers were determined in sera of sacrificed mice.

Statistics

Statistical comparisons between experimental groups for HI titers were made with a one-way ANOVA followed by a Tukey-Kramer test for multiple comparisons. Statistical comparisons between experimental groups for IgG titers were made with a one-way or two-way ANOVA test followed by Bonferroni correction for multiple comparisons. Probability (p) values ≤ 0.05 were considered significant. Statistics were performed using GraphPad Prism 6.02 software for Windows (GraphPad Software).

RESULTS

Liquid influenza vaccine characterization

Non-reducing SDS-PAGE (Figure 2) analysis showed that WIV and split vaccines contained all viral proteins including nucleoprotein (NP) and matrix protein 1 (M1). In contrast, virosomal and subunit vaccines contained hemagglutinin and, compared to WIV and split, a reduced amount of neuraminidase.

The particle sizes of WIV and virosome vaccines were found to be 137 and 131 nm respectively and relatively monodisperse (PDI close to 0), as shown in Table 1. Split vaccine had a particle size of 520 nm but with a high PDI, indicating a relatively heterodisperse particle size of the antigens. Subunit vaccine had a similar high PDI, with a particle size of 103 nm, indicating an organization smaller than influenza virus particles.

Lyophilized influenza vaccine characterization

Bioneedles were successfully filled and freeze-dried with influenza vaccines. To establish the recovery of the vaccine in terms of HA content after freeze-drying, vaccine formulations lyophilized in vials were reconstituted and analyzed for HA1 content by RP-HPLC and for antigenicity by surface plasmon resonance (Table 1). HA1 content and antigenicity of liquid vaccines were set as 100% recovery and were compared with lyophilized vaccines. The relative standard deviations of these methods were in the range of 4-11 % (RP-HPLC) and 1-7 % (SPR), respectively. The recovery of HA1 content from the lyophilized vaccines ranged from 110% for virosomal vaccine to 137% for WIV vaccine. Similarly, antigenic recovery ranged from 95% for split vaccine to 118% for virosomal vaccine. These recoveries were not significantly different compared to the starting materials, indicating that all vaccine formulations retained their HA1 content and antigenicity after freeze-drying.

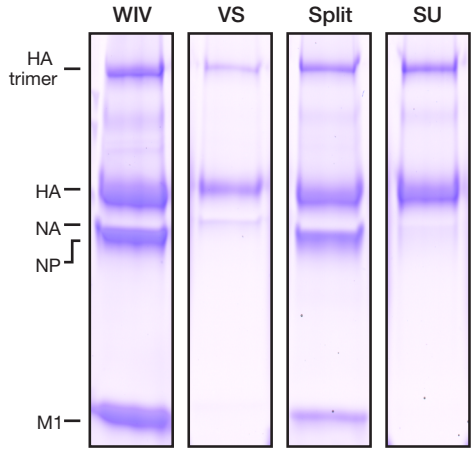

Figure 2. Protein composition of the liquid influenza vaccines. Formulations were analyzed by non-reducing SDS-PAGE using a 12% pre-cast gel stained with Coomassie Brilliant Blue. From left to right: WIV, virosome (VS), split and subunit (SU). HA = hemagglutinin (76 kDa); NP = nucleoprotein (60 kDa); NA = neuraminidase (58 kDa) and M1 = matrix protein 1 (25 kDa). Identity of the bands was confirmed by mass spectrometry.

Table 1. Characteristics of liquid and lyophilized influenza vaccine formulations. Size and polydispersity index (PDI) are shown for liquid formulations and reconstituted lyophilized formulations. Recovery of HA1 and antigenicity of lyophilized vaccines are shown as a percentage of vaccines before freeze-drying. Data represent mean ± SD (n = 3).

Vaccine	Liquid		Lyophilized			
	Size (nm)	PDI	Size (nm)	PDI	HA1 recovery (%)	Antigenic recovery (%)
WIV	137 ± 1	0.07	146 ± 3	0.09	137 ± 11	111 ± 1
Virosome	131 ± 2	0.13	151 ± 9	0.24	110 ± 9	118 ± 4
Split	520 ± 14	0.63	150 ± 4	0.43	115 ± 4	95 ± 7
Subunit	103 ± 2	0.41	131 ± 4	0.32	115 ± 11	100 ± 2

Dynamic light scattering showed that the particle size of lyophilized WIV vaccine was 146 nm after reconstitution, which is slightly higher compared to the particle size of liquid WIV vaccine. The PDI remained low, indicating that the virus particles did not aggregate or disintegrate during or after freeze-drying. Virosomal and subunit vaccines both showed an increased particle size and PDI compared to the liquid formulations. In contrast, the particle size of split vaccine was decreased after lyophilization, while retaining a high PDI, indicating that the vaccine still had a heterodisperse particle distribution.

Humoral immune responses

The immune responses induced by the vaccines delivered with Bioneedles were compared with those induced after subcutaneous administration of conventional liquid influenza vaccines. Liquid WIV and subunit vaccines were also administered via the intramuscular route in control groups. Intramuscular vaccination is the standard route for administration to humans; as a result, the i.m. control groups are used as standard for the *in vivo* evaluation of the influenza Bioneedle vaccine concepts.

Serum HI titers were undetectable three weeks after the first immunization (day 21) with virosomal, split or subunit vaccine administered either i.m., s.c. or by Bioneedles (data not shown). Only influenza WIV vaccine induced low HI titers (^{2}log(HI) = 2 to 7) after a single immunization, regardless of delivery method.

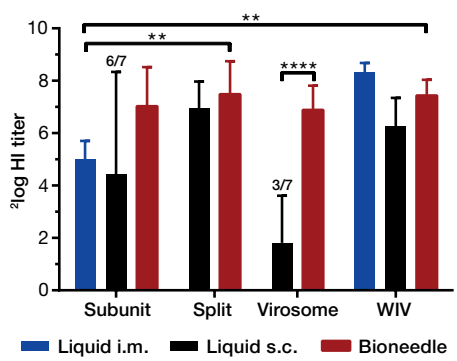

Figure 3. HI titers one week after the second vaccination with liquid or Bioneedle influenza vaccines. Data represent geometric mean ± SD (n = 7). For groups with mice having an undetectable HI titer, the number of responders is indicated above the bars. Asterisks indicate titers are significantly (**$p < 0.01$, ****$p < 0.0001$) different from each other.

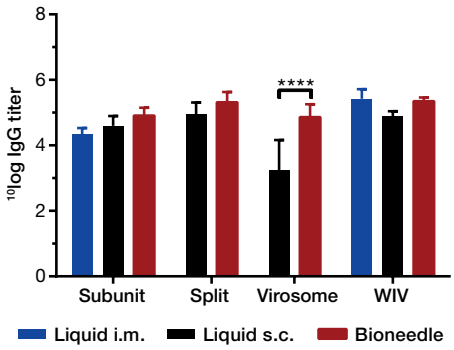

Figure 4. Antigen-specific serum IgG titers one week after boost vaccinations. Data represent mean ± SD (n = 7). Asterisks indicate titers are significantly (****$p < 0.0001$) higher than those of mice s.c. immunized with liquid virosome vaccine.

Bioneedle-delivered vaccines were directly compared to the s.c. liquid vaccines. The HI titers in sera taken one week after the second immunization (day 28) were assessed by an HI assay (Figure 3). The HI titers found in mice after vaccination with subunit, split or WIV vaccine delivered by Bioneedles did not differ significantly from the HI titers induced by s.c. vaccination with liquid influenza vaccines. In contrast, serum HI titers induced by virosomal vaccine delivered by Bioneedles were significantly higher than HI titers induced by s.c. administered liquid virosome vaccine. Liquid virosome vaccine induced poor HI titers in general, with four out of seven animals being non-responders.

Next, Bioneedle delivered influenza vaccines were compared to intramuscular administered subunit vaccine, currently a standard influenza vaccine. Subunit vaccine delivered by Bioneedles induced high HI titers compared to HI titers induced by i.m. subunit vaccine. Subunit and virosomal vaccines delivered by Bioneedles showed similar HI titers compared to the respective liquid formulations. Furthermore, HI titers in mice immunized with split or WIV vaccine delivered by Bioneedles were significantly higher compared to those induced by i.m. subunit vaccine. Thus, influenza vaccines delivered by Bioneedles elicited equal or superior HI titers after vaccination compared to the standard i.m. administered subunit vaccine.

In addition to HI titers, antigen-specific serum IgG titers from one week after the second immunization (day 28) were determined (Figure 4). WIV, split and subunit vaccines all elicited equally high IgG titers after either i.m. or s.c. immunization, or administration by Bioneedles. In contrast, s.c. administered virosomal vaccine induced IgG titers that were significantly lower than those induced by virosomal vaccine delivered by Bioneedles, further indicating that Bioneedles have a positive effect on vaccine immunogenicity.

Cell-mediated immune responses

In order to investigate whether immunization with the different influenza vaccines induced cell-mediated immune responses, the antigen-specific frequencies of both IFN-γ and IL-4 cytokine-producing splenocytes of immunized mice were assessed (Figure 5).

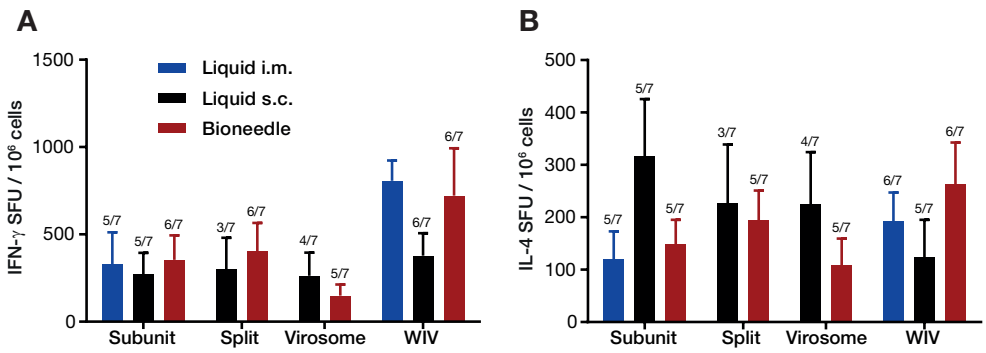

Figure 5. Numbers of IFN-γ (A) and IL-4 (B) cytokine producing splenocytes of immunized mice after *ex-vivo* stimulation with influenza subunit. Data represent mean ± SEM (n = 7). The number of responders, if less than 7, is indicated above the bars.

Overall, both subcutaneous and Bioneedle administration of subunit, split and virosome vaccine formulations induced comparable frequencies of IFN-γ producing splenocytes. Bioneedle groups however did have less non-responders than subcutaneous groups. In contrast, i.m. liquid WIV and WIV vaccine delivered by Bioneedles induced high levels of IFN-γ producing cells compared to the other influenza vaccines. The difference between s.c. administered liquid WIV vaccine and Bioneedle administered WIV vaccine was not significant. Bioneedle influenza vaccines showed frequencies of IL-4 producing splenocytes after vaccination that did not differ significantly from responses induced after i.m. administered subunit or WIV vaccines. Subcutaneous delivered liquid subunit, split and virosome vaccines however induced frequencies of IL-4 producing cells that did not differ significantly compared to their Bioneedle counterparts. Thus, delivery of influenza vaccines by Bioneedles did not significantly alter the type of immune response.

To further assess the quality of the cell-mediated immune response, the IgG subtype profile in the sera was determined. The ratio between IgG1 and IgG2c, the C57BL/6 analog of IgG2a [17], was determined for each individual mouse after immunization (Figure 6). Mice immunized with influenza subunit, split or virosomal vaccine all showed a mixed IgG2c/IgG1 ratio regardless of delivery method. Thus, in line with the data on the cellular immune response, delivery of influenza vaccines by Bioneedles did not alter the type of immune response. Additionally, WIV vaccine, independent

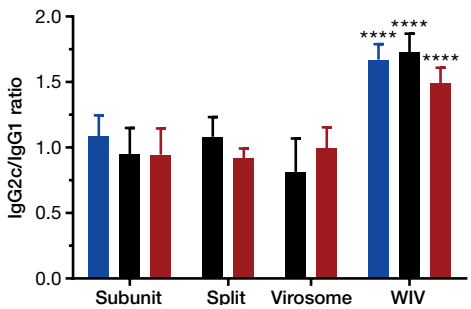

Figure 6. IgG2c/IgG1 subtype ratios in serum of mice one week after boost vaccinations. Data represent mean ± SD (n = 7). Asterisk indicate IgG2c/IgG1 ratio of WIV groups are significantly (****$p < 0.0001$) higher than ratios of other vaccine formulation groups.

of delivery method, exhibited an IgG2c/IgG1 ratio that was significantly favoring the IgG2c subtype compared to subunit, split or virosomal vaccines.Heat stability of liquid and lyophilized influenza vaccines

The heat stability of lyophilized influenza vaccines was determined by storing liquid and lyophilized influenza vaccines in glass vials at 4°C, 24°C, 37°C and 60°C for one month. For both liquid and lyophilized vaccines, the recovery of HA1 and antigenicity was determined by RP-HPLC and SPR respectively (Table 2). Additionally, the residual moisture content was determined for lyophilized vaccines and used for calculation of the predicted glass transition temperature (T_g) with the Gordon-Taylor equation for a binary water-trehalose system as described in literature [18].

The residual moisture content may affect the mobility of biopharmaceuticals entrapped in glassy matrixes such as lyophilized (glassy) trehalose. In general, a higher moisture content indicates a lower T_g and consequently may increase molecular movement at lower storage temperatures giving risk to destabilization of the entrapped biopharmaceutical. The residual moisture content was found to be 2.1% or less for all four lyophilized influenza vaccines. The predicted T_g at a residual moisture content of 2.1% was calculated to be approximately 95°C, which is well above normal or elevated storage conditions.

Both liquid and lyophilized influenza vaccine formulations were not affected by storage at 4°C and 24°C for a month. All vaccines showed a recovery of both HA_1 and antigenicity that ranged between 90 to 110% (data not shown). After one-month storage at 37°C, freeze-dried vaccines show HA1 recoveries of approximately 100%, whereas antigenicity slightly decreased to approximately 80%. In contrast, antigenicity of liquid subunit, split and WIV vaccines was lowered below 70% after one month at 37°C, while liquid virosomal vaccines retained 81% of their original antigenicity. HA1 recoveries for liquid vaccines after one-month storage at 37°C ranged from 84 to 103%. After

Table 2. Heat stability of lyophilized influenza vaccine formulations. Residual moisture content (RMC) of lyophilized vaccine formulations was determined directly after lyophilization. Glass transition temperatures (T_g) were calculated from RMCs. Lyophilized vaccines were stored for one month at 37°C and 60°C. Recovery of HA1 and antigenicity was determined after reconstitution and is shown as a percentage of initial recovery after lyophilization. Data represent mean ± SD (n = 3).

Vaccine	Formulation	RMC (%)	T_g (°C)	HA1 recovery (%)		Antigenic recovery (%)	
				37°C	60°C	37°C	60°C
WIV	Liquid	n.a.[a]	n.a.[a]	99 ± 5	n.d.[b]	69 ± 5	n.d.[b]
	Lyophilized	1.3 ± 0.1	104	96 ± 20	91 ± 12	82 ± 7	83 ± 25
Virosome	Liquid	n.a.	n.a.	103 ± 6	n.d.	81 ± 6	n.d.
	Lyophilized	1.8 ± 0.2	99	104 ± 7	84 ± 6	87 ± 13	93 ± 11
Split	Liquid	n.a.	n.a.	89 ± 3	n.d.	64 ± 16	n.d.
	Lyophilized	1.4 ± 0.2	103	105 ± 8	83 ± 7	85 ± 8	103 ± 9
Subunit	Liquid	n.a.	n.a.	84 ± 1	n.d.	69 ± 3	n.d.
	Lyophilized	2.1 ± 0.4	95	109 ± 5	82 ± 10	74 ± 12	96 ± 3

[a] Not applicable. [b] Not detectable; detection limit = ~1 µg/ml (equal to 0.1% recovery).

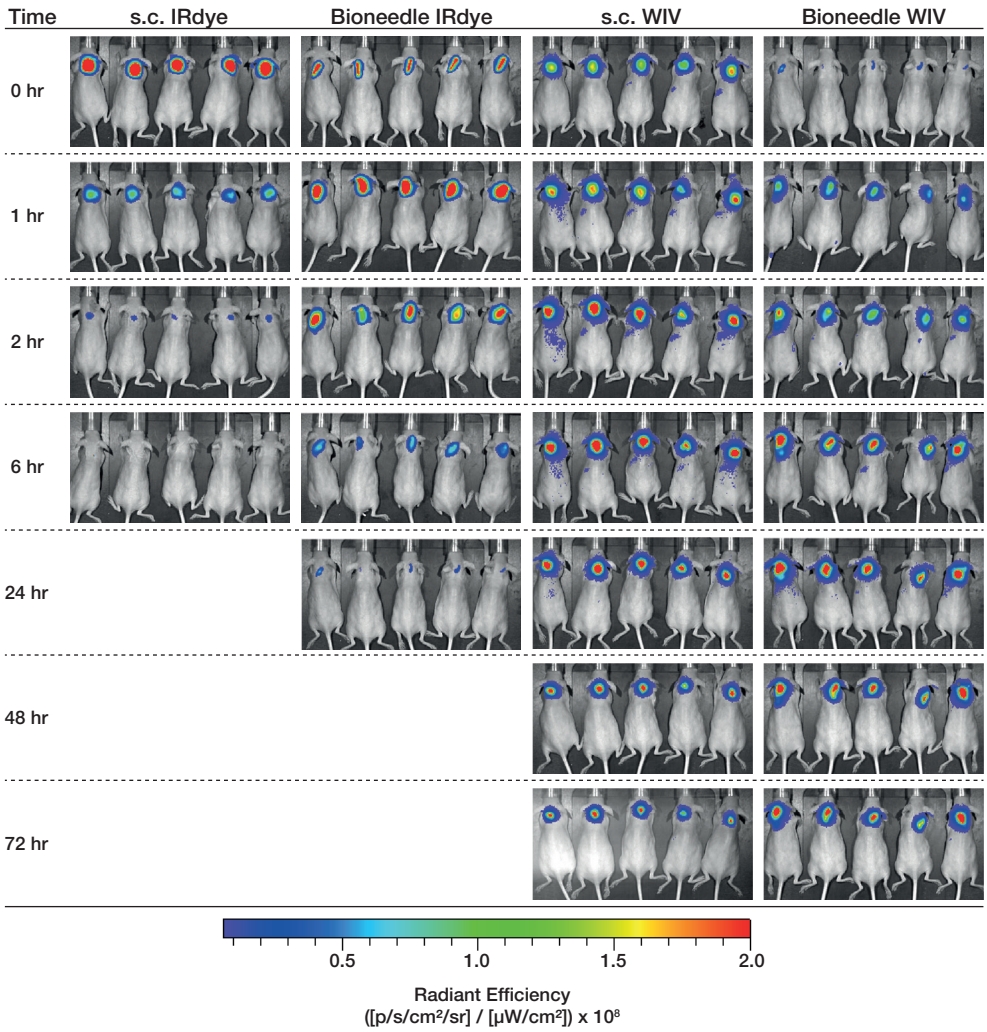

Figure 7. *In vivo* imaging of vaccine-filled Bioneedles. IRdye and labeled WIV were administered either s.c. or by Bioneedles in mice, after which the fluorescence was tracked over time.

one-month storage at 60°C, lyophilized influenza vaccines were minimally affected, with HA1 and antigenicity recoveries being higher than 83%. Strikingly, liquid influenza vaccines showed no detectable recoveries of either HA_1 or antigenicity after one-month storage at 60°C, indicating that the liquid vaccines were completely deteriorated.Biodistribution of vaccine-filled Bioneedles

To investigate the vaccine release profile of influenza vaccine-filled Bioneedles, an infrared dye was either filled in Bioneedles or bound to influenza WIV vaccine before filling and subsequent freeze-drying. Nude mice received either IRdye or IRdye-labeled WIV s.c. or by Bioneedle. The release of IRdye was tracked over time with a bioimaging system. The images are presented in

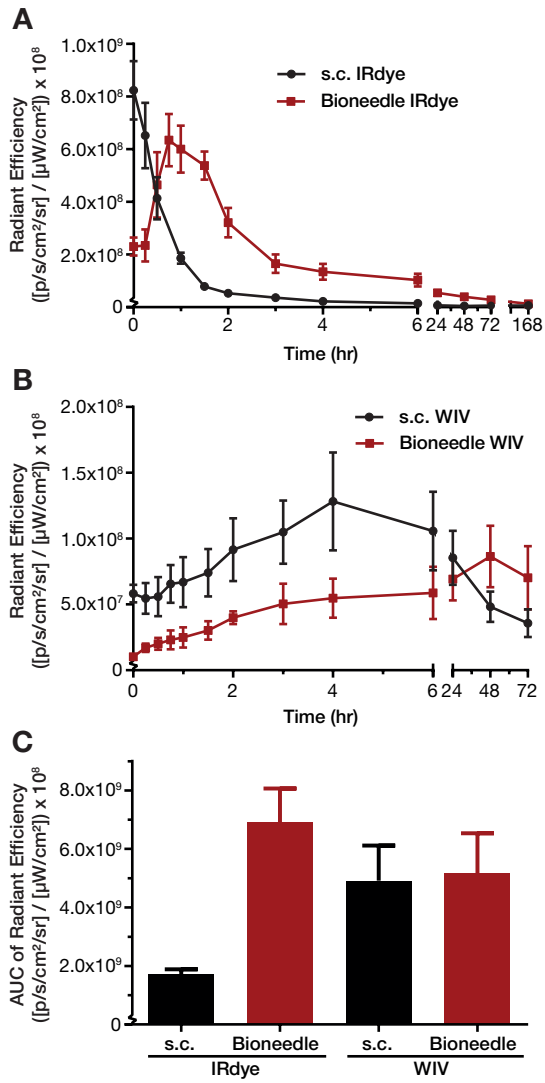

Figure 8. Quantitative fluorescence at the site of injection over time for both IRdye (A) and WIV (B). Area under the curve (AUC) was calculated for each mice (C). Data represents mean ± SD (n = 5).

Figure 7. Subcutaneous administered IRdye rapidly disappears from the site of injection (SOI), and is completely undetectable after 6 hours at the SOI. In contrast, Bioneedle-delivered IRdye remained visible at the SOI up to 24 hours post administration, indicating that the IRdye was slowly released from the Bioneedle (Figure 8A). Surprisingly, s.c. administered WIV remained at the SOI for a prolonged period of time, similar to Bioneedle-delivered WIV (Figure 8B), although Bioneedle-delivered WIV maintained a steady antigen presence at the SOI even after 72 hours. Total release of WIV over time did not differ between s.c. and Bioneedle groups (Figure 8C).

DISCUSSION

This study demonstrates that influenza vaccines delivered by Bioneedles elicit equal or improved immune responses in C57BL/6 mice compared to conventional liquid vaccines. Furthermore, lyophilization of the different influenza vaccine types formulated with trehalose and HBS greatly improved the heat stability of the influenza vaccines.

The four influenza vaccine types were selected for their differences in compositions and particulate organization. All vaccines were produced from a single virus batch, enabling a fair comparison between the vaccines. Comparative immunogenicity studies remain few [8, 19], and studies on subcutaneous influenza vaccines are mostly limited to a single vaccine type. This knowledge gap makes it difficult to preselect the most suitable influenza vaccine candidate for delivery with Bioneedles. Therefore, a head-to-head comparison between these four vaccines was performed in this study. Characterization of the produced influenza vaccines confirmed that WIV and split vaccines contained all viral components, whereas virosome and subunit vaccines only contained the membrane proteins HA and NA. Determination of the particle size and polydispersity of the vaccines showed that WIV and virosome vaccines do have a relatively monodisperse particle size. In contrast, the produced split and subunit vaccines showed heterodisperse particle sizes, indicating that the vaccines contained viral components in a less organized fashion. WIV and virosome vaccine retained their particle size after freeze-drying, while split and subunit vaccines displayed still a heterodisperse particle size. These differences however did not influence the survival of the trehalose/HBS stabilized vaccines after freeze-drying in vials; a complete recovery of HA1 protein (HPLC) and vaccine antigenicity (SPR) was observed after freeze-drying. This indicates that influenza vaccine containing Bioneedles can be produced without the loss of vaccine structure and antigenicity.

Immunization of C57BL/6 mice with all influenza vaccine-filled Bioneedles induced strong systemic humoral responses. Serum HI titers induced by vaccines delivered by Bioneedles were higher than the HI titers induced by intramuscular administered subunit vaccine and comparable to HI titers induced by intramuscular administered WIV vaccine. Overall, Bioneedle-delivered influenza vaccines induced similar HI titers as their liquid counterparts, indicating that this alternative method of administration could be used for influenza vaccines. The HI titers induced after i.m. immunization with WIV or subunit vaccine were comparable to those found in the study by Hagenaars et al [8]. This might indicate that in terms of HI titers, subcutaneous and intramuscular administered liquid influenza vaccines elicit comparable responses.

The current correlate of protection (CoP) for human influenza vaccines is indicated by an HI titer higher than 40 in humans after vaccination [20]. However, previous studies have shown that cellular immune responses are important as well, especially for pandemic vaccine candidates [21]. Additionally, it is believed that cellular responses may play an important role in the elderly, in which vaccines fail to elicit adequate antibody responses [22]. Analysis of the cellular immune responses

and IgG subtype profiling revealed that subunit, split and virosomal vaccines induced comparable mixed TH1/TH2 responses. In contrast, immunization with WIV vaccines resulted in an IgG subtype profile that was significantly skewed towards IgG2c, indicating a cellular response skewed towards TH1 [23]. This observation can be explained by the presence of viral ssRNA in the WIV vaccine, which is not present in subunit, split and virosomal vaccines [24, 25]. Administration by Bioneedles did not alter the cellular or IgG subtype profile significantly, which indicates that the route and method of Bioneedle administration have no impact on the quality or type of immune response induced by the influenza vaccines.

From the data it can be concluded that influenza vaccine-filled Bioneedles can induce immune responses that are similar to responses induced by subcutaneous and intramuscular influenza vaccines. Considering the virosome vaccine, there is an indication that Bioneedles might actually improve the immunogenicity of influenza vaccines. This could be explained by several mechanisms. A previous study by Hirschberg et al showed a dose sparing effect after immunization with tetanus toxoid-filled Bioneedles [11]. Influenza vaccines might benefit from a dose sparing effect by Bioneedles. To investigate the dose-sparing potential of influenza Bioneedles, dose-response studies are warranted. Furthermore, while trehalose is present as lyoprotectant in the lyophilized vaccine formulations, it has shown no adjuvant activity in influenza vaccines before [26, 27].

The Bioneedles may cause a depot effect at the SOI, which might result in an increased immunogenicity of the Bioneedle-delivered influenza vaccines. To investigate what happens to the Bioneedles at the SOI, a biodistribution study was performed with infrared-labeled WIV. Initially, the Bioneedle groups show little infrared signal, most likely due to the solid, freeze-dried state of the contents in the Bioneedle. After 1 hour, the infrared signal at the SOI significantly increases, indicating that the Bioneedle was dissolved. After this initial delay in vaccine release time, the infrared signal of Bioneedle-delivered IRdye is parallel to the signal of s.c. administered liquid IRdye. Studies with infrared-labeled WIV showed similar results, with an initial lag time caused by the reconstitution process of the Bioneedle. Surprisingly, s.c. administered WIV vaccine showed a prolonged presence at the SOI. Similarly, Bioneedle-delivered WIV vaccine also remained visible at the SOI after 3 days. These results are similar to data obtained from a study with inactivated polio vaccine (IPV), which showed that both liquid and Bioneedle-delivered IPV remained at the SOI for several days [28]. Contrarily, a study found that ovalbumin was cleared within 3 days at the SOI [29], suggesting that the residence time of antigens may vary. Physicochemical characteristics of the antigen such as size and surface charge might influence the residence time at the SOI. Furthermore, antigens may interact with the Bioneedle material after Bioneedle reconstitution, which may affect antigen residence time. However, the current data does not suggest that the Bioneedle significantly increases the residence time of the antigen. Thus, the observed increased immunogenicity of Bioneedle-delivered influenza vaccines was not the result of a depot effect at the SOI. Further studies thus should be conducted to elucidate this observation.

The vaccine antigens in the produced influenza Bioneedles were expected to possess increased vaccine stability. The stability of the different types of influenza vaccines after lyophilization was assessed in vials. In order to retain the vaccine antigenicity after lyophilization and subsequent storage, trehalose was chosen as stabilizer. Trehalose is known for its excellent lyoprotective capacities in influenza vaccine formulations [26, 30, 31]. Lyophilized vaccines had low residual moisture contents, positively affecting the glass transition temperature of the vaccine product [18]. Glass transition temperatures calculated were above 95°C, indicating that the glassy matrix provided by trehalose was physically stable, and retained its glassy structure with low molecular mobility at the storage conditions evaluated in this study. Lyophilization of the influenza vaccines resulted in vaccine formulations that were heat stable at 60°C for at least one month. In sharp contrast, conventional liquid influenza vaccines showed a decrease in stability after one-month storage at 37°C. The liquid vaccines lost all HA1 content and antigenicity after storage at 60°C for one month, indicating that conventional liquid vaccines have limited heat stability. It should be noted that antigenic recoveries of freeze-dried vaccines dropped slightly after one-month storage at 37°C and 60°C, which could have an impact on immunogenicity of the product. However, a previous study by Geeraedts et al concluded that storage of liquid WIV vaccine at 40°C for three months resulted in a significant loss of immunogenic potency of the vaccine [27], whereas lyophilized WIV vaccine remained stable under the same conditions. These results show a similar trend in vaccine degradation compared to this study, and demonstrates that freeze-dried influenza vaccines are still immunogenic after storage at elevated temperatures.

In case of Bioneedles, a previous study with hepatitis B antigen demonstrated that heat-stressed Bioneedles induced similar immune responses compared to unstressed Bioneedles [12], which underlines the stability of freeze-dried Bioneedles. For the current study, it should be noted that the lyophilized vaccine product in the Bioneedles might not behave exactly similar in terms of stability as the formulations lyophilized in vials. A previous study by Hirschberg et al compared the heat stability of liquid tetanus toxoid with tetanus toxoid that was lyophilized in glass vials or Bioneedles [11]. Tetanus toxoid showed high antigenicity recoveries after lyophilization in both glass vials and Bioneedles. Tetanus toxoid lyophilized in Bioneedles showed a 60% recovery when incubated for 3 weeks at 60°C, whereas liquid tetanus toxoid lost all activity after 1 week at 60°C. Whether influenza vaccine in Bioneedles follows this same trend can be determined once the influenza vaccine stability can directly be measured from vaccine material in Bioneedles. If needed, the lyophilization process for influenza Bioneedles can be optimized further to increase vaccine recovery and stability.

CONCLUSION

This study demonstrates the potential of Bioneedles as an alternative delivery system for influenza vaccines. The immune responses induced by four influenza vaccine formulations were compared to determine the optimal influenza vaccine for Bioneedle vaccine development. All influenza vaccine formulations delivered by Bioneedles induced immune responses that were non-inferior to liquid formulations. WIV was determined as the best influenza vaccine formulation for use in Bioneedles, due to its ease of formulation and ability to induce both strong humoral and cellular immune responses. The freeze-dried state of the vaccine in the Bioneedle makes it suitable for long-term storage outside the cold chain, and enables easy stockpiling. To continue development, challenge studies with influenza Bioneedle vaccine should be performed to confirm the induction of protective immune responses after vaccination. Finally, the potential of Bioneedles for influenza vaccine delivery must be confirmed in non-inferiority and/or superiority studies in human. This study confirmed that Bioneedles could serve as a promising alternative delivery system for influenza vaccines.

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Chapter 8

Summary and perspectives

SUMMARY

The development of cross-reactive influenza vaccines remains a challenging ordeal. Several vaccine design approaches to tackle this problem are currently being followed, as described in **Chapter 2**. One of these approaches is the induction of influenza-specific T cell responses that lead to clearance of virus-infected cells. T cell peptides covering conserved epitopes of influenza are able to induce such T cell responses. However, these peptides are poorly immunogenic and require additional formulation with one or more adjuvants (i.e., a delivery system and/or immunopotentiator).

The aim of this thesis was to develop novel formulations that increase the immunogenicity of influenza T cell peptides. Three different types of peptide-based influenza vaccine formulations were investigated in this thesis; peptide-loaded virosomes, whole inactivated influenza virus (WIV)-adjuvanted peptides and peptide-loaded liposomes. Additionally, an alternative vaccine delivery system, the Bioneedle, was evaluated in this thesis for use in influenza vaccination.

Chapter 2 provides an extensive overview of formulation and production methods for current and future influenza vaccines. Key problems concerning current seasonal influenza vaccines, such as lack of cross-reactivity, lack of efficacy in high-risk populations and limited production capacity, were identified and possible solutions were suggested. Potentially cross-reactive immune responses and their formulation strategies for their respective antigens, which may lead to a new generation of influenza vaccines, were described. Finally, several novel influenza vaccine production methods were reviewed, and the production feasibility of novel influenza concepts was assessed.

Chapter 3 focused on the development of influenza virosomes as a delivery system for influenza peptide antigens. The influenza peptide GILGFVFTL was successfully associated with virosomes, without affecting virosome size, surface charge or fusogenicity. The immunogenicity of the peptide-loaded virosomes was tested in HLA-A2.1 transgenic mice. Peptide-loaded virosomes were able to induce peptide-specific T cells, and addition of Toll-like receptor (TLR) 9 agonist CpG-ODN 1826 to the virosomes significantly increased the T cell response. This formulation was also able to decrease morbidity and increase the recovery rate of mice infected with a heterologous influenza strain, which indicates that CpG-adjuvanted peptide-loaded virosomes are a promising vaccine formulation for the induction of effective T cells. Association between peptide and virosome as well as virosome fusogenicity were found to be necessary for effective uptake by dendritic cells and the subsequent induction of T cell responses.

The use of WIV as an adjuvant for peptides was investigated in **Chapter 4**. A physical mixture of WIV and the influenza peptide GILGFVFTL was able to induce high peptide-specific T cell responses in HLA-A2.1 transgenic mice. The dose for both WIV and peptides was optimized in a following study, and a prediction model could be generated from the dose-finding study. Next, it was found that WIV and peptide has to be co-localized at the site of injection to induce a T cell response; when WIV and peptide were injected at separate sites, the T cell response decreased significantly. Interestingly,

WIV did not have to be fusogenic to retain its adjuvanticity. Finally, WIV was used as an adjuvant for peptide pools of three wild-type influenza peptides (GILGFVFTL, FMYSDFHFI and NMLSTVLGV), or three chemically enhanced peptide ligands (CPL) derived from the three WT peptides. WIV was able to increase the immunogenicity of both WT and CPL GILFGFVFTL and FMYSDFHFI, while incomplete Freund's adjuvant was only effective for the GILGFVFTL peptide and its CPL derivative. The results of this study indicate that WIV is an effective adjuvant for influenza peptides and might also be useful as an adjuvant with other antigens.

In **Chapter 5** the influence of the lipid composition on liposome size, surface charge and liposome-induced dendritic cell maturation was studied by using a design of experiments (DoE) approach. Liposome size and surface charge could be modeled and accurately predicted. The surface charge of the liposome was mostly influenced by the inclusion of DOTAP, a cationic lipid, in the lipid composition. The other cationic lipid included in the study, DC-Chol, had much less influence on the liposomal surface charge. Aside from physicochemical characteristics of the liposome, the ability to mature dendritic cells (DCs) by the liposomes was investigated. CD40, CD80, CD83 and CD86 maturation responses could be modeled as a function of lipid composition. The DOTAP lipid positively affected CD40 and CD80 expression, while the other lipids did not influence the expression of these maturation factors. CD83 expression was controlled by the presence of DC-Chol and DOTAP, while DOPE negatively affected CD83 expression. None of the lipids had a significant impact on the CD86 expression. The prediction models for all four maturation markers were able to accurately predict the DC maturation responses induced by a liposome composed of a previously untested lipid composition. Liposome size and surface charge as function of lipid composition also could be accurately predicted. This method could therefore be a valuable tool to rapidly screen the immunogenicity of various liposomal formulations *in vitro*, using a minimal number of experiments with the DoE approach.

The current state of the development of T cell-based influenza vaccines is reviewed in **Chapter 6**. In contrast to antibody responses, influenza-specific T cell responses have the potential to be cross-reactive against many epidemic and pandemic influenza strains. Many different concepts are currently under development, such as whole virion-based vaccines (live-attenuated and whole inactivated influenza vaccines), and small subunit antigens such as short or long influenza peptides. However, certain aspects of T cell responses have yet to be elucidated. The priming status of the vaccinated individual seems to be of great importance for the induction of T cells; naïve individuals generate better cellular responses than individuals who are already primed with antibody-inducing influenza vaccines. Moreover, the location of the cellular response is important; local T cell responses at the site of infection (i.e., the lungs) are more effective than systemic T cell responses. Furthermore, there are some concerns about the induction of cellular responses, because they can lead to severe lung inflammation and pneumonia. Therefore, the induction of cellular responses by vaccines should be adequate but not excessive. Furthermore, it should be noted that T cell responses do not

provide protection against influenza infection, but merely accelerate viral clearance and decrease disease morbidity after infection. Thus, T cell-based influenza vaccines have great potential, but special attention should be given during their development to the immunological aspects of such vaccines.

Chapter 7 describes the development of an alternative delivery method for influenza vaccines, the Bioneedle. Bioneedles were successfully filled and freeze-dried with four types of inactivated influenza antigens (WIV, virosome, split and subunit), while maintaining vaccine antigenicity. The immunogenicity of the influenza antigen-filled Bioneedles was assessed in C57BL/6 mice and compared to that of intramuscular and subcutaneous administered influenza vaccines. Bioneedle-delivered vaccines induced high HI and IgG titers, comparable to i.m. administered vaccines. Bioneedle-delivered virosome vaccine performed even better than s.c. administered virosome vaccine, which indicates a beneficial effect of Bioneedle delivery. It was also found that, in line with previous literature, WIV was able to induce influenza-specific T cell responses, contrarily to the other vaccine formulations. An accelerated stability study showed that vaccine-filled Bioneedles remained antigenicity after exposure to 60°C for one month, indicating that Bioneedles are superior to liquid vaccines under harsh environmental conditions, which can be beneficial in developing countries.

PERSPECTIVES

Peptide-based influenza vaccines

Peptide antigens are an interesting group of antigens for influenza vaccines. Compared to traditional protein antigens, they are relatively small, which comes with an inherent poor immunogenicity. However, peptide antigens come with several benefits. They encompass one or several epitopes, which can be arranged at will for the most optimal immune response. This gives researchers the opportunity to design the ideal peptide antigen, which can induce both humoral or cellular immune responses. This is especially useful for T cell-inducing influenza vaccines, since priming with antibody-inducing influenza vaccines can even affect the efficacy of subsequent cellular-based vaccines, as demonstrated with LAIV in children and adults [1]. Furthermore, peptide antigens can be produced synthetically, while most other antigens are produced on biological platforms. Synthetic-based vaccines can be changed more easily from a production point of view than vaccine produced on biological platforms such as cell lines. This offers more flexibility in terms of altering the epitopes to be included in the peptide vaccine.

Selection of the epitope(s) to be included in the peptide is arguably the most crucial for the effectiveness of the vaccine, but remains a tricky business. While many epitope discovery strategies exist, e.g., *in silico* prediction, mass spectrometry-based discovery and other methods [2-4], not a single immunological parameter, such as epitope immunodominance, is yet correlated to protective T cell epitopes [5]. Indeed, this was recently demonstrated by Keskin et al., who concluded that the $M1_{58-66}$ influenza peptide, which was the peptide of choice in this current thesis because of its immunodominance, induced T cell responses with such a poor avidity that the T cells did not recognize influenza infected epithelium [6]. With this knowledge, careful consideration should be given to pursue further optimization of epitopes, such as increasing epitope binding properties [7].

Nonetheless, peptide-based influenza vaccines remain promising. Concepts that use long peptides comprised of multiple influenza epitopes have recently seen considerable success, with Multimeric-001, Flu-v and FP-01.1 passing phase I studies [8-10]. The efficacy of these vaccines however has not been evaluated yet in humans, and phase II and III studies with these vaccines are thus highly anticipated.

Short peptide antigens still remain in the preclinical phase of development as of yet. Due to their poor intrinsic immunogenicity, short peptides need additional formulation with drug delivery systems and/or other adjuvants to be effective. In this thesis, three types/categories of formulations for short peptide antigens have been studied. In the following sections, the prospects for each of these formulation types will be briefly discussed.

Influenza virosomes as peptide carriers

Virosomes were originally developed as an inactivated influenza vaccine [11]. While subunit and split influenza vaccines dominate the market, they are unable to induce a cellular response, which

greatly reduces their cross-reactivity. Virosomes are able to induce both humoral and cellular responses, similar to WIV formulations. Virosomes were initially marketed by Berna Biotech as a seasonal influenza vaccine in Europe since 1997 [12], but have recently been discontinued for reasons yet to be disclosed.

Next to the use as an antigen, influenza virosomes have been also identified as a possible vaccine delivery system [13]. Virosomes have been extensively studied as a carrier for malaria peptide vaccines [14]. Furthermore, hepatitis C peptides were tested in combination with influenza virosomes [15]. Next to a publication of Arkema et al. [16], this thesis contains the only other study utilizing influenza virosomes as a delivery system for influenza T cell peptides [17]. The formulation was able to decrease disease morbidity and accelerate recovery in mice. However, the low encapsulation efficiencies and extensive formulation procedures may make the virosome concept less attractive for the delivery of influenza peptide antigens. Especially the inclusion of several peptide antigens, which is necessary to ensure proper HLA coverage [18], could prove to be too challenging. Thus, while the preclinical performance of peptide-loaded virosomes is promising, it is unlikely that this concept will be a vaccine formulation viable for human use.

WIV as a vaccine adjuvant

Whole inactivated influenza vaccine was the first influenza vaccine formulation that entered the market in the 1940s [19]. WIV was eventually replaced by split, subunit and virosome vaccines, because the use of WIV was associated with a higher incidence of adverse effects. However, improvements in the production and purification processes have made WIV equally safe as split vaccines [20]. The immunogenicity of WIV is however still superior to that of other inactivated influenza vaccines, most likely because it contains the viral ssRNA, which is a natural TLR7 ligand [21, 22]. This inherent immunogenicity of WIV was the rationale behind the study using WIV as an adjuvant for peptide antigens, as described in this thesis. WIV proved to be an effective adjuvant for most of the tested influenza epitopes, both for immunodominant and less dominant epitopes. It is likely that WIV can increase the immunogenicity of non-influenza antigens as well, for both humoral and cellular responses. WIV should therefore be combined with other vaccine antigens in future studies to investigate its effect on non-influenza antigens.

The ease of formulation (simple mixing) of WIV makes it an attractive adjuvant. Formulations such as virosomes and liposomes are more sophisticated, but require formulation steps that are sometimes difficult to scale up for industrial scale production. Simple mixing of adjuvant and antigen means that both components can be produced separately. Furthermore, the components can be mixed just moments prior to administration, which is useful when adjuvant and antigen combined are only temporarily stable. It thus might be feasible to use WIV as both an antigen and an adjuvant in a combination vaccine.

Liposomes as peptide carriers

Liposomes have been used extensively as delivery systems for various pharmaceuticals and biologics, including vaccine antigens. How liposomes affect the immune system themselves is however unclear. Liposome formulations that include PAMPs in the lipid layer, such as CAF01, clearly serve as an immunopotentiator. However, studies have shown that cationic liposomes induce DC maturation without the inclusion of PAMPs or other immunostimulatory molecules [23, 24]. In this thesis, we attempted to evaluate and model the effect of the liposomal lipid composition on the expression of DC surface markers. While the mechanism(s) behind this immunostimulatory effect of cationic lipids on DCs were not investigated, this study confirmed that cationic liposomes can activate DCs on their own, and that the DC maturation responses can be predicted when the lipid composition is known. The method described in this thesis could be an excellent tool to extensively screen liposomal formulations *in vitro* with minimal formulation efforts.

Influenza Bioneedles

Many alternative administration methods for influenza vaccines have been developed and studied over the years [25]. One of these alternatives are Bioneedles, which are hollow injectable implants which can be filled with a vaccine formulation. Bioneedles have been tested with numerous antigens, including alum-adjuvanted tetanus toxoid [26], LpxL1-adjuvanted hepatitis B surface antigen [27], CAF01-adjuvanted BCG and inactivated polio vaccine [28, 29]. In this thesis, Bioneedles were made with four influenza vaccine types [30]. Influenza vaccine-filled Bioneedles proved to be remarkably thermostable (minimal loss of antigenicity after expose to 60°C for 1 month). This could be especially useful in developing countries, where the logistics for vaccine refrigeration are often unreliable, hampering the distribution of vaccines. Furthermore, if an appropriate implantation device is developed, Bioneedles can be quickly administered by untrained personnel, which is useful in mass vaccination campaigns. Bioneedles are therefore a promising alternative to conventional needle-based vaccination, and further development of this system should be pursued.

Universal influenza vaccines

T cell-inducing influenza vaccines are just one of the concepts that are currently in development to reach the ultimate goal: a universal influenza vaccine, which protects against all influenza strains with a limited number of immunizations. Other concepts involve induction of mucosal IgA at the lungs or cross-reactive antibodies directed against HA stalk regions or M2e protein. While all concepts are promising, it is unlikely they will result in a truly universal influenza vaccine in the next 5 to 10 years. However, existing seasonal vaccines might be supplemented with additional antigens that induce cross-reactive T cells or antibodies to expand the breadth of the induced immune response. Indeed, already two different T cell-inducing vaccines have been combined with seasonal influenza vaccines in preclinical and clinical studies [31-33]. Such combinations of seasonal and T cell vaccines have the potential to be the next generation of influenza vaccines.

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Appendix

Nederlandse samenvatting
List of publications
Curriculum vitae

NEDERLANDSE SAMENVATTING

Blauwgedrukte woorden zijn opgenomen in de verklarende woordenlijst op pagina 186.

Griep

Het influenzavirus (ofwel griepvirus) is een RNA-virus van de Orthomyxoviridae familie, dat de luchtwegen kan infecteren. Er bestaan drie types griepvirussen; influenza A, B en C, waarvan influenza A en B mensen kunnen infecteren. Naast mensen zijn er verschillende andere gastheren voor griepvirussen, waaronder vogels, varkens, honden en paarden. Typische symptomen tijdens een griepvirus infectie, zijn hoesten, verstopte neus, hoofdpijn, koorts, spierpijn en vermoeidheid. In ernstige gevallen kan een griepinfectie leiden tot een primaire virale longontsteking, secundaire bacteriële longontsteking of infectie van de sinussen, welke allen potentieel dodelijk kunnen zijn in zwakke individuen.

Het griepvirus verspreid zich gewoonlijk in de vorm van jaarlijkse epidemieën die rond het winterseizoen vallen. Schattingen van de Wereldgezondheidsorganisatie (WHO) geven aan dat jaarlijks wereldwijd 3 tot 5 miljoen mensen opgenomen moeten worden in het ziekenhuis door griep-geassocieerde ziekte, waarvan ongeveer een half miljoen mensen sterft aan directe of indirecte gevolgen van een griepinfectie. Naast invloed op de volksgezondheid hebben deze epidemieën ook negatieve invloed op de economie, door stijgingen in ziekteverzuim en ziektekosten. Het is dus essentieel dat griepepidemieën ingeperkt worden.

Griepvaccins

Een griepvirus heeft meestal een ronde vorm met een grootte van 100 tot 150 nanometer. Het virus bestaat uit grofweg twee delen: 1) de virale envelop, bestaande uit fosfolipiden en de membraaneiwitten hemagglutinine (HA) en neuraminidase (NA), en 2) het nucleocapside, de kern die de interne eiwitten en het RNA bevat (zie Figuur 1 in Hoofdstuk 1). De membraaneiwitten HA en NA worden onderverdeeld in verschillende subtypen (18 voor HA, 11 voor NA), die samen het subtype van het griepvirus aanduiden (bijv. H1N1 of H3N2). Deze membraaneiwitten fungeren ook als **antigenen**. Het immuunsysteem maakt vervolgens antigeen-specifieke **antilichamen** aan die het virus kunnen neutraliseren.

Vaccinatie is de enige manier, naast een griepinfectie zelf, om deze antilichamen op te wekken. De antigenen van griepvirus ondergaan echter voortdurend veranderingen door **antigene drift** en shift, waardoor de opgewekte antilichamen niet meer aan het virus kunnen binden. Om voor deze veranderingen te compenseren, worden de huidige seizoenale griepvaccins elk jaar aangepast, aan de hand van epidemiologische voorspellingen van de antigene drift. Hierdoor bevatten de griepvaccins HA en NA subtypen die meestal identiek zijn aan de griepvirussen die in de betreffende winter circuleren. De veranderingen die veroorzaakt worden door antigene shift zijn echter niet te voorspellen. Een antigene shift duidt een kruising aan tussen twee verschillende virussubtypen; meestal een combinatie van een humaan griepvirus en een vogel- of varkensgriepvirus. Dit

kan gebeuren wanneer er intensief contact is tussen mens en dier, zoals pluimveebedrijven en varkensstallen, waar de virussen de kans krijgen om één dezelfde gastheer te infecteren om vervolgens te recombineren tot een nieuw virussubtype. Dit kan leiden tot een pandemische griepstam, zoals de varkensgriep (ook wel Mexicaanse griep genoemd) in 2009 (H1N1). Seizoenale griepvaccins zijn over het algemeen niet effectief tegen nieuwe griepstammen, omdat de vaccin-geïnduceerde antilichamen niet **kruisreageren** met andere HA en NA subtypen. Er zijn dus nieuwe griepvaccins nodig die immuunresponsen opwekken die onafhankelijk van virussubtype bescherming bieden.

T cel-activerende griepvaccins

Om nieuwe griepvaccins te ontwikkelen die **kruisbeschermende** immuunresponsen opwekken, is kennis van immunologie noodzakelijk. Centraal hierin staan de **antigeen-presenterende cellen** (APCs), waaronder **dendritische cellen** (DCs). APCs kunnen (vaccin) antigenen herkennen en opnemen, waarna ze vervolgens bepaalde **epitopen** van het antigeen presenteren aan T cellen via **MHC moleculen**. Tijdens dit proces moeten de DCs ook **gematureerd** zijn, om de antigeenpresentatie en activatie van T cellen goed te laten verlopen. Maturatie van DCs wordt meestal geïnduceerd door **pathogen-associated molecular patterns** (PAMPs), **immuunstimulatoren** die aanwezig zijn in bacteriën of virussen, zoals viraal RNA. T cellen zijn onder te verdelen in twee hoofdtypes: T helper cellen (T_H) en **cytotoxische T cellen** (CTLs). T helper cellen hebben verschillende functies, zoals de activatie van B cellen (die antilichamen produceren) en de ondersteuning van CTLs. CTLs zijn in staat om geïnfecteerde cellen te lyseren (zie Figuur 1D, Hoofdstuk 2), en vormen naast antilichamen de belangrijkste tak van specifieke immuniteit.

In dit proefschrift leggen we de focus op het ontwikkelen van een griepvaccin dat CTLs induceert. CTLs zijn in staat om griepvirus-geïnfecteerde cellen te herkennen aan delen van het virus die aan het celoppervlak geëxposeerd worden. In tegenstelling tot antilichamen, die alleen epitopen aan de buitenkant van het virus herkennen, kunnen T cellen ook epitopen van interne virale eiwitten herkennen, die minder snel muteren. Dit zorgt ervoor dat T cellen onafhankelijk van het virussubtype kunnen reageren op een griepinfectie en daarmee mogelijk kunnen leiden tot kruisbescherming.

T cellen opwekken met een vaccin is echter niet eenvoudig. Antigenen die de epitopen van interne virale eiwitten bevatten, zoals **peptiden**, hebben een zwakke **immunogeniciteit**. Dit is voornamelijk te wijten aan twee factoren: 1) peptiden zijn klein en worden slecht herkend en opgenomen door DCs, en 2) door het gebrek aan PAMPs matureren de DCs niet, waardoor T cellen niet geactiveerd worden. Deze twee aspecten kunnen verbeterd worden door het gebruik van **adjuvantia**.

De opname van peptiden door DCs kan verbeterd worden door zogenaamde **delivery systemen** als adjuvantia te gebruiken; deeltjes die het peptide antigeen naar de DCs kunnen transporteren en ín de DC via de juiste route op MHC moleculen naar het celoppervlak kunnen sturen, zogenaamde

kruispresentatie. De maturatie van DCs kan door verschillende soorten immunostimulatoren geïnduceerd worden. Het **formuleren** van het antigeen met adjuvantia is dus een belangrijk aspect van het ontwikkelen van een peptide-gebaseerd griepvaccin. Het hoofddoel van dit promotieonderzoek is dan ook het ontwikkelen van nieuwe formuleringen voor influenza peptide vaccins.

Virosomen als delivery systeem

In **Hoofdstuk 3** werden virosomen gebruikt als delivery systeem voor het influenza peptide **GILGFVFTL**. Virosomen zijn lege virusmantels die bestaan uit de eiwitten en lipiden die in de virale envelop zitten (zie Figuur 2, Hoofdstuk 2). GILGFVFTL werden vervolgens geassocieerd met de virosomen, wat in peptide-geladen virosomen (P-V) resulteerde. Nadat was bewezen dat de associatie met peptiden de eiwitsamenstelling, de deeltjesgrootte en lading van de virosomen niet beïnvloedde, werd de immunogeniciteit van P-V getest in **HLA-A2.1 transgene muizen**. Uit de dierstudies bleek dat P-V beter in staat waren om peptide-specifieke T cellen op te wekken dan peptide alleen (zonder virosomen) of peptide gemixt met virosomen (dus niet geassocieerd). De additie van **CpG**, een immunostimulator, bleek de immunogeniciteit van P-V zelfs verder te verbeteren. Verder bleek dat de **fusogeniciteit** (de eigenschap van een virus of virosoom om met een **endosomaal membraan** te fuseren en zo zijn inhoud af te geven in het **cytoplasma**) van virosomen belangrijk was om een goede T cel respons op te wekken. Vervolgens werd de effectiviteit van de P-V als vaccin getest door muizen eerst te vaccineren met P-V, om ze vervolgens te infecteren met een **heteroloog griepvirus**. Uit deze studie bleek dat muizen gevaccineerd met P-V minder gewichtsverlies leden, en uiteindelijk sneller herstelden van de virusinfectie. Virosomen zijn dus een effectief delivery systeem voor peptide vaccins, en de P-V met CpG formulering is in staat om griep-specifieke CTLs op te wekken in muizen, die het herstel van een heterologe virusinfectie versnellen.

Geïnactiveerd influenza virus als adjuvant

In **Hoofdstuk 4** onderzochten we de potentie van geïnactiveerd influenza virus (WIV) als een adjuvant. Zoals eerder gezegd, zijn immunostimulatoren meestal afgeleid van bacteriële of virale componenten. WIV bevat viraal RNA, wat ook een immunostimulator is. Het influenza peptide GILGFVFTL werd geformuleerd met WIV, en vervolgens ingebracht in HLA-A2.1 transgene muizen. Dit resulteerde in een verbeterde peptide-specifieke T cel respons vergeleken met peptide of WIV alleen. Om het belang van **co-localisatie** van WIV en het peptide te bestuderen, werden beide componenten ook apart op verschillende locaties in de muis ingebracht. Hieruit bleek dat wanneer WIV en peptide apart op verschillende locaties in de muis werden ingebracht, het adjuvant effect van WIV teniet werd gedaan. Tevens bleek dat de fusogeniciteit van WIV niet belangrijk was voor de adjuvanticiteit van WIV in combinatie met GILGFVFTL. Naast het GILGFVFTL peptide, werden ook twee andere influenza peptiden, FMYSDFHFI en NMLSTVLGV, geadjuveerd met WIV. Ook werden er chemisch gemodificeerde versies van deze drie peptiden geïncludeerd in de studie, die een betere bindingsaffiniteit hadden met MHC moleculen. Een verhoogde bindingsaffiniteit zou eventueel

kunnen leiden tot een betere immuunrespons. De resultaten van de dierstudie wezen uit dat vooral de gemodificeerde versie van FMYSDFHFI beter T cellen induceerde dan ongemodificeerd FMYSDFHFI. WIV had een adjuverend effect voor alle peptiden. Uit deze studie konden we dan ook concluderen dat WIV veel potentie heeft als een adjuvant voor T cel-inducerende peptide vaccins.

Liposomen als op maat gemaakte delivery systemen

Liposomen zijn vesicles van lipiden die als delivery systeem te gebruiken zijn voor antigenen. Er kunnen talloze lipiden gebruikt worden om liposomen te maken, die elk andere eigenschappen aan het liposoom kunnen geven. Liposomen kunnen daardoor op maat gemaakt worden voor een specifiek doeleinde; de grootte en electrische lading kunnen bijvoorbeeld gevarieerd worden, wat eventueel tot een immuunstimulerend effect kan leiden. In **Hoofdstuk 5** bestudeerden wij welke lipiden het beste gebruikt konden worden in liposomen om ***in vitro*** DCs te laten matureren, wat weer een maat is voor adjuvant effect van de liposomen. Om te evalueren welke lipiden een invloed hadden op DC maturatie en de andere eigenschappen van het liposoom (zoals grootte en lading), gebruikten we **design of experiments** (DoE). Met behulp van DoE konden we statistische predictiemodellen voor elke uitleesparameter (liposoom grootte, lading, en de DC **maturatiemarkers** CD40, CD80, CD83 en CD86) genereren, afhankelijk van de lipiden in het liposoom. Deze predictiemodellen werden vervolgens gevalideerd door de uitleesparameters voor een nog niet gemaakt liposoom te voorspellen, om vervolgens het liposoom te maken en daadwerkelijk alle parameters te meten. Hieruit bleek dat de predictiemodellen zeer accuraat waren; alle DC maturatiemarkers waren correct voorspeld. Deze methode kan wellicht gebruikt worden om de ontwikkeling van liposoomformuleringen als adjuvantia voor vaccins te versnellen, aangezien DoE een relatief klein aantal experimenten benodigd om een predictiemodel te genereren.

Thermostabiele Bionaalden voor toediening van griepvaccins

Griepvaccins worden normaliter intramusculair (i.m.) toegediend via een injectienaald, of in sommige gevallen met een intranasale spray. Aan i.m. toediening kleven echter enkele nadelen, zoals de noodzaak van getraind personeel, de kans op prikaccidenten en daarmee op besmettingen met bijvoorbeeld hepatits B en naaldafval wat hergebruik niet uitsluit. Deze punten zijn vooral problematisch in ontwikkelingslanden. Tevens wordt de vraag naar vervanging van de naald in de westerse wereld steeds groter om de acceptatie van vaccinatie te verhogen. Vloeibare vaccins zijn tevens afhankelijk van de **koude keten**; ze zijn gevoelig voor hoge temperaturen. Om deze redenen wordt er gezocht naar alternatieve toedieningsvormen voor (influenza) vaccins. In **Hoofdstuk 7** onderzochten wij het gebruik van Bionaalden voor de toediening van verschillende griepvaccins. Bionaalden zijn holle implantaten gemaakt van biodegradeerbaar zetmeel, welke gevuld kunnen worden met een gevriesdroogd vaccin (zie Figuur 1, Hoofdstuk 7). Na een onderhuidse toediening (idealiter onder hoge druk met een injector), lost de Bionaald met vaccin op. In onze studie vulden wij Bionaalden met vier verschillende typen griepvaccins (WIV, virosomen, split en subunit; zie Figuur 2, Hoofdstuk 1), en vaccineerden vervolgens muizen met deze Bionaalden. Dit resulteerde

in het algemeen in goede influenza-specifieke antilichaam responsen voor alle groepen; alleen de virosoom en subunit groepen waren iets minder immunogeen. Tevens waren deze Bionaald vaccins in staat om influenza-specifieke T cellen op te wekken. Vervolgens werd er ook gekeken naar de thermostabiliteit van de Bionaalden. Vloeibare vaccins moeten normaliter constant gekoeld worden, maar vaccins in de vaste fase (zoals gevriesdroogd vaccin) zijn in het algemeen stabieler. Na een maand blootstelling aan een temperatuur van 60°C waren de griepvaccins in de Bionaalden nog steeds intact, terwijl vloeibare griepvaccins binnen enkele dagen hun **antigeniciteit** verliezen. Hieruit kunnen we concluderen dat Bionaalden geschikt zijn als een alternatieve toedieningsvorm voor griepvaccins, en ook zeer stabiel zijn buiten de koude keten.

Conclusies en vooruitblik

Er zijn in dit proefschrift verschillende adjuvantiaontwikkeld voor gebruik met influenza peptiden. Virosomen waren in combinatie met CpG in staat om de immunogeniciteit van het GILGFVFTL peptide significant te verhogen. Dit leidde tot een verhoging van het aantal peptide-specifieke CTLs in muizen. De geïnduceerde CTLs waren vervolgens in staat om de ernst van een heterologe griepinfectie te verminderen en het herstel na infectie te bevorderen. Tevens werd aangetoond dat eigenschappen van het virosoom, zoals peptide associatie en fusogeniciteit, cruciaal waren voor de inductie van T cellen. Virosomen hebben dus de potentie om een goed delivery systeem te zijn voor peptide vaccins.

WIV bleek een effectief adjuvant te zijn voor verschillende peptiden. Het was in staat om de T cel responsen tegen de peptiden significant te verhogen, mits WIV en peptide antigeen op dezlefde plek toegediend werden. Verder bleek dat modificatie van de peptiden om de bindingsaffiniteit te bevorderen de T cel respons kon verhogen. WIV bleek dus een effectief adjuvant te zijn voor peptide vaccins.

De toepassing van de design of experiments methode bleek zeer nuttig te zijn om liposoomformuleringen te optimaliseren. Ook bleek dat deze methode in staat was om biologische responsen geïnduceerd door liposomen, zoals *in vitro* DC maturatie, te voorspellen aan de hand van de liposoomsamenstelling. Deze methode kan dus in de toekomst gebruikt worden om de ontwikkeling van liposoomformuleringen voor vaccins te versnellen.

Uit de studie met Bionaalden bleek dat verscheidene influenza vaccins in Bionaalden te formuleren waren. Deze bleken immunogeen en thermostabiel te zijn. Influenza vaccins zijn dus met Bionaalden op een alternatieve wijze toe te dienen, zonder het gebruik van conventionele injectienaalden.

Het hoofddoel van het onderzoek in dit proefschrift was de ontwikkeling van een universeel griepvaccin. De studies in dit proefschrift zijn bijna allemaal uitgevoerd met één influenza peptide antigen, GILGFVFTL. Een vaccin gebaseerd op een enkel peptide is echter voor een humaan vaccin

niet genoeg; er komen meerdere HLA varianten voor in de humane populatie die allen andere epitopen binden. Er zijn dus meerdere peptiden nodig om een voldoende brede basis te leggen voor T cel immuniteit in de gehele humane populatie. Er moeten dus nog flinke stappen gezet worden voordat peptide vaccins daadwerkelijk op de markt komen.

Daarentegen zijn er op dit moment wel enkele andere universele griepvaccinconcepten in de klinische ontwikkelingsfase. De eerste resultaten zijn enigszins hoopgevend; er kunnen influenza-specifieke T cellen worden geïnduceerd. Of deze immuunresponsen daadwerkelijk genoeg zijn om heterologe griepinfecties tegen te gaan moet nog blijken. Tevens zouden deze vaccins de huidige seizoenale vaccins kunnen aanvullen om bredere immuunresponsen te verkrijgen. De komende jaren zullen uitwijzen of een **universeel griepvaccin** daadwerkelijk haalbaar is.

VERKLARENDE WOORDENLIJST

Adjuvantia	Stoffen die de immunogeniciteit van antigenen versterken. Kunnen delivery systemen en/of immuunstimulatoren zijn.
Antigeen	Een molecuul dat een immuunreactie kan opwekken.
Antigeen-presenterende cellen (APCs)	Cel die antigenen opnemen, om vervolgens epitopen daarvan aan T cellen te presenteren via MHC moleculen.
Antigene drift	Spontane mutaties in het genoom van influenza virus, die de epitopen van voornamelijk HA en NA veranderen.
Antigene shift	Kruising tussen twee afzonderlijke virusstammen, waardoor een nieuw griepvirus ontstaat.
Antigeniciteit	De mate waarin een antigeen zijn correcte structuur, welke antistoffen herkennen, behoudt.
Antilichaam	Eiwit dat aan lichaamsvreemde stoffen kan binden, om ze onschadelijk te maken.
Co-localisatie	Toediening van antigeen en adjuvant op dezelfde locatie.
CpG	Een herhalend DNA patroon wat voorkomt in verschillende pathogenen, zoals virussen en bacteriën. Is een immuunstimulator.
Cytoplasma	Ruimte van een cel die het cytosol en de organellen bevat. Antigenen die in het cytoplasma terecht komen kunnen via MHC-I moleculen gepresenteerd worden.
Cytotoxische T cel (CTL)	T cel die geïnfecteerde cellen (of tumorcellen) kan lyseren.
DC maturatie	Een gematureerde DC heeft karakteristieke dendrieten, en brengt verschillende costimulatoire moleculen tot expressie die nodig zijn voor een succesvolle activatie van T cellen.
Delivery systeem	Deeltjes die het antigeen kunnen vervoeren en op de juiste plek (meestal APCs) afleveren.
Dendritische cel (DC)	Een van de meest voorkomende antigeen-presenterende cellen.
Design of experiments (DoE)	Methode om systematisch de invloeden van bepaalde factoren te screenen. Kan ook gebruikt worden om predictiemodellen te genereren.
Endosomaal membraan	Membraan van het endosoom. Lichaamsvreemde stoffen kunnen door DCs geïnternaliseerd worden in endosomen, losse blaasjes (vesikels) bestaande uit het celmembraan.
Epitoop	Een deel van een antigeen dat herkend kan worden door antilichamen, B cellen of T cellen.
Formulering	De samenstelling van hulpstoffen die in een vaccin zitten, waaronder stabilisatoren en adjuvantia.

Fusogeniciteit	De eigenschap van een virus of virosoom om met een endosomaal membraan te fuseren en zo zijn inhoud af te geven in het cytoplasma.
GILGFVFTL	Peptide antigeen (en epitoop) afgeleid van influenza Matrix eiwit 1. Kan alleen gepresenteerd worden via humane HLA-A2.1 moleculen.
Heterologe virusinfectie	Een infectie met een griepstam die niet hetzelfde is als de griepstam in het vaccin.
HLA-A2.1 transgene muizen	Muizen met een humaan MHC (HLA-A2.1) molecuul.
Immunogeniciteit	Het vermogen van een antigeen om een immuunrespons op te wekken.
Immuunstimulator	Stoffen die het immuunsysteem activeren.
In vitro	Een test buiten een levend organisme; vaak op cellijnen.
Koude keten	Voorzieningen, zoals gekoelde transport en opslag, nodig om (vloeibare) vaccins tussen de 2°C en 8°C te houden, omwille van de vaccinstabiliteit.
Kruisbescherming	Zie kruisreageren.
Kruispresentatie	Normaliter worden door de DC alle opgenomen antigenen via MHC-II moleculen gepresenteerd. Voor de activatie van CTLs moeten antigenen echter via MHC-I moleculen gepresenteerd worden. Door kruispresentatie kunnen peptide antigenen via MHC-I moleculen gepresenteerd worden.
Kruisreageren	Een immuunrespons die tegen meerdere griepvirusstammen kan reageren, ongeacht virussubtype.
Lyseren	Het doden van een cel door het breken van het celmembraan.
Maturatiemarkers	Moleculen die door gematureerde DCs tot expressie worden gebracht. Deze zijn cruciaal voor een goede T cel activatie.
MHC moleculen	Major histocompability complex; eiwitten aan het oppervlak van (immuun)cellen die epitopen presenteren aan T cellen. Menselijke MHC moleculen worden humaan leukocytenantigeen (HLA) genoemd.
Pathogen-associated molecular pattern (PAMP)	Moleculen afkomstig van pathogenen, die als immuunstimulatoren kunnen fungeren.
Peptide	Keten van een klein aantal aminozuren; in dit geval rond de 10. Kan vaak ook een epitoop bevatten.
Universeel griepvaccin	Griepvaccin dat tegen elke griepstam effectief is.

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CURRICULUM VITAE

Peter Soema was born on the 3rd of July in 1987 in The Hague. After graduating from the Gymnasium Haganum in The Hague in 2005, he started his study Bio-Pharmaceutical Sciences at Leiden University. After obtaining his Bachelor's degree in 2009, he continued his studies for a Master's degree. During his study he completed two internships. The first internship was performed at the division for Drug Delivery Technology of the Leiden Academic Centre for Drug Research (LACDR), during which he investigated polymer-protein conjugates to be used for nasal vaccination. Subsequently, he went to the Faculty of Pharmaceutical Sciences of the University of British Columbia for his second internship, where he studied magnetic stem cell targeting to the eye. In 2011 he obtained his Master's degree with honors. In the same year, he started his PhD project at the Institute for Translational Vaccinology (Intravacc) under the supervision of Prof. dr. Gideon Kersten, Prof. dr. Wim Jiskoot (Leiden University) and Dr. Jean-Pierre Amorij, which resulted in this thesis. He is currently working at the same company as a scientist on vaccine delivery.