European Journal of Pharmaceutics and Biopharmaceutics 94 (2015) 251–263

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review Article

Current and next generation influenza vaccines: Formulation and production strategies

Peter C. Soema [a,b], Ronald Kompier [a,c], Jean-Pierre Amorij [a,*], Gideon F.A. Kersten [a,b]

[a] Intravacc (Institute for Translational Vaccinology), Bilthoven, The Netherlands
[b] Division of Drug Delivery and Technology, Leiden Academic Centre for Drug Research, Leiden University, The Netherlands
[c] FluConsult, Noordwijk, The Netherlands

ARTICLE INFO

Article history:
Received 10 April 2015
Revised 27 May 2015
Accepted in revised form 28 May 2015
Available online 3 June 2015

Keywords:
Influenza vaccines
Vaccine formulation
Vaccine production
Novel influenza vaccines
Influenza vaccine limitations
Universal influenza vaccine

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.
© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. 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.

* Corresponding author.
E-mail address: jean-pierre.amorij@intravacc.nl (J.-P. Amorij).

http://dx.doi.org/10.1016/j.ejpb.2015.05.023
0939-6411/© 2015 The Authors. Published by Elsevier B.V.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Table 1
Limitations of current influenza vaccines and potential solutions.

Limitation	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

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 toward the development of cross-reactive influenza 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.

2. 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 (Fig. 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.

2.1. 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 (Fig. 1A). Other stalk-reactive antibodies disrupt viral membrane fusion (Fig. 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.

2.2. 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

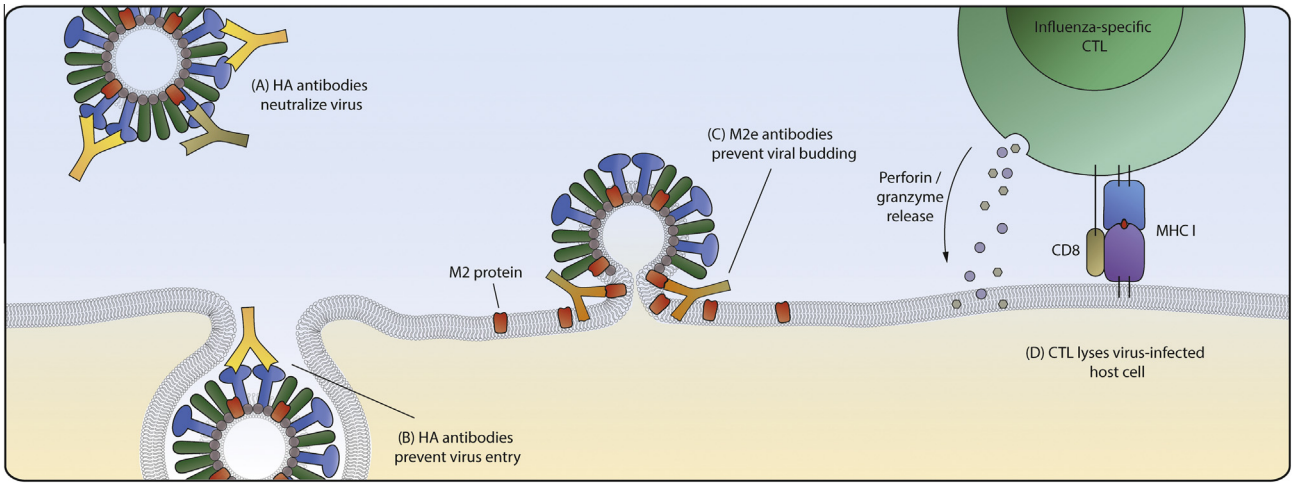


Fig. 1. Immune responses against influenza virus. (A) HA head- or stalk-reactive antibodies neutralize the virus. (B) HA-specific antibodies prevent virus attachment and entry to host cells. (C) M2e-specific antibodies prevent the budding and release of virus particles produced in infected host cells.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

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 (Fig. 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.

2.3. 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 (Fig. 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.

3. 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.

3.1. 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 Fig. 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

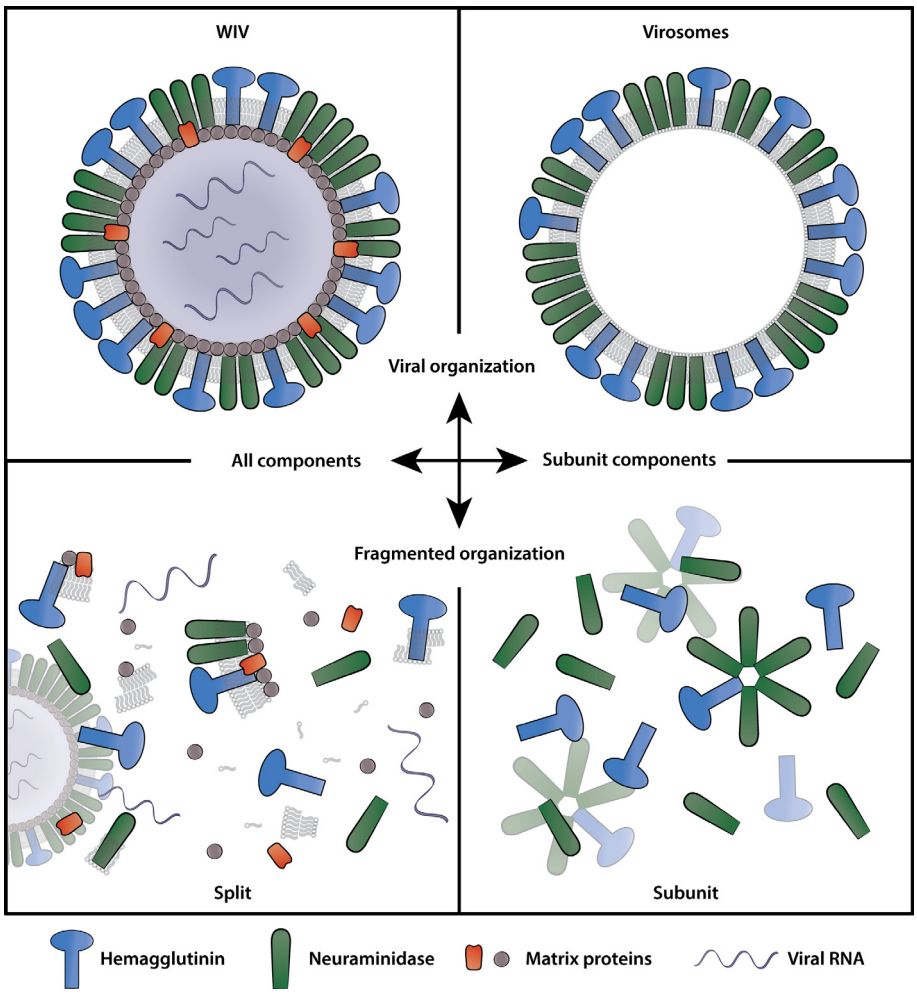


Fig. 2. Composition of inactivated influenza vaccines. The four different compositions of influenza vaccine differ in antigen components and structural organization. These differences also have an impact on the immunogenicity of the vaccine.

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.

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.

3.2. 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.

3.3. 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.

3.4. 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.

4. Formulation strategies for influenza vaccines: antigens, adjuvants and excipients

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.

4.1. 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 toward T_{H1} responses, which support the induction of cytotoxic lymphocytes. This additional immune response could further broaden the protection of these vaccine concepts.

4.2. 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.

4.3. 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-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 $HA2_{76–130}$ (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.

4.4. 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.

4.5. 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 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 toward 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 [73], 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 [74–77]. Additionally, MF59 has similar immunostimulatory effects in combination with prepandemic vaccine formulations [78]. 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 [79,80]. Furthermore, AS03-adjuvanted influenza vaccines were also able to confer seroprotection in immunocompromised patients infected with HIV-1 [81]. In contrast, adjuvanted vaccines failed to increase seroprotection rates in other immunocompromised groups, such as transplant recipients or patients with lymphoid malignancies [82,83].

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 [84]. 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 [85]. Clinical studies with ISCOM-adjuvanted influenza split vaccines revealed accelerated antibody responses in individuals who received ISCOM-adjuvanted influenza vaccines [86]. Furthermore, this coincided with a notable increase of influenza-specific $CD8^+$ T cell responses [87]. A third generation of saponin based adjuvants, named Matrix-M, was evaluated in a clinical study in combination with a pandemic virosomal influenza vaccine [88]. 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 [89].

Table 2
Adjuvants for influenza vaccines.

Adjuvant category	Adjuvant	Antigen(s)	Stage of development	Reference
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 sulfate esters)	WIV (pandemic)	Animal model	[123]
Saponins	Iscomatrix	WIV (seasonal)	Clinical development	[86]
	Matrix-M	Virosomes (pandemic)	Clinical development	[88]
Glycolipids	Alpha-GalCer (alpha-galactosylceramide)	LAIV (seasonal)	Animal model	[124]
		DNA (HA-encoding)		[125]
Liposomes	CCS/c (cationic liposomes of ceramide carbamoyl-spermine/cholesterol)	Subunit (seasonal)	Animal model	[126]
	CAF01 (cationic liposomes of DDA/TDB)	Split (seasonal)	Animal model	[127]
	Vaxfectin (cationic liposomes of GAP-DMORIE/DPyPE)	Split (seasonal), WIV (pandemic)	Animal model (split, WIV)	[128]
		DNA (HA-encoding)	Clinical development (DNA)	[70]
Bacterial components	CTA1-DD (Cholera toxin subunit A)	Peptide (M2e-based)	Animal model	[129]
	LT patch (*Escherichia coli* enterotoxin)	Split (pandemic)	Clinical development	[92]
	Salmonella and *Escherichia coli flagellins*	rHA (pandemic)	Clinical development	[91]
		rM2e		[130]
Cytokines	IL-12, IL-23	WIV (laboratory strain)	Animal model	[131]
	GM-CSF (Granulocyte-Monocyte Colony Stimulating Factor)	DNA (HA-encoding)	Animal model	[132]
	Type 1 IFN (IFNα)	Subunit (seasonal)	Animal model	[133]
TLR agonists/ immunomodulators	GLA (glucopyranosyl lipid A) (TLR4)	rHA (pandemic)	Clinical development	[134]
	Bacterial flagellins (TLR5)	rHA (pandemic)	Clinical development	[91]
		rM2e		[130]
	CpG oligodeoxynucleotide (TLR9)	Split (seasonal)	Clinical development	[135]
	PolyI:C (TLR3)	Split (seasonal)	Animal model	[136]
		LAIV (laboratory strain)		[137]
	IC31 oligodeoxynucleotide (TLR9)	Subunit (seasonal)	Animal model	[138]
	sLAG-3 (IMP321) (MHC class II ligand)	Split (seasonal)	Clinical development	[139]
Polymers	Chitosan	Subunit (laboratory strain)	Animal model	[140]
		DNA (M2 and NP encoding)		[141]
	PCPP (poly[di(carboxylatophenoxy)phosphazene])	Subunit (pandemic)	Animal model	[142]
	Advax (delta inulin)	Split (pandemic)	Clinical development	[143]
		rHA (pandemic)		[94]

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 [90,91]. 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 [92].

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 [93]. 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 [94]. 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 [95]. 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 [96]. A recent study identified higher amounts of structurally altered influenza NP protein in the Pandemrix vaccine than Arepanrix, another AS03-adjuvanted influenza vaccine [97]. 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 [98]. 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.

4.6. 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 a 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 [99–102]. This proves that the addition of stabilizing excipients can greatly enhance influenza vaccine stability under extremely unfavorable conditions.

5. 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 [64,103], indicating that these concepts are more prone to supplement rather than to replace existing seasonal vaccines.

6. Production strategies for influenza vaccines

6.1. 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 Fig. 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 [104]. 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 2012–2013 influenza season [105]. Decreasing dependence on egg-based influenza propagation

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

Targeted response	Concept	Status	Registration number	References
M2e antibodies	VAX102 (recombinant M2e fused to flagellin)	Phase I/II completed	NCT00603811, NCT00921947, NCT00921206	[130]
	VAX102 + seasonal vaccine (coadministered)	Phase I completed	NCT00921973	Unpublished data
	ACAM FLU-A (recombinant M2e fused to hepatitis B core protein)	Phase I completed	NCT00819013	Unpublished data
	VGX-3400X (DNA plasmid encoding for HA, NA and M2e-NP of H5N1 delivered by electroporation)	Phase I completed	NCT01184976, NCT01142362	Unpublished data
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	Unpublished data
	FP-01.1 combined with seasonal vaccine (prime) + FP-01.1 (boost)	Phase I completed	NCT01701752	Unpublished data
	Flu-v (long peptides containing multiple T cell epitopes)	Phase I completed	NCT01226758, NCT01181336	[144]
	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	[20,48,145]
	MVA-NP + M1 + seasonal vaccine (coadministered)	Phase I completed	NCT01465035	[103]
	ChAdOx1 NP + M1 (simian adenovirus vectored vaccine containing multiple T cell epitopes) + MVA-NP + M1 (mixed prime/boost)	Phase I ongoing	NCT01818362	[50]

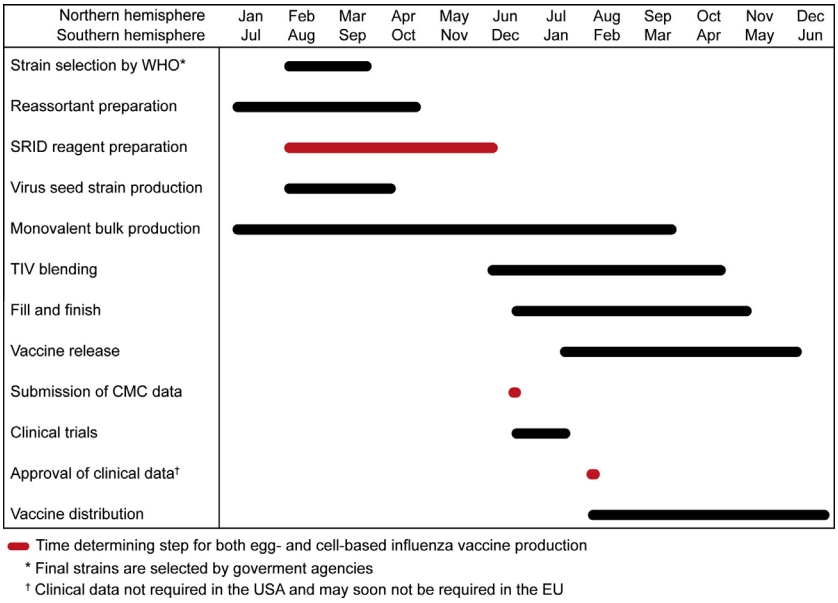


Fig. 3. Timeline of seasonal influenza vaccine production. While some novel production methods can grow influenza viruses faster, the vaccine production timeline contains several steps that are time determining (red lines). This means that while the vaccine can be produced faster, the time from strain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

is thus a crucial step toward 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 [106]. 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 [104].

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 [107]. Most WT human influenza viruses grow efficiently in MDCK cells, and existing egg-adapted reassortant strains can grow to similar titers [108]. These advantages make MDCK cells an acceptable substitute for

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

egg-based influenza virus production. Vero cells have also been used as a production platform for both seasonal and prepandemic vaccines [109,110]. Influenza virus cultivation in laboratory-scale bioreactors was compared between MDCK and Vero cells, but production yields between cell lines were not significantly different [111]. 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 [112]. 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 [113], 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 for influenza production such as Human Embryonic Kidney (HEK)-293 and Amniocyte Derived (CAP) cell lines are currently still in preclinical development [114,115]. 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 [116].

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 [117]. 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 [118]. 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 [119], expanding the number of influenza vaccine producers and increasing influenza vaccine production capacity in the world.

6.2. 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 [120]. 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 [121]. 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 [122]. 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.

7. 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.

Acknowledgment

This work was supported by the Center for Translational Molecular Medicine grant AMPVACS.

References

[1] K.M. Kunisaki, E.N. Janoff, Influenza in immunosuppressed populations: a review of infection frequency, morbidity, mortality, and vaccine responses, Lancet Infect. Dis. 9 (8) (2009) 493–504.
[2] J.E. McElhaney et al., The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines, Vaccine 30 (12) (2012) 2060–2067.
[3] P.L. Fraaij, T. Heikkinen, Seasonal influenza: the burden of disease in children, Vaccine 29 (43) (2011) 7524–7528.
[4] G. Neumann, T. Noda, Y. Kawaoka, Emergence and pandemic potential of swine-origin H1N1 influenza virus, Nature 459 (7249) (2009) 931–939.
[5] J.C. de Jong et al., A pandemic warning?, Nature 389 (6651) (1997) 554
[6] R. Gao et al., Human infection with a novel avian-origin influenza A (H7N9) virus, N. Engl. J. Med. 368 (20) (2013) 1888–1897.
[7] J.P. Amorij et al., Needle-free influenza vaccination, Lancet Infect. Dis. 10 (10) (2010) 699–711.
[8] C. van Els et al., Fast vaccine design and development based on correlates of protection (COPs), Hum. Vaccines Immunother. 10 (7) (2014) 1935–1948.
[9] B. Brandenburg et al., Mechanisms of hemagglutinin targeted influenza virus neutralization, PLoS One 8 (12) (2013) e80034.
[10] R.H.E. Friesen et al., New class of monoclonal antibodies against severe influenza: prophylactic and therapeutic efficacy in ferrets, PLoS One 5 (2) (2010) e9106.
[11] R.H. Friesen et al., A common solution to group 2 influenza virus neutralization, Proc. Natl. Acad. Sci. USA 111 (1) (2014) 445–450.
[12] S. Khurana et al., Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease, Sci. Transl. Med. 5 (200) (2013) 200ra114.
[13] R.A. Lamb, S.L. Zebedee, C.D. Richardson, Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface, Cell 40 (3) (1985) 627–633.
[14] M. Schotsaert et al., Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments, Expert Rev. Vaccines 8 (4) (2009) 499–508.
[15] P.C. Soema et al., Development of cross-protective influenza A vaccines based on cellular responses, Front. Immunol. 6 (2015) 237.
[16] H. Hamada et al., Multiple redundant effector mechanisms of CD8+ T cells protect against influenza infection, J. Immunol. 190 (1) (2013) 296–306.
[17] S. Sridhar et al., Cellular immune correlates of protection against symptomatic pandemic influenza, Nat. Med. 19 (10) (2013) 1305–1312.
[18] Z. Wang et al., Recovery from severe H7N9 disease is associated with diverse response mechanisms dominated by CD8(+) T cells, Nat. Commun. 6 (2015) 6833.
[19] T.M. Wilkinson et al., Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans, Nat. Med. 18 (2) (2012) 274–280.
[20] P.J. Lillie et al., Preliminary assessment of the efficacy of a T cell-based influenza vaccine, MVA-NP+M1, in humans, Clin. Infect. Dis. 55 (1) (2012) 19–25.
[21] A. Al-Mazrou et al., Comparison of adverse reactions to whole-virion and split-virion influenza vaccines in hospital personnel, Can. Med. Assoc. J. 145 (3) (1991) 213–218.
[22] R.A. van Boxtel et al., Safety and immunogenicity of influenza whole inactivated virus vaccines: a phase I randomized clinical trial, Hum. Vaccines Immunother. 11 (4) (2015) 983–990.
[23] F. Geeraedts et al., Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by Toll-like receptor signalling, PLoS Pathog. 4 (8) (2008) e1000138.
[24] F. Ansaldi et al., Fluzone((R)) intradermal vaccine: a promising new chance to increase the acceptability of influenza vaccination in adults, Expert Rev. Vaccines 11 (1) (2012) 17–25.
[25] P. Auewarakul et al., Antibody responses after dose-sparing intradermal influenza vaccination, Vaccine 25 (4) (2007) 659–663.
[26] S. Squarcione et al., Comparison of the reactogenicity and immunogenicity of a split and a subunit-adjuvanted influenza vaccine in elderly subjects, Vaccine 21 (11–12) (2003) 1268–1274.
[27] R. Baxter et al., Evaluation of the safety, reactogenicity and immunogenicity of FluBlok(R) trivalent recombinant baculovirus-expressed hemagglutinin influenza vaccine administered intramuscularly to healthy adults 50–64 years of age, Vaccine 29 (12) (2011) 2272–2278.
[28] J.C. King Jr. et al., Evaluation of the safety, reactogenicity and immunogenicity of FluBlok trivalent recombinant baculovirus-expressed hemagglutinin influenza vaccine administered intramuscularly to healthy children aged 6–59 months, Vaccine 27 (47) (2009) 6589–6594.
[29] C. Herzog et al., Eleven years of Inflexal V – a virosomal adjuvanted influenza vaccine, Vaccine 27 (33) (2009) 4381–4387.
[30] W.E.P. Beyer et al., Immunogenicity and safety of inactivated influenza vaccines in primed populations: a systematic literature review and meta-analysis, Vaccine 29 (34) (2011) 5785–5792.
[31] W.E. Beyer, A.M. Palache, A.D. Osterhaus, Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: a review and meta-analysis of the literature, Clin. Drug Investigat. 15 (1) (1998) 1–12.
[32] M.I. Barria et al., Localized mucosal response to intranasal live attenuated influenza vaccine in adults, J. Infect. Dis. 207 (1) (2013) 115–124.
[33] N.J. Carter, M.P. Curran, Live attenuated influenza vaccine (FluMist(R); Fluenz): a review of its use in the prevention of seasonal influenza in children and adults, Drugs 71 (12) (2011) 1591–1622.
[34] P.K. Tosh, T.G. Boyce, G.A. Poland, Flu myths: dispelling the myths associated with live attenuated influenza vaccine, Mayo Clin. Proc. 83 (1) (2008) 77–84.
[35] C.S. Ambrose, Levin, M.J., The rationale for quadrivalent influenza vaccines, (2164-554X (Electronic)).
[36] L. Manzoli et al., Meta-analysis of the immunogenicity and tolerability of pandemic influenza A 2009 (H1N1) vaccines, PLoS One 6 (9) (2011) e24384.
[37] J.K. Yin et al., Immunogenicity and safety of pandemic influenza A (H1N1) 2009 vaccine: systematic review and meta-analysis, Influenza Other Respir. Viruses 5 (5) (2011) 299–305.
[38] G.L. Chen, K. Subbarao, Live attenuated vaccines for pandemic influenza, Curr. Top. Microbiol. Immunol. 333 (2009) 109–132.
[39] J.-P. Amorij et al., Towards tailored vaccine delivery: needs, challenges and perspectives, J. Control. Release 161 (2) (2012) 363–376.
[40] J.P. Amorij et al., Development of stable influenza vaccine powder formulations: challenges and possibilities, Pharm. Res. 25 (6) (2008) 1256–1273.
[41] J. Steel et al., Influenza virus vaccine based on the conserved hemagglutinin stalk domain, MBio 1 (1) (2010).
[42] A. Schneemann et al., A virus-like particle that elicits cross-reactive antibodies to the conserved stem of influenza virus hemagglutinin, J. Virol. 86 (21) (2012) 11686–11697.
[43] M. Kanekiyo et al., Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies, Nature 499 (7456) (2013) 102–106.
[44] B.S. Shim et al., Sublingual immunization with M2-based vaccine induces broad protective immunity against influenza, PLoS One 6 (11) (2011) e27953.
[45] J. Denis et al., Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform, Vaccine 26 (27–28) (2008) 3395–3403.
[46] N.V. Petukhova et al., Immunogenicity and protective efficacy of candidate universal influenza A nanovaccines produced in plants by Tobacco mosaic virus-based vectors, Curr. Pharm. Des. 19 (31) (2013) 5587–5600.
[47] A.F. Altenburg et al., Modified vaccinia virus Ankara (MVA) as production platform for vaccines against influenza and other viral respiratory diseases, Viruses 6 (7) (2014) 2735–2761.
[48] R.D. Antrobus et al., A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years, PLoS One 7 (10) (2012) e48322.
[49] M. Gurwith et al., Safety and immunogenicity of an oral, replicating adenovirus serotype 4 vector vaccine for H5N1 influenza: a randomised, double-blind, placebo-controlled, phase 1 study, Lancet Infect. Dis. 13 (3) (2013) 238–250.
[50] R.D. Antrobus et al., Clinical assessment of a novel recombinant simian adenovirus ChAdOx1 as a vectored vaccine expressing conserved Influenza A antigens, Mol. Ther. 22 (3) (2014) 668–674.

[51] R. Calcedo et al., Host immune responses to chronic adenovirus infections in human and nonhuman primates, J. Virol. 83 (6) (2009) 2623–2631.
[52] S. Rosendahl Huber et al., T cell responses to viral infections – opportunities for peptide vaccination, Front. Immunol. 5 (2014) 171.
[53] A. Ninomiya et al., Intranasal administration of a synthetic peptide vaccine encapsulated in liposome together with an anti-CD40 antibody induces protective immunity against influenza A virus in mice, Vaccine 20 (25–26) (2002) 3123–3129.
[54] M. Matsui et al., A CTL-based liposomal vaccine capable of inducing protection against heterosubtypic influenza viruses in HLA-A*0201 transgenic mice, Biochem. Biophys. Res. Commun. 391 (3) (2010) 1494–1499.
[55] T. Ichihashi et al., Cross-protective peptide vaccine against influenza A viruses developed in HLA-A*2402 human immunity model, PLoS One 6 (9) (2011) e24626.
[56] M. Taneichi et al., Liposome-coupled peptides induce long-lived memory CD8 T cells without CD4 T cells, PLoS One 5 (11) (2010) e15091.
[57] E.B. Day et al., The context of epitope presentation can influence functional quality of recalled influenza A virus-specific memory CD8+ T cells, J. Immunol. 179 (4) (2007) 2187–2194.
[58] T. Ichihashi et al., Emulsified phosphatidylserine, simple and effective peptide carrier for induction of potent epitope-specific T cell responses, PLoS One 8 (3) (2013) e60068.
[59] A. Arkema et al., Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes, Vaccine 18 (14) (2000) 1327–1333.
[60] P.C. Soema et al., Influenza T cell epitope-loaded virosomes adjuvanted with CpG as a potential influenza vaccine, Pharm. Res. 32 (4) (2015) 1505–1515.
[61] J.N. Francis et al., A novel peptide-based pan-influenza A vaccine: a double blind, randomised clinical trial of immunogenicity and safety, Vaccine (2014).
[62] J.H. Ma et al., An M2e-based synthetic peptide vaccine for influenza A virus confers heterosubtypic protection from lethal virus challenge, Virol. J. 10 (2013) 227.
[63] J. Atsmon et al., Safety and immunogenicity of multimeric-001 – a novel universal influenza vaccine, J. Clin. Immunol. 32 (3) (2012) 595–603.
[64] J. Atsmon et al., Priming by a novel universal influenza vaccine (Multimeric-001) – a gateway for improving immune response in the elderly population, Vaccine 32 (44) (2014) 5816–5823.
[65] T.T. Wang et al., Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes, Proc. Natl. Acad. Sci. USA 107 (44) (2010) 18979–18984.
[66] S. Jones et al., DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial, Vaccine 27 (18) (2009) 2506–2512.
[67] J.E. Ledgerwood et al., DNA priming and influenza vaccine immunogenicity: two phase 1 open label randomised clinical trials, Lancet Infect. Dis. 11 (12) (2011) 916–924.
[68] C.J. Wei et al., Induction of broadly neutralizing H1N1 influenza antibodies by vaccination, Science 329 (5995) (2010) 1060–1064.
[69] C.-J. Wei et al., Elicitation of broadly neutralizing influenza antibodies in animals with previous influenza exposure, Sci. Transl. Med. 4 (147) (2012) 147ra114.
[70] L.R. Smith et al., Phase 1 clinical trials of the safety and immunogenicity of adjuvanted plasmid DNA vaccines encoding influenza A virus H5 hemagglutinin, Vaccine 28 (13) (2010) 2565–2572.
[71] B. Petsch et al., Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection, Nat. Biotechnol. 30 (12) (2012) 1210–1216.
[72] A. Hekele et al., Rapidly produced SAM(®) vaccine against H7N9 influenza is immunogenic in mice, Emerg. Microbes Infect. 2 (8) (2013) e52.
[73] S. Calabro et al., The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect, Vaccine 31 (33) (2013) 3363–3369.
[74] D.I. Bernstein et al., Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults, J. Infect. Dis. 197 (5) (2008) 667–675.
[75] P.G. Van Buynder et al., The comparative effectiveness of adjuvanted and unadjuvanted trivalent inactivated influenza vaccine (TIV) in the elderly, Vaccine 31 (51) (2013) 6122–6128.
[76] G. Della Cioppa et al., Trivalent and quadrivalent MF59((R))-adjuvanted influenza vaccine in young children: a dose- and schedule-finding study, Vaccine 29 (47) (2011) 8696–8704.
[77] T. Vesikari et al., Enhanced immunogenicity of seasonal influenza vaccines in young children using MF59 adjuvant, Pediatr. Infect. Dis. J. 28 (7) (2009) 563–571.
[78] G. Galli et al., Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine, Proc. Natl. Acad. Sci. USA 106 (19) (2009) 7962–7967.
[79] T.F. Schwarz et al., Single dose vaccination with AS03-adjuvanted H5N1 vaccines in a randomized trial induces strong and broad immune responsiveness to booster vaccination in adults, Vaccine 27 (45) (2009) 6284–6290.
[80] J.E. McElhaney et al., AS03-adjuvanted versus non-adjuvanted inactivated trivalent influenza vaccine against seasonal influenza in elderly people: a phase 3 randomised trial, Lancet Infect. Dis. 13 (6) (2013) 485–496.
[81] C. Durier et al., Long-term immunogenicity of two doses of 2009 A/H1N1v vaccine with and without AS03(A) adjuvant in HIV-1-infected adults, Aids 27 (1) (2013) 87–93.
[82] T. Fairhead et al., Poor seroprotection but allosensitization after adjuvanted pandemic influenza H1N1 vaccine in kidney transplant recipients, Transpl. Infect. Dis. 14 (6) (2012) 575–583.
[83] D. Villa et al., Evaluation of 2009 pandemic H1N1 influenza vaccination in adults with lymphoid malignancies receiving chemotherapy or following autologous stem cell transplant, Leuk. Lymphoma 54 (7) (2013) 1387–1395.
[84] I. Mbawuike, Y. Zang, R.B. Couch, Humoral and cell-mediated immune responses of humans to inactivated influenza vaccine with or without QS21 adjuvant, Vaccine 25 (17) (2007) 3263–3269.
[85] G.F. Kersten et al., On the structure of immune-stimulating saponin-lipid complexes (iscoms), Biochim. Biophys. Acta 1062 (2) (1991) 165–171.
[86] G.F. Rimmelzwaan et al., A randomized, double blind study in young healthy adults comparing cell mediated and humoral immune responses induced by influenza ISCOM vaccines and conventional vaccines, Vaccine 19 (9–10) (2000) 1180–1187.
[87] F.A. Ennis et al., Augmentation of human influenza A virus-specific cytotoxic T lymphocyte memory by influenza vaccine and adjuvanted carriers (ISCOMS), Virology 259 (2) (1999) 256–261.
[88] R.J. Cox et al., Evaluation of a virosomal H5N1 vaccine formulated with Matrix M adjuvant in a phase I clinical trial, Vaccine 29 (45) (2011) 8049–8059.
[89] L.F. Fries, G.E. Smith, G.M. Glenn, A recombinant virus like particle influenza A (H7N9) vaccine, N. Engl. J. Med. 369 (26) (2013) 2564–2566.
[90] J.J. Treanor et al., Safety and immunogenicity of a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults, Vaccine 28 (52) (2010) 8268–8274.
[91] D.N. Taylor et al., Development of VAX128, a recombinant hemagglutinin (HA) influenza-flagellin fusion vaccine with improved safety and immune response, Vaccine 30 (39) (2012) 5761–5769.
[92] G.M. Glenn et al., Safety and immunogenicity of an influenza vaccine A/H5N1 (A/Vietnam/1194/2004) when coadministered with a heat-labile enterotoxin (LT) adjuvant patch, Vaccine 27 (Suppl. 6) (2009) G60–G66.
[93] R.C. Layton et al., Delta inulin polysaccharide adjuvant enhances the ability of split-virion H5N1 vaccine to protect against lethal challenge in ferrets, Vaccine 29 (37) (2011) 6242–6251.
[94] D.L. Gordon et al., Randomized clinical trial of immunogenicity and safety of a recombinant H1N1/2009 pandemic influenza vaccine containing Advax polysaccharide adjuvant, Vaccine 30 (36) (2012) 5407–5416.
[95] L. Wijnans et al., The incidence of narcolepsy in Europe: before, during, and after the influenza A(H1N1)pdm09 pandemic and vaccination campaigns, Vaccine 31 (8) (2013) 1246–1254.
[96] M. Partinen et al., Increased incidence and clinical picture of childhood narcolepsy following the 2009 H1N1 pandemic vaccination Campaign in Finland, PLoS One 7 (3) (2012) e33723.
[97] O. Vaarala et al., Antigenic differences between AS03 adjuvanted influenza A (H1N1) pandemic vaccines: implications for Pandemrix-associated narcolepsy risk, PLoS One 9 (12) (2014) e114361.
[98] L. Jacob et al., Comparison of Pandemrix and Arepanrix, two pH1N1 AS03-adjuvanted vaccines differentially associated with narcolepsy development, Brain Behav. Immun. (2014).
[99] J.P. Amorij et al., Rational design of an influenza subunit vaccine powder with sugar glass technology: preventing conformational changes of haemagglutinin during freezing and freeze-drying, Vaccine 25 (35) (2007) 6447–6457.
[100] J. de Jonge et al., Inulin sugar glasses preserve the structural integrity and biological activity of influenza virosomes during freeze-drying and storage, Eur. J. Pharm. Sci. 32 (1) (2007) 33–44.
[101] F. Geeraedts et al., Preservation of the immunogenicity of dry-powder influenza H5N1 whole inactivated virus vaccine at elevated storage temperatures, AAPS J. 12 (2) (2010) 215–222.
[102] P.C. Soema et al., Solid bioneedle-delivered influenza vaccines are highly thermostable and induce both humoral and cellular immune responses, PLoS One 9 (3) (2014) e92806.
[103] R.D. Antrobus et al., Coadministration of seasonal influenza vaccine and MVA-NP+M1 simultaneously achieves potent humoral and cell-mediated responses, Mol. Ther. 22 (1) (2014) 233–238.
[104] M.L. Perdue et al., The future of cell culture-based influenza vaccine production, Expert Rev. Vaccines 10 (8) (2011) 1183–1194.
[105] D.M. Skowronski et al., Low 2012–2013 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not antigenic drift in circulating viruses, PLoS One 9 (3) (2014) e92153.
[106] J.A. Tree et al., Comparison of large-scale mammalian cell culture systems with egg culture for the production of influenza virus A vaccine strains, Vaccine 19 (25–26) (2001) 3444–3450.
[107] A. Doroshenko, S.A. Halperin, Trivalent MDCK cell culture-derived influenza vaccine Optaflu (Novartis Vaccines), Expert Rev. Vaccines 8 (6) (2009) 679–688.
[108] M.S. Lee, A.Y. Hu, A cell-based backup to speed up pandemic influenza vaccine production, Trends Microbiol. 20 (3) (2012) 103–105.
[109] C.Y. Chan, P.A. Tambyah, Preflucel(R): a Vero-cell culture-derived trivalent influenza vaccine, Expert Rev. Vaccines 11 (7) (2012) 759–773.
[110] G.L. Plosker, A/H5N1 prepandemic influenza vaccine (whole virion, vero cell-derived, inactivated) [Vepacel(R)], Drugs 72 (11) (2012) 1543–1557.
[111] Y. Genzel et al., MDCK and Vero cells for influenza virus vaccine production: a one-to-one comparison up to lab-scale bioreactor cultivation, Appl. Microbiol. Biotechnol. 88 (2) (2010) 461–475.

[112] M.G. Pau et al., The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines, Vaccine 19 (17–19) (2001) 2716–2721.
[113] R.J. Cox et al., A phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine, Vaccine 27 (13) (2009) 1889–1897.
[114] A. Le Ru et al., Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing, Vaccine 28 (21) (2010) 3661–3671.
[115] Y. Genzel et al., CAP, a new human suspension cell line for influenza virus production, Appl. Microbiol. Biotechnol. 97 (1) (2013) 111–122.
[116] I.W. Li et al., Differential susceptibility of different cell lines to swine-origin influenza A H1N1, seasonal human influenza A H1N1, and avian influenza A H5N1 viruses, J. Clin. Virol. 46 (4) (2009) 325–330.
[117] R.D. Hess et al., Regulatory, biosafety and safety challenges for novel cells as substrates for human vaccines, Vaccine 30 (17) (2012) 2715–2727.
[118] M.M.J. Cox, Recombinant protein vaccines produced in insect cells, Vaccine 30 (10) (2012) 1759–1766.
[119] J. Hendriks et al., An international technology platform for influenza vaccines, Vaccine 29 (Suppl. 1) (2011) A8–A11.
[120] C. Babin, N. Majeau, D. Leclerc, Engineering of papaya mosaic virus (PapMV) nanoparticles with a CTL epitope derived from influenza NP, J. Nanobiotechnol. 11 (2013) 10.
[121] Y. Cai, S. Rodriguez, H. Hebel, DNA vaccine manufacture: scale and quality, Expert Rev. Vaccines 8 (9) (2009) 1277–1291.
[122] B.L. Bray, Large-scale manufacture of peptide therapeutics by chemical synthesis, Nat. Rev. Drug Discov. 2 (7) (2003) 587–593.
[123] R. Bodewes et al., A single immunization with CoVaccine HT-adjuvanted H5N1 influenza virus vaccine induces protective cellular and humoral immune responses in ferrets, J. Virol. 84 (16) (2010) 7943–7952.
[124] S.A. Kopecky-Bromberg et al., Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine, Vaccine 27 (28) (2009) 3766–3774.
[125] J.T. Hung et al., Potent adjuvant effects of novel NKT stimulatory glycolipids on hemagglutinin based DNA vaccine for H5N1 influenza virus, Antiviral Res. 107 (2014) 110–118.
[126] O. Even-Or et al., A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS). II. Studies in mice and ferrets and mechanism of adjuvanticity, Vaccine 29 (13) (2011) 2474–2486.
[127] C.J. Martel et al., CAF01 potentiates immune responses and efficacy of an inactivated influenza vaccine in ferrets, PLoS One 6 (8) (2011) e22891.
[128] L.R. Smith et al., Preclinical evaluation of Vaxfectin-adjuvanted Vero cell-derived seasonal split and pandemic whole virus influenza vaccines, Hum. Vaccines Immunother. 9 (6) (2013) 1333–1345.
[129] D.G. Eliasson et al., CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine, Vaccine 26 (9) (2008) 1243–1252.
[130] C.B. Turley et al., Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults, Vaccine 29 (32) (2011) 5145–5152.
[131] T. Khan et al., Membrane-bound IL-12 and IL-23 serve as potent mucosal adjuvants when co-presented on whole inactivated influenza vaccines, Virol. J. 11 (2014) 78.
[132] P.T. Loudon et al., GM-CSF increases mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into the epidermis of non-human primates, PLoS One 5 (6) (2010) e11021.
[133] E. Proietti et al., Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model, J. Immunol. 169 (1) (2002) 375–383.
[134] J.J. Treanor et al., Evaluation of safety and immunogenicity of recombinant influenza hemagglutinin (H5/Indonesia/05/2005) formulated with and without a stable oil-in-water emulsion containing glucopyranosyl-lipid A (SE+GLA) adjuvant, Vaccine 31 (48) (2013) 5760–5765.
[135] C.L. Cooper et al., Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine, Vaccine 22 (23–24) (2004) 3136–3143.
[136] T. Ichinohe et al., PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants, Vaccine 27 (45) (2009) 6276–6279.
[137] J.V. Perez-Giron et al., Mucosal polyinosinic–polycytidylic acid improves protection elicited by replicating influenza vaccines via enhanced dendritic cell function and T cell immunity, J. Immunol. 193 (3) (2014) 1324–1332.
[138] K. Riedl et al., The novel adjuvant IC31 strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice, Vaccine 26 (27–28) (2008) 3461–3468.
[139] C. Brignone et al., IMP321 (sLAG-3) safety and T cell response potentiation using an influenza vaccine as a model antigen: a single-blind phase I study, Vaccine 25 (24) (2007) 4641–4650.
[140] A.J. Mann et al., Intranasal H5N1 vaccines, adjuvanted with chitosan derivatives, protect ferrets against highly pathogenic influenza intranasal and intratracheal challenge, PLoS One 9 (5) (2014) e93761.
[141] C. Sawaengsak et al., Intranasal chitosan-DNA vaccines that protect across influenza virus subtypes, Int. J. Pharm. 473 (1–2) (2014) 113–125.
[142] A.K. Andrianov et al., PCPP-formulated H5N1 influenza vaccine displays improved stability and dose-sparing effect in lethal challenge studies, J. Pharm. Sci. 100 (4) (2011) 1436–1443.
[143] Y. Honda-Okubo, F. Saade, N. Petrovsky, Advax, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses, Vaccine 30 (36) (2012) 5373–5381.
[144] O. Pleguezuelos et al., Synthetic Influenza vaccine (FLU-v) stimulates cell mediated immunity in a double-blind, randomised, placebo-controlled Phase I trial, Vaccine 30 (31) (2012) 4655–4660.
[145] T.K. Berthoud et al., Potent CD8+ T cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1, Clin. Infect. Dis. 52 (1) (2011) 1–7.