Infection (2014) 42:251–262
DOI 10.1007/s15010-013-0546-4

REVIEW

Development of universal influenza vaccines based on influenza virus M and NP genes

M. Zheng · J. Luo · Z. Chen

Received: 25 July 2013 / Accepted: 15 October 2013 / Published online: 1 November 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract
Purpose Vaccination is the safest and most effective measure against influenza virus infections. However, traditional influenza vaccines cannot respond effectively to an unforeseen epidemic or pandemic caused by a virus with antigenic drifts or antigenic shifts. Therefore, developing a universal influenza vaccine (UIV) that induces broad-spectrum and long-term immunity has become a major trend in influenza vaccine research and development.
Methods This article reviews the development of UIVs based on these conserved influenza virus proteins.
Results and Conclusion The matrix protein (M1, M2) and nucleoprotein (NP) of influenza viruses have highly conserved sequences, and they become the major target antigens of current UIV studies.

Keywords Influenza virus · Universal vaccine · Matrix protein · Nucleoprotein · Broad spectrum

M. Zheng and J. Luo contributed equally.

M. Zheng · J. Luo · Z. Chen (✉)
Shanghai Institute of Biological Products,
Shanghai 200052, China
e-mail: chenze2005@hotmail.com; chenze2005@263.net

Z. Chen
State Key Laboratory of Virology, Wuhan Institute of Virology,
Chinese Academy of Sciences, Wuhan 430071, Hubei, China

Z. Chen
College of Life Sciences, Hunan Normal University,
Changsha 410081, Hunan, China

Introduction

Influenza is a severe acute respiratory tract disease caused by influenza virus infection, and vaccination is the most economical and effective means against influenza virus infections. During the early development of influenza vaccine, the main immunization strategy involved the injection of an inactivated whole-virus vaccine. However, because these vaccines could result in more adverse effects through vaccination, they are rarely used for the current seasonal influenza epidemic [1]. Influenza vaccines used in the present human populations are primarily 'split-virus' vaccines, which are produced months in advance of an influenza season in embryonated eggs or cells using vaccine strains carrying particular surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) [2, 3]. However, HA and NA are prone to antigenic drift and it is difficult to ensure that antigens of vaccine strains match those in the strains circulating in the subsequent season. Therefore, developing an effective and long-term universal influenza vaccine (UIV) has become a research priority. Since influenza A virus, which often causes flu epidemics or pandemics, has a broader host range and superior variability, thus, UIV development is currently focused mainly on influenza A virus.

Antigens with highly conserved antigenic epitopes are the basics of UIVs. So far, the matrix protein M, nucleoprotein NP, and conserved epitopes of other influenza proteins have been explored. These influenza virus proteins have been shown to induce adequate antibody levels after vaccination, similar to that induced by whole-virus vaccines [1]. Moreover, recombinant protein vaccines have excellent safety in humans because they cause slighter side effects and fewer local reactions than inactivated whole-virus vaccines [1]. For example, VAX125, an influenza

vaccine consisting of the globular head of the HA1 domain of the A/Solomon Islands/3/2006 (H1N1) influenza virus, is well tolerated and it causes no serious adverse events [4]. Furthermore, in the event of a newly emerging pandemic influenza virus, recombinant vaccines are quick and easy to produce: an antigenic protein of the new virus strain could be rapidly expressed and purified with molecular biology techniques, and when proportionally amplified in a bioreactor, numerous doses of a vaccine could be manufactured within a short period of time [5, 6].

However, if a new pandemic influenza virus breaks out, a single vaccination with a recombinant protein vaccine seems to be insufficient, and two or more doses will be required [7]. In addition, recombinant protein vaccines require adjuvants to improve their ability of eliciting an effective immune response [8–10]. The use of a safe adjuvant with a vaccine will enhance immune response and reduce the antigen dose required. For example, adding Al or MF59 adjuvant into a vaccine administered by intramuscular (i.m.) injection could induce humoral immunity and Th2-biased immune responses, and adding mucosa adjuvants, such as chitosan, lipids, and *Escherichia coli* heat-labile toxin (LT), into a vaccine given via the intranasal (i.n.) route could induce a mixed Th1/Th2 type immune response [11].

Therefore, the development of a universal influenza vaccine capable of inducing broad-spectrum and long-term immunity has become an important trend in the current research and development efforts on influenza vaccines. In the present article, we review the progress of UIV research and development based on the conserved influenza virus M and NP proteins.

Characteristics of influenza virus M and NP proteins

The matrix protein (M) was encoded by segment 7 of influenza viral RNA, which encodes at least two proteins, M1 and M2. M1, the main structural protein, is highly conserved [12]. Therefore, the M1 protein of influenza A virus might be a suitable target as UIVs. In recent years, epitopes of the M1 protein have been intensively investigated. M1 peptides derived from proteinase hydrolysis were analyzed for immune reactivity to monoclonal antibodies (MAbs). The N-terminus 1/3 region binds to the viral lipoid layer, and the C-terminus 2/3 region binds to ribonucleoprotein (RNP). At least one B cell epitope was found in each of the amino acid positions 8–89, 80–109, and 129–164 of the A/WSN/33 (H1N1) influenza virus, and at least two B cell epitopes and two RNA binding sites were found in amino acid positions 89–141 of the A/WSN/33 (H1N1) influenza virus [13]. The carboxyl terminal polypeptide QAYQKRMGVQMQRFK constitutes a $CD4^+$ T cell epitope [14]; amino acids in positions 58–66 (GILGFVFTL) of the A/PR8/34 (H1N1) influenza virus constitute a $CD8^+$ cytotoxic T cell epitope [15]; and amino acids in positions 62–70 (FVFTLTVPS) constitute a $CD4^+$ T cell epitope [16]. A recombinant vaccinia virus vaccine containing multiple T epitopes of M1, NS1, NP, PB1, and PA proteins of influenza virus was found to increase the number of influenza virus-specific IFN-γ secreting spleen cells, and this virus vaccine also protected hosts against influenza virus infection [17].

M2 is a multifunction protein; it could serve as an ion channel, and its changes directly impact the virus replication process [18]. The N-terminus 24aa of the M2 extracellular domain (M2e) is highly conserved. In particular, the N-terminal amino acid positions 2–9 epitope (SLLTEVET) of the A/WSN/33 (H1N1) influenza virus has no mutations in all influenza A virus strains that have been identified [19]. Thus, M2e has attracted great attention in research. M2-related immune response and anti-influenza immunity was studied as early as 1988 when Zebedee et al. [20] first demonstrated in vitro that MAbs against the M2 protein could inhibit influenza virus replication in cells. Subsequently, Treanor et al. [21] demonstrated, via an antibody transfer experiment, that the M2-specific MAb could inhibit the replication of influenza virus in mice. The relation between M2- or M2e-specific immune response and protective immunity against influenza virus has been confirmed by several further studies [22, 23].

The influenza virus NP is highly conserved and has low mutation rates throughout evolution. NP proteins of influenza viruses of the same type have over 90 % amino acid homology [24]. NP is one of the main determinants of species specificity, and it show a lower degree of homology between type A and type B influenza viruses, despite them both serving the same function in RNP. Following infection by an influenza virus, NP is the main antigen recognized by host cytotoxic T lymphocytes (CTL). Through recognizing NP antigen peptides presented by MHC-I molecules on the surface of virus-infected cells, CTL then destroys the virus-infected cells, thereby eliminating the virus [25]. Cross-reactive CTL aimed at NP is a key factor in controlling influenza virus infection. In vitro experiments by Gschoesser et al. [26] revealed that, in the presence of IL-2, the recombinant influenza virus NP could induce the proliferation of $CD4^+$ and $CD8^+$ T cells.

Development of UIVs

Development of M-based influenza vaccines

The matrix proteins M1 and M2 have maintained highly stable structures over the evolution of the influenza virus. Therefore, some research groups have focused their UIV

studies on the M genes. Okuda et al. [27] constructed a recombinant eukaryotic expression plasmid containing the M1 and M2 genes of the influenza A virus (A/PR/8/34), and following i.m. or i.n. administration to mice, specific antibodies without neutralizing activity were detected in the sera of mice. However, after challenge with the homologous virus strain A/PR/8/34 and the heterologous virus strain A/WSN/33, the immunized mice were protected against the two viruses, indicating that matrix proteins have good immunogenicity, and could induce cross-protective immunity. We also found in a previous study that i.m. administration plus electroporation of an M1 DNA vaccine into mice provided partial protection against infection of homologous virus [28]. Some investigators have demonstrated that DNA vaccines encoding M1 or M2 proteins do not prevent infection or symptoms of disease, but it could protect against a lethal influenza challenge. M1- or M2-based vaccines were proposed to have the ability to induce antibodies or T cell responses which limit viral replication and spread by interfering with virus budding. In addition, antibodies that bind before budding of the virions could hinder them from infecting cells or could enhance the uptake by phagocytic cells via the Fc receptor [29].

For quite a long time, only DNA vaccine studies have been performed for M genes. No studies on M1 subunit vaccines have been published until our laboratory first reported that immunization with recombined M1 protein as a subunit vaccine could induce immunity against homologous virus and provide some level of protection [8]. We found that i.n. administration of M1 protein vaccine formulated with chitosan provided full protection in mice against homologous virus and partial protection against heterologous virus, respectively. The study indicated that the cross-protection might be due to cell-mediated immunity specific to highly conserved M protein, as well as high levels of anti-M IgA in respiratory mucosa. Secretory IgA could bind to the influenza virus during transcytosis in the infected epithelial cells. IgA antibodies may also inhibit intracellular replication or virus assembly of the virus by interfering with a function of the newly synthesized viral protein M1 [8]. Therefore, the M1 protein is a candidate antigen for a broad-spectrum influenza vaccine (Table 1).

As a highly conserved protein, M2 has long been considered a main candidate antigen for an ideal UIV that is capable of inducing cross-protective immunity against different influenza virus variants. Current multiple forms of M2-based influenza vaccines have been validated in animal models, including recombinant protein vaccines, DNA vaccines, and viral-vectored vaccines [2, 30]. Compared with the full-length M2 protein, the M2e peptide has weaker immunogenicity and, therefore, when M2e is chosen as the antigen, some modifications are required for vaccine design. Meanwhile, we also reported that immunization with 15 µg sM2 (M2 without transmembrane domain) protein with adjuvant chitosan could completely protect mice against the homologous virus, and also offered 90 and 30 % protection against heterologous H1N1 and H5N1 viruses, respectively [9]. Similar to our M1 study, this cross-protection was found to be associated with cell-mediated immunity induced by the highly conserved M2 protein. Moreover, such protection might also be associated with high anti-M2 IgA titer in respiratory mucosa. Furthermore, M2-specific MAbs could inhibit the replication of influenza virus in mice [21] and prevent virus release from infected cells.

An overview of M2- or M2e-based UIVs reported thus far is given in Table 2 [2, 9, 23, 30–46]. These include a baculovirus-expressed influenza M2 protein, a fusion protein of M2e (23 aa) and core antigen of hepatitis B virus (HBc) (5–183aa) M2e-HBc, and various other M2e fusion proteins or conjugates. M2-based polypeptide vaccines were explored in earlier studies. Although such a vaccine has high purity and stability, its antigenicity is poor because the polypeptide is expressed in linear form, which lacks the ability to fold [9]. Consequently, the main challenge faced by scientists is to convert the usually non-immunogenic or weakly immunogenic M2e peptide into a highly immunogenic vaccine antigen. Genetic engineering or chemical conjugation methods were used to construct M2e into a suitable vector, such as virus-like particle, HBc, and various chemical conjugation groups [glutathione S-transferase (GST), keyhole hemocyanin (KLH), outer membrane protein complex (OMPC), multiple antigenic peptide (MAP) and liposome, and so on]. For example, immunization using M2e peptide attached to an HBc subunit provides higher immunogenicity, and M2e-HBc can be efficiently expressed in *E. coli*. [3]. Animals immunized with these M2 proteins via the intraperitoneal (i.p.) or i.n. route resisted the challenge by the homologous virus strain, and were also cross-protected against a challenge by heterologous viruses [2].

Development of NP-based influenza vaccines

The NP of influenza A virus is a UIV component preferred by researchers. Numerous studies have found that NP-based DNA vaccines and viral-vectored vaccines could not only protect against homologous influenza virus but also provide partial protection against challenges by heterologous viruses. We found that mice immunized with NP DNA alone at least three times showed a 60–80 % survival rate [28]. Our results suggested that NP-based vaccines could induce antibodies or T cell responses which limit viral replication and spread.

NP vaccines based on viral vectors have been extensively reported. These NP vaccines include recombinant

Table 1 Pre-clinical study of M- or NP-based universal influenza vaccine (Chen et al. [8–10, 28])

Target antigen	Dose (μg)	Animal model	Route of immunization	Adjuvant	Immunization times	Specific IFN-γ secreted from splenocytes (stimulator)	Homologous challenge virus strain (protection)	Heterologous challenge virus strain subtype (protection)	Ref. (year)
M1 (H9N2) protein	100	Mouse	i.n.	Chitosan	3	++++ (M1 peptide)	H9N2 (100 %)	H1N1 (70 %) H5N1 (30 %)	[8] (2010)
				–		+++ (M1 peptide)	H9N2 (50 %)	–	
			i.p.	Chitosan		–	H9N2 (30 %)	–	
				–		–	H9N2 (20 %)	–	
sM2 (H9N2) protein	15	Mouse	i.n.	Chitosan	3	–	H9N2 (100 %)	H1N1 (90 %) H5N1 (30 %)	[9] (2010)
				–		–	H9N2 (50 %)	–	
NP (H1N1) protein	100	Mouse	i.n.	CTB*	3	+++ (NP peptide)	H1N1 (100 %)	H9N2 (90 %) H5N1 (100 %)	[10] (2010)
				–		++ (NP peptide)	H1N1 (40 %)	–	
			i.p.	CTB*		–	H1N1 (0 %)	–	
M1 DNA	50	Mouse	i.m.	–	3	++ (M1 peptide)	H5N1 (0/20)	–	[28] (2009)
					4	++ (M1 peptide)	H5N1 (3/20)		
					5	+++ (M1 peptide)	H5N1 (5/20)		
NP DNA	50	Mouse	i.m.	–	3	+++ (NP peptide)	H5N1 (12/20)	–	
					4	+++ (NP peptide)	H5N1 (12/20)		
					5	++++ (NP peptide)	H5N1 (17/20)		
M1+NP DNA	50 + 50	Mouse	i.m.	–	3	+++ (M1 peptide) +++ (NP peptide)	H5N1 (14/20)	–	
					4	+++ (M1 peptide) ++++ (NP peptide)	H5N1 (17/20)		
					5	+++ (M1 peptide) +++++ (NP peptide)	H5N1 (19/20)		

–, none

+, specific IFN-γ secreted from splenocytes, 0–50 SFC/10^6 cells

++, specific IFN-γ secreted from splenocytes, 51–100 SFC/10^6 cells

+++, specific IFN-γ secreted from splenocytes, 101–250 SFC/10^6 cells

++++, specific IFN-γ secreted from splenocytes, 251–500 SFC/10^6 cells

+++++, specific IFN-γ secreted from splenocytes, >501 SFC/10^6 cells

poxvirus, vaccinia virus, or adenovirus vector, as well as NP-based DNA vaccines. However, these vaccines show weak immunogenicity upon vaccination. As early as 1987, Wraith et al. [47] purified the NP protein of influenza virus X31 (H3N2) and immunized mice by i.p. injection. They found that the vaccine could protect 75 % of the immunized mice against a lethal challenge by heterosubtypic influenza virus A/PR/8/34(H1N1), and such a protection

Table 2 M2- or M2e-based universal influenza vaccines in research

Name of candidate vaccine (vaccine type)	Target antigen	Vector/molecular adjuvant	Form of fusion	Animal model	Route of vaccination	Adjuvant	Subtype of challenge virus stain	Ref. (year)
M2e-HBc (protein) M2e-HBc-particle 1818 (VLP)	M2e	HBcAg	Gene recombination	Mouse	i.p. i.n.	No adjuvant CTA1-DD	H3N2	[23, 31, 32] (2006)
GST-M2e (protein)	M2e	GST	Chemical coupling and gene recombination	Mouse	i.p. s.c.	–	H1N1 H2N2 H3N2	[33] (1999)
M2e-KLH (protein) M2e-OMPC (protein)	M2e	Keyhole hemocyanin and group B Nm outer membrane protein complex	Chemical coupling	Mouse Ferret Macaque	s.c. i.m.	QS-21	H1N1 H3N1	[34] (2004)
M2e-MAP (protein)	M2e	–	Chemical coupling	Mouse	i.n.	CpG-ODN CT	H3N2	[35] (2003)
Liposomal-M2eA (protein)	M2e	Liposome	Chemical coupling	Mouse	s.c. i.n. i.p.	–	H1N1, H5N1, H6N2, H9N2	[36] (2006)
M2-DNA (DNA) M2-Ad (VLP)	M2	–	–	Mouse	i.p.	–	H1N1 H5N1	[30] (2007)
PapMV-CP-M2e (VLP)	M2e	Papaya mosaic virus coat protein	Gene recombination	Mouse	s.c.	Al adjuvant	H1N1	[37] (2008)
CTA1-M2e-DD (protein) CTA1-3M2e-DD(protein)	M2e	CTA1-DD	Gene recombination	Mouse	i.n.	–	H3N2	[38] (2008)
M2e-tGCN4 (protein)	M2e	CN4 leucine zipper	Gene recombination	Mouse	i.p. i.n.	Trehalose + monophosphoryl lipid A adjuvant; alumina gel; CTA1-DD	H3N2	[39] (2008)
M2-HPV VLP (VLP)	M2e	HPV L1 protein	Chemical coupling	Mouse	i.m.	Al adjuvant	H1N1 H3N2	[40] (2006)
Qbeta-VLP-M2 (VLP)	M2	Qβ phage	Gene recombination	Mouse	i.n.	–	H1N1	[41] (2008)
STF2.4×M2e (protein)	M2e	Flagellin	Gene recombination	Mouse	s.c. i.n. i.m.	–	H1N1	[42] (2008)
LBM2eHBc+ (protein)	M2e	HBcAg major immunodominant region	Gene recombination	Mouse	i.n.	LTB (fusion expression)	–	[43] (2009)

Table 2 continued

Name of candidate vaccine (vaccine type)	Target antigen	Vector/molecular adjuvant	Form of fusion	Animal model	Route of vaccination	Adjuvant	Subtype of challenge virus stain	Ref. (year)
RASV (VLP)	M2e	Attenuated *Salmonella*	Gene recombination	Mouse	oral i.n.	–	H1N1	[44] (2010)
H5N1-M2e-MAP (protein)	M2e	–	Chemical coupling	Mouse	s.c. i.m. i.p. i.n.	Alum adjuvant	H5N1 H1N1	[45] (2010)
SM2 (protein)	M2 without transmembrane domain	–	Gene recombination	Mouse	i.n.	Chitosan	H9N2 H1N1 H5N1	[9] (2010)
M2e-ASP-1 (protein) M2e3-ASP-1 (protein)	M2e	ASP (molecular adjuvant)	Gene recombination	Mouse	i.m.	–	Multiple H5N1 strains	[46] (2010)

–, none

may be attributed to CTL with cross-protective activity. Tamura et al. [48] further demonstrated that i.n. immunization with rNP protein of A/PR/8/34 expressed by insect cells could accelerate nasal clearance of the homologous virus, thereby promoting the recovery of infected mice. No related studies on influenza virus NP-based vaccines were reported for a long time following this investigation. In 2009, we reported that the nasal administration of NP in combination with CTB* [CTB containing a trace amount (0.1 %) of CT] could provide protection for mice with three immunizations [10], indicating that both mucosal immunity and cell-mediated immunity were induced, and possible acceleration of virus clearing from the nasal cavity and promotion of recovery from infection. These data indicate the potential of NP protein as a UIV component for controlling epidemics caused by emerging influenza viruses. Moreover, recent studies have suggested that IgG antibodies against the NP of influenza virus might have antiviral activity [49].

Researchers also attempted to develop a novel NP-based vaccine to improve the protective effect. NP-based vaccines of various forms have been developed, including DNA vaccines of different designs, genetically engineered recombinant vaccines based on insect baculovirus expression or prokaryotic expression, and virus-vectored vaccines [10, 50]. All these experimental vaccines could induce a certain level of immune response that is effective against heterologous subtypes, but the cross-protection is not yet satisfactory, and further optimization is required in the vaccine design or immunization strategy. Among these approaches, the DNA prime/adenovirus boost strategy greatly improves the cross-protective effect of NP-based vaccines [51]. UIVs with NP as the sole antigen have not been tested in clinical trials thus far.

UIVs based on a combination of multiple proteins or epitopes

Studies have found that influenza vaccines based on M1, M2, or NP proteins alone cannot provide adequate protection, and that immunizing mice with two or more influenza virus antigens is better than using a single antigen. Immunization with the M1, M2, or NP proteins does not elicit 'neutralizing' antibodies. Hence, the protective immunity is mainly mediated by M1-, M2-, or NP-specific CTL immune response and non-neutralizing functions of specific antibodies. An ideal vaccine can induce humoral, cellular, and even innate immune response. To fully mobilize the antiviral immunity of the body and further improve protection by UIVs, researchers tried to achieve the desired goals through the combined use of multiple target antigens.

Previous studies demonstrated that UIVs containing HA, M1, and/or NP genes from different influenza virus strains (H5N1, H3N2, H9N2, etc.) can provide effective cross-protection against a lethal challenge of different subtypes of influenza viruses [52, 53–56]. Donnelly et al. [57] constructed a fusion DNA vaccine containing HA, M1, and NP genes from different influenza virus strains. They found that this vaccine can effectively protect animals against a lethal challenge of an antigen shift mutant strain. Jeon et al. prepared a polypeptide containing three influenza A virus epitopes in tandem, $HA_{91–108}$ (B cell epitope), $NP_{55–69}$ (Th cell epitope), and $NP_{147–158}$ ($CD8^{+}$ T cell epitope), and fused with the flagellin protein of *Salmonella*. They found that mucosally immunized BALB/C mice with this vaccine show a protective effect against an influenza virus of a different subtype [58]. Zhou et al. [59] constructed several adenovirus-vectored vaccines carrying a fusion gene containing M2e and NP from three different influenza virus strains. They found that the immunization of mice with these vaccines results in a robust immune response and successful protection against a high dose of an influenza virus of a different subtype.

Immunizing mice with a DNA vaccine carrying the influenza virus HA gene plus the NP or M gene confers immunity against heterologous subtypes [28, 60]. M1 or NP DNA partially protected the mice, whereas M2 DNA failed to effectively protect them. However, the co-immunization of M1 DNA with NP DNA not only improved vaccine protection but also enabled resistance to challenges by influenza viruses of different subtypes (Table 1). These data suggest that a multicomponent vaccine with an appropriate immunization schedule can be an alternative approach to UIVs against potential influenza virus pandemics.

The combination of multiple proteins or epitopes has been used as a basis for designing various vaccine forms, including recombinant subunit vaccine, DNA vaccine, and virus vectored vaccine, to elicit a comprehensive immune response against influenza viruses of different subtypes. Identifying whether the conserved genes of influenza virus can be utilized to develop a combination vaccine for stable and long-term protection is indispensable. However, regardless of target antigen (M1, M2, or NP), none of the UIVs have achieved the same protection level as the current inactivated vaccine. Influenza viruses can still cause pulmonary infections even after UIVs vaccination. Moreover, the antibodies and cellular immunity induced by UIVs can only clear infected cells to prevent the dissemination of influenza viruses. That is, they cannot prevent infection. Therefore, these UIVs can lower mortality but cannot effectively lower the incidence of influenza.

UIV-related adjuvants

As described above, as yet, there has not been a UIV, whether based on M1, M2, or NP alone, that might be sufficient and achieved a similar level of immune response as the current inactivated vaccine. Therefore, at present, researchers are trying the addition of adjuvants to promote the induction of more antibodies and to improve the effectiveness of vaccines.

Using a safe adjuvant with a vaccine can enhance immunogenicity and achieve effective immune response with lower antigen dose. Some adjuvants can enhance the effectiveness of protein vaccines. Aluminum hydroxide and MF59 can be used with inactivated influenza vaccines. Moreover, mucosa adjuvants can also be added into a vaccine to induce mixed Th1/Th2 immune response following mucosal administration. To date, the most effective mucosa adjuvants identified by studies are cholera toxins (CT) and heat-labile toxins (LT). However, these toxins target ganglioside receptors, and affect nearly all nucleated cells, including nerve cells. Therefore, they are not suitable for use in humans. Chitosan, a deacetylated product of chitin, is often used as a mucosal adjuvant. Previous studies found that the nasal administration of chitosan as an adjuvant with vaccines can enhance the humoral and cellular immune responses of mice and guinea pigs against influenza, pertussis, diphtheria, and tetanus [8, 9]. Chitosan is non-toxic, bioadhesive, biodegradable, non-irritant, and not allergic for humans; in addition, the U.S. Food and Drug Administration (FDA) has already approved the use of chitosan in drugs and food [11]. Previous experiments showed that CTB co-administered with CT to antigens could efficiently induce immune responses compared to CTB alone. Thus, the CTB* adjuvant was used in our studies.

We found that i.n. immunization of mice with a certain dose of sM2, M1, or NP protein and chitosan or CTB* adjuvant can not only can provide full protection against a challenge by the homologous influenza virus, but can also confer cross-protection against heterologous influenza viruses (Table 1) [8–10]. Chitosan or CTB*, as a mucosal adjuvant, can enhance T cell responses and, thus, trigger strong systemic and mucosal immune responses by antigens. To some extent, cellular immunity can exceed the restrictions of virus subtypes and provide cross-protection, especially when humoral immunity offers no cross-protection. Therefore, enhancing T cell response is important to improve the protective effect of vaccines. Many studies have confirmed that cell-mediated immune response is important in clearing influenza A viruses in mice and humans (Fig. 1). We also found that mucosal immunization is more effective than other parenteral administration routes in clearing viruses infecting the respiratory tract.

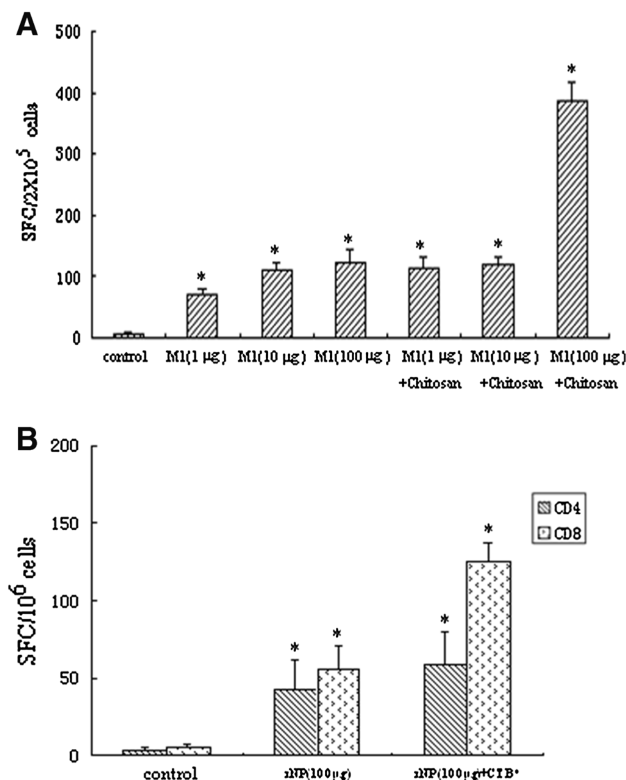

Fig. 1 Detection of cell-mediated immunity by ELISPOT assays of influenza virus M1 or NP protein. **a** Mice were immunized intranasally with M1 alone or in combination with chitosan two times with an interval of 3 weeks. At 2 weeks after the second immunization, the numbers of IFN-γ secreting cells in the spleen were detected and stimulated with 2 μg/ml of M1 peptide. **b** Mice were immunized intranasally with 100 μg rNP alone or in combination with CTB* three times with an interval of 3 weeks. At 2 weeks after the last immunization, the numbers of IFN-γ secreting cells in the spleen were detected and stimulated with peptides recognized by $CD4^{+}$ or $CD8^{+}$ T cells. The results of test groups were evaluated by Student's *t*-test; $p < 0.05$ was considered to indicate statistical significance. *Significant difference compared with the mice in the control group ($p < 0.05$). *SFC* spot-forming cells

This finding can be attributed to the fact that mucosal immunization can rapidly recruit immune cells to infection sites and can induce high levels of local IgA antibodies, which is critical for mucosal immunity. Our experiments also demonstrated that the survival of mice is related to the IgA antibody level. Secretory IgA antibodies possibly inhibit the intracellular replication or assembly of the virus by interfering with the function of the newly synthesized viral protein [8–10, 61, 62–65].

Adjuvants have been widely used in studies of various influenza vaccines, because of their capacity to enhance the immunogenicity of vaccines. Adjuvant safety and effectiveness are still key factors limiting their clinical application. More studies are needed in order to expand the availability of new adjuvants for commercial influenza vaccines.

Clinical trials of UIVs

The safety and effectiveness of a few UIVs previously researched in pre-clinical studies were subsequently tested in randomized controlled clinical trials (Table 3).

ACAM-FLU-A, developed by Acambis (a subsidiary of Sanofi-Aventis) in the 1990s, is the first UIV tested in a clinical trial. It is a recombinant vaccine that uses HBc protein to present M2e. HBcAg and M2e were expressed as a fusion protein in *E. coli*, and then the property of HBcAg to self-assemble into VLP was used to display M2e on the VLP surface. The VLP was purified and developed as a candidate vaccine called ACAM-FLU-A. In pre-clinical studies, the protective effect of ACAM-FLU-A against the Vietnam 2004 strain of H5N1 avian influenza (bird flu) virus was tested in mice. The results showed that the vaccine group showed a protection rate of 70 %, while all the mice in the placebo group died. This vaccine entered a phase I clinical trial in 2007 involving 79 healthy volunteers aged 18–40 years [66]. The clinical trial proved the safety and immunogenicity of the vaccine [67]. Meanwhile, the highest immune response was observed in the group vaccinated with ACAM-FLU-A plus QS-21. No severe adverse effect was observed on the vaccinated population.

VAX102 (STF2.4×M2e) is an M2e-based candidate vaccine developed by VaxInnate (US). This vaccine is a recombinant fusion protein that comprises *Salmonella typhimurium* flagellin type 2 (STF2 or fljB), a ligand for Toll-like receptor 5 (TLR5), fused to four tandem repeats of M2e at its C-terminus. Flagellin can bind to TLR5 on the surface of human innate immune cells to activate innate immunity; thus, unlike M2e alone, the vaccine can produce a significant immune response in humans [68]. This vaccine started phase I and phase II trials in 2009 involving 80 healthy volunteers aged 18–49 years [69, 70]. The trials results showed that the vaccine is safe and well-tolerated, while inducing good immune responses in humans [71].

Multimeric-001 [72] is a candidate UIV developed by BiondVax (Israel). This vaccine was developed through the expression and purification of a fusion protein consisting of conserved epitopes from influenza type A and type B virus strain genes HA, NP, and M in *E. coli*. Multimeric-001 had completed phase I/II clinical trials in young adults (aged 18–49 years), older adults (aged 55–65 years), and the elderly (aged >65 years). The clinical trials demonstrated that the vaccine is safe and elicits humoral and cellular immune responses in healthy humans [70, 73, 74]. Currently, the vaccine has entered a phase II clinical trial in the elderly to assess the safety and immunogenicity of Multimeric-001 followed by the administration of inactivated influenza vaccine (TIV); all clinical trials are estimated to be completed in 2013 [75].

Table 3 Overview of clinical trials of M- or/and NP-based universal influenza vaccines

Vaccine name (vaccine type)	Age	*n*	Study design (route of immunization)	Title	Phase	Ref. (year)
ACAM-FLU-A (VLP)	18–40	79	Randomized, placebo-controlled, double-blind (i.m. injection)	Safety study of recombinant M2e influenza-A vaccine in healthy adults (FLU-A)	Phase I	[66, 67] (2007)
VAX102(STF2.4×M2e) (protein)	18–49	80	Multicenter, double-blinded, randomized, placebo-controlled (i.m. injection)	Safety and immunogenicity of VAX102 universal influenza vaccine when given in the same arm with the standard influenza vaccine in healthy adults	Phase I Phase II	[68, 69, 71, 83] (2007–2011)
Multimeric-001 (protein)	18–49 55–57	60	Randomized, single-blinded, placebo-controlled (i.m. injection)	A double-dose safety study of an influenza vaccine (Multimeric-001)	Phase I Phase II	[70, 72–75] (2009–2011)
N8295 (protein)	18–40	54	Randomized, placebo-controlled, open-label (i.m. injection)	Clinical evaluation of N8295, a universal influenza A vaccine containing M2e and NP antigens conjugated to an oligonucleotide immunostimulatory sequence	Phase I	[76, 77] (2010–2011)
MVA-NP+M1 (VLP)	18–70 18–50	58 27	Non-randomized, parallel assignment, open-label (i.m. injection)	A study to assess the safety and efficacy of a new influenza candidate vaccine MVA-NP+M1 in healthy adults	Phase I Phase II	[52, 78–80] (2008–2012)
VGX-3400 (DNA)	20–39 Male	30	Non-randomized, parallel assignment, open-label (i.m. injection)	Study Of VGX-3400X, H5N1 avian influenza virus DNA plasmid + electroporation in healthy adults	Phase I	[81, 82] (2010–2012)
V512 (protein)	18–35	187	Randomized, parallel assignment, double-blinded (i.m. injection)	A study of a bivalent influenza peptide conjugate vaccine in healthy adults	Phase I	[84] (2006–2009)
VCL-IPT1, VCL-IPM1 (DNA)	18–45	56	Randomized, parallel assignment, double-blinded (i.m. injection)	A phase 1, double-blinded study to evaluate the safety, tolerability, and immunogenicity of pandemic influenza plasmid DNA vaccines	Phase I	[85] (2007–2008)

N8295 is a candidate vaccine developed by Dynavax (US); this vaccine uses both NP and M2e proteins as target antigens. A fusion protein of eight copies of M2e and one NP protein was expressed in *E. coli*. The purified product was covalently linked to an immunostimulatory sequence (ISS) to form M2e/NP-ISS, which can combine with a conventional influenza vaccine. The ISS is an agonist of TLR-9 and can enhance innate immune response in humans to improve vaccine efficacy and enable antigen sparing. This vaccine entered a phase I clinical trial involving 54 volunteers in July 2010 [76, 77]. The results showed that N8295 alone or in combination with the H5N1 (A/Turkey/Turkey/1/2005) vaccine is safe and generally well tolerated, that is, no serious adverse events were observed.

MVA-NP+M1 is a modified vaccinia virus Ankara (MVA) vector-based candidate vaccine designed by Oxford University. The M1 and NP genes of the influenza virus A/Panama/2007/99 (H3N2) were inserted into the MVA vector [52]. The vaccine was prepared by purifying the recombinant virus grown in chicken embryo fibroblasts. This vaccine started its phase I clinical trial in August 2008 in healthy volunteers aged 18–50 years. At 6 months after vaccination, MVA-NP+M1 showed good safety in humans, and induced strong immune responses, particularly cellular immune response. In 2011, the safety and immunogenicity of the co-administration of the candidate influenza vaccine MVA-NP+M1 and seasonal influenza vaccine was confirmed in healthy volunteers aged 50 years and older [78, 79]. In a phase IIa clinical trial, a challenge experiment was performed in 12 subjects at 1 month after i.m. injection of the vaccine to evaluate the protective effect of the vaccine [80]. These studies confirmed the safety and immunogenicity of the vaccine; they also provided preliminary evidence of the vaccine's efficacy.

VGX-3400 is a DNA vaccine developed by VGX (US). This DNA vaccine includes a plasmid encoding the consensus sequences of HA, NA, and M2e-NP antigen derived from multiple strains of the H5N1 avian influenza virus. The vaccine was delivered by electroporation to increase the immune responses in animal models. The VGX-3400 vaccine had completed a phase I clinical trial in 2011 in 32 human subjects; the clinical trial evaluated the safety and immunogenicity of three doses of VGX-3400 vaccine in healthy adults [81, 82].

Conclusions

M- and/or NP-based universal influenza vaccine strategies have appealing advantages in theory. Studies on M1, M2, and NP candidate universal vaccines have demonstrated the feasibility of these strategies. These universal vaccines have highly conserved proteins and clearly defined components. Moreover, these vaccines can achieve a level of immune response similar to that of the current inactivated vaccine when immunized with an adjuvant and sometimes with other conserved proteins or components. Universal vaccines produced by genetic engineering, such as *E. coli,* have the advantages such as low cost, high yield, easy scale-up in production, and no need for switching vaccine strains every year. Therefore, this could be a way to avoid frequently changing the vaccine strains switching in current seasonal flu vaccines. This method can effectively control an influenza epidemic of a new virus strain, when matching vaccine strains are not available. In clinical trials of UIVs, most candidate influenza vaccines are administered in a parenteral manner. However, influenza infection starts at mucosal sites of the upper respiratory tract. Thus, many groups have worked on developing mucosal vaccines. Mucosal immunity can act at the early stages of influenza virus invasion to prevent the virus from passing through the mucosa and to stop virus replication. Therefore, mucosal immunization might be more effective than other parenteral routes of immunization in virus clearance. Moreover, mucosal immunization is easy to operate, does not need specialized injection tools (such as syringes), and could be a mode of self-vaccination. Highly immunogenic and safe UIVs might be developed in the future to prevent seasonal and pandemic influenza. New vaccination techniques need be developed to fully realize versatile and robust immune responses of UIVs. More research efforts are needed in order to create the availability of a commercial universal vaccine.

Acknowledgments This study was supported by the National Natural Science Foundation of China (Project Nos. 81172738, 81071346), the National High-Technology Research and Development Program of China (863 Program 2010AA022905, 2010AA022908, 2010AA022910), and the Shanghai Science and Technology Support Program, China (12431901701).

Conflict of interest All authors declare that they have no competing interest.

References

1. Kamps BS, Hoffmann C, Preiser W, editors. Influenza report 2006. ISBN 3-924774-51-X. http://www.influenzareport.com.
2. Slepushkin VA, Katz JM, Black RA, Gamble WC, Rota PA, Cox NJ. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine. 1995;13:1399–402.
3. Fiers W, De Filette M, El Bakkouri K, Schepens B, Roose K, Schotsaert M, Birkett A, Saelens X. M2e-based universal influenza A vaccine. Vaccine. 2009;27:6280–3.
4. Treanor JJ, Taylor DN, Tussey L, Hay C, Nolan C, Fitzgerald T, Liu G, Kavita U, Song L, Dark I, Shaw A. Safety and immunogenicity of a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults. Vaccine. 2010;28:8268–74.
5. Song L, Nakaar V, Kavita U, Price A, Huleatt J, Tang J, Jacobs A, Liu G, Huang Y, Desai P, Maksymiuk G, Takahashi V, Umlauf S, Reiserova L, Bell R, Li H, Zhang Y, McDonald WF, Powell TJ, Tussey L. Efficacious recombinant influenza vaccines produced by high yield bacterial expression: a solution to global pandemic and seasonal needs. PLoS One. 2008;3:e2257.
6. Ambrozaitis A, Groth N, Bugarini R, Sparacio V, Podda A, Lattanzi M. A novel mammalian cell-culture technique for consistent production of a well-tolerated and immunogenic trivalent subunit influenza vaccine. Vaccine. 2009;27:6022–9.
7. Kemble G, Greenberg H. Novel generations of influenza vaccines. Vaccine. 2003;21:1789–95.
8. Sui Z, Chen Q, Fang F, Zheng M, Chen Z. Cross-protection against influenza virus infection by intranasal administration of M1-based vaccine with chitosan as an adjuvant. Vaccine. 2010;28:7690–8.
9. Sui Z, Chen Q, Wu R, Zhang H, Zheng M, Wang H, Chen Z. Cross-protection against influenza virus infection by intranasal administration of M2-based vaccine with chitosan as an adjuvant. Arch Virol. 2010;155:535–44.
10. Guo L, Zheng M, Ding Y, Li D, Yang Z, Wang H, Chen Q, Sui Z, Fang F, Chen Z. Protection against multiple influenza A virus subtypes by intranasal administration of recombinant nucleoprotein. Arch Virol. 2010;155:1765–75.
11. Kim TJ, Kim KH, Lee JI. Stimulation of mucosal and systemic antibody responses against recombinant transferrin-binding protein B of Actinobacillus pleuropneumoniae with chitosan after tracheal administration in piglets. J Vet Med Sci. 2007;69:535–9.
12. Helenius A. Unpacking the incoming influenza virus. Cell. 1992;69:577–8.
13. Ye ZP, Baylor NW, Wagner RR. Transcription-inhibition and RNA-binding domains of influenza A virus matrix protein mapped with anti-idiotypic antibodies and synthetic peptides. J Virol. 1989;63:3586–94.
14. Adler S, Reay P, Roy P, Klenk HD. Induction of T cell response by bluetongue virus core-like particles expressing a T cell epitope of the M1 protein of influenza A virus. Med Microbiol Immunol. 1998;187:91–6.
15. Plotnicky H, Cyblat-Chanal D, Aubry JP, Derouet F, Klinguer-Hamour C, Beck A, Bonnefoy JY, Corvaïa N. The immunodominant influenza matrix T cell epitope recognized in human induces influenza protection in HLA-A2/K(b) transgenic mice. Virology. 2003;309:320–9.
16. Linnemann T, Jung G, Walden P. Detection and quantification of CD4($^{+}$) T cells with specificity for a new major histocompatibility complex class II-restricted influenza A virus matrix protein epitope in peripheral blood of influenza patients. J Virol. 2000;74:8740–3.
17. Goodman AG, Heinen PP, Guerra S, Vijayan A, Sorzano CO, Gomez CE, Esteban M. A human multi-epitope recombinant vaccinia virus as a universal T cell vaccine candidate against influenza virus. PLoS One. 2011;6:e25938.
18. Lamb RA, Lai CJ, Choppin PW. Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc Natl Acad Sci USA. 1981;78:4170–4.

19. Ito T, Gorman OT, Kawaoka Y, Bean WJ, Webster RG. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol. 1991;65:5491–8.
20. Zebedee SL, Lamb RA. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J Virol. 1988;62:2762–72.
21. Treanor JJ, Tierney EL, Zebedee SL, Lamb RA, Murphy BR. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol. 1990;64:1375–7.
22. Saelens X. The influenza matrix protein 2 as a vaccine target. Future Virol. 2008;3:167–78.
23. Schotsaert M, De Filette M, Fiers W, Saelens X. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. Expert Rev Vaccines. 2009;8:499–508.
24. Yewdell JW, Bennink JR, Smith GL, Moss B. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci USA. 1985;82:1785–9.
25. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. Emerg Infect Dis. 2006;12:48–54.
26. Gschoesser C, Almanzar G, Hainz U, Ortin J, Schonitzer D, Schild H, Saurwein-Teissl M, Grubeck-Loebenstein B. $CD4^{+}$ and $CD8^{+}$ mediated cellular immune response to recombinant influenza nucleoprotein. Vaccine. 2002;20:3731–8.
27. Okuda K, Ihata A, Watabe S, Okada E, Yamakawa T, Hamajima K, Yang J, Ishii N, Nakazawa M, Okuda K, Ohnari K, Nakajima K, Xin KQ. Protective immunity against influenza A virus induced by immunization with DNA plasmid containing influenza M gene. Vaccine. 2001;19:3681–91.
28. Chen Q, Kuang H, Wang H, Fang F, Yang Z, Zhang Z, Zhang X, Chen Z. Comparing the ability of a series of viral protein-expressing plasmid DNAs to protect against H5N1 influenza virus. Virus Genes. 2009;38:30–8.
29. Heinen PP, Rijsewijk FA, de Boer-Luijtze EA, Bianchi AT. Vaccination of pigs with a DNA construct expressing an influenza virus M2–nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. J Gen Virol. 2002;83:1851–9.
30. Tompkins SM, Zhao ZS, Lo CY, Misplon JA, Liu T, Ye Z, Hogan RJ, Wu Z, Benton KA, Tumpey TM, Epstein SL. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg Infect Dis. 2007;13:426–35.
31. Neirynck S, Deroo T, Saelens X, Vanlandschoot P, Jou WM, Fiers W. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med. 1999;5:1157–63.
32. De Filette M, Ramne A, Birkett A, Lycke N, Löwenadler B, Min Jou W, Saelens X, Fiers W. The universal influenza vaccine M2e-HBc administered intranasally in combination with the adjuvant CTA1-DD provides complete protection. Vaccine. 2006;24:544–51.
33. Frace AM, Klimov AI, Rowe T, Black RA, Katz JM. Modified M2 proteins produce heterotypic immunity against influenza A virus. Vaccine. 1999;17:2237–44.
34. Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, Fu TM, Joyce J, Przysiecki CT, Keller PM, Garsky VM, Ionescu R, Rippeon Y, Shi L, Chastain MA, Condra JH, Davies ME, Liao J, Emini EA, Shiver JW. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine. 2004;22:2993–3003.
35. Mozdzanowska K, Feng J, Eid M, Kragol G, Cudic M, Otvos L Jr, Gerhard W. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. Vaccine. 2003;21:2616–26.
36. Ernst WA, Kim HJ, Tumpey TM, Jansen AD, Tai W, Cramer DV, Adler-Moore JP, Fujii G. Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. Vaccine. 2006;24:5158–68.
37. Denis J, Acosta-Ramirez E, Zhao Y, Hamelin ME, Koukavica I, Baz M, Abed Y, Savard C, Pare C, Lopez Macias C, Boivin G, Leclerc D. Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. Vaccine. 2008;26:3395–403.
38. Eliasson DG, El Bakkouri K, Schön K, Ramne A, Festjens E, Löwenadler B, Fiers W, Saelens X, Lycke N. CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine. Vaccine. 2008;26:1243–52.
39. De Filette M, Martens W, Roose K, Deroo T, Vervalle F, Bentahir M, Vandekerckhove J, Fiers W, Saelens X. An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. J Biol Chem. 2008;283:11382–7.
40. Ionescu RM, Przysiecki CT, Liang X, Garsky VM, Fan J, Wang B, Troutman R, Rippeon Y, Flanagan E, Shiver J, Shi L. Pharmaceutical and immunological evaluation of human papillomavirus viruslike particle as an antigen carrier. J Pharm Sci. 2006;95:70–9.
41. Bessa J, Schmitz N, Hinton HJ, Schwarz K, Jegerlehner A, Bachmann MF. Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: implications for vaccine design. Eur J Immunol. 2008;38:114–26.
42. Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, Tang J, McDonald W, Song L, Evans RK, Umlauf S, Tussey L, Powell TJ. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine. 2008;26:201–14.
43. Zhang GG, Li DX, Zhang HH, Zeng YM, Chen L. Enhancement of mucosal immune response against the M2eHBc+ antigen in mice with the fusion expression products of LTB and M2eHBc+ through mucosal immunization route. Vet Res Commun. 2009;33:735–47.
44. Ameiss K, Ashraf S, Kong W, Pekosz A, Wu WH, Milich D, Billaud JN, Curtiss R 3rd. Delivery of woodchuck hepatitis virus-like particle presented influenza M2e by recombinant attenuated Salmonella displaying a delayed lysis phenotype. Vaccine. 2010;28:6704–13.
45. Zhao G, Sun S, Du L, Xiao W, Ru Z, Kou Z, Guo Y, Yu H, Jiang S, Lone Y, Zheng BJ, Zhou Y. An H5N1 M2e-based multiple antigenic peptide vaccine confers heterosubtypic protection from lethal infection with pandemic 2009 H1N1 virus. Virol J. 2010;7:151.
46. Zhao G, Du L, Xiao W, Sun S, Lin Y, Chen M, Kou Z, He Y, Lustigman S, Jiang S, Zheng BJ, Zhou Y. Induction of protection against divergent H5N1 influenza viruses using a recombinant fusion protein linking influenza M2e to Onchocerca volvulus activation associated protein-1 (ASP-1) adjuvant. Vaccine. 2010;28:7233–40.
47. Wraith DC, Vessey AE, Askonas BA. Purified influenza virus nucleoprotein protects mice from lethal infection. J Gen Virol. 1987;68:433–40.
48. Tamura S, Miyata K, Matsuo K, Asanuma H, Takahashi H, Nakajima K, Suzuki Y, Aizawa C, Kurata T. Acceleration of influenza virus clearance by Th1 cells in the nasal site of mice immunized intranasally with adjuvant-combined recombinant nucleoprotein. J Immunol. 1996;156:3892–900.
49. Wang W, Huang B, Jiang T, Wang X, Qi X, Gao Y, Tan W, Ruan L. Robust immunity and heterologous protection against influenza in mice elicited by a novel recombinant NP-M2e fusion protein expressed in E. coli. PLoS One. 2012;7:e52488.
50. Roose K, Fiers W, Saelens X. Pandemic preparedness: toward a universal influenza vaccine. Drug News Perspect. 2009;22:80–92.

51. Lo CY, Wu Z, Misplon JA, Price GE, Pappas C, Kong WP, Tumpey TM, Epstein SL. Comparison of vaccines for induction of heterosubtypic immunity to influenza A virus: cold-adapted vaccine versus DNA prime-adenovirus boost strategies. Vaccine. 2008;26:2062–72.
52. Lillie PJ, Berthoud TK, Powell TJ, Lambe T, Mullarkey C, Spencer AJ, Hamill M, Peng Y, Blais ME, Duncan CJ, Sheehy SH, Havelock T, Faust SN, Williams RL, Gilbert A, Oxford J, Dong T, Hill AV, Gilbert SC. Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. Clin Infect Dis. 2012;55:19–25.
53. Xu K, Ling ZY, Sun L, Xu Y, Bian C, He Y, Lu W, Chen Z, Sun B. Broad humoral and cellular immunity elicited by a bivalent DNA vaccine encoding HA and NP genes from an H5N1 virus. Viral Immunol. 2011;24:45–56.
54. Pandey A, Singh N, Vemula SV, Couëtil L, Katz JM, Donis R, Sambhara S, Mittal SK. Impact of preexisting adenovirus vector immunity on immunogenicity and protection conferred with an adenovirus-based H5N1 influenza vaccine. PLoS One. 2012;7:e33428.
55. Li Z, Gabbard JD, Mooney A, Chen Z, Tompkins SM, He B. Efficacy of parainfluenza virus 5 mutants expressing hemagglutinin from H5N1 influenza A virus in mice. J Virol. 2013;87: 9604–9.
56. Boyd AC, Ruiz-Hernandez R, Peroval MY, Carson C, Balkissoon D, Staines K, Turner AV, Hill AV, Gilbert SC, Butter C. Towards a universal vaccine for avian influenza: protective efficacy of modified Vaccinia virus Ankara and Adenovirus vaccines expressing conserved influenza antigens in chickens challenged with low pathogenic avian influenza virus. Vaccine. 2013;31: 670–5.
57. Donnelly JJ, Friedman A, Ulmer JB, Liu MA. Further protection against antigenic drift of influenza virus in a ferret model by DNA vaccination. Vaccine. 1997;15:865–8.
58. Jeon SH, Ben-Yedidia T, Arnon R. Intranasal immunization with synthetic recombinant vaccine containing multiple epitopes of influenza virus. Vaccine. 2002;20:2772–80.
59. Zhou D, Wu TL, Lasaro MO, Latimer BP, Parzych EM, Bian A, Li Y, Li H, Erikson J, Xiang Z, Ertl HC. A universal influenza a vaccine based on adenovirus expressing matrix-2 ectodomain and nucleoprotein protects mice from lethal challenge. Mol Ther. 2010;18:2182–9.
60. Chen Z, Yoshikawa T, Kadowaki S, Hagiwara Y, Matsuo K, Asanuma H, Aizawa C, Kurata T, Tamura S. Protection and antibody responses in different strains of mouse immunized with plasmid DNAs encoding influenza virus haemagglutinin, neuraminidase and nucleoprotein. J Gen Virol. 1999;80:2559–64.
61. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG. Intracellular neutralization of virus by immunoglobulin A antibodies. Proc Natl Acad Sci USA. 1992;89:6901–5.
62. Mazanec MB, Coudret CL, Fletcher DR. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. J Virol. 1995;69:1339–43.
63. Yan H, Lamm ME, Björling E, Huang YT. Multiple functions of immunoglobulin A in mucosal defense against viruses: an in vitro measles virus model. J Virol. 2002;76:10972–9.
64. Huang YT, Wright A, Gao X, Kulick L, Yan H, Lamm ME. Intraepithelial cell neutralization of HIV-1 replication by IgA. J Immunol. 2005;174:4828–35.
65. Wright A, Yan H, Lamm ME, Huang YT. Immunoglobulin A antibodies against internal HIV-1 proteins neutralize HIV-1 replication inside epithelial cells. Virology. 2006;356:165–70.
66. Safety study of recombinant M2e influenza-A vaccine in healthy adults (FLU-A). http://clinicaltrials.gov/ct2/show/NCT00819013.
67. Acambis reports positive data from trials of universal influenza A vaccine. FierceBiotech. http://www.fiercebiotech.com/press-releases/acambis-reports-positive-data-trials-universal-influenza-vaccine#ixzz13KPQYAE2.
68. Talbot HK, Rock MT, Johnson C, Tussey L, Kavita U, Shanker A, Shaw AR, Taylor DN. Immunopotentiation of trivalent influenza vaccine when given with VAX102, a recombinant influenza M2e vaccine fused to the TLR5 ligand flagellin. PLoS One. 2010;5:e14442.
69. Safety and immunogenicity of VAX102 universal influenza vaccine when given in the same arm with the standard influenza vaccine in healthy adults. http://clinicaltrials.gov/ct2/show/NCT00921973?term=VAX102&rank=2.
70. Atsmon J, Kate-Ilovitz E, Shaikevich D, Singer Y, Volokhov I, Haim KY, Ben-Yedidia T. Safety and immunogenicity of multimeric-001—a novel universal influenza vaccine. J Clin Immunol. 2012;32(3):595–603.
71. Turley CB, Rupp RE, Johnson C, Taylor DN, Wolfson J, Tussey L, Kavita U, Stanberry L, Shaw A. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. Vaccine. 2011;29:5145–52.
72. Ben-Yedidia T. Multimeric-001: BiondVax's universal flu vaccine. Interview by Duc Le. Expert Rev Vaccines. 2010;9:241–2.
73. A double-dose safety study of an influenza vaccine (Multimeric-001) injected to elderly volunteers. http://www.clinicaltrials.gov/ct2/show/NCT01010737?term=Multimeric-001&rank=1.
74. Further investigation of an intramuscular influenza vaccine (Multimeric-001). http://www.clinicaltrials.gov/ct2/show/NCT01146119?term=Multimeric-001&rank=4.
75. A double-dose safety study of an influenza vaccine (Multimeric-001). http://clinicaltrials.gov/ct2/show/NCT00877448?term=Multimeric-001&rank=3.
76. Pipeline: flu vaccine. http://www.dynavax.com/flu.html.
77. Janssen R, Ervin J, Martin T. Clinical evaluation of N8295, a universal influenza A vaccine containing M2e and NP antigens conjugated to an oligonucleotide immunostimulatory sequence. http://www.who.int/vaccine_research/diseases/influenza/18_feb_2011_Robert_Janssen_ws.pdf.
78. Antrobus RD, Berthoud TK, Mullarkey CE, Hoschler K, Coughlan L, Zambon M, Hill AV, Gilbert SC. Coadministration of Seasonal influenza vaccine and MVA-NP+M1 Simultaneously achieves potent humoral and cell-mediated responses. Mol Ther. 2013.
79. Antrobus RD, Lillie PJ, Berthoud TK, Spencer AJ, McLaren JE, Ladell K, Lambe T, Milicic A, Price DA, Hill AV, Gilbert SC. A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. PLoS One. 2012;7:e48322.
80. A study to assess the safety and efficacy of a new influenza candidate vaccine MVA-NP+M1 in healthy adults. http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00993083.
81. Study Of VGX-3400X, H5N1 avian influenza virus DNA plasmid + electroporation in healthy adults. http://www.clinicaltrials.gov/ct2/show/NCT01142362?term=VGX-3400&rank=1.
82. VGX-3400: Phase I data. http://www.biocentury.com/weekinreview/clinicalresults/2011-07-25/vgx-3400-phase-i-data-314011.
83. Comparative safety and immunogenicity of 1.0 μg intramuscular (i.m.) and 2.0 μg subcutaneous (s.c.) dosing with VAX102 (M2e-flagellin) universal influenza vaccine in healthy adults. http://www.clinicaltrials.gov/ct2/show/NCT00921947?term=STF2.4xM2e&rank=2.
84. A study of a bivalent influenza peptide conjugate vaccine in healthy adults. http://www.clinicaltrials.gov/ct2/show/NCT00851266?term=influenza+M2&rank=3.
85. A Phase 1, double-blinded study to evaluate the safety, tolerability, and immunogenicity of pandemic influenza plasmid DNA vaccines. http://www.clinicaltrials.gov/ct2/show/NCT00709800?term=influenza+M2&rank=10.