

The challenges of creating a universal influenza vaccine

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ABSTRACT

The lack of population immunity to the periodically emerging pandemic influenza strains makes influenza infection especially dangerous. The fragmented nature of the influenza virus genome contributes to the formation of influenza virus reassortants containing genomic fragments from different strains. This mechanism is the main reason for the natural influenza virus antigenic diversity as well as for the occurrence of influenza pandemics. Vaccination is the best measure to prevent the spread of influenza infection, but the efficacy of existing vaccines is not sufficient, especially for the elderly and small children. Specific immunity, developed after disease or immunization, poorly protects against infection by influenza viruses of another subtype. In this regard, there is an urgent need for a more effective universal influenza vaccine that provides a long-lasting broad cross-protective immunity, and is able to protect against influenza A and B viruses of all known subtypes. The basic approaches to as well as challenges of creating such a vaccine are discussed in this review.

INTRODUCTION

Of all the viral respiratory diseases, influenza causes the most severe pathology and leads to the greatest damage to the population health and economy. The lack of population immunity to the periodically emerging new pandemic influenza strains makes influenza infection especially dangerous. It is known that the Spanish flu caused the death of 30 to 50 million people in 1918. Currently, according to the World Health Organization (WHO) data, each year approx. 20% of the population worldwide, including 5-10% of adults and 20-30% of children become ill with influenza during seasonal epidemics. Severe disease forms are recorded for 3-5 million cases, and 250,000 to 500,000 cases are lethal [1-3]. Economic losses caused by influenza and other acute respiratory viral infections account for approx. 77% of the total damage from all infectious diseases. Significant losses are related both to the direct costs of patients' treatment and rehabilitation, as well as to the indirect losses caused by a decrease in productivity and a reduction in corporate profits. Influenza and acute respiratory viral infections account for 12-14% of the total number of temporary disability cases [2, 4].

Furthermore, influenza infection can cause hidden damage, such as serious clinical complications associated with the nervous and cardiovascular systems, as well as the exacerbation of chronic diseases (diabetes, heart failure, chronic obstructive pneumonia, etc.) and lead to delayed death, especially in children under two years old, the elderly and people with poor health [5].

The influenza virus belongs to the family of Orthomyxoviridae, which includes five genera: influenza A, B, C, D (Thogotovirus) and Isavirus. Genomes of

influenza A and B viruses are structurally similar, and consist of eight RNA genome segments of negative polarity. These genome segments encode 12 proteins and are named according to the product translated from the primary open reading frame: PB1, PB2, PA, HA, NP, NA, M, and NS [6]. The polymerase complex PB2, PB1, and PA transcribes one mRNA from each genomic fragment, which is translated to the corresponding protein. In addition to M1 and NS1 proteins, two other proteins - M2 and NEP - are coded by the two corresponding mRNA genomic segments M and NS by means of splicing. For some strains from the alternative reading frame of the PB1 segment PB1-F2 protein is translated [7]. All proteins except NS1 and PB1-F2 are structural components of a virus particle. Nonstructural protein NS1 accumulates in the cytoplasm of infected cells and acts as an interferon antagonist. It is assumed that the PB1-F2 protein works as a proapoptotic factor that inhibits the function of immunocompetent cells [8].

The segmented structure of the influenza virus genome is an inexhaustible source of new strains that are the result of the reassortment process. Reassortants contain genomic segments from different strains of the same genus. Reassortment is one of the mechanisms that form the basis of the natural antigenic diversity of influenza viruses, eventually leading to periodic influenza pandemics. The antigenic properties of influenza virus are determined by the surface glycoproteins - hemagglutinin (HA or H) and neuraminidase (NA or N). The HA and NA form spikes composed by HA trimers and NA tetramers on the surface of the virion. In the process of viral replication, the HA is cleaved (HA activation) by

cellular proteases into two subunits - HA1 and HA2 - that remain connected by a disulfide bond [9]. HA ectodomain consists of two parts: a globular part comprising HA1 subunit and the stem region, which is formed mainly by HA2, and by a small part of HA1 subunits. The globular part includes the receptor-binding site and five antigenic sites, and serves as the main target for the formation of antibodies. Antibodies that block virus binding to the cell receptor are neutralizing [10]. The HA1 subunit is characterized by high variability. In contrast, the stem of the HA that is located in close proximity to the viral membrane is highly conservative and characterized by low immunogenicity [11]. The main function of the HA2 subunit is to ensure the fusion of the viral and the endosomal membranes by means of the fusion peptide. According to the antigenic specificity, 18 subtypes of HA and 11 subtypes of NA are known to date for the influenza A virus [12]. The subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18 belong to the first group (I) and the subtypes H3, H4, H7, H10, H14, and H15 belong to the second group (II). At the same time, only the subtypes H1, H2, and H3 of influenza virus A and different antigenic variants of influenza virus B, that are circulating in the human population, are causing the pandemics and seasonal influenza epidemics.

The specific immunity generated after the disease or after vaccination by one subtype of influenza A virus poorly protects from infection by the other virus subtypes. The immunity to any influenza virus A subtype does not protect from the infection by influenza virus B, and vice versa - immunization against the influenza virus B is not effective in regard to influenza virus A.

Vaccination is the best measure to prevent the spread of influenza infection. During the last 60 years, a lot of vaccines were developed that have certain advantages and shortcomings. However, none of the existing vaccines can solve the problem of influenza morbidity control. The poor efficiency of the influenza vaccines can be explained by the high variability of the pathogen. Two mechanisms enable the extremely high variability of the influenza virus and, therefore, its ability to escape from the neutralizing antibodies: 1) accumulation of the point mutations leading to the change in the antigenic structure of the surface glycoproteins (antigenic drift) and 2) reassortment of the genomic segments coding surface antigens (antigenic shift). The latter might lead to the emergence of new subtypes of viruses that can cause pandemics.

All of the existing influenza vaccines have low efficiency in elderly and infants [13-16]. Furthermore, these vaccines can protect against the circulating virus only if the vaccine virus has the same antigenic properties as the epidemic strain. Thus, the high variability of the virus surface antigens - HA and NA - makes the annual vaccination necessary. It should be mentioned that seasonal vaccines that are developed according to the WHO recommendations are not effective in the case of the occurrence of a new influenza pandemic virus strain that is fundamentally different from all of the circulating strains, as it happened in 2009 when the pandemic virus A/California/7/2009 (H1N1pdm09) emerged. One

more example could be the low efficiency of the H3N2 component of the seasonal vaccine 2014 due to the emergence of the new antigenic variant of this virus subtype as a result of antigenic drift [17]. Additionally, there are known cases of human infection by the avian viruses of the H5N1, H7N9, H9N2, H6N1, H7N3, and H10N8 subtypes. The possibility of the avian virus transmission from human to human has not been proved yet. However, in ferrets it was shown that only several mutations in genes coding the PB1, PB2, and HA proteins could lead to the emergence of the transmitting virus [18-20]. Viruses of H5N1 and H7N9 subtypes are highly pathogenic and lead to lethality up to 50% in humans. The spreading of these viruses is extremely dangerous. In this regard, there is an urgent need for the development of an effective universal influenza vaccine that provides a long-lasting broad cross-protective immunity and is able to protect against the influenza A and B viruses of all known subtypes. Several examples of the cross-reactive antibodies that neutralize the HA of groups I and II of influenza viruses A as well as the antibodies that react with A and B viruses are known to date [21]. These results indicate that the creation of the universal influenza vaccine is possible.

The protective role of antibodies against the conservative influenza virus antigens

The main humoral immune response during the influenza infection is directed against the HA surface glycoprotein and primarily against its most variable globular part [22]. The HA protein has two main functions: binding to the cell membrane in order to ensure the virus entry into the cell and the fusion of the virus and the cell membranes that takes place in endosomes [23]. The acidification of endosomes leads to the HA conformational change resulting in the extension of the fusion peptide and emergence of the previously hidden parts of the subunit HA2 on the surface of the HA trimer. In spite of the continuous HA variability the stem region, responsible for membrane fusion, has been conserved for decades and has kept its functionality. In addition to the HA2 subunit, the inner influenza virus proteins (polymerases, NP, M1, M2, and NS1) are also conservative. The most conservative regions of these proteins could be used for the creation of the universal influenza vaccine. Actually, the majority of antibodies directed against the conservative regions of the inner proteins do not have the neutralizing activity and do not react *in vitro* in the hemagglutination inhibition (HAI) or neutralization assays [24]. Although these specific antibodies do not prevent the infection of humans with a virus, they could slow down the infection process. The antibodies against the HA stem region could block the fusion of the viral and endosomal membranes [25], while the antibodies against the structural proteins could induce the complement dependent cell lysis and the antibody-dependent cell cytotoxicity (ADCC) [26]. The interaction of the natural killers (NK) CD16 with the antibody's Fc-region leads to the release of granzyme B or perforin and to the elimination of the infected cells by apoptosis [27]. Thus, the antibodies participating in ADCC could induce the accelerated virus clearance from

the cells of the respiratory tract that leads to the fast recovery of the influenza patients.

The cell immunity to the influenza infection

The cell immunity also does not prevent the infection of the humans with influenza virus, although it could have a significant influence on the virus reproduction leading to a less severe course of illness and decreased mortality. The cross-protective immune response is possible if the epitopes of the conservative influenza virus proteins that are known to be predominantly recognized by the T-lymphocytes (CD4⁺ and CD8⁺) are presented effectively [28]. As was shown in animal models, the cell immune response might have a protective role in case of homological and heterological infections [29-35]. The results of the adaptive transfer of T-cells from the primed mice to the naive recipients prove the protective function of the CD4⁺ and CD8⁺ cells [36]. The protective function of the preexisting virus specific T-cells CD4⁺ and CD8⁺ was also shown in humans in case of experimental [37, 38] and natural infection by the pandemic virus H1N1 of 2009 [39]. T-cells CD4⁺ were shown to control the influenza infection by the production of the anti-inflammatory cytokines and chemokines as well as by the lysis of the infected cells and synergy with naive B-lymphocytes or CD8⁺ cells [27, 40, 41]. Furthermore, the CD4⁺ cells play an important role in the formation and support of memory cells [42]. It was shown that CD8⁺ T-cells determine the accelerated virus clearance from the body [43]. Therefore, the universal influenza vaccine should evoke the CD8⁺ T-cell response [44]. In order to induce the effective CD8⁺ response, virus proteins should be synthesized in the infected cells with the consecutive presentation of the CD8⁺ epitopes by the class I molecules of the major histocompatibility complex (MHC I). The influenza virus NP and M1 proteins play the most important role in the formation of CD8⁺ T-cell response in the case of influenza infection [45].

The peptide vaccination as well as the vaccination with the whole virion or split inactivated vaccines causes the antibody and CD4⁺ T-cell response due to the antigen presentation by the MHC II molecules. In contrast to peptides, the use of the replicating virus for vaccination leads to the processing and presentation of antigens by class I and class II MHC molecules due to the synthesis of the viral proteins providing both humoral and cell immunity. In case of the intranasal immunization, the mucosal immunity is induced in addition to the humoral and cell immunity. Therefore, inducing a full immune response that is close to a natural infection response is only possible to attain in the case of vaccination with live attenuated vaccines or with vectors that assure the synthesis of the viral proteins in cytoplasm. Moreover, the induction of the effective T-cell response could be achieved by the cross-priming mechanism using such adjuvant as the Toll-like receptors (TLR) ligands [46].

The main approaches to the creation of a universal vaccine

The function of all the licensed influenza vaccines – inactivated (whole virion, split, or subunit) or live

(attenuated cold adapted) – is to generate the immunity to the globular part of HA. Antibody titers, determined by HAI or by neutralization assay, are considered to be the main surrogate marker of vaccine efficacy.

In contrast to the variable globular part, the HA stem part of influenza A (groups I and II) and B viruses is much more conservative. There are known several mechanisms of direct and indirect neutralization for the antibodies induced to this part of HA. One of the mechanisms of the direct neutralization contributes to the prevention of HA cleavage to HA1 and HA2 subunits. That is achieved by the interaction of antibodies with the HA part that is located in the vicinity of the cleavage site [47]. The second mechanism of direct neutralization contributes to the prevention of the HA conformational change that is necessary for the fusion peptide release and the subsequent fusion of the endosomal and viral membranes in order to deliver the viral genome into the cytosol. The antibody dependent and complement dependent cytotoxicity are involved in the mechanisms of indirect neutralization [48, 49].

Vaccination practically does not induce the antibodies to the HA stem region, while after the natural infection a small quantity of these antibodies could be detected [22, 50-54]. The exception is the pandemic virus H1N1pdm09: in that case, the generation of the cross-reactive antibodies directed to the subunit HA2 conservative sites after the disease and vaccination was shown [54, 55].

It should be mentioned that the antibodies to the HA stem region could play not only the protective role but they could also provoke the infection. Thus, the interaction of the complex virus-antibody with the cell Fc-receptor led to the facilitation of the virus penetration into the cell and consequently to the intensification of the infection process [56]. The negative role of the cross-reactive neutralizing antibodies to the HA stem region *in vivo* was shown during the repeated infection by the antigenically heterologous influenza virus. The enhancement of the respiratory tract infection was recorded after the vaccination of animals and humans with the inactivated vaccine and consequent infection by the heterologous influenza strain [57-60]. Thus after the double immunization of pigs by whole virion inactivated vaccine against H1N1 virus with adjuvant, the enhancement of the lung pathology was recorded in case of a consequent infection by H1N1pdm09 virus. As it was found out in the course of epitopes mapping, the antibodies contributing to the infection enhancement are directed to the HA2 region from 32 to 77 amino acid residues that is located in close proximity to the fusion peptide [61]. It should be mentioned that after the vaccination of pigs with the live attenuated vaccine (cold adapted or containing the truncated NS gene), the pathology enhancement in the case of infection with H1N1pdm09 virus was not observed [62, 63].

The majority of the currently being developed approaches to the generation of the universal vaccine are targeting the conservative regions of the influenza virus proteins. The data on the major viral targets, corresponding vaccines, and their assumed mechanisms of action, presented by the WHO at the Vaccines Advisory Committee (PD-VAC) meeting – 2015, are shown in

Table 1 [64]. The analysis of the proteins' primary structure of different virus strains showed that 80% of the virus strains of human and avian viruses contain five of eleven proteins (PB2, PB1, PA, NP, and M1) with the homologous regions from 9 to 58 amino acids long. It is known that the HA protein has the 9 amino acid region that is a part of the fusion peptide (FGAIAGFIA) and is identical for all the influenza A viruses [65].

The antibodies directed to the conservative proteins PB2, PB1, PA, NP, and M1 do not have neutralizing activity but could play an important role in virus elimination by means of ADCC. It was shown that the NP protein could be temporarily expressed on the surface of the cell and the antibodies induced by this protein could also possess the neutralizing activity [66]. Moreover, the NP protein peptides, presented on MHC class I molecules, represent the most important targets for the cytotoxic CD8⁺ cells [28].

It was discovered that the M2 protein, which forms the ion transmembrane channel, also has significantly conservative structure. The part of M2 protein, which is located on the surface of the virus particle and consists of 23 amino acids (M2e), is considered to be an attractive target for the construction of the universal vaccine [67, 68]. The antibodies directed to M2e also do not neutralize the virus but promote the virus elimination from the infected cells by the ADCC mechanism. The most developed preparation that is now in phase I clinical trials has several fragments of M2e connected to the hepatitis B virus core (HBc) fusion protein [69, 70].

Several examples of generating a universal vaccine are based on HA2 subunit. The triple immunization of mice with peptides representing the ectodomain HA2 (23-185 amino acid residues) or the fusion peptide (1-38 amino acid residues) conjugated to the keyhole limpet hemocyanin (KLH) induced the cross-reactive immunity leading to a decrease in the animal mortality when challenged with a lethal dose of heterologous virus strain [71].

More effective protection was developed in the case of vaccination with chimeric HA constructs. Krammer *et al.* showed that heterosubtypic humoral immunity is induced in mice immunized with chimeric proteins, containing the HA globular parts from the viruses of different subtypes in combination with the HA stem region of the same virus [72-76]. Thus, animal immunization with the chimeras containing the HA globular part of H9, H6, and H5 viruses and the stem region from the H1N1 virus (PR8/34, group I) protected the animals from the weight loss and mortality when challenged with the viruses of the I group of H1N1, H5N1, and H6N1 subtypes, but did not protect them from

the virus of the II group of H3N2 subtype. Similar vaccines, containing the HA stem region from the virus of H3N2 subtype and the globular part from viruses of H4, H5, and H7 subtypes, protected the animals that were infected with different H3N2 viruses as well as with H7N1 and H10N7 viruses of group II [51]. The study of the molecular mechanism of immunity by the passive transfer of the antibodies from the immune to naive animals showed that antibodies play the major role in the protection of animals. The complicated immunization scheme that includes the animals electroporation using DNA and double intramuscular and intranasal immunization with the protein constructs supplemented with the adjuvant poly (I: C) are the shortcomings of this approach.

The use of the stabilized structures (mini-HA) generated by means of gene engineering, based on the amino acid sequence of the HA stem region of the H1N1 virus, serves as an example of a different approach to the generation of the universal influenza vaccine. Only the structures with the highest affinity to the antibodies that have a broad range of neutralizing activity were selected from the large library. The immunization of mice with these structures also protected the animals from death when challenged with highly pathogenic avian influenza virus of H5N1 subtype (group I) [77]. The complete protection of mice from death was achieved by the double intramuscular immunization with 30 µg of the purified mini-HA protein supplemented with the Matrix-M adjuvant produced by Novavax. The protective efficacy of this preparation was also proved in *cynomolgus monkeys (Macaca fascicularis)*. The animals that were immunized three times intramuscularly with 150 µg of the protein together with 50 µg of adjuvant Matrix-M (which is not licensed for humans) demonstrated a significantly lower body temperature than the control animals after infection with a sublethal dose of the A/Mexico/InDRE4487/2009 (H1N1) virus.

The other prospective direction in the development of the universal influenza vaccine is based on the design of the self-assembling nanoparticles that significantly enhance the immunogenic properties of HA [78]. The nanoparticles were generated based on the nucleotide sequence of the HA2 subunit of the A/New Caledonia/20/1999 virus. The HA stabilized-stem immunogen lacked the transmembrane and cytoplasmic regions of HA2 but contained additional mutations that stabilize the HA2 trimer structure. In order to ensure the self-assembling of nanoparticles, the subunit of the ferritin protein, isolated from the *Helicobacter pylori*, was

Table 1. Viral targets for broadly reactive influenza vaccines (reprinted with permission of WHO)

Protein antigen	Targeted function	Proposed mechanism of protection
Hemagglutinin (HA)	Receptor binding and membrane fusion	Inhibition of fusion, maturation of HA, ADCC
Ectodomain of M2 (M2e)	Ion channel	Complement-mediated lysis, ADCC, antibody-dependent NK cell activity
Neuraminidase (NA)	Cleaves sialic acid releasing virus from the surface of infected cells	Inhibition of viral spread
Matrix 1 (M1)	T cell stimulation	Cell lysis by CD8 ⁺ cytotoxic T lymphocytes (CTLs), CD4 ⁺ T lymphocyte-mediated cytolysis and B cell stimulation
Nucleoprotein (NP)		

attached to the C-end of the obtained HA stabilized-stem immunogen [79]. As a result, the spherical particles with 8 spikes on the surface were obtained. Their protective properties were studied in mouse and ferret models. The animals were immunized 2 or 3 times intramuscularly with nanoparticles supplemented with the new adjuvant SAS (Sigma Adjuvant System). The specificity of antibodies was determined by ELISA. It was shown that the induced antibodies were binding to the influenza viruses of group I (H1, H2, H5 and H9) and to a lesser extent - to the viruses of group II (H3 and H7). Although there were no antibodies revealed by HAI test, the antibodies neutralizing the viruses A/California/04/2009 and A/Singapore/6/1986 of the H1N1 subtype were detected. The antibodies to the viruses of H5N1, H2N2, and H9N2 subtypes were neither detected in mice nor in ferrets. In spite of the lack of the neutralizing antibodies to the virus of the H5N1 subtype in mice as well as in ferrets, these animals turned out to be completely protected from death when infected with a highly pathogenic H5N1 avian virus.

One of the modern technologies for the generation of live vaccine is based on the construction of the corresponding vaccine vector that enables to express the antigens of one virus by the other virus. Different DNA-containing viruses, namely: adenovirus [80], herpesvirus [81], baculovirus [82], or poxvirus [83], are used as the vectors for the expression of influenza antigens. Thus, the experiments with the adenovirus vector showed that the triple immunization with plasmid (50 µg) containing the sequences of the influenza virus A conservative proteins NP and M2, followed by intranasal infection with the two adenovirus vectors that express the same proteins, led to the complete protection of mice and ferrets from death and weight loss against challenge with the virus A/FM/1/47 (H1N1) or with the highly pathogenic strain of the avian influenza virus of the H5N1 subtype [84, 85]. Several adenovirus vectors that express the conservative influenza virus antigens developed for the intranasal immunization in humans are known to date [86].

All of the discussed approaches prove the possibility of the generation of a vaccine that will protect from infection with influenza viruses of group I as well as group II. However, none of the preparations described above present a vaccine that ensures protection from influenza virus B. Initially, influenza viruses A and B were separated based on the antigenic differences in NP and M proteins. The sequence homology of these proteins between influenza viruses A and B reaches only 36% and 27%, respectively. The sequence homology of the HA and NA proteins is even lower and makes up 18% and 20%, respectively. The only HA region almost completely identical in both types of viruses is the fusion peptide that is represented by the sequence (L/I/F)FGAIAGFIE(G/N)GW. It was shown that the antibodies generated after the immunization with this peptide attach to virus A (H1-H13) HA as well as to B viruses [85]. A protective epitope was also discovered in the HA stem region that induces the antibodies reacting with both influenza A and B viruses. The monoclonal antibody CR9114, directed to this epitope, protected mice from infection with influenza A and B viruses [87]. In addition, the T-cell CD4⁺ [69] and

CD8⁺ epitopes that recognize the HLA-A2.1 motif [88] were identified in the fusion peptide.

CONCLUSION

Based on the literature data analysis, it is possible to assume that the generation of the universal influenza vaccine that is capable of ensuring protection from all known antigenic variants of influenza A and B viruses is feasible. A number of such preparations are currently being developed. The progress of these research projects is under the control of the WHO. The main companies that are designing and developing the universal influenza vaccines and the principals of action of these vaccines are shown in the Table 2 [89].

Ideally, the universal influenza vaccine should induce the antibody and T-cell immune response to the conservative influenza virus epitopes. It is obvious that the efficacy of this vaccine would depend on the variety of the involved epitopes and on the Th1 polarization of the immune response. The analysis of the experimental results led the author to the conclusion that the generation of live intranasal vector vaccines using the influenza virus itself as a vector for the expression of the conservative antigenic determinants appears to be the most prospective approach in current research. These vaccine candidates are capable of inducing the enhanced Th1 systemic and the local mucosal immune response at the infection entry. The current research data prove that the vector technology based on the modification of the NS segment of influenza virus enables the generation of highly immunogenic recombinant virus strains that express the foreign sequences from the NS1 reading frame [90-96]. Clinical trials showed that viruses with the modified NS gene are safe and highly immunogenic [97]. The single immunization of mice with the vector strain based on the influenza virus A that express HA2 segments of the A and B influenza viruses from the NS1 open reading frame ensures the protection of animals from death not only after being challenged with influenza A viruses of other subtypes, but also after infection with the influenza virus B/Lee/40 (*unpublished results*). Therefore, the formation of the influenza vectors that additionally express the conservative epitopes together with the NS1 protein appears to be the prospective direction toward the generation of a universal influenza vaccine.

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Table 2. Development Status of Current Vaccine Candidates (reprinted with permission of WHO)

Organization Identifier	Approach, Target, Adjuvant	Pre-clinical	Phase 1	Phase 2	Phase 3
Novartis (USA)	Use of MF-59 adjuvant to achieve broadly cross-reactive antibody response.				X (and Phase 4)
	Rational antigen design (HA) based on preferential presentation of conserved epitopes for antibody response.	X			
VaxInnate (USA)	Fusion protein between influenza M2e and bacterial flagellin (TLR5 ligand). Self- adjuvanted. Proposed to be used with conventional TIV.			X	
Medicago (Canada)	Recombinant HA expressed as virus-like particle in tobacco plants. Requires adjuvant.			X	
Immune Targeting Systems (UK)	Six long peptides from four core influenza proteins conjugated to fluorocarbon chain, elicits strong T cell response, proposed to be used with conventional TIV.			X	
BiondVax Pharmaceuticals (Israel)	Proposed as “universal primer” to be followed by conventional TIV boost to potentiate HAI responses. Consists of a mixture of peptides comprising nine B and T cell conserved linear epitopes derived from three influenza proteins HA, M1, and NP.			X	
SEEK (formerly PepTcell, U.K.)	Mixture of 4 chemically synthesized peptides targeting conserved T cell epitopes present in M1, NPA, NPB and M2. Proposed to be used with oil-in-water adjuvant			X	
Flanders Institute (Belgium)	Fusion between M2e and hepatitis B virus core protein for virus-like particle expression and antibody-directed response.		X		
Inovio (USA)	DNA plasmids encoding consensus sequences of HA, NA, and NP delivered by intradermal electroporation for eliciting antibody and T cell responses.		X		
Dynavax (USA)	Fusion protein comprised of two highly conserved influenza antigens, NP, and M2e, covalently linked to proprietary immunostimulatory sequence. Envisioned to be used with conventional TIV.	X			
Antigen Express (USA)	Synthetic peptides derived from conserved B cell epitopes from HA modified with MHC Class 2 for facilitated Th activity. Envisioned to be combined with traditional seasonal vaccine for improved response.		X		
National Institute of Allergy and Infectious Diseases (USA)	Adenovirus encoding HA to prime followed by inactivated vaccine (TIV) boost.		X		
	Fusion protein between self-assembling ferritin protein and HA for nanoparticle presentation of HA.	X			
Jenner Institute, University of Oxford (UK)	Replication-deficient modified vaccinia Ankara (MVA) virus expressing both NP and M1. Designed for strong cross-reactive T cell response. Self -adjuvanted.		X		
	Replication-deficient simian adenovirus expressing both NP and M1. Designed for strong cross-reactive T cell response.		X		
	MVA expressing NP, M1 and conserved portion of HA.		X		
Wistar Institute (USA)	Fusion protein between M2e and NP, expressed in chimpanzee adenovirus vector.	X			
Gamma Vaccines (Australia)	Whole virion gamma-irradiated virus for intranasal application. Elicits B and T cell responses which are cross-protective. Self-adjuvanted.	X			
Florida Vaccine and Gene Therapy Institute (USA)	Computer optimized consensus HA sequence. Elicits broad antibody response. Alum adjuvanted.	X			
FluGen (USA)	Single-replication influenza virus which is not attenuated but unable to shed and designed to elicit humoral, mucosal, and cell mediated immunity.	X			
University of Maryland, College Park (USA)	Rearranged genome of influenza virus permitting expression of two HA on the same virus, while also attenuating it.	X			

Organization Identifier	Approach, Target, Adjuvant	Pre-clinical	Phase 1	Phase 2	Phase 3
Icahn School of Medicine at Mount Sinai (USA)	Various approaches to target conserved broadly reactive epitopes on HA stalk, such as “headless” HA or functional chimeric HA (comprised of non-matched “head” and “stalk”) expressed either in the context of whole virus or as a rHA. Use of recombinant cHA protein requires adjuvant.	X			
CureVac (Germany)	Synthetic mRNA encoding HA and NP. Temperature-stable product, elicits both B and T cell response, self-adjuvanted.	X			
University of Pennsylvania (USA)	Adenovirus expressing broadly-neutralizing monoclonal antibody against HA delivered by intranasal administration.	X			
Sanofi Pasteur (USA)	Multiple, including support for Flanders Institute and the Vaccine and Gene Therapy Institute. Internal work attempts to develop sequence-optimized HA.	X			
Georgia State University (USA)	M2e expressed in a virus-like particle (VLP).	X			
Merck (USA)	M2e-based vaccine comprised of peptide fusion to KLH carrier protein.	X			
Bionor (Norway)	Peptide-based approach targeting conserved epitopes.	X			
VBI (formerly Variation Biotechnologies)	Unique technology using a mixture of 8 to 32 peptides which represent hypervariable epitopes of HA to elicit polyclonal immune response	X			
University of Wisconsin (USA)	“Headless” HA expressed together with NA and M1 in <i>Drosophila</i> S2 cell line for induction of anti-stalk antibodies	X			

REFERENCES

- Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos AM, Posey DL, Klimov A, Lindstrom SE, Balish A, Medina MJ, Wallis TR, Guarner J, Paddock CD, Shieh WJ, Zaki SR, Sejvar JJ, Shay DK, Harper SA, Cox NJ, Fukuda K, Uyeki TM. Influenza-associated deaths among children in the United States, 2003-2004. *N Engl J Med* 2005; 353, 2559-67.
- Molinari NA, Ortega-Sanchez IR, Messonnier ML, Thompson WW, Wortley PM, Weintraub E, Bridges CB. The annual impact of seasonal influenza in the US: measuring disease burden and costs. *Vaccine* 2007; 25, 5086-96.
- Estimates of deaths associated with seasonal influenza --- United States, 1976-2007. *MMWR Morb Mortal Wkly Rep* 2010; 59, 1057-62.
- Girard MP, Cherian T, Pervikov Y, Kieny MP. A review of vaccine research and development: human acute respiratory infections. *Vaccine* 2005; 23, 5708-24.
- Shah NS, Greenberg JA, McNulty MC, Gregg KS, Riddell J, Mangino JE, Weber DM, Hebert CL, Marzec NS, Barron MA, Chaparro-Rojas F, Restrepo A, Hemmige V, Prasadthrasint K, Cobb S, Herwaldt L, Raabe V, Cannavino CR, Hines AG, Bares SH, Antiporta PB, Scardina T, Patel U, Reid G, Mohazabnia P, Kachhdiya S, Le BM, Park CJ, Ostrowsky B, Robicsek A, Smith BA, Schied J, Bhatti MM, Mayer S, Sikka M, Murphy-Aguilu I, Patwari P, Abeles SR, Torriani FJ, Abbas Z, Toya S, Doktor K, Chakrabarti A, Doblecki-Lewis S, Looney DJ, David MZ. Severe Influenza in 33 US Hospitals, 2013-2014: Complications and Risk Factors for Death in 507 Patients. *Infect Control Hosp Epidemiol* 2015; 36, 1251-60.
- Zheng W, Tao YJ. Structure and assembly of the influenza A virus ribonucleoprotein complex. *FEBS Lett* 2013; 587, 1206-14.
- Buehler J, Navi D, Lorusso A, Vincent A, Lager K, Miller CL. Influenza A virus PB1-F2 protein expression is regulated in a strain-specific manner by sequences located downstream of the PB1-F2 initiation codon. *J Virol* 2013; 87, 10687-99.
- Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. *PLoS Pathog* 2005; 1, e4.
- Bullough PA, Hughson FM, Skehel JJ, Wiley DC. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 1994; 371, 37-43.
- Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. *Science* 2009; 324, 246-51.
- Krystal M, Elliott RM, Benz EW, Jr., Young JF, Palese P. Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proc Natl Acad Sci U S A* 1982; 79, 4800-4.
- Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson

- JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO. New world bats harbor diverse influenza A viruses. *PLoS Pathog* 2013; 9, e1003657.
13. Jefferson T, Rivetti A, Di Pietrantonj C, Demicheli V, Ferroni E. Vaccines for preventing influenza in healthy children. *Cochrane Database Syst Rev* 2012; 8, CD004879.
 14. Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. *Lancet Infect Dis* 2011; 12, 36-44.
 15. Pfliegerer M, Trouvin JH, Brasseur D, Granstrom M, Shivji R, Mura M, Cavaleri M. Summary of knowledge gaps related to quality and efficacy of current influenza vaccines. *Vaccine* 2014; 32, 4586-91.
 16. Andersohn F, Bornemann R, Damm O, Frank M, Mittendorf T, Theidel U. Vaccination of children with a live-attenuated, intranasal influenza vaccine - analysis and evaluation through a Health Technology Assessment. *GMS Health Technol Assess* 2014; 10, Doc03.
 17. Skowronski DM, Chambers C, Sabaiduc S, De Serres G, Dickinson JA, Winter AL, Drews SJ, Fonseca K, Charest H, Gubbay JB, Petric M, Krajden M, Kwindt TL, Martineau C, Eshaghi A, Bastien N, Li Y. Interim estimates of 2014/15 vaccine effectiveness against influenza A(H3N2) from Canada's Sentinel Physician Surveillance Network, January 2015. *Euro Surveill* 2015; 20.
 18. Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 2012; 336, 1534-41.
 19. Linster M, van Boheemen S, de Graaf M, Schrauwen EJ, Lexmond P, Manz B, Bestebroer TM, Baumann J, van Riel D, Rimmelzwaan GF, Osterhaus AD, Matrosovich M, Fouchier RA, Herfst S. Identification, characterization, and natural selection of mutations driving airborne transmission of A/H5N1 virus. *Cell* 2014; 157, 329-39.
 20. Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 2012; 486, 420-8.
 21. Terajima M, Babon JA, Co MD, Ennis FA. Cross-reactive human B cell and T cell epitopes between influenza A and B viruses. *Virology* 2013; 10, 244.
 22. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng NY, Mays I, Garman L, Helms C, James J, Air GM, Capra JD, Ahmed R, Wilson PC. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. *Nature* 2008; 453, 667-71.
 23. Skehel JJ, Bayley PM, Brown EB, Martin SR, Waterfield MD, White JM, Wilson IA, Wiley DC. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc Natl Acad Sci U S A* 1982; 79, 968-72.
 24. Carragher DM, Kaminski DA, Moquin A, Hartson L, Randall TD. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *J Immunol* 2008; 181, 4168-76.
 25. Vareckova E, Mucha V, Wharton SA, Kostolansky F. Inhibition of fusion activity of influenza A haemagglutinin mediated by HA2-specific monoclonal antibodies. *Arch Virol* 2003; 148, 469-86.
 26. Terajima M, Co MD, Cruz J, Ennis FA. High Antibody-Dependent Cellular Cytotoxicity Antibody Titers to H5N1 and H7N9 Avian Influenza A Viruses in Healthy US Adults and Older Children. *J Infect Dis* 2015; 212, 1052-60.
 27. Jegaskanda S, Job ER, Kramski M, Laurie K, Isitman G, de Rose R, Winnall WR, Stratov I, Brooks AG, Reading PC, Kent SJ. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. *J Immunol* 2013; 190, 1837-48.
 28. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. *Emerg Infect Dis* 2006; 12, 48-54.
 29. Kreijtz JH, de Mutsert G, van Baalen CA, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. *J Virol* 2008; 82, 5161-6.
 30. Kreijtz JH, Bodewes R, van Amerongen G, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. *Vaccine* 2007; 25, 612-20.
 31. van de Sandt CE, Kreijtz JH, de Mutsert G, Geelhoed-Mieras MM, Hillaire ML, Vogelzang-van Trierum SE, Osterhaus AD, Fouchier RA, Rimmelzwaan GF. Human cytotoxic T lymphocytes directed to seasonal influenza A viruses cross-react with the newly emerging H7N9 virus. *J Virol* 2013; 88, 1684-93.
 32. Hillaire ML, Vogelzang-van Trierum SE, Kreijtz JH, de Mutsert G, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Human T-cells directed to seasonal influenza A virus cross-react with 2009 pandemic influenza A (H1N1) and swine-origin triple-reassortant H3N2 influenza viruses. *J Gen Virol* 2012; 94, 583-92.
 33. Bodewes R, Kreijtz JH, Geelhoed-Mieras MM, van Amerongen G, Verburgh RJ, van Trierum SE, Kuiken T, Fouchier RA, Osterhaus AD, Rimmelzwaan GF. Vaccination against seasonal influenza A/H3N2 virus reduces the induction of heterosubtypic immunity against influenza A/H5N1 virus infection in ferrets. *J Virol* 2011; 85, 2695-702.

34. Florek NW, Weinfurter JT, Jegaskanda S, Brewoo JN, Powell TD, Young GR, Das SC, Hatta M, Broman KW, Hungnes O, Dudman SG, Kawaoka Y, Kent SJ, Stinchcomb DT, Osorio JE, Friedrich TC. Modified vaccinia virus Ankara encoding influenza virus hemagglutinin induces heterosubtypic immunity in macaques. *J Virol* 2014; 88, 13418-28.
35. Nakayama M, Shichinohe S, Itoh Y, Ishigaki H, Kitano M, Arikata M, Pham VL, Ishida H, Kitagawa N, Okamatsu M, Sakoda Y, Ichikawa T, Tsuchiya H, Nakamura S, Le QM, Ito M, Kawaoka Y, Kida H, Ogasawara K. Protection against H5N1 highly pathogenic avian and pandemic (H1N1) 2009 influenza virus infection in cynomolgus monkeys by an inactivated H5N1 whole particle vaccine. *PLoS ONE* 2014; 8, e82740.
36. Hillaire ML, van Trierum SE, Kreijtz JH, Bodewes R, Geelhoed-Mieras MM, Nieuwkoop NJ, Fouchier RA, Kuiken T, Osterhaus AD, Rimmelzwaan GF. Cross-protective immunity against influenza pH1N1 2009 viruses induced by seasonal influenza A (H3N2) virus is mediated by virus-specific T-cells. *J Gen Virol* 2011; 92, 2339-49.
37. Wilkinson TM, Li CK, Chui CS, Huang AK, Perkins M, Liebner JC, Lambkin-Williams R, Gilbert A, Oxford J, Nicholas B, Staples KJ, Dong T, Douek DC, McMichael AJ, Xu XN. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. *Nat Med* 2012; 18, 274-80.
38. McMichael AJ, Gotch FM, Noble GR, Beare PA. Cytotoxic T-cell immunity to influenza. *N Engl J Med* 1983; 309, 13-7.
39. Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, Bean T, Barclay W, Deeks JJ, Lalvani A. Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med* 2013; 19, 1305-12.
40. McKinstry KK, Strutt TM, Kuang Y, Brown DM, Sell S, Dutton RW, Swain SL. Memory CD4+ T cells protect against influenza through multiple synergizing mechanisms. *J Clin Invest* 2012; 122, 2847-56.
41. Boonnak K, Subbarao K. Memory CD4+ T cells: beyond «helper» functions. *J Clin Invest* 2012; 122, 2768-70.
42. Altenburg AF, Rimmelzwaan GF, de Vries RD. Virus-specific T cells as correlate of (cross-)protective immunity against influenza. *Vaccine* 2014; 33, 500-6.
43. LaMere MW, Lam HT, Moquin A, Haynes L, Lund FE, Randall TD, Kaminski DA. Contributions of antinucleoprotein IgG to heterosubtypic immunity against influenza virus. *J Immunol* 2011; 186, 4331-9.
44. Hillaire ML, Osterhaus AD, Rimmelzwaan GF. Induction of virus-specific cytotoxic T lymphocytes as a basis for the development of broadly protective influenza vaccines. *J Biomed Biotechnol* 2011; 2011, 939860.
45. Gianfrani C, Oseroff C, Sidney J, Chesnut RW, Sette A. Human memory CTL response specific for influenza A virus is broad and multispecific. *Hum Immunol* 2000; 61, 438-52.
46. Taylor DN, Treanor JJ, Sheldon EA, Johnson C, Umlauf S, Song L, Kavita U, Liu G, Tussey L, Ozer K, Hofstaetter T, Shaw A. Development of VAX128, a recombinant hemagglutinin (HA) influenza-flagellin fusion vaccine with improved safety and immune response. *Vaccine* 2012; 30, 5761-9.
47. Steel J, Lowen AC, Pena L, Angel M, Solorzano A, Albrecht R, Perez DR, Garcia-Sastre A, Palese P. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. *J Virol* 2009; 83, 1742-53.
48. Terajima M, Cruz J, Co MD, Lee JH, Kaur K, Wrarmert J, Wilson PC, Ennis FA. Complement-dependent lysis of influenza A virus-infected cells by broadly cross-reactive human monoclonal antibodies. *J Virol* 2011; 85, 13463-7.
49. Jegaskanda S, Weinfurter JT, Friedrich TC, Kent SJ. Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques. *J Virol* 2013; 87, 5512-22.
50. Moody MA, Zhang R, Walter EB, Woods CW, Ginsburg GS, McClain MT, Denny TN, Chen X, Munshaw S, Marshall DJ, Whitesides JF, Drinker MS, Amos JD, Gurley TC, Eudailey JA, Foulger A, DeRosa KR, Parks R, Meyerhoff RR, Yu JS, Kozink DM, Barefoot BE, Ramsburg EA, Khurana S, Golding H, Vandergrift NA, Alam SM, Tomaras GD, Kepler TB, Kelsoe G, Liao HX, Haynes BF. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. *PLoS ONE* 2011; 6, e25797.
51. Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, Palucka K, Garcia-Sastre A, Palese P, Treanor JJ, Krammer F. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. *J Virol* 2013; 87, 4728-37.
52. Corti D, Suguitan AL, Jr., Pinna D, Silacci C, Fernandez-Rodriguez BM, Vanzetta F, Santos C, Luke CJ, Torres-Velez FJ, Temperton NJ, Weiss RA, Sallusto F, Subbarao K, Lanzavecchia A. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. *J Clin Invest* 2010; 120, 1663-73.
53. Krammer F, Pica N, Hai R, Tan GS, Palese P. Hemagglutinin Stalk-Reactive Antibodies Are Boosted following Sequential Infection with Seasonal and Pandemic H1N1 Influenza Virus in Mice. *J Virol* 2012; 86, 10302-7.
54. Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, Tan GS, Krause JC, Moran T, Stein CR, Banach D, Wrarmert J, Belshe RB, Garcia-Sastre A, Palese P. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. *Proc Natl Acad Sci U S A* 2012; 109, 2573-8.

55. He XS, Sasaki S, Baer J, Khurana S, Golding H, Treanor JJ, Topham DJ, Sangster MY, Jin H, Dekker CL, Subbarao K, Greenberg HB. Heterovariant cross-reactive B-cell responses induced by the 2009 pandemic influenza virus A subtype H1N1 vaccine. *J Infect Dis* 2012; 207, 288-96.
56. Tamura M, Webster RG, Ennis FA. Antibodies to HA and NA augment uptake of influenza A viruses into cells via Fc receptor entry. *Virology* 1991; 182, 211-9.
57. Gauger PC, Vincent AL, Loving CL, Henningson JN, Lager KM, Janke BH, Kehrli ME, Jr., Roth JA. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. *Vet Pathol* 2012; 49, 900-12.
58. Gauger PC, Vincent AL, Loving CL, Lager KM, Janke BH, Kehrli ME, Jr., Roth JA. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (delta-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. *Vaccine* 2011; 29, 2712-9.
59. Kitikoon P, Nilubol D, Erickson BJ, Janke BH, Hoover TC, Sornsen SA, Thacker EL. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. *Vet Immunol Immunopathol* 2006; 112, 117-28.
60. Vincent AL, Lager KM, Janke BH, Gramer MR, Richt JA. Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine. *Vet Microbiol* 2008; 126, 310-23.
61. Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Henningson J, Vincent AL, Golding H. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. *Sci Transl Med* 2013; 5, 200ra114.
62. Vincent AL, Ma W, Lager KM, Richt JA, Janke BH, Sandbulte MR, Gauger PC, Loving CL, Webby RJ, Garcia-Sastre A. Live attenuated influenza vaccine provides superior protection from heterologous infection in pigs with maternal antibodies without inducing vaccine-associated enhanced respiratory disease. *J Virol* 2012; 86, 10597-605.
63. Gauger PC, Loving CL, Khurana S, Lorusso A, Perez DR, Kehrli ME, Jr., Roth JA, Golding H, Vincent AL. Live attenuated influenza A virus vaccine protects against A(H1N1)pdm09 heterologous challenge without vaccine associated enhanced respiratory disease. *Virology* 2014; 471-473, 93-104.
64. WHO Product Development for Vaccines Advisory Committee (PD-VAC) meeting – 2015. Available (June 27, 2016): http://who.int/immunization/research/meetings_workshops/pdvac/en/
65. Heiny AT, Miotto O, Srinivasan KN, Khan AM, Zhang GL, Brusci V, Tan TW, August JT. Evolutionarily conserved protein sequences of influenza A viruses, avian and human, as vaccine targets. *PLoS ONE* 2007; 2, e1190.
66. Virelizier JL, Allison AC, Oxford JS, Schild GC. Early presence of ribonucleoprotein antigen on surface of influenza virus-infected cells. *Nature* 1977; 266, 52-4.
67. 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.
68. Stepanova LA, Kotlyarov RY, Kovaleva AA, Potapchuk MV, Korotkov AV, Sergeeva MV, Kasianenko MA, Kuprianov VV, Ravin NV, Tsybalova LM, Skryabin KG, Kiselev OI. Protection against multiple influenza A virus strains induced by candidate recombinant vaccine based on heterologous M2e peptides linked to flagellin. *PLoS ONE* 2015; 10, e0119520.
69. Neirynek 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.
70. De Filette M, Ramne A, Birkett A, Lycke N, Lowenadler 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.
71. Stanekova Z, Kiraly J, Stropkova A, Mikuskova T, Mucha V, Kostolansky F, Vareckova E. Heterosubtypic protective immunity against influenza A virus induced by fusion peptide of the hemagglutinin in comparison to ectodomain of M2 protein. *Acta Virol* 2011; 55, 61-7.
72. Krammer F, Palese P, Steel J. Advances in universal influenza virus vaccine design and antibody mediated therapies based on conserved regions of the hemagglutinin. *Curr Top Microbiol Immunol* 2014; 386, 301-21.
73. Krammer F, Hai R, Yondola M, Tan GS, Leyva-Grado VH, Ryder AB, Miller MS, Rose JK, Palese P, Garcia-Sastre A, Albrecht RA. Assessment of influenza virus hemagglutinin stalk-based immunity in ferrets. *J Virol* 2014; 88, 3432-42.
74. Krammer F, Palese P. Universal influenza virus vaccines: need for clinical trials. *Nat Immunol* 2013; 15, 3-5.
75. Krammer F, Palese P. Influenza virus hemagglutinin stalk-based antibodies and vaccines. *Curr Opin Virol* 2013; 3, 521-30.
76. Krammer F, Pica N, Hai R, Margine I, Palese P. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. *J Virol* 2013; 87, 6542-50.
77. Impagliazzo A, Milder F, Kuipers H, Wagner MV, Zhu X, Hoffman RM, van Meersbergen R, Huizingh J, Wannigen P, Verspuij J, de Man M, Ding Z, Apetri A, Kukrer B, Sneekes-Vriese E, Tomkiewicz D, Laursen NS, Lee PS, Zakrzewska A, Dekking L, Tolboom J, Tettero L, van Meerten S, Yu W, Koudstaal W, Goudsmit J,

- Ward AB, Meijberg W, Wilson IA, Radosevic K. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science* 2015; 349, 1301-6.
78. Kanekiyo M, Wei CJ, Yassine HM, McTamney PM, Boyington JC, Whittle JR, Rao SS, Kong WP, Wang L, Nabel GJ. Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. *Nature* 2013; 499, 102-6.
79. Yassine HM, Boyington JC, McTamney PM, Wei CJ, Kanekiyo M, Kong WP, Gallagher JR, Wang L, Zhang Y, Joyce MG, Lingwood D, Moin SM, Andersen H, Okuno Y, Rao SS, Harris AK, Kwong PD, Mascola JR, Nabel GJ, Graham BS. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med* 2015; 21, 1065-70.
80. Coughlan L, Mullarkey C, Gilbert S. Adenoviral vectors as novel vaccines for influenza. *J Pharm Pharmacol* 2015; 67, 382-99.
81. Dudek T, Knipe DM. Replication-defective viruses as vaccines and vaccine vectors. *Virology* 2006; 344, 230-9.
82. He F, Madhan S, Kwang J. Baculovirus vector as a delivery vehicle for influenza vaccines. *Expert Rev Vaccines* 2009; 8, 455-67.
83. Draper SJ, Cottingham MG, Gilbert SC. Utilizing poxviral vectored vaccines for antibody induction-progress and prospects. *Vaccine* 2013; 31, 4223-30.
84. Price GE, Soboleski MR, Lo CY, Mispilon JA, Pappas C, Houser KV, Tumpey TM, Epstein SL. Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. *Vaccine* 2009; 27, 6512-21.
85. Chun S, Li C, Van Domselaar G, Wang J, Farnsworth A, Cui X, Rode H, Cyr TD, He R, Li X. Universal antibodies and their applications to the quantitative determination of virtually all subtypes of the influenza A viral hemagglutinins. *Vaccine* 2008; 26, 6068-76.
86. Sedova ES, Shcherbinin DN, Migunov AI, Smirnov Iu A, Logunov D, Shmarov MM, Tsybalova LM, Naroditskii BS, Kiselev OI, Gintsburg AL. Recombinant influenza vaccines. *Acta Naturae* 2013; 4, 17-27.
87. Dreyfus C, Laursen NS, Kwaks T, Zuijdgheest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, van der Vlugt R, Lamrani M, Korse HJ, Geelen E, Sahin O, Sieuwerts M, Brakenhoff JP, Vogels R, Li OT, Poon LL, Peiris M, Koudstaal W, Ward AB, Wilson IA, Goudsmit J, Friesen RH. Highly conserved protective epitopes on influenza B viruses. *Science* 2012; 337, 1343-8.
88. Kosor Krnic E, Gagro A, Drazenovic V, Kuzman I, Jeren T, Cecuk-Jelicic E, Kerhin-Brkljacic V, Gjenero-Margan I, Kaic B, Rakusic S, Sabioncello A, Markotic A, Rabatic S, Mlinaric-Galinovic G, Dekaris D. Enumeration of haemagglutinin-specific CD8+ T cells after influenza vaccination using MHC class I peptide tetramers. *Scand J Immunol* 2008; 67, 86-94.
89. Status of Vaccine Research and Development of Universal Influenza Vaccine Prepared for WHO PD-VAC. 2015. Available (June 27, 2016): http://who.int/immunization/research/meetings_workshops/Universal_Influenza_VaccineRD_Sept2014.pdf, 7-9.
90. Tabynov K, Sansyzybay A, Kydyrbayev Z, Yespembetov B, Ryskeldinova S, Zinina N, Assanzhanova N, Sultankulova K, Sandybayev N, Khairullin B, Kuznetsova I, Ferko B, Egorov A. Influenza viral vectors expressing the Brucella OMP16 or L7/L12 proteins as vaccines against *B. abortus* infection. *Virology* 2014; 11, 69.
91. Sereinig S, Stukova M, Zabolotnyh N, Ferko B, Kittel C, Romanova J, Vinogradova T, Katinger H, Kiselev O, Egorov A. Influenza virus NS vectors expressing the mycobacterium tuberculosis ESAT-6 protein induce CD4+ Th1 immune response and protect animals against tuberculosis challenge. *Clin Vaccine Immunol* 2006; 13, 898-904.
92. Stukova MA, Sereinig S, Zabolotnyh NV, Ferko B, Kittel C, Romanova J, Vinogradova TI, Katinger H, Kiselev OI, Egorov A. Vaccine potential of influenza vectors expressing Mycobacterium tuberculosis ESAT-6 protein. *Tuberculosis (Edinb)* 2006; 86, 236-46.
93. Kittel C, Ferko B, Kurz M, Voglauer R, Sereinig S, Romanova J, Stiegler G, Katinger H, Egorov A. Generation of an influenza A virus vector expressing biologically active human interleukin-2 from the NS gene segment. *J Virol* 2005; 79, 10672-7.
94. Kittel C, Sereinig S, Ferko B, Stasakova J, Romanova J, Wolkerstorfer A, Katinger H, Egorov A. Rescue of influenza virus expressing GFP from the NS1 reading frame. *Virology* 2004; 324, 67-73.
95. Ferko B, Stasakova J, Sereinig S, Romanova J, Katinger D, Niebler B, Katinger H, Egorov A. Hyperattenuated recombinant influenza A virus nonstructural-protein-encoding vectors induce human immunodeficiency virus type 1 Nef-specific systemic and mucosal immune responses in mice. *J Virol* 2001; 75, 8899-908.
96. Ferko B, Katinger D, Grassauer A, Egorov A, Romanova J, Niebler B, Katinger H, Muster T. Chimeric influenza virus replicating predominantly in the murine upper respiratory tract induces local immune responses against human immunodeficiency virus type 1 in the genital tract. *J Infect Dis* 1998; 178, 1359-68.
97. Wacheck V, Egorov A, Groiss F, Pfeiffer A, Fuereder T, Hoeflmayer D, Kundi M, Popow-Kraupp T, Redlberger-Fritz M, Mueller CA, Cinatl J, Michaelis M, Geiler J, Bergmann M, Romanova J, Roethl E, Morokutti A, Wolschek M, Ferko B, Seipelt J, Dick-Gudenus R, Muster T. A novel type of influenza vaccine: safety and immunogenicity of replication-deficient influenza virus created by deletion of the interferon antagonist NS1. *J Infect Dis* 2009; 201, 354-62.