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REVIEW

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Influenza vaccine: Where are we and where do we go?

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Summary

The alarming rise of morbidity and mortality caused by influenza pandemics and epidemics has drawn attention worldwide since the last few decades. This life-threatening problem necessitates the development of a safe and effective vaccine to protect against incoming pandemics. The currently available flu vaccines rely on inactivated viral particles, M2e-based vaccine, live attenuated influenza vaccine (LAIV) and virus like particle (VLP). While inactivated vaccines can only induce systemic humoral responses, LAIV and VLP vaccines stimulate both humoral and cellular immune responses. Yet, these vaccines have limited protection against newly emerging viral strains. These strains, however, can be targeted by universal vaccines consisting of conserved viral proteins such as M2e and capable of inducing cross-reactive immune response. The lack of viral genome in VLP and M2e-based vaccines addresses safety concern associated with existing attenuated vaccines. With the emergence of new recombinant viral strains each year, additional effort towards developing improved universal vaccine is warranted. Besides various types of vaccines, microRNA and exosome-based vaccines have been emerged as new types of influenza vaccines which are associated with new and effective properties. Hence, development of a new generation of vaccines could contribute to better treatment of influenza.

KEYWORDS

adverse effects, immunity, viral vaccines

1 | INACTIVATED INFLUENZA VACCINE

Monovalent influenza vaccine was first produced after the isolation of influenza A virus (H1N1) from outbreak-associated cases of influenza in Puerto Rico in 1934. Subsequently, in 1942, 2 years after isolation of type B influenza virus, first bivalent influenza vaccine was tested for efficacy in military recruits. Then, this whole-virus inactivated vaccine was approved for use in civilian populations of the United States in 1945. Trivalent influenza vaccine was introduced in 1977 and contains two representative strains of type A (ie, H1N1, H3N2) and one of

Abbreviations: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; AEs, Adverse events; AV, Attenuated vaccine; BMDCs, Bone marrow dendritic cells; BMMΦ, Bone marrow macrophage; CaMKIV, Calcium/calmodulin-dependent protein kinase IV; CRP, C-reactive protein; CTLs, Cytotoxic T lymphocytes; DCs, Dendritic cells; dsRNA, Double strand RNA; HA, Hemagglutinin; IL-6, *Interleukin-6*; IRF1, Interferon regulatory factor 1; LAIV, Live attenuated influenza vaccine; LncRNA, Long noncoding RNA; *M2e*, Matrix protein 2; MHC, Major histocompatibility complex; MRE, *MiRNA response element*; MΦ, Human macrophage; NA, Neuraminidase; PAMP, Pathogen-associated molecular pattern; PEI, Polyethylenimine; rRNA, Ribosomal RNA; SAM, Self-amplifying mRNA; saRNA, Small activating RNAs; ssRNA, Single-stranded RNA virus; TCR, T-cell antigen receptor; TLR, Toll-like receptor; TNFα, Tumor necrosis factor alpha; VLP, Virus like particle

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the two strains of type B. Quadrivalent influenza vaccines were developed during 2013-14 influenza season in the United States and contain the same A strains and two strains of influenza B lineages.[1] These vaccines have high intrinsic immunogenicity that makes them effective against pandemic.[2,3]

International surveillance is performed annually by the World Health Organization[4] to choose appropriate virus strains for inclusion in seasonal influenza vaccines. These selected strains provide HA and NA genes for reassortant vaccine viruses, which also possess internal genes from A/Puerto Rico/8/34 (PR8). PR8 is a strain that confers high viral yield in embryonated eggs. Formalin or β-propiolactone is used to inactivate influenza viruses. Thiomersal or formaldehyde is used in vaccines as a preservative in order to inhibit bacterial or fungal growth. Some manufacturers may also use aminoglycoside antibiotics. Some vaccines contain additives like gelatine to stabilize them in unfavorable conditions. Residual egg proteins are also present in low quantities in egg-derived inactivated vaccines.[5,6] Although virus multiplication on embryonated chicken eggs remains the most common method for production of influenza vaccines, it contains several limitations. For instance, securing sufficient qualified eggs for mass vaccine production of the vaccine is very challenging, especially in context of influenza outbreak among poultry.[7] Furthermore, optimization of influenza wild type strains for growth in eggs requires recombination of these strains with high-yield laboratory strains. Thereupon, mutations in the egg-adapted reassortant strain can contribute to a mismatch between the vaccine strain and the circulating strain which was reported recently.[8]

Trivalent inactivated vaccination can induce local and systemic immune responses (Figure 1). Influenza-specific antibodies including IgG especially IgG1 (high concentration), IgA, and IgM (low concentration) are detectable 2 to 6 days post-vaccination and peak 2 to 3 weeks after vaccination in primed subjects.[9-13] Upon vaccination, antibody production is typically type specific. However, it can be highly cross-reactive which results in protection towards earlier and newer viral strains.[14,15]

It has been shown that Influenza inactivated vaccines up-regulate three activation markers of MHC II (CD40, CD80, and CD86), while exposure to active virus and subunit (SU) vaccine has a less similar effect on CD86 expression. Studies show that treatment of DCs with SU vaccines results in similar levels of IL-6 and TNFα. In contrast, in influenza attenuated vaccine (AV) and IV-treated DC cultures, IL-6 and TNFα levels were clearly increased.[16]

Live or inactivated influenza virus can induce production of IFN-α and inflammatory cytokines from plasmacytoid DC (pDCs) in a Toll-like receptor (TLR7) and MyD88-dependent manner by recognition of viral single-stranded RNA virus (ssRNA).[17] Signaling of TLR7 in pDCs has an important role in inducing productive antibody response by virion RNA-containing split vaccine[18] and inactivated whole virus vaccine.[19] Conversely, following influenza virus infection in the lungs, cytokine production did not require TLR7-signaling in pDCs.[20]

Similar to live viral vaccines, the live attenuated influenza vaccine (LAIV) induces the expression of several interferon-related genes. Whereas, the TIV vaccine induces a signature composed of genes highly expressed in plasma B cells. In the case of TIV, there are 44 genes identified to accurately predict the outcome of immunization as either high or low antibody titres. Of these, the calcium/calmodulin-dependent protein kinase IV (CaMKIV) gene has no known role in regulating immunity, but has a negative correlation with antibody titres. In agreement with this finding, high antibody titres were observed in the CAMKIV-deficient mice after vaccination.[21]

There are comprehensive data about safety properties of inactivated influenza vaccines. Soreness at the vaccination site is a very common local reaction. These kinds of reactions are self-limiting and require no intervention. Systemic reactions such as fever, headache, myalgia, or any physical unease happen mostly in children and due to initial exposure to influenza vaccine antigens. These mild adverse events (AEs) are found within 6 to 12 hours post-immunization and last for 1 to 2 days.[22] Figure 2 and Table 1 illustrate various forms of influenza vaccines.

2 | LIVE ATTENUATED INFLUENZA VACCINE

Customary live influenza vaccines are usually attenuated by adapting them to replicate at lower temperatures. These cold adapted virions

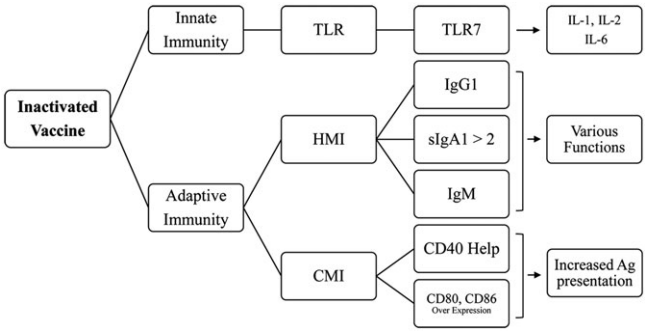

FIGURE 1 Inactive influenza vaccine. Trivalent inactivated vaccines can induce innate and adaptive immune responses. Inactivated influenza virus induced high level production of IFN-α and inflammatory cytokines such as IL6, IL1, and TNF-a from pDCs in a TLR7-and MyD88-dependent manner by recognition of viral ssRNA. Signaling of TLR7 in pDCs has an important role for inducing productive. Influenza-specific IgG antibodies especially IgG1 (high concentration), IgA, and IgM (low concentration) produced in primed subjects. SIgA1 with lower concentrations of SIgA2 are the major antibody response in the oral fluid. Inactivated vaccination results in obvious up-regulation of each of the three activation markers of MHC II (CD40, CD80, and CD86); high expression of this molecules on antigen presenting cells (ex: DC, MQ) results in increased antigen presentation process and then high activation of innate and adaptive immune responses.

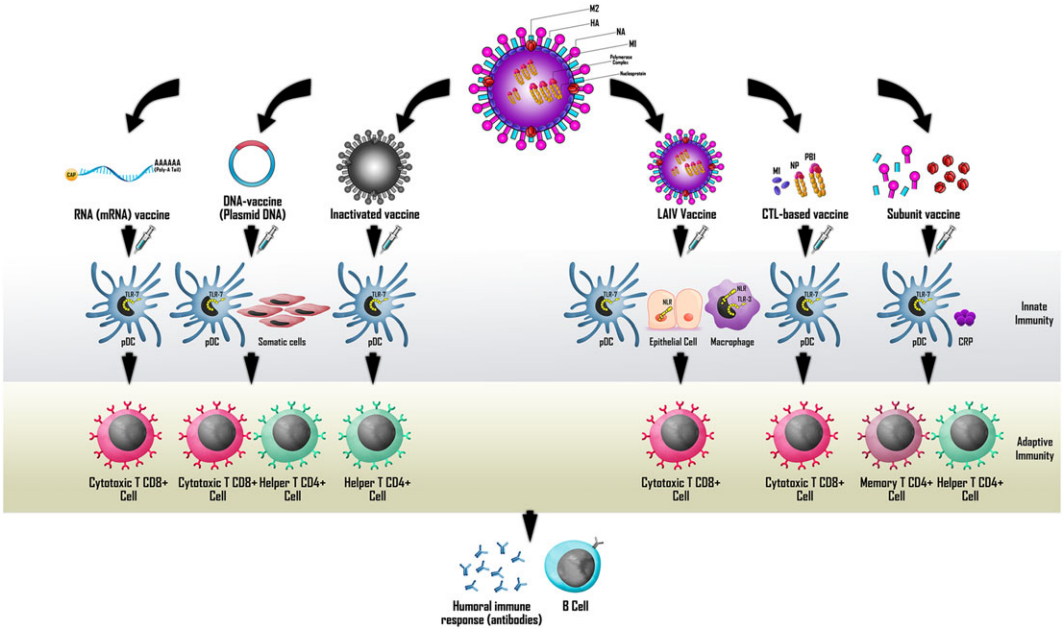

FIGURE 2 Various forms of influenza vaccine. Application use of the vaccine of the complete influenza virus (LAIV), surface proteins (subunit vaccine), nucleoproteins (CTL-based vaccine), viral genomes (DNA vaccine), and inactive viruses (inactivated vaccine) is shown in the figure. Looking at which distinct types of vaccines, different immunological pathways are induced in innate and adaptive immunity. Finally, the stimulation of the B lymphocytes and the production of specific antibodies secreting plasma cells.

are generated by re-assortment of a cold-adapted virus with seasonal influenza A virus. There are additional methods for virus attenuation. For example, the NS1-based escape mutants of influenza virus can be applied to create live-attenuated influenza viruses by elimination of NS1 through truncation or deletion of the corresponding gene.[23-25] Another way is modification of viral M1 protein, which can induce protective humoral and cellular immune responses against homologous and heterologous influenza viruses in mice.[26]

Live attenuated vaccination induces both secretory (mucosal response) and systemic immune responses, which is closely similar to immune response observed upon natural infection. Previously, in young children, live AVs were administered by nasal drops[27]; currently, this vaccine is administered intranasally. However, immunogenicity between the two approaches is similar. Mucosal response induced by live attenuated vaccination is characterized by IgA antibodies in nasal secretions, which peak 2 to 11 weeks after vaccination and slowly decrease by 6 months to 1 year post-vaccination in children (Figure 3).[28,29]

Live attenuated (cold-adapted) influenza vaccines are likely to activate TLR3 and -7 during viral replication intracellularly leading to the up-regulation of inflammatory cytokines, and thus adjuvants are not needed.[30]

Studies have shown that influenza virus infection induces the expression and activation of NLRP3 inflammasome components (NLRP3, ASC, and Caspase-1).[31] The NLRP3 inflammasome components mediate IL-1β and IL-18 production in different cell types in vitro including mice bone marrow dendritic cells (BMDCs) and bone marrow macrophage (BMMΦ), human macrophage (MΦ), nasal airway epithelial cells, and monocytic cell line THP-1.[32-35] For this reason, the NLRP3 inflammasome-deficient mice did not produce IL-1β and IL-18 following the high lethal dose influenza virus infection. Hence, the reduced protective inflammation including the suppressed accumulation of neutrophils and monocytes to the lungs and airways and consequently higher mortality upon influenza virus were observed in NLRP3-deficient mice.[35]

Due to the previously mentioned side effects, it seems that inactivated influenza vaccines are no longer such safe options for immunization, and, as a result, researchers are trying to develop alternative vaccines such as live AVs and VLPs, which are expected to have lower adverse effects; however, some studies have reported opposite results. Higher rate of respiratory AEs and hospitalizations was observed among children receiving LAIV compared with trivalent inactivated influenza vaccine recipients.[36] A study indicates that despite of higher rate of AEs like respiratory inflammatory responses in LAIV vaccinated individuals in comparison with TIV vaccines, Guillain-Barre Syndrome and paralysis are significantly more abundant in TIV recipients.[37] Although fever, abdominal pain, and other symptoms are reported after LAIV vaccination, it is indicated that overall various AEs like asthma/wheezing do not increase significantly among LAIV vaccinated children compared with controls.[38]

3 | RECOMBINANT SUBUNIT VACCINES

The use of recombinant SU influenza vaccines can solve the problems associated with the use of chicken embryos and the urgency to attenuate strains of the influenza virus. One of the new methods to the manufacture of SU influenza vaccines is use of different expression systems for rapid production of individual viral proteins in preparative levels. Baculoviral expression systems produced influenza antigens in insect cells by using baculoviral vectors that transport the genes of the target antigens. The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) genome is used in Sf9 cell lines obtained from *Spodoptera frugiperda* for production of different

TABLE 1 Advantages and disadvantages of different influenza vaccines

Vaccine Type	Advantage	Disadvantage
Inactive vaccine	a) All age groups (except children under 6 months) with no contraindications can receive inactivated influenza vaccine b) Safe in pregnant women c) Use in immunocompromised patients	a) Soreness at the vaccination site,fever, headache, myalgia, or any physical unease happen mostly in children b) Allergy c) In rare case autoimmune disorders
Live attenuate	a) Safe in cystic fibrosis patients b) No systemic allergic reactions such as urticaria, angioedema, rhinitis, and eczema	a) Mild to moderate symptoms including runny nose, sneezing, nasal discomfort, fever and headache b) Is not recommended to be routinely used in pregnant women
Recombinant	a) High safety profile without involving infectious viruses b) Rapid, stable c) Induces humoral and cellular immune responses	a) Low immunogenicity b) Require appropriate adjuvants
DNA vaccine	a) Induce all three arms of adaptive immunity, CTLs, antibodies, and helper T cells b) Possible mucosal delivery and thus may stimulate innate immunity	a) Lower immunogenicity, low level of T-cell, and B-cell memory due to b) Integration of DNA vaccine genetic material into cellular or host DNA, c) Development of autoimmune disorders against host DNA
Universal vaccine	M2e: a) Induces M2e-specific humoral and cellular immune responses; b) Elicits broad cross-protection against divergent virus strains Epitope-base: a) They are considered to be safe, easy to produce, and stable. b) Can induce B-cell and T-cell in the same formulation	a) Single M2e molecule induces lower immune responses a) The main disadvantage of the epitope-based vaccine is that algorithms may fail to predict all the appropriate epitopes
CTL inducing vaccine	a) Target conserved influenza virus proteins and improve recovery and inhibit disease progression	a) Need to have an epitope that can be recognized by all major histocompatibility complex (MHC)
RNA vaccine	a) Safety b) Efficacy c) Higher potency (especially with self-amplifying RNA vaccines)	a) Possibility of adverse consequences like thrombus and/or edema b) Limited availability in cases of pandemic and endemic diseases.

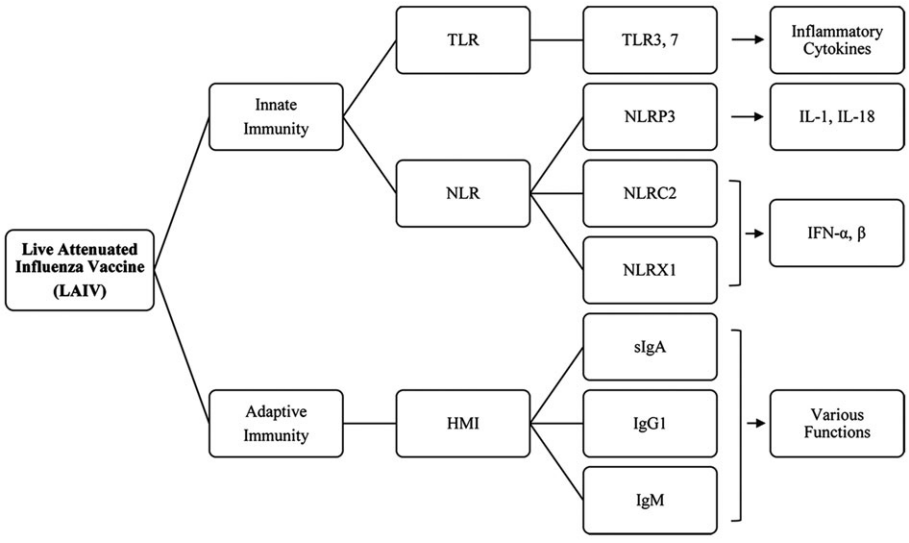

FIGURE 3 Live attenuated influenza vaccine. Live attenuated vaccination induces both innate and adaptive immune response. Among all receptors in the innate immune system, TLRs and NLRs have an important role. Live attenuated influenza vaccines activate TLR3 and 7 leading to the up-regulation of inflammatory cytokines. NLRs have a differential role in response to influenza virus infection in order to innate immunity balance. Expression and activation of NLRP3 inflammasome components (NLRP3, ASC, and Caspase-1) mediate IL-1β and IL-18 production in different cell types. NLRC2 (or NOD2) recruit MAVS adaptor protein and type I IFN production in DCs and MΦ after recognizes the viral genomic ssRNA in response to influenza virus. In addition, NLRX1 promotes both MΦ survival as well as type I IFN signaling in mice after binding to viral protein PB1-F2. Mucosal response induced by live attenuated vaccination is characterized by IgA antibodies in nasal. Systemic immune response against live attenuated vaccine characterized by production of IgA and IgM in serum. IgG1 is the major IgG subclass produced by live attenuated vaccination in the serum.

antigens of the influenza A virus. The main disadvantage of this recombinant SU vaccine is low immunogenicity; therefore, the need for repeated vaccination and use of adjuvants are recommended.[39,40]

As mentioned, use of adjuvants in the composition of SU vaccines can resolve this problem. The recombinant protein STF2.4 × M2e that includes flagellin and produced in *Escherichia coli* cells has protected mice to a lethal dose of the influenza virus. In another study, safety and efficacy of a vaccine based on this construct were illustrated in adult volunteers. Immunization of mice with the recombinant fusion protein 4 × M2e. HSP70c demonstrates a reduced viral titer in the lungs, significant decrease in weight loss, and a less pronounced manifestation of the symptoms of the disease after challenge mice groups with a lethal dose of the influenza A H1N1, H3N2, or H9N2 viruses.[41]

The most favorable candidate for influenza SU vaccines is M2 protein (the ion channel-forming protein). M2 is a highly conserved protein expressed on the virion's surface. The M2 protein ectodomain (M2e) is regarded as a candidate for designing broad-spectrum vaccines. Because of low immunogenicity, using this type of vaccines needs adjuvants and repeated vaccination (Figure 4). Molecular adjuvants such as ligands of several receptors of the innate immunity system can be used in combination with SU vaccines. One study shows that the recombinant protein StF2.4 × M2e produced in *Escherichia coli* cells, which includes flagellin (the toll-like receptor 5 (tLr-5) ligand), causes immunization against a lethal dose of the influenza virus in mice.[42]

4 | VIRUS LIKE PARTICLE (VLP) VACCINE

In recent years, VLP vaccines are under precise study as potential candidates for vaccination against influenza. Produced VLPs are devoid of viral genomes which reflects high safety of these vaccines. Glycoproteins of these particles are not exposed to destructive fixatives, and their membrane-anchored state imitates the conformation of native viral glycoproteins. VLPs can effectively stimulate APCs especially dendritic cells and also induce both humoral and cellular responses as well.[43-49] These vaccines are able to induce immune responses of $CD4^+$ T-cells as well as cytotoxic T-cells.[48,50,51] Headless HA2 protein expressed on VLPs can induce cross-reactive antibody response which is effective against heterologous influenza strains in vivo.[52] Immune responses to NA and M2 antigens of influenza are relatively low which is due to immune dominancy of HA antigen and higher number of this antigen on influenza virions. However, by presenting less immunogenic viral proteins on separate VLPs, these antigens can avoid the immune-dominant effect of HA and thereupon will be more immunogenic.[53,54] In addition, VLP vaccines can become more effective by direct incorporation of adjuvant molecules.[55-58]

Several surveys also have demonstrated a satisfactory safety profile for VLP vaccines. In a blinded, randomized, placebo-controlled trial about safety and immunogenicity of a pandemic influenza A (H1N1) 2009 VLP vaccine generated by recombinant baculovirus culture in Sf9 cells, participants showed only mild local reactions with no vaccine-related systemic AEs and the vaccine seemed to be well-tolerated and safe enough. This method of VLP production is considered safer than other expression systems.[59] Another clinical trial also has evaluated a VLP vaccine produced by the same method and side effects were mostly mild. However, adjuvanted VLP vaccine could raise local and systemic reactions.[60] Also, a phase I clinical trial on previously healthy non-immune adults exhibited remarkable safety results for a bacterially produced VLP vaccine (gH1-Qbeta). Most of the reported local and systemic AEs were mild in severity and no serious AEs were occurred.[61]

5 | INFLUENZA DNA VACCINE

Although conventional influenza vaccines in preventing seasonal influenza viruses are successful, they have some of the problems including

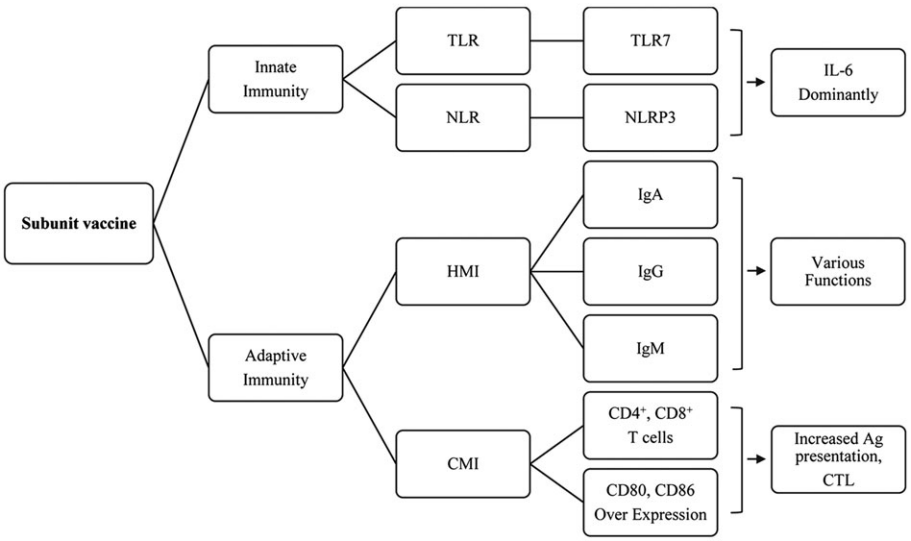

FIGURE 4 Subunit vaccine. Subunit vaccination induces both innate and adaptive immune response. Innate immune system receptors such as TLRs and NLRs have an important role in the production of inflammatory cytokines significantly IL6 after subunit vaccination. Among this receptors TLR7 and NLRP3 are more important from other receptors. Production of antibodies such as IgA, IgM, and IgG is important in providing mucosal and systemic immunity to vaccine. Subunit vaccination can induce both T $CD4^+$ and T $CD8^+$ cells. Expression of APC markers including CD80/86 and MHC II molecules results in high activation of these cells and increased antigen presentation by APCs.

allergic reactions and strain-specific in some patients. Therefore, the current influenza vaccines need to be reformulated every year to act efficient against sudden epidemics and pandemics. The production process of a conventional influenza vaccine is time-consuming and so the need to design newer vaccines is of utmost importance. To solve the disadvantages of conventional influenza vaccines, DNA influenza vaccines are a promising approach. These vaccines are able to induce humoral and cellular immune responses via incorporating a gene encoding an antigen, in transfected host cells. Actually, DNA vaccines unlike protein base vaccines can provide anti-influenza T-cell-mediated protection.[62-64]

Since DNA vaccine are egg-free and can be produced more expeditiously, thus reducing the delay from vaccine production to clinical use and can be produced rapidly in response to epidemics or pandemics. One of the concerns about the use of DNA vaccines is possible risk of integration into the human genome but that advantages make it pave for human application. Bacterial plasmids, recombinant viral vectors, and bacterial vectors are existing DNA-based antigen delivery platforms which are used for DNA vaccines manufacturing.[65,66]

It has been shown that intranasal DNA vaccination induces potent mucosal and systemic immune responses and cross-protective immunity against influenza viruses (Figure 5). New strategies for stimulation of nasal immune response have been reported with PEI/DNA complexes. Polyethylenimine (PEI), a synthetic polycation, was previously shown to improve the efficacy of gene delivery both in vitro and in vivo.[67,68]

In recent reports, adaptor molecules in TLR signaling pathway such as MyD88 (myeloid differentiation primary response gene) and TRIF (Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β) were incorporated into plasmid DNA as genetic adjuvants and improved humoral immune responses against plasmid-encoded antigen.[69] These studies suggest that TLR agonists may act as DNA vaccine adjuvants. Flagellin, as a TLR5 agonist, activates innate immune responses. Dermal injection of plasmids encoding flagellin and influenza A virus nucleoprotein results in both humoral and cellular immune responses. The flagellin vaccine adjuvant induces antigen-specific IgA production and enhances protective immunity to lethal influenza A virus infection. These findings indicate that expression of DNA-encoded TLR agonists can improve the immunogenicity of DNA vaccines.[70]

Furthermore, IRF1, 3, and 7 are other genetic adjuvants for influenza virus DNA vaccines. IRF1 genetic adjuvant strongly stimulates humoral immune responses. Conversely, IRF3 induces stronger cellular immune responses. Meanwhile, IRF7 genetic adjuvant enhances both humoral and cellular immune responses.[71] These findings suggest that IRF genetic adjuvants can improve both humoral and/or cellular immune responses. Also, constitutive active forms of IRF3 and IRF7, as DNA vaccine adjuvants, elicit both humoral and cellular immune responses against virus infection.[72] In addition, studies showed that DNA binding domain-lacked IRF1 (ΔIRF1) genetic adjuvant enhanced cellular immune responses.[73]

6 | UNIVERSAL VACCINE

Genes encoding influenza virus hemagglutinin (HA) and neuraminidase (NA) proteins have the high mutation rate, therefore yearly vaccination against circulating seasonal influenza virus strains is of great importance. Running research priorities include the development of a universal influenza vaccine that could evoke humoral and cellular responses, be safe, manufactured rapidly in large amounts, and provides long-lasting and cross-strain protection. Moreover, efforts are being made to design M2e-based or stalk-based vaccines, since these proteins (the type-2 matrix protein and the stalk domain of HA, respectively) are entirely well conserved from an evolutionary standpoint and can elicit immune response against influenza virus.[74]

6.1 | M2e-based vaccine

Universal influenza A vaccines focus on using highly conserved sequences among influenza virus subtypes in order to provoke cross-reacting antibody responses. Major targets consist of ectodomain of matrix protein 2 and conserved epitopes of some other influenza proteins.[75] Recombinant multimeric M2e proteins can be incorporated with several adjuvants in order to induce specific antibodies.[76] Vaccines containing M2e antigen coupled with carrier proteins or adjuvants are able to induce strong cross-protection against influenza in mice. Some reports indicate a trend towards Th1 responses after vaccination, leading to induction of cytotoxic lymphocytes.[77,78] This proton-selective ion channel protein of influenza virus is required to stimulate the NLRP3 inflammasome pathway. M2

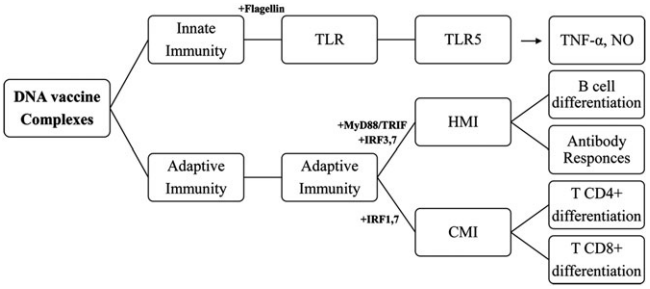

FIGURE 5 DNA vaccine. DNA vaccines have been created in complex with different stimulant components that stimulate different pathways. DNA/Flagellin complex, in addition to cellular immunity, stimulates TLR5 in the innate immunity leading to TNF and NO production. DNA/IRF3.7 complexes and DNA/TRIF and MYD88 complexes stimulate the B lymphocytes differentiation and specific antibody secretion. On the other hand, the DNA/IRF1.7 complexes mainly stimulate the pathway of cellular immunity and differentiate T lymphocytes.

localization to the trans-Golgi network is important for NLRP3 activation.[79] Due to lacking its proton selectivity, the mutant influenza virus M2 protein enables the transport of other cations (Na^+ and K^+), which mediates increased IL-1β secretion compared with the wild-type M2 protein.[79]

An acceptable safety profile has been revealed for these vaccines. ACAM-FLU-A,a M2e_HBc vaccine manufactured by Sanofi Pasteur was tested in phase I clinical trial and showed no serious side effects.[80] STF2.4xM2e, M2e vaccine with flagellin as an adjuvant, was reported to be safe, but two high doses of the vaccine were observed to be associated with raised levels of C-reactive protein (CRP).[81] Another universal influenza vaccine, Multimeric-001, was announced to be safe in phase I/II clinical trial, and no severe AE was reported.[82]

6.2 | Epitope base vaccine

Progression in biotechnology methods has provided the emergence of new approaches towards rational design of vaccines. One important approach is the use of epitopes corresponding to immunogenic, conserved sequences of microbial proteins. Focuses on the minimal component of these microbial pathogens that activates the lymphocyte in the epitope-based approach are very important. Short peptides with 8 to 10 amino acids truly activating T-cells and peptides up to 20 amino acids induce B-cells responses. Such conserved epitopes are found in M, NP, and even HA protein of influenza virus.[83,84] Moreover, perception of the molecular basis of antigen recognition and human leukocyte antigen (HLA) binding motifs has ameliorate selection of peptides predicted to bind to human class I or class II MHC molecules. The immunological efficacy of peptide-based vaccines versus infectious diseases is very important, and it is demonstrated in animal models and clinical studies. The most important challenges associated with peptide vaccines are low immunogenicity and the need for valid and simple assays to measure the T-cell responses. On the other hand, the range of production options is a distinct and notable advantage of peptide-based vaccines.[85,86]

7 | CYTOTOXIC T-LYMPHOCYTE-INDUCING VACCINES

Cytotoxic T lymphocytes (CTLs) have the ability to target more conserved influenza virus proteins. Unlike antibody-mediated responses, this response is induced mainly by the internal viral proteins.[87-89] CTL responses improve recovery and inhibit disease progression and clearance of the virus.[90] As a problem, CTL vaccines need to have an epitope that can be recognized by all major histocompatibility complex (MHC) subclasses. Assarsson et al showed that although epitope recognition varied across individuals, it is possible to identify epitope regions that are recognized by all six HLA super- types.[91] Their study and a study by Lee et al resulted in identification of highly conserved epitopes in the M1, NP, and PB1 proteins from more than 17 strains across six different subtypes which are targeted by CD4 and CD8 T cells.[92]

An immunodominant CTL vaccine epitope is likely to reduce the extent of response in primed vaccinated subjects. Thus, epitope choice can also alter the outcome of the response, depending on the binding avidity of the T-cell antigen receptors (TCRs) with the MHC peptide.[93]

For a strong CD8 T-cell response, the antigen should be processed via the MHC class I processing pathway of dendritic cells (DCs). During infection, the viral antigen is loaded onto DCs by direct entry of the virus or through uptake of infected cells undergoing apoptosis.[94] Hence, CTL responses during vaccination are different. In other words, LAIV induces a strong CD8 T-cell response, while the conventional SU TIV is less effective than the whole-virus vaccine at inducing CTLs.[90] Thus, taking all of these considerations is needed in the successful design and execution of a CTL-based vaccination approach.

8 | INFLUENZA RNA VACCINE

Owing to fewer regulatory tests and being products with invariant base regardless of the type of pathogen, a large body of evidence have nominated nucleic acid-based vaccines as encouraging vaccine candidates.[95]

Particularly, a great deal of effort, accompanied by technological innovation, have enhanced qualification of mRNA, thereby promoting therapeutic potential in the fields of vaccine development.[96] The capability of RNA vaccines to trigger strong, protective immune responses against a wide spectrum of pathogens has led to a large number of studies focusing on the use of these vaccines against influenza virus. As recently approved influenza vaccines demonstrated variable and inadequate protection, a promising efficacy has been shown for several more recently RNA vaccines types in preclinical models.[97]

Several features have made mRNA vaccines superior over the other types of vaccines. First, safety: since mRNA is a non-infectious and has a platform which does not integrate into the genome, there would be no possibility for infection or mutagenesis. Additionally, the safety of mRNA vaccines is substantially enhanced by down-modulation of their immunogenicity. Second, efficacy: the high stability and translatability of mRNA are a result of a variety of modifications. Rapid and efficient uptake and expression of mRNA are guaranteed through formulating it into carrier molecules. The characteristic of mRNA as a minimal genetic vector impedes the anti-vector immunity, allowing mRNA vaccines to be administered repeatedly. Third, production: mRNA vaccines are capable of being produced in a rapid, cheap, and scalable manner, mainly because of the high yields of in vitro transcription reactions.[96] As toxic chemicals are not used in manufacturing of mRNA, there is no possibility of common risks associated with other vaccine platforms.[95] However, possible undesired consequences such as fever can originate from the extreme induction of type I interferons and proinflammatory cytokines, mediated by some RNA vaccines. Also, extracellular RNA arisen from vaccine may result in safety concerns via mediating the formation of pathological thrombus or edema.[97] Despite the good antiviral protection, mRNA vaccines require a great amount of synthetic mRNA material, probably

limiting the accessibility of vaccine in cases of epidemics and pandemics. Owing to the greater length of constructed products, challenges remained to be addressed in terms of the production process and stability of the mRNA products, especially with self-amplifying RNA vaccines.[95]

There are two leading types of RNA that are recently employed as vaccines: non-replicating mRNA and virally derived, self-amplifying RNA. Containing 5′ and 3′ UTRs, non-replicating mRNA-based vaccines encode the antigen of interest, while self-amplifying RNAs encode both the antigen and the viral replication machinery, allowing the amplification of intracellular RNA and substantial protein expression.[96]

8.1 | Self-amplifying mRNA vaccines

A majority of recently used self-amplifying mRNA (SAM) vaccines are based upon the genome of an alphavirus genome. The full-length RNA with an approximate length of 9 kb can be readily yielded by in vitro transcription (IVT) from a DNA template. Intracellular replication of RNA which encodes antigen guarantees the highly effective antigen production from an extremely small dose of self-amplifying RNA vaccine. SAM vaccines are capable of self-production of adjuvants, intermediates of replication, and the rest of contributory motifs in their high potency.[95]

dsRNA is well known as a powerful pathogen-associated molecular pattern (PAMP), identified by pattern recognition receptors in various cellular compartments. Robust type I interferon responses are driven by Recognition of dsRNA-contaminated IVT mRNA. In addition to dsRNA contaminants, exogenously delivered single-stranded mRNA molecules are in turn a PAMP. Single-stranded oligoribonucleotides and the products of their degradation are recognized by the endosomal sensors Toll-like receptor 7 (TLR7) and TLR8, leading to type I interferon production.[96] In addition, trivalent saRNA (small activating RNAs) vaccination could result in anti-H1N1 and H3N2 IgG responses.[97]

A number of delivery formulation platforms have been explained to enhance the RNA vaccination, including PEI-based delivery vehicles.[95] Furthermore, a validated adjuvant strategy is TriMix, which is formed by a combination of mRNAs encoding three immune activator proteins: CD70, CD40 ligand (CD40L), and constitutively active TLR4. TriMix mRNA prolonged the immunogenicity of naked, unmodified, unpurified mRNA and was especially linked with increased DC maturation and cytotoxic T lymphocyte (CTL) responses. Substantial activation of TLR7 (mouse and human) and TLR8[98] and yields of type I interferon, pro-inflammatory cytokines, and chemokines was demonstrated following intradermal immunization with the RNActive vaccines. Unlike the immunization with protein, several mRNA vaccines foster potent CD8+ T cell responses, probably through efficient presentation of endogenously produced antigens on MHC class I molecules, besides strong CD4+ T cell responses. Additionally, unlike DNA immunization, neutralizing antibody responses with much lower doses of mRNA vaccines have been generated. Consequently, mRNA vaccines have induced protective immunity against various infectious agents in preclinical models.[96]

9 | MICRORNA AND EXOSOME-BASED VACCINE

MicroRNAs are small non-coding RNAs which act as epigenetic regulators.[99-106] These molecules play critical roles in regulation of gene expression in the RNA and protein levels.[22,107-113] It has been shown that deregulation of microRNAs is associated with initiation and progression of various diseases such as infectious diseases, cardiovascular diseases, diabetes, neurogenerative diseases, and cancer.[112,114-134]

Exosomes are other particles which have critical roles in the pathogenesis of various diseases.[122,135-137] Exosomes are nano-carriers which carry many cargos such as DNAs, RNAs, microRNAs, LncRNAs, and proteins. Targeting these cargos to host cells could lead to change behaviors of cells and contribute to progression of physiological and pathological processes.[138,139]

As vaccine preparation through classical temperature-based attenuation method was a time-consuming procedure, the need for urgent control of influenza outbreaks emerge from mutant viruses, highlighting the importance of preparing vaccines based on other mechanisms such as (microRNA) miRNA and exosome technologies.[140] MiRNA-based strategy for influenza virus attenuation could act through sequence-specific gene silencing mechanism or using miRNA/siRNA to suppress virus replication or infection.

According to previous studies, inserting miRNA response element (MRE) of viral microRNAs into Influenza virus genome could limit tissue tropism, and incorporating miRNA target site into viral NP or HA segment resulted to attenuate influenza A virus.[99,141,142] Further, in numerous studies, specific miRNAs have been recruited to demonstrate which gene targeting segments have maximal potential for viral attenuations. For example, a recent study on mice models demonstrated that inserting of miR-let-7b target sequence into 2009 pandemic H1N1virus (H1N1) genome successfully produced a recombinant virus which was slow to grow and attenuated in mice respiratory system.[143]

Another in vivo study found that incorporating miR-93 target sites into H1N1 and H5N1 viruses could result in viral attenuation in human lines and mouse lungs.[141] Similarly, fusing of miR-192 target sequence into IAV genome could attenuate influenza pathogenicity in mice.[142] Furthermore, the ability of miRNA-based attenuation method to determine virus fitness through introducing various number and location of target sites is superiority over classical viral attenuation methods.[144] Together, these evidences suggested that the engineering flu genome virus containing the target sites of deregulated miRNAs in a species-specific manner could be employed as a high-throughput strategy which is able to provide a new class of flu IAV vaccines. Fortunately, at the moment, a MRE design web server is available in which users could design various number of MRE tools for Influenza A genome to produce LAVs and help reduce experimental time and costs.[145]

Exosomes are two membrane-enclosed nano-vesicles that are actively secreted by a variety of cells into the extracellular space.

These vesicles exchange information between cells and transfer biologically active proteins, lipids, and various nucleic acids including mRNA, miRNA, ribosomal RNA (rRNA), long noncoding RNA (lncRNA), and variably some DNA.[146-149] Through different studies conducted on distinct techniques such as stem-loop PCR and microfluidic microarray, it was found that more than 274 miRNAs were secreted by different cell types via exosomes.[150,151] The secretion of miRNAs through exosomes in the extracellular fluids guarantees their half time and keeps them away from degradation by body fluid enzymes.[148,150,151] Based on previous studies, exosome-mediated extracellular delivery also exists in viral life cycles. Transition of viral components including viral mRNA, miRNA, and genomic RNA, as well as genetic regulatory through exosomes, increased viral persistence, skipping host immune system and spread to uninfected cells.[146] These findings are promising that an expressional exosomal miRNA could be used in therapeutic and diagnostic fields. However, exosomes which are produced naturally in tissues are not filled with a great amount of proteins or miRNAs; therefore, to prepare more efficient targeted delivery exosomes, it is crucial to develop an efficient purification method to enrich specific miRNA in isolated exosome.

In two recent studies,[152,153] exosomes were enriched with a selected miRNA using a modified calcium-chloride mediated or electroporation. The results show these exosomes as cargo could successfully deliver a great deal of miRNA mimic(s) or inhibitor(s) to the target cells and lead to overexpression or deletion of the designed miRNA in recipient cells as well as alter cellular function.

These findings suggested that engineered exosomes as natural vehicles could represent a powerful tool for targeted delivery. Hence, exosomes could be clinically suitable applications for enhancing patient condition. Despite these advances, it seems there are still many challenges ahead, and existing techniques in exosome-mediated drug delivery need further development.

10 | CONCLUSION

In conclusion, most studies indicate that each type of influenza vaccines has particular excellences compared with another. For example, LAIV and VLP vaccines in addition to induction of antibody responses are able to stimulate effective cellular immune responses and in this respect are better than inactivated vaccines. However, newer vaccines such as VLPs can be more advanced and effective choices for extensive immunization of different populations. Influenza vaccines also can cause various inevitable adverse effects which for some vaccines with more severe local and systemic side effects may lead to uncertainty for clinical applications. Up to now among available influenza vaccines, emerging VLP vaccines seem to have the lowest reported AEs, although universal utilization of these vaccines needs more comprehensive clinical surveys. Overall, if advantages can overcome disadvantages, a vaccine will be suitable for immunization programs. Future studies can offer more ideal products by assembling the advantages of existing vaccines in a unique influenza vaccine.

CONFLICT OF INTEREST

None declared.

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