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REVIEW

Influenza vaccines: Past, present, and future

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Summary

Globally, infection by seasonal influenza viruses causes 3–5 million cases of severe illness and 290,000–650,000 respiratory deaths each year. Various influenza vaccines, including inactivated split- and subunit-type, recombinant and live attenuated vaccines, have been developed since the 1930s when it was discovered that influenza viruses could be cultivated in embryonated eggs. However, the protection rate offered by these vaccines is rather low, especially in very young children and the elderly. In this review, we describe the history of influenza vaccine development, the immune responses induced by the vaccines and the adjuvants applied. Further, we suggest future directions for improving the effectiveness of influenza vaccines in all age groups. This includes the development of an influenza vaccine that induces a balanced T helper cell type 1 and type 2 immune responses based on the understanding of the immune system, and the development of a broad-spectrum influenza vaccine that can increase effectiveness despite antigen shifts and drifts, which are characteristics of the influenza virus. A brighter future can be envisaged if the development of an adjuvant that is safe and effective is realized.

KEYWORDS
adjuvants, influenza viruses, vaccines

1 | INTRODUCTION

Influenza viruses belong to the family *Orthomyxoviridae*, and their genomes consist of segments of negative-sense RNA.[1] They are divided into A, B, C and D types, the latter of which was isolated from pigs exhibiting influenza-like symptoms in April 2011.[2] In humans, mainly the A and B types cause disease, and the A type causes more severe illness than the B type.[3,4] Influenza A viruses are further categorized according to the antigenicity of their surface antigens, haemagglutinin (HA) and neuraminidase (NA), and there are 18 HA and 11 NA serotypes.[5,6] Influenza B viruses have diverged into only two antigenically distinguishable lineages, Yamagata and Victoria, since the 1970s.[3] Current influenza viruses circulating in humans are mainly A/H1N1 and A/H3N2 and the B/Yamagata and B/Victoria lineages.[7] Yearly, seasonal influenza causes 3–5 million cases of severe illness and 290,000–650,000 respiratory deaths globally.[8] Mortality rates and severe cases are higher in the elderly (>65 years) and children younger than 5 years, and in immunosuppressed people.[8–10]

Antiviral drugs to treat influenza include oseltamivir, zanamivir, peramivir and baloxavir.[11] However, vaccination is considered the most effective method for controlling influenza.[12] Through continuous antigenic drift, that is, the accumulation of point mutations in the surface antigens, influenza viruses can escape immunity,[13,14] which is why yearly vaccination is required. Seasonal influenza vaccines have been steadily developed since the 1940s, and currently

Abbreviations: APCs, antigen-presenting cells; CTLs, cytotoxic T lymphocytes; HA, haemagglutinin; IIVs, inactivated influenza vaccines; iTregs, induced regulatory T cells; LAIVs, live-attenuated influenza vaccines; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MDCK, Madin–Darby canine kidney; MHC, major histocompatibility complex; MPLA, monophosphoryl lipid A; NA, neuraminidase; TGF-β, transforming growth factor-beta; Th, T helper; TLR, Toll-like receptor; TNF-α, tumour necrosis factor alpha; VPL, virus-like particle.

marketed preventive vaccines differ in type (whole, split, recombinant and subunit inactivated, and live-attenuated types) and the substrate used for production (embryonated eggs or cells).[15]

A study on vaccine effectiveness by the USA Centers for Disease Control and Prevention showed that their protection rate remains low (40%–60%), despite being antigenically matched with circulating strains.[16] Of concern, vaccines do not effectively elicit immune responses among very young children (6–35 months) and elderly people (>65 years),[17,18] the two age groups more vulnerable to influenza virus infection and with higher mortality rates. Therefore, there is an urgent need for effective influenza vaccines for all ages.

2 | DEVELOPMENTAL HISTORY OF AND IMMUNE RESPONSES INDUCED BY INFLUENZA VACCINES

2.1 | History of influenza vaccine development

Methods for culturing influenza viruses were developed in the 1930s. In 1933, the human influenza virus was transmitted to ferrets by intranasal instillation of a specimen obtained from throat washings collected from a patient.[19] In 1935, Wilson Smith suggested a method for cultivating influenza virus in the chorioallantoic membrane of embryonated eggs.[20] This method yielded substantially higher virus concentrations than previous methods based on virus extraction from the lungs of infected animals. Embryonated eggs are still used today to produce influenza vaccines. In addition, cultivation methods using cells and medium have been developed since the mid-1930s.[21,22] With the ability to isolate and culture influenza viruses, research on influenza vaccine development took off. From the mid-1930s to the early 1940s, vaccine effects of activated and formalin-inactivated influenza viruses obtained from allantoic fluid of embryonated eggs or extracts of infected animal organs were studied in animals and humans by monitoring antibody production.[23–25] It was found that antigenic matching between the vaccine and circulating strains is important to guarantee vaccine efficacy[26] and that concentrated vaccine is more effective than the unconcentrated vaccine, whether or not the virus is inactivated.[27,28] In 1942, immune responses induced by an inactivated whole bivalent influenza vaccine consisting of the PR8 strain of influenza type A and the Lee strain of influenza type B and produced in embryonated eggs were evaluated in a clinical study.[29] In 1943, a larger clinical study by the Commission on Influenza of the U.S. Armed Forces showed that inactivated whole trivalent vaccine including the A-subtype PR8 and Weiss strains and the B-type Lee strain protected against influenza.[30] Vaccine doses were established through clinical trials, and in 1945, the first inactivated influenza vaccine (IIV) was licensed in the United States.[31]

Since the development of the first inactivated whole virus vaccine in embryonated chicken eggs in the 1940s, production methods for IIVs were continuously improved, and in the 1950s, the current IIV manufacturing process using embryonated eggs was developed.[32] IIVs included whole-virus, split-virus disrupted by a detergent and further purified subunit vaccines composed of surface antigen, HA and NA.[33] The whole-virus vaccine, the first developed IIV, induced good immune responses even in unprimed individuals.[34] However, there were concerns about pyrogenicity and adverse side effects.[35] To overcome these problems, in the 1960s, split virus vaccines were developed by treating the virus with ether or detergent,[36] which made them safe for children.[37] In the 1970s, purified subunit influenza vaccines mainly based on HA and NA were developed, which further improved safety and reduced reactogenicity.[34,35,38–41]

Live-attenuated influenza vaccines (LAIVs), prepared by successive passages of influenza virus in ferrets and mice or embryonated eggs, have also been studied since the 1930s.[42] These host-range variant vaccines protect against influenza without causing flu symptoms in humans. However, they have some drawbacks, including low virus titres and difficulties in maintaining constant attenuation and antigenicity levels.[43] In the 1960s, a new method was adopted to attenuate influenza virus through consecutive passages in embryonated eggs at low temperatures, yielding cold-adapted, temperature-sensitive variants.[44] Since cold-adapted, temperature-sensitive influenza virus replicates best at lower temperatures, viral replication was enacted in the nasal cavity and not in the respiratory tract. These viruses were safer than those attenuated by previous methods and induced an immune response. Accordingly, they were further developed as donor viruses for LAIVs.[45]

As RNA viruses, influenza viruses lack proofreading activity and therefore are genetically unstable; thus, antigenic mutations occur at a high frequency.[46] To increase the protection rate of influenza vaccines, it is important to accurately predict the influenza viruses that will circulate and to manufacture vaccines with those strains. To this end, the World Health Organization's (WHO) Global Influenza Surveillance and Response System has conducted global influenza surveillance since 1952, and based on the monitoring results, the WHO annually announces recommended influenza virus vaccine compositions for the northern (February) and southern (September) hemispheres.[47,48] Initially, the WHO recommended three influenza virus strains, including A/H1N1, A/H3N2, and either the B/Victoria or the B/Yamagata lineage for trivalent vaccines; however, since co-circulation of the two B lineages was observed at a high frequency, B/Victoria as well as B/Yamagata are being recommended for quadrivalent influenza vaccines.[49] However, since IIVs and LAIVs are manufactured using the recommended candidate viruses, they will not be effective because of antigenic mismatch if the prediction is not accurate. Thus, broad-spectrum or universal influenza vaccines are being actively researched. These vaccine types target a conserved region of the influenza virus, such as the stalk region of HA, M2e, M1 or nucleoprotein instead of the globular head of HA, which is immunodominant, but variable and strain-specific (Figure 1).[15,50] These vaccines are prepared by using a viral vector, DNA vector, virus-like particle (VLP), nanoparticle or a peptide that directly stimulates T cells.[51] Among them, nanoparticle and VLP platform show the most visible results while completing the phase III clinical trial in 2020 (NCT04120194, NCT03301051 and NCT03739112). Both nanoparticle (Novavax) and VLP influenza vaccine (Medicago) showed results inducing cross-reactive antibody and T-cell response.[52,53]

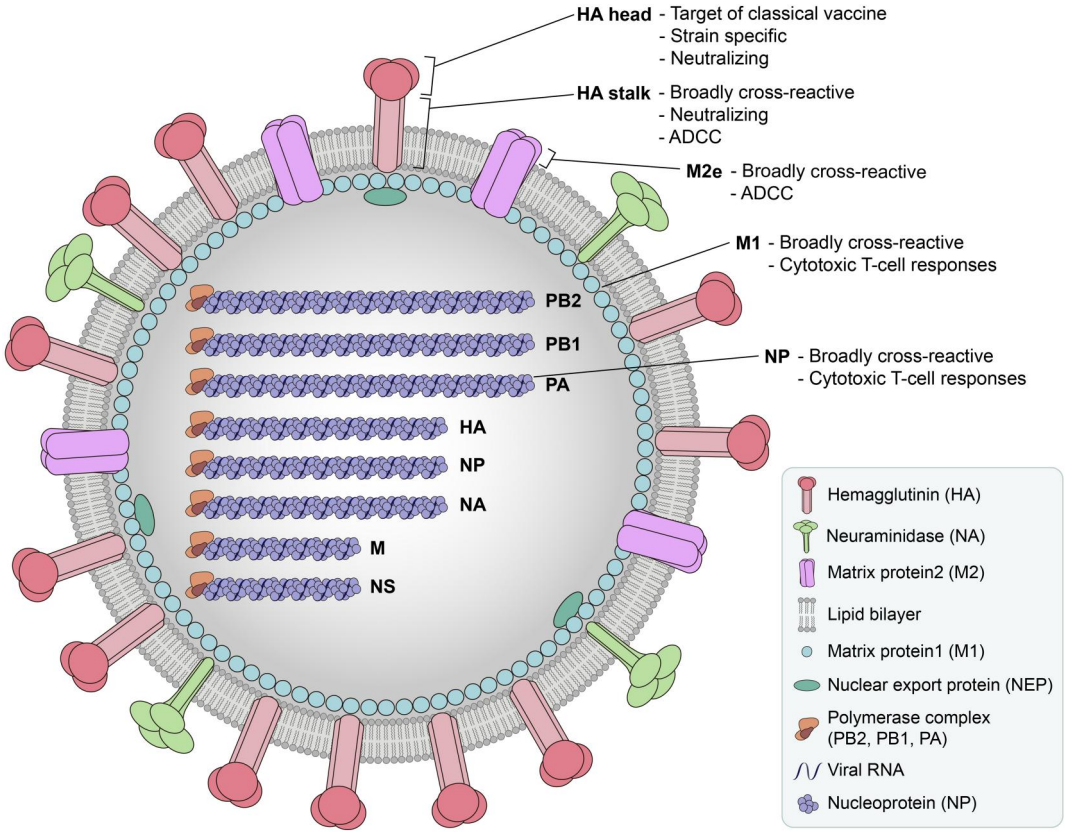

FIGURE 1 Targets of broad-spectrum or universal influenza vaccine. Broad-spectrum or universal influenza vaccines target antigens that can elicit broadly cross-reactive immune responses. Antibodies induced by HA stalk and ectodomain of the M2 ion channel (M2e), which are highly conserved regions, can mediate antibody-dependent, cell-mediated cytotoxicity (ADCC). Antibodies against HA stalk is neutralizing; while antibodies against M2e is not. M1 and NP proteins possess conserved regions and are internal proteins. Therefore, they mainly induce cytotoxic T cell responses

2.2 | Substrates for influenza vaccine production

Although the conventional method using embryonated eggs is still predominantly used worldwide, it has significant drawbacks. Egg-based vaccines may cause an allergic reaction to albumin, and when the demand for embryonated eggs suddenly increases, for example, during a pandemic, the supply may be insufficient, hampering timely vaccine production.[54,55] Most importantly, consecutive virus passaging in embryonated eggs, especially in the case of H3N2, can result in egg-adaptive mutations of the antigenic site, leading to altered antigenicity and thus reduced vaccine effectiveness.[56–58] To overcome these shortcomings, cell-culture-based vaccine production technologies were developed. Cell lines used include Madin–Darby canine kidney (MDCK) by Solvay and Seqirus (formerly Novartis),[59,60] PER.C6 by Sanofi Pasteur (formerly Crucell),[61] Vero by Baxter[62] and Sf9 insect cells combined with baculovirus vectors by Protein Science (recombinant HA), and Novavax (VLP vaccine).[63,64] Thus, influenza vaccine development is gradually moving away from the conventional egg-based platform to the cell culture (Figure 2), though efforts to increase yield and lower production costs of the latter are needed.

2.3 | FDA-licensed influenza vaccines and the immune responses they trigger

The immune responses triggered by vaccines differ according to whether the inoculum is an inactivated antigen or a live virus. In the case of an inactivated viral vaccine, the injected inactivated extracellular antigen is engulfed through phagocytosis by antigen-presenting cells (APCs), such as dendritic cells. The antigen is then degraded to peptides in the lysosomes of APCs, and the peptides are presented on the surface of APCs by major histocompatibility complex (MHC) II molecules. The MHC II–antigen complex is recognized by T helper (Th) cells, also known as CD4+ T cells, which causes the activation of naïve Th cells. The activated Th cells are further differentiated into T-helper 1 (Th1) cells, Th2 cells, Th17 cells, follicular Th (Tfh) cell, induced regulatory T cells (iTregs), and others.[65]

Among the various subsets of CD4+ effector T cells, Th1, Th2 and Th17 cells play major roles in defense against pathogens. Th1 cells, which induce a cell-mediated immune response, secrete IFN-γ and tumour necrosis factor-alpha (TNF-α), which activate macrophages and neutrophils that eliminate intracellular pathogens

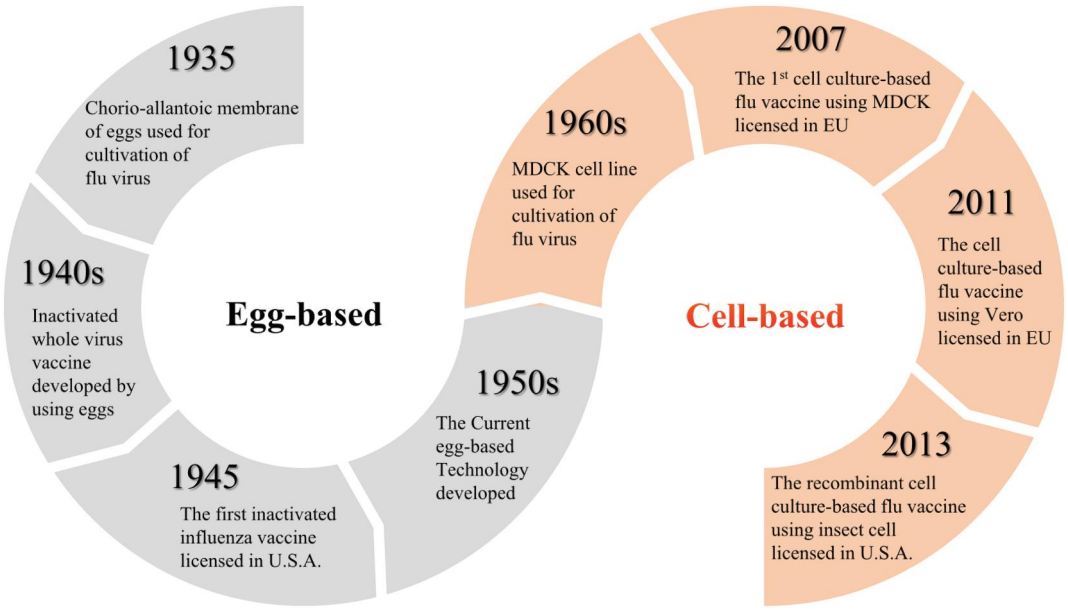

FIGURE 2 Evolution of influenza vaccine substrates from embryonated eggs to cells. In the 1930s, a method for cultivating influenza viruses in embryonated eggs was developed, and until the 1950s, methods for manufacturing influenza vaccines using embryonated eggs were continuously developed. Since the MDCK cell line was established in the late 1950s, the facts that the influenza viruses could be cultivated in various cells such as MDCK and Vero cells were revealed, and gradually the substrate for manufacturing of the influenza vaccine began to change from embryonated eggs to cells. MDCK, Madin–Darby canine kidney

through increased phagocytosis and inflammation. Th2 cells, which induce a humoral immune response, mainly secrete IL-4, IL-5 and IL-13. Lastly, Th17 cells secrete IL-17 and IL-22, which are pro-inflammatory cytokines, as well as IL-21, thereby stimulating immune responses against extracellular pathogens and fungi.[65–69]

In contrast, a live virus or attenuated live virus vaccine infects cells and produces viral proteins in the cytosol of infected cells. The viral proteins are degraded to peptides by the proteasome, and the peptides are presented on the surface of infected cells in complex with MHC I molecules. The MHC I–antigen complex is recognized by CD8+ T cells, which subsequently differentiate into cytotoxic T lymphocytes (CTLs) with other signals and the help of Th cells. Dendritic cells are not easily infected by viruses, but play an important role in the activation of CD8+ T cells through cross-presentation. In this pathway, virus-infected cells are ingested by dendritic cells leading to the release of viral antigens in the cytosol of dendritic cells. Subsequent processes from degradation in the proteasome to the presentation are identical to the MHC I pathway described above.[70] When CTLs recognize the MHC I–antigen complex on infected cells, they release cytotoxic proteins, perforin and granzyme, which enter the infected cells and induce apoptosis. CTLs can also kill infected cells through the interaction of Fas ligand expressed on CTLs and Fas expressed on target cells.[67,69] Various types of influenza vaccines have been approved by the FDA (Table 1), and they cause different immune responses. Based on the mechanism of immune response induction, influenza vaccines are largely categorized into IIVs and LAIVs, and IIVs are further classified into whole, split and subunit types.

As the active substances of IIVs are three (trivalent IIVs) or four (quadrivalent IIVs) inactivated antigens, inoculated antigens are mainly presented on the surface of APCs through the MHC II pathway and thus CD4+ T cells will be mainly stimulated; however, the immune responses following IIV inoculation are not yet fully understood.[71] As mentioned above, CD4+ T cells can differentiate into several subtypes, but IIVs predominantly stimulate Th2 immune responses, inducing a humoral immune response rather than a cellular immune response. A haemagglutination inhibition titre, which correlates with influenza-specific antibodies, of more than 1:40 induced by IIV provided a 50% protection rate against influenza infection in adults.[72,73] However, IIV does not effectively induce mucosal immunity and cellular immunity, which plays an important role in respiratory viral infection. Unlike IIVs, LAIVs are administered via the intranasal route. They infect cells of the upper respiratory tract and are more efficiently processed through the MHC I pathway, and thus induce mucosal immunity and cellular immunity. Further, Th1 immune responses are well activated by CD4+ T cells in the case of LAIVs.[74,75] Although LAIV can be considered as an ideal vaccine type for influenza because it induces balanced Th1/Th2 immune responses, it has safety concerns and cannot be administered in very young children and immunocompromised individuals.[76] Moreover, it is not easy to develop LAIVs for avian influenza viruses since they show greater tropism in the lower than in the upper human respiratory tract.[77]

TABLE 1 The list of FDA-licensed seasonal influenza vaccines for use in the United States

Manufacturing platform	Vaccine type	Trade name	Manufacturer	Dose	Age	Remark
Egg platform	Split	Afluria (TIV, QIV)	Seqirus Pty Ltd.	15 μg/strain	≥6 months old	-
		FluLaval (TIV/QIV)	ID Biomedical Corporation of Quebec (a division of GlaxoSmithKline)	15 μg/strain	≥6 months old	-
		Fluarix (TIV/QIV)	GlaxoSmithKline Biologicals	15 μg/strain	TIV: ≥3 years old, QIV: ≥6 months old	-
		Fluzone (TIV/QIV)	Sanofi Pasteur, Inc	15 μg/strain	≥6 months old	-
		Fluzone intradermal (TIV/QIV)	Sanofi Pasteur, Inc	9 μg/strain	18–64 years old	0.1 ml dose
		Fluzone high-dose (TIV/QIV)	Sanofi Pasteur, Inc	60 μg/strain	≥65 years old	-
	Subunit	Agriflu (TIV)	Seqirus Inc.	15 μg/strain	≥18 years old	-
		Fluvirin (TIV)	Seqirus vaccines limited	15 μg/strain	≥4 years old	-
		FLUAD (TIV/QIV)	Seqirus, Inc.	15 μg/strain	≥65 years old	MF59C.1 adjuvant
	Live attenuated	FluMist (TIV/QIV)	MedImmune, LLC	$10^{6.5-7.5}$ FFU/strain	2–49 years old	-
Cell platform	Subunit	Flucelvax (TIV/QIV)	Seqirus, Inc.	15 μg/strain	≥4 years old	MDCK cells
	Recombinant	Flublok (TIV/QIV)	Protein Sciences Corporation	45 μg/strain	≥18 years old	Insect cells (expresSF+®)

Abbreviations: FFU, fluorescent focus units; TIV, trivalent; QIV, quadrivalent.

TABLE 2 The list of FDA-licensed adjuvanted influenza vaccines for use in the United States

Vaccine type	Product name	Trade name	Manufacturer	Dose	Age	Adjuvant
Pandemic	Influenza virus vaccine, H5N1[a]	-	Sanofi Pasteur, Inc.	90 μg/strain	18–64 years old	-
	Influenza A (H5N1) virus monovalent vaccine, adjuvanted	-	ID Biomedical Corporation of Quebec	1.9 μg/strain	6 months–17 years old	AS03
				3.75 μg/strain	≥18 years old	
	Influenza A (H5N1) monovalent vaccine, adjuvanted	AUDENZ	Seqirus Inc.	7.5 μg/strain	≥6 months old	MF59C.1
Seasonal	Influenza vaccine, adjuvanted	FLUAD (TIV/QIV)	Seqirus, Inc.	15 μg/strain	≥65 years old	MF59C.1

Abbreviations: TIV, Trivalent; QIV, Quadrivalent.

[a]Reference for comparing dose with adjuvanted vaccine.

3 | STATUS OF INFLUENZA VACCINE ADJUVANT DEVELOPMENT

3.1 | The need for adjuvanted influenza vaccines

Adjuvants are agents that boost the immune response of a vaccine, inducing higher antibody production and longer-lasting protection with lower amounts of antigen per dose,[78] thereby reducing the burden of vaccine production.

Unlike seasonal influenza viruses, where the same subtypes circulate continuously in the human population, influenza viruses that cause pandemics are new reassortant or variant viruses against which humans have no pre-existing antibodies, thus resulting in higher mortality rates.[79] Non-adjuvanted pandemic influenza vaccines against H5N1 and H7N9 induced weak immune responses, possibly because most people did not have pre-existing immunity against those subtypes, and pandemic vaccines are not administered yearly.[80,81] Thus, pandemic influenza vaccines are usually adjuvanted, especially because they improve immune responses in individuals who are immunocompromised due to chronic disease, obesity, HIV infection or transplant treatment.[82–85] Furthermore, unlike unadjuvanted IIV, adjuvanted IIV can confer a balanced Th1/Th2 immune response and mucosal immunity, which protect against influenza infection.[86]

3.2 | Immune mechanism of licensed adjuvants for influenza vaccine

Adjuvants currently used in influenza vaccines include aluminium phosphate ($AlPO_4$) gel, aluminium hydroxide ($Al[OH]_3$), AS03 and MF59.[86] The oldest and most commonly used adjuvants are $AlPO_4$ and $Al(OH)_3$, commonly called alum (although strictly speaking, alum refers to potassium aluminium sulphate ($KAl[SO_4]_2$). Influenza vaccines containing MF59 or AS03, which are oil-in-water emulsion adjuvants developed later than alum, were approved in 1997 and 2009, respectively.[87,88] These adjuvants are more effective than alum in IIVs.[89,90] Two out of the three H5N1 pandemic influenza vaccines that have been licensed by US FDA contain AS03 or MF59 adjuvant. The third vaccine does not contain adjuvant, but contains 12–24 times more HA than the adjuvanted vaccines (Table 2). The FDA-approved adjuvanted seasonal influenza subunit vaccine (Seqirus, FluAd®) contains MF59 and is intended for use in the elderly (>65 years), who have poorer immune responses than young adults (Table 2).[86,91]

While alum has since long been widely used as an adjuvant, its mechanism remains unknown.[92] Originally, alum was thought to enhance immune responses by slowly and continuously exposing the antigen to APCs through the so-called antigen depot effect.[93] However, recent studies showed that alum also recruits various types of innate immune cells, including neutrophils and monocytes, to the injection site, thereby activating innate immune responses.[94–96] Studies on the cellular and molecular mechanisms have reported that alum triggers the activation of the NLRP3 inflammasome through phagosomal destabilization via alum phagocytosis by APCs, resulting in the secretion of the pro-inflammatory cytokine IL-1β.[97–100] In addition, alum causes necrosis at the injection site, resulting in the release of damage-associated molecular patterns, such as uric acid and DNA, which activate the NLRP3 inflammasome.[95,101] Alum can enhance the immune response through prostaglandin E_2 production, which is involved in inducing a Th2 immune response, via ITAM-Syk-PI3Kδ signalling.[102] These results suggest that alum induces a Th2 response rather than a Th1 response.

MF59 is an oil-in-water adjuvant consisting of squalene and two surfactants, polysorbate 80 (Tween 80) and sorbitan trioleate (Span 85). Since its first application in influenza vaccines in Europe in 1997, substantial research has been done to clarify its immune-boosting mechanism. MF59 was shown to have no depot effect,[103,104] which was supported by the finding that it showed an adjuvant effect even when injected 24 h before to 1 h after antigen injection.[105] Instead, MF59 activates monocytes, macrophages and granulocytes at the injection site, resulting in the secretion of chemokines, followed by immune cell recruitment, increased antigen uptake, differentiation of monocytes into immature dendritic cells and enhanced antigen transport to the draining lymph nodes.[106,107] The differentiation of monocytes into immature dendritic cells induced by MF59 facilitates their migration to the draining lymph nodes, where they stimulate T cells and B cells, leading to the enhancement of the adaptive immune

response with a balanced Th1/Th2 response to the administered vaccine antigen.[108] In addition, MF59-adjuvanted antigen was presented on various cells, including B cells, monocytes and neutrophils in the lymph nodes of immunized mice, whereas alum-adjuvanted antigen was presented only on dendritic cells,[96] indicating that MF59 is more efficient in enhancing the immune response than alum. MF59 causes muscle cells at the injection site to release ATP and exerts its adjuvant function through MyD88.[109,110] In an animal model, hydrolysis of ATP by locally administered apyrase led to reduced T-cell responses and haemagglutination inhibition titres in response to MF59-adjuvanted trivalent IIV.[111] Thus, ATP plays an important role in MF59 adjuvant function; however, the exact signalling pathway requires further study.

AS03 is an oil-in-water adjuvant consisting of polysorbate 80 (Tween 80) and two biodegradable oils, squalene and α-tocopherol, which is the most bioavailable form of vitamin E.[112] Dietary vitamin E supplementation has an immunostimulatory effect.[113] Accordingly, α-tocopherol exhibits an adjuvant-like function, whereas the MF59 components do not have an adjuvant effect per se. A study in mice showed that immune responses were weakened when the antigen was administered with AS03 lacking α-tocopherol.[114] Unlike MF59, AS03 requires spatiotemporal co-localization with the antigen to function as adjuvant. Similar to MF59, AS03 promotes immune cell recruitment to the injection site, and antigen uptake and transport to the draining lymph nodes.[114] In detail, AS03 induces a local and transient increase in NF-κB and enhances cytokine release and immune cell recruitment.[115,116] Subsequently, activated and antigen-loaded APCs activate CD4+ T cells in the draining lymph nodes, which directly stimulate antigen-specific B cells. Thus, AS03 strengthens the immune response by inducing a high number of memory B cells and antibody-secreting plasma cells.[116]

3.3 | Development status of influenza vaccine adjuvants

Adjuvants are widely studied worldwide. In what follows, we discuss the mechanisms and immune responses of influenza vaccine adjuvants other than alum, MF59 and AS03 that have reached clinical trials, including TLR ligands, cytokines, and micro- and nano-emulsions. In addition, there are immunostimulators of which the mechanisms are not precisely known. Among these, TLR agonists and formulations are the most studied (Table 3).

TLRs stimulate innate immune responses by recognizing pathogen-associated molecular patterns. Based on their localization, they are classified into cell-surface TLRs and intracellular TLRs. Cell-surface TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, mostly recognize components derived from microbial membranes. Intracellular TLRs, which are localized in the endosome, including TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13, recognize self and non-self nucleic acids.[132] Given that various TLRs can recognize unique ligands, several TLR agonists are in clinical trials as adjuvants. The TLR4 agonists AS01 and AS04 are licensed, while other TLR4 agonists, including glucopyranosyl lipid adjuvant, monophosphoryl lipid (MPLA), and LPS, are actively being studied.[118,119] In addition, dsRNA, flagellin, imiquimod and CpG are being developed as TLR3, TRL5, TLR7 and TLR9 agonists, respectively.[117,120–122]

Emulsion particles such as MF59 and AS03 are sometimes combined with other substances such as saponin, DNA and MPLA. Various emulsion-type adjuvants with different components and ratios are in clinical trials (Table 3).[123–126] Other particle types, such as virosome or VLP, may also have an adjuvant function.[127] Several immunostimulators of which the mechanisms of action are not yet known are also being studied.[128–130] By the way, cytokine, which stimulates the Th1 immune response and B lymphocyte differentiation in mice, showed no adjuvant effect in phase I clinical trials.[131] Thus, it is important to develop a non-clinical system that can accurately predict adjuvant effects. In a recent study, an IIV formulated with a single-stranded RNA adjuvant induced cross-protection against heterologous influenza virus infection and mucosal immune response.[133] The detailed mechanism and safety aspects remain to be studied.

4 | DIRECTIONS FOR INFLUENZA VACCINE DEVELOPMENT

4.1 | Current status and adverse events associated with influenza vaccines

The influenza vaccines currently on the market can be administered to very young children (≥6 months of age), although the recommended age for vaccination differs for each product, and most of them are inactivated vaccines. They primarily induce Th2 immune responses and lead to the production of specific antibodies against the administered influenza virus strains, thereby conferring immune protection. However, the effectiveness of the influenza vaccines investigated over the last decade is not high, with an average protection rate of 42% (range, 19%–60%),[16] and generally is even lower in young children and the elderly.

To overcome the low antibody production rate in young children and the elderly, vaccine manufacturers have increased the standard HA antigen content of seasonal influenza vaccines two times for young children (≥6 months; Flulaval Trivalent/Quadrivalent, Fluarix Quadrivalent, Fluzone Quadrivalent) and four times for the elderly (≥65 years; Fluzone High-Dose) or have applied an adjuvant (only for the elderly; FLUAD). These products are FDA-licensed, and the improved efficacy or effectiveness has been proven.[134–136] However, the required increase in antigen production can pose a burden to the manufacturers, especially in emergencies such as pandemics.

Alternatively, immune responses to influenza vaccines can be enhanced by administering LAIV instead of IIV as mentioned earlier, or by applying new strategies, such as using the intradermal route instead of injection (Sanofi Pasteur's Fluzone® Intradermal influenza vaccine), as the skin is rich in APCs. Despite the same IIV, the intradermal route induced a non-inferior protective immune response

TABLE 3 Adjuvants for influenza vaccine in clinical trials

Adjuvant type	Adjuvant name	Adjuvant description	Effect on immune response	Status	Registration number	Reference
TLRs ligand	dsRNA	TLR3 agonist	Improved protection effect in challenge study compared to non-adjuvanted group	Phase II	NCT02918006	117
	GLA-AF	TLR4 agonist	Increased HI titer	Phase I	NCT01657929	118
	MPLA	TLR4 ligand monophosphoryl lipid A	Dose-sparing effect	Phase I	NCT01111968	119
	ND002	Pantoea agglomerance-derived LPS (TLR4 agonist)	Data not shown	Phase I	NCT02955030	N/A
	Vax128	Haemagluttinin-flagellin fusion (TLR5 agonist)	Induced immune response (no non-adjuvanted group)	Phase I	NCT01172054	120
	Imiquimod	a synthetic TLR7 agonist	Significantly improved immunogenicity	Phase III	NCT02103023	121
	CpG7909	TLR9 agonist	Dose-sparing effect	Phase I	NCT00559975	122
Micro- and nanoemulsion	JVRS-100	Cationic liposome-DNA complexes	Data not shown	Phase II	NCT00936468	N/A
	IB160	Squalene based oil-in-water emulsion	Data not shown	Phase I	NCT03330899	N/A
	MAS-1	Nanoparticular, emulsion-based	Data not shown	Phase I	NCT02500680	N/A
	Matrix-M1	Saponins formulated with cholesterol and phospholipids into nanoparticles	Enhanced antibody response	Phase III	NCT04120194	123
	CCS/C	Polycationic sphingolipid complexed with cholesterol	Data not shown	Phase II	NCT00915187	N/A
	ISCOMATRIX™	A particulate adjuvant comprising cholesterol, phospholipid and saponin	Data not shown	Phase I	NCT00851266	N/A
	AS25, AS50,	Oil-in-water emulsion containing MPL,	Increased influenza-specific	Phase II	NCT00318149	N/A
	AS01B, AS01E	Liposomal adjuvant containing MPL-A	CD4 T cell responses			
	SE	2% oil-in-water stable emulsion	Dose-sparing effect	Phase I/II	NCT02464163	124
	Montanide ISA-51	Water-in-oil	Induced immune response (no non-adjuvanted group)	Phase II	NCT03180801, NCT02962908	125
	W805EC	Nanoemulsion-based adjuvant	Improved mucosal immunity	Phase I	NCT01333462	126
	Endocine™	Liposome-based adjuvant	Data related to immune response not shown	Phase I/II	NCT02998996	N/A
	PAL[a]	Papaya mosaic virus nanoparticle	Dose-sparing, improved CMI response	Phase I	NCT02188810	127
Immuno-stimulator	LT Adjuvant patch	Heat labile enterotoxin from *E. coli*	Enhanced immune response	Phase I/II	NCT00532792	128
	AD07010	Heat-labile enterotoxin (LT)-derived from *E. coli*	Improved mucosal immunity	Phase II	NCT03784885	129

TABLE 3 (Continued)

Adjuvant type	Adjuvant name	Adjuvant description	Effect on immune response	Status	Registration number	Reference
	Advax-CpG55.2	Combination adjuvant (Advax + CpG55.2)	N/A (active, not recruiting)	Phase I	NCT03945825	N/A
	Advax	Polysaccharide adjuvant based on delta inulin	Increased seroprotection rate	Phase I/II	ACTRN 12609000674235[&]	130
Cytokine	IFN-α	Type I interferon	No adjuvant effect	Phase I	NCT00436046	131

Note: Searched at ClinicalTrials.gov (Search criteria: Condition or disease is influenza vaccine and other terms is adjuvant) except for [&], Australia New Zealand Clinical Trial Registry. When there were multiple studies using the same adjuvant, only the higher clinical stage was indicated.

[a]Although PAL is not an emulsion type, it has a similar form as nanoparticle, so it is classified in this category.

with a smaller amount of HA antigen (9 μg per strain) compared to an intramuscular route.[137,138] However, FDA-licensed products using these approaches are also not yet applicable to young children or the elderly (Table 1). The LAIV FluMist is approved for use in persons 2–49 years of age, and Fluzone intradermal, which is injected intradermally, is approved for use in persons 18–64 years of age.

The adverse events associated with the influenza vaccine vary from mild symptoms, such as erythema from the shot, headache, fever, nausea, and myalgia to unusual events, such as severe allergic reaction, Guillain–Barré syndrome and oculo-respiratory syndrome. Most of the adverse events associated with influenza vaccines are mild and easy to recover.[139,140] However, the Pandemrix vaccine against the 2009 H1N1 influenza pandemic is considered to be associated with narcolepsy,[141,142] and this narcolepsy caused by Pandemrix is linked to autoimmune disease, but the mechanism remains unknown.[143,144]

4.2 | Directions for influenza vaccine development

For influenza vaccines, seroprotection, seroconversion and geometric mean titre ratio are criteria considered for obtaining approval as a commercial product. When evaluating the efficacy of a vaccine, the focus is on a Th2 immune response rather than a Th1 immune response. However, Th1 and mucosal immune responses also play important roles in the defence against respiratory viruses such as influenza virus.[145] Therefore, an influenza vaccine that induces balanced Th1/Th2 immune responses, including cell-mediated and antibody responses and mucosal immune responses involving secretory IgA in all age groups, including infants and the elderly, could significantly reduce the influenza mortality and morbidity rates.

As mentioned earlier, many attempts have been made to increase the efficacy of influenza vaccines using various platforms, including viral vectors, DNA vectors, VLP or peptide vaccines, which are expected to overcome the limitations of current influenza vaccines, for example, by inducing a more dominant Th1 response and a memory response. Moreover, considering that the antigenicity of influenza vaccine strains changes almost yearly and a wide variety of influenza subtypes can cause outbreaks due to antigen shift and drift, universal influenza vaccines are being actively studied.[146] One candidate, a recombinant protein M-001 containing nine conserved epitopes from influenza A and B, is currently in a phase III clinical trial (NCT03450915), which is expected to be completed in December 2020. mRNA-based vaccines established their potential during the SARS-CoV-2 pandemic[147,148]; therefore, they are worth considering against an influenza pandemic as well. mRNA vaccines for influenza virus exhibited protective effects in mice, immunogenic responses and safety in human clinical trials, and cross-protection effect by eliciting influenza virus HA stalk-specific antibodies.[149,150] Adjuvants, such as AS03 and MF59, are already applied in influenza vaccines. However, influenza vaccines are inoculated yearly, and therefore, substantial effort should be made to develop new adjuvants with good safety for repeated administration and efficacy, such as

RNA-based adjuvants. These are as easily degradable as TLR3 or TLR7/8 ligands[133] and are relatively safe, when compared to other adjuvants that remain in the body for long time.

5 | CONCLUSIONS

Influenza vaccines are somewhat complex. They induce different immune responses depending on the vaccine type, such as IIV versus LAIV, and the age at vaccination and their mechanisms in inducing immune responses have not been completely clarified. Most of the licensed vaccines induce mainly Th2-type immune responses, and in young children and the elderly, they induce weaker immune responses than in adults. Therefore, efforts are needed to develop influenza vaccines that induce stronger and more balanced Th1/Th2 immune responses, based on our understanding of the immune system, which differs according to age. Current approval criteria for influenza vaccines, which focus on Th2 responses, will have to be modified accordingly to include confirmation of T-cell-mediated protection. Improved immune responses can also be achieved by using adjuvants, and thus, safer and more effective adjuvants should be developed. Finally, effective and reliable tools for predicting immune responses to vaccines and adjuvants would greatly help increase the protection rate against influenza virus infection, not only in adults but also in young children and the elderly.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Yun-Hee Kim wrote and designed the first draft, Kee-Jong Hong and Hun Kim provided advice for improving the manuscript, and Jae-Hwan Nam edited and re-wrote the manuscript. All authors have read and approved the submitted version.

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