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Current and future influenza vaccines

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Although antiviral drugs and vaccines have reduced the economic and healthcare burdens of influenza, influenza epidemics continue to take a toll. Over the past decade, research on influenza viruses has revealed a potential path to improvement. The clues have come from accumulated discoveries from basic and clinical studies. Now, virus surveillance allows researchers to monitor influenza virus epidemic trends and to accumulate virus sequences in public databases, which leads to better selection of candidate viruses for vaccines and early detection of drug-resistant viruses. Here we provide an overview of current vaccine options and describe efforts directed toward the development of next-generation vaccines. Finally, we propose a plan for the development of an optimal influenza vaccine.

One hundred years have passed since the first recorded influenza pandemic was caused by an influenza A(H1N1) virus—the 1918 Spanish flu (Boxes 1 and 2). Since then, there have been three other pandemics caused by A(H2N2), A(H3N2), and A(H1N1)pdm09 viruses—the 1957 Asian flu, the 1968 Hong Kong flu, and the 2009 swine-origin flu, respectively[1]. Currently, A(H1N1)pdm09 and A(H3N2) viruses together with influenza B virus (Yamagata and Victorian lineages) cause epidemics as seasonal influenza, but A(H1N1) and A(H2N2) viruses have disappeared[1].

To understand and contend with influenza virus, a considerable amount of research has been conducted, and this effort has yielded a vast amount of information. For example, functional analysis of influenza virus proteins in vitro has revealed fundamental virological properties of influenza, resulting in the establishment of a method to generate influenza viruses entirely from plasmids[2]. This method has been and continues to be used to understand the biology of influenza viruses and to improve influenza countermeasures. Many host proteins have now been shown to contribute to virus propagation, revealing part of the complicated virus–host interaction[3,4]. Viral proteins and amino acid residues involved in the pathogenicity of influenza virus have also been identified, and the experimental procedures used to assess them are now well-established, leading to rapid risk assessment of newly emerged influenza viruses[5–7]. Several neuraminidase (NA) and polymerase inhibitors, which target virus proteins, have been developed and are efficacious when used early after onset, and rapid influenza diagnostic kits, which can provide results in 5–20 minutes, are now also available[8–11]. Seasonal influenza vaccines are available prior to every influenza season, and prepandemic vaccines against particular virus subtypes with pandemic potential have also been prepared[12]. Nevertheless, the control of seasonal influenza remains suboptimal, and there is always the risk of a pandemic caused by a virus to which the majority of human populations have no immunity.

To understand virus properties, viral genomic sequences have been analyzed since the late 1970s using the Sanger sequencing method, also known as the dideoxy chain termination method[13]. Recently, deep-sequencing technology has allowed us to determine the whole genomic sequence of many isolates, resulting in the accumulation of a large number of virus sequences in public databases. This wealth of information now makes it possible to monitor viruses circulating worldwide[14,15], to predict virus fitness in silico[16], and to assess the pandemic risk of virus isolates[17].

Here, we review the understanding of viral evolution and spread and the current vaccine situation, and we describe future prospects for the development of next-generation vaccines.

Influenza virus and current epidemics

Influenza viruses cause a respiratory illness with symptoms such as fever, cough, sore throat, runny nose, muscle or body aches, headaches, and/or gastrointestinal symptoms (vomiting and diarrhea). The virus annually causes 3–5 million severe cases, 0.3–0.6 million deaths, and subsequent economic losses[18]. Currently, the influenza A virus subtypes H1N1pdm09 and H3N2, as well as influenza B viruses of the Yamagata and Victoria lineages, are as globally prevalent among humans as seasonal influenza viruses (Box 1). Global year-round surveillance is conducted by the World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS), which includes the National Influenza Centres, the WHO Collaborating Centres for Reference and Research on Influenza, and the Essential Regulatory Laboratories, to monitor changes in the virus genome, especially in hemagglutinin (HA) and NA (http://www.who.int/influenza/gisrs_laboratory/flunet/en/). On the basis of the phylogenetic similarity of the nucleotide sequence of HA, epidemic viruses are classified into a 'clade' or 'subclade.' A real-time snapshot of the current populations of these viruses is available at the website nextstrain.org[15].

Emergence and subsequent evolution of pandemic viruses in humans

Pandemic influenza is caused by the emergence of a virus with an HA protein to which the majority of human populations do not have immunity[19]. Previously, it was thought that a pandemic occurs when a virus whose HA subtype is different from that of viruses circulating in humans emerges; however, this concept was challenged when the A(H1N1)pdm09 virus caused a pandemic in 2009 even though A(H1N1) and A(H3N2) viruses were cocirculating. Influenza A viruses of a variety of subtypes are naturally maintained in avian species, especially aquatic birds, and are the typical source of the current HA. In contrast, influenza B viruses are unlikely to cause a pandemic because their antigenic diversity is limited. Reassortment (i.e., the exchange of genes between two or more influenza viruses

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Box 1 | Types of influenza

There are four types of influenza virus: types A, B, C, and D. Influenza A and B viruses cause seasonal epidemics in humans. While influenza A virus circulates in humans and a variety of animals in addition, such as birds, pigs, dogs, and horses, influenza B virus infection is limited to humans and seals[120]. Influenza C virus causes a mild respiratory illness only in humans. Influenza D virus has not been shown to cause illness in humans.

The four types of influenza virus belong to the family Orthomyxoviridae, in which viruses possess negative-sense, single-stranded, segmented RNAs as their genome. All influenza A viruses encode at least ten major viral proteins (PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, and NS2), and some isolates express several additional proteins, including PB1-F2, PA-X, M42, NS3, PB2-S1, PB1-N40, PA-N155, and PA-N182 (ref. [121]). On the basis of the similarity of the major antigenic hemagglutinin (HA) and neuraminidase (NA) sequences, influenza A viruses are classified into 18 HA subtypes (H1 through H18) and 11 NA subtypes (N1 through N11) in various combinations[122]. These subtypes are further divided into two or three groups: group 1 HA (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 HA (H3, H4, H7, H10, H14, and H15), or group 1 NA (N1, N4, N5, and N8), group 2 NA (N2, N3, N6, N7, and N9) and group 3 NA (N10 and N11)[122]. The trimeric type I transmembrane glycoprotein HA is produced as HA0, which is proteolytically split into HA1 and HA2. The HA1–HA2 monomer assembles as trimers consisting of a cytoplasmic domain, a transmembrane domain, an apical globular head region, which contains the receptor-binding site (RBS), and a stem region, which possesses a fusion peptide[123]. HA is essential for virus entry into host cells because its globular head and stem regions are involved in binding to the cellular receptor sialyloligosaccharides and in membrane fusion, respectively. The tetrameric type II transmembrane glycoprotein NA comprises several domains: a cytoplasmic domain, a transmembrane domain, a catalytic head domain, which is formed by six antiparallel β-sheets in a propeller-like arrangement and possesses the sialidase active site, and a stalk domain that connects the head and transmembrane domains[124]. The sialidase activity of NA cleaves off the sialic acid, allowing release of the progeny virions from the cell surface[125]. The enzymatic activity of NA also contributes to virus entry by removing receptor decoys within the airways[126].

Box 2 | Historic pandemics

The first recorded pandemic, which began in 1918, was caused by the Spanish influenza virus A(H1N1), which killed 50–100 million people worldwide in 1918–1919. Nucleotide sequence analysis suggested a 'considerable evolutionary distance between the source of the 1918 NP and the currently sequenced virus strains in wild birds'[127]. However, avian viruses whose proteins (with the exception of HA and NA) differ by less than 10 amino acids from those of the 1918 virus are still circulating in nature[128].

The second recorded pandemic began in 1957 and was caused by the Asian influenza virus A(H2N2); this pandemic caused 1.1 million deaths globally from 1957–1959[129]. The Asian influenza virus was a human–avian reassortant that possessed H2 HA, N2 NA, and PB1 segments derived from an avian virus and its other five segments from the Spanish A(H1N1) virus.

In 1968, the third recorded pandemic was caused by the Hong Kong influenza virus A(H3N2), which was a human–avian reassortant that possessed H3 HA and PB1 segments of an avian virus and its other six segments from the Asian A(H2N2) virus.

The latest pandemic, caused by the swine-origin influenza virus A(H1N1)pdm09, was first identified in Mexico in 2009[130]. More than 18,000 deaths among the laboratory-confirmed cases were reported to the World Health Organization (http://www.who.int/csr/don/2010_08_06/en/). Genomic composition analysis revealed that this swine-origin virus resulted from the reassortment of North American triple-reassortant swine viruses (PB2, PB1, PA, H1 HA, NP, and NS segments) with Eurasian avian-like swine viruses (N1 NA and M segments)[131].

upon co-infection of cells) between human, swine, and/or avian viruses in pigs and the direct interspecies transmission of an avian virus to humans led to the latest four pandemics[1] (Box 2). One of the most important facts that we have learned from these past pandemics is that no one knows when, where, or which subtype of influenza virus will cause the next pandemic.

After each pandemic, all four pandemic viruses continued to circulate in humans as a seasonal influenza virus after competing out previous seasonal viruses. Since inhibitory antibodies against HA and NA are usually elicited upon influenza virus infection in infected individuals[20–22], it is viruses with amino acid mutations in HA and NA that are responsible for antigenic changes for evasion from such antibodies, so-called 'antigenic drift' (Fig. 1). The mutations in HA mostly accumulate around the receptor-binding site (RBS) because antibodies that recognize the area around the RBS efficiently inhibit the binding of HA to its receptor, resulting in the neutralization of virus infectivity[23,24]. The five major antigenic sites, Ca1, Ca2, Cb, Sa, and Sb for H1 HA and A through E for H3 HA, have been mapped by X-ray crystallography, comparative sequence analysis, and characterization of mutant viruses that escaped from neutralizing mouse monoclonal antibodies[25–28]. Antigenic cartography suggests that antigenic drift of human influenza viruses occurs in clusters; while nucleotide changes continue to occur, clusters of antigenically similar variants exist for several years until they are replaced by viruses that form a novel cluster[29,30], meaning that the genetic evolution is continuous, whereas antigenic evolution is punctuated. The 'cluster-transitions' of A(H3N2) virus over a 35-year period were predominantly caused by single amino acid substitutions that occurred at only seven positions (position 145 at antigenic site A and positions 155, 156, 158, 159, 189, and 193 at antigenic site B) adjacent to the RBS[31]. Similarly, mutations in NA are frequently identified around the enzymatic active center[32,33]. Antibodies that recognize these epitopes interfere with the sialidase activity of NA, resulting in the suppression of virus release from infected cells[34]. Studies with monoclonal antibodies and amino acid sequence analysis have revealed two to three antigenic sites in the NA protein[35].

Current influenza vaccines

To reduce the burden attributed to seasonal and pandemic influenza, multiple approaches, including vaccines and antiviral drugs, have been developed. Since a fully effective vaccine, if available, would be able to prevent influenza completely, vaccination is an appropriate option to combat influenza virus. Currently, three kinds of vaccines (inactivated, live attenuated, and recombinant HA vaccines) are licensed in various countries, and each type of vaccine has advantages and drawbacks[36]. For all of these vaccines, the vaccine seed viruses must be replaced periodically to match their antigenicity to that of the circulating viruses. Since antigenic mismatch causes low vaccine efficacy, the WHO biannual influenza vaccine composition meetings (one for the Northern hemisphere and the other for the Southern hemisphere) try to select the correct ones on the basis of the genetic and antigenic characteristics of the circulating viruses and epidemiologic information from individual

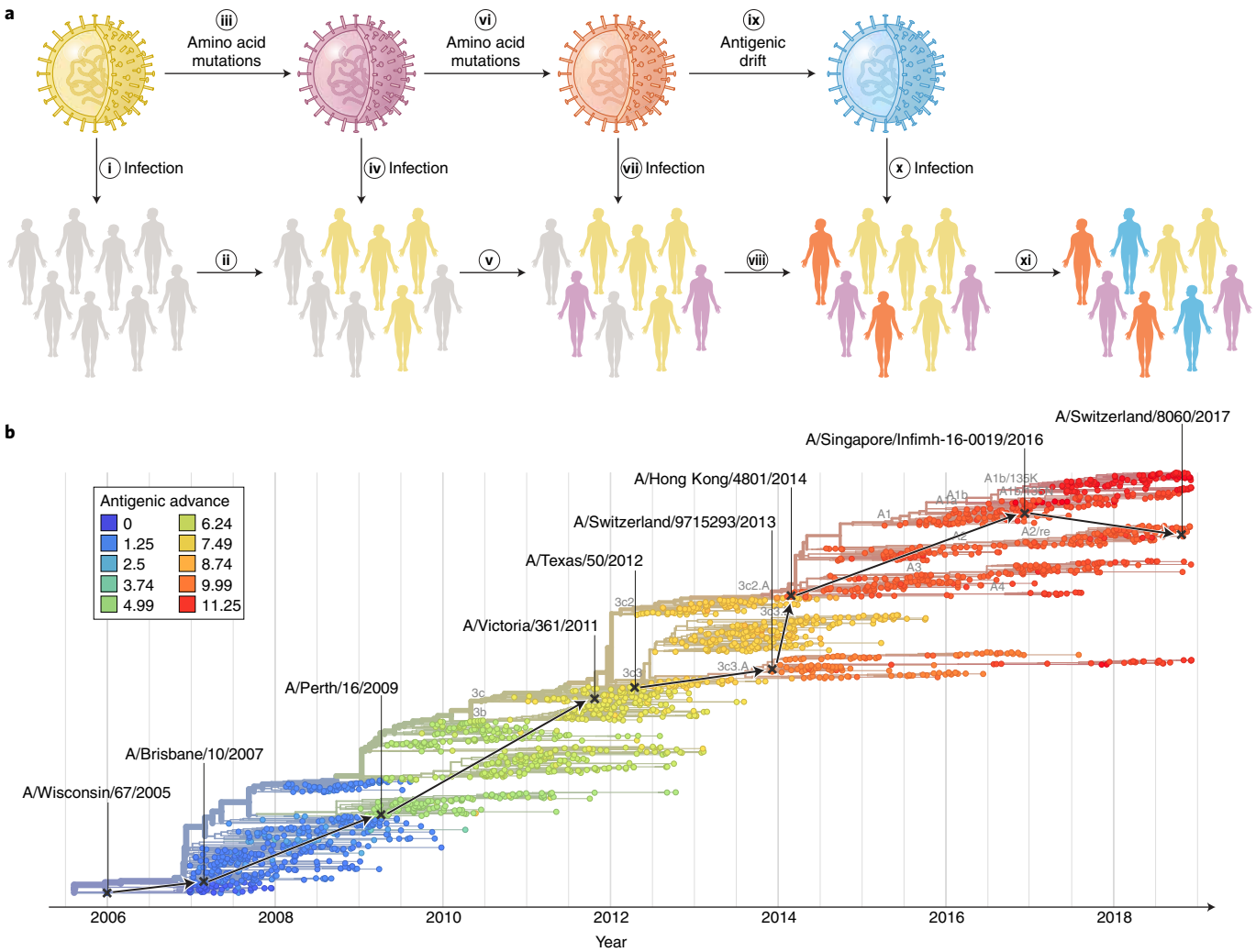

Fig. 1 | Antigenic drift. Immunologically naive human populations are infected with an influenza virus (i). Infected individuals acquire immunity against influenza virus (yellow) (ii). Viruses accumulate amino acid mutations in their antigenic HA during replication (iii). Some individuals who do not possess immunity against the initial virus are infected by the mutated virus (iv). Infected individuals acquire immunity against this virus (purple) (v). The virus further accumulates amino acid mutations in its HA (vi). The remaining naive individuals are attacked by this further mutated virus (vii). The majority of the individuals in human populations eventually acquire immunity against these viruses (yellow, purple, orange). They are then protected from influenza viruses with similar antigenicity (viii). The virus obtains one or two mutations in its HA that substantially alter antigenicity (i.e., antigenic drift) (xi). The antigenically drifted virus can infect individuals who possessed immunity against the previously circulating viruses (x). The individuals infected with the antigenically drifted virus mount immunity to this virus (blue). The cycle continues (xi). **b**, Real-world tracking of influencza viral epidemics allows the development of appropriate vaccines. Note that the virus evoles gradually, whereas antigenic shift occurs in steps. This is a screenshot of a phylogenic tree based on the HA sequences of human A(H3N2) viruses isolated between 2006 and 2018 from next strain (https://nextstrain.org/). Subclades (3b, 3c, 3c2, 3c3, and 3c2A, etc.) are indicated by grey letters, and antigenic advance of isolates is indicated by each color. Vaccine seed A(H3N2) viruses indicated in the tree were changed in response to antigenic advance of circulating virusese. The labels are the names of virus isolates. For example, in A/Texas/50/2012, 'A' means influenza A virus, '50' is sample number, and '2012' is collection year. Credit: Debbie Maizels/Springer Nature

countries. Since the vaccine seed viruses are determined more than 6 months before each epidemic season (http://www.who.int/influenza/vaccines/virus/recommendations/consultation201802/en/), antigenic mismatch between vaccine candidates and circulating strains occurs occasionally[37].

Inactivated vaccines. Inactivated vaccine, produced by growing the vaccine seed virus in chicken embryonated eggs, is the most popular approach in the world because of relatively low production costs and high safety. Vaccinations with inactivated vaccines begin at 6–12 months of age, and annual vaccinations are needed because the immunity conferred by the vaccine does not last long[38]. There are three types of inactivated vaccines: whole-virion vaccines, split-virion vaccines, and subunit vaccines. Whole-virion vaccine is prepared by purification of virions that have been chemically inactivated with formaldehyde or β-propiolactone. In the split-virion vaccine, the virus envelope of the whole virion is disrupted by diethyl ether or detergent treatment. Subunit vaccines contain HA and NA that are further purified by exclusion of the viral ribonucleoprotein (vRNP), M1, and viral envelope (lipid). Despite low immunogenicity and a narrow range of protection, split-virion and subunit vaccines are used more commonly than whole-virion vaccines to vaccinate humans against seasonal influenza. Another key issue for inactivated vaccines is that influenza A virus, especially the recent A(H3N2) virus, requires many passages in eggs to achieve high titers because the initial isolates replicate poorly in eggs. Excessive

passages in eggs can change the antigenicity of HA, resulting in an antigenic mismatch with the epidemic isolates[39–41].

To avoid egg-adaptive mutations in HA, cultured cell lines (such as Madin-Darby canine kidney (MDCK) and Vero cells) can be used for virus propagation[42]. However, the titers of vaccine seed viruses in such cell lines grown under serum- and animal-component-free conditions and in suspension or a bioreactor are lower than those in eggs, resulting in high cost and low productivity[43]. Therefore, the use of cell-culture-based inactivated seasonal vaccines has been limited.

Live attenuated vaccines. Live attenuated vaccines are available in the United States, Canada, and several European countries. Vaccines derived from cold-adapted and temperature-sensitive master donor viruses[44–46] are propagated in eggs, causing egg-adaptive mutations in HA. Because live attenuated vaccines mimic a natural infection without causing major adverse reactions, they can elicit both IgA, which is the principal isotype in secretions at the mucous membrane and which operates mainly on epithelial surfaces, in the upper respiratory tract as well as IgG, which is the principal isotype in blood and extracellular fluid and which operates mainly in body tissues, in serum, providing cross-reactive immune responses at the initial replication site[47,48]. However, the live attenuated vaccine is not recommended for use in children younger than 2 years of age, pregnant women, and people with certain underlying illnesses or a compromised immune system because the vaccine viruses may replicate to higher titers in these individuals, leading to some side effects.

Recombinant HA vaccines. The recombinant HA vaccine is produced by a recombinant-protein-expressing system using insect cells and baculovirus[49] and is approved by the Food and Drug Administration (FDA) for use in the United States. Since this system does not use live influenza viruses, HA protein is obtained that lacks the unwanted mutations that can be introduced during egg adaptation. Furthermore, recombinant HA vaccine can be manufactured within 2 months, indicating that this vaccine would be suitable for the prevention of pandemic influenza viruses. Although the mechanism of action of this vaccine is similar to that of inactivated vaccines, the commercial formulation of the recombinant HA vaccine contains three times the amount of HA as the inactivated influenza vaccines to induce antibody titers equivalent to those obtained with conventional inactivated vaccines[50]. Since the elicited immunity is HA- and strain-specific, the HA must be updated frequently to match the antigenicity of the epidemic strains. The recombinant HA vaccine is limited to use in individuals 18–49 years of age because of its low immunogenicity, especially in children.

Development of next-generation vaccines

Although vaccines are used in many countries, seasonal influenza epidemics have not been controlled. To improve the effectiveness of vaccines, advances must be made in five major areas: selection of the vaccine seed virus, targeting the vaccine, use of cultured cells instead of eggs for vaccine virus preparation, increasing the NA content of vaccines, and development of novel classes of adjuvants.

Selection of vaccine seed virus. To improve vaccine selection, in silico and in vitro studies have been conducted. Current epidemics can be visualized by integrating sequence data with epidemiologic information[14] or by the continuous updating of databases to monitor the rise and decline of virus clades[15,51] (Fig. 2). In silico modelling using past epidemic patterns together with information on viral fitness[16] or the relative distances of amino acid sequences in the multidimensional scaling-constructed 3D space[52] are used to predict the future direction of influenza virus evolution. To model which viruses may circulate in the future, viruses possessing random mutations in the HA head are first generated by reverse genetics. Of these, the viruses that could escape from neutralizing antibodies against HA are identified by using antisera obtained from ferrets that are experimentally infected with the parental virus, or sera obtained from humans who were exposed to influenza virus infection or vaccination[53]. These approaches allow the antigenicity of future epidemic strains to be determined and, in some cases, the exact amino acid changes that may occur can be predicted. Therefore, scientists are getting closer to identifying viruses that are antigenically similar to those that may circulate in nature before they emerge.

The next challenge for the selection of better vaccine components is to identify emerging viruses with antigenic drift. Antigenically similar viruses circulate in humans for several years without antigenic change[31]. During this period, the viruses first accumulate amino acid mutations in their HA that do not affect antigenicity and then acquire mutations that do affect the antigenicity of the virus, resulting in the emergence of antigenically drifted viruses (Fig. 1). Therefore, we need to learn what triggers the emergence of the latter amino acid changes. Studies on the antibody landscape (i.e., population dynamics of the levels of antibodies against the circulating strains) may help to solve this problem.

Vaccine targeting. The Center for Disease Control (CDC) recommends influenza vaccinations for all age groups (https://www.cdc.gov/flu/protect/whoshouldvax.htm). Individuals with a relatively higher risk for influenza, such as young children, the elderly, pregnant women, and people with chronic medical conditions, are highly encouraged to get vaccinated[54–56]. For these high-risk groups, vaccination tailored to specific targets may improve protection. For young children, the inactivated vaccine and live attenuated vaccine are available for individuals >6 months of age and >2 years of age, respectively (see above). Although annual vaccination for those who are 6 months of age and older is recommended to reduce the risk of influenza, it is unclear how best to induce 'good' immunologic imprinting in these individuals (see below). For pregnant women, vaccination benefits both the pregnant woman and her unborn baby[54].

Improvement of cell-based vaccine productivity. To address problems with low productivity, efforts have been made in two distinct areas: modification of the virus and amelioration of the cells in which it is produced. For virus modification, several sets of a vaccine backbone are prepared by optimizing the polymerase activity and efficiency of genome packaging and virion release of the influenza A or B vaccine viruses to achieve a high virus titer in MDCK and/or Vero cells[57–60]. A mutation that increases the fidelity of the virus polymerase may be useful for genetic stability of the virus for vaccine production[61,62]. Such backbones, together with HA and NA segments derived from circulating isolates, can be utilized for virus candidate production by using plasmid-driven reverse genetics[2]. For cell amelioration, researchers pick up high-virus-producing clones from parental cells[63], downregulate host protein expression that suppresses virus growth[64], and upregulate expression of human-type virus receptor[65,66]. Although these improvements show promise, they have not yet been incorporated into actual vaccine production.

NA content. The protection afforded by current inactivated vaccines is thought to be primarily mediated by HA because HA is a major target for protective antibodies. Therefore, the HA content of inactivated vaccines is standardized and measured. Despite the fact that antibody responses to NA have been shown to be the only independent immune correlate of all assessed measures of protection in human challenge models[67], the NA content of inactivated vaccines is not quantified and is suboptimal, resulting in a lack of immune response against NA in vaccinees[68]. In infected patients, NA can elicit protective antibodies, most of which possess neuraminidase

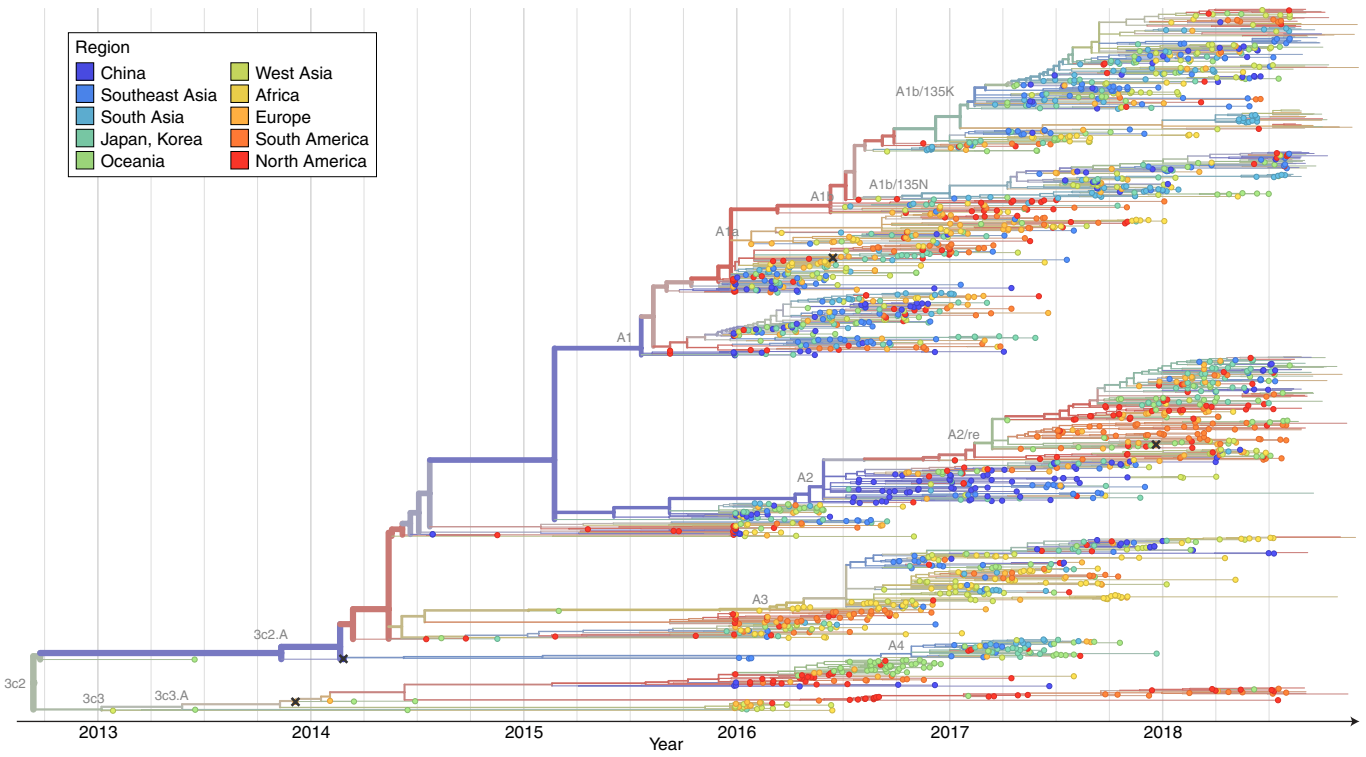

Fig. 2 | Real-time tracking of influenza virus epidemics. Real-time tracking of influenza A is now possible, which allows tracking of strains across the globe. This is a screen shot of a phylogenic tree based on the HA sequences of human A(H3N2) viruses isolated between 2016 and 2018 generated at https://nextstrain.org/. Subclades (3c2, A1, A1b, and A1a, etc.) are indicated by grey letters, and different colors indicate where the isolate was isolated. Credit: Debbie Maizels/Springer Nature

inhibition (NI) activity[68]; some antibodies without NI activity also protect against influenza infection via Fc-mediated effector cell activation[69]. Although antigenic drift and immunologic imprinting of NA have been reported[70,71], only NA antibodies that recognize the epitopes around the enzymatic active site and inhibit sialidase activity have been studied. Therefore, there is a need for analyses of antibodies against the NA head that lack sialidase inhibitory activity and of anti-NA stalk antibodies to fully understand the importance of NA as a vaccine antigen. In addition, a cross-reactive anti-NA antibody that binds and inhibits N1 through N9 NA activity was shown to be partially protective against H1N1 and H3N2 virus infection in mice[72], suggesting that an NA-targeted vaccine may have the potential to induce cross-protective antibodies and that the NA content in inactivated vaccines should be increased and standardized.

Adjuvants. Another improvement in vaccine efficacy may come from novel classes of adjuvant. An adjuvant is a substance that is formulated as part of a vaccine to enhance its ability to induce protection via activation of the immune system, allowing the antigens in vaccines to induce long-term protective immunity. The current adjuvanted vaccines normally cause local and general side effects, including pain, fatigue, headache, and myalgia, more frequently than nonadjuvanted vaccines because the adjuvant fundamentally promotes immune responses by mimicking the infection and causing inflammation. Mild adverse effects are acceptable, but severe adverse effects should be avoided. The severe adverse reaction of an increased risk of narcolepsy has been reported to be associated with the currently licensed adjuvant AS03 (refs. [73,74]).

To reduce the possibility of the occurrence of serious adverse effects, researchers have developed novel classes of adjuvant with a clear mechanism of action. Toll-like receptor (TLR) ligands are the best understood of these adjuvants. TLRs are members of a family of pattern recognition receptors that recognize common motifs of pathogens. TLR4 agonists MPLA (monophosphoryl lipid A) and GLA (glucopyranosyl lipid adjuvant), a TLR7/8 agonist (imiquimod), a TLR3 agonist (rintatolimod), a TLR9 agonist (CpG ODN (CpG oligodeoxynucleotide)), and a TLR5 agonist (flagellin) have been evaluated as influenza vaccine adjuvants[75]. Cytokines are well-characterized cell signaling molecules. Since cytokine induction is an essential action for most adjuvants, representative cytokines involved in immune responses, such as the T cell activator IL-2, the dendritic cell activator granulocyte-macrophage colony-stimulating factor (GM-CSF), and type I interferon, have been incorporated as adjuvants into vaccines that are currently being developed[12].

Other formulations and immunostimulators have been developed as adjuvants, but their mechanisms of action have not yet been characterized; the simplest approach to improving the host immune response via an adjuvant could be usage of the whole virion as the vaccine antigen. Whole-virion vaccines, but not split vaccines, elicit high neutralizing antibodies in the early T-cell-independent response, which requires B-cell–intrinsic TLR7 signaling activated by viral RNAs within the whole-virion vaccine[76].

Evaluation of host immune responses to vaccines

Vaccine efficacy is assessed according to the level of protection from infection the vaccine provides. However, surrogates, such as hemagglutinin-inhibition (HI) antibody titers in sera and virus-specific cytotoxic T lymphocyte (CTL) levels have been used to evaluate vaccine immunogenicity. Since a serum HI antibody titer of $\geq$1:40 is associated with a significant reduction in influenza incidence, serum HI antibody titer induction is used as a measure of vaccine efficacy in clinical trials[77]. The inactivated vaccine elicits HI antibodies against viruses that vaccinees have encountered over their

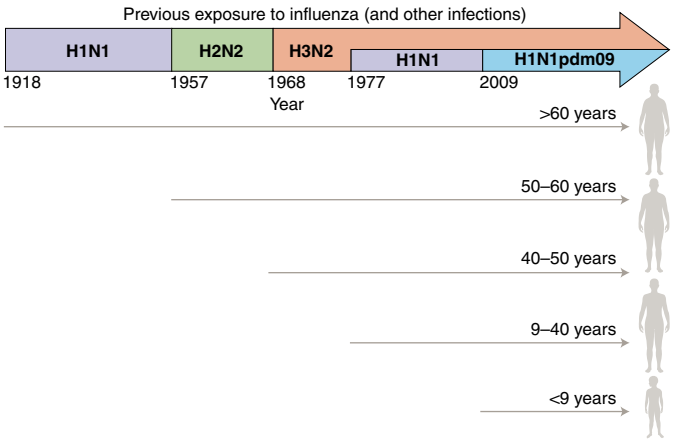

Fig. 3 | Immunologic imprinting by influenza A viruses during childhood. Immunologic imprinting varies from generation to generation because the circulating influenza A viruses in childhood differ. The first encounters of individuals born between 1918 and 1957, between 1957 and 1968, between 1968 and 1977, between 1977 and 2009, or after 2009 with an influenza virus would have been with H1N1, H2N2, H3N2, H1N1 or H3N2, or H1N1pdm09 or H3N2 virus, respectively. Even though the virus subtypes were identical between these periods, the antigenicity of the viruses changed over time. Therefore, infections during childhood immunologically imprinted the infected individuals in a variety of ways. This imprinting affects immune responses to subsequent virus infection and vaccination. Credit: Debbie Maizels/Springer Nature

lifetime, not just the antigens in the vaccines, an effect known as the 'back-boost'[78]. Such serologic evaluation can measure the quantity of HI antibodies, especially the most potent neutralizing antibodies that recognize the epitopes around the HA RBS.

Recent studies have revealed that infection- and vaccine-induced human in vitro neutralizing and non-neutralizing antibodies against the HA stem, which mostly recognize heterosubtypic HA[79–81], show in vivo protective efficacy via Fcγ-receptor-medicated activation of natural killer (NK) cells (antibody-dependent cellular cytotoxicity; ADCC), macrophages (antibody-dependent cellular phagocytosis; ADCP), and neutrophils (antibody-dependent neutrophil-mediated phagocytosis; ADNP)[69,82,83]. Antibodies against the HA head or stem also inhibit virus particle release from infected cells[81,84]. Thus, antibodies other than HI antibodies should be measured to evaluate vaccine immunogenicity in future trials. Accordingly, it is important to develop methods to evaluate different types of immunity that can serve as immune correlates for protection.

Since vaccination induces antibodies that protect vaccinees by different mechanisms, analyses using sera that contain a mixture of many different antibodies are not useful for developing a mechanistic understanding of vaccine protection. Antibody responses have been qualitatively analyzed by determining B cell receptor sequences to reveal the antibody repertoire at the molecular level. An unbiased antibody repertoire analysis revealed that the inactivated vaccine elicits antibody production from memory and naive B cells[85]. Of the elicited antibodies, many cross-reactive neutralizing clones that recognized the HA RBS and many cross-reactive non-neutralizing protective clones that recognized the lateral surface of the HA head were detected in some individuals[85,86]. However, the antibody response to a vaccine differs among individuals owing to differences in their history of influenza infection and vaccination[87–89].

Generation of broadly protective vaccines

Current inactivated vaccines provide some protection to vaccine recipients from viruses that are antigenically similar to the vaccine viruses. However, such vaccines fail to suppress infections caused by antigenically drifted viruses and offer no protection against an antigenic shifted virus that has the potential to cause a pandemic. Therefore, there is a need for a vaccine capable of inducing immune responses that last for a long time and that protect against a wide range of viruses, ideally all influenza A and B viruses[90]. Several approaches have been taken to produce such vaccines (so-called universal vaccines) based on the concept of inducing immune responses against the conserved protective epitopes in virus proteins. The targets of universal vaccine candidates include the HA stem, the RBS of HA, the extracellular domain of M2 (M2e), and the CTL epitopes in M1 and NP[36].

Although the antigenicity of the HA head varies between HA subtypes, that of the HA stem is highly conserved among HA group members[79,91]. Several epitopes in the HA stem are common across groups 1 and 2 (ref. [80]), and an epitope conserved in both type A and B viruses has also been reported[81]. Antibodies against the stem are typically heteroreactive and suppress virus replication by inhibiting membrane fusion and virus release as well as the activation of Fc-region-mediated cytotoxicity[84,92,93]. Therefore, several approaches have been proposed to elicit anti-HA stem antibodies, including immunization with headless HA[94–97], sequential chimeric or heterosubtypic HA[98–100], synthetic fragments or peptides of the HA stem[101,102], and hyperglycosylated HA[103].

M2 is a relatively conserved tetrameric type III transmembrane protein that functions as a proton-selective ion channel. M2e has been extensively investigated as a target for a universal vaccine. Antibodies against M2e do not interfere with virus entry but prevent virus release and activate effector cells via an Fc–receptor interaction[104]. Since M2e per se is poorly immunogenic, various strategies such as multimerization, display on virus-like particles or phages, and fusion with a carrier protein or a protein with adjuvant activity are being tested to improve the host immune response[105]. Animals vaccinated with M2e were shown to be protected from homologous and heterologous challenge infection[105,106]. Although several kinds of M2e vaccine have been evaluated in early-phase clinical trials, no M2e vaccine is as of yet available on the market. To overcome some of the limitations of the M2e-based vaccines, combination with other conserved proteins is now under consideration[105].

Despite the high variability of the HA head, the RBS is functionally conserved because sialic acid receptor recognition is an essential step for influenza virus entry. Antibodies against the RBS mimic the binding mode of sialic acid to some extent, resulting in a high cross-neutralizing capability[107–109]. Although the RBS could be a target for a broadly protective vaccine, such a vaccine is not being actively investigated because of the lack of an optimally designed antigen. However, efforts towards this goal are underway, as are efforts towards the development of antiviral drugs targeting the RBS[110,111].

NP and M1 are highly conserved among influenza A viruses; however, they are generally considered unsuitable targets for antibody-inducing vaccines owing to their lack of exposure on the virion surface, although NP has been detected on the cell surface[112]. Therefore, the conserved epitopes in these proteins are targets for CTLs, resulting in a broadly cross-reactive response. In fact, NP is the major target for the CTL response in humans[113]. Since activated $CD8^+$ T cells attack infected cells and enhance virus clearance, the CTL-inducing vaccines reduce disease severity and mortality but do not prevent infection. A modified vaccinia Ankara (MVA) expressing NP and M1 (MVA-NP + M1)[114] or a mixture of synthetic polypeptides derived from M1 and NP[115] has been used to induce CTL activation. These vaccines were evaluated in phase 1b or 2a trials and induced a good CTL response in humans[116,117].

If the next-generation vaccines are expected to induce antibodies possessing HI activity, it would seem to be appropriate to evaluate these vaccines by using the current HI assay. However, most of the next-generation vaccines currently under development

target areas other than the major antigenic sites of the HA head. Therefore, HI antibody titers are not a suitable measure for assessing such vaccines. Although antibody titers against the HA stem, M2e, or NA, or CTL activation against NP or M1 can be measured in humans and animals, we do not know whether they can serve as immune correlates of protection. Moreover, the evaluation method and threshold values used differ among vaccines. Therefore, regulatory science to control and evaluate next-generation vaccines needs to be established.

Optimal protection by understanding imprinting

Many candidate next-generation vaccines are under investigation in clinical or preclinical trials. One essential issue, 'immunologic imprinting' (or the so-called original antigenic sin), remains to be understood and managed. In 1960, it was proposed that the initial exposure to an influenza virus affects the antibody response to subsequent virus exposures[118].

Recent epidemiological research regarding H5N1 and H7N9 viruses has demonstrated that lifelong immunologic imprinting, which is elicited by infection with the influenza strain circulating during one's childhood, helps protect against unfamiliar HA subtypes from the same HA group[119]. This immunologic imprinting varies among individuals depending on the year of their birth and the virus strains they encountered, and it likely impacts how individuals will respond to the antigens of next-generation vaccines as well as current inactivated split vaccines (Fig. 3). Therefore, this necessitates a complete understanding of immunological imprinting by analyzing its establishment in an individual's childhood. It may be possible to optimally immunologically imprint individuals in childhood and to induce optimal immune responses in adults and the elderly by avoiding the original antigenic sin. Moreover, scientists must establish animal models for evaluation of immunologic imprinting; most animal experiments are conducted using animals that have never been infected with influenza virus. Optimal immunologic imprinting by vaccination of animals needs to be established to avoid unwanted immunologic imprinting.

Concluding remarks

Many kinds of next-generation vaccines are under development for clinical use. To establish truly universal vaccines, several of these vaccines may need to be combined. Although it will not be easy to develop a universal influenza vaccine, it may be possible with time, money, wisdom, and collaboration between laboratories, companies, and countries.

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Competing interests

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Additional information

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