Advances in the development of influenza virus vaccines

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Abstract | Influenza virus infections are a major public health concern and cause significant morbidity and mortality worldwide. Current influenza virus vaccines are an effective countermeasure against infection but need to be reformulated almost every year owing to antigenic drift. Furthermore, these vaccines do not protect against novel pandemic strains, and the timely production of pandemic vaccines remains problematic because of the limitations of current technology. Several improvements have been made recently to enhance immune protection induced by seasonal and pandemic vaccines, and to speed up production in case of a pandemic. Importantly, vaccine constructs that induce broad or even universal influenza virus protection are currently in preclinical and clinical development.

Antigenic drift
A mechanism by which influenza viruses escape from human 'herd immunity'. The RNA-dependent RNA polymerase of influenza viruses is relatively error prone and has no proofreading mechanism, resulting in a high frequency of point mutations. Immunologic pressure in the human population then selects for mutants that can escape from this herd immunity. The globular head domain of haemagglutinin is — owing to its immuno-dominance and high plasticity — most affected by antigenic drift.

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Seasonal influenza virus epidemics are estimated to cause 2–5 million cases of severe illness and up to 250,000–500,000 deaths per year worldwide[1]. Global annual infection rates are estimated to be 5–10% in adults and 20–30% in children[1]. Although current influenza virus vaccines are an effective countermeasure against disease, the vaccines induce narrow and strain-specific immunity (see BOX 1 for mechanisms of anti-influenza immunity) and have to be updated in a complex, costly and time-consuming process almost every year because of antigenic drift. Four distinct types of influenza viruses are currently co-circulating in the human population: two are influenza A viruses (the 2009 H1N1 pandemic strain and H3N2) and the other two are divergent lineages of the influenza B virus[2]. Vaccine formulations have to contain at least the two influenza A virus strains and one influenza B virus strain, which further complicates the manufacturing process of such vaccines[2].

In addition to seasonal epidemics, influenza viruses cause pandemics at irregular intervals. The influenza virus pandemic of 1918 claimed approximately 40 million lives and was caused by an H1N1 virus[3,4]. Since then, pandemics have been caused by H2N2 in 1957, by H3N2 in 1968 and again by H1N1 in 2009 (REFS 3,5). Pandemics are caused by influenza viruses that have crossed the species barrier from the animal reservoir (for example, avian species and swine) and acquire the ability to efficiently grow in humans and transmit among the population (BOX 2). Importantly, these viruses are often reassortants of haemagglutinin and neuraminidase (HA and NA) genomic segments from animal viruses and several internal genomic segments from human, or at least mammalian, virus origin[3]. Seasonal influenza virus vaccines are usually ineffective against novel pandemic viruses; therefore, a strain-specific vaccine has to be produced (FIG. 1). Unfortunately, the production of a strain-specific vaccine is time-consuming and the vaccine might be distributed and administered too late, as was the case in 2009 in the United States[6].

Here, we describe improvements that have been made in the production process of both seasonal and pandemic influenza virus vaccines to overcome these problems. Furthermore, we discuss novel vaccine constructs, vaccination regimens and adjuvants that induce broader and sustained protection. Finally, we review novel findings regarding the immune response towards haemagglutinin and neuraminidase, and provide an overview of several universal influenza virus vaccine approaches that could lead to vaccines with lifelong protection from any type of influenza virus[7].

Improving seasonal influenza virus vaccines

Vaccines against influenza A and B viruses were invented in the 1940s. These early vaccines, termed whole-virus inactivated vaccines, were generated in embryonated chicken eggs (a technology that is still predominant today) and consisted of crudely purified whole virus inactivated with formalin and phenylmercuric nitrate[8,9]. The effect of antigenic drift made it necessary to reformulate vaccines after only 2 years of use, and the World Health Organization soon established an influenza surveillance network for the early detection

Box 1 | **Immune responses that protect against influenza virus infections and disease**

Several components of the immune system contribute to protection against influenza virus infection and disease. Antibodies act early on in an infection. Mucosal antibodies can neutralize viruses before an infection is established. Systemic antibody responses have a similar potency and might also be able to prevent infection (sterilizing immunity). Antibodies might neutralize viruses via several mechanisms, including antibody-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity (see FIG. 2) — activities that are not readily measured in neutralization assays. The breadth of the antibody response varies from narrow, strain-specific responses (for example, those induced by inactivated influenza vaccines) to broadly neutralizing heterosubtypic responses (for example, those induced by stalk-based universal influenza virus vaccines or natural infection). Cytotoxic T cell responses are another important immune component that mediates substantial protection against development of disease following influenza infection. T cells act later in an infection than antibodies but can exhibit broad reactivity owing to the conservation of internal influenza virus proteins, which possess strong T cell epitopes. Virus-vectored vaccines, live attenuated influenza vaccines and natural infection induce strong cytotoxic T cell responses, whereas inactivated influenza vaccines fail to do so.

of drifted strains[10,11]. The 1968 pandemic led to the development of trivalent inactivated vaccines (TIVs) against influenza viruses[12]. Furthermore, studies on reactogenicity to different vaccine formulations in children ultimately led to the development of split and subunit vaccines[13]. These vaccines are split using ether and/or detergent, and haemagglutinin and neuraminidase are, in the case of subunit vaccines, purified and enriched[2]. In addition to inactivated influenza vaccines (IIVs), live attenuated influenza vaccines (LAIVs) are also used. LAIVs are usually temperature-sensitive and cold-adapted and will efficiently replicate in the upper respiratory tract but not in the lower respiratory tract. LAIVs, which are administered by nasal spray, have been developed in parallel in Russia (licensed in 1980) and in the United States (licensed in 2003)[14–16].

A recent study that evaluated 34 randomized clinical trials concluded that the vaccine efficacy of LAIVs in children (the age group for which this type of vaccine is indicated and thought to be most effective) is approximately 83% and the efficacy of TIVs in adults is approximately 75% (REF. 17). Furthermore, a study on the use of IIVs in pregnant women in Bangladesh showed that vaccination reduced the incidence of influenza virus infection in mothers and newborns, and also significantly decreased the number of stillbirths and increased birth weight[18,19]. Collectively, these studies demonstrate that current seasonal influenza virus vaccines confer good protection against infection and are an important public health tool. However, a vaccine efficacy of 75% is far from optimal and drops sharply in the elderly who are more susceptible to influenza virus infection[20,21]. Furthermore, the duration of protection is short[22,23]. Mismatches between vaccine strains and circulating strains also occasionally occur and are usually associated with lower vaccine efficacy[24].

Recently, improvements in vaccine formulations have been made with the goal of eliciting better protection against seasonal influenza virus strains. To induce a stronger, broader and more sustained immune response — specifically in the elderly — several novel formulations have been tested (TABLE 1). These formulations range from high-dose vaccines for the elderly, which have been licensed in the United States[25,26], to the development of several adjuvanted vaccines. Two of the most advanced adjuvant formulations — MF59 and AS03 — have been tested with seasonal influenza virus vaccines and were able to enhance the efficacy of the vaccines[27]. MF59 adjuvanted seasonal vaccines for the elderly population have been licensed and marketed in more than 25 countries under the brand name Fluad (Novartis)[27,28]. AS03 adjuvanted influenza vaccines are also under consideration for use in the elderly population[29]. An additional improvement in seasonal influenza virus vaccines is the inclusion of a second influenza B virus strain. The novel quadrivalent influenza virus vaccine is now licensed in the United States as an IIV and a LAIV, but debate regarding the added value of these vaccines compared with TIVs is ongoing[30–32].

Another strategy that can be used to induce a broader and more sustained immune response against seasonal influenza virus strains is based on heterologous prime–boost regimens. This type of regimen has been tested in mice, in ferrets and in nonhuman primates. A DNA vaccine expressing a haemagglutinin from a seasonal influenza virus is administered first (prime), and a typical TIV is subsequently administered (boost). Mice that received the prime–boost regimen showed broader immunity and had a more than 50-fold higher neutralizing titre than that induced by TIVs only[33]. Pre-existing immunity to influenza virus, which occurs in humans, did not have a negative effect on this vaccination regimen[34]. Several clinical trials that translated these findings into humans have recently been completed (ClinicalTrials.gov identifiers: NCT01609998, NCT01676402, NCT00995982 and NCT01498718). Another study showed that vaccination with ferritin particles displaying influenza virus haemagglutinin trimers induced stronger and broader immune responses than TIVs[35].

Improvements on the vaccine production side include the US licensure of the first recombinant influenza virus vaccine (FluBlok; Protein Sciences Corporation) and the US licensure of the first cell-culture-derived seasonal influenza virus vaccine (Flucelvax; Novartis)[36,37]. These developments in vaccine production have also had a high impact on improving the speed at which pandemic influenza virus vaccines can be produced (FIG. 1).

Improving pandemic preparedness

During the past decades, several avian influenza viruses have caused zoonotic outbreaks in the human population. These outbreaks were sporadic and were usually

Haemagglutinin
(HA). A homotrimeric viral surface glycoprotein that mediates the attachment of influenza viruses to cells by binding to sialic acids on glycan structures of cellular receptors. Haemagglutinin also mediates the fusion of viral and endosomal membranes, which causes the release of the viral genome into the cytosol. Haemagglutinin is the major antigen of the virus.

Neuraminidase
(NA). A viral homotetrameric viral surface glycoprotein with sialidase activity. Neuraminidase helps transport the virus trough mucosal surfaces and mediates the release of budding viruses from the cell surface.

Whole-virus inactivated vaccines
Whole-virus inactivated vaccines are based on intact virions that have been chemically (for example, with formalin or β-propiolactone) or physically (for example, with ultraviolet light) inactivated. Treatment of these virions with detergent leads to split vaccines. Further (partial) purification of the haemagglutinin and neuraminidase of viruses results in subunit vaccines.

Haemagglutination inhibition
(HI). Haemagglutination activity is the standard correlate of protection used for influenza virus vaccines, and haemagglutination inhibition describes the ability of antibodies to block the binding of the haemagglutinin globular head domain to cellular receptors. Stalk-reactive antibodies are generally haemagglutination inhibition negative.

associated with close contact to infected poultry or other avian species. Highly pathogenic H5N1 viruses in humans were first detected in Hong Kong in 1997 and reappeared in 2003 (REFS 38,39). These viruses express a haemagglutinin with a multibasic cleavage site and are therefore able to replicate to high titres in many tissues in infected birds[40]. The H5N1 virus is now distributed over Eurasia and Africa and has evolved into a number of antigenically distinct clades[39]. Humans have been occasionally infected and the high fatality rate of the infection, together with the wide geographical spread of the H5N1 virus, has raised concerns about its pandemic potential[41] (see The WHO Influenza Monthly Risk Assessment Summaries; Influenza at the Human–Animal Interface (in Further information)). However, serological data suggest that a high number of infections with the virus — for example, in Southeast Asia — remain subclinical in humans[42]. Furthermore, the H5N1 virus expresses an N1 subtype of neuraminidase that is closely related to the neuraminidase of the currently circulating pandemic H1N1 virus[43]. As such, the human population would not be completely naive to a pandemic strain of H5N1.

Many other zoonotic viruses, including H5N6, H6N1, H7N9 and H10N8, have recently caused morbidity and mortality in humans in Asia[44–48]. In addition, H3N2 variant viruses that transmit from pigs to humans, seal H3N8 and H10N7 viruses, and highly pathogenic avian H5N8 and H7N3 viruses have raised concerns about their potential to spread in the human population in Europe and in North America[49–53]. It is difficult to predict the strain or subtype that will cause the next influenza virus pandemic. As the human population expands, the interface between the animal reservoir of influenza viruses and the human population grows. This expanded interface makes it more likely for a virus to cross the species barrier. Therefore, the development of vaccines for influenza virus strains with pandemic potential is warranted to improve our pandemic preparedness.

Currently, there are two major problems relating to pandemic influenza vaccines that need to be addressed. The first is the lag between pandemic virus identification and vaccine development and distribution. When a novel pandemic virus is identified, it takes months to develop, test, distribute and administer the new vaccine. After vaccination of an individual, it takes an additional 2–3 weeks until a protective immune response is mounted (FIG. 1). A stark example of this problem is the situation in 2009, when the majority of the pandemic H1N1 vaccine was distributed only after the second wave of the pandemic hit the US population[6]. The second issue is low immunogenicity. For example, current pandemic candidate vaccines against H5N1 and H7N9 induce relatively weak immune responses as measured by the traditional correlate of protection, the haemagglutination inhibition (HI) titre[54–57]. The cause of this low immunogenicity is currently debated, and vaccine formulations and regimens to overcome this problem are being developed.

Vaccine candidates for potentially pandemic viruses have been developed using a range of different production platforms. The IIV platform — in the split and whole virus format — has advanced the furthest, and vaccines made using this platform have been used for stockpiling[58,59]. In the case of vaccines against highly pathogenic H5N1 strains, seed strains have been generated using reverse genetics to remove the multibasic cleavage site of the haemagglutinin and to change the backbone to that of a high-growth A/Puerto Rico/8/1934 H1N1 strain[59]. These modifications render the vaccine strains safer and production possible because highly pathogenic influenza A viruses usually kill embryonated eggs, resulting in low yields of the vaccine[59]. A number of these H5N1 and H7 vaccines have been tested in humans and a high antigen dose or the use of an adjuvant (or a combination of both) was necessary to induce reliable haemagglutination inhibition titres above 1:40, which is the titre needed for approval by US and European regulatory authorities[59,60]. Even under these conditions, immune responses were low. A recent clinical trial of a H7N9 vaccine candidate resulted in a vaccine efficacy of approximately 60% despite the use of an adjuvant[61]. As described below, it has been hypothesized that vaccination with H5 (group 1 haemagglutinin) or H7 (group 2 haemagglutinin) vaccines primarily boosts antibodies against the conserved stalk domain of the haemagglutinin structure to which humans have low levels of pre-existing immunity[62–64]. This might explain why adjuvants and multiple vaccinations are necessary to yield sufficient vaccine efficacy.

As described above, two LAIV backbones (cold adapted A/Ann Arbor/6/1960 and A/Leningrad/134/17/1957) are currently available. Both backbones, as well as experimental LAIV constructs, have been used to generate and test pre-pandemic vaccines, including H2-, H5-, H6- and H7-expressing candidates[65–74]. Immune responses measured upon vaccination with these constructs in humans are moderate to weak depending on the ability of the vaccine virus to replicate in the upper respiratory tract[65–73]. The inability of vaccine viruses to replicate in the upper respiratory tract may be due to the absence of a specific glycan structure in this part of the anatomy of humans[75].

A novel strategy that can improve the efficacy of pandemic vaccines is the use of a LAIV or DNA vaccine prime followed by an IIV boost. The LAIV or DNA

Box 2 | **Pandemic influenza viruses**

Currently, 16 haemagglutinin subtypes and 9 neuraminidase subtypes have been detected in the animal reservoir, mostly in avian species. In addition, H17N10 and H18N11 have recently been identified in bats (although these haemagglutinin subtypes do not haemagglutinate and the corresponding neuraminidase subtypes have no neuraminidase activity). Viruses from the animal reservoir can occasionally cross the species barrier and cause morbidity and mortality in humans (for example, H5N1 and H7N9) but these incidents do not usually result in a pandemic. Viruses that cause pandemics need to transmit efficiently in humans and are mostly reassorted viruses that have genomic segments from both human and animal viruses. After a pandemic, the virus that caused it usually establishes itself in the population, starts to drift antigenically owing to human herd immunity and becomes a seasonal influenza virus. Influenza B viruses lack substantial animal reservoirs and therefore do not cause pandemics.

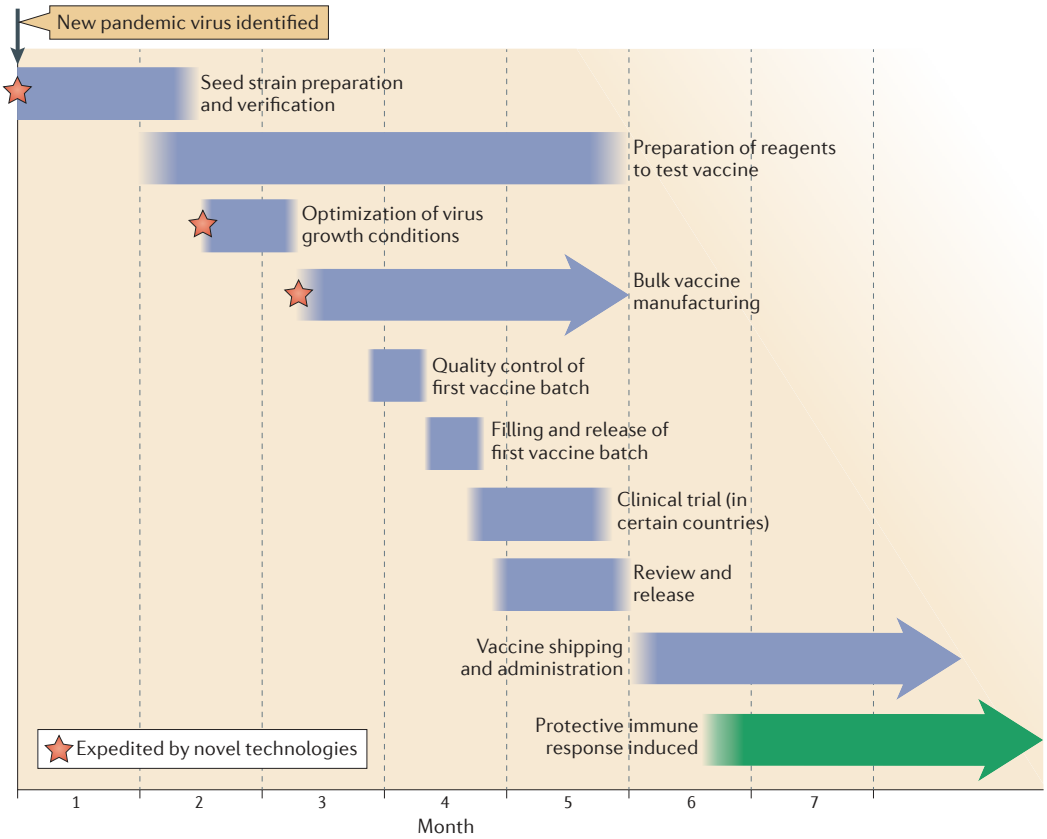

Figure 1 | **Advances in the pandemic influenza virus vaccine production process.** This figure shows the vaccine production process in response to a new pandemic. Orange stars indicate steps that could be accelerated by using novel technology. For example, seed strain preparation and verification can be facilitated by using gene synthesis, reverse genetics and deep sequencing. Once seed strains are prepared, viruses can be rescued in a backbone that has been optimized for growth on the selected substrate. This strategy can reduce the time required for growth optimization. Bulk manufacturing can be expedited by using novel production technologies that are easy to scale up (for example, cell culture or recombinant protein technologies). Adapted from REF. 227, World Health Organization.

vaccine immunologically primes subjects — often without a measurable seroconversion — and this immune response can subsequently be recalled by administering an IIV boost. Several clinical trials have demonstrated the value of this approach[76–78]. Strategies to prime particular groups of the human population (for example, health-care workers) with H5 or H7 LAIVs to induce a rapid and strong recall of the immune response in case of a pandemic are currently being discussed.

Novel platforms for rapid vaccine production. Rapid vaccine production in response to a novel pandemic influenza virus strain is vital for reducing global morbidity and mortality. Several novel technologies that improve the vaccine production process have been described in recent years (FIG. 1). The use of cellular substrates could make influenza virus vaccine production independent of the global embryonated egg supply and enable easy scaling up of the process. Several cell lines, including Madin–Darbey canine kidney cells, Vero cells (African green monkey) and Per.C6 cells (human), have been tested and established for influenza virus vaccine production[55,79,80]. In addition, novel gene synthesis technologies combined with influenza virus reverse genetics now enable the generation of custom-made seed strains within very short time frames[80,81]. These novel technologies can be used for both IIV and LAIV candidates, abolish the need for time-consuming classical reassortment and could significantly shorten their production time.

Recombinant protein expression has several advantages for the production of pandemic influenza virus vaccines. These include rapid vaccine production, the absence of infectious virus during production, the independence from egg supplies, the ease of scale up, the ability to use sequences derived directly from clinical specimens without egg- or cell-culture passage history and — for many recombinant expression systems — the low cost of production. A disadvantage of this approach is the reliance on one influenza virus antigen, usually haemagglutinin. These vaccines therefore lack the multifaceted immune response against other influenza virus proteins that might confer protection.

Numerous recombinant protein vaccines, mostly haemagglutinin-based, are currently in preclinical and clinical development. Additionally, the trivalent seasonal recombinant haemagglutinin vaccine FluBlok, which is

Table 1 | **Overview of established and novel influenza virus vaccine technologies**

Technology	Type of immunity	Breadth of protection	Development stage	Comments	Refs
LAIVs (seasonal or pandemic)	Humoral, cellular and mucosal	Strain-specific but broader than inactivated vaccines	Licensed (seasonal), clinical (pandemic)	Mucosal administration	14–16, 65–74
IIVs (seasonal or pandemic)	Predominantly humoral	Strain-specific	Licensed (seasonal and pandemic)	–	2
Quadrivalent influenza vaccines (seasonal; as IIVs or LAIVs)	Dependent on the platform used	Strain-specific	Licensed (seasonal)	Protects against both influenza B lineages	30–32
Recombinant insect-cell-produced HA vaccines (seasonal as TIVs or pandemic)	Predominantly humoral	Predominantly strain-specific	Licensed (seasonal), clinical (pandemic)	Rapid production, no infectious virus during production process, no antigenic changes during production or passaging, does not rely on egg supply	37
High-dose IIV	Predominantly humoral	Strain-specific	Licensed	Higher dosage used to induce better immune responses in the elderly	25,26
Adjuvanted IIV (seasonal or pandemic)	Predominantly humoral	Strain-specific but broader than inactivated vaccines	Licensed in several countries	Broader and stronger immune responses compared to regular IIVs, dose sparing	27–29
Cell-culture-derived IIVs (seasonal or pandemic)	Predominantly humoral	Strain-specific	Licensed (seasonal), clinical (pandemic)	Rapid production, does not rely on egg supply	36,55, 79,80
Heterologous prime–boost regimens (seasonal or pandemic)	Predominantly humoral	Broad	Clinical	Combinations of LAIV or DNA prime vaccinations with IIV or recombinant protein booster vaccinations	33,34, 76–78
DNA vaccines (seasonal or pandemic)	Predominantly humoral	Strain-specific	Clinical	Highly cost-effective, easy scale-up	103
Insect-cell-derived VLPs (seasonal or pandemic)	Humoral and cellular immunity	Strain-specific	Clinical	Rapid production, no infectious virus during production process, no antigenic changes during production or passaging, does not rely on egg supply	94,101
Plant-derived influenza virus vaccines (seasonal or pandemic)	Predominantly humoral but also cellular immunity (VLPs)	Strain-specific	Clinical	Rapid production, no infectious virus during production process, no antigenic changes during production or passaging, does not rely on egg supply	84,99, 100,102
Bacterial-expressed influenza vaccines (seasonal or pandemic)	Predominantly humoral	Strain-specific	Clinical	Rapid production, no infectious virus during production process, no antigenic changes during production or passaging, does not rely on egg supply, highly cost-effective	85,86,92
MVA-vectored vaccines (pandemic)	Humoral and cellular	Strain-specific	Clinical	Does not rely on egg supply, no antigenic changes during production or passaging, safe vaccine platform	105–109, 111
MVA-vectored vaccines (universal)	Cellular	Broad, universal	Clinical	Strong cellular immune responses, also considered as an additive to seasonal IIVs	209–213
M2e (universal)	Humoral (ADCC)	Broad, universal	Clinical	Tested in different forms of fusion proteins and VLPs	200–205
Epitope or peptide vaccines (universal)	Cellular	Broad, universal	Clinical	Developed as an additive to IIVs	222,223
Headless HA (universal)	Humoral	Broad, universal	Preclinical	Induces broadly reactive antibodies to the HA stalk domain	169–174
Chimeric HA (universal)	Humoral	Broad, universal	Preclinical	Induces broadly neutralizing antibodies to the HA stalk domain, production platform independent	7,62–64, 175–181
Centralized HA (broad seasonal)	Humoral	Broad, seasonal	Preclinical	Production platform independent	182–185, 188
Ferritin nanoparticle-based vaccines (broad, seasonal)	Humoral	Broad, seasonal	Preclinical	–	35

ADCC, antibody-dependent cell-mediated cytotoxicity; HA, haemagglutinin; IIV, inactivated influenza vaccine; LAIV, live attenuated influenza vaccine; M2e, 22–23-amino-acid short ectodomain of M2; MVA, vaccinia virus Ankara; TIV, trivalent inactivated vaccine; VLP, virus-like particle.

produced in insect cells, has already been licensed by the US Food and Drug Administration and paved the way for pandemic vaccines to be produced in the same manner[37]. Popular expression systems for influenza virus vaccines and vaccine candidates include the following: baculovirus and insect cell expression systems[82,83]; *Agrobacterium* species-driven expression in plants such as the *Nicotiana* species[84]; and bacterial expression in *Escherichia coli*[85,86]. Furthermore, vaccine candidates have been expressed in *Lactobacillus* species[87], algae[88], yeast[89,90] and cell-free expression systems[91]. Haemagglutinins expressed in insect and plant cell expression systems are relatively similar to those expressed in mammalian cells, with the exception of the N-linked glycosylation pattern, and are usually correctly folded. By contrast, haemagglutinin expressed in *E. coli* is not glycosylated, forms inclusion bodies and has to be refolded[85,92].

Another platform developed for the production of influenza virus vaccines is the use of virus-like particles (VLPs). VLPs can be produced by co-expression of influenza virus structural proteins in mammalian cells, insect cells or plants[83,93–100]. Pandemic influenza VLP vaccines have been clinically tested and have shown good safety and efficacy profiles[94,101,102].

In addition, several DNA and virus-vectored pandemic influenza virus vaccines are currently in preclinical and clinical development[103,104]. Many virus-vectored vaccines are based on modified vaccinia virus Ankara (MVA) because of its excellent safety profile. Several H5N1 and H7N9 MVA constructs have been tested in animal models and can induce strong cellular and humoral immune responses[105–110]. The efficacy of these vaccines in humans is currently being tested in clinical trials[111].

Immunity to haemagglutinin and neuraminidase

Recent advances in human monoclonal antibody (mAb) technology, including phage library technology and expression cloning of antibodies from plasmablast and memory B-cell populations, have made it possible to gain new insight into the immune responses towards the influenza virus surface glycoproteins haemagglutinin and neuraminidase[112–117] (FIG. 2). The rediscovery of haemagglutinin stalk-reactive antibodies that was facilitated by these techniques was a major milestone towards the development of a universal influenza virus vaccine. The first stalk-reactive antibody, mAb C179, was isolated in 1992 using traditional murine hybridoma technology[118]. However, stalk-reactive antibodies are rare in humans, and the first human antibodies with this specificity — CR6261, F10 and a small number of mAbs generated from an antibody library of Turkish H5N1 survivors — were only isolated in 2008–2009 (REFS 115,116,119). In addition to haemagglutinin stalk-reactive antibodies, several broadly reactive antibodies against the haemagglutinin globular head domain and neuraminidase have been discovered[120–125].

Haemagglutinin stalk-reactive antibodies. Stalk-reactive antibodies are particularly interesting because they bind epitopes on the membrane proximal, conserved portion of haemagglutinin and therefore show broad binding to divergent haemagglutinins. The binding pattern of most stalk-reactive antibodies follows the phylogeny of the influenza virus haemagglutinins and they bind to either group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) or group 2 (H3, H4, H7, H10, H14 and H15) haemagglutinins[116,118,126–129]. However, some stalk mAbs have a narrower binding pattern and only recognize haemagglutinin of one subtype (for example, mAb 6F12 shows pan-H1 binding, and mAb 12D1 shows pan-H3 binding), whereas other exceptionally rare antibodies bind to all influenza A haemagglutinins or even cross-react between influenza A and B haemagglutinins[130–134]. Importantly, most stalk-reactive antibodies seem to bind preferentially to conformational epitopes but do not recognize denatured haemagglutinin[116,126,135]. Furthermore, they do not show haemagglutination inhibition activity[136]. In contrast to antibodies with haemagglutination inhibition activity (FIG. 2), which mostly neutralize by inhibiting the interaction between haemagglutinin and sialic acid residues on cellular receptors, stalk-reactive antibodies may protect through several mechanisms (FIG. 2).

Upon binding to haemagglutinin, stalk-reactive antibodies lock the haemagglutinin trimer in a pre-fusion conformation and prevent pH-triggered conformational change when the virus is taken up into the endosome (FIG. 2). Therefore, no fusion of the viral and endosomal membranes can occur and the virus is trapped in the endosome[116,126,130,137]. In addition, antibody binding sterically blocks access of proteases to the basic cleavage site between the HA1 and HA2 subunits of haemagglutinin, which is located in the stalk domain[126,137] (FIG. 2). Uncleaved haemagglutinin (HA0) is unable to undergo the necessary conformational changes for fusion, and this mechanism might also contribute to the protection against infection. Finally, stalk-reactive antibodies also retain newly formed haemagglutinin on the cell surface and may inhibit virus budding[129] (FIG. 2). In addition to mechanisms that directly neutralize the virus, other mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity might contribute to protection conferred by stalk-reactive antibodies *in vivo*[138–142] (FIG. 2). Specifically, ADCC is an important factor and can potentiate the protective efficacy of stalk-reactive antibodies *in vivo*[139].

Stalk-reactive antibodies are not induced at significant levels by currently used IIVs. The stalk domain seems to be immunosubdominant compared to the immunodominant globular head domain to which most antibodies are directed[63,113,114]. However, natural infection is able to induce a baseline level of these antibodies in mice and humans[143–145]. Interestingly, stalk-reactive antibody levels were boosted significantly by infection with the 2009 pandemic H1N1 virus, and these antibodies were also isolated from individuals who survived an H5N1 infection[119,146,147]. This led to the hypothesis that exposure to haemagglutinins that have a divergent head domain to which humans are naive (for example, H5N1 or pH1N1) and to stalk domains with conserved epitopes can boost stalk-reactive antibody titres. Additional support for this hypothesis comes from the

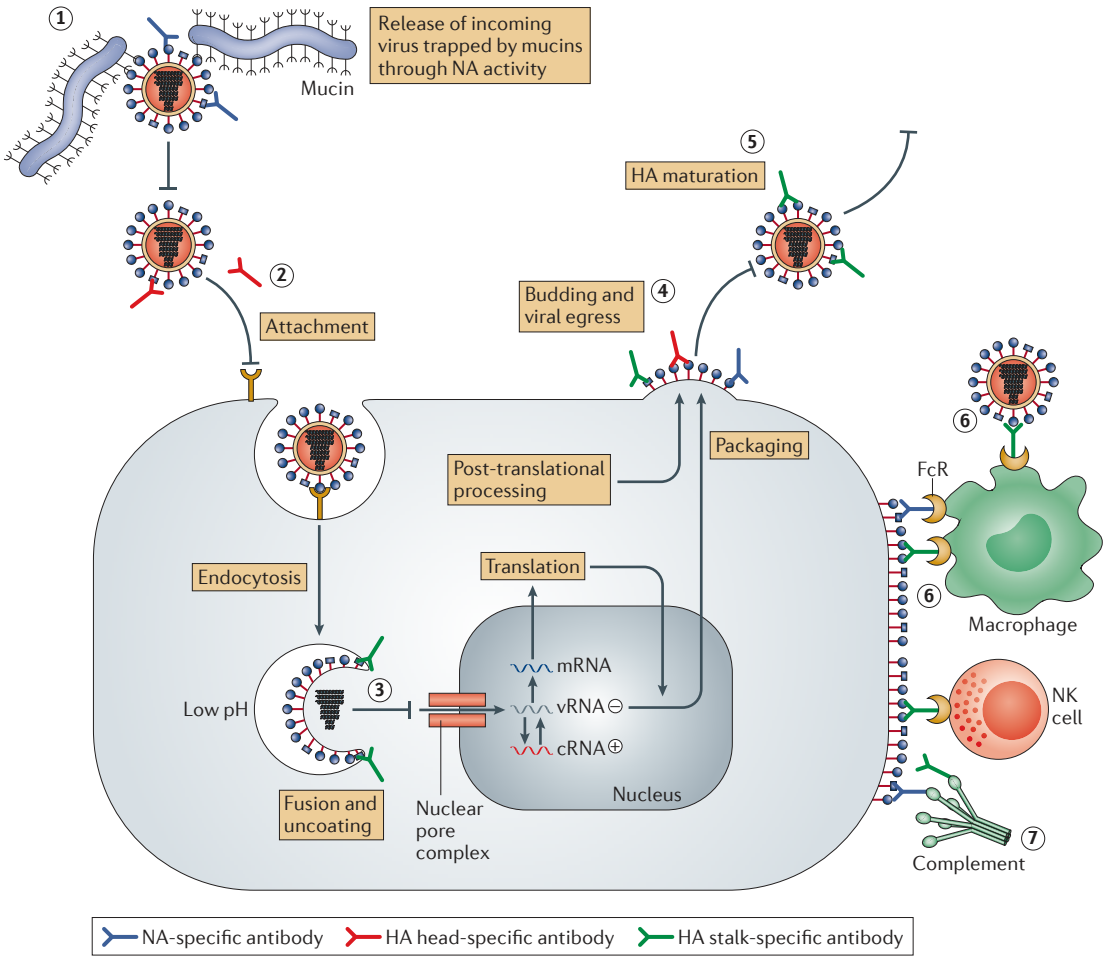

Figure 2 | **Mechanism of action of haemagglutinin-specific and neuraminidase-specific antibodies.** Antibodies directed against the haemagglutinin (HA) globular head domain (red) and the stalk domain (green) or against neuraminidase (NA) (blue) may confer protection via a number of mechanisms. As viruses enter the host and come in contact with mucosal surfaces they are trapped by highly glycosylated innate defence proteins called mucins (step 1). NA helps the virus to pierce through this layer, and this activity may be inhibited by NA-specific antibodies. Antibodies that bind to the globular head domain of HA sterically block interactions between HA and sialic acid on cellular receptors, effectively inhibiting attachment of the virus to the cell (step 2). In rare cases, NA-specific antibodies can also have this function (also working through steric hindrance). Stalk-reactive antibodies bind to the HA on virus particles and prevent the fusion of viral and endosomal membranes by blocking the rearrangement of the HA fusion machinery (step 3). Head-, stalk- and NA-reactive antibodies may inhibit budding and viral egress (step 4). Stalk-reactive antibodies bound to HA sterically inhibit HA maturation (step 5). Stalk-reactive antibodies and possibly NA-reactive antibodies also act through antibody-dependent cell-mediated cytotoxicity (step 6) or complement-dependent cytotoxicity (step 7). cRNA, complementary RNA; FcR, Fc receptor; NK, natural killer; vRNA, viral RNA.

analysis of clinical trials with pandemic vaccine candidates — including H5N1, H7N1 and swine-origin H1N1 strains — which induced preferentially stalk-reactive antibodies[62–64,148–150].

Interestingly, studies with H5N1 vaccines showed that the first vaccine administration induces high levels of stalk-reactive antibodies, whereas the second vaccination with the same vaccine formulation predominantly induces a response against the globular head domain[63,64]. This result indicates that the globular head domain regains immunodominance over the stalk domain once the immune system is primed for these novel head domain epitopes. Importantly, polyclonal anti-stalk responses induced by H5N1 vaccines are highly crossreactive towards group 1 haemagglutinins but do not significantly crossreact with group 2 haemagglutinins when measured using quantitative methods[63,64].

Broadly reactive antibodies against the haemagglutinin globular head domain and neuraminidase. In addition to broadly neutralizing stalk-specific antibodies, a small number of human antibodies that can neutralize a broad panel of influenza viruses through binding to the haemagglutinin head domain have been isolated[121–124]. Some of these antibodies bind to the receptor-binding site of haemagglutinin by mimicking sialic acid, the substrate

to which haemagglutinin binds[122–124]. This molecular mimicry explains the binding breadth of these antibodies, which sometime spans several subtypes. However, the antibodies need to insert one of their binding loops deep into the receptor-binding site, and the addition of glycans on the rim around the receptor-binding site can sterically prevent binding without forcing the virus to change the conserved receptor-binding domain. Most of these antibodies are exceptionally rare but some light has been shed recently on the induction of broadly neutralizing antibodies against the H1 head domain of haemagglutinin[151,152]. Similar to stalk-reactive antibodies, these antibodies seem to be mostly induced when individuals are exposed to highly divergent H1 haemagglutinins over time. In this context, the specific exposure history of an individual, and especially the virus to which the individual was first exposed, seem to have a major role[151,152].

Several antibodies against the second surface glycoprotein, neuraminidase, have also shown exceptional breadth[153]. A rabbit mAb against a conserved linear epitope on neuraminidase showed a broadly inhibitory effect on divergent neuraminidases from influenza A and B viruses and showed limited protection in passive transfer experiments[154,155]. However, it is unclear whether similar antibodies are induced by natural infection or influenza virus vaccination. In addition, murine antibodies with broad reactivity to the N1 subtype of neuraminidase have been reported recently[120]. Several of these have neuraminidase inhibition (NI) activity (FIG. 2) and are able to reduce virus cell-to-cell spread *in vitro*. In general, neuraminidase inhibition activity seems to correlate with *in vivo* protection for these antibodies. However, protection was also seen in cases in which mAbs did not have neuraminidase inhibition activity against the challenge virus, suggesting that alternative mechanisms such as ADCC and complement-dependent cytotoxicity might also have a role *in vivo*[120].

Glycans: in the context of broadly reactive immune responses, size matters. Both the influenza virus haemagglutinin and neuraminidase are glycoproteins that have several putative N-glycosylation motifs, and glycosylation might have an important role in the folding and biology of these proteins[156] (FIG. 3). Haemagglutinin has a variable number of glycosylation sites in the head domain, whereas glycosylation sites in the stalk domain are relatively conserved across haemagglutinin groups[156]. Haemagglutinin glycosylation has a strong influence on the pathogenicity and antigenicity of haemagglutinin, whereas the role of N-linked glycosylation on neuraminidase is less well understood[157]. Recent studies suggest that the number and size of glycans on haemagglutinin also influence the breadth of the immune response. Pre-pandemic seasonal H1, pandemic H1 or H5 haemagglutinins that were enzymatically treated to reduce the number of glycan structures to one *N*-acetylglucosamine showed broader immune responses and protection against challenge with heterologous strains than fully glycosylated haemagglutinins[158,159]. However, complete deglycosylation led to reduced protection, which is probably due to the loss of important conformational epitopes.

Reduction of the glycan size seems to lead to stronger immune responses against conserved epitopes that are probably less accessible when shielded by large glycans. This hypothesis is supported by studies showing that binding of broadly neutralizing stalk-reactive antibodies to fully glycosylated haemagglutinin is inhibited at low temperature (4 °C), which is when glycan structures are becoming rigid[160]. Interestingly, this effect was not seen with haemagglutinin produced in insect cells, which has smaller paucimannose-like non-complex glycan structures.

Glycan size on haemagglutinin is strongly influenced by the production method. Mammalian-cell-derived haemagglutinins (on average 12 monosaccharide units, sialylated if expressed without neuraminidase) have the largest glycans followed by egg-derived haemagglutinins (8–9 monosaccharide units, highly branched, no sialic acid). Insect-cell-derived haemagglutinins have glycans that are 5–6 monosaccharide units in length[161] (FIG. 3). Therefore, vaccines made in production platforms that produce haemagglutinins with smaller glycans — such as insect cells[83] — might be more suitable for inducing broad immune responses. However, some insect cell lines are known to add α-1,3-linked fucose to their glycans, which can be allergenic[162].

Large glycan structures can shield epitopes from immune recognition on haemagglutinin[157]. The introduction of additional glycosylation sites on the immunodominant head domain might therefore be used to skew the immune response towards immunosubdominant epitopes in the stalk domain. A recent study demonstrated that hyperglycosylated H1 haemagglutinin produced in mammalian cells induces broadly protective immune responses against the stalk domain[163]. Similar results have been reported with prime–boost H5 vaccine strategies with vaccine constructs that had additional glycosylation sites grafted on the head domain[164,165].

Development of universal influenza virus vaccines

Stalk-based vaccine constructs. Attempts to construct vaccines based on the stalk domain by removing the immunodominant head domain (producing a headless haemagglutinin) were made as early as 1983 (Ref166). The methodology used to remove the head domain, or more specifically the HA1 subunit of the haemagglutinin, involved an acid treatment followed by treatment with a reducing agent. However, this treatment induced significant conformational changes in the HA2 portion of the stalk domain and completely removed the HA1 portion of the stalk domain, therefore destroying important conformational epitopes. Following the discovery of the stalk-reactive mAb C179, a genetic approach to remove the globular head domain was developed[167]. A modified H2 haemagglutinin was expressed in mammalian cells and used to vaccinate mice, where it achieved limited protection against a heterosubtypic H1N1 challenge[167]. Another approach with an H1-based headless haemagglutinin displayed on VLPs showed success in the mouse model and was able to induce antibodies that crossreacted with H2 and H5 haemagglutinin[168]. Several other stalk-only and headless haemagglutinin constructs

Neuraminidase inhibition (NI). NI describes the ability of antibodies to block the sialidase function of neuraminidase.

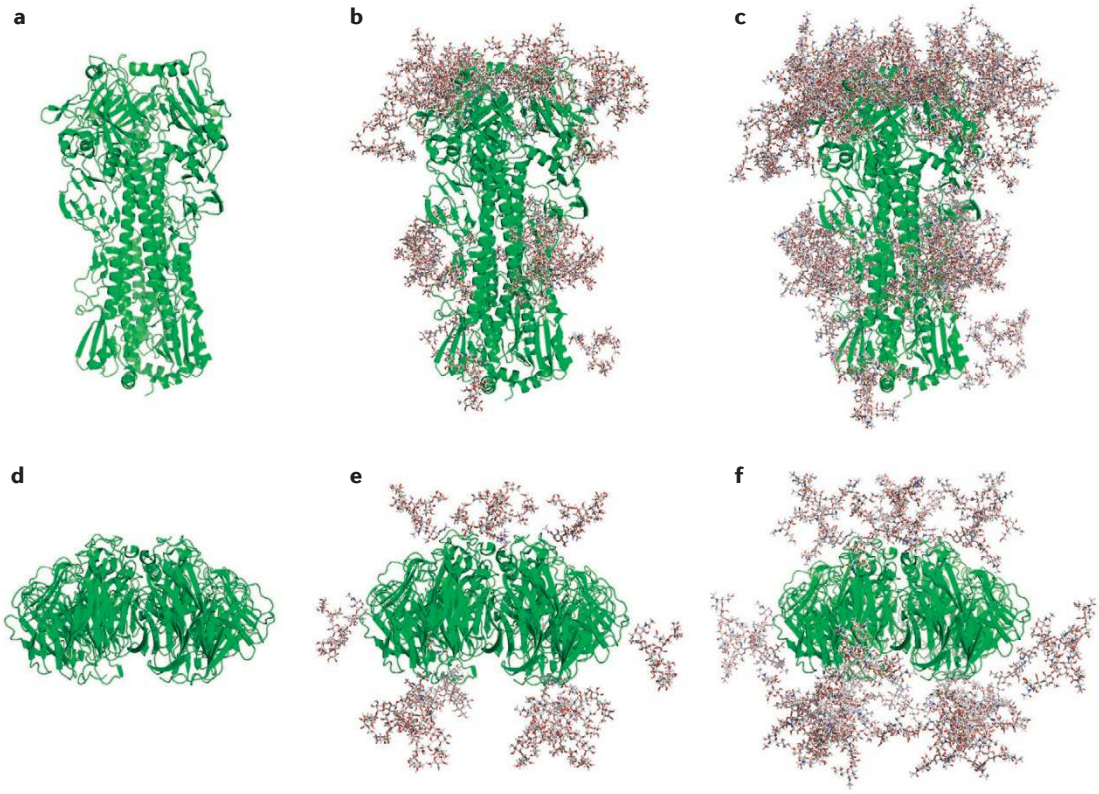

Figure 3 | **Glycosylated influenza virus haemagglutinin and neuraminidase molecules. a**–**c** | Haemagglutinin trimers without glycan structures attached (part **a**), with oligomannose glycan structures attached (part **b**) and with complex glycan structures attached (part **c**). Structures are based on the H3 subtype of haemagglutinin of a recent seasonal H3N2 isolate, A/Victoria/361/2011 (RCSB Protein Data Bank (PDB) ID: 4O5N[228]), which is heavily glycosylated (12 potential N-glycosylation sites). **d**–**f** | Structure of the neuraminidase tetramer of the 2009 pandemic H1N1 virus (PDB ID: 3NSS[229]) (part **d**). This neuraminidase possesses eight potential N-linked glycosylation sites, which are shown with oligomannosidic structures (part **e**) and with complex glycans (part **f**). These glycan structures, although flexible at physiological temperatures, might restrict the access of B cell receptors and antibodies to haemagglutinin and may shield important antigens. Glycans were attached to the haemagglutinin and neuraminidase structures using GlyProt and visualized using PyMOL[230].

have been designed and expressed in *E. coli* and cell-free expression systems and have shown limited efficacy in a mouse model with low challenge doses[169–174]. The main obstacle to overcome for the development of successful headless haemagglutinin constructs is the correct folding of conformational neutralizing epitopes, and better approaches to design stable structures are needed.

A novel approach to induce high levels of stalk-reactive antibodies is based on chimeric haemagglutinins (cHAs)[7,175,176] (FIG. 4a). Chimeric haemagglutinins consist of H1 (group 1), H3 (group 2) or influenza B haemagglutinin stalk domains in combination with 'exotic' globular head domains, mostly of avian origin. A disulfide bond between Cys52 and Cys277 (H3 numbering) forms the demarcation line between stalk and head domains. Amino acids between these two cysteine residues belong to the membrane distal globular head domain, whereas amino acids of the haemagglutinin ectodomain that are N-terminal of Cys52 and C-terminal of Cys277 belong to the stalk domain. Importantly, the stalk domain includes parts of the HA1 and the HA2 subunits. The presence of an exotic head domain on these chimeric haemagglutinins stabilizes important conformational epitopes in the stalk domain. Chimeric haemagglutinins are fully functional, and recombinant influenza viruses expressing them grow to high titres in embryonated eggs and in cell cultures[175].

Chimeric haemagglutinins with different head domains have been used in a sequential vaccination regimen to induce stalk-reactive antibodies. After the first exposure to a chimeric haemagglutinin — for example, cH6/1 HA (an H6 head on top of an H1 stalk) — the immune system induces a strong primary response against the exotic head domain but only a weak, almost undetectable, response against the stalk domain. Sequential vaccination with a second chimeric haemagglutinin that expresses a different head domain — for example, cH5/1 HA (an H5 head on top of an H1 stalk) — induces a primary response against the novel head domain but boosts antibodies against the stalk domain because both chimeric haemagglutinins have this domain in common. A third vaccination with yet another different chimeric haemagglutinin — for example, cH8/1 HA (an H8 head on top of an H1 stalk) — again boosts stalk-reactive antibodies

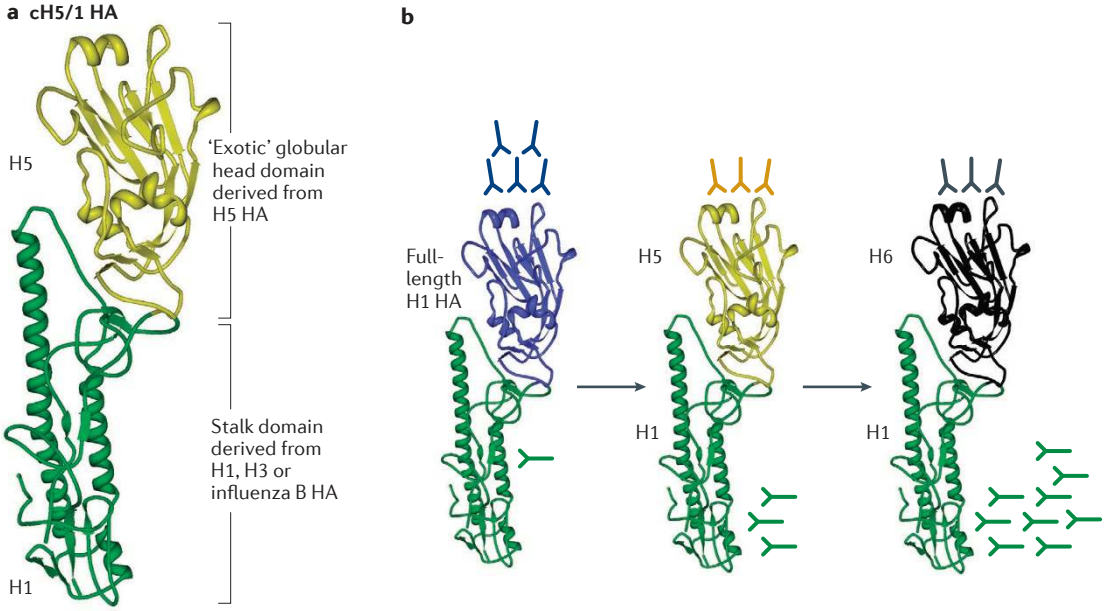

Figure 4 | **Chimeric haemagglutinin-based universal influenza virus vaccine strategies.** Chimeric haemagglutinins (cHAs) consist of 'exotic' globular head domains (for example, from H5 or H6 HA) in combination with H1, H3 or influenza B virus stalk domains. **a** | An example of a cH5/1 HA (an H5 head (yellow) on top of an H1 stalk (green)). **b** | Humans have pre-existing immunity to H1 (group 1), H3 (group 2) and influenza B viruses, which is mostly directed against the HA head domains but also includes low levels of anti-stalk immunity. Upon vaccination with a cHA — for example, a cH5/1 HA — antibody levels against the stalk domain are boosted, whereas only a primary response is induced against the novel globular head domain to which humans are naive. A boost with a second cHA that possesses the same stalk domain but a different head domain (for example, cH6/1 HA) could further increase stalk-specific antibody levels. Structures are based on RCSB Protein Data Bank ID 1RU7, and were visualized using Protein Workshop[231].

whereas only a primary response against the H8 head domain is mounted (FIG. 4b). Using this strategy, it is possible to break the immunodominance of the head domain and to induce high titres of stalk-reactive antibodies.

As discussed above, the breadth of stalk-reactive antibodies is mostly restricted to one haemagglutinin group (group 1, group 2 or B haemagglutinins). Therefore, a successful chimeric haemagglutinin-based universal vaccine candidate needs a group 1 component, a group 2 component and an influenza B haemagglutinin component. Group 1 constructs based on the H1 stalk domain have so far been successfully tested in mice and ferrets and protect from heterologous (H1N1) and heterosubtypic challenge (for example, H5N1 and H6N1), but not from challenge with group 2 viruses (for example, H3N2)[177,178]. Group 2 constructs based on the H3 stalk domain can protect against various H3N2 viruses and against heterosubtypic challenge viruses such as H7N1 and H7N9 (REFS 179,180). Although most of these studies were performed using experimental DNA and recombinant protein vaccines, it should be mentioned that the chimeric haemagglutinin technology is platform independent and can potentially be used in the form of IIVs, LAIVs, virus vectors, recombinant protein vaccines, VLPs, DNA vaccines, and other forms. As described above, adults already have low levels of B cells with specificities against the stalk domain and would therefore probably only require boosting of these B cell populations with chimeric haemagglutinin constructs to increase the production of virus-specific antibodies (FIG. 4b). Evidence for this hypothesis comes from trials with H5N1 and H7N1 vaccine candidates[62–64]. As described above, these vaccines, which possess exotic head domains but have conserved group 1 or group 2 stalk domains, induced high levels of stalk-reactive antibodies in humans. However, one of these trials showed that the immune response against the stalk domain in the context of inactivated vaccines was as short lived as the immune response against the head domain, with titres returning to baseline 6 months post-vaccination[64].

Clearly, a universal influenza virus vaccine that is protective for only a short duration is of limited use. However, it should be noted that stalk-directed immune responses induced by natural infection (and potentially by whole-virus inactivated vaccines) have long half-lives[143,148]. Moreover, adjuvants can drastically improve the immune response induced by chimeric haemagglutinin-based vaccines[179,181]. An adjuvanted chimeric haemagglutinin vaccine, possibly in the context of a heterologous prime–boost regimen (for example, an LAIV followed by an IIV or a DNA vaccine, followed by an IIV) could therefore be used to induce a long-lasting anti-stalk immune response. Clinical trials to test this hypothesis have been initiated. Importantly, novel potency assays and correlates of protection have to be established for these vaccine candidates because current assays and correlates are focused on globular-head-directed immunity.

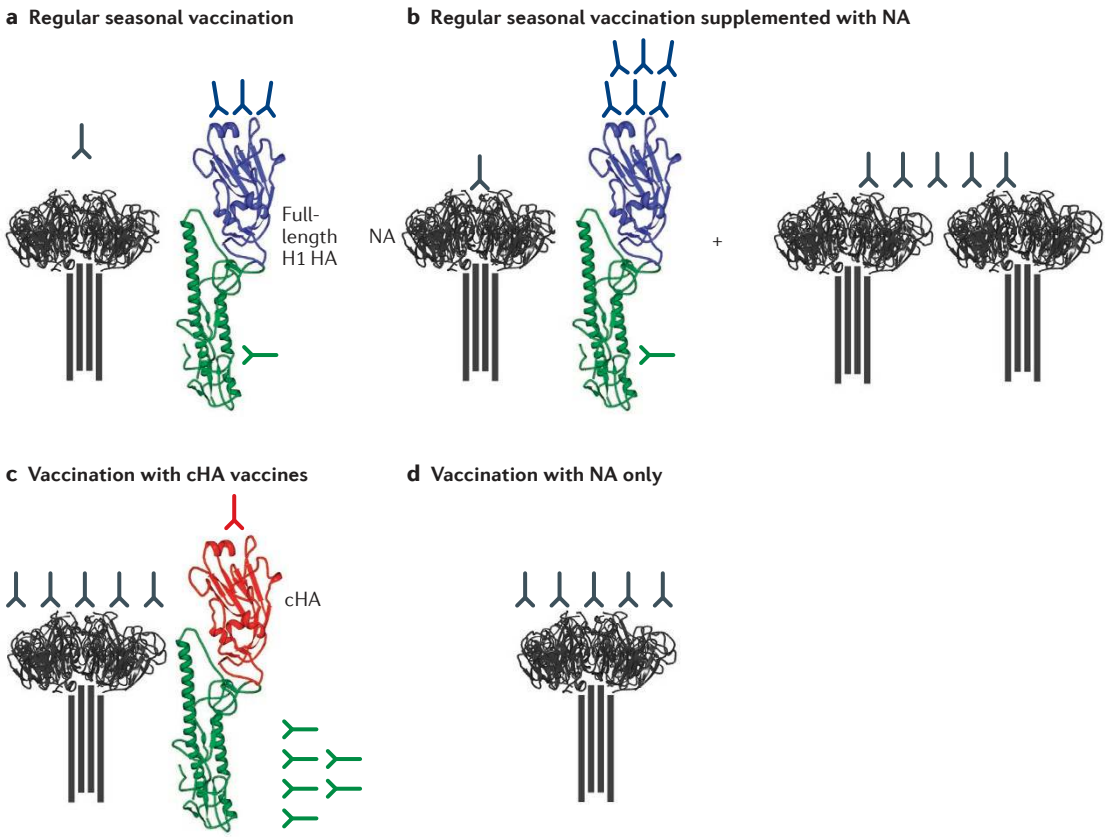

Figure 5 | **Strategies to enhance neuraminidase-based immunity.** In association with haemagglutinin (HA) (the globular head domain is coloured blue and the stalk domain is coloured green), the viral neuraminidase (NA; black) is immunologically subdominant and the majority of the immune response is directed against the HA globular head domain (part **a**). Strategies to improve the immunogenicity of NA include supplementing seasonal trivalent inactivated vaccines with recombinant NA (part **b**), the use of chimeric HA (cHA)-based vaccines (red and green) that break the immunodominance of the HA globular head domain (part **c**) or vaccination with recombinant or purified NA only (part **d**). Structures are based on RCSB Protein Data Bank IDs 1RU7 (HA) and 3B7E (NA), and were visualized using Protein Workshop[231,232].

Broadly protective vaccines based on the globular head domain of haemagglutinin, neuraminidase or M2e. In addition to universal vaccine approaches that are based on the conserved stalk domain, approaches to induce a broader response towards the globular head domain are in development[182–184]. These approaches are restricted to a subtype or even to specific clades within a subtype but could still result in vaccines that last for several years, which is a clear advantage over current vaccines that have to be reformulated almost every year. This concept is based on 'centralized' sequences[182], ancestral sequences[184] or computationally optimized broadly reactive antigens (COBRAs), which are synthetic haemagglutinins representing an optimized merged sequence of representative strains[183,185]. COBRA-based vaccines have been shown to successfully induce protection against highly pathogenic H5N1 viruses in mice, ferrets and nonhuman primates[186–188]. Candidates for seasonal influenza viruses are currently in development. Similar to chimeric haemagglutinin constructs, these COBRA-based haemagglutinins are fully functional and vaccine platform independent.

As described above, crossprotective mAbs against the second surface glycoprotein of the influenza virus, neuraminidase, demonstrate that neuraminidase-based immunity has the potential to confer at least intra-subtypic crossprotection. This is also supported by the fact that neuraminidase antigenic drift rates are generally lower than antigenic drift rates of the globular head domain of haemagglutinin[189–191]. However, the immune response to homologous neuraminidase after influenza virus vaccination and infection is not well characterized and understood[153]. IIVs are not standardized for their neuraminidase content, and the functionality and correct folding of the neuraminidase in these vaccines is not assessed on a regular basis. Recent efforts to gain a better understanding of the neuraminidase content in IIVs and the immune response that they induce showed marked differences in neuraminidase content and anti-neuraminidase immune responses for commercially available vaccines. Immune responses in mice varied from no induction to neuraminidase inhibition titres of 1:1,280 (REF. 192). However, it has been demonstrated that neuraminidase-based immunity drastically reduces

Box 3 | **Heterosubtypic immunity**

Heterosubtypic immunity refers to the protection against a specific influenza virus subtype (for example, H1N1) that is induced by another influenza virus subtype (for example, H3N2). This protection can be mediated by cellular immunity or by humoral immunity. Cellular heterosubtypic immune responses are usually directed towards the internal proteins of the virus, such as the nucleoprotein, the M1 protein or the polymerases, which are highly conserved. Effective humoral heterosubtypic immunity is rare and mostly based on antibodies against the haemagglutinin stalk domain. Natural heterosubtypic immunity for neuraminidase has so far not been observed. Heterosubtypic immune responses against internal proteins lack significant protective or neutralizing efficacy, probably because they are directed against proteins that are usually not accessible on the cell or virus membrane. Heterosubtypic protection should not be confused with heterologous protection against divergent viruses of the same subtype. A broadly protective vaccine usually refers to broad protection within a subtype, whereas a universal influenza virus vaccine refers to protection from multiple (or all) subtypes of the virus.

viral replication and clinical signs of infection in humans[193]. In general, it is assumed that neuraminidase, similar to the stalk domain of haemagglutinin, is immunosubdominant if it is associated with an immunodominant haemagglutinin globular head domain[194,195] (FIG. 5a–c). However, it is possible to restore neuraminidase immunogenicity by using neuraminidase-only vaccines[195–197] (FIG. 5d).

Recent studies in ferrets using neuraminidase-only immunogens that induce high titres of anti-neuraminidase immunity clearly showed crossprotection to viruses expressing divergent N1 neuraminidases[198]. An alternative strategy to increase neuraminidase immunity would be to decrease the immunodominance of the associated haemagglutinin globular head. H7N2 vaccines can boost anti-neuraminidase immunity to high titres in humans, whereas control H3N2 vaccines have failed to do so[153,199]. As discussed above, the H7 globular head domain appears to be less immunodominant in humans who are naive to this subtype. It could be hypothesized that LAIV-based or IIV-based chimeric haemagglutinin vaccines that have an associated neuraminidase could also induce high titres of anti-neuraminidase immunity. In such a scenario, the immunodominance of the haemagglutinin head domain is also reduced (FIG. 5c). In conclusion, vaccine approaches that induce strong anti-neuraminidase immune responses could improve protection against homologous and heterologous influenza virus strains and would certainly represent a valuable addition to the armamentarium to fight influenza virus infections.

M2 is the third influenza virus surface transmembrane protein and is also of interest for the development of broadly protective influenza virus vaccines. Specifically, the 22–23-amino-acid short ectodomain of M2 (M2e) is promising because of its high conservation and surface exposure[200]. The development of M2e-based vaccines began in 1999 (REF. 201) and since then many M2e vaccine constructs, including tetrameric M2e, VLP-displayed M2e, flagellin-fused M2e and multimeric M2e, have been successfully tested for efficacy against a panel of divergent influenza viruses[201–206]. M2 is present at very low copy numbers on virions but is abundant on infected cells. Protection conferred by M2e-based vaccines is probably mediated by ADCC[200,207]. M2e-specific antibodies are usually non-neutralizing and do not induce sterilizing immunity; however, passive transfer studies in humans demonstrated a reduction in clinical signs and nasal wash virus titres upon challenge with a human H3N2 influenza virus isolate[208].

T-cell- or epitope-based universal influenza virus vaccines. Recently, a number of virus-vectored universal vaccine candidates have been developed. Several of these vaccines are based on MVA, which is an excellent platform to induce strong CD4 and CD8 T cell responses and is therefore preferentially used to boost cellular immunity. An MVA vector expressing a fusion protein of the conserved matrix (M1) and nucleoprotein has been tested in clinical trials and was found to be safe and effective in inducing cellular immune responses against influenza viruses[209,210]. However, the vaccine showed only weak protection in human challenge studies with an H3N2 strain[211]. Importantly, this study only assessed protection from mild upper respiratory infections, and the vaccine — owing to the nature of T-cell-based immunity — probably has a much stronger effect on lower respiratory infections with long durations (the study was stopped on day 5 post-infection using the antiviral drug oseltamivir)[211]. The same vaccine candidate is now being tested as an additive to a TIV and shows promising results in this context in preclinical experiments and clinical studies[212,213]. In addition, a prime–boost regimen with MVA and an adenovirus expressing M1-nucleoprotein showed successful induction of heterosubtypic immunity (BOX 3) in mice[214]. A similar approach used an MVA vector expressing several influenza virus proteins — including haemagglutinin, neuraminidase, nucleoprotein, M1 and M2 — from H5N1 strains and interleukin-15 as a molecular adjuvant[215]. Challenge studies in mice showed antibody-independent heterosubtypic immunity against H1N1, H3N2 and H7N7 with an efficacy of 80–100% (REF. 215). However, the mice experienced relatively high weight loss (between 15% and 20% of their initial weight)[215].

In addition to viral vectors, numerous vaccine candidates, based on influenza viruses that are either severely attenuated or restricted to single-cycle replication, have been tested in recent years[216–218]. Heterosubtypic immunity has been demonstrated for these constructs — mostly in the absence of neutralizing antibodies — suggesting that T-cell-based protection was induced. Several vaccine candidates composed of single or multiple B- or T-cell epitopes are also in development[219–221]. A vaccine based on an *E. coli*-expressed fusion peptide containing different epitopes, Multimeric-001, has been tested in clinical trials and was found to be safe[222]. This vaccine candidate was also assessed in combination with regular TIV and was shown to induce T cell responses and increased haemagglutination inhibition responses to TIV strains in the elderly[223].

Although many of these T-cell-based approaches might have the potential to protect from severe morbidity and mortality[224–226], it is unclear whether they would also protect from the upper respiratory infection

that drives transmission of the virus. Furthermore, it is unclear how long protective T cell responses against influenza viruses last. These questions will most likely be addressed in future clinical trials.

Conclusions

Both seasonal and pandemic influenza virus vaccines and vaccine production processes have been significantly improved since the 2009 H1N1 pandemic. Novel production platforms that enable rapid production have been established and several improved influenza virus vaccines have been licensed by the US Food and Drug Administration. Furthermore, the development of novel technologies for a detailed analysis of the human immune response to influenza virus infection and vaccination has led to an improved understanding of protection against influenza. It is now imperative to translate this knowledge into vaccines that provide broad protection from influenza virus infection and, ideally, lifelong universal coverage against all influenza A and B virus strains.

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Competing interests statement
The authors declare competing interests: see Web version for details.

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