EXPERT REVIEWS

Prospects for antibody-based universal influenza vaccines in the context of widespread pre-existing immunity

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Influenza inflicts significant global mortality and morbidity that can be combated by effective immunization. However, the protective efficacy of current vaccines is limited by both the significant antigenic diversity of the viral hemagglutinin protein and the capacity for rapid antigenic change. This necessitates global influenza surveillance efforts, frequent vaccine reformulation and annual readministration. There is, therefore, tremendous interest in the development of novel strategies to elicit broad and durable protection against both seasonal and pandemic infection. This review presents an overview of candidate universal influenza vaccines designed to elicit cross-protective antibody responses to hemagglutinin. In particular, we focus on the potential impact that widespread pre-existing influenza immunity may play upon the design, testing and deployment of universal influenza vaccines.

KEYWORDS: antigenic sin • influenza • pandemic • universal • vaccine

Influenza-A (IAV) and influenza-B (IBV) viruses are globally significant pathogens that cause respiratory infections associated with high morbidity and mortality, particularly among more vulnerable populations such as children and the elderly. IAV naturally infects aquatic birds and a broad range of potential hosts, including rodents, bats, horses, domestic poultry and pigs. On the basis of hemagglutinin (HA) sequence identity, IAVs encompass up to 18 reported subtypes [1], further divided into two major phylogenetic groupings (FIGURE 1): group 1 (H1, H2, H5, H6, H8, H9, H11, H13, and H16–18) and group 2 (H3, H4, H7, H10, and H14–15). Transmission into human populations of influenza viruses containing avian or porcine HA and/or neuraminidase (NA) genes has led to four major pandemics in the past 100 years: 1918- (H1N1), 1957–1968 (H2N2), 1968- (H3N2) and 2009- (H1N1) [2]. IBV infection is limited to humans and seals [3], and two major IBV serotypes have been described: Victoria-like and Yamagata-like. H1N1, H3N2 and both IBV serotypes remain endemic, cocirculate and cause seasonally recurrent human infection.

Immunization remains the most cost-effective mechanism to combat widespread influenza infection. The first influenza vaccines were inactivated monovalent formulations targeting H1N1 and entered human use during the Second World War. Since the 1970s, trivalent (IAV H1N1, H3N2 and a single B strain) and recently quadrivalent (IAV H1N1, H3N2 and both IBV serotypes) influenza vaccines (TIV and QIV, respectively) have been widely distributed and administered within developed countries, particularly among at-risk subpopulations. However, the availability of influenza vaccines remains limited in most developing countries, leaving the vast majority of the global population susceptible to infection. Three classes of multivalent influenza vaccines are currently in production. Inactivated 'split' vaccines can be produced using egg-based (Afluria®, Fluarix®, Fluzone® and others) or cell culture-based (Flucelvax®) methods, consisting primarily of purified HA proteins isolated from disrupted influenza virions, and can be administered intramuscularly or intradermally. Naming conventions in the USA have been recently revised for this class

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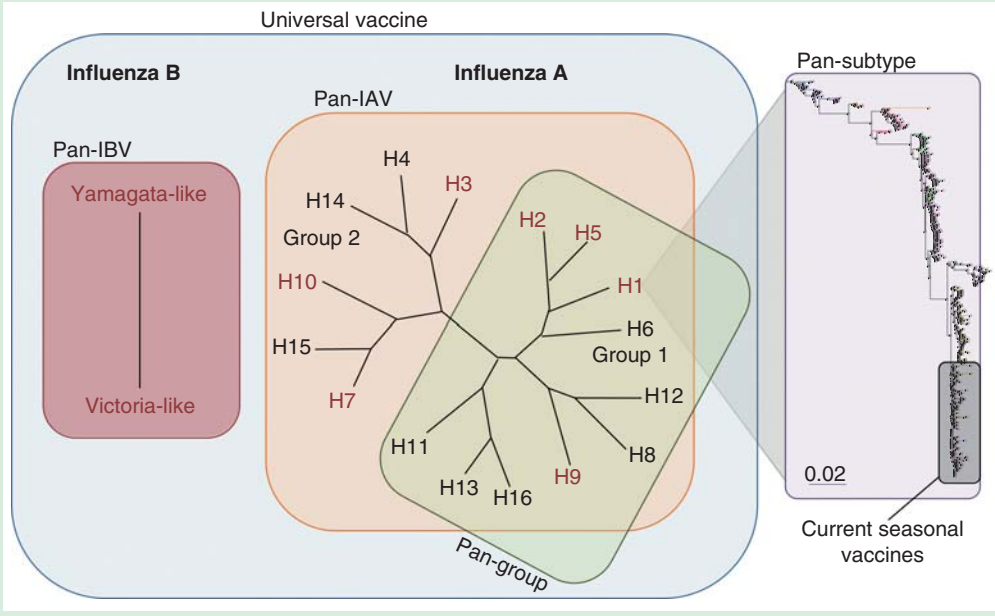


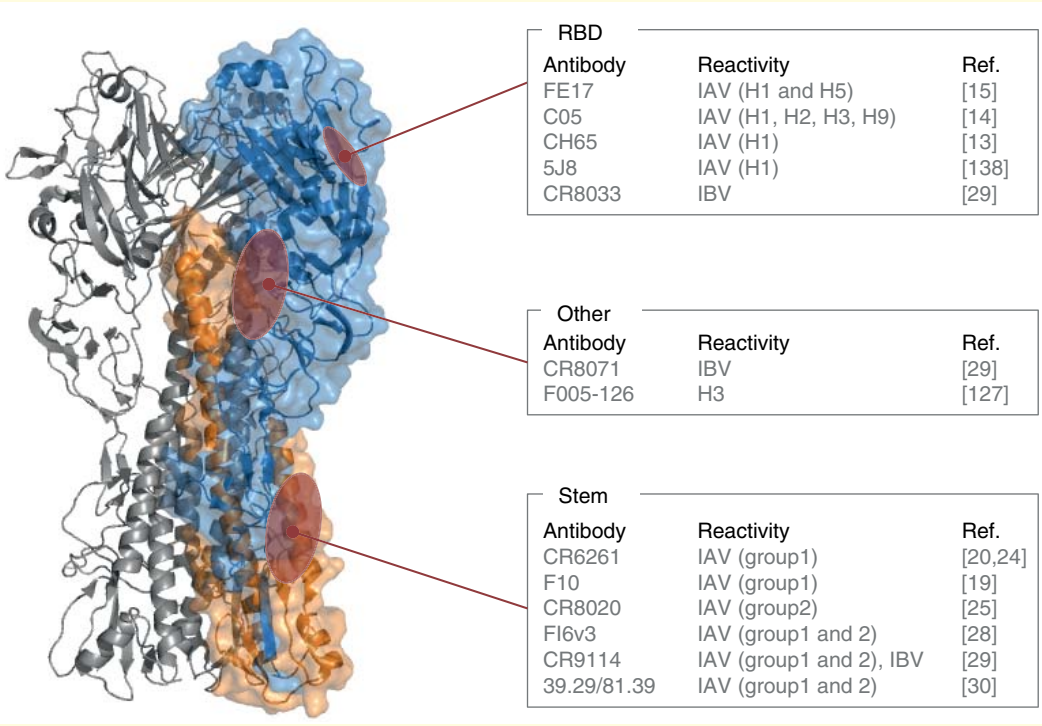
Figure 1. Antigenic diversity of influenza HA and degrees of broad protection. The high antigenic diversity of viral hemagglutinin confounds influenza vaccination efforts. Influenza A and B viruses can be divided into multiple distinct subtypes, many of which are capable of causing human infection (shown in red). Currently available vaccines elicit narrow protection against closely matched strains within a single subtype. This is illustrated upon a phylogenetic tree showing selected circulating H1N1 sequences isolated from the USA from 1978 to 2008, with the 2008/2009 vaccine only neutralizing the subset of H1 strains shown in the dark gray box. Although a universal vaccine is the ultimate goal, any vaccine capable of increasing protective breadth – whether pan-subtype, pan-group, pan-IAV or pan-IBV – would constitute a major medical advance.

of vaccines to inactivated influenza vaccine (IIV), IIV3 for TIV and IIV4 for QIV. Alternatively, intranasal administration is possible with a live attenuated influenza vaccine (LAIV; FluMist®), which uses temperature-sensitive viral mutants to limit replication and spread *in vivo*. Most recently, a third class of vaccines based upon recombinant HA protein (FluBlok®) has been licensed in the USA, foregoing the dependence upon embryonated eggs for production. Regardless of production or delivery methods, all three classes of seasonal vaccines elicit significant immunity with protection primarily mediated by humoral responses targeting antigenic sites surrounding the receptor-binding domain (RBD) of HA. These antibodies potently inhibit interactions between the virus and the sialylated host cell receptors and can readily be measured in the serum using established hemagglutination inhibition assays.

Despite being a critical tool in combating influenza infection worldwide, the use of currently available influenza vaccines is severely limited by the ability of influenza viruses to evade humoral immune responses. The gradual accumulation of point mutations (antigenic drift) in the viral hemmagglutin leads to eventual escape from antibody recognition and a consequent loss of vaccine effectiveness. This necessitates an annual reassessment of circulating influenza strains for changes in antigenicity and frequently vaccine reformulation. A second limitation of current seasonal vaccines is their ineffectiveness against serologically novel viral variants. The segmented genome of influenza viruses facilitates periodic reassortment of HA or NA genes with environmental avian and/or porcine viral strains. This major and dramatic change in viral composition (antigenic shift) can result in complete evasion of prevailing immunity and drive the emergence of pandemic influenza. The underlying vulnerability of human populations was highlighted by the emergence of pandemic 'swine' H1N1 (pdmH1N1) in 2009 [4]. A reassorted virus comprising human, swine and avian influenza genes [5], pdmH1N1 spread rapidly from a North American epicenter to affect all continents within 4 months, eventually infecting an estimated 11–21% of the global population [6] and causing over 284,500 deaths [7]. Interestingly, 80% of respiratory and cardiovascular deaths were reported in subjects <65 years old when compared with 19% in an average pre-2009 season [8]. These observations underscore that current seasonal influenza immunization elicits insufficient immunity to cross-reactive B epitopes to limit infection with newly emergent viral strains. This has become a growing concern in recent years given unpredictable zoonotic transmission from animal reservoirs has led to human infections with variant H3N2 [9], H10N8 [10], H7N9 [11] and H5N1 [12] – with infection by these novel strains often associated with unusually high pathogenicity and mortality.

Universal influenza vaccines

To overcome the limited breadth of seasonal vaccines, there has been significant interest in developing novel vaccines that elicit broad and long-lived immunity against diverse viral



RBD

Antibody	Reactivity	Ref.
FE17	IAV (H1 and H5)	[15]
C05	IAV (H1, H2, H3, H9)	[14]
CH65	IAV (H1)	[13]
5J8	IAV (H1)	[138]
CR8033	IBV	[29]

Other

Antibody	Reactivity	Ref.
CR8071	IBV	[29]
F005-126	H3	[127]

Stem

Antibody	Reactivity	Ref.
CR6261	IAV (group1)	[20,24]
F10	IAV (group1)	[19]
CR8020	IAV (group2)	[25]
FI6v3	IAV (group1 and 2)	[28]
CR9114	IAV (group1 and 2), IBV	[29]
39.29/81.39	IAV (group1 and 2)	[30]

Figure 2. Structure of influenza hemagglutinin and binding sites of broadly neutralizing antibodies. The influenza HA [149]; (PDB ID - 1RUZ) is a heterotrimer of two domains – HA1 (blue) and HA2 (orange, only ectodomain pictured). The HA1 domain contains the receptor binding domain (RBD), a major target for neutralizing antibody that acts by blocking interaction of HA with sialylated cellular proteins. The HA2 domain, often termed the stalk or stem, contains the fusion machinery. Human monoclonal antibodies with broad neutralization activity can bind the RBD, stem or alternative cross-reactive epitopes. Selected examples are shown at each site and the breadth of influenza recognition indicated.

subtypes – so-called universal influenza vaccines (UIV). Ideally, these would offer life-long protection against seasonal influenza infection analogous to current vaccines for other viral pathogens such as measles, hepatitis B or polio. Crucially, such vaccines would convey some level of pandemic preparedness against emergent influenza outbreaks, potentially preventing initial zoonotic transmission or, at a minimum, slowing spread to facilitate timely pandemic-specific vaccine production. Cross-reactive T-cell responses between diverse influenza isolates have been widely reported and could provide universal protection against influenza disease (extensively reviewed in [13–15]. However cross-reactive antibody responses hold great promise for sterilizing protection from the acquisition of influenza infection and are the focus of this review. An overarching strategy for antibody-based UIV development consists of two primary goals. First, to identify conserved sites of vulnerability common to diverse influenza strains. And second, to develop immunization modalities to specifically target humoral immune responses onto these sites to generate serum titers sufficiently high to provide lasting protection.

UIV targeting HA

Many studies to date have focused on the viral entry protein HA. Given the remarkable diversity of influenza HA, a vaccine able to broaden protective efficacy would be a tremendous medical advance (Figure 1). Virion-associated HA is a heterotrimer of two polypeptide chains termed HA1 and HA2 (Figure 2). HA1 encodes the highly glycosylated globular head that characteristically displays low-sequence conservation between subtypes. Antibodies that bind proximal to the sialic acid RBD of HA1 can mediate potent viral neutralization and are currently the primary means of vaccine-elicited protection. However, escape from antibody recognition at the RBD readily occurs by antigenic drift or antigenic shift. Nevertheless, selected RBD-specific monoclonal antibodies with some degrees of breadth have been isolated from humans (Figure 2) [16–19].

HA2, often termed the HA stem or stalk, is relatively conserved between subtypes and contains transmembrane domains that anchor HA into the viral membrane and the viral fusion machinery. The isolation of the murine monoclonal antibody C179 [20] initially established the stem as a potential site of broad virus neutralization, with reactivity against group 1 H1, H2, H5 and H6 viruses [20,21]. Subsequently, many groups have reported the isolation of human stem binding antibodies with analogous pan-group 1 neutralizing activity (Figure 2) [18,22–25]. Crystallographic studies revealed a common epitope based around a conserved hydrophobic pocket in the stem [22,26,27]. An alternative, proximal stem epitope was

Table1. Selected potential pathways towards a universal influenza vaccine.

Strategy	Target	Description	Comment	Ref.
Viral vectors or adjuvanted vaccines	HA	Novel vectors or adjuvants can significantly improve the immunogenicity of current seasonal or pandemic influenza vaccines	Increased immunogenicity may raise the effective titers of antibodies targeting subdominant epitopes within HA with broad protective potential	[44,46–48,51,52]
Consensus HA antigens	HA	Synthetic HA designed to maximize antigenic conservation between diverse viral isolates	The ability to induce broad immunity within a subtype has been shown for H5N1, H1N1 and H7N2. The utility of consensus HA for bridging greater antigenic distances between subtypes remains unclear	[53–58]
'Headless' HA	HA2	Stabilized variants of the HA2 domain lacking HA1	HA2-based immunogens may bypass antigenic competition from the immunodominant HA1 domain	[61–64]
Chimeric HA	HA1 and HA2	Chimeric HA1 domains (usually of avian origin) fused to a common and conserved HA2 domain	Sequential immunization with chimeric HA may allow simultaneous priming of protective head responses against potentially pandemic strains, while also promoting the expansion of HA2 antibodies with broad influenza specificity	[75–79]
Targeting conserved influenza proteins	NA, NP or M2	Other viral proteins with less antigenic diversity than HA may provide potential targets for protective T cell and antibody responses	Vaccines targeting NA, NP or M2 may provide some protection against influenza pathogenesis through T cell immunity and/or antibody effector functions such as ADCC	[88,89,91,97,99–101]

ADCC: Antibody-dependent cellular cytotoxicity; HA: Hemagglutinin; M2: Matrix protein 2; NA: Neuraminidase; NP: Nucleoprotein.

identified in group 2 viruses by the isolation of CR8020 [28], and subsequently other groups have reported similar antibodies [29,30]. The existence of human antibodies with exceptionally broad specificity (intergroup) was initially confirmed by the discovery of the broadly neutralizing antibody FI6 [31]. Subsequently, other antibodies were isolated: CR9114 [32], and 39.29 and 81.39 [33]. All these antibody lineages bind an overlapping epitope surrounding the hydrophobic pocket in HA2 and neutralize diverse group 1 and group 2 IAV isolates, extending to IBV reactivity in the case of CR9114 [32]. The infrequent reports to date of monoclonal antibodies with intergroup or IAV/IBV cross-reactivity suggests such specificities may be rare in humans. The neutralization activity of stem-binding antibodies *in vitro* appears due to antibody-mediated inhibition of viral fusion and/or the prevention of HA1/HA2 polypeptide cleavage [27–29,34]. However, protection *in vivo* using a murine passive infusion model was largely reliant upon Fc interactions, suggesting an essential role for antibody-dependent cellular cytotoxicity (ADCC) or other antibody effector functions [35].

The wealth of monoclonal antibodies characterized to date clearly demonstrate that the human humoral immune system is capable of generating antibodies with broad heterosubtypic influenza specificity (reviewed in [36]). Moreover, passive infusion into animal models confirm both the RBD and the HA stem comprise highly conserved targets for antibody-mediated protection [30–33,37]. It remains an open question how to augment or elicit broadly protective HA antibodies by vaccination?

A number of strategies currently under development that endeavor to broaden humoral immune responses to influenza are discussed further below and are summarized in Table 1. In the general population, antibodies with heterosubtypic neutralizing activity are found only at low serological concentrations thought insufficient to be protective [38]. This is somewhat paradoxical, as repeated and sequential exposure to antigenically distinct HA, such as conceivably seen by periodic infection or annual TIV administration, might be expected to drive immunity toward common epitopes. Influenza infection in mice [39] and humans [25,39–41] reproducibly elicits cross-reactive antibody responses, including those targeted to the HA stem, albeit with low absolute titers. In contrast, TIV is manifestly poor at eliciting heterosubtypic immunity, both in terms of serum antibody [18,39,42] and memory B-cell responses [18]. Similarly, infection but not TIV administration raised H1N1 cross-reactive ADCC responses in macaques [43]. It is generally accepted that conventional vaccines may lack both the antigen loads and the 'danger signals' associated with natural infection. Therefore, strategies that increase the immunogenicity of current influenza vaccines may raise subdominant antibody

responses at cross-reactive epitopes to potentially protective levels. Evidence supporting this comes from studies pairing influenza vaccines with new adjuvants or improved vaccine delivery vectors.

Coformulation of TIV with the potent oil-in-water adjuvant MF59 has been shown in several clinical studies to increase the potency of antibody responses against heterovariant influenza strains compared with nonadjuvanted vaccines [44–47]. Similar results have been reported with monovalent avian H5N1 vaccines, with MF59 increasing humoral cross-reactivity against heterologous H5N1 strains [48,49]. A range of new adjuvant formulations, including agonists for toll-like receptors-3, -4, -5, -8 and -9, are currently undergoing clinical assessment (reviewed in [50]), raising hope for a straightforward mechanism to broaden protection from seasonal vaccines. However as seasonal vaccines are extensively used in children and the elderly, tolerance for excessive vaccine reactogenicity is exceedingly low and adjuvanted influenza vaccines are primarily being developed for pandemic-type avian strains. Other groups have pursued improved delivery modalities to increase vaccine immunogenicity. Gene-based prime-boost immunization regimens in mice, ferrets and macaques often elicit broad neutralization of variant H1 viruses, with evidence of expanded antistem antibodies in comparison with TIV protein immunization alone [51]. Similarly, vaccination of mice or ferrets with HA arrayed upon ferritin-based nanoparticles raised immune serum with potent neutralizing activity against a broad spectrum of H1N1 viruses [52]. Notably, this approach raised antibodies to highly conserved HA epitopes in both the stem and proximal to the RBD.

One strategy to combat HA diversity and generate broader responses has been to generate synthetic consensus HA encoding highly conserved amino acids residues at each position. Mice vaccinated with a DNA vaccine expressing a consensus H5N1 HA displayed broad serum neutralizing activity and were protected against challenge with homologous and heterologous H5N1 viruses [53]. Similar findings have subsequently been reported for consensus H1N1 [54] and H7N9 [55] antigens. Computational approaches have recently been developed to aid the rational design of consensus H5 antigens [56]. Importantly, such proteins presented upon a virus-like particle platform retain the physiologic functions of native HA [56] and can elicit protective immunity from lethal H5N1 challenge in mice, ferrets [56,57] and nonhuman primates [58]. Vaccination with virus-like particles generated antibodies with broader specificity for heterologous H5N1 strains compared with animals vaccinated with polyvalent mixtures of HA. Therefore, consensus antigens appear to elicit broader subtype-specific humoral immunity. It remains to be seen whether the greater antigenic distances between influenza subtypes can be successfully bridged.

HA stem-based UIV approaches

Antibodies to epitopes in the HA stem have clear heterosubtypic neutralizing potential, driving historical interest in the development of 'headless' HA2-based immunogens [59,60]. In one such approach, HA1 was replaced with a glycan linker allowing the production of headless HA2 constructs that elicited group 1 or 2 heterosubtypic antibody responses in mice [61]. However, only partial protection to homologous viral challenge was observed. More recently, modifications to stabilize HA stem-based immunogens have seen increased protein expression and improved fidelity of folding to maintain correct antigenicity. Such stabilized HA2 immunogens have demonstrated increased protective potential in small animals [62–65].

An alternative approach to 'headless' constructs has been efforts that refocus immune responses onto the subdominant stem region. It has been widely reported that a marked boost in serum titers of HA2-specific neutralizing antibodies can been observed in subjects following infection with pdmH1N1 [25,66]. Similar findings have been described after immunization with either H5N1 [24,67–70] or pdmH1N1 vaccines [38,41,42,71,72]. Therefore, exposure to immunologically novel (antigen-shifted) HA has the potential to augment humoral immunity specific for the HA stem [73,74]. Strategies to translate these observations into universal influenza vaccine candidates are under investigation. Chimeric HA molecules have been constructed consisting of a conserved HA2 domains paired with antigenically distinct HA1 domains that few humans have been exposed to, usually of avian origin [75]. Sequential vaccination with heterologous, chimeric HA antigens elicits high titers of HA2-specific antibody and can protect mice and ferrets from diverse heterologous group 1 [76,77] or group 2 challenge [78,79]. In a related approach, targeted glycan addition to mask prominent antigenically variable epitopes on HA1 leads to increased stem antibody responses and protection from heterovariant H5N1 challenge in mice [80,81]. Collectively, these studies highlight the promise of stem-based influenza vaccines that maximize the immune exposure of stem epitopes by avoiding immunodominant HA1-directed recall responses.

The functional importance of the viral fusion machinery most likely accounts for the high-sequence conservation of the HA stem. However, while antigenic escape from neutralizing antibody recognition occurs rapidly at the RBD, less is known about the evolution and tolerability of antigenic variation within the stem domain. Escape mutations can be induced *in vitro* under monoclonal antibody selection in some studies [20,23,28] but not in others [22,82]. Moreover, broadly cross-reactive stem monoclonal antibodies display subtype or strain specificity in epitope recognition [28,31,32], suggesting that HA2 sequence diversity in natural viral isolates may confer resistance to some modes of antibody-mediated neutralization. However, circulating stem-specific B cells in humans are highly polyclonal in nature and drawn from multiple diverse immunoglobulin gene families [16,70]. Therefore, redundancy in recognition of the stem epitope may act to constrain pathways of immune escape from vaccine-elicited humoral responses targeting HA2.

UIV targeting other viral proteins

Given the high intrinsic diversity of influenza HA, targeting less diverse viral proteins may also be a pathway toward broad protection and universal vaccine approaches have been advanced targeting conserved matrix proteins 1 and 2 (M1 and M2) and nucleoprotein (NP) (extensively reviewed in [83–85]). The viral M2 protein is a proton-selective ion channel, of which the 23 amino acid extracellular domain (M2e) protrudes past the virion membrane providing a potential target for humoral immune responses. High-sequence conservation among influenza A viruses makes M2e an attractive immunogen, and it has been incorporated into a wide range of vaccine platforms (reviewed in [86]). Immunization of mice with M2e-based vaccines can significantly enhance survival from lethal influenza challenge by eliciting M2e-specific antibodies [87–89]. M2e specific antibody is not directly antiviral [90], with protection thought to be dependent upon Fc effector functions such as ADCC leading to clearance of virally infected cells [91,92]. Monoclonal antibodies targeting M2e are generally not effective in preventing influenza infection, but can enhance survival and/or ameliorate disease symptoms during experimental influenza infection in animal models [93–97] and humans [98]. Vaccines based on M2e alone, or in combination with NP, are progressing through human clinical trials and appear safe and immunogenic [85,99–101]; however, the elicitation of broadly protective humoral immunity remains to be assessed.

The observation that antibodies to NA from seasonal vaccines could protect against avian H5N1 in mice [102] highlighted that conservation of NA between diverse influenza serotypes may provide a common immunologic target. Broadly cross-reactive monoclonal antibodies binding NA have been identified [103–105] and can protect from influenza challenge [103,106]. While the NA content of seasonal vaccines is difficult to estimate, TIV does reliably elicit anti-NA antibody responses [107]. Vaccines designed to augment NA-specific antibody are immunogenic in humans [108]. However, the serological titers of NA-specific antibody required to prevent disease remain poorly defined, in part, due to the lack of a standardized assay for the measurement of NA activity.

Taken in isolation, humoral immune responses to M2, NA or internal proteins, such as NP [85], clearly afford some protection against influenza in experimental animal models. Moreover, polyclonal antibody responses to these antigens can meditate ADCC and other Fc-mediated effector functions (reviewed in [109]) potentially aiding viral clearance and ameliorating disease. However, it is important to recognize that the rapid spread of pdmH1N1 took place despite the totality of potential cross-reactive responses previously elicited by seasonal vaccination or infection. While baseline cross-protective T-cell immunity to NP and/or other conserved proteins negatively correlated with symptomatic influenza disease [110,111], pdmH1N1 infections were atypically high within healthy middle-aged adults, suggesting limited impact upon acquisition. Testing candidate NP and/or M2 vaccines in ferrets have yielded conflicting results, with reports of effective cross-protection [112], while other studies suggest only vaccines containing an HA component could effectively protect from heterosubtypic H5N1 challenge [113]. These findings suggest that the potency of HA-specific antibody may still hold the greatest promise for vaccine-elicited sterilizing protection from influenza acquisition.

UIV in the context of pre-existing influenza immunity

Pre-existing influenza immunity within a given host population can significantly affect responsiveness to influenza vaccines. The phenomena of 'original antigenic sin' [114] was initially described during studies into recall responses to HA – whereby secondary vaccination with an antigenically related (drifted) influenza HA preferentially increases the magnitude and affinity of antibodies raised to the primary immunogen [115,116]. More recently, this concept has been refined to account for multiple sequential influenza exposures and termed 'antigenic seniority' [117,118], where the order of exposure to influenza drift variants may program a hierarchy of serological responses, with the highest titers reported to the earliest encounters with HA [117,119]. This historical baseline of influenza immunity can suppress the development of novel humoral immune responses following vaccination with antigenically related HA [120]. Moreover, pre-existing serum antibody responses negatively correlate with the magnitudes of circulating influenza-specific plasmablast expansion and the development of vaccine-specific serum antibody following seasonal immunization [120,121]. These observations highlight that the complex immunological histories seen in human populations will be an important consideration for UIV development.

Candidate UIV vaccines will almost certainly undergo initial clinical assessment in healthy adults with substantial pre-existing immunity. In contrast, preclinical development of UIVs is largely assessed using influenza-naive small animal models. Most humans appear to be naturally infected by approximately 7 years of age [122], then experience regular twice per decade infection events [123] and probably have multiple additional subclinical exposures. Regular seasonal TIV immunization will additionally increase exposure to HA antigenic drift variants. Efforts to mimic the complex human immune history with influenza in small animals models have used prior infection or sequential vaccination. Pre-existing immunity can markedly influence susceptibility to subsequent pandemic infection. For example, prior infection of ferrets or guinea pigs with seasonal influenza can prime cross-reactive and partially protective immune responses against pdmH1N1, with protection greatest when antigenic distance between primary infection and challenge viruses was smallest [124–127]. Baseline immunity can also affect responses to influenza vaccines. Prior H1N1 infection increased humoral responses in ferrets following prime-boost vaccination with a heterovariant influenza strain [128]. Notably, this effect was strain specific and related to the antigenic distance between infecting and vaccination strains. Similarly, priming of ferrets with TIV enhanced the immunogenicity and efficacy of subsequent immunization with a

pdmH1N1 vaccine [129]. Taken together, these observations highlight that the nature (vaccination or infection), frequency and specific strains used to establish immune history all influence humoral responses and outcomes during secondary influenza exposure. Thus, a single infection or exposure to TIV is unlikely to recapitulate the diverse CD4 and B-cell repertoires expected in human adults. Efforts to recapitulate the depth of human-like influenza immunity in small animals may be limited by the limited lifespan of mice and ferrets. Moving to models of influenza infection in non-human primates may combine the advantages of a lung physiology similar to humans [130], ample immunological reagents and longevity. Macaques are readily infected with human influenza isolates; however, disease pathogenesis can vary based upon the route of inoculation, infecting viral strain and species of macaque [131]. Regarding the assessment of humoral immune responses, macaque and human immunoglobulin genes share high homology [132]; however, the effector function of the various immunoglobulin subtypes may exhibit some species-specific differences [133]. One important caveat may be the absence of a macaque ortholog of the human *ighv1-69* gene, commonly used among HA stem-specific neutralizing antibodies. Consistent with observations in small animal models, priming macaques with seasonal H1N1 led to more rapid viral clearance following pdmH1N1 challenge [134] and elicited cross-reactive antibody responses with ADCC activity [135]. Despite the obvious cost and time limitations, repeated TIV immunization and periodic infections of nonhumans primates will more accurately model the complex influenza-immune histories of adult humans.

Better animal models are urgently required to clarify the interplay between host immunity and novel UIV. However, both potential benefits and potential disadvantages might be envisaged. One benefit may be the ability to harness immunologic memory to jump start vaccine responsiveness. Exposure to circulating influenza viruses seeds a long-lived pool of influenza-specific memory B cells [136]. With increasing age, it has been suggested that memory B cells and non-naïve B cells are preferentially recruited into secondary immune responses following vaccination [137,138]. Therefore, the memory pool provides substrate for future humoral immune responses and UIV strategies that target memory B cells may bypass potentially limiting frequencies of naïve precursor lymphocytes. For example, expansion of stem-specific memory B cells elicited by prior infection or seasonal immunization presumably facilitates the rapid expansion of HA2 serological reactivity following immunization with antigenically distant HA [24]. Furthermore, prior affinity maturation during encounter with the primary immunogen means maturation pathways of memory B cells to achieve high-affinity binding and potentially more potent anti-influenza activity may be shorter than from a naïve base. Similar effects might be anticipated for influenza-specific CD4+ T cells. While CD4+ T cells mediate important antiviral activity in their own right, they also play a critical role supporting the generation of high-affinity humoral responses. Follicular helper T cells (Tfh) are a subset of antigen specific CD4+ T cells localized to B-cell follicles within secondary lymphoid tissues where cross-signaling with cognate B cells triggers the formation and maintenance of germinal center reactions [139]. A strong correlation between neutralizing antibody responses following TIV vaccination and the expansion of a circulating CD4+ T-cell population with a Tfh-like phenotype has been reported [140,141]. Furthermore, broadly cross-reactive memory CD4+ T cells capable of recognizing antigenic shift variants can be primed by seasonal influenza exposure [142–144]. This population of HA-specific CD4+ memory cells may constitute an expanded pool of Tfh to provide cognate help for HA-binding B cells to subsequent immunization. Thus, the wide prevalence of broadly cross-reactive influenza-specific memory T- and B-cell populations mean that a putative UIV need only boost, or refocus, pre-existing immune specificities.

Alternatively, prior priming of the immune system may comprise a significant disadvantage for UIVs. Establishment of immunodominance patterns within both the B-cell and CD4+ T-cell compartments by infection or vaccination may end up favoring suboptimal epitopes without the capacity for broad protection. For instance, the HA globular head is generally considered immunodominant with regard to humoral responses to HA. Consistent with the concepts of antigen sin, shifting the dominance hierarchies once established can be difficult and will likely require novel HA immunogens, such as the aforementioned chimeric HA constructs, to limit the immune exposure of prominent head epitopes. The mechanisms underpinning antigenic sin are likely multifactorial, but likely related to the diminished capacity of naïve B cells to compete effectively with higher affinity memory B cells for limiting CD4+ T-cell help, previously shown to modulate the recruitment and maintenance of B cells within germinal centers [139]. Furthermore, B cells compete with serum antibody for antigen binding and as a consequence, established cross-reactive antibody may suppress B-cell responsiveness either through epitope occlusion or through antigen depletion *in vivo* via opsonization or alternative antibody effector mechanisms.

Neutralizing antibody responses as measured using hemagglutination inhibition assays are universally recognized as an important marker of serological protection from influenza acquisition. However, numerous studies spanning decades have established that other immune effector arms contribute to protection from influenza infection and pathogenesis. This includes both CD4+ and CD8+ T-cell responses (reviewed in [14]), and polyclonal non-neutralizing humoral responses to HA and other viral proteins, which can mediate antiviral activity or aid viral clearance via opsonization, ADCC or antibody-dependent phagocytosis (reviewed in [109]). However preclinical testing of UIV in the absence of prior immunity fails to account for any combinatorial effects of responses drawn from multiple immune arms. This could potentially lead to an overestimation of the protective threshold that a candidate UIV must achieve, without the added benefit of widespread cellular and humoral influenza-specific immunity.

In summary, while a vaccine that elicits broad, durable and potent universal protection is ultimately the goal, in reality any influenza vaccine capable of increasing protective breadth – whether pan-subtype, pan-group, pan-IAV or pan-IBV – would constitute a major medical breakthrough. Furthermore, eliminating the cycle of annual reformulation and administration will significantly offset economic and logistical hurdles that prevent the deployment of effective influenza immunization regimes in resource poor countries around the world. However, the interplay between host influenza immunity and putative UIVs remains difficult to predict. Further experimentation will provide clarification critical to speed the deployment and maximize the chances of success, for the many promising UIV candidates currently under development.

Expert commentary & five-year view

The continuing development of novel adjuvants and delivery vectors suggest immunogenic improvements to current TIV and QIV formulations are well within reach. However, it seems likely that iterative improvements to current vaccines will not be sufficient to provide broad, life-long immunity to seasonal and pandemic influenza strains and more unconventional approaches will be required. We highlight three key areas where further research will significantly benefit UIV development.

First, there is an urgent need for improved non-human primate models to increase the informative value of preclinical assessments of UIV candidates. While mouse and ferret models are well established, macaques have a lifespan and physiology that is more suitable for establishing a human-relevant immune baseline of influenza exposure. Sequential influenza infection provides a means to establish immune history. However, consistent with the concept of antigenic seniority, optimization of the specific strains, order and timing of influenza exposures will be required to best model influenza immunity to a given target human population. As an adjunct to the development of improved nonhuman primate models, the protective potential of novel UIV platforms could be rapidly clarified using small-scale clinical trials, preferably in the context of experimental influenza challenge(s).

Second, a sizable focus for recent UIV development has been strategies targeting the highly conserved HA2 domain of HA. However, deconvolution of the respective contributions from RBD- versus stem-binding antibodies to vaccine-elicited neutralizing activity and subsequent protection has so far been difficult. Recent advances in stabilizing 'headless' stem immunogens should enable the immunogenic properties of the HA stem domain to be assessed in the absence of antigenic competition. In addition, clinical studies are currently underway examining whether passive infusion of stem-specific monoclonal antibodies can ameliorate experimental influenza infection (ClinicalTrials.gov identifiers: [145–147]). These studies will provide invaluable guidance as to the protective potential of stem neutralizing antibodies, especially with regard to recent reports about potential for enhancement of influenza pathogenesis by HA2-specific antibody [148].

Finally, a greater understanding of the mechanistic basis for B-cell immunodominance is needed. The direct molecular interactions during infection or vaccination between influenza HA and components of the humoral immune system are poorly defined. While the epitopes within the HA head domain clearly appear dominant over other sites within HA such as the stem, the role that primary sequence, conformation, density and localization of HA play in establishing patterns of immunodominance *in vivo* require further characterization. This is particularly relevant given interest in UIV strategies to overcome or bypass antigenic sin and increase the prominence of subdominant epitopes with heterosubtypic protective potential. Establishing guidelines that allow the manipulation of established immunodominance hierarchies to favor broadly cross-protective epitopes will speed rational UIV design and will also be informative for vaccine development for other viral pathogens, such as HIV, where high antigenic diversity poses a significant barrier to effective vaccine development.

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Key issues

- Antigenic diversity of HA limits the effectiveness of current vaccines.
- Novel universal vaccines are required to elicit broad and lifelong immunity.
- Several vaccine candidates in development look encouraging in animal models of influenza infection.
- However, currently, preclinical development largely fails to account for pre-existing influenza immunity prevalent in human population.
- Baseline influenza immunity may markedly alter vaccine responsiveness and eventual effectiveness.
- More comprehensive animal models are required to fully account for influenza immunity in target populations.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1. Tong S, Zhu X, Li Y, et al. New world bats harbor diverse influenza A viruses. PLoS Pathog 2013;9:e1003657
2. Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe 2010;7:440-51
3. Bodewes R, Morick D, de Mutsert G, et al. Recurring influenza B virus infections in seals. Emerg Infect Dis 2013;19:511-12
4. Novel Swine-Origin Influenza A, Dawood FS, Jain S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009;360:2605-15
5. Garten RJ, Davis CT, Russell CA, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science 2009;325:197-201
6. Kelly H, Peck HA, Laurie KL, et al. The age-specific cumulative incidence of infection with pandemic influenza H1N1. 2009 was similar in various countries prior to vaccination. PloS one 2011;6:e21828
7. Dawood FS, Iuliano AD, Reed C, et al. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. Lancet Infect Dis 2012;12:687-95
8. Simonsen L, Spreeuwenberg P, Lustig R, et al. Global mortality estimates for the 2009 Influenza Pandemic from the GLaMOR project: a modeling study. PLoS medicine 2013;10:e1001558
9. Jhung MA, Epperson S, Biggerstaff M, et al. Outbreak of variant influenza A (H3N2) virus in the United States. Clin Infect Dis 2013;57:1703-12
10. To KK, Tsang AK, Chan JF, et al. Emergence in China of human disease due to avian influenza A(H10N8)–cause for concern? J Infect 2014;68:205-15
11. Lam TT, Wang J, Shen Y, et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature 2013;502:241-4
12. Guan Y, Smith GJ. The emergence and diversification of panzootic H5N1 influenza viruses. Virus Res 2013;178:35-43
13. Epstein SL, Price GE. Cross-protective immunity to influenza A viruses. Expert Rev Vaccines 2010;9:1325-41
14. Quinones-Parra S, Loh L, Brown LE, et al. Universal immunity to influenza must outwit immune evasion. Front Microbiol 2014;5:285
15. Valkenburg SA, Rutigliano JA, Ellebedy AH, et al. Immunity to seasonal and pandemic influenza A viruses. Microbes Infect 2011;13:489-501
16. Whittle JR, Zhang R, Khurana S, et al. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. Proc Nat Acad Sci USA 2011;108:14216-21
17. Ekiert DC, Kashyap AK, Steel J, et al. Cross-neutralization of influenza A viruses mediated by a single antibody loop. Nature 2012;489:526-32
18. Corti D, Suguitan AL, Pinna D, et al. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J Clin Invest 2010;120:1663-73
19. Benjamin E, Wang W, McAuliffe JM, et al. A broadly neutralizing human monoclonal antibody directed against a novel conserved epitope on the influenza virus H3 hemagglutinin globular head. J Virol 2014;88:6743-50
20. Okuno Y, Isegawa Y, Sasao F, et al. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J Virol 1993;67:2552-8
21. Smirnov YA, Lipatov AS, Gitelman AK, et al. An epitope shared by the hemagglutinins of H1, H2, H5, and H6 subtypes of influenza A virus. Acta Virol 1999;43:237-44
22. Sui J, Hwang WC, Perez S, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biology 2009;16:265-73
23. Throsby M, van den Brink E, Jongeneelen M, et al. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 2008;3(12):e3942
24. Whittle JRR, Wheatley AK, Wu L, et al. Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. J Virol 2014;88:4047-57

• **Describes the derivation of recombinant HA probes that allow the direct interrogation of human memory B cell populations for cross-subtype specificities.**

25. Wrammert J, Koutsonanos D, Li GM, et al. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J Exp Med 2011;208:181-93
26. Dreyfus C, Ekiert DC, Wilson IA. Structure of a classical broadly neutralizing stem antibody in complex with a pandemic H2 influenza virus hemagglutinin. J Virol 2013;87:7149-54
27. Ekiert DC, Bhabha G, Elsliger MA, et al. Antibody recognition of a highly conserved influenza virus epitope. Science 2009;324:246-51
28. Ekiert DC, Friesen RH, Bhabha G, et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science 2011;333:843-50
29. Friesen RH, Lee PS, Stoop EJ, et al. A common solution to group 2 influenza virus neutralization. Proc Natl Acad Sci USA 2014;111:445-50
30. Tan GS, Lee PS, Hoffman RM, et al. Characterization of a broadly neutralizing monoclonal antibody that targets the fusion domain of group 2 influenza A virus hemagglutinin. J Virol 2014;88:13580-92
31. Corti D, Voss J, Gamblin SJ, et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science 2011;333:850-6
32. Dreyfus C, Laursen NS, Kwaks T, et al. Highly conserved protective epitopes on influenza B viruses. Science 2012;337:1343-8

•• **Describes the isolation and characterization of broadly cross-reactive antibodies to influenza B viruses. These include CR9114, which showed extraordinarily broad neutralization activity and recognition of 14 of 16 IAV subtypes and both IBV subtypes.**

33. Nakamura G, Chai N, Park S, et al. An in vivo human-plasmablast enrichment technique allows rapid identification of therapeutic influenza A antibodies. Cell Host Microbe 2013;14:93-103

•• **Describes a highly novel, *ex-vivo* enrichment procedure for human plasmablasts using SCID mice that facilitated the isolation of broadly neutralizing antibodies with activity against group1 and group2 viruses.**

34. Brandenburg B. Koudstaal W, Goudsmit J, et al. Mechanisms of Hemagglutinin

Targeted Influenza Virus Neutralization. PLoS One 2013;8(12):e80034

35. DiLillo DJ, Tan GS, Palese P, et al. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcγR interactions for protection against influenza virus in vivo. Nat Med 2014;20:143-51

•• **Demonstrated that Fc-mediated effector mechanisms are essential for HA stem, but not RBD, -binding neutralizing antibodies for passive protection from challenge in a small animal model.**

36. Air G. M. Influenza virus antigenicity and broadly neutralizing epitopes. Current opinion in virology 2015;11:113-21

37. Tan G, Krammer F, Eggink D, et al. A pan-H1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. J Virol 2012;86:6179-88

38. Sui J, Sheehan J, Hwang WC, et al. Wide prevalence of heterosubtypic broadly neutralizing human anti-influenza A antibodies. Clin Infect Dis 2011;52: 1003-9

•• **This study highlights the wide prevalence of serum antibody binding the hemagglutitin stem in human populations.**

39. Margine I, Hai R, Albrecht RA, et al. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J Virol 2013;87:4728-37

40. Moody MA, Zhang R, Walter EB, et al. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. PLoS One 2011;6(10):e25797

41. Thomson CA, Wang Y, Jackson LM, et al. Pandemic H1N1 Influenza Infection and Vaccination in Humans Induces Cross-Protective Antibodies that Target the Hemagglutinin Stem. Front Immunol 2012;3:87

42. He XSS, Sasaki S, Baer J, et al. Heterovariant cross-reactive B-cell responses induced by the 2009 pandemic influenza virus A subtype H1N1 vaccine. J Infect Dis 2013;207:288-96

43. Jegaskanda S, Amarasena TH, Laurie KL, et al. Standard trivalent influenza virus protein vaccination does not prime antibody-dependent cellular cytotoxicity in macaques. J Virol 2013;87:13706-18

44. Giudice G, Hilbert A, Bugarini R, et al. An MF59-adjuvanted inactivated influenza vaccine containing A/Panama/1999 (H3N2) induced broader serological protection against heterovariant influenza virus strain A/Fujian/2002 than a subunit and a split influenza vaccine. Vaccine 2006;24(16): 3063-5

45. Orsi A, Ansaldi F, de Florentiis D, et al. Cross-protection against drifted influenza viruses: options offered by adjuvanted and intradermal vaccines. Human vaccines & immunotherapeutics 2013;9:582-90

46. Ansaldi F, Zancolli M, Durando P, et al. Antibody response against heterogeneous circulating influenza virus strains elicited by MF59- and non-adjuvanted vaccines during seasons with good or partial matching between vaccine strain and clinical isolates. Vaccine 2010;28:4123-9

47. Ansaldi F, Bacilieri S, Durando P, et al. Cross-protection by MF59-adjuvanted influenza vaccine: neutralizing and haemagglutination-inhibiting antibody activity against A(H3N2) drifted influenza viruses. Vaccine 2008;26(12):1525-9

48. Banzhoff A, Gasparini R, Laghi-Pasini F, et al. MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic antibody responses in non-elderly and elderly adults. PLoS One 2009;4(2):e4384

49. Alberini I, E, Del Tordello et al. Pseudoparticle neutralization is a reliable assay to measure immunity and cross-reactivity to H5N1 influenza viruses. Vaccine 2009;27:5998-6003

50. Even-Or O, Samira S, Ellis R, et al. Adjuvanted influenza vaccines. Expert Rev Vaccines 2013;12:1095-108

51. Wei CJJ, Boyington JC, McTamney PM, et al. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. Science 2010;329:1060-4

52. Kanekiyo M, Wei JJ, Yassine HM, et al. Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. Nature 2013;499:102-6

53. Chen MWW, Cheng RJ, Huang Y, et al. A consensus-hemagglutinin-based DNA vaccine that protects mice against divergent H5N1 influenza viruses. Proc Natl Acad Sci USA 2008;105:13538-43

54. Weaver EA, Rubrum AM, Webby RJ, Barry MA. Protection against divergent influenza H1N1 virus by a centralized influenza hemagglutinin. PLoS One 2011; 6(3):e18314

55. Yan J, Villarreal DO, Racine T, et al. Protective immunity to H7N9 influenza viruses elicited by synthetic DNA vaccine. Vaccine 2014;32:2833-42

56. Giles BM, Ross TM. A computationally optimized broadly reactive antigen (COBRA) based H5N1 VLP vaccine elicits broadly reactive antibodies in mice and ferrets. Vaccine 2011;29:3043-54

57. Giles BM, Bissel SJ, Dealmeida DR, et al. Ross. Antibody breadth and protective efficacy are increased by vaccination with computationally optimized hemagglutinin but not with polyvalent hemagglutinin-based H5N1 virus-like particle vaccines. Clin Vaccine Immunol 2012;19:128-39

58. Giles BM, Crevar CJ, Carter DM, et al. A computationally optimized hemagglutinin virus-like particle vaccine elicits broadly reactive antibodies that protect nonhuman primates from H5N1 infection. J Infect Dis 2012;205:1562-70

59. Graves PN, Schulman JL, Young JF, Palese P. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. Virology 1983;126:106-16

60. Sagawa H, Ohshima A, Kato I, et al. The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular region. J Gen Virol 1996; 77(7):1483-7

61. Steel J, Lowen AC, Wang TT, et al. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. mBio 2010;1(1):e00018-0

62. Bommakanti G, Lu X, Citron MP, et al. Design of Escherichia coli-expressed stalk domain immunogens of H1n1 hemagglutinin that protect mice from lethal challenge. J Virol 2012;86(24):13434-44

63. Bommakanti G, Citron MP, Hepler RW, et al. Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. Proc Natl Acad Sci USA 2010;107(31): 13701-6

64. Mallajosyula VVA, Citron MP, Ferrara F, et al. Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. Proc Natl Acad Sci USA 2014;111(25):E2514-23

65. Lu Y, Welsh JP, Swartz JR. Production and stabilization of the trimeric influenza hemagglutinin stem domain for potentially broadly protective influenza vaccines. Proc Natl Acad Sci USA 2014;11(1):125-30

66. Pica N, Hai R, Krammer F, et al. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a

mechanism for the extinction of seasonal H1N1 viruses. Proc Natl Acad Sci USA 2012;109:2573-8

67. Ellebedy AH, Krammer F, Li GM, et al. Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. Proc Natl Acad Sci USA 2014;111:13133-8
68. Nachbagauer R, Wohlbold TJ, Hirsh A, et al. Induction of broadly reactive anti-hemagglutinin stalk antibodies by an H5N1 vaccine in humans. J Virol 2014;88:13260-8
69. Ledgerwood JE, Zephir K, Hu Z, et al. Prime-boost interval matters: a randomized phase 1 study to identify the minimum interval necessary to observe the H5 DNA influenza vaccine priming effect. J Infect Dis 2013;208:418-22
70. Wheatley AK, Whittle JR, Lingwood D, et al. H5N1 Vaccine-Elicited Memory B Cells Are Genetically Constrained by the IGHV Locus in the Recognition of a Neutralizing Epitope in the Hemagglutinin Stem. J Immunol 2015;195(2):602-10
71. Miller MS, Tsibane T, Krammer F, et al. 1976 and 2009 H1N1 influenza virus vaccines boost anti-hemagglutinin stalk antibodies in humans. J Infect Dis 2013;207:98-105
72. Li GM, Chiu C, Wrammert J, et al. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. Proc Natl Acad Sci USA 2012;109:9047-52
73. Krammer F, Palese P. Universal influenza virus vaccines: need for clinical trials. Nature immunology 2014;15:3-5
74. Krammer F, Palese P. Advances in the development of influenza virus vaccines. Nat Rev Drug Discov 2015;14:167-82
75. Hai R, Krammer F, Tan GS, et al. Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. J Virol 2012;86:5774-81

- **Describes the construction of chimeric HA, based on heterologous HA1 domains fused to conserved HA2. These constructs promote the focusing of the humoral immune responses onto potentially protective HA stem epitopes in small animal immunization models.**

76. Krammer F, Hai R, Yondola M, et al. Assessment of influenza virus hemagglutinin stalk-based immunity in ferrets. J Virol 2014;88:3432-42
77. Krammer F, Pica N, Hai R, et al. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. J Virol 2013;87:6542-50
78. Krammer F, Margine I, Hai R, et al. H3 stalk-based chimeric hemagglutinin influenza virus constructs protect mice from H7N9 challenge. J Virol 2014;88:2340-3
79. Margine I, Krammer F, Hai R, et al. Hemagglutinin stalk-based universal vaccine constructs protect against group 2 influenza A viruses. J Virol 2013;87:10435-46
80. Lin SCC, Liu C, Jan T, Wu SCC. Glycan masking of hemagglutinin for adenovirus vector and recombinant protein immunizations elicits broadly neutralizing antibodies against H5N1 avian influenza viruses. PLoS One 2014;9(3):e92822
81. Lin S-C, Lin YF, Chong P, Wu SC. Broader neutralizing antibodies against H5N1 viruses using prime-boost immunization of hyperglycosylated hemagglutinin DNA and virus-like particles. PLoS One 2012;7(6):e39075
82. Wang TT, Tan GS, Hai R, et al. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. PLoS Pathog 2010;6(2):e1000796
83. Deng L, Cho KJ, Fiers W, Saelens X. M2e-based universal influenza A vaccines. Vaccines 2015;3:105-36
84. Zhang N, Zheng J, Lu L, et al. Advancements in the development of subunit influenza vaccines. Microbes Infect 2015;17:123-34
85. Zheng M, Luo J, Chen Z. Development of universal influenza vaccines based on influenza virus M and NP genes. Infection 2014;42:251-62
86. Schotsaert M, De Filette M, Fiers W, Saelens X. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. Expert Rev Vaccines 2009;8:499-508
87. Liu X, Guo J, Han S, et al. Enhanced immune response induced by a potential influenza A vaccine based on branched M2e polypeptides linked to tuftsin. Vaccine 2012;30:6527-33
88. Huleatt JW, Nakaar V, Desai P, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 2008;26:201-14
89. Neirynck S, Deroo T, Saelens X, et al. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 1999;5:1157-63
90. Muto NA, Yoshida R, Suzuki T, et al. Inhibitory effects of an M2-specific monoclonal antibody on different strains of influenza A virus. Jpn J Vet Res 2012;60:71-83
91. Kim MCC, Lee JS, Kwon M, et al. Multiple heterologous M2 extracellular domains presented on virus-like particles confer broader and stronger M2 immunity than live influenza A virus infection. Antiviral Res 2013;99:328-35
92. El Bakkouri K, Descamps F, De Filette M, et al. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. J Immunol 2011;186:1022-31
93. Grandea AG III, Olsen OA, Cox TC, et al. Human antibodies reveal a protective epitope that is highly conserved among human and nonhuman influenza A viruses. Proc Natl Acad Sci USA 2010;107:12658-63
94. Treanor JJ, Tierney EL, Zebedee SL, et al. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J Virol 1990;64:1375-7
95. Song A, Myojo K, Laudenslager J, et al. Evaluation of a fully human monoclonal antibody against multiple influenza A viral strains in mice and a pandemic H1N1 strain in nonhuman primates. Antiviral Res 2014;111:60-8
96. Wang R, Song A, Levin J, et al. Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. Antiviral Res 2008;80:168-77
97. Stepanova LA, Kotlyarov RY, Kovaleva AA, et al. Protection against Multiple Influenza A Virus Strains Induced by Candidate Recombinant Vaccine Based on Heterologous M2e Peptides Linked to Flagellin. PLoS One 2015;10:e0119520
98. Ramos EL, Mitcham JL, Koller TD, et al. Efficacy and safety of treatment with an anti-m2e monoclonal antibody in experimental human influenza. J Infect Dis 2015;211:1038-44
99. Turley CB, Rupp RE, Johnson C, et al. Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. Vaccine 2011;29:5145-52
100. Talbot HK, Michael TR, Casey J, et al. Immunopotentiation of trivalent influenza vaccine when given with VAX102, a

recombinant influenza M2e vaccine fused to the TLR5 ligand flagellin. PLoS One 2010; 5(12):e14442

101. Antrobus RD, Lillie PJ, Berthoud TK, et al. A T cell-inducing influenza vaccine for the elderly: safety and immunogenicity of MVA-NP+M1 in adults aged over 50 years. PLoS One 2012;7:e48322
102. Sandbulte MR, Jimenez GS, Boon AC, et al. Cross-reactive neuraminidase antibodies afford partial protection against H5N1 in mice and are present in unexposed humans. PLoS Med 2007;4(2):e59
103. Wan H, Yang H, Shore DA, et al. Structural characterization of a protective epitope spanning A(H1N1)pdm09 influenza virus neuraminidase monomers. Nature communications 2015;6:6114
104. Gravel C, Li C, Wang J, et al. Quantitative analyses of all influenza type A viral hemagglutinins and neuraminidases using universal antibodies in simple slot blot assays. J Vis Exp 2011;50:2784
105. Gui X, Ge P, Wang X, et al. Identification of a highly conserved and surface exposed B-cell epitope on the nucleoprotein of influenza A virus. J Med Virol 2014;86: 995-1002
106. Wan H, Gao J, Xu K, et al. Molecular basis for broad neuraminidase immunity: conserved epitopes in seasonal and pandemic H1N1 as well as H5N1 influenza viruses. J Virol 2013;87:9290-300
107. Couch RB, Atmar RL, Keitel WA, et al. Randomized comparative study of the serum antihemagglutinin and antineuraminidase antibody responses to six licensed trivalent influenza vaccines. Vaccine 2012;31:190-5
108. Kilbourne ED, Couch RB, Kasel JA, et al. Purified influenza A virus N2 neuraminidase vaccine is immunogenic and non-toxic in humans. Vaccine 1995;13:1799-803
109. Jegaskanda S, Reading PC, Kent SJ. Influenza-specific antibody-dependent cellular cytotoxicity: toward a universal influenza vaccine. J Immunol 2014;193: 469-75
110. Sridhar S, Begom S, Bermingham A, et al. Cellular immune correlates of protection against symptomatic pandemic influenza. Nat Med 2013;19:1305-12
111. Hayward AC, Wang L, Goonetilleke N, et al. Natural T Cell-mediated Protection against Seasonal and Pandemic Influenza. Results of the Flu Watch Cohort Study. Am J Respire Crit Care Med 2015;191:1422-31
112. Price GE, Soboleski MR, Lo Y, et al. Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. Vaccine 2009;27:6512-21
113. Rao SS, Kong P, Wei J, et al. Comparative efficacy of hemagglutinin, nucleoprotein, and matrix 2 protein gene-based vaccination against H5N1 influenza in mouse and ferret. PLoS One 2010;5(3):e9812
114. Francis T. On the doctrine of original antigenic sin. Proc Am Philos Soc 1960;104:572-8
115. Fazekas de St G, Webster RG. Disquisitions on Original Antigenic Sin. II. Proof in lower creatures. J Exp Med 1966;124: 347-61
116. Fazekas de St G, Webster RG. Disquisitions of Original Antigenic Sin. I. Evidence in man. J Exp Med 1966;124:331-45
117. Lessler J, Riley S, Read JM, et al. Evidence for antigenic seniority in influenza A (H3N2) antibody responses in southern China. PLoS Pathog 2012;8(7):e1002802
118. Miller MS, Gardner TJ, Krammer F, et al. Neutralizing antibodies against previously encountered influenza virus strains increase over time: a longitudinal analysis. Sci Transl Med 2013;5(198):198ra107
119. Davenport FM, Hennessy AV, Francis T. Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. J Exp Med 1953;98:641-56
120. Andrews SF, Kaur K, Pauli NT, et al. High preexisting serological antibody levels correlate with diversification of the influenza vaccine response. J Virol 2015;89:3308-17

•• **This study demonstrates that pre-existing humoral immunity may suppress the elicitation of antibody-secreting cells and memory B cell responses following subsequent vaccination.**

121. Sasaki S, He SXS, Holmes TH, et al. Influence of prior influenza vaccination on antibody and B-cell responses. PLoS One 2008;3(8):e2975
122. Bodewes R, de Mutsert G, van der Klis FR, et al. Prevalence of antibodies against seasonal influenza A and B viruses in children in Netherlands. Clin Vaccine Immunol 2011;18:469-76
123. Kucharski AJ, Lessler J, Read JM, et al. Estimating the life course of influenza A (H3N2) antibody responses from cross-sectional data. PLoS Biol 2015;13(3): e1002082
124. Steel J, Staeheli P, Mubareka S, et al. Transmission of pandemic H1N1 influenza virus and impact of prior exposure to seasonal strains or interferon treatment. J Virol 2010;84:21-6
125. Min JY, Chen GL, Santos C, et al. Classical swine H1N1 influenza viruses confer cross protection from swine-origin 2009 pandemic H1N1 influenza virus infection in mice and ferrets. Virology 2010;408:128-33
126. O'Donnell CD, Wright A, Vogel LN, et al. Effect of priming with H1N1 influenza viruses of variable antigenic distances on challenge with 2009 pandemic H1N1 virus. J Virol 2012;86:8625-33
127. Carter DM, Bloom CE, Nascimento EJ, et al. Sequential seasonal H1N1 influenza virus infections protect ferrets against novel 2009 H1N1 influenza virus. J Virol 2013;87:1400-10
128. Wei CJJ, Yassine HM, McTamney PM, et al. Elicitation of broadly neutralizing influenza antibodies in animals with previous influenza exposure. Sci Transl Med 2012;4(147):147ra114
129. Del Giudice G, Stittelaar KJ, van Amerongen G, et al. Seasonal influenza vaccine provides priming for A/ H1N1 immunization. Sci Transl Med 2009;1(12):12re1
130. Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (Macaca fascicularis). Vet Pathol 2003;40: 304-10
131. O'Donnell CD, Subbarao K. The contribution of animal models to the understanding of the host range and virulence of influenza A viruses. Microbes Infect 2011;13:502-15
132. Sundling C, Phad G, Douagi I, et al. Isolation of antibody V(D)J sequences from single cell sorted rhesus macaque B cells. J Immunol Methods 2012;386:85-93
133. Hogarth PM, Anania JC, Wines BD. The FcγR of humans and non-human primates and their interaction with IgG: implications for induction of inflammation, resistance to infection and the use of therapeutic monoclonal antibodies. Curr Top Micrbiol Immunol 2014;382:321-52
134. Weinfurter JT, Brunner K, Capuano SV III, et al. Cross-reactive T cells are involved in rapid clearance of 2009 pandemic H1N1 influenza virus in nonhuman primates. PLoS pathogens 2011;7:e1002381
135. Jegaskanda S, Weinfurter JT, Friedrich TC, Kent SJ. Antibody-dependent cellular cytotoxicity is associated with control of

pandemic H1N1 influenza virus infection of macaques. J Virol 2013;87:5512-22

136. Yu X, Tsibane T, McGraw PA, et al. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature 2008;455:532-6

137. Dormitzer PR, Galli G, Castellino F, et al. Influenza vaccine immunology. Immunol Rev 2011;239:167-77

138. Jiang N, He J, Weinstein JA, et al. Lineage structure of the human antibody repertoire in response to influenza vaccination. Sci Transl Med 2013;5:171ra119

139. Victora GD, Nussenzweig MC. Germinal centers. Annu Rev Immunol 2012;30: 429-57

140. Nayak JL, Fitzgerald TF, Richards KA, et al. CD4+ T-cell expansion predicts neutralizing antibody responses to monovalent, inactivated 2009 pandemic influenza A(H1N1) virus subtype H1N1 vaccine. J Infect Dis 2013;207: 297-305

141. Bentebibel SE, Lopez S, Obermoser G, et al. Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. Sci Transl Med 2013;5:176ra132

142. Richards KA, Nayak J, Chaves FA, et al. Seasonal Influenza Can Poise Hosts for CD4 T-Cell Immunity to H7N9 Avian Influenza. J Infect Dis 2014;212(1):86-94

143. Ge X, Tan V, Bollyky PL, et al. Assessment of seasonal influenza A virus-specific CD4 T-cell responses to 2009 pandemic H1N1 swine-origin influenza A virus. J Virol 2010;84:3312-19

144. Richards KA, Topham D, Chaves FA, Sant AJ. Cutting edge: CD4 T cells generated from encounter with seasonal influenza viruses and vaccines have broad protein specificity and can directly recognize naturally generated epitopes derived from the live pandemic H1N1 virus. J Immunol 2010;185:4998-5002

145. Evaluation of the Protective Efficacy and Safety of CR8020 in an Influenza Challenge. Available from: https://clinicaltrials.gov/ct2/show/NCT01938352

146. Study in Healthy Volunteers to Evaluate the Efficacy and Safety of CR6261 in an H1N1 Influenza Healthy Human Challenge Model. Available from:https://clinicaltrials.gov/ct2/show/NCT02371668

147. A Study of MHAA4549A in Healthy Volunteers in an Influenza Challenge Model. Available from: https://clinicaltrials.gov/ct2/show/NCT01980966 [Last accessed 3 March 2015]

148. Khurana S, Loving CL, Manischewitz J, et al. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. Sci Transl Med 2013;5(200):200ra114

149. Gamblin SJ, Haire LF, Russell RJ, et al. The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science 2004;303(5665): 1838-42