Epitope-focused vaccine design against influenza A and B viruses

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Abstract

The threat of influenza A and B variants via antigenic drift and emerging novel influenza A and B strains in the human population via antigenic shift has spurred research efforts to improve upon current seasonal influenza vaccines. In recent years, a wave of novel technological breakthroughs has led to the identification of many broadly anti-influenza hemagglutinin (HA) monoclonal antibodies (mAbs) and the elucidation of the conserved epitopes recognized by these mAbs in both the head and the stem of HA as well as the mechanisms of inhibition. These discoveries along with an improved understanding of how the immune system responds to influenza infection and vaccination has spurred great efforts on stem-based cross-subtype ("universal") vaccine design as well as RBS-based HA subtype-specific vaccine design.

Introduction

Current seasonal influenza vaccines are efficacious when vaccine strains are matched with circulating influenza A and B strains. However, they need to be reformulated frequently to elicit protective antibody responses against viral genetic variants that arise via antigenic drift. They also do not protect humans from pandemics and outbreaks of newly emerging strains via antigenic shift. Thus, the holy grail of influenza vaccine research is to design immunogen(s) that not only protect current strains, but also future strains resulting from antigenic drift and/or antigenic shift.

Protection elicited by current seasonal influenza vaccines or other licensed vaccines against viral diseases are antibody-mediated. These antibodies bind to viral surface proteins that mediated virus entry or budding from host cells, leading to neutralization, opsonization and/or complement fixation. CD4 T helper cells are required for such antibody responses during the induction, expansion, differentiation and maturation of viral surface protein-specific B cells.

There are many challenges in eliciting desirable antibody responses against viral surface proteins. First, these proteins are extremely genetically diverse. Thus, the elicitation of antibody responses against sequence- and structure-conserved epitopes that could protect diverse virus strains is a high priority. Second, these proteins may exist in different forms and shapes, depending on whether the viral particles are mature or immature. As a result, some epitopes may be exposed in one form or shape, but not in others. Third, these proteins often go through a series of conformational changes when they interact with their receptor and coreceptor or when viruses are endocytosed into the low pH endosomal compartment. As a result, some epitopes may expose transiently in one conformation, but not in others. Fourth, these proteins often assemble into higher-order structures. Some epitopes exist in the interface between two subunits within one protomer, but other quaternary epitopes exist in more than one protomer. And fifth, some epitopes on these proteins contain specific glycans. Because of this, antigenicity and immunogenicity of these epitopes may be strongly influenced by the heterogeneity of glycans produced by different expression systems. Thus, viral surface protein-based vaccine design should take into consideration all these challenges.

Three vaccine design strategies have been developed in dealing with the genetic diversity of these proteins. The first strategy is reverse vaccinology, i.e. to analyze protein sequence diversity

and then design ancestral, consensus, mosaic and multivalent immunogens [1]. The second strategy is serologic vaccinology, i.e. to analyze cross-reactivity of antibody responses among subtypes or clades of a given virus and then design immunogens based on antigenic clusters [2]. And the third strategy is analytic vaccinology (also coined as reverse vaccinology 2.0), i.e. to analyze the human immune responses from infected or vaccinated individuals, isolate neutralizing antibodies, determine the neutralization epitopes and then design immunogens based on epitope structures [3]. During the past 10 years, a wave of new technological breakthroughs, such as antibodies isolated from single B cells or plasma cells in vaccinated or infected individuals, structural determination of antibody-antigen complex, computational design for epitope-focused vaccines, deep RNA sequencing for longitudinal antigen and antibody evolution, have greatly facilitated analytic vaccinology [3].

In this review we will focus on epitope-focused vaccine design against influenza hemagglutinin (HA). We will summarize conserved epitopes in influenza A and B HA molecules and discuss how they are being applied to the stem-based cross-subtype ("universal") vaccine design and the receptor-binding site (RBS)-based subtype-specific vaccine design. Due to the page limitation, we apologize many interesting studies could not be cited in this review.

Conserved epitopes in influenza A and B HA

Influenza A and B viruses are members of the family *Orthomyxoviridae*. Naturally influenza A virus infects many avian species and some mammalian species including humans; while influenza B virus infects only humans. HA on the virion surface forms a trimer of covalently linked HA1/HA2 heterodimers. HA1 binds to sialic acid receptors on host cells and allows virus to be endocytosed and HA2 mediates viral and endosomal membrane fusion. HA in influenza A virus has 18 subtypes, which are divided into two phylogenetically distinct groups [4-7]. Group 1 comprises of H1, H2, H5, H6, H8, H9, H11, H13, H16, H17 and H18 and Group 2 consists of H3, H4, H7, H10, H14 and H15. HA within a subtype has up to 15% sequence diversity, whereas HA subtypes within a group or between two groups have 40% or 60% sequence diversity, respectively. Influenza B virus has two antigenically distinct lineages Victoria and Yamagata [8].

During the past several years, quite a few monoclonal antibodies (mAbs) against the head and stem with various degrees of cross-reactivity have been isolated and their corresponding conserved epitopes have been elucidated. From these studies, several conclusions can be drawn.

First, the stem contains more conserved epitopes than the head. Among them, some are conserved within the HA subtypes of group 1 [9-14] and the other of group 2 [15,16]. Occasionally, the epitopes are conserved in both groups 1 and 2 [17] and even between influenza A and B viruses [18]. But the latter are very rare and require the special mode of action [17,18]. Second, the head also contains some conserved epitopes present in diverse strains of different subtypes [19-21]. But their conservation appears more limited than the conserved epitopes in the stem. Third, the head contains epitopes that are conserved in diverse strains within a HA subtype. These epitopes are located in the RBS [22-27] or outside the RBS [27-32]. The RBS binding antibodies are common in vaccine-induced antibody responses. They have diverse V_H/V_L gene usages and utilize sialic acid mimicry on their HCDR3 loops to interact with the RBS [33]. How common outside RBS-binding antibodies are in vaccine-induced antibody responses is currently unknown. Recently we identified four conserved epitopes in the H5 HA head, and three of them were located outside RBS. Interestingly, two convalescent serum samples tested had antibody responses against all four of these epitopes [32]. Fourth, an antibody recognizes the epitopes located at the HA head/stem interface [34], which are similar to anti-HIV-1 antibody 35O22 [35] or anti-EBOV GP antibody 2G4 [36]. Two antibodies recognize the quaternary epitopes [31,37], which are similar to anti-HIV-1 antibody PGT151 [38] or anti-RSV prefusion F antibody AM14 [39]. Fifth, the antibody repertoire against the epitopes in the head is much more diverse than the antibody repertoire against the epitopes in the stem [40]. This could be due to the occlusive nature of the epitopes in the stem on virion, in which only a few antibodies with specific mode of action can interact with these epitopes [41]. As a result, antibody responses against the head are more potent and dominant than those against the stem. [Table 1](#) summarizes the conserved epitopes revealed by mAbs.

Stem-based vaccine design that aims to elicit cross-subtype ("universal") antibody responses

The discovery of broadly neutralizing mAbs against conserved epitopes in the stem has spurred great efforts on HA stem-based cross-subtype ("universal") vaccine design (there is an excellent review by Neu et al. in this issue that highlights the rationales and the feasibility for HA stem-based "universal" vaccines). Basically, two approaches have been tested ([Table 2](#)). One approach used sequential infection with different influenza strains [42] or sequential prime-boosts with

head/stem chimera (cHA), in which head from different HA subtypes were fused with a common stem [43-45]. Sequential prime-boosts with cHA containing a common H1 stem were able to cross-protect mice from lethal challenge of H5N1 and H6N1 viruses (group 1) and to cross-neutralize H2N2 virus (group 1) [44]; whereas sequential prime-boosts with cHA containing a common H3 stem were able to cross-protect mice from lethal challenge of H7N1 and H7N9 (group 2) [45]. Recently, Tran et al. [46] reported the first three-dimensional visualization of cHA (cH5/1) displayed on the virion surface. They found that the cH5/1 structure differs from those of native HA by displaying a more open head and a dramatically twisted head/stem arrangement. Despite this unusual spatial relationship between head and stem, viruses expressing the cH5/1 are fully infectious and display a high glycoprotein density. Currently, cHAs for sequential immunization are in preparation for clinical trials.

Another approach was to develop stem-only (or headless) HA (Table 2). Earlier studies to remove HA head chemically or genetically resulted in limited success [47-49]. This was largely due to the misfolding of the headless HA leading to concomitant loss of conserved conformational epitopes. Bommakanti et al. [50] reported that a headless H1 HA construct produced in *E. Coli* elicited protection in mice against a heterologous H1 virus, but not heterosubtypic virus. Two follow up studies by the same group reported an improved headless H1 HA construct that elicited 30% cross-H3 protection in mice [51]; and a prefusion mini headless H5 HA construct that elicited the protection against lethal challenge of H5 and H1 (group 1) and H3 (group 2) viruses [52]. A recent study by Lu et al. [53] reported a multi-step rational design approach that resulted in the headless HA displays correctly folded conserved conformational epitopes, but with no report on its *in vivo* efficacy. Another recent study by Wohlbold et al. [54] showed that a soluble headless H1 HA produced by insect cells provided protection against lethal challenge of H1, H5 and H6 viruses, although the protein did not bind to a panel of stem-specific antibodies as measured by ELISA.

Impagliazzo et al. [55] recently reported several stable H1 HA stem structures derived by using multiple cycles of structure-based design. Among them a H1 HA stem trimer #4900 maintains conserved conformational epitopes. Mice immunized with this stem trimer plus a Matrix M adjuvant had binding antibodies against H1, H5, and H9 (group 1) as well as H3 and H7 (group 2), and neutralizing antibodies against divergent H1 strains as well as H5 strains. Importantly,

mice immunized twice or three times with this stem trimer plus the adjuvant were completely protected from the lethal dose challenges of heterologous H1N1 and heterosubtypic H5N1 virus, respectively. When cynomolgus monkeys were immunized with the stem trimer plus the adjuvant followed by sublethal heterosubtypic H5N1 challenge, reduced fever, but no reduction of tracheal viral loads, was observed. Similarly, through multiple cycles of structure-based design, Yassine et al. [56] recently reported stable H1 HA stem (HA-SS) immunogens. They then generated a self-assembled nanoparticle (HA-SS-np) by linking HA-SS with *H. pylori* ferritin. Structural studies indicated that the conserved conformational epitopes were maintained in H1 HA-SS-np. Mice and ferrets immunized with H1 HA-SS-np plus Ribi adjuvant had binding antibody responses against H1, H2, H5, and H9 (group 1) as well as H3 and H7 (group 2); decent neutralizing antibody responses against divergent H1 strains, but much lower or undetectable neutralization of heterosubtypic H5, H2, H7 and H9 strains. Importantly, when immunized mice and ferrets were challenged with a high lethal dose of H5N1 strain, complete and partial protection was observed in mice and in ferrets, respectively.

Although the successful design of stable, correctly folded headless HA is an important advancement towards "universal" influenza vaccine, many questions remain. First, both constructs contain heterologous elements: leucine zipper motif and *H. pylori* ferritin. These elements may induce autoimmunity in humans because similar leucine zipper motifs exist in human proteins and human ferritin has similar overall structure even though it shares little sequence identity. Second, in an influenza infection swine model vaccine-induced anti-stem antibodies, in the absence of anti-RBS neutralizing antibodies, have been shown to promote virus fusion and enhance influenza virus respiratory disease [57]. Thus, it is important to determine whether antibody-mediated disease enhancement (ADE) exists during the evaluation of stem-based vaccines. Third, both studies showed that although these modified H1 HA stem constructs bind properly with broadly neutralizing anti-stem antibodies and induce stem-reactive antibody responses, they cross-protect heterosubtypic H5 challenge with no robust neutralizing antibody responses. It has been known that phylogenetically H5 HA is much closer to H1 HA than most other HA subtypes of group 1. Thus, it will be very interesting to know the breadth of heterosubtypic protection these modified H1 HA stems might actually elicit.

RBS-based vaccine design that aims to elicit HA subtype-specific antibody responses

Although the HA head is less conserved than the HA stem and prone to antigenic drift, the discovery of many anti-RBS antibodies that cross-neutralize diverse strains within subtypes H1, H3, H5 and H7 [22-24,27,31,32] and within influenza B viruses [18] has spurred efforts on RBS-based HA subtype-specific vaccine design (Table 2). The rationale behind this approach is that currently only H1 and H3 subtypes of influenza A viruses and both lineages of influenza B viruses circulate in human population and H5, H7 and H9 subtypes of influenza A viruses are occasionally transmitted from avian species to humans. Thus if RBS-based vaccines can elicit subtype-specific protective antibody responses against these subtypes, one may take care of antigenic drift problem.

Towards this goal, Kanekiyo et al. [58] designed self-assembling nanoparticles by genetically linking the ectodomain of HA from H1 (1999 NC or 2009 CA), H3 (2009 Perth) and influenza B (2006 FL) to *H. pylori* non-haem ferritin. Amazingly, the HA-ferritin fusion protein produced by a mammalian expression system spontaneously self-assembled into 24 mer nanoparticles, each displaying eight trimeric HA spikes protruding from the ferritin core. Immunogenicity studies indicate that the self-assembled HA-ferritin nanoparticles plus Ribi adjuvant elicits HI titers more than 10-fold higher than the licensed inactivated vaccine. Neutralizing antibodies elicited by this vaccine regimen target both conserved epitope on the stem and the RBS on the head. Importantly, antibodies elicited by H1 HA-nanoparticles from 1999 NC strain neutralized H1 viruses from 1934 to 2007 and protected ferrets from a heterologous H1 challenge.

Previously, we showed that priming mice twice with DNA plasmid encoding H5 HA from a 2004 TH strain and boosting once with virus-like particles (VLP) derived from the same strain (notated as TH DDV) induced antibody responses that cross-neutralize all clades and subclades H5 viruses tested. We also showed that although TH DDV sera bind to both the head and the stem of H5 HA, neutralization activity mainly recognizes the head [59]. More recently, we mapped three conformational neutralization epitopes AS1 (RBS), AS3 (outside RBS) and AS4 (outside RBS) in the HA head recognized by TH DDV sera and only one conformational neutralization epitope AS1 (RBS) recognized by HK5052 (another H5N1 strain) DDV sera. We then replaced the narrowly focused, but highly potent neutralization AS1 (RBS) epitope in the HK5052 HA (recipient) with the broad, yet moderately potent neutralization AS1 (RBS) epitope from the TH HA (donor). Remarkably, the resulting graft elicits significantly more potent and

broader AS1 (RBS) epitope-specific neutralizing antibody responses than either donor or recipient HA, which completely cross-protect heterologous H5 and heterosubtypic H1 challenges, and partially cross-protect heterosubtypic H9 and H3 challenge in both active and passive immunizations (Wang et al. unpublished data). Recently, from characteristics of H1 HA RBS-directed antibodies, Schmidt et al. [40] devised a signature motif in HCDR3 region to search for similar antibodies from three TIV vaccinees. They identified 107 candidates encoded by eleven different V_H gene families. X-ray studies indicate that these antibodies mimic binding of sialic acid receptor to engage the RBS by supplying a critical dipeptide on their projecting HCDR3 region. Thus, it is extremely interesting to test whether anti-H5 HA RBS antibodies in TH AS1 HK5052 sera also have a similar signature motif in their HCDR3 to engage RBS binding as the HCDR3 of anti-H1 HA RBS antibodies reported by Schmidt et al [40]. This comparative study may shed some lights on what actually determines HA subtype specificity in the RBS-binding antibodies.

Summary and outlook

In the past several years significant progress has been made in epitope-focused vaccine designs against influenza A and B viruses. Many conserved epitopes with various degrees of sequence- and structure-conservation have been identified. This has spurred both stem-based cross-subtype ("universal") vaccine design and RBS-based subtype-specific vaccine design. In the stem-based vaccine design, stable, correctly folded stems with improved cross-subtypic antibody responses and protection have been developed through multiple cycles of structure-based designs. But their safety and breadth of cross-protection still need to be improved. The feasibility of RBS-based subtype-specific vaccine design has been demonstrated in H1 and H5 subtypes. However, more studies need to be done to determine whether these approaches can protect all current strains as well as block the occurrence of antigenic drift. Historically, conserved epitopes outside RBS in HA head have been least studied. New tools need to be developed to study to what extent they exist and what is their relative contribution to the elicitation of HA subtype-specific antibody responses.

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Papers of particular interests, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

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3 • A concise review for epitope-focused vaccine designs.

32 • This study shows the H5 HA head contains four conserved neutralization epitopes: one in RBS and the other three outside RBS. Two convalescent serum samples tested have antibody responses against all four of these epitopes

33 •• This elegant structural study shows that RBS-binding antibodies with diverse germline origins mimic binding of sialic acid receptor to engage the RBS by supplying a critical dipeptide on their projecting HCDR3 region.

42 • This study shows that the sequential infection with divergent H1N1 viruses induces HA stem-reactive antibodies.

44 • This study shows that sequential prime-boosts with chimeric HA containing a common H1 stem cross-protected mice from lethal challenge of heterosubtypic group 1 virus challenge. (group 1)

45 • This study shows that sequential prime-boosts with chimeric HA containing a common H3 stem cross-protected mice from lethal challenge of heterosubtypic group 2 virus challenge. (group 1)

55 •• This elegant study reported a structure-based design for making a stable, correctly folded H1 stem trimer #4900, which elicited heterosubtypic antibody responses and protection in mice and cynomolgus monkeys.

- 56 ••** This elegant study reported a structure-based design for making a self-assembled nanoparticle H1 HA-SS-np, in which H1 stem is correctly folded. H1 HA-SS-np plus Ribi adjuvant elicited heterosubtypic antibody responses and protection in mice and ferrets.
- 57 •** This study shows that in an influenza infection swine model vaccine-induced anti-stem antibodies, in the absence of anti-RBS neutralizing antibodies, promote virus fusion and enhance influenza virus respiratory disease.
- 58 ••** This elegant study first reported a structure-based design for making a self-assembled nanoparticle (H1 HA-np) from 1999 NC strain, which elicits much higher HI titers than the licensed inactivated vaccine, neutralized H1N1 viruses from 1934 to 2007, and protected ferrets from a heterologous H1 challenge.
- 59 •** This study shows that a heterologous prime-boost strategy (TH DDV) elicits antibody responses that cross-neutralize all clades and subclades of H5 viruses and the neutralization activity is directed to HA head.

Table 1 Conserved epitopes recognized by anti-HA mAbs

	Antibodies	Cross-reactivity	Source and methods	Ref.
Stem				
Epitope 1 Centered on the A α -helix of the HA2 region	CR9114	Influenza A and B*	TIV, phage display, X-ray structure**	[18]
	FI6v3	H1, H3, H5, H7, H2, H4, H6, H8, H9, H10	H1 and TIV, single-cell PCR, X-ray structure	[17]
	C179	H1, H2, H5, H6, H9	H2 vaccinated mice, hybridoma, X-ray structure	[9,13,60]
	F10	H1, H2, H5, H6, H8, H9, H11	A 27 billion member human phage display, X-ray structure	[12]
	CR6261	H1, H2, H5, H6, H8, H9, H12, H13, H16	TIV, phage display, X-ray structure	[10,11,18]
	3.1	H1, H2, H5, H6	TIV, phage display, X-ray-structure	[14]
Epitope 2 Located at the upper part of the long α -helix CD in the HA2 region	CR8020	H3, H4, H7, H10, H14, H15	TIV, immortalized B cells by genetic programming, X-ray structure	[15,18]
	CR8043	H3, H10, H4, H7, H14, H15	TIV, immortalized B cells by genetic programming, X-ray structure	[16]
	9H10	H3, H10	Sequential infection, mouse hybridoma, functional mapping	[61]
Epitope 3 Located at the base of the stem spanning regions of the fusion peptide and helix-capping loops	12D1	6/7 H3 except for A/Equine/KY/02 strain	Sequential DNA vaccination, mouse hybridoma, functional mapping	[62]
	1F2/1F4/1E1	H1, H3, H5, H7, H9	2009 pandemic H1N1vaccinees, single cell PCR, functional mapping	[63]
Epitope 4 Located near the C terminal of the HA1	5A7	B (Yamagata and Victoria)	TIV, hybridoma, functional mapping	[64]
Head				
RBS	S139/1	H1, H2, H3, H13, H16, H5, H6, H9, H13	H3 vaccinated mice, hybridoma, X-ray structure	[20,65]
	C05	H1, H2, H3, H9, H12	H1/H3 Infected donors, phage display, X-ray structure	[21]
	F045-092	H1, H2, H3, H5, H13	Healthy donor, phage display, X-ray structure	[19,66]
	2D1	Pandemic H1 and A/Swine/Iowa/15/30	PBMC from volunteers born in 1915 or earlier, EBV transformation/hybridoma, X-ray structure	[67,68]
	5J8	H1N1 strains from 1918 to 1977 and pandemic 2009, but not seasonal H1N1 strains from 1999 to 2007	Healthy donor, EBV transformation/hybridoma, X-ray structure	[25,69]

	CH65	30/36 of H1 strains tested	TIV, single cell PCR, X-ray structure	[23]
	CH67	19/23 of H1 strains tested	TIV, single cell PCR, X-ray structure	[24]
	2G1	7/8 H2 strains and 1/4 H3 strains tested	Healthy donors, EBV transformation/hybridoma, X-ray structure	[26,70]
	8F8/8M2	7/8 H2 strains tested	Healthy donors, EBV transformation/hybridoma, X-ray structure	[26,70]
	B-1	H3 vaccine strains during 1968–2005	TIV, hybridoma, functional mapping	[22]
	D-1	H3 vaccine strains during 1989–2005	TIV, hybridoma, functional mapping	[22]
	AVFluIgG03	11/16 H5 strains tested	H5 infected donor, phage library, X-ray structure	[32,71]
	1B2	H7 Euroasian and North America strains	H7 vaccinated mice, hybridoma, functional mapping	[27]
	3A2/10C4	B (Yamagata lineage)	TIV, hybridoma, functional mapping	[64]
	CR8033	B (Yamagata and Victoria)	TIV, phage display, x-ray structure	[18]
Outside RBS	65C6	19/20 H5 strains tested	H5 infected donor, EBV transformation, X-ray structure	[28,32]
	100F4	19/20 H5 strains tested	H5 infected donor, EBV transformation, X-ray structure	[29,32]
	H5M9	H5 clades 0, 1, 2.3.4, and 7	H5 vaccinated mice, hybridoma, X-ray structure	[30,72]
	F005-126	H3, H1	Healthy donors, phage display, X-ray structure	[31]
	1H5	H7 Euroasian strains	H7 vaccinated mice, hybridoma, functional mapping	[27]
	CR8059/CR8071	B (Yamagata and Victoria)	TIV, phage display, X-ray structure	[18]

*Black color: neutralization; blue color: binding.

**X-ray structure implies that both crystal structure of HA/Fab complex and functional mapping have been used to determine epitope composition and structure.

Table 2 Summary of studies on HA stem-based cross-subtype "universal" and RBS-based subtype-specific vaccine designs

	Immunogens/source	Platform	Antigenicity	Cross-reactivity	Cross-protection		Ref.
					Heterologous	Heterosubtypic	
Stem-based cross-subtype "universal" vaccine design							
Sequential infection or vaccination	1999 NC (H1) 2006 SI (H1) 2009 CA (H1)	Embryonated eggs	ND	cH6/1, cH5/1*	ND	Mouse H5N1, 80%	[42]
	cH6/1 (DNA) cH9/1 (protein) cH5/1 (protein) stem (1934 PR)	Baculovirus expression system (High Five cells)	Recognized by 6F12	H1, H2, H5	Mouse H1N1, 100%	Mouse H5N1, 100% H6N1, 100% H3N2, 10%	[44,46]
	cH4/3 (DNA) cH5/3 (protein) cH7/3 (protein) stem (2009 Perth)	Baculovirus expression system(High Five cells)	ND	H3, H7, H10, H14, H15	Mouse H3N2, 100%	Mouse H7 (90%) H10 (lung titer)	[45]
Headless HA	From H1 (1934 PR) and others	Subviral particles	ND	heterosubtypic HA	ND	ND	[47]
	From H2 (1957 Okuda)	Transfected cells	Recognized by C179	ND	ND	Mouse, H1N1, 70%	[48]
	From H1 (1934 PR) and (1968HK)	VLP	Not detected	H1, H2, H3, H5	Mouse, 100%	ND	[49]
	From H1 (1934 PR), (1999 NC), (2009 CA)	Bacterial expression system	Recognized by CR6261	H1, H5	Mouse, 100% (homologues) 90% (heterologous)	ND	[50]
	From H1 (2009 CA)	Synthesized protein	Recognized by CR6261, FI6 and C179	ND	ND	ND	[53]
	From H1 (1934 PR)	Bacterial expression system	Recognized by CR6261, FI6v3 and F10	H1, H3, H5	Mouse, 100%(PR8)	Mouse H3N2, 30%	[51]
	From H1 (1934 PR)	Baculovirus expression system(sf9)	No binding to stem-directed antibodies	H1, H2, H5	Mouse (PR8) 100%	Mouse H5N1 60% H6N1, 100%	[54]

	A stem trimer #4900 from H1 (2007 Brisbane)	Mammalian expression system	Recognized by CR9114 and CR6261	H1, H3, H5, H7, H9	Mouse, 100% NHP, reduced fever, but no reduced viral loads	Mouse, H5N1, 100%	[55]
	HA-SS-np from H1 (1999 NC)	Mammalian expression system	Recognized by C179, CR6261, CR9114, F10 and 70-5B03	H1, H2, H3, H5, H7, H9	ND	Mouse, H5N1: 100% Ferrets, H5N1: 67%	[56]
	Pre-fusion mini stem from H5 (2004 VN) and H1 (1934 PR)	Bacterial expression system	Recognized by CR6261, F10 and FI6v3	H1, H3, H5, H7	Mouse, 80%	Mouse (H1F): H5N1, 30%; H3N2, 30%. Mouse (H5F): H1N1, 60%; H3N2, 60%	[52]
RBS-based subtype-specific vaccine design							
HA-ferritin nanoparticles	H1 (1999 NC or others)	Mammalian expression system	Recognized by CR6261 and F10 as well as anti-head antibodies	H1 (6/7)	Ferrets, yes	ND	[58]
Heterologous prime-boost	H5 (2004 TH)	DNA and VLP	ND	H5 (17/17)	Mouse, 100%	ND	[59]

*Black color: neutralization; blue color: binding. ND: not done.