

# Recent advances in “universal” influenza virus antibodies: the rise of a hidden trimeric interface in hemagglutinin globular head

Yulu Wang, Dan Hu, Yanling Wu (✉), Tianlei Ying (✉)

MOE/NHC/CAMS Key Laboratory of Medical Molecular Virology, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai 200032, China

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**Abstract** Influenza causes seasonal outbreaks yearly and unpredictable pandemics with high morbidity and mortality rates. Despite significant efforts to address influenza, it remains a major threat to human public health. This issue is partially due to the lack of antiviral drugs with potent antiviral activity and broad reactivity against all influenza virus strains and the rapid emergence of drug-resistant variants. Moreover, designing a universal influenza vaccine that is sufficiently immunogenic to induce universal antibodies is difficult. Some novel epitopes hidden in the hemagglutinin (HA) trimeric interface have been discovered recently, and a number of antibodies targeting these epitopes have been found to be capable of neutralizing a broad range of influenza isolates. These findings may have important implications for the development of universal influenza vaccines and antiviral drugs. In this review, we focused on the antibodies targeting these newly discovered epitopes in the HA domain of the influenza virus to promote the development of universal anti-influenza antibodies or vaccines and extend the discovery to other viruses with similar conformational changes in envelope proteins.

**Keywords** influenza virus; neutralizing antibody; hemagglutinin; globular head region; trimeric interface

## Introduction

Influenza virus, a member of the *Orthomyxoviridae* family of viruses, is an enveloped virus with a negative-sense single-stranded RNA genome [1]. Influenza viruses can be classified according to the diversity of the surface proteins hemagglutinin (HA) and neuraminidase (NA) [2]. Currently, four types of influenza viruses have been identified: A, B, C, and D types [3]. Influenza A and B viruses are the most dominant among all types, and they circulate, disseminate, and cause seasonal influenza epidemics [4], which may lead to high mortality and morbidity rates in vulnerable populations ([https://www.who.int/news-room/fact-sheets/detail/influenza-\(seasonal\)](https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal))). The World Health Organization has identified 18 HA and 11 NA types in influenza A viruses. They can be further classified into two separate groups on the basis of HA type: group 1, which includes H (1, 2, 5, 6, 8, 9, 11, 12, 13, 16, 17, and 18); and group 2, which includes H (3, 4, 7, 10, 14, and 15). Each

group has many subtypes that can infect humans, pigs, sea mammals, and birds [5,6].

Influenza viruses have high mutation rates, which cause resistance to existing drugs; in addition, the development of “universal” protective antibodies and universal vaccine candidates to induce such antibodies is limited by the diversity and rapid evolution of influenza viruses [7]. Some avian influenza viruses, such as H5N1 [8], H7N9 [9,10], and H10N8 [11], can infect humans. In 1996, the H5N1 virus, which was isolated from a domestic goose, infected humans and caused 18 influenza cases and 6 deaths. Outbreaks of human infection were reported from 1998 to 2003; for example, H5N1 was reported in Hong Kong, China, H9N2 [12] was reported in Guangdong, China, and H7N7 [13] was reported in the Netherlands. In 2013, a new H7N9 avian influenza virus emerged in China and infected about 1500 individuals (<http://www.who.int/csr/don/01-may-2017-ah7n9-china/en/>). The spread of the virus from one species to another is a severe risk factor to human health. The diversity and rapid evolution of influenza viruses are also serious problems that limit the development of “universal” protective antibodies and universal vaccine candidates to induce antibodies [14]. Furthermore, some highly conserved epitopes in the HA

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Correspondence: Tianlei Ying, [tlying@fudan.edu.cn](mailto:tlying@fudan.edu.cn);  
Yanling Wu, [yanlingwu@fudan.edu.cn](mailto:yanlingwu@fudan.edu.cn)

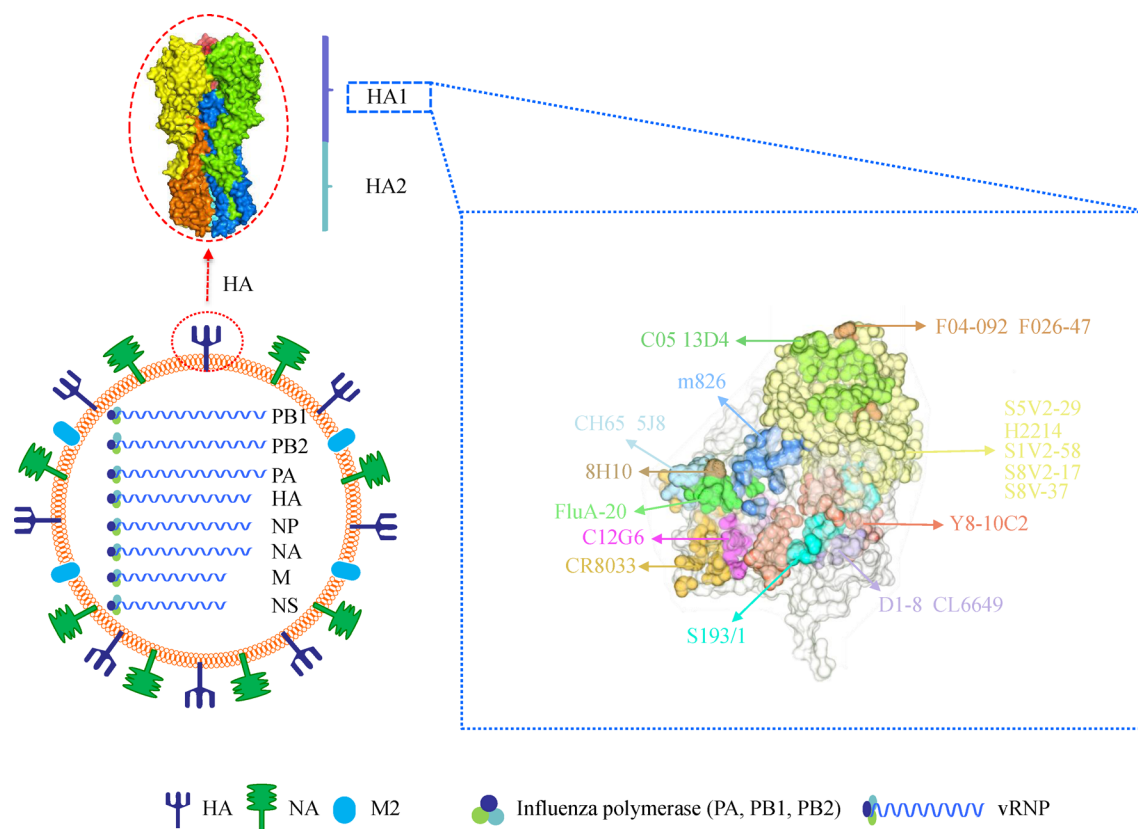
might have low immunogenicity, and they cannot easily induce antibody response. Thus, the key difficulty may be the development of new strategies to strengthen specific immunization and improve specific antibody selection. Yu *et al.* isolated mAb m826 as a novel epitope in the HA trimeric interface that exerted highly therapeutic effects on mice with a lethal challenge of H7N9 [15]. This interface was previously considered to be hidden but can be exposed according to the recently proposed HA “breathing” theory [16]. Consequently, panels of broadly neutralizing antibodies targeting these epitopes were identified [16–18]. In the present work, we review the recent progress on these novel “universal” antibodies and discuss the potential implications of existing studies on influenza drug and vaccine development.

## Targets of influenza virus antibodies

Influenza viruses primarily consist of eight gene segments, which encode ten identified proteins (Fig. 1). Among these

proteins, three surface proteins, namely, HA, NA, and M2 (Fig. 1), are the most dominant and could be “observed” by the humoral immune system. Therefore, almost all antibodies that are known to inhibit viral proliferation target these proteins.

M2 is the influenza virus surface protein that acts as a proton channel at low pH, following the uncoating of the viral ribonucleoprotein complex from the endosome to the cytoplasm [19,20]. However, antibodies against M2 are not neutralizing and generally do not confer protection by promoting effector functions on the basis of their Fc regions [21]. Furthermore, the antibody responses toward M2 are typically weak and/or transient after natural influenza infections in humans [22,23]. NA, another viral surface protein, acts as a receptor-destroying enzyme and removes sialic acid residues from the surface of infected cells to release and spread budding virions [24]. Although NA has lower variability than HA, it also undergoes a certain degree of antigenic drift, which may affect the intensity of NA antibody response to viruses or vaccines. The HA protein facilitates viral infection into host cells and



**Fig. 1** Overview of influenza virus structure and epitopes recognized by neutralizing antibodies in the HA1 domain. The genomes of the influenza virus comprise eight gene segments, including polymerase basic 1 (PB1), polymerase basic 2 (PB2), polymerase acidic (PA), nucleoprotein (NP), matrix (M), nonstructural (NS), HA, and NA. The dominant glycoprotein on the viral surface, HA, mediates receptor binding, and membrane fusion is divided into HA1 and HA2. HA1 is the main target of neutralizing antibodies and antibody binding sites in the HA1 domain (indicated by different colors).

influences the humoral immune response by shielding receptor binding sites (RBSs) and the process of antigenic drift. The antibodies targeting HA could directly neutralize viruses or stimulate the effector functions to eliminate infected host cells [25]. An analysis of blood samples collected from influenza virus-infected or immunized individuals shows that most broadly neutralizing antibodies target HA [26–30]. Therefore, the present work mainly focuses on the antibodies that target the highly conserved regions of HA.

## Influenza virus HA protein: structure and function

HA, a type 1 glycoprotein on the virus surface, forms a homotrimer that measures approximately 220 kDa and is responsible for the virus binding to the host cell receptors. The mature influenza HA monomer consists of two subunits [31], namely, HA1 and HA2 (Fig. 1); these subunits are connected by disulfide bonds and functions differently [32–34]. The HA1 region contains a highly antigenic domain, and the HA2 region is the conservative membrane fusion domain that aids viral and cell membrane fusion within the endosome.

The HA structure is metastable and changes dynamically through pH adjustment [35] because pH influences the neutral pH conformation of the protein found on the virus and induces viral and cell membrane fusion. During influenza infection, HA aids the influenza virus in binding to the host cell membrane via an HA/sialic acid interaction, which allows the internalization of viral particles via endocytosis. The HA protein is cleaved enzymatically into the N-terminal HA1 polypeptides with a distal domain of globular shape and C-terminal HA2 polypeptides with part of the HA1 polypeptide during endocytosis. Within the low pH (5–6) environment of the endosome, HA2 undergoes structural rearrangements, and the viral envelope fuses with host endosomal membranes, thereby allowing the virus to be internalized and the viral RNA to be released within the host cell [36].

HA is an appropriate target for inhibiting influenza viruses [37] because it is not only the most abundant protein antigen on the viral surface but also a critical factor that facilitates viral infection. The HA domain is diverse; however, almost all HA domains, except the newly identified H17 and H18 HAs from bats, play the same role of aiding the binding of viruses to host cells and inducing the fusion of viruses and host cell membranes [38–41]. Thus, blocking the RBS or inhibiting the process of HA maturation and membrane fusion are two suitable ways to prevent influenza viral infection. The two types of influenza viral antibodies target the globular head (HA1) or stem (HA2) regions of HA [29].

## Conventional anti-HA antibodies: what we have learned so far

Most neutralizing antibodies against influenza viruses are directed to the conformational epitopes on HA, particularly the globular head domain. A vast number of influenza-neutralizing antibodies against the HA globular domain are strain-specific and can efficiently prevent infection by blocking the HA-mediated attachment to cells. Examples of such antibodies include the H1-specific antibody 5J8, the H2-specific antibody 8M2, the H5-specific antibody 13D4, and the H7-specific antibody H7.5 [18,42–44], whose binding sites are close to or overlap with RBSs. In 2018, an H1-specific antibody CL6649 identified another conserved site on the HA head side of the H1 subtype; this site is called a “lateral patch” [45]. However, strain-specific antibodies bind to epitopes with high mutation rates, thereby allowing antibody binding sites to change and escape from antibody-mediated neutralization.

Identifying the conserved sites in the variable head domain is difficult [32,46], and only a few anti-HA head domain antibodies have been described as broadly neutralizing antibodies. In 2009, mAb S139/1 was isolated from immunized mice with H3 viruses, and it exhibited neutralization activity against the H1, H2, and H3 strains. Its epitope is located in the antigen site B near the RBS [47]. A human monoclonal antibody called CH65 was obtained by isolating it from a subject immunized with the 2007 trivalent influenza vaccine, and it neutralized 30 out of 36 H1N1 strains. An analysis of the CH65–H1N1 complex showed that CH65 binds to the receptor binding pocket on HA1 through HCDR3, mimicking the physiologic interaction between sialic acid and HA [48]. Using phage antibody libraries from a human donor, Ekiert *et al.* isolated mAb (C05), which binds directly to the RBS on HA and neutralizes strains from multiple subtypes of the influenza A virus, including H1, H2, and H3 [49]. In addition, some broadly neutralizing antibodies whose epitopes are far from the RBS have been described. Two anti-head mAbs, F045-092 and F026-427, were identified from human B lymphocytes by screening with the H3N2 virus; these antibodies showed activity against H1N1, H3N2, and H5N1 viruses [50]. D1-8 is a human mAb targeting the antigenic site D and exhibits potent, broadly neutralizing activity across antigenically diverse influenza H3 subtype viruses [26]. Moreover, 429B01 is particularly broad and binds to groups 1 and 2 viruses [51]. Antibodies CR8033 and CR8071 recognize a distinct conserved epitope in the HA head domain of the influenza B virus, and they have broad protection against influenza A and B viruses [52]. C12G6 targets a conserved epitope that overlaps with the RBS in the HA region of the influenza B virus and therefore protects against influenza B infection [53]. These previous studies indicated that most anti-HA

head regions neutralizing antibodies only offer a narrow line of protection and do not neutralize all influenza subtypes due to the high level of sequence diversity in the HA heads between subtypes, as well as the incorporation of large amounts of glycans in this region.

Broadly neutralizing antibodies usually target the stem region of HA due to the low variability of the stem domain. Such stem-targeted bnAbs recognize the highly conserved regions of the stem and inhibit the conformational changes necessary for viral fusion. Therefore, these bnAbs, e.g., CR9114 and MEDI8852, have more extensive heterotype-neutralizing activity compared with most head-targeted antibodies; some can even target almost all HA types and groups [54–57]. Recently, panels of neutralizing stem-directed human-derived bnAbs have been reported. A human monoclonal antibody called CR9114 recognizes a conserved epitope in the HA stem and protects against lethal challenges with the influenza A and B viruses [52]. Neutralizing antibodies CR6261, F10, or 70-1F02 against group 1 influenza types target highly conserved pocket regions in the stem of HA using only heavy chains due to a phenylalanine in position 54 at the HCDR2 region unique to the VH<sub>1-69</sub> gene [55,58]. Prior studies have shown that these antibodies can be elicited by vaccination [59–62]. However, VH<sub>1-69</sub>-derived antibodies generally do not neutralize groups 1 and 2 strains of influenza A, and only CR9114 can neutralize both groups [63]. By contrast, group 2-specific antibodies, such as CR8020, bind to epitopes closer to virus membranes [64]. As the most extensive heterotype-neutralizing antibodies that recognize both groups of HAs, mAb FI6 and FI6V3 can bind to conserved epitopes in the F subdomain [65]. Moreover, bnAbs specific for the HA stem region usually require FcγR-mediated effector functions, which act via the interactions of the Fc region of the antibodies with FcγR receptors, to protect against influenza virus *in vivo* and kill virus-infected target cells by attracting immune cells, such as NK cells, macrophages, and neutrophils [66]. However, the humoral response of the HA stem region is generally weaker than that of the more immunogenic HA trimer globular head region [17], leading to the vast generation of anti-HA globular head domain antibodies during infection with an influenza virus. Therefore, developing a universal vaccine that promotes the generation of anti-stem antibodies or using anti-stem antibodies via passive immunization in infected individuals is difficult.

Designing a universal influenza vaccine that is sufficiently immunogenic to induce universal antibodies capable of neutralizing a broad range of influenza isolates is difficult partly because of the lack of highly conserved epitopes in the HA globular head region [16,23]. With the advances in antibody discovery-related technologies, a growing number of antibodies are being identified, and some of them target critical functional regions that are not highly sensitive to antigenic drifts in the HA domain

[67,68]. Particular attention has been paid to some novel epitopes hidden in the influenza trimeric HA head interface [69,70]. These studies have rekindled the interest in the development of “next-generation” universal influenza virus antibodies and corresponding vaccine immunogens.

## Recent advances in anti-HA antibodies

In the past decade, extensive efforts have been made to identify the extremely conserved epitopes and corresponding universal influenza antibodies [17,68,71,72]. Several strategies focused on the rational design of HA antigens that could expose conserved epitopes to generate bnAbs [73–76]. The glycosylation in the HA domain is involved in the antigenic drift of influenza viruses; influenza viruses have been found to introduce or remove glycans to change the viral structure and shield the sites near the RBS on its surface to reduce or evade host immune response [77–79]. A number of studies engineered glycans to expose the hidden conserved epitopes in the HA domain. For instance, one article reported that modifying glycosylation sites in the HA stem region leads to unmasked sites by N-glycans that elicit effective broadly neutralizing antibodies [80]. Such studies confirmed the potential role of engineering in changing the immunogenicity of antigens and exposing conserved sites, which are the key to induce broadly neutralizing antibodies [76,81,82].

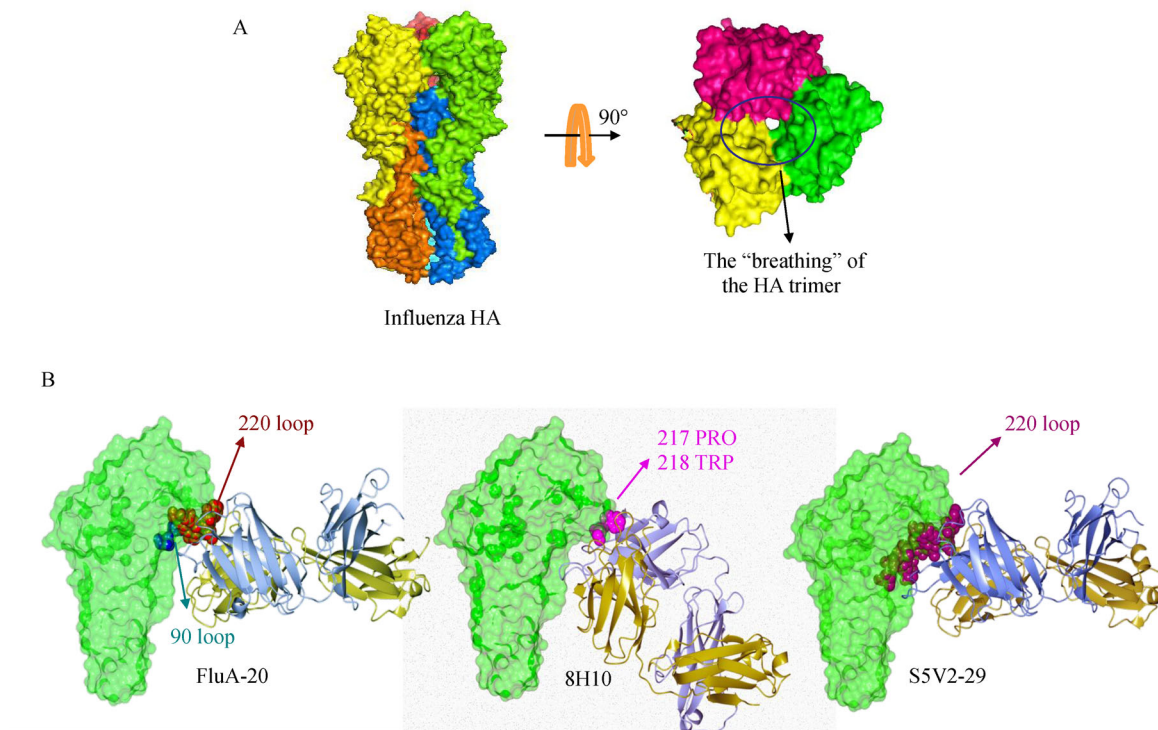
In a recent research article, the authors engineered a number of hyperglycosylated versions of HA and used them to immunize animals [76]. In their work, the glycans changed the initially diverse antibody response into a focused one. Three monoclonal antibodies were identified and designated as 8H10, FL-1056, and FL-1066. The structural studies showed that the binding sites of the three antibodies belonged to the HA head domain in the same epitope that is ordinarily occluded on a prefusion structure and hidden in the trimeric HA head interface. With the introduction of non-native putative N-linked glycosylation sites, such occluded and highly conserved epitopes were exposed in the glycosylated HA molecules; such condition allowed for the broad (but not complete) antibody mAb 8H10 to recognize all of the identified H3 types and a representative H4 type [16,76]. The article proposed that hyperglycosylated HA could block some epitopes from exposure to occluded epitopes and elicit broadly protective responses.

A class of anti-influenza virus antibodies targeting the interface of HA trimer has been identified. In 1988, a monoclonal antibody Y8-10C2 was described. Y8-10C2 recognized an epitope located at the interface of adjacent subunits and exposed to antibody binding only after acid treatment of HA [83–85]. In 2016, a panel of H1 + H3 cross-reactive antibodies, discovered by Georgiou’s group

and identified in multiple donors who received the trivalent seasonal influenza vaccine, all bound to a highly conserved epitope on monomeric HA located on the RBS but occluded in the intact HA trimer. Moreover, these antibodies conferred prophylactic and therapeutic protection against challenge by group 1 and group 2 strains despite their complete lack of neutralization activity *in vitro* [86]. However, the precise mechanism for protection remains under investigation. Recently, some novel epitopes that are targeted by therapeutic antibodies but are difficult to be accessed in intact trimeric HA have been identified. The H7N9-specific antibody, m826, is isolated from a large phage-displayed library and binds to HA1 with subnanomolar affinity. Interestingly, m826 exhibits pH-sensitive binding to trimeric HA. It binds to trimeric HA with subnanomolar affinity at pH 5.0; binding at pH 7.4 is about 10-fold weaker. The crystal structure of Fab m826 in the complex with the H7N9 HA1 fragment shows that m826 binds to a unique epitope on H7 HA that is buried in the trimeric structure. The mechanism of action could be deciphered by the “breathing” of the HA protomers [15]. Turner *et al.* also found this mechanism by observing the process of HA binding to a potent

influenza H7 HA head-directed mAb through cryo-electron microscopy. The epitope of the antibody is not solvent and accessible in the compact prefusion conformation but is transiently exposed during “breathing” [18]. In 2019, a study discovered a naturally occurring human monoclonal antibody, FluA-20, which can protect against almost all influenza types [87]. Structural studies revealed the novel epitopes of FluA-20 that are extremely well conserved on the non-RBS side across diverse types. The novel epitopes are positioned in the 220 loop and the adjacent 90 loop, which is usually buried in the native HA trimer (Fig. 2).

These findings suggest that the HA trimer is dynamic [88–90] and that the interface can be opened, perhaps transiently or partially, to expose conserved sites. The “breathing” of the interface of the HA head domain has also been reported [16–18,87]. HA0 is the HA precursor protein. HA is assembled into a trimer by protein folding in the endoplasmic reticulum and is then transported to the cell surface for maturation [91,92]. HA cleavage is required for the infectivity of influenza viruses [93,94]. A previous study demonstrated that HA cleavage alters the HA trimer dynamics, influences the presentation of the



**Fig. 2** HA interface is the critical binding site for neutralizing antibodies. (A) Trimeric HA structure seen from the side and top in space filling. The HA trimer is dynamic, and the partially open structure is observed after a change in conformation, which resembles “breathing.” (B) The epitopes are presented by the representative structure of targeting HA-interfaced antibody-HA complexes (FluA-20, 8H10, and S5V2-29). The “breathing” process of the HA trimer exposes a concealed epitope, which can be recognized by protective and well-conserved antibodies. The 220 loop is targeted by antibodies FluA-20 and H3-S5V2-29 partially for 8H10.

FluA-20 epitope, and reduces antibody function. Exposure of HA residues allowed these residues to be recognized by FluA-20, thereby confirming the presence of previously unnoticed dynamic features of the HA trimer [87].

Another study reported the isolation of S5V2-29, H2214, S1V2-58, S8V2-17, and S8V2-37 from the human donors and identified epitope S5-C1 that is extremely conserved on the non-RBS hidden HA globular head region and covers residues between 91 and 206 and between 219 and 230 [17]. All these five human antibodies own the collective breadth covering group 1 and almost all of group 2 HA (H1, H2, H3, H5, H7, H9, and H14). S5-C1-lineage B cell receptors (BCRs) have a similar gene composition. The complex structure of the antibodies bound to monomeric HA has been described [17]. At neutral pH, the epitope on the HA trimer interface recognized by antibodies is normally hidden in the shield to prevent access by the antibody or BCR [88]. However, the HA trimer could undergo conformational changes dynamically with pH adjustment. The HA trimer is more dynamic in the floating pH environment, and if the transiently exposed epitopes encounter B cells, the BCRs can recognize the virus, thus allowing for the induction of the primary immune response against the influenza virus [17,95]. Although BCRs or antibodies can bind to virus particles, the transient nature of the exposure site results in a binding rate lower than that for fully exposed epitopes. Failure to prevent the initial infection of cells *in vitro* indicates that the HA trimer’s conformational fluctuations expose the epitope at the interface to a certain extent [17,91,96]. Thus, only a small fraction of the head interface epitopes could be exposed [97–99]. These studies also showed that although most BCRs are specific for HA epitopes, only a minority of these broadly cross-reactive memory B cells recognize RBS epitopes. Instead, numerous cross-reactive memory B cells recognize non-RBS epitopes. Therefore, systematic investigations into HA head epitopes that have not been identified to date are important. Such epitopes may prove to be immunogenic components of a broadly protective influenza virus drugs and vaccines.

In summary, three independent groups have reported similar results in the identification of important epitopes on the non-RBS HA globular head region, as well as the related universal antibodies that are protective against many influenza virus types [17,76,87]. All antibodies bind to a hidden interface in the HA trimer and have no neutralizing activities, but they protect mice against challenge with influenza viruses via strongly Fc-dependent mechanisms or disruption of the HA trimer. The characteristics of some of these antibodies are briefly summarized in Table 1.

The new protective epitopes recognized by the antibodies are transiently or partially exposed under certain conditions; this property is reminiscent of the pH-mediated

reversible “breathing” of HA that has been supported by recent HA kinetic studies [16,88]. The viral surface envelope protein is dynamic and always changing, thereby resembling “breathing” and showing that exposed cryptic epitopes can be targeted by protective antibodies. This “breathing” phenomenon has also been observed in envelope glycoproteins from other viruses, e.g., MERS-CoV, dengue virus, Zika virus, and HIV [100–103]. Moreover, a number of antibodies targeting possibly hidden conserved epitopes in these viruses have been identified in humans. Hence, these epitopes are promising candidates for developing “universal” vaccines.

## Summary and perspectives

The discovery of broadly neutralizing antibodies targeting highly conserved epitopes has renewed the hope of developing universal influenza drugs and vaccines. Recent data have shown that antibody and memory B cell responses against the variable epitopes of the HA head are much higher than the responses of antibodies and memory B cells directed against the conserved HA stem cell region [17,70,104]. However, the most widely reported anti-HA head region antibodies do not have broadly neutralizing activity and only offer a narrow line of protection due to the high level of sequence diversity and incorporation of large amounts of glycans in this region.

The recent studies reviewed herein provided comprehensive analyses and showed that novel epitopes hidden in the influenza trimeric HA head interface, which are conserved across most influenza groups, may not be affected by the immune pressure driving the antigenic drift of viruses. Therefore, such “breathing” interface might represent an ideal immunogenic candidate and should be considered as a potential site to be used in the development of drugs and vaccines against influenza virus infections.

Unlike anti-stem antibodies, most anti-HA head antibodies do not rely on ADCC activity, and they exhibit exceptional breadth and potency even in the Fab (antibody without the Fc domain) format [105]. This finding suggests the potential to develop small antibody constructs, such as single domain antibodies [106], which could more easily access the “breathing” subdomain or other hidden epitopes than large-sized IgG antibodies. With the establishment and further development of the HA “breathing” theory, an increasing number of antibodies targeting the hidden trimeric interface are expected to be discovered in the near future.

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**Table 1** Monoclonal antibodies specific for HA stalk and trimeric interface

	Antibody	Year	Epitope	Activity		Phase
				<i>In vitro</i>	<i>In vivo</i>	
Anti-stalk	CR6261 [55]	2008	A highly conserved helical region in the membrane-proximal stem of HA1 and HA2	Neutralization group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16)	In mice: H2N2-1.7mg/kg-100% survival rate; H2N3-1.7mg/kg-100% survival rate In ferret: H5N1-30mg/kg-100% survival rate	Phase 2
	CR8020 [64]	2011	A highly conserved epitope in the HA stalk close to the virus membrane	Neutralization group 2 (H3, H4, H7, H10, H14, H15)	Mice challenges H3N3-3mg/kg-100% survival rate; H7N7-3mg/kg-100% survival rate	Phase 2
	MEDI8852 [57]	2016	A highly conserved epitope encompassing a hydrophobic groove in the fusion domain and a large portion of the fusion peptide	Neutralization group 1 (H1, H2, H5, H6, H9) and group 2 (H3, H7)	Mice and ferret challenge studies In mice: H5N1-10 mg/kg-100% survival rate; H7N9-10 mg/kg-100% survival rate In ferret: H5N1-12.5 mg/kg-86% survival rate	Phase 1b/2a
Anti-trimeric interface	FluA-20 [87]	2019	An exceedingly conserved protective determinant in the influenza HA head domain trimer interface	No neutralization but mediates ADCC against group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H16) and group 2 (H3, H4, H7, H10, H14, H15)	Mice challenge studies: H1N1-10 mg/kg-100% survival rate; H3N2-10 mg/kg-80% survival rate; H5N1-10 mg/kg-100% survival rate	Preclinical
	S5V2-29 [17]	2019	The epitope, occluded in the pre-fusion form of HA, is at the contact surface between HA head domains	No neutralization but the Fc-dependent protection against group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16) and group 2 (H3, H4, H7, H10, H14, H15)	Mice challenge studies: H3N2-100 µg-100% survival rate; H1N1-100 µg-90% survival rate	Preclinical

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## Compliance with ethics guidelines

Yulu Wang, Dan Hu, Yanling Wu, and Tianlei Ying declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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