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Heads, stalks and everything else: how can antibodies eradicate influenza as a human disease?

Karlynn E. Neu[1], **Carole J. Henry Dunand**[2], and **Patrick C. Wilson**[1,2]

[1]Committee on Immunology, The University of Chicago, Chicago, IL 60637, USA

[2]The Department of Medicine, Section of Rheumatology, The Knapp Center for Lupus and Immunology Research, The University of Chicago, Chicago, Illinois 60637, USA

Abstract

Current seasonal influenza virus vaccines are effective against infection but they have to be reformulated on a regular basis to counter antigenic variations. The majority of the antibodies induced in response to seasonal vaccination are strain-specific. However, antibodies targeting conserved epitopes on the hemagglutinin protein have been identified and they offer broad protection. Most of these antibodies bind the hemagglutinin stalk domain and are generated from preexisting memory B cells. Broadly protective stalk-biased responses induced by antigenically divergent influenza strains, in concert with prior immunity, are sufficient to eradicate seasonally circulating strains. Future vaccine trials should aim to harness and maintain such a response with the realistic goal of developing a universal influenza vaccine.

Introduction

Influenza virus epidemics contribute to 250,000 to 500,000 deaths per year worldwide [1]. Current seasonal influenza virus vaccines are effective against infection with some limitations, such as the need to be reformulated most years to counter antigenic variations, also called antigenic drift [2]. Due to the timely production of the vaccine, the strains composing the seasonal vaccine have to be determined based on prediction and surveillance; mismatches between vaccine and circulating strains occasionally occur [3]. Furthermore such vaccines do not protect against novel pandemic strains, which are occasionally introduced into the human population, typically due to antigenic shift [4]. Seasonal vaccination generally induces a narrow, strain-specific response against the highly variable head domain of hemagglutinin (HA) and thus antibodies targeting the globular head quickly lose efficacy against drifted strains [5,6]. The stalk domain, in contrast, is more conserved among influenza A (group 1 and 2) and B viruses allowing antibodies that target this region to neutralize a wide spectrum of influenza virus subtypes [7–9]. Such antibodies are relatively rare in the human population but novel approaches to enhance these antibodies are

Corresponding authors: caroledunand@uchicago.edu and wilsonp@uchicago.edu.

currently being developed [10,11]. Importantly, it is believed that targeting such conserved epitopes is the key to the elimination of seasonal influenza strains. Broadly neutralizing stalk-reactive antibodies are emerging therapeutic tools against influenza virus infections and are a promising prospect for the development of a universal influenza virus vaccine. A key issue in the field is whether or not an antibody response to HA stalk epitopes could sufficiently protect and sustain for permanent immunity to all, or most, circulating influenza strains. We argue herein that indeed a properly designed stalk-based vaccine could provide broad immunity.

Antibody responses to influenza virus

The influenza virus has two main surface glycoproteins: HA and neuraminidase (NA) [12]. HA is a trimeric protein with an immunodominant head domain that is preferentially mutated during immune evasion [4,13,14]. There is a receptor-binding site within the head domain that binds to sialic acid moieties on the surface of host cells to facilitate viral infection [15]. Antibodies blocking this binding site are characterized by their ability to prevent influenza virus mediated agglutination; *in vitro* these antibodies can be identified using a hemagglutination-inhibition assay (HAI) [12]. The HA stalk domain is composed of three helical bundles and is functionally required for the pH induced conformational changes involved in membrane fusion during viral entry and exit from the host cell [8,14,16,17]. Antibodies specific for this region can be identified by their ability to block viral cell infection independently of HAI activity, using *in vitro* microneutralization or plaque assay. NA, on the other hand, is required for cleaving the HA-sialic acid tethering to release new virions, allowing for viral spread [18,19]. Potentially protective NA-reactive antibodies are identified by their ability to block NA cleavage [20,21].

Influenza A viruses are subtyped based on the sequence and antigenic divergence of the HA and NA surface proteins. A total of 18 HA and 11 NA subtypes have been identified so far, with the type of HA expressed splitting influenza A viruses into two phylogenetic groups (Group 1: H1, H2, H5, H6, H8, H9, H11, H13, H16, H17, H18; and Group 2: H3, H4, H7, H10, H14, H15) [22–25]. Influenza B viruses are divided into two antigenically different lineages (Victoria and Yamagata) [26]. The majority of protective antibodies generated in response to influenza target the HA protein [27]. Less is known about how the antibody response to NA alters the course of an influenza infection, although NA-inhibitors such as Oseltamivir (Tamiflu), Zanamivir (Relenza), Laninamivir (Inavir), and Peramivir (Rapivab) have some efficacy in reducing severity if used early during the course of infection [28,29]. This review focuses on the antibody response to HA.

Conserved protective epitopes on HA

Despite the fact that the majority of the protective antibodies targeting HA recognize the head domain and display a high level of strain specificity [6], a number of head specific antibodies have been identified with varying levels of cross-reactivity between influenza strains [30–42]. All of these antibodies identified thus far, target one of two cross-protective head epitopes (Figure 1). Antibodies that target epitope A must overcome the extreme variability of the HA head, by forming key interactions within the highly conserved

receptor-binding site [30–39,42]. An extensive study of antibodies binding to this epitope revealed that they are fairly common in a vaccine response, have diverse V(D)J gene usage and utilize sialic acid mimicry on their HCDR3 loops to directly engage the receptor binding site [42]. These interactions are sufficient to overcome the extreme strain-to-strain variation present in the surrounding contact residues [15,42]. Epitope B is protective in both B strain lineages and includes the vestigial esterase domain at the base of the HA head [31].

In contrast to the HA-head variability, the stalk domain displays a much higher level of conservation across influenza strains with some central residues being identical across all subtypes [7–9]. Three protective epitopes, with varying levels of cross-reactivity between group 1 and 2 influenza strains, have been identified within the stalk portion of influenza A HA (Figure 1) [8,9,31,43–47]. Epitope 1 is centered on the A α-helix of the HA2 region of HA [8,9,31,44,48]. Targeting this epitope is also protective against B strains, but the antibody must have unique properties to accommodate key modifications helping to obscure the epitope surface [31]. Epitopes 2 and 3 are protective across group 2 influenza A subtypes. Epitope 2 includes the upper portion of the long alpha helix CD in HA2 [49], whereas epitope 3 is located at the base of the HA2 stalk spanning regions of the fusion peptide and helix-capping loops [43]. The fourth protective stalk epitope is located in the C terminal portion of HA1 and offers broad protection across both B strain lineages [50]. Generating a strong antibody response against any of these conserved epitopes can offer broader and more durable protection against influenza by circumventing reliance on epitopes prone to antigenic drift.

The antibodies specific for the conserved epitopes within the head and stalk are protective due to their neutralizing capacity. Recently we, and others, discovered a novel group of non-neutralizing protective antibodies. We identified these antibodies following H7N9 vaccination in humans. They are broadly cross-reactive, harbor high level of somatic mutations and target new epitopes on the HA protein conserved between group 1 and 2 [51]. Such antibodies have also been isolated after H7 immunization in mice [52]. Both studies showed that protection by these non-neutralization antibodies is partially dependent on Fc-FcγR interactions. The discovery of such antibodies is reinforced by serum studies showing that cross-reactive antibodies were found in influenza seropositive humans in the absence of neutralization [53] and in the absence of HAI activity [54]. Moreover passive transfer of both neutralizing and non-neutralizing antibodies from vaccinated individual sera improved virus clearance in a mouse model [55]. Targeting antibodies against any of these conserved protective epitopes would offer significantly improved protection against circulating influenza strains. Most hope is currently being placed on antibodies specific for the stalk domain because it is so widely conserved and can be independently targeted as an immunogen.

Occurrence of broadly neutralizing stalk-reactive antibodies

Broadly neutralizing stalk-reactive responses have been characterized following natural infection with seasonal H1N1 and H3N2 strains, but are more rare after seasonal influenza vaccination [56,57]. However, plasmablasts with HA stalk specificity have been reported after vaccination with the seasonal trivalent vaccine [58] and more recently, we found that

group 2 cross-reactive stalk antibodies induced by seasonal vaccination were not uncommon [45]. Studies are now revealing that broadly neutralizing stalk-reactive antibodies are boosted more efficiently in humans upon exposure to antigenically divergent head HA domains, which was the case with the 2009 pandemic H1N1 strain [36,59–63]. Interestingly, this pandemic H1N1 virus led to the disappearance of all pre-pandemic H1N1 strains in the human population. In nature, the replacement of existing seasonal viruses by novel pandemic strains in the human population is a recurrent phenomenon. Over the past 60 years, multiple instances of seasonal circulating viruses being eradicated by the emergence of pandemic influenza viruses have occurred. In 1957, the H2N2 pandemic virus replaced the seasonal H1N1 strains that were previously circulating in humans [64]. In 1968, a boost in antibodies against conserved epitopes on neuraminidase after the introduction of the H3N2 strain might have caused the extinction of human H2N2 strains [65]. Both the 1957 and 2009 pandemic H1N1 virus expressed divergent head domains compared to the previous circulating strains but had conserved stalk domains. These observations lead to the hypothesis that antibodies against conserved epitopes, regulated by the immune status of the general population, could be responsible for the extinction of circulating seasonal influenza viruses after the emergence of novel pandemic strains [66]. In support of this, an elegant study using chimeric hemagglutinin proteins revealed that anti-stalk antibodies generated after the pandemic H1N1 infection played a substantial role in the disappearance of the existing seasonal H1 viruses [67].

Zoonotic reservoirs are the source of pandemic influenza strains [68]. Our documented history of influenza infections only spans 100 years and it is likely that many more subtypes than are currently appreciated have existed throughout human history. We discussed above how broad protection induced from exposure to novel strains in conjunction with existing partial immunity to antigenically drifted epitopes could cause circulating strains to be eliminated. The current evidence suggests that targeted vaccines that induce broad immunity on a wide scale could eliminate all currently circulating influenza strains. However, the progeny of past strains, or genetic reassortants thereof, are always available within these zoonotic reservoirs to re-enter human populations as reoccurring seasonal strains (Figure 2). People who have been exposed to similar antigens in the past will meet these strains with broad protective immunity, but the majority of the population will be vulnerable due to loss of antigen breadth and herd immunity over time. This cycle will only be broken by removal of zoonotic reservoirs, which is likely not possible, or by widespread and durable immunization against conserved epitopes. This could come in the form of a broadly protective vaccination, which eliminates currently circulating strains, and is reformulated upon cyclic re-entry from zoonotic reservoirs. These issues will need to be addressed in the design of a universal influenza vaccine.

Memory origin of stalk-reactive antibodies

Humans have an extensive immune history and upon antigen re-exposure, antigen-specific memory B cells are recalled in the immune response. The antibody producing plasmablast population bursts after vaccination, or infection, and is mostly comprised of antigen-specific cells [6]. It is now well appreciated that the adult influenza vaccine response is driven by activation of preexisting memory B cells, which can be identified by extensively mutated

variable region genes [6,36,48]. By analyzing the B cell response to vaccination in adult subjects who received the trivalent influenza vaccine over consecutive years, we showed that memory B cells are the predominant precursors to the plasmablast influenza response [69]. Based on their substantial mutation load and their binding affinity, stalk-reactive antibodies appear to be pre-existent within the memory compartment [36,48,69]. Protective H7N9 stalk-reactive antibodies have even been identified in people who have never been exposed to this virus, due to presence of group 2 cross-reactive memory B cells [45]. Additionally, an H7 vaccine study showed the generation of a vigorous, high-affinity, stalk-specific antibody response with a consistent increase in circulating memory B-cell frequencies [70]. Finally, geriatric populations with extensive memory B cell compartments but limited naïve B cells, showed a stalk-biased serum antibody response following seasonal vaccination [71]. These studies suggest that the key to inducing broad protection against influenza is to activate cross-reactive memory B cells.

How to boost stalk-reactive antibody responses

It is known that immunological memory acquired against influenza strains alters the response to subsequent viral encounters [72], but how sequential exposure to antigenically distinct influenza strains shapes the humoral immune response remains poorly understood. Two recent longitudinal studies provide important new insights. The first study is an analysis of antibody titers against various pandemic and seasonal influenza strains spanning a 20-year period, before the pandemic 2009 H1N1. HAI neutralizing titers specific to pandemic viruses in human circulation between 1957 and 2008 (H2N2, H3N2, and H1N1) exhibited sustained increases over the course of study. Interestingly group 1 and 2 stalk-reactive antibodies also rose modestly over the same period of time, even in the absence of major antigenic shift. However, group 1 HA stalk-reactive antibody titers were greatest in individuals who were exposed to the most diverse group 1 viruses [73]. An antigenically more stable virus, human cytomegalovirus, did not induced sustained increase in neutralizing antibody titers, suggesting that antigenic variation of influenza A viruses play a role in shaping the humoral response. The second study analyzed the B cell response to the pandemic 2009 H1N1 strain at the monoclonal antibody level upon first or second exposure [69]. Only individuals with low preexisting serological levels of pandemic H1N1-specific antibodies generated a broadly neutralizing plasmablast response directed toward the HA stalk. This observation confirmed that in the context of exposure to divergent influenza strains, immune history directly determines the likelihood of generating a broadly protective response (Figure 3A). Moreover maintaining a sustainable broadly neutralizing stalk-biased response upon subsequent exposures is a challenge, as re-exposure to the pandemic 2009 H1N1 strain by vaccination induced an HA head–biased response [69].

The effects of immune history on induction of these broadly neutralizing antibodies result from characteristics of the virus and the antibodies themselves (Figure 3B). Firstly, there is an immuno-dominance towards epitopes located on the globular head. One reason proposed for sub-dominance of the stalk HA epitopes is a limited access due to steric shielding by the HA globular head and/or because of their proximity to the viral envelope [74,75]. However, structural studies have demonstrated that the HA stalk epitopes are accessible for antibody binding [76]. Although the epitopes are accessible, we found that stalk-reactive antibodies

have reduced affinity to whole virus but a similar affinity to soluble HA protein compared to head-reactive antibodies. [69]. The structure of HA on whole virions must be the limiting factor to antibody binding. In addition to these steric restrictions there are also molecular/biochemical constrains imposed by the epitopes themselves, with most antibodies specific for the conserved HA stalk epitope 1 preferring the VH1-69 and VH1-18 genes [8,9,43,69,77]. This is attributed to three conserved hydrophobic residues located within the HCDR2 & HCDR3 loops of this V gene, which are required for the heavy chain mediated interaction [8,9,43,78]. Less common antibodies specific for this epitope have been identified without gene restriction and they utilize the more canonical antigen binding mediated by both the heavy and the light chain [44]. Broad immunoglobulin variable gene usage has also been identified for the other stalk epitopes [43,79]. These observations suggest that the limited accessibility of the HA stalk epitopes imposes molecular constraints on antibodies, leading to restricted VH usage of neutralizing HA stalk–reactive antibodies.

Anti-HIV antibodies that bind the gp140 glycoprotein have been shown to be polyreactive. The antibodies have one high-affinity binding site on gp140 and one low-affinity binding site on another molecule at the surface of HIV virus. This mechanism, referred to as heteroligation, demonstrably increases the apparent affinity of polyreactive antibodies to HIV and improves viral neutralization [80,81]. Interestingly, broadly neutralizing stalk-reactive antibodies have also been reported to have higher levels of polyreactivity [69]. We found that polyreactivity is a specific characteristic of antibodies capable of binding broadly protective epitopes on the HA stalk, independently of the VH usage. Therefore, immune checkpoints that curb possible self-reactivity, including polyreactivity [82] may also contribute to the scarcity of these broadly protective cells, further contributing to HA head immuno-dominance (Figure 3B).

New approaches towards a universal influenza virus vaccine

Two main avenues are currently being explored to modify the seasonal influenza vaccine to induce a more protective stalk-biased response. The first approach utilizes immunizations with recombinant HA proteins; either a stabilized headless version or a chimeric HA with a conserved stalk region combined with a diverse HA head [74,83–85]. The second focuses on modifying the current vaccine to include an adjuvant or to incorporate a live attenuated influenza vaccine boost prior to the inactivated vaccine [70,86,87]. These approaches have been successful at biasing the antibody response against the conserved stalk domains in animal models, and coincide with the literature on recalling stalk antibodies from within the pre-existing memory compartment. The chimeric-HA immunization strategy is in preparation for clinical trials. It will be quite interesting to see the results of this trial vaccine when placed in the context of a diverse immune memory/history [88,89].

Summary and outlook

We have learned from nature that the eradication of particular influenza viruses is possible. Further, the discovery of stalk-reactive antibodies has been a catalyst for the goal of a universal influenza virus vaccine. Understanding the impact of immune memory to conserved influenza virus epitopes in humans is critical for the induction of a broadly stalk-

reactive antibody response and its sustainability over time. Future clinical trials with vaccine candidates targeting such a response in humans will demonstrate how realistic a universal influenza vaccine is.

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Highlights

1. Conserved epitopes on the influenza HA protein offer broad protection
2. Antibodies specific for these conserved epitopes are induced by exposure to antigenically distinct strains
3. Immune history directly determines the likelihood of generating a broadly protective response
4. Current vaccine trials are underway to harness this response with the realistic goal of eradicating seasonal influenza strains

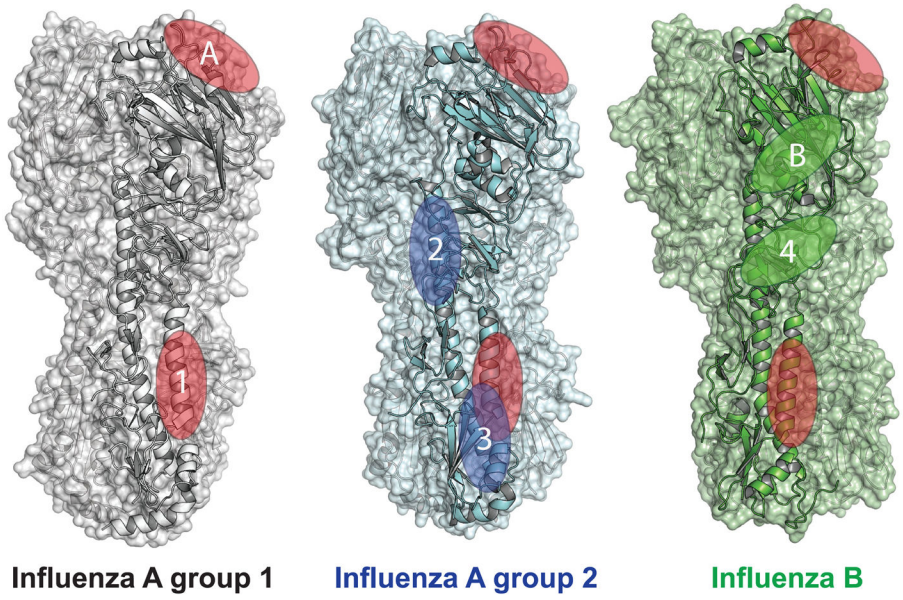

Figure 1. Conserved protective hemagglutinin (HA) epitopes

Epitopes shared between all three influenza groups are indicated by red ovals, influenza A group 2 are blue and influenza B epitopes are green. Two conserved epitopes have been identified within the HA head domain. Epitope A includes the receptor-binding site, which is conserved across all influenza subtypes. Epitope B contains the vestigial esterase domain and is conserved across both B strain lineages. Four conserved epitopes have been identified within the HA stalk domain. Epitope 1, which includes the A α helix of HA2, is conserved across influenza A group 1, group 2 and influenza B strains. Stalk epitope 2 consists of the CD α helix in HA2, while epitope 3 encompasses regions of at the base of the HA2 stalk. Both of these epitopes are conserved within influenza A group 2. Epitope 4 is conserved between both B lineages and is located in the C terminal portion of HA1. Each epitope is only indicated on a single monomer within the HA trimer. Accession numbers: Group 1 H5N1 (2FK0), Group 3 H3 (3ZTJ) and influenza B/Brisbane/60/2008 (4FQM).

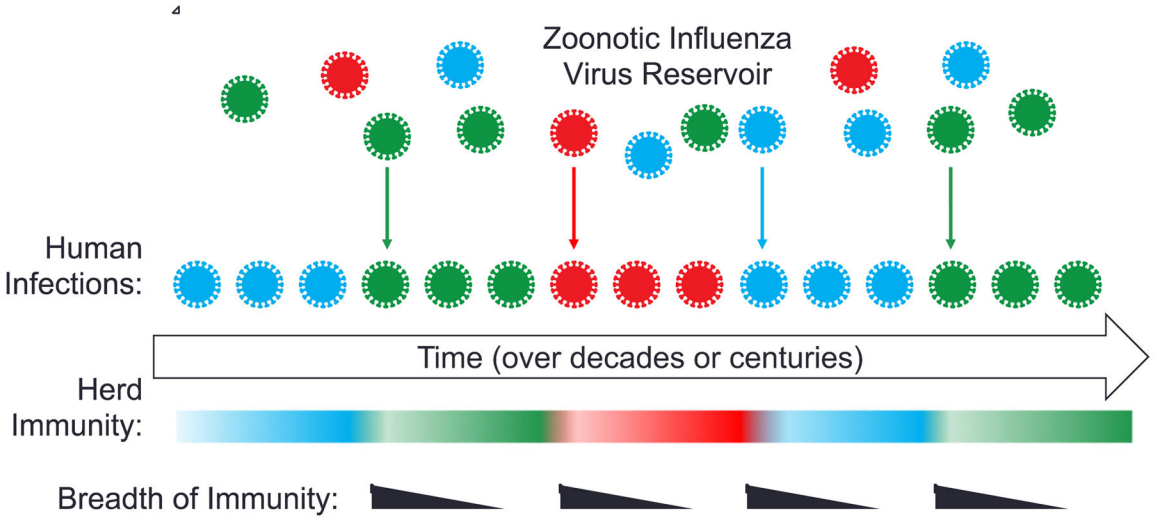

Figure 2. Model for cyclic re-entry of zoonotic influenza strains to humans

Diverse strains of influenza persist in zoonotic reservoirs and can re-enter the human population. Introduction of novel zoonotic strains, most often due to genetic reassortment, boosts immunity preferentially to conserved epitopes. The breadth of this response decreases over time with subsequent exposures. Herd immunity to past circulating strains is lost over generations and they can again become infectious to humans. Both sources of novel influenza strains result in a never-ending cycle of re-entry. Inducing broad immunity on a wide scale, and maintaining it indefinitely at the population level, could eradicate influenza infections of humans.

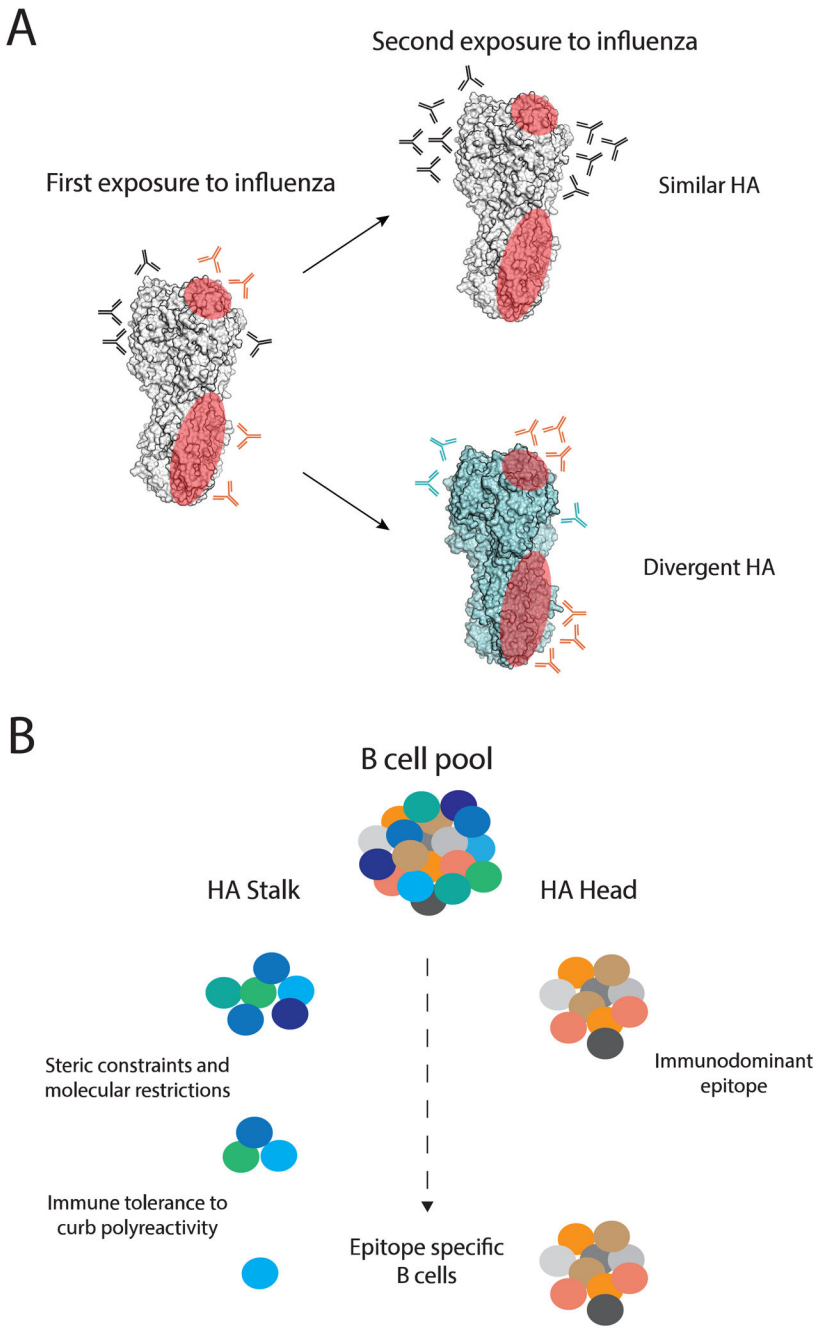

Figure 3. Broadly protective antibodies

(A) Antibodies against conserved epitopes, shown in red on a single monomer of the HA trimer, are generated at a low frequency during a primary exposure. The B cells generating these antibodies are recalled upon secondary exposure to an antigenically distinct influenza strain, and contribute significantly when only these protective epitopes are conserved. Unfortunately, due to the immuno-dominant nature of the HA-head, when secondary exposure is against an antigenically similar influenza strain the stalk antibodies will be lost in the crowd of strain specific HA-head antibodies. (B) There are multiple factors proposed

to be responsible for the relative scarcity of broadly protective stalk antibodies. First, the subdominant immunogenicity of the stalk domain has been attributed to steric constrains, which impose harsh molecular restrictions on the antibodies recognizing these epitopes. Second, immune tolerance to curb polyreactivity may also select against these cells.