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# Generating Broadly Protecting Immune Responses to Viral Influenza

Evan Engelhardt

## Abstract

Modern influenza vaccines are very effective against the strains they are composed of. However, variations between flu strains easily bypasses the induced immunity, rendering the vaccine outdated by the time the next flu season comes around. Current research into a “universal” influenza vaccine that can elicit a broadly protective response from the immune system has made great progress. Most methods are designed to induce broadly neutralizing antibodies to conserved sequences within surface glycoproteins. These methods can be adapted to induce broad cellular immunity as well. Memory to conserved sequences grants the immune system protection against divergent strains of influenza that share these sequences of conservation. The strategies explored in this review have the potential to become the next breakthrough in viral pathogen vaccination.

Discussed are methods including direct conserved antigen administration, modified glycoprotein vaccines, and sequential vaccination designs for DNA vaccines and antigenically similar constructs. Additionally, designs adapted from related viral pathogen studies including virus-like particle vaccines, COBRA vaccines, and immunity from vectored immunoprophylaxis. The strategies reported have shown to be effective in animal studies, however, continued experimentation is necessary before clinical trials.

## Introduction

Common influenza, influenza A and B virus (IAV and IAB), infects millions of people every year and causes hundreds of thousands of deaths. Influenza A virus, in particular, has several different subtypes and has been the cause of many pandemics in the past. IAV finds its natural host mainly in wild aquatic birds. Through a few small mutations, IAV subtypes can find themselves transmitted across multiple animal species until ending up in a human. In 1918, the world faced its most severe flu outbreak in what was commonly called the “Spanish flu”. A strain of H1N1, mutated from birds to pigs, that caused millions of deaths worldwide [1]. In 2009, a new strain of H1N1 spread. A strain that no seasonal flu vaccine of the time provided any protection against. Young adults and children showed no initial cross-reactive antibody response to the 2009 strain [2]. Their immune systems were almost entirely naïve to that particular strain of H1N1, modern flu vaccines could not prepare them for it.

The best known defense against influenza is a vaccine. Vaccinating healthy individuals provides protection for both the individual and the immunocompromised via herd immunity. Individuals are recommended to get a flu vaccine every year due to its variability and, subsequent, evasive nature to our immune system. The flu’s high mutation rate and resulting antigenic drift and shift make vaccine development an endless game of cat and mouse.

Modern influenza vaccines target surface glycoproteins, particularly, hemagglutinin (HA). This protein is responsible for the virus' attachment and entry into host cells, another protein, neuraminidase (NA), also has a role in this. Current vaccines elicit an antibody response to the HA head domain, a highly immunogenic and variable region [3]. Binding blocks receptor interaction and cell entry. High antigenic drift of this region is the driving factor behind annual vaccine updates and low antibody cross-reactivity to related strains.

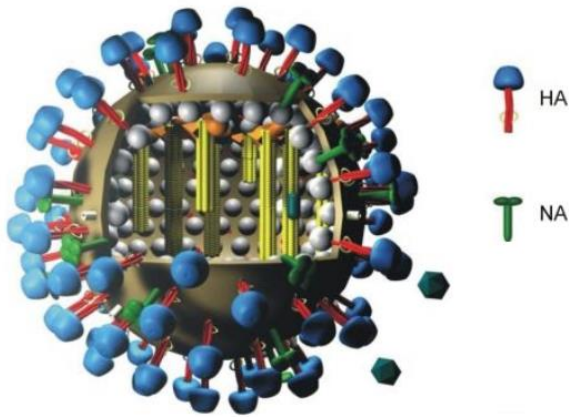


Figure 1: Influenza structure; Hemagglutinin (HA) and Neuraminidase (NA) shown on the surface [4]

More recent approaches to vaccine development have shifted attention away from the variable HA head domain. In order to provide a vaccine that is more broadly protective and more potent against influenza, researchers are targeting the more conserved, less immunogenic HA stalk domain, surface NA glycoproteins, and even internal proteins [3]. A vaccine against conserved, invariant domains of HA would be cross-reactive to many different subtypes, and the benefit of targeting internal proteins like nucleoproteins is a more robust T-cell response to eliminate the virus more efficiently. This review will look at the current scene of influenza vaccination and cross-reactive antibodies. Focus will also be given to the early days of the universal influenza vaccine and chart varied approaches to its development and production.

### **The Current State of Vaccines and Cross-Reactive Antibodies**

Currently, an individual can go to their healthcare provider or local pharmacy and receive the seasonal flu vaccine. This vaccine, statistically, will be 45% effective against this season's influenza A and B viruses [5]. The breakdown of this number gives a 37% effectiveness against H1N1. While the number does not seem high, current vaccination methods are the most effective defense against seasonal flu infections. However, seasonal flu vaccinations are greatly limited in effectiveness against only the strains included in the vaccine. Many findings have shown that seasonal vaccines are not significantly capable of eliciting cross-reactive antibodies that can neutralize divergent strains of influenza [6,7]. Unfortunately, infection is the best way to induce cross-reactive antibodies, specifically those against HA epitopes [8]. Research into the nature of

these antibodies has laid the foundation for universal flu vaccine development. Elucidating their induction and their binding affinity for HA is essential.

Hemagglutinin is a homotrimeric integral membrane protein [9]. Composed of two domains: HA1, the head domain; HA2, the stalk (stem) domain. Early research of the HA glycoprotein uncovered conserved sequences within the HA2 subunit [10,11]. Antibodies against these conserved antigenic sequences could exhibit cross-reactivity to different HA subtypes.

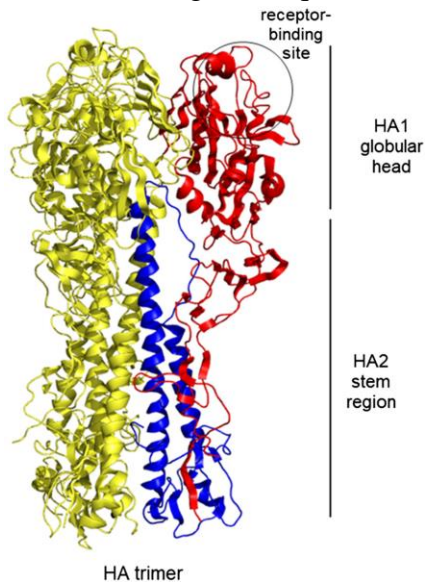


Figure 2: HA trimeric structure, including head and stem (stalk) domain and receptor binding site (RBS) [12]

The first cross-reactive antibody obtained was monoclonal antibody C179 by Okuno et al. in 1993 [13]. Through mouse vaccine trials with H2N2, C179 was found to neutralize multiple influenza subtypes via conserved epitopes in the HA stalk. These epitopes were discovered when stains of H1 and H2 were grown in the presence of C179. Antigenic variations resulted in variants that were not recognized by C179, those were then subjected to sequencing. Nucleotide changes in a single residue in HA1 and a single residue in HA2 were the cause of the antibody's inability to recognize them. When the sequences from each strain were compared, both HA1 and HA2 contained series of residues that were identical in all H1 and H2 strains [13]. In the case of C179, several of the residues in these regions are responsible for the recognition across multiple subtypes.

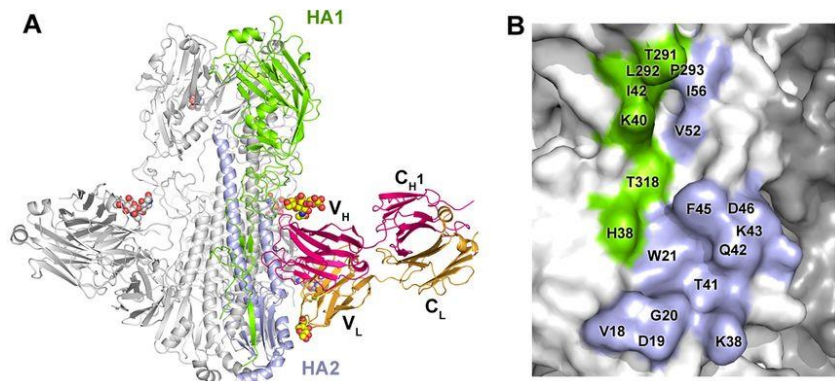


Figure 3: Model of C179 bound to HA stalk epitope; A) H2 influenza strain HA protein in complex with C179 antibody, HA1 in green, HA2 in blue. B) Residues of both HA1 (green) and HA2 (blue) that are interacting with C179 [14]

Smirnov et al. demonstrated the effectiveness of C179 against H5 influenza virus. Significant results were obtained from trials involving C179 and a challenge dose of H5N2 in mice. The mice that received C179 displayed a survival rate of 75-80% after 2 weeks, while the control group (0.9% NaCl solution) had a 100% mortality rate. This indicates C179 is capable of both neutralizing H5 strains and decreasing mortality even after infection was initiated [10].

Continued research into broadly neutralizing antibodies (bnAbs) has shed light onto promising human bnAbs like CR6261 and F10. These antibodies show broad neutralizing ability against several group 1 HAs. Similar to C179, CR6261 and F10 both bind conserved epitopes in the HA stalk [15,16]. Also like C179, binding to the stalk blocks fusion of the virus with endosomal membranes, preventing RNA injection. These antibodies have also shown great promise in mouse models, opening the door for human therapies [58].

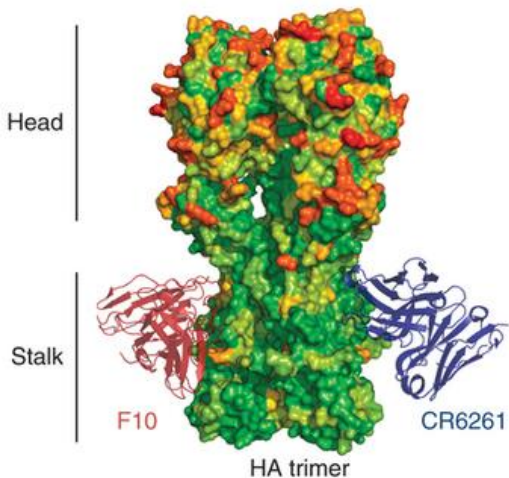


Figure 4: Model of broadly neutralizing antibodies F10 and CR6261 bound to HA stalk domain [58]

Studies into cross-reactive antibodies for group 2 HA proteins have yielded a human bnAb called CR8020. A relatively recent discovery, CR8020 was acquired from a memory B cell activated by a seasonal influenza vaccine [17]. Trials assessing CR8020's safety and efficacy have been administered; however, the results have yet to be officially disclosed [17]. Going further, antibodies that can neutralize both influenza A and influenza B have been found. CR9114 binds a highly conserved epitope that is present across almost all influenza A and B subtypes [18]. For this reason, it is effective at binding and neutralizing numerous subtypes, including those from H1, H2, and H3 groups [19]. The epitope it binds is typically obscured by conserved glycan structures from the head domain, however. Designs for getting expression of CR9114 would confer the broadest protection, therefore making it one of the most ideal antibodies to express. The production of a universal flu vaccine requires a method for effectively inducing these cross-reactive and neutralizing antibodies against the conserved epitopes of the HA glycoprotein.

### **HA Glycoprotein Research/Application**

Focus on the HA stalk domain kicked off research into its structure and relevance in viral infection. Studies found that its primary function involves triggering the fusion of the virus to the endosomal membrane, this allows for RNA release, completing the infection [20]. Many early ideas involved direct vaccination of HA stalk domain. When used with the right adjuvants, it could prove a very potent vaccine. Considering the head domain is the immunodominant portion of HA, developing a headless stalk domain would alleviate some immunogenic interference. Production methods for the HA stalk would require proper folding of the trimer in order to expose the crucial epitopes. The stem's trimeric form would need to remain stable throughout its life, from manufacturing to administration, in order to be effective. Mice studies with headless stalk domains have shown to elicit cross-reactive antibodies for both heterosubtypic strains and cross-group strains [21,22]. Similar methods of HA stalk production have yielded so-called HA "mini-stems" which are polypeptides that are expressed bacterially. The mini-stem is an economic version of a wild-type HA stalk that maintains immunogenic epitopes by mimicking HA's trimeric conformation [23]. This HA mini-stem was shown to bind known bnAbs with high affinity and elicited broad cross-reactivity to multiple group 1 HAs with high affinity as well [23,24,25]. Another promising method for "economic" HA constructs is mosaic design. This involves combining known antigenic sites from different flu subtype HAs onto one diverse, mosaic HA. The aim is to maximize cross-reactive responses, while minimizing genetic differences. This also allows some of the more conserved, immunosubdominant sequences to get more interaction, as illustrated in Figure 5. One benefit is with antibody interaction, specifically bnAbs, because the head domain is a much easier site to access. The mosaic method has shown to elicit cross-reactive antibodies as well as significant T-cell responses [26,27,28,29]. Similarly, mosaic designs using nanoparticles to present a range of HA epitopes have shown confirming results [30].

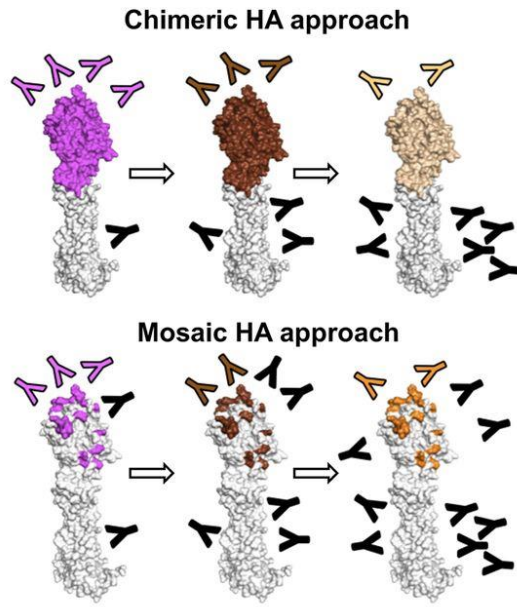


Figure 5: Example of mosaic design; the chimeric approach involves influenza B HA stalks with substituted influenza A HA heads, the mosaic approach involves influenza B HAs that have major antigenic sequences from their heads replaced with sequences from influenza A HAs [26]

A vaccine design based on the HA stalk as the primary immunogen could be administered as a booster, given that most immunocompetent people maintain some level of stalk reactive antibody from memory [31]. Importantly, manufactured HA stalks have exhibited strong resistance to physical and chemical stressors. Added benefits of bacteria-based production means fewer restrictions for growth and better product stability, as opposed to expression via eukaryotic systems. This benefit is seen largely in transportation potential, as these methods would likely eliminate conditions like cold chains.

Conserved HA epitopes are not exclusive to the stalk, however. While most “universal” vaccine research investigates the stalk, some studies have found sequences of conservation across influenza subtypes in the head domain as well. More recent research has elucidated areas of interface between trimeric head domains that bind human antibodies [32,33]. The antibodies come from human memory B cells from previously vaccinated or infected adults. Because of the epitopes’ awkward location, antibodies can only bind at specific time periods when the interface is widened. This exposure typically happens when the HA proteins are displayed on the surface of infected cells, therefore, action by antibodies consists of inhibition of spread and antibody-dependent cellular cytotoxicity. The very broadly conserved nature of these epitopes, much like HA stalk epitopes, makes them prime targets for study. The discovery of human antibody FluA-20 by Bangaru et al. is one such example. This naturally occurring antibody binds with high affinity and breadth, recognizing almost all HA head domains across varying influenza A

subtypes [33]. Subsequent tests in mice conferred protection from lethal challenges of diverse influenza A strains.

### **VLPs/COBRA**

Another promising application of the HA glycoprotein is its use with virus-like particles (VLPs). These molecules are similarly structured to live virions, but are not infectious due to their inability to replicate. Most are capable of self-assembling within a system and can be engineered to display any epitope of interest. A great advantage to VLPs is that they do not necessitate an adjuvant, although many designs involve one, they can be highly immunogenic and conducive to APC uptake alone [34]. Most vaccine studies with VLPs involve either a cocktail of VLPs, each displaying a different subtype strain of HA, or a concentration of homogenous VLP all displaying multiple HA subtypes. In sequential vaccination trials with HA VLPs from groups 1 and 2 influenza phylogeny, heterosubtypic IgG levels were induced, and mucosal immunity was greatly enhanced when administration was intranasal. The mice that received the heterogenous VLP vaccinations exhibited protection from lethal challenges of heterogenous influenza subtypes [35]. Similar studies by Schwartzman et al. with mice receiving cocktails of VLPs, each displaying a different HA subtype (H1, H3, H5, and H7) confirmed these findings. Mice that received the VLP cocktail presented significant protection from lethal challenges of 8 different strains with 7 different HA subtypes. Heterosubtypic protection was also seen when the mice were challenged with matching pandemic strains and avian strains, highlighting the broad protection induced by the VLP vaccine. When mice were tested 6 months after they received the vaccination/boost, they still showed a 100% survival rate from the heterogenous challenges [36]. A tangential VLP strategy for eliciting this broad protection involves designing a single VLP expressing multiple HA epitopes. Again, enhanced protection from matching influenza subtype challenges was seen in mice, further lending credence to HA VLPs as a vaccination method [37,38].

Building off of VLP vaccine strategies, research into Computationally Optimized Broadly Reactive Antigens (COBRA) to develop heterogenous HA antigen-based vaccines has shown a lot of promise. COBRA methods involve multiple rounds of consensus sequence generation to produce HA proteins that can represent the current circulating strain or strains of influenza with high fidelity. These COBRA protein sequences are then expressed on the surface of VLPs. Experimentation with COBRA HA sequences have been able to induce monoclonal antibodies that have broad neutralizing capabilities against multiple HA strains [39,40].



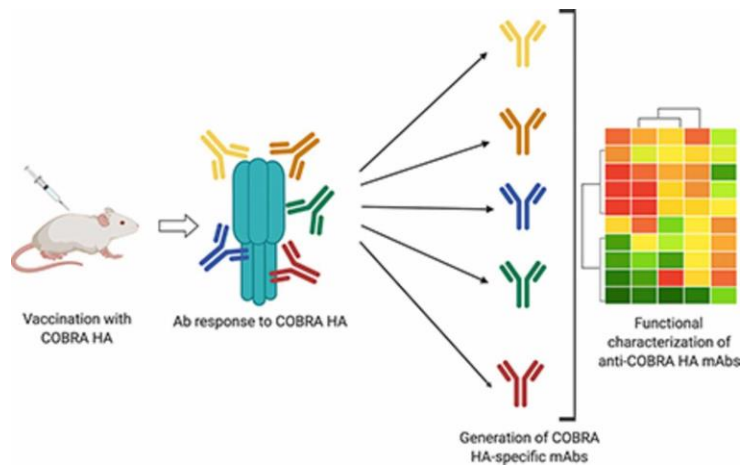


Figure 6: Diagrammed vaccination with COBRA HA; the optimized antigen induces broadly reactive antibodies [39]

Research by Wong et al. went further with 17 COBRA HA antigens designed for human H3N2. These antigens were expressed on VLPs and tested on mice for antibody responses. This method was able to elicit broadly neutralizing antibodies against multiple co-circulating strains of H3N2. The COBRA design was generated using thousands of human H3N2 HA amino acid sequences, these were narrowed down into 17 COBRA sequences [40]. This gave rise to sequences that were optimized to prime immunity to multiple subtypes and induce the most effective antibodies against the strain of interest. Similar studies have been done with the NA glycoprotein that have also induced bnAbs [41].

### Modified Glycan Vaccines

One particular mechanism of variability in HA is the modification of N-glycan structures [42]. These structures themselves are typically not immunogenic, however, they can mask more immunogenic sequences beneath [43]. Research into modifying these glycan structures has uncovered the presence of more conserved sequences underneath that are capable of eliciting more broad immune protection. Modifying the structure to be monoglycosylated has shown significant evidence of inducing broadly neutralizing antibodies [44,45,46]. Stripping the glycan structures back to a single residue is the minimum requirement to maintain HA structure and stability [44]. Recombinant monoglycosylated hemagglutinin constructs (HA<sub>mg</sub>) have shown to be very reactive antigens to B cells. Studies in mice show the induction of cross-strain protection via bnAbs and elevated production of cytokines [44,45]. Additional studies have also seen enhanced CD8<sup>+</sup> cytotoxicity effects [45].

Tseng et al. gives evidence for a potential vaccine method based on similar research. They produced a monoglycosylated inactivated split H1N1 virus vaccine from chicken eggs. Tests in mice showed increased cross-strain protection against lethal challenges of influenza. The vaccine also elicited more stem-specific antibodies and antibody-secreting splenocytes as well as enhanced ADCC activity against strain-specific and cross-strain HA-expressing cells. The virus

is modified using kifunensine to inhibit glycosylation, then endoglycosidase H is used to trim down existing glycan structures to a single residue (Figure 7) [46].

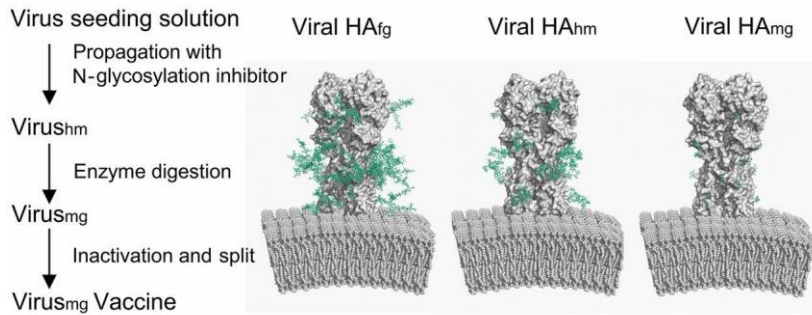


Figure 7: Series of events in producing monoglycosylated HA (HA<sub>mg</sub>); glycan structures are represented in green [46]

This recent vaccine design has great potential due to its similar production to the current procedure. It can be produced via simple modifications to the chicken egg-based procedure, and it is capable of providing much broader protection against H1N1 strains.

### Sequential Vaccination/Infection

In line with the strategies involving conserved sequences, research continued on modifications to the current vaccination methods to be more effective and elicit a more broad antibody response. The challenge came from the latter: broadly neutralizing antibodies are not easily elicited from traditional vaccination methods. A relatively simple variation on the current model that involves consecutive administration of distinct antigens. Wei et al. attempted to develop plasmid-vaccines that would be able to prime immune responses, soon followed with a booster. The experiment tested a DNA primer taken from a strain of flu virus paired with either a booster from a seasonal flu vaccine or a replication-defective adenovirus 5 (rAd5) encoding viral HA [47]. Early results indicated that boosting the DNA vaccine with a seasonal vaccine or rAd5 for matching HA showed increased neutralization capacity for more subtypes than the one expressed in the DNA primer and booster. Further tests were run to ensure the specificity of the neutralizing antibodies to the conserved stem. Sera from mice, ferrets, and non-human primates were run on a neutralization assay along with competitors that included a WT H1-influenza HA trimer (WT) and a matched stem mutant protein. The assay included different strains of virus, the binding activity of the sera antibodies was observed. In all cases, the sera from the DNA primer + booster elicited a higher titer of neutralizing antibodies to the WT HA trimer than the stem mutant. In the cases of the DNA primer + rAd5 boost, the titer elicited was even greater than just the seasonal vaccine booster. Because the WT HA trimer was preferentially neutralized compared to the stem mutant and whole virus, the conclusion can be drawn that the antibodies are binding the conserved region within the WT trimer. This indicates strong cross-reactive capabilities from the antibodies elicited in the DNA/HA plasmid vaccine.

Further research into the prime-boost model of eliciting cross-reactive antibodies showed that sequential infection of H1N1 strains yields similar results. After the 2009 pandemic H1N1 virus, isolated monoclonal antibodies from those infected were shown to be matured and reactive to various subtypes of influenza virus [48]. This specific sequential model involves initial exposure to seasonal H1N1 followed by a boost from the pandemic H1N1. Upon exposure to the virus with an antigenically novel HA, memory B cells were boosted toward portions of HA that were conserved between the strains [48, 49,50]; a method that sees use in many other vaccination strategies for this reason.

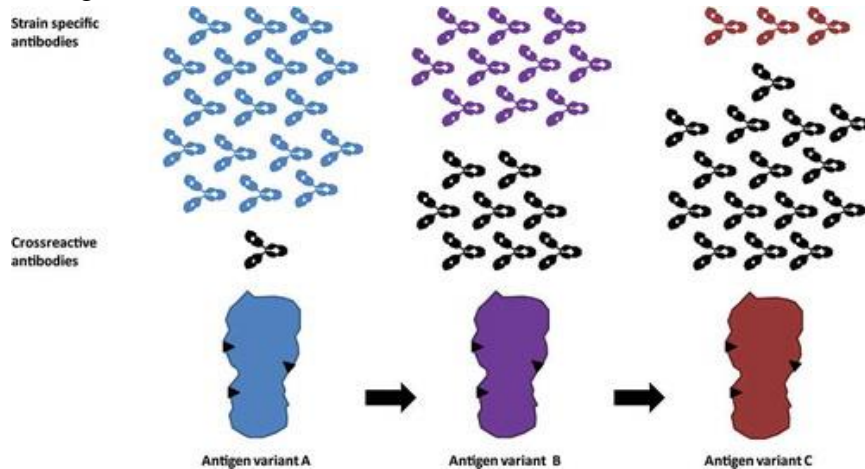


Figure 8: Diagram of sequential vaccination concept; as different antigens with the same conserved sequences are consecutively administered, the antibody titer to the area of conservation increases [43]

The greatest bnAb induction is seen when the booster antigen is one that the person has little pre-existing antibodies for. In fact, when boosted with the same antigens, immunity is shifted back to anti-head domain responses [49]. The key is heterogenous immunization with HA from non-circulating influenza strains to increase stalk-specific immune responses. The boost in affinity also comes with enhanced neutralization capabilities to antigenically distinct HA [50]. Additional studies have found a similar result from the sequential infection of chronologically separated H1N1 strains. It was found that this boost brings added protection to novel H1N1 strains [52,53]. The likely reason behind this significant boost comes down to a wild-type virus' replication abilities. Appreciable induction of anti-stalk antibodies is made possible by very high doses of virus. More replication means more antigen presentation. Attenuated viruses and DNA vaccines alone have much lower replication levels than wild-type, thus more would be required to induce the same antibody response [48]. This goes along with the data from the previous paragraph, in which the DNA primer vaccine would be boosted by a modified adenovirus. Introducing the host to a novel virus via DNA vaccine primes the immune system to the HA epitopes. This is built upon by the subsequent administration of a virus with an antigenically distinct HA head domain, allowing the host's immune system to find the pattern between the two immunogens. Evidence of this has been seen in trials with H3 viruses, where the bnAbs that are

induced have affinity for the regions conserved between the sequential virus vaccines [51]. Thus, the memory B cells are boosted towards the conserved stem domain that the subtypes share.

### **IgA/Mucosal Immunity**

Current vaccine models are mainly designed for intramuscular or subcutaneous administration to induce an adaptive immune response. This approach is effective on the whole, and with the proper vaccine, can elicit a strong immune response. This model is effective primarily in inducing IgG antibodies against the target antigen. The induction of IgA is minimal at best, leaving a figurative hole in immune defenses on the mucosal front. This should raise alarms given that mucosal pathways like the nose and throat are the most likely entry points for influenza. Resident IgA antibodies are able to provide immediate immunity to most pathogens by clearing them before they can even pass the mucosal barrier [54]. IgA is shown to be effective at disarming viruses in virus-infected secretory epithelial cells [55]. Responses by IgA are non-inflammatory, utilization of IgA in highly pathogenic strains of influenza could eliminate complications that come from uncontrolled inflammatory responses [56].

Harnessing the potential of IgA in a universal flu vaccine could serve to strengthen immunity by bolstering mucosal defenses and adding further cross-reactive memory. Tests with IgA antibodies have shown a lot of promise in terms of both binding affinity and neutralization. When variable regions of mouse antibodies known to bind HA stalks were cloned into human IgG or IgA backbones, the IgA backbone antibodies showed significantly greater binding and neutralization of the virus [57]. The practicality of designing a vaccine with such antibodies aside, IgA's potential contribution to future vaccines is great. Trials using isolated, stimulated B cells from human donors have highlighted the body's natural ability to produce stalk-specific IgA: from equal pools of IgG and IgA, the IgA population had a significantly higher proportion of stalk-specific antibodies [57]. The key to production is proper immune activation. Intranasal administration of inactivated influenza virus vaccines in mice have been shown to induce cross-protective immunity [58]. The same research showed that systemic IgG was induced in addition to mucosal IgA. Intranasal immunization with an inactivated whole virus vaccine is capable of inducing a broad spectrum of heterosubtypic immunity in mice [58]. The immune responses against influenza A virus-specific proteins are vital to this protection. The caveat to inducing mucosal immunity is finding the right adjuvant for the vaccine. Many inactivated virus vaccines or subunit virus vaccines given intranasally break down before they are able to be picked up by antigen presenting cells. Promise has been seen in formalin-inactivated virus vaccines due to the liposome-like formation of the inactivated virus particles (e.g. internal proteins) [58]. Preserving the internal proteins for presentation is paramount for creating cross-reactive immunity to varying influenza subtypes. The overarching idea is, then, to make the respiratory pathway the route for the broadly inducing vaccine. Including a mucosal component in a universal flu vaccine would grant enhanced protection from all forms of entry by the virus.

The methods described in this and previous sections involve conferring enhanced protection in otherwise healthy immune systems. They require a response from the individual's immune system to generate memory in order to defend from subsequent infection. The methods can fall short when considering the elderly population and the immunocompromised. Thus, the need for a vaccination method that can produce a broadly neutralizing immune response in both healthy individuals and those with weakened immune systems is immense.

### Vectored Immunoprophylaxis

Traditional vaccines and most novel approaches to vaccination involve the immune system responding to an antigen by inducing lymphocytes, specifically humoral lymphocytes, to fight the foreign agent. The idea behind vectored immunoprophylaxis (VIP) is to bypass the humoral immune system and generate broadly neutralizing antibodies via non-hematopoietic cells. By means of adeno-associated viruses (AAVs), vectors encoding such bnAbs as F10, CR6261, or CR9114 are administered intramuscularly to elicit expression. Essentially, a form of passive immunization, a synthetic induction of antibodies by having the body translate the administered vectors.

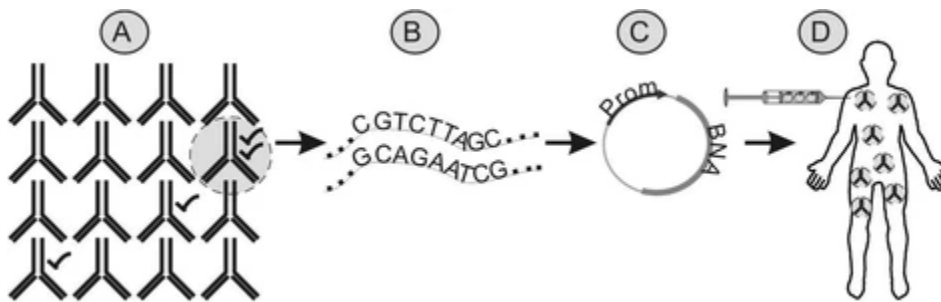


Figure 9: Diagram of VIP vaccine development; A) Most effective bnAb is selected (circled). B) Genetic sequence of bnAb is determined. C) Genetic sequence of bnAb is inserted on a vector, downstream of Promoter. D) The vector vaccine is administered where needed to be translated and confer protection locally or systemically [60]

Studies by Balazs et al. in mice have shown the induction of detectable levels of expression of both antibodies. Neutralization assays with sera from the vaccinated mice exhibited neutralization of multiple influenza subtypes. Further tests of VIP with intramuscular injection gave confirming results. Challenge doses of three different H1N1 strains caused no significant signs of illness in the mice who received VIP treatment, as opposed to the control groups which showed rapid weight loss. Moreover, these results were seen again in tests with older and/or immunocompromised mice. The tests with H1N1 also showed that endogenous humoral immunity could be induced in the vaccinated mice after infection [59]. This is important when considering the long-term effectiveness of a vaccine by this design. While administration of a vector encoding bnAbs may seem like a quick, short-term fix, the potential for long-term memory to be conferred would validate its use as a vaccine. Balazs et al. notes that the antibodies generated remained detectable in the mice throughout the 64-week study. Although the

neutralizing capabilities of those antibodies at the end of the study had diminished, they still showed relatively enhanced protection from intranasal infection compared to controls [59].

Vectored immunoprophylaxis is a prospective method for quick induction of broadly neutralizing antibodies. Vaccines made by this design would allow for highly selective and enhanced antibodies, as well as vectors that can be engineered to target specific cells and tissues. This would offer localized and systemic immunity options [60,61]. Most importantly, VIP vaccines would grant significant protection to older or immunocompromised individuals, as this method does not require an immune response to generate bnAbs. However, research on vectored immunoprophylaxis as a vaccine for influenza is insufficient. Most experimental data on VIP is for use with HIV infections, which has shown a lot of promise [62,63]. Elucidation of VIP use against influenza in animal models is needed, but could prove to be fruitful.

### Cellular Immunity

In line with the enlistment of IgA for influenza protection, cellular immunity from T-cells adds another layer to systemic defense. T-cells have the added benefit of recognizing the internal proteins of viruses. Similarly to the humoral immune response to conserved influenza epitopes, T-cells have been shown to be stimulated by conserved peptides from multiple strains of influenza [64]. The body's cellular response is able to confer memory to the conserved epitopes from different strains: CD8<sup>+</sup> T-cells induced from infection with seasonal H1N1 or H3N2 show cross-reactivity to pandemic H1N1 and swine-origin, reassorted H3N2 [65]. Primary targets for T-cell responses are internal viral proteins nucleoprotein (NP) and matrix protein 1 (M1), both of which are highly conserved [3]. These regions for T-cells are analogous to the HA stalk region for antibodies and could be the basis for broad T-cell protection. When used in conjunction with antibody epitopes, this collection of conserved viral regions is capable of stimulating both a humoral and cellular response [64]. Specific methods of co-expressing an NP+M1 fusion protein and different chimeric HAs using vector vaccines induced antibodies to the HA stalk and induced a T-cell response to NP and M1 [3]. This was built upon by Stepanova et al. in their design of a flagellin-fused protein that contains conserved epitopes for humoral and cellular responses (Figure 10).

Flg-HA2-2-4M2ehs:



FlgSh-HA2-2-4M2ehs:

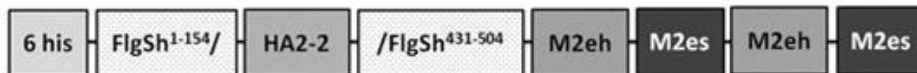


Figure 10: Vaccinia virus vaccine design; flagellin protein with two immunogenic sequences attached; HA2 consensus sequences from two different influenza A strains, and 4 copies of internal influenza protein M2e [66]

The epitopes were derived from both conserved HA proteins and conserved internal influenza proteins. The construct was able to activate neutralizing antibodies as well as enhance specificity of both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells to various influenza challenges [66].

Other methods of broad T-cell induction involve much the same methods as broad antibody induction. A range of conserved influenza proteins known to be recognized by T-cells during infection are congregated into a single vaccine design. Studies with engineered virus vaccines expressing several influenza-derived proteins have resulted in the induction of broadly reactive T-cells. Valkenburg et al. designed a vaccinia virus-based H5N1 influenza vaccine that did just this. When the vaccine was used with IL-15 as a molecular adjuvant, significant protection was seen in mice challenged with a variety of heterosubtypic strains of influenza [67,68]. The derived proteins were internal, and they activated both CD8<sup>+</sup> and CD4<sup>+</sup> T-cells with cross-reactivity. A technique by Eickhoff et al. used immunoinformatic tools to elucidate possible T-cell epitopes within conserved motifs. The motifs investigated were important in HLA binding during antigen presentation. The DNA vaccines that were then designed were shown to induce protection against diverse influenza strains in HLA transgenic mice. Building off this, they designed specific HLA immunogenic consensus sequences that selectively stimulated CD4<sup>+</sup> T-cells. Tests showed that the memory cells generated by this method in mice conferred broad protection. The memory cells induced were broad in the sense that they were capable of facilitating the induction of bnAbs and broadly effective CD8<sup>+</sup> T-cells [69].

Difficulties in T-cell memory generation lies in durability. CD8<sup>+</sup> T-cells for smallpox are known to last decades after vaccination; however, not enough is known regarding CTL's durability for influenza. Some research has shown T-cells in the blood diminishing only a few months after infection, indicating a possible half-life of 2-3 years [70]. Even so, conferring memory T-cells with a half-life of a few years would prove beneficial in the long-term, considering that the vaccinated individual would retain cross-reactivity in that time.

### **Discussion/Conclusion**

Research into a “universal” influenza vaccine has exposed several areas of attack against seasonal and pandemic influenza. The elucidation of conserved proteins within the virus has created an opportunity to design a vaccine that can be effective across many strains of influenza. The initial problem of targeting these proteins is their lessened immunogenicity compared to other more immunodominant, but variable, proteins. However, novel approaches to vaccine design and increased knowledge of immune system responses to influenza have helped circumvent this issue to an extent. New methods for targeting conserved viral proteins within the HA glycoprotein or within the virus itself have made great strides towards the goal of a “universal” vaccine. A vaccine that can induce broad, cross-reactive protection from both humoral and cellular immune responses. A vaccine capable of conferring immunity to strains of

influenza beyond the seasonal offender. A vaccine with the potential to be more cost-effective and easy to produce and handle in the event of an outbreak.



## References

1. History of 1918 Flu Pandemic. (2018, March 21). Retrieved from <https://www.cdc.gov/flu/pandemic-resources/1918-commemoration/1918-pandemic-history.htm>
2. Schnirring, L. (2009, May 21). Some immunity to novel H1N1 flu found in seniors. Retrieved from <http://www.cidrap.umn.edu/news-perspective/2009/05/some-immunity-novel-h1n1-flu-found-seniors>
3. Arunkumar, G. A., et al. (2019). Vaccination with viral vectors expressing NP, M1 and chimeric hemagglutinin induces broad protection against influenza virus challenge in mice. *Vaccine*, 37(37), 5567–5577. doi: 10.1016/j.vaccine.2019.07.095
4. Eickmann, M. (2005). *Structure of swine influenza virus showing different types of antigens present on and inside the capsid*.
5. Crawford, Chris. “CDC Releases Interim Flu Vaccine Effectiveness Report.” *AAFP Home*, American Academy of Family Physicians (AAFP), 26 Feb. 2020, [www.aafp.org/news/health-of-the-public/20200226interimfluve.html](http://www.aafp.org/news/health-of-the-public/20200226interimfluve.html).
6. Centers for Disease Control and Prevention (CDC). Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. *MMWR. Morbidity and Mortality Weekly Report*. 2009 May;58(19):521-524.
7. Hancock, Kathy, et al. “Cross-Reactive Antibody Responses to the 2009 Pandemic H1N1 Influenza Virus.” *New England Journal of Medicine*, vol. 361, no. 20, Dec. 2009, pp. 1945–1952., doi:10.1056/nejmoa0906453.
8. Jens Wrammert, et al.; Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection . *J Exp Med* 17 January 2011; 208 (1): 181–193. doi: <https://doi.org/10.1084/jem.20101352>
9. Wilson, I., Skehel, J. & Wiley, D. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature* 289, 366–373 (1981). <https://doi.org/10.1038/289366a0>
10. Smirnov, Y., Lipatov, A., Gitelman, A. *et al.* Prevention and treatment of bronchopneumonia in mice caused by mouse-adapted variant of avian H5N2 influenza A virus using monoclonal antibody against conserved epitope in the HA stem region. *Arch. Virol.* 145, 1733–1741 (2000). <https://doi.org/10.1007/s007050070088>
11. Rong Hai, et al.; “Influenza Viruses Expressing Chimeric Hemagglutinins: Globular Head and Stalk Domains Derived from Different Subtypes”. *Journal of Virology* Apr 2012, 86 (10) 5774-5781; DOI: 10.1128/JVI.00137-12
12. Han, L., et al. (2019). Structural Insights for Anti-Influenza Vaccine Design. *Computational and Structural Biotechnology Journal*, 17, 475–483. doi: 10.1016/j.csbj.2019.03.009
13. Okuno, Y., Isegawa, Y., Sasao, F., & Ueda, S. (1993). A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *Journal of Virology*, 67(5), 2552–2558. doi: 10.1128/jvi.67.5.2552-2558.1993
14. Dreyfus, C., Ekiert, D. C., & Wilson, I. A. (2013). Structure of a Classical Broadly Neutralizing Stem Antibody in Complex with a Pandemic H2 Influenza Virus Hemagglutinin. *Journal of Virology*, 87(12), 7149–7154. doi: 10.1128/jvi.02975-12
15. Ekiert, Damian C, and Ian A Wilson. “Broadly Neutralizing Antibodies against Influenza Virus and Prospects for Universal Therapies.” *Current Opinion in Virology*, vol. 2, no. 2, 2012, pp. 134–141., doi:10.1016/j.coviro.2012.02.005.
16. Lingwood, D., McTamney, P., Yassine, H. *et al.* Structural and genetic basis for development of broadly neutralizing influenza antibodies. *Nature* 489, 566–570 (2012). <https://doi.org/10.1038/nature11371>
17. Sedeyn, Koen, and Xavier Saelens. “New Antibody-Based Prevention and Treatment Options for Influenza.” *Antiviral Research*, vol. 170, 2019, p. 104562., doi:10.1016/j.antiviral.2019.104562.
18. Dreyfus, C., et al. (2012). Highly conserved protective epitopes on influenza B viruses. *Science (New York, N.Y.)*, 337(6100), 1343–1348. <https://doi.org/10.1126/science.1222908>

19. Sutton, T. C., et al. (2017). In Vitro Neutralization Is Not Predictive of Prophylactic Efficacy of Broadly Neutralizing Monoclonal Antibodies CR6261 and CR9114 against Lethal H2 Influenza Virus Challenge in Mice. *Journal of Virology*, *91*(24). doi: 10.1128/jvi.01603-17
20. Lu, Y., Welsh, J. P., & Swartz, J. R. (2013). Production and stabilization of the trimeric influenza hemagglutinin stem domain for potentially broadly protective influenza vaccines. *Proceedings of the National Academy of Sciences*, *111*(1), 125–130. doi: 10.1073/pnas.1308701110
21. Mallajosyula, V. V. A., et al. (2014). Influenza hemagglutinin stem-fragment immunogen elicits broadly neutralizing antibodies and confers heterologous protection. *Proceedings of the National Academy of Sciences*, *111*(25). doi: 10.1073/pnas.1402766111
22. Steel, J., et al. (2010). Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain. *MBio*, *1*(1). doi: 10.1128/mbio.00018-10
23. Valkenburg, S., Mallajosyula, V., Li, O. *et al.* Stalking influenza by vaccination with pre-fusion headless HA mini-stem. *Sci Rep* *6*, 22666 (2016). <https://doi.org/10.1038/srep22666>
24. Impagliazzo, A., et al. (2015). A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science*, *349*(6254), 1301–1306. doi: 10.1126/science.aac7263
25. Joan E. M. Van Der Lubbe, et al. (2018). Mini-HA Is Superior to Full Length Hemagglutinin Immunization in Inducing Stem-Specific Antibodies and Protection Against Group 1 Influenza Virus Challenges in Mice. *Frontiers in Immunology*, *9*. doi: 10.3389/fimmu.2018.02350
26. Sun, W., et al. (2019). Development of Influenza B Universal Vaccine Candidates Using the “Mosaic” Hemagglutinin Approach. *Journal of Virology*, *93*(12). doi: 10.1128/jvi.00333-19
27. Kamlangdee, A., et al. (2014). Broad Protection against Avian Influenza Virus by Using a Modified Vaccinia Ankara Virus Expressing a Mosaic Hemagglutinin Gene. *Journal of Virology*, *88*(22), 13300–13309. doi: 10.1128/jvi.01532-14
28. Li, S. Q., et al. (1992). Influenza A virus transfectants with chimeric hemagglutinins containing epitopes from different subtypes. *Journal of Virology*, *66*(1), 399–404. doi: 10.1128/jvi.66.1.399-404.1992
29. Barouch, D. H., et al. (2010). Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. *Nature Medicine*, *16*(3), 319–323. doi: 10.1038/nm.2089
30. Kanekiyo, M., et al. (2019). Mosaic nanoparticle display of diverse influenza virus hemagglutinins elicits broad B cell responses. *Nature Immunology*, *20*(3), 362–372. doi: 10.1038/s41590-018-0305-x
31. Wohlbold, T. J., et al. (2015). Vaccination with soluble headless hemagglutinin protects mice from challenge with divergent influenza viruses. *Vaccine*, *33*(29), 3314–3321. doi: 10.1016/j.vaccine.2015.05.038
32. Watanabe, A., et al. (2019). Antibodies to a Conserved Influenza Head Interface Epitope Protect by an IgG Subtype-Dependent Mechanism. *Cell*, *177*(5). doi: 10.1016/j.cell.2019.03.048
33. Bangaru, S., et al. (2019). A Site of Vulnerability on the Influenza Virus Hemagglutinin Head Domain Trimer Interface. *CellPress*, *177*(5), 1136–1152. Doi: doi.org/10.1016/j.cell.2019.04.011
34. Ludwig, C., & Wagner, R. (2007). Virus-like particles—universal molecular toolboxes. *Current Opinion in Biotechnology*, *18*(6), 537–545. doi: 10.1016/j.copbio.2007.10.013
35. Luo, Y., Mohan, T., Zhu, W. *et al.* Sequential Immunizations with heterosubtypic virus-like particles elicit cross protection against divergent influenza A viruses in mice. *Sci Rep* *8*, 4577 (2018). <https://doi.org/10.1038/s41598-018-22874-w>
36. Schwartzman, L. M., et al. (2015). An Intranasal Virus-Like Particle Vaccine Broadly Protects Mice from Multiple Subtypes of Influenza A Virus. *MBio*, *6*(4). doi: 10.1128/mbio.01044-15
37. Pushko, P., et al. (2011). Influenza virus-like particle can accommodate multiple subtypes of hemagglutinin and protect from multiple influenza types and subtypes. *Vaccine*, *29*(35), 5911–5918. doi: 10.1016/j.vaccine.2011.06.068
38. Wang, B.-Z., et al. (2012). Enhanced Influenza Virus-Like Particle Vaccines Containing the Extracellular Domain of Matrix Protein 2 and a Toll-Like Receptor Ligand. *Clinical and Vaccine Immunology*, *19*(8), 1119–1125. doi: 10.1128/cvi.00153-12

39. Sautto, G. A., et al. (2019). A Computationally Optimized Broadly Reactive Antigen Subtype–Specific Influenza Vaccine Strategy Elicits Unique Potent Broadly Neutralizing Antibodies against Hemagglutinin. *The Journal of Immunology*, 204(2), 375–385. doi: 10.4049/jimmunol.1900379
40. Wong, T. M., et al. (2017). Computationally Optimized Broadly Reactive Hemagglutinin Elicits Hemagglutination Inhibition Antibodies against a Panel of H3N2 Influenza Virus Cocirculating Variants. *Journal of virology*, 91(24), e01581-17. <https://doi.org/10.1128/JVI.01581-17>
41. Job, E. R., et al. (2018). Broadened immunity against influenza by vaccination with computationally designed influenza virus N1 neuraminidase constructs. *Npj Vaccines*, 3(1). doi: 10.1038/s41541-018-0093-1
42. Bajic, G., et al. (2019). Influenza Antigen Engineering Focuses Immune Responses to a Subdominant but Broadly Protective Viral Epitope. *Cell Host & Microbe*, 25(6). doi: 10.1016/j.chom.2019.04.003
43. F Krammer (2017) Strategies to induce broadly protective antibody responses to viral glycoproteins, *Expert Review of Vaccines*, 16:5, 503-513, DOI: [10.1080/14760584.2017.1299576](https://doi.org/10.1080/14760584.2017.1299576)
44. Wang, C.-C., et al. (2009). Glycans on influenza hemagglutinin affect receptor binding and immune response. *Proceedings of the National Academy of Sciences*, 106(43), 18137–18142. doi: 10.1073/pnas.0909696106
45. Chen, J.-R., et al. (2014). Vaccination of monoglycosylated hemagglutinin induces cross-strain protection against influenza virus infections. *Proceedings of the National Academy of Sciences*, 111(7), 2476–2481. doi: 10.1073/pnas.1323954111
46. Tseng, Y.-C., et al. (2019). Egg-based influenza split virus vaccine with monoglycosylation induces cross-strain protection against influenza virus infections. *Proceedings of the National Academy of Sciences*, 116(10), 4200–4205. doi: 10.1073/pnas.1819197116
47. Wei, C.-J., et al. (2010). Induction of Broadly Neutralizing H1N1 Influenza Antibodies by Vaccination. *Science*, 329(5995), 1060–1064. doi: 10.1126/science.1192517
48. Krammer, F., et al. (2012). Hemagglutinin Stalk-Reactive Antibodies Are Boosted following Sequential Infection with Seasonal and Pandemic H1N1 Influenza Virus in Mice. *Journal of Virology*, 86(19), 10302–10307. doi: 10.1128/jvi.01336-12
49. Ellebedy, A. H., et al. (2014). Induction of broadly cross-reactive antibody responses to the influenza HA stem region following H5N1 vaccination in humans. *Proceedings of the National Academy of Sciences*, 111(36), 13133–13138. doi: 10.1073/pnas.1414070111
50. Ledgerwood, J. E., et al. (2011). DNA priming and influenza vaccine immunogenicity: two phase 1 open label randomised clinical trials. *The Lancet Infectious Diseases*, 11(12), 916–924. doi: 10.1016/s1473-3099(11)70240-7
51. Wang, T. T., et al. (2010). Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS pathogens*, 6(2), e1000796. <https://doi.org/10.1371/journal.ppat.1000796>
52. Pica, N., et al. (2012). Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. *Proceedings of the National Academy of Sciences*, 109(7), 2573–2578. doi: 10.1073/pnas.1200039109
53. Kirchenbaum, G. A., Carter, D. M., & Ross, T. M. (2015). Sequential Infection in Ferrets with Antigenically Distinct Seasonal H1N1 Influenza Viruses Boosts Hemagglutinin Stalk-Specific Antibodies. *Journal of Virology*, 90(2), 1116–1128. doi: 10.1128/jvi.02372-15
54. Riet, E. V., et al. (2012). Mucosal IgA responses in influenza virus infections; thoughts for vaccine design. *Vaccine*, 30(40), 5893–5900. doi: 10.1016/j.vaccine.2012.04.109
55. Brandtzaeg, P. (2007). Induction of secretory immunity and memory at mucosal surfaces. *Vaccine*, 25(30), 5467–5484. doi: 10.1016/j.vaccine.2006.12.001
56. Yel, L. Selective IgA Deficiency. *J Clin Immunol* 30, 10–16 (2010). <https://doi.org/10.1007/s10875-009-9357-x>

57. He, W., et al (2015). Broadly Neutralizing Anti-Influenza Virus Antibodies: Enhancement of Neutralizing Potency in Polyclonal Mixtures and IgA Backbones. *Journal of Virology* Mar 2015, 89 (7) 3610-3618; DOI: 10.1128/JVI.03099-14
58. Takada, Ayato, et al. (2003). "Intranasal Immunization with Formalin-Inactivated Virus Vaccine Induces a Broad Spectrum of Heterosubtypic Immunity against Influenza A Virus Infection in Mice." *Vaccine*, vol. 21, no. 23, 2003, pp. 3212–3218., doi:10.1016/s0264-410x(03)00234-2.
59. Balazs, A. et al. (2013). Broad protection against influenza infection by vectored immunoprophylaxis in mice. *Nature Biotechnology*, 31(7), 647–652. doi: 10.1038/nbt.2618
60. Sanders, J. W., & Ponzio, T. A. (2017). Vectored immunoprophylaxis: an emerging adjunct to traditional vaccination. *Tropical Diseases, Travel Medicine and Vaccines*, 3(1). doi: 10.1186/s40794-017-0046-0
61. Deal, C., et al. (2014). Vectored antibody gene delivery protects against *Plasmodium falciparum* sporozoite challenge in mice. *Proceedings of the National Academy of Sciences*, 111(34), 12528–12532. doi: 10.1073/pnas.1407362111
62. Balazs, A., et al. (2012). Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission. *Retrovirology*, 9(S2). doi: 10.1186/1742-4690-9-s2-p42
63. Priddy, F. H., et al. (2019). Adeno-associated virus vectored immunoprophylaxis to prevent HIV in healthy adults: a phase 1 randomised controlled trial. *The Lancet HIV*, 6(4). doi: 10.1016/s2352-3018(19)30003-7
64. Testa, J. S., et al. (2012). MHC Class I-Presented T Cell Epitopes Identified by Immunoproteomics Analysis Are Targets for a Cross Reactive Influenza-Specific T Cell Response. *PLoS ONE*, 7(11). doi: 10.1371/journal.pone.0048484
65. Hillaire, M. L. B., et al. (2013). Human T-cells directed to seasonal influenza A virus cross-react with 2009 pandemic influenza A (H1N1) and swine-origin triple-reassortant H3N2 influenza viruses. *Journal of General Virology*, 94(3), 583–592. doi: 10.1099/vir.0.048652-0
66. Stepanova, L. A., et al. (2018). Flagellin-fused protein targeting M2e and HA2 induces potent humoral and T-cell responses and protects mice against various influenza viruses a subtypes. *Journal of Biomedical Science*, 25(1). doi: 10.1186/s12929-018-0433-5
67. Valkenburg, S. A., et al. (2019). Vaccine-induced T cell protection from influenza viruses. *Hong Kong Med J*, 25(5), 33–36.
68. Valkenburg, S. A., et al. (2018). Protection by universal influenza vaccine is mediated by memory CD4 T cells. *Vaccine*, 36(29), 4198–4206. doi: 10.1016/j.vaccine.2018.06.007
69. Eickhoff, C. S., et al. (2019). Highly conserved influenza T cell epitopes induce broadly protective immunity. *Vaccine*, 37(36), 5371–5381. doi: 10.1016/j.vaccine.2019.07.033
70. Sridhar, S. (2016). Heterosubtypic T-Cell Immunity to Influenza in Humans: Challenges for Universal T-Cell Influenza Vaccines. *Frontiers in Immunology*, 7. doi: 10.3389/fimmu.2016.00195