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REVIEW

Universal influenza virus vaccines: what can we learn from the human immune response following exposure to H7 subtype viruses?

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Abstract **Several universal influenza virus vaccine candidates based on eliciting antibodies against the hemagglutinin stalk domain are in development. Typically, these vaccines induce responses that target group 1 or group 2 hemagglutinins with little to no cross-group reactivity and protection. Similarly, the majority of human anti-stalk monoclonal antibodies that have been isolated are directed against group 1 or group 2 hemagglutinins with very few that bind to hemagglutinins of both groups. Here we review what is known about the human humoral immune response to vaccination and infection with H7 subtype influenza viruses on a polyclonal and monoclonal level. It seems that unlike vaccination with H5 hemagglutinin, which induces antibody responses mostly restricted to the group 1 stalk domain, H7 exposure induces both group 2 and cross-group antibody responses. A better understanding of this phenomenon and the underlying mechanisms might help to develop future universal influenza virus vaccine candidates.**

Keywords universal influenza virus vaccine; hemagglutinin stalk; H7N9

Introduction

Influenza viruses cause annual epidemics and, in irregular intervals, pandemics in the human population. Viral infection in humans leads to respiratory disease that can be associated with severe morbidity and mortality with up to half a million deaths caused by seasonal influenza viruses every year globally [1]. Pandemics are usually associated with higher numbers of deaths. An example is the 1918/19 H1N1 pandemic which caused an estimated 40 million deaths worldwide [2]. Influenza viruses are a significant challenge for vaccine design due to their ability to mutate and escape antibody-based immunity through introduction of antigenic changes in their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). The HA, specifically the immunodominant, membrane distal globular head domain, is the major target of influenza virus vaccines (Fig. 1A) [2]. The globular head domain harbors the receptor binding site of the virus and antibodies that block this site prevent the virus from attaching to host cells and therefore neutralize it. Unfortunately, this domain has a very high plasticity [3,4] and the virus escapes the antibody response by introducing point mutations that lead to changes in the major antigenic sites on the head domain, a phenomenon called antigenic drift. Therefore, seasonal influenza virus vaccines need to be updated frequently [5]. The situation is even worse for emerging pandemic viruses for which matched vaccines need to be manufactured, a process that usually takes up to six months during which the population is vulnerable to infection [2]. These problems have led to major initiatives to develop a universal influenza virus vaccine that would protect from all antigenically drifted seasonal influenza viruses, zoonotic influenza virus infections, and pandemic influenza viruses [6,7]. Several conserved targets for such a vaccine have been proposed including the membrane proximal stalk domain (Fig. 1A) of the HA which evolves much slower than the head domain [8]. In fact, antibodies against this domain have been isolated from mice and humans and can neutralize a

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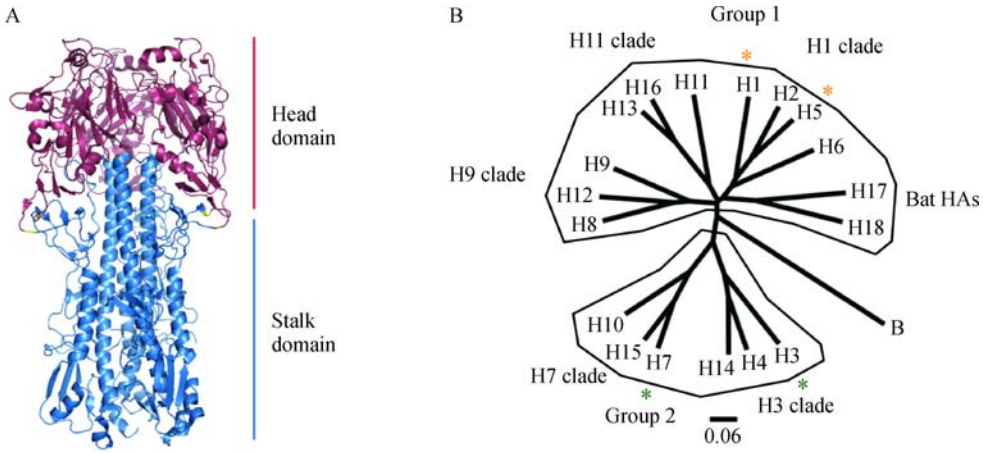

Fig. 1 Structure of influenza A virus hemagglutinin and phylogenetic tree of influenza A and B hemagglutinins. (A) An HA timer with the membrane distal globular head domain visualized in dark red and the membrane proximal stalk domain shown in blue. Cysteines 52 and 277 (H3 numbering), which are the demarcation line between head and stalk, are shown in yellow. The figure is based on PDB # 1TI8 [27]. (B) Phylogenetic tree of influenza A and B hemagglutinins based on amino acid sequence. Influenza A HAs are separated into groups 1 and 2 based on their sequence. HA clades and subtypes are annotated. H1 and H5 (group 1) and H3 and H7 (group 2) are marked by stars. The scale bar represents % amino acid difference. The sequences were assembled in Clustal Omega and visualized in FigTree.

broad panel of influenza virus subtypes and strains. The majority of these broadly neutralizing antibodies bind to either the stalk of influenza A group 1 HAs (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, H18) [9–11] or to the stalk of influenza A group 2 HAs (H3, H4, H7, H10, H14, H15) [12,13] with some notable exceptions that bind to both groups [14–16] or even all influenza A and B HAs [17]. It has been noted that exposure of humans with pre-existing immunity to pre-pandemic seasonal H1N1 to novel influenza virus strains/subtypes from group 1 that feature drastically different head domains, but conserved epitopes in the stalk domain leads to preferential induction of these broadly neutralizing stalk-reactive antibodies. This has been shown for infection and vaccination with 2009 pandemic H1N1 (which has a very different head domain compared to pre-pandemic seasonal H1N1) and for vaccination with H5N1 vaccines [18–25]. These observations have led to vaccine strategies based on chimeric HAs which have "exotic" head domains, but stalk domains from e.g. H1 or H3 HAs. Sequential vaccination with these constructs in animal models also leads to the preferential induction of broadly neutralizing antibodies [26]. While several studies have elucidated the immune response after sequential exposure to divergent group 1 HAs in humans, studies about sequential exposure to group 2 HAs are sparse. Here we will review what is known about the immune response after H7 (group 2) exposure in humans that have been primed by infection and/or vaccination with seasonal H3N2 (group 2) viruses. Lessons learned from these studies might inform further development of group 2 targeted universal influenza virus vaccine candidates.

Pre-existing immunity to H7 HA on a polyclonal and monoclonal level

The human population is constantly exposed to seasonal H3N2 viruses through both vaccination and infection. H3N2 has been circulating in humans ever since it caused a pandemic in 1968 ("Hong Kong Flu") and can cause severe morbidity and also mortality. Therefore, the human population is primed for group 2 HAs. However, sera from human individuals have little or no baseline neutralizing activity to H7 subtype viruses [28–30]. This is not very surprising since most of the immune response induced by H3N2 exposure is directed toward the globular head domain [31] and H3 and H7 globular head domains only share approximately 30% amino acid identity [32]. In addition, H3 and H7 HAs are relatively far apart phylogenetically within group 2 HAs, with H3 being a member of the H3 clade (with H4 and H14) and H7 being a member of the H7 clade (with H10 and H15) (Fig. 1B). However, H3N2 infections (and potentially to a lower degree, vaccination) induces low levels of group 2 stalk-reactive antibodies in humans [28,33]. Of note, these levels are substantially lower than group 1 stalk reactive antibodies, likely because circulation of very different group 1 HA expressing viruses (H1N1, H2N2, pandemic H1N1) has boosted stalk titers against group 1 HAs to higher levels [34,35]. In fact, the reactivity to H7 is lower than most other group 2 or group 1 HA independently of the age group (Fig. 2). Nevertheless, some cross-reactivity to H7 HA has been detected in serum of human individuals [28]. This also translates to the monoclonal level. In a

recent study Henry Dunand and colleagues found that of 83 H3 reactive antibodies isolated from plasmablasts after vaccination only six reacted to H7 [36]. Three of these six antibodies showed neutralizing activity and protected mice from a lethal challenge with H7N9 virus. Interestingly, two of the mAbs also reacted with group 1 HAs while one had a pan-group 2 profile. It is interesting to note that two of the mAbs were isolated after seasonal influenza virus vaccination (containing H1N1, H3N2 and influenza B components) while the third, cross-group mAb was isolated after vaccination with monovalent pH1N1 vaccine. The cross-group mAbs both featured variable heavy (VH) segments from the VH1-18 heavy chain germline [37]. Similarly, low numbers of H7 reactive memory-B cells were found in the same study. While human serum has low reactivity to H7, this reactivity is boosted to some degree after H3N2 infection [28]. However, even then antibody levels remain low. In summary, low levels of antibodies and B cells with specificities to H7 HA exist in humans, and the majority of this reactivity is likely induced by H3N2 exposure and directed to the stalk domain of HA. Of note, recent work by Gostic and colleagues hypothesized that group 2 imprinting by H3N2 infection during childhood could explain protection of certain segments of the population from severe infections and death caused by H7N9 viruses [38]. The authors speculate that this could be driven by antibodies to the group 2 stalk. This is an intriguing thought and while reactivity to H7 HA is very

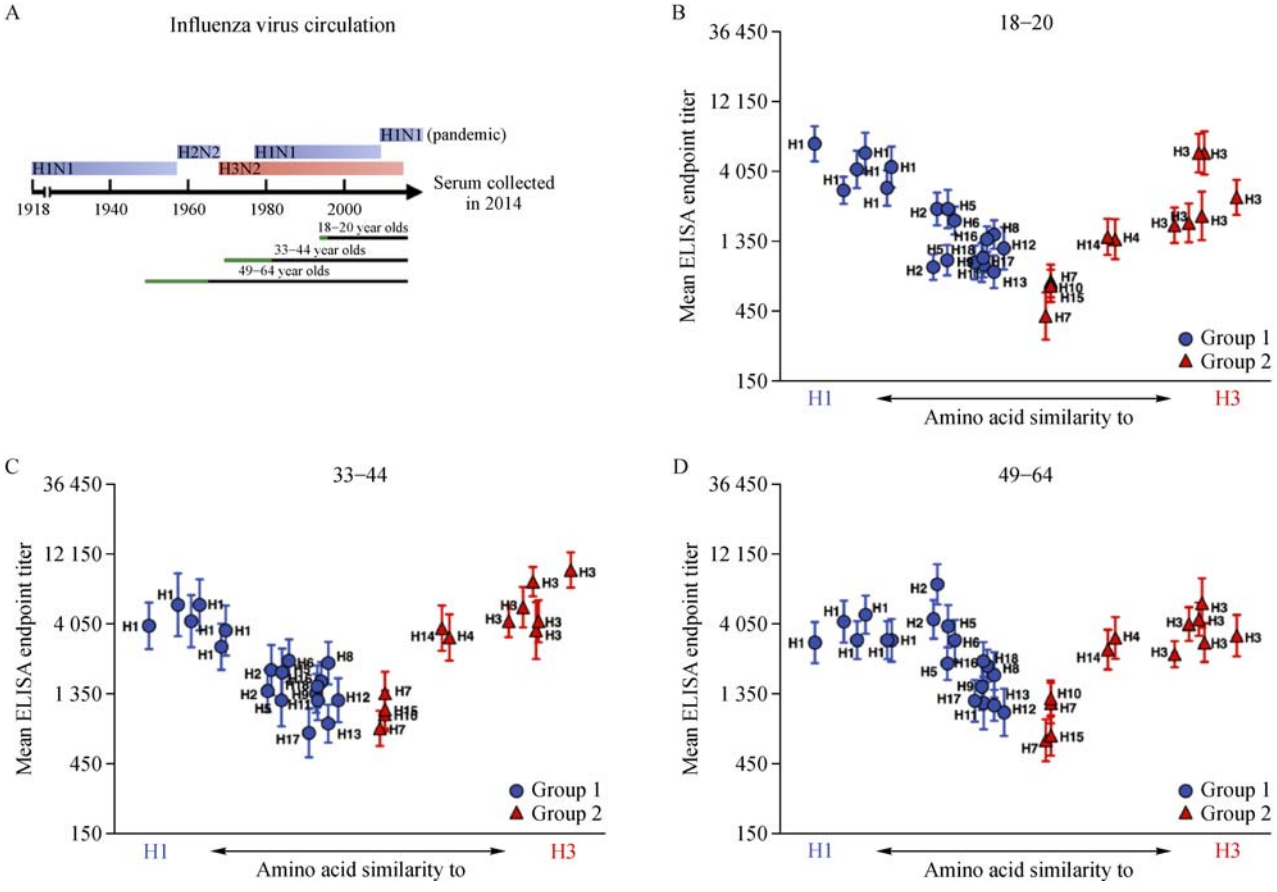

Fig. 2 Reactivity of human sera of different age groups to diverse influenza virus HAs. (A) Human serum samples from three different age cohorts were tested by ELISA against recombinant HA proteins including all influenza A subtypes. The birth ranges for each cohort are indicated in green for 18 – 20 year olds (n = 30), 33 – 44 year olds (n = 30) and 49 – 64 year olds (n = 30). The blue bars indicate circulating group 1 viruses and the red bars indicate circulating group 2 viruses. Serum samples were collected in 2014. (B–D) The ELISA endpoint titers are shown on the y-axis. Each point indicates the geometric mean titer of 30 individuals. The error bars show the 95% confidence intervals. Blue circles show group 1 HAs and red triangles show group 2 HAs. The x-axis indicates the difference of the analyzed HAs to both H1 (A/New Caledonia/20/99; NC99) and H3 (A/Philippines/2/1982; Phil82). The percent similarities for each strain were calculated and the percent difference to Phil82 was subtracted from the percent difference to NC99 for each HA. This resulted in an alignment that shows HAs more closely related to H1, but more distantly related to H3 on the left side and vice versa. HAs that are distantly related to both H1 and H3 are shown toward in the middle of the graph. (B) Sera from 18 to 20 year olds. (C) Sera from 33 to 44 year olds. (D) Sera from 49 to 64 year olds. Figures are adapted from Ref. [28].

low in all age groups (including those likely first exposed in life to H3N2; Fig. 2) this phenomenon might be driven by memory B cells for which antibody products are not readily detected in serum.

Breadth of the polyclonal immune response after H7 vaccination

Before H7N9 emerged in 2013 [39,40], very few vaccine trials with H7 HA had been conducted. The general consensus from these trials was that H7 HAs are of very low immunogenicity, even lower than H5 HAs [29,41,42]. Similar observations have been made with H7N9 vaccines. However, the use of adjuvants [30], virus-like particles [43], or heterologous prime-boost regimens [44,45] improved the immunogenicity but results still lagged behind what is typically observed for seasonal influenza virus vaccines. Importantly, the typical readout used in these trials is the hemagglutination inhibition (HI) assay. In addition, microneutralization (MN) assays are now used to assess the induction of H7 specific immunity, but results from these assays — unlike results from the HI assay— are not accepted by regulatory agencies as "surrogates" or "correlates" of protection. The HI assay only detects antibodies against the globular head domain, while the MN assay detects mostly antibodies against the head domain but might detect stalk-reactive antibodies as well, when they are present at high levels. Both assays usually miss the induction of binding but non-neutralizing antibodies as well as levels of neutralizing antibodies below the limit of detection. Importantly, these types of antibodies might still provide protection, e.g. through effector functions, as demonstrated by several studies [46–49]. Enzyme-linked immunosorbent assays (ELISAs) were used to assess the immune response in only very few studies. These assays detect all antibodies that bind a certain HA, including antibodies that are not detected in HI and MN assays. A study based on an H7N1 inactivated vaccine prime-boost regimen found strong cross-reactivity induced by ELISA, while HI or MN activity was negligible [50]. The antibody response also extended to H15 and an induction of group 2 stalk-reactive antibodies was detected (albeit at low levels). An induction of antibodies to seasonal H1 HA was not detected. Another study examined the antibody response in humans vaccinated twice with an H7N7 live attenuated vaccines (LAIV) followed by an H7N7 inactivated vaccine (IIV) [51]. An antibody response against both the H7 head domain as well as the stalk domain was detected by several methods including analysis of memory B cell frequency and by mapping via a phage library. The memory B cell frequency against pandemic H1 HA was also measured but no significant increase was detected. From these limited data it can be concluded that H7 vaccines induce detectible levels of group 2 stalk-reactive antibodies.

Breadth of the polyclonal immune response after H7N9 infection

So far, two studies have analyzed the breadth of the antibody response after H7N9 infection in humans. In an early paper in 2014 Guo and colleagues analyzed sera from samples from 21 patients and found an increase of H7 binding antibodies, HI and MN titers post H7N9 infection [52]. Interestingly, they also detected an increase in H3 and pandemic H1 antibody binding by ELISA. The increase was higher and longer lasting for H3, which would be expected since it can be hypothesized that H7 exposure in H3 experienced individuals would boost stalk-reactive antibodies. The induction of antibodies to pandemic H1 HA however is highly unusual. A second, more detailed study that examined sero-reactivity of 18 individuals infected with H7N9 was recently published [53]. Liu and colleagues tested binding to all HA subtypes and found that H7N9 infection boosted binding antibody titers (measured by ELISA) against group 1 and group 2 HAs (but not influenza B virus HA). As expected, overall stronger induction (and higher absolute titers) was observed for group 2 HAs but reactivity against several group 1 HAs, specifically pandemic H1, was strongly induced as well. Of note, this was also reflected in neutralization titers which increased against many subtypes including pandemic H1N1.

These are important findings since they suggest that infection with H7N9 induces a much broader immune response than infection with H3N2 or even vaccination with H7 vaccines, which induce some group 2 specific stalk antibodies on a polyclonal basis. The response was comparable to the primary response to infection with pandemic H1N1 in humans previously exposed to seasonal HIN1, which can also trigger cross-group HA responses [28]. A direct comparison with the immune response after H5N1 vaccination cannot be made since only very limited data from H5N1 survivors exists [54].

It is important to keep in mind that the analyzed cohorts typically consisted of elderly individuals who were likely exposed to influenza viruses many times. In addition, many of the H7N9 infected individuals frequented wet markets, which increases the chance that they were exposed to avian influenza viruses of different subtypes in the past and had therefore a skewed immune response already. Finally, most of these individuals suffered from co-morbidities which might have influenced their immune responses in unknown ways.

Analysis of the monoclonal immune response after H7N9 vaccination

Three studies have so far analyzed the monoclonal antibody response to H7N9 vaccination. An initial study

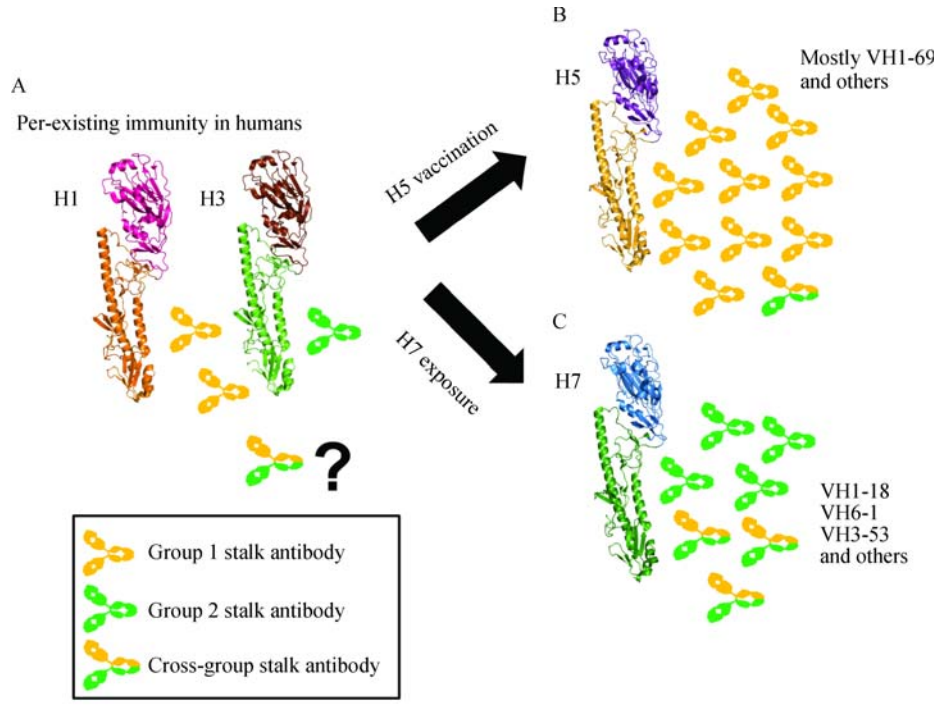

Fig. 3 The immune response of human individuals with pre-existing H1 and H3 immunity to H5 vaccination or H7 exposure. Hemagglutinins (H1, H3, H5, H7) are shown as monomers (based on PDB # 1TI8) for simplicity. (A) Low levels of pre-existing anti-stalk immunity to H1 (pink head domain, orange stalk domain, group 1 HA) and to H3 (brown head, green stalk, group 2) exists in the human population. Typically anti-group 1 HA stalk antibody (yellow) levels are higher than anti-group 2 HA stalk antibody (green) levels. The baseline level of cross-group stalk antibodies (yellow and green) is unclear and likely very low. Antibodies binding to the globular head domain are not shown. (B) Vaccination with H5 HA (purple head, orange stalk) leads to a strong increase of mostly group 1 reactive stalk antibodies (biased toward the VH1-69 germline) and very few cross-group stalk antibodies. (C) Exposure to H7 HA (blue head, green stalk) induces fewer anti-stalk antibodies overall but a relatively larger proportion of cross-group reactive stalk antibodies (focused toward VH1-18, VH6-1 and other germlines).

analyzed mAbs derived from memory B cells of 12 donors who had received an inactivated split H7N9 vaccine (adjuvanted with MF59 or AS03) twice. Twenty hybridomas were obtained and 11 mAbs were further characterized [55]. Eight mAbs showed binding that was restricted to H7 HAs, with 5 of them having classical HI activity. Three mAbs showed broader binding activity with two binding broadly to group 2 HAs and one binding to both group 2 and group 1 HAs. One of the group 2 cross-reactive mAbs showed strong neutralization against H3N2 while the other two seemed to be non-neutralizing antibodies, with the caveat that neutralizing activity was only tested up to a concentration of 2 μg/mL. This concentration might not be in the range of many anti-stalk antibodies which are typically less potent neutralizers as compared to HI-active antibodies [56,57]. Another study analyzed mAbs isolated from plasmablasts after a vaccination regimen that included two H7N9 LAIV inoculations followed by an inactivated H7N9 vaccine [46]. Of 12 analyzed mAbs, eight showed specificity (albeit mostly broad) for H7 and had HI and/or MN activity. One of these mAbs, an HI active antibody, cross-reacted to H15. Three mAbs had no neutralizing activity but showed broad binding to group 2 HAs, and in two cases to group 1 HAs. While these mAbs did not neutralize, they were still capable of protecting mice from a lethal challenge with H7N9 virus. The last antibody showed no HI activity but was neutralizing, and was identified as bona fide anti-stalk mAb. It bound strongly to group 2 HAs as well as pandemic H1 HA (and with lower affinity to some other group 1 HAs). Of note, this mAb featured a VH from the VH3-53 germline (see below). A third, very recent study [58] reported mAbs derived from memory B cells from a vaccine trial that tested H7 DNA prime-H7N9 inactivated vaccine boost vaccination regimens (with H7N9 inactivated vaccine prime-boost regimens or DNA plus inactivated vaccine twice as controls) [45]. Cells were obtained from all three vaccine groups and the authors stated that no difference was found between the groups. Memory B cells were sorted for reactivity to H7 and other HAs. The authors reported that the overall amount of cross-reactive cells was lower for H7 as compared to an H5 trial with a similar setup. However, while H5N1 vaccination induced approximately 50% group 1 stalk-reactive antibodies and very

little cross-group reactivity, the percentage of cross-group reactive antibodies was close to 25% in the H7 vaccinees. The response after H5N1 vaccination was skewed toward the VH1-69 germline, which is heavily over-represented in group 1 stalk-reactive antibodies. After H7N9 vaccination the response was skewed toward a VH1-18 response. In this study, several mAbs were cloned out and analyzed for signatures. mAbs with a VH1-18 germline, or a VH6-1 germline typically showed cross-group reactivity. However, while VH6-1 mAbs neutralized both H1N1 and H3N2, VH1-18 mAbs either neutralized H1N1 or H3N2 (more often). Another signature found were VH3-53 mAbs, which showed pan group 2 activity and neutralized H3N2, but also had an affinity toward pandemic H1 HA (with no binding to seasonal H1 and very little binding to other group 1 HAs). Interestingly these VH1-18 and VH3-53 mAbs with similar signatures had also been isolated in earlier studies from plasmablast responses to seasonal trivalent, pandemic H1N1 and H7N9 vaccines [36,46].

These data are highly interesting and suggest that the human immune system is capable of reacting to H7 exposure with both group 2 specific as well as cross-group anti-stalk responses. Of note, some bona fide group 2 stalk antibodies (VH3-53) also seem to have an unusual, selective affinity for pandemic H1— a signature that was also detected in the polyclonal response after H7N9 infection.

Conclusions

Based on the limited data available it becomes clear that the human immune response to H7 HA is — like the response to H5 HA — strongly influenced by H1 and H3 primed pre-existing memory-B cells that have specificities for conserved epitopes which are shared between human and avian influenza viruses. While H5N1 vaccination seems to produce strong responses of stalk antibodies that cross-react between group 1 HAs (with a skewed, VH1-69 signature), exposure to H7 HA seems to trigger a slightly different response. H7 vaccination, as analyzed so far, seems to induce a polyclonal serological response that mostly targets H7 and other group 2 HAs with a substantial proportion of group 2 stalk antibodies present. H7N9 infection triggers a polyclonal serum response that is much broader than the one induced by vaccination and includes induction of antibodies against group 2 and group 1 HAs with a notable reactivity to H1. On a monoclonal level, broadly reactive mAbs with VH1-18 signatures can be isolated from plasmablasts even after vaccination with seasonal trivalent inactivated or monovalent pH1N1 vaccine. Plasmablast responses after H7N9 vaccination also reveal that neutralizing (VH3-53) and non-neutralizing broadly protective antibodies are induced. Similarly, analysis of the memory B cell compartment after H7N9 vaccination revealed broadly binding and neutralizing mAbs, in many cases spanning group 1 and group 2 (VH6-1, VH1-18) or with pan-group 2 plus pH1 reactivity (VH3-53). After infection and on a cellular, monoclonal level these responses seem to be much broader than the VH1-69 dominated group 1 stalk response after H5N1 vaccination. Currently, it is unclear why there is a difference in the response to H5 as compared to the response to H7. The stalk structure of group 1 and group 2 HAs is slightly different and the positions of conserved glycans differ as well. It might be that germlines like VH1-18, VH3-53 and VH6-1 have structure/sequence constellations that facilitate interactions with the H7 (group 2) stalk while binding is not well supported by the VH1-69 germline.

It is important to realize that both plasmablast and memory B responses — on which the current data are mostly based — are not necessarily reflective of long-term serum antibody responses that are driven by long lived plasma cells in the bone marrow. Just because a memory B cell or plasmablast response produces antibodies with broad specificities does not necessarily mean that the same cells (or clonally related cells) will migrate to the bone marrow to become long lived plasma cells that provide constant levels of serum antibody. Additional serological analysis of H7N9 vaccine trials (including heterologous prime-boost regimens and adjuvanted vaccination) are needed to reach a better understanding of the long-term serum response. Nevertheless, these data — although based on only a handful of studies — suggest that a group 2 stalk antigen might be a viable option to induce broad protection against group 2 HA expressing viruses including H3N2, H7N9 and H10N8. These limited data also suggest that a very strong stalk-based immunity induced by a group 2 construct might provide some protection against group 1 HA expressing viruses, specifically pH1N1.

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Compliance with ethics guidelines

Daniel Stadlbauer declares no conflict of interest. Raffael Nachbagauer is named as inventor on patent applications filed by the Icahn School of Medicine at Mount Sinai regarding influenza virus vaccines. Philip Meade declares no conflict of interest. Florian Krammer is named as inventor patent on applications filed by the Icahn School of Medicine at Mount Sinai regarding influenza virus vaccines. 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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