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Novel universal influenza virus vaccine approaches

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Abstract

Seasonal influenza virus vaccines have to be re-formulated and re-administered on an annual basis due to antigenic drift of the influenza virus surface glycoproteins. In addition, seasonal vaccines show limited efficacy against novel pandemic influenza virus strains, and producing tailored vaccines for these strains in a timely manner is challenging. Several novel broadly protective vaccine candidates targeting the conserved stalk domain of the viral hemagglutinin have been developed. Here we review these novel constructs and discuss several important findings and considerations regarding the protective efficacy of stalk-based vaccines.

Introduction

Influenza virus infections cause significant morbidity and mortality worldwide [1]. Current influenza virus vaccines provide good protection against disease but have to be re-formulated and re-administered on an annual bases due to antigenic drift of the virus [2]. This antigenic drift is caused by human herd immunity, which is mostly directed against the globular head domain of the viral hemagglutinin (HA, Figure 1). Antibodies against this immunodominant, membrane distal HA head domain are potent neutralizers of the virus. However, the high plasticity of the head domain [3] makes it easy for the virus to escape immune pressure. The membrane proximal stalk domain of the HA (Figure 1) is more conserved and antibodies that target this domain have been shown to broadly neutralize influenza viruses across several subtypes [4–12]. Unfortunately, the stalk domain is immunosubdominant compared to the head domain and is usually not targeted by the immune system following exposure to influenza virus vaccines. In the past it has been difficult to design vaccines that target the stalk domain due to the immunosubdominant and fragile nature of the conformational epitopes to which most neutralizing anti-stalk antibodies bind.

Stalk-based vaccine approaches

Two major strategies to induce stalk-based immunity have been developed so far. The first focuses on removal of the entire immunosubdominant head domain to construct headless

HAs [13]. (Table 1) Graves and colleagues recognized in the 1980s that the HA2 subunit (which forms the majority of the stalk domain) is more conserved than the HA1 subunit (which includes the globular head domain) [14]. In order to unmask the HA2 on the viral surface they treated virus preparations with acid (to induce a post-fusion conformation) and then removed the HA1 using a reducing agent [15]. Unfortunately, this treatment most likely destroyed the conformational epitopes which can induce neutralizing anti-stalk antibodies. In the 1990s, the first anti-stalk antibody, mAb C179 was isolated [8], and cells expressing a construct including the HA2 domain were used as immunogens in mice providing partial protection against heterosubtypic H1N1 challenge [16]. Steel and colleagues expressed their headless HA construct on virus-like particles and achieved homologous protection [17]. A construct based on the same design but expressed as soluble protein in insect cells showed full homologous and partial heterosubtypic protection following challenge of vaccinated mice [18]. Several other constructs were developed and provided protection against viral challenge in the mouse model [19–21]. However, the structural integrity of these constructs with respect to complex, conformational stalk epitopes was most likely suboptimal. Lu and colleagues improved on these constructs using an iterative design process and a cell free expression platform [22]. They were the first to show binding of broadly-neutralizing stalk mAbs to their immunogen (in an ELISA). However, animal studies with this headless HA construct have not been published. Recently, Yassine *et al.* and Impagliazzo *et al.* independently reported stable, correctly folded headless immunogens [23,24]. Interestingly both groups used a similar strategy to stabilize their respective stalk structures. Removal of the globular head domain exposes an area at the membrane distal part of the stalk at the very end of the HA2 long alpha helix (LAH) that is usually covered by the head domain. In both studies, this membrane distal part of the stalk was stabilized by a trimerization domain. While Yassine *et al.* used an HIV gp41 trimerization domain that was later removed, Impagliazzo *et al.* replaced the upper part of the LAH with a helical leucine zipper trimerization domain (which is present in the final construct). In addition, Yassine *et al.* fused their construct to a bacterial ferritin, which forms nanoparticles. This strategy was chosen to further stabilize the stalk and to make the construct more immunogenic. Structures based on X-ray crystallography and electron microscopy show binding of stalk mAbs to both constructs suggesting that their structure closely resembles the native HA stalk with respect to conformational stalk epitopes. Both constructs induced stalk-reactive antibodies in animal models and protected from challenge with highly pathogenic H5N1 viruses. Interestingly, despite robust protection, neutralizing antibody titers against homologous viruses were low and titers against heterosubtypic viruses were almost undetectable, a finding that will be further discussed below.

The second major strategy seeks to break the immunodominance of the head domain by sequential exposure of the immune system to chimeric HAs (cHAs) [14,25,26] (Table 1). cHAs consist of stalk domains from H1 (group 1), H3 (group 2) or influenza B viruses in combination with head domains of exotic – mostly avian – influenza virus subtypes [27–29]. By sequential vaccination with cHAs that have different head domains but the same stalk domain, it is possible to refocus the immune response towards the (usually) subdominant stalk domain (Figure 2A). This concept has proven successful in mouse and ferret models, using constructs from both group 1 and group 2 HAs [30–35]. Importantly, this strategy

provided complete protection against a heterosubtypic challenge using H5N1, H6N1 or H7N9 viruses [30,31]. cHA vaccination also reduced lung titers in mice challenged with H3N8, H10N7, and non-lethal H3N2 variant viruses [32] and reduced transmission of pandemic H1N1 virus in the ferret model [36]. Typically, three sequential vaccinations with cHAs that have the same stalk domain but different head domains are necessary in naive animals to induce protective immunity. However, humans already have low levels of B-cells and antibodies with specificities for stalk epitopes and are therefore already primed [12,37–40]. This pre-existing immunity is most likely induced by natural infection with influenza viruses and/or influenza vaccinations [41–44]. Therefore, it is likely that the administration of one or two cHA-based vaccines induces high titers of stalk-reactive antibodies. It remains to be tested if these titers will be sufficiently high to confer protection. Clinical studies with pre-pandemic avian influenza virus vaccines provide evidence for this hypothesis. When subtype H5 or H7 vaccines are administered in clinical studies these vaccines significantly boost anti-stalk titers [38,39,45,46]. Importantly, these subtypes have completely different head domains in combination with stalk domains that express conserved group 1 (H5) or group 2 (H7) stalk epitopes. To some extent, this phenomenon of an enhanced stalk response also occurred during the H1N1 pandemic of 2009 [28,40,47–50]. While the 2009 pandemic H1N1 and the pre-pandemic seasonal H1N1 strains both share a highly conserved stalk domain, the HA head domains of the two strains are largely antigenically distinct. In contrast, vaccination with seasonal influenza virus vaccines or repeated administration of the pandemic H1N1 strain results in more narrow and head-specific responses [38,42,51–53].

In addition to headless HA and cHA immunogens, a number of other antigen designs and strategies have been proposed (Table 1). These include the presentation of stalk epitopes on virus-like particles [54,55], peptide based approaches [56,57], strategies that shield the HA head domain from recognition by the immune system [58,59] and heterologous prime-boost strategies [60–64] that consist of a prime with DNA, a live virus-vector or live-attenuated virus followed by a protein or inactivated vaccine boost.

Vaccination with cHA and headless HA immunogens may result in different antibody profiles

The type of antibody response induced by headless and chimeric HAs on a monoclonal level is currently unknown. cHAs are full length HAs that have H1, H3 or influenza B stalk domains combined with 'exotic' globular head domains and can be produced using traditional influenza vaccine production platforms as inactivated or live-attenuated vaccines. Stalk antibodies induced by cHAs will likely bind and affect wild type HAs on virions and infected cells (Figure 2A and B). Antibodies induced against the 'exotic' globular head domains will be irrelevant against currently circulating strains (but may be useful in protecting against pandemic viruses of the matched HA subtype). Headless HA constructs, however, may induce antibodies that bind to the immunogen but not to wild type HA on virions (Figure 2C and D). This scenario is likely since headless HAs expose areas on the top of the stalk domain that are not accessible on wild type HAs because they are covered by the head domain. Furthermore, the absence of the head domain might allow for angles of antibody binding which are not possible in the presence of a head domain on infectious

virus. An immune response against headless HAs might therefore result in the induction of antibodies that do not bind to wild type HAs and therefore cannot contribute to protection.

Stalk neutralization titers are typically low in mice

It has been noted that neutralization titers induced by stalk-based vaccines are low in animal models specifically mice. Although this observation is complicated by the different types of assays used in the studies, low or undetectable neutralization titers appear to be common [18,23,24,30,31,42]. This is in stark contrast to studies in humans, in which stalk-based neutralization titers can be readily measured when individuals have experienced pandemic H1N1 infections or H5N1 vaccinations (both scenarios have shown to stimulate anti-stalk antibodies) [38,39,41,65]. Several reasons may explain these findings. First, the *in vitro* neutralizing potency of stalk reactive antibodies is generally lower than that of head-reactive hemagglutination inhibition (HI) antibodies. As an example, He and colleagues [66] reported a potency difference between murine head and stalk antibodies of 4–6 logs, a finding confirmed by Dilillo and colleagues [67]. This divergence between the potency of murine head and stalk antibodies is not observed for human head and stalk antibodies (Figure 3). The latter are generally more similar with respect to their potency when unbiased data from plasmablast responses are considered [47,68]. For example Li and colleagues report 23 HI active, head reactive and 3 stalk-reactive mAbs recovered from plasmablasts after pandemic H1N1 vaccination. While only 8 (34.8%) of their recovered anti-head mAbs had neutralization IC_{50} values of below 1 ug/ml (with the remaining 15 HI active mAbs having IC_{50} values between 32 and 1 ug/ml) all three recovered anti-stalk antibodies showed IC_{50} values below 1 ug/ml [47]. Second, the serum IgG concentration of human blood is approximately 10 fold higher than that of standard laboratory mouse strains. IgG serum concentrations in human adults range from 4–22 mg/ml serum with averages between 11–12 mg/ml depending on age, ethnicity and gender [69–71]. The level of serum IgG in mice usually used in vaccine studies (6–20 weeks) has been shown to be between 1–2 mg/ml [72,73]. Third, inherent differences in CDR composition and length between humans and mice might influence neutralization potency as well [74]. Based on these considerations, anti-stalk neutralization titers should be readily measured in adult human sera while very high levels of anti-stalk antibody levels would be needed in the mouse model to reach the limit of detection in common microneutralization assays.

Stalk antibodies show enhanced protective potency *in vivo* as compared to *in vitro*

While anti-head antibodies show higher potency than anti-stalk antibodies *in vitro*, it has been demonstrated that the protective effect of both types of antibodies is almost equal *in vivo* in murine challenge models with mouse-adapted and non-mouse-adapted viruses (Figure 3) [67,75,76]. As discussed above differences between head- and stalk-stalk antibodies in *in vitro* potency can be more than thousand fold [66,67,76]. Monoclonal antibody CR9114 shows an even stronger difference and displays no neutralizing activity against influenza B viruses *in vitro*, while affording robust protection against challenge with divergent influenza B strains in the mouse model [75]. This enhancement of potency of anti-

stalk antibodies *in vivo* is most likely caused by Fc-mediated mechanisms like antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) which are not readily measured in (classical) *in vitro* neutralization assays. Recent studies have clearly demonstrated the efficacy-enhancing effect of ADCC on cross-reactive antibodies both on a monoclonal [67] as well as a polyclonal level [77–80]. A possible effect of CDC was also reported *in vitro* [81] but the significance of mediating complement activity for anti-stalk antibodies *in vivo* has not yet been elucidated. In addition to ADCC and CDC, more complex mechanisms like interactions between alveolar macrophages, CD8+ T-cells and antibodies may play a role as well [82]. Finally, it cannot be ruled out that the neutralizing activity of anti-stalk antibodies is enhanced *in vivo* by yet to be described interactions with host defense proteins, specific cell types or by the micro-environment of the lung architecture.

How broad is 'broad protection'?

An important feature of stalk-reactive antibodies is their breadth of binding and neutralization. Typical stalk-reactive antibodies bind to HAs within the phylogenetic group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17 and H18) or group 2 (H3, H4, H7, H10, H14, H15) [7–9,83–85]. Exceptions are mAbs 12D1 [11] and 6F12 [10] which are restricted to one subtype but bind broadly within this subtype and several antibodies that are capable of cross-group binding [6,12,86–88] with one mAb even recognizing influenza B HAs [75]. Polyclonal antibodies induced by stalk-immunogens in mice [23,24,30] or vaccination with pandemic influenza virus vaccines [38,39,45,46] in humans usually follow the same trend with good group 1 cross-reactivity induced by H1 stalks and good group 2 cross-reactivity induced by H3 stalks. Cross-group reactivity at a very low level can be detected but does not provide full protection from challenge [30]. It is important to note that reactivity against different viruses within the same group varies. While there is still considerable cross-reactivity and cross-protection towards members within the same HA group, cross-reactivity in both animals [18,23,24,30,32,33] and humans [38,39] is highest towards the stalks that were used to induce the antibodies. For, example, Impagliazzo and colleagues reported approximately 100–1000 ELISA units (EU)/ml reactivity against various H1 strains, 10 EU/ml against H5 and 0.1 EU against H9 for sera from mice vaccinated with their H1-based stalk construct [24]. Reactivity to group 2 HAs (H3, H7) was close to or below the limit of detection. This makes sense, since stalk epitopes change only slightly from subtype to subtype and affinity maturation will likely result in antibodies that bind most efficiently to HAs that the immune system actually encountered. Nevertheless, lower titers against heterosubtypic HAs can still confer full protection as demonstrated by several studies [18,23,24,30–32]. These data suggests that a vaccine formulation comprised of a group 1 stalk, a group 2 stalk and an influenza B HA stalk component will make an effective universal influenza virus vaccine for humans.

Longevity of broadly protective vaccines

The primary goal for the development of universal influenza virus vaccines is to induce a broadly protective immune response. However, it is crucial that this immune response is long-lived. One of the most significant caveats of current inactivated influenza virus

vaccines is that the immune response they elicit is relatively short lived and may wane over the course of one influenza season [89]. A universal influenza virus vaccine that would induce short-lived immunity could be an appealing tool in case of a new pandemic but would be less useful in protecting against seasonal influenza viruses since it would have to be given annually like current vaccines. It is not well understood why current inactivated influenza virus vaccines induce relatively short-lived immune responses. Natural influenza virus infection can certainly induce lifelong immunity against a specific strain (which is not necessarily an advantage due to antigenic drift) [90]. A better understanding of the difference - in quality and quantity - of the immune responses to vaccination versus natural infection will be very important in order to design a novel generation of vaccines that induce long-lasting immunity. Several strategies to enhance the longevity of the immune response to influenza have been tested and might be suitable for stalk-based vaccines as well [34,91].

Conclusions

The discovery of stalk-reactive antibodies has spurred the development of universal influenza virus vaccine candidates. Several new designs for stalk-based immunogens and vaccination strategies have been proposed and successfully tested in pre-clinical studies. Future clinical trials will show if these vaccine candidates perform well in humans in terms of safety, immunogenicity and efficacy. Along the way we will learn important lessons that will help us in better understanding the mechanisms of immune responses to conserved influenza virus epitopes in humans.

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Conflict of interest

The Icahn School of Medicine at Mount Sinai has filed several patents regarding influenza virus vaccines.

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Highlights

- Two different types of stalk-based universal influenza virus vaccine candidates - chimeric hemagglutinins and headless hemagglutinins - are currently in development
- The two types of vaccines might induce a different antibody profile
- Efficacy of anti-stalk antibodies *in vivo* is high despite low *in vitro* neutralization potential
- Long lasting immunity is important for universal influenza virus vaccines

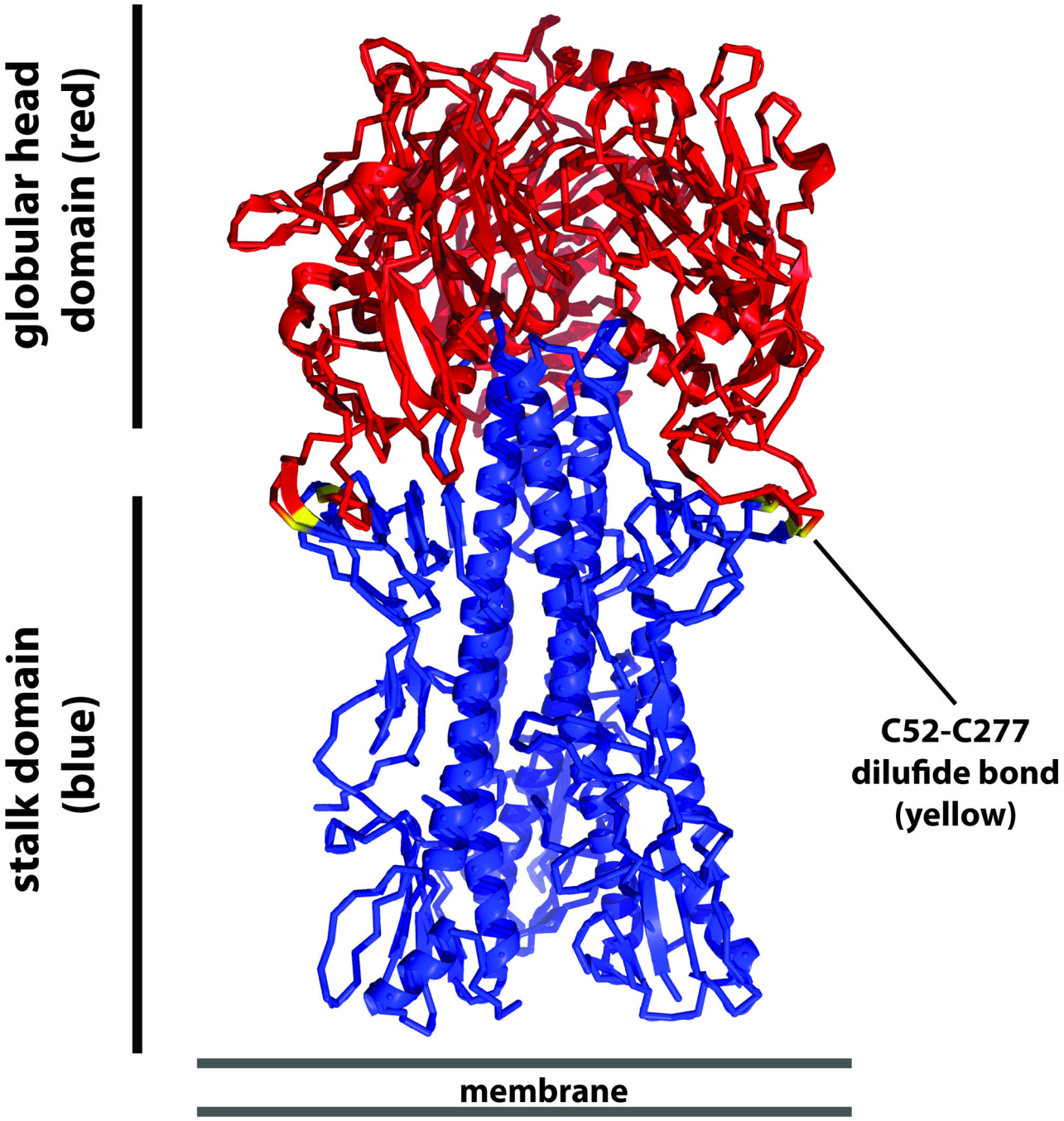

Figure 1. Schematic of the trimeric influenza virus HA

The membrane distal globular head domain is shown in red and the membrane proximal stalk domain is shown in blue. Cysteines 52 and 277 - which form a disulfide bond that demarcates head and stalk domain - are shown in yellow. The schematic is based on the H1 HA of A/PR/8/34 (PDB 1RU7 as described in [92]).

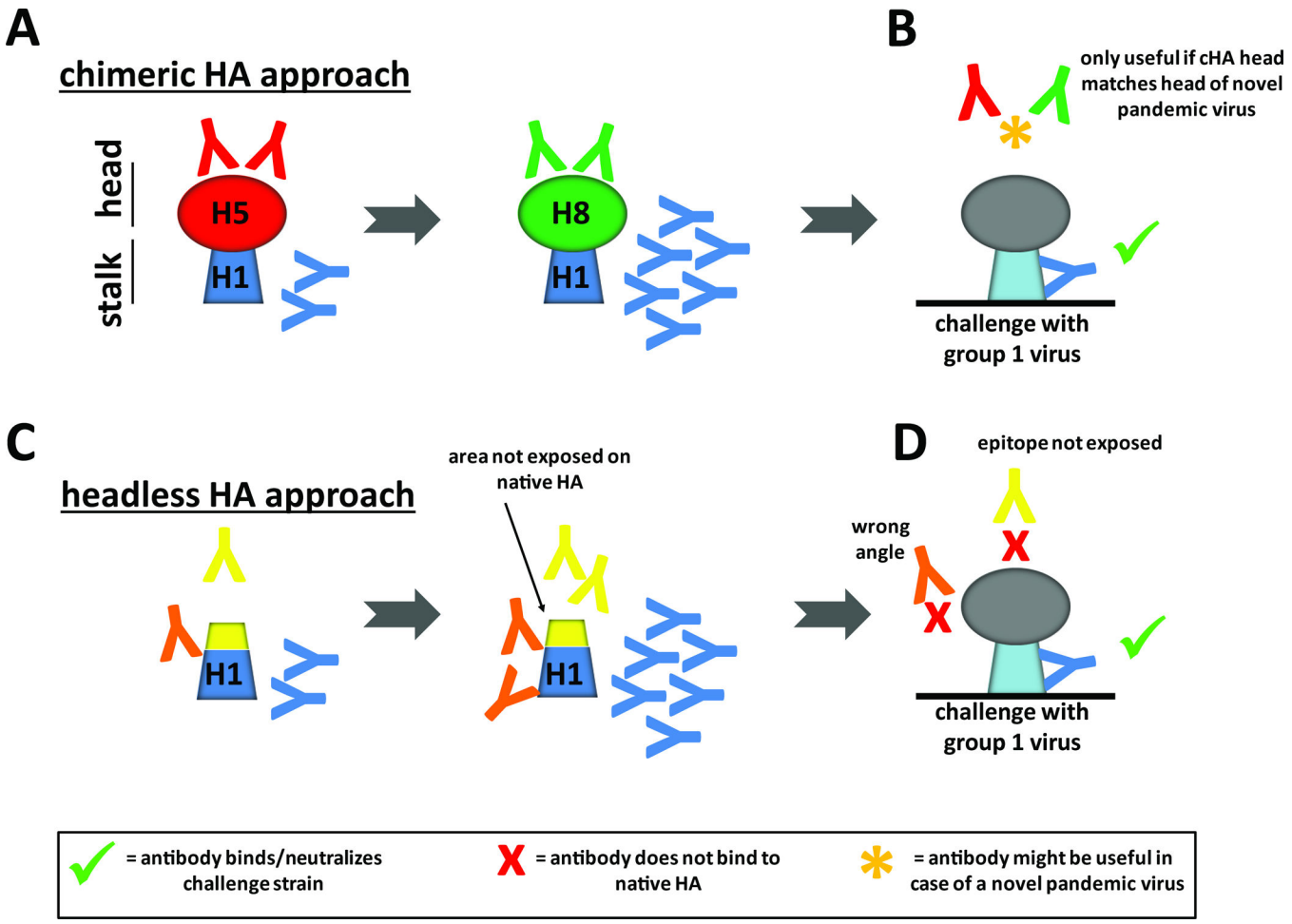

Figure 2. Vaccination with cHA and headless HA immunogens may result in different antibody profiles

A cHA vaccination induces stalk-reactive antibodies by sequential exposure of the immune system to constructs with a conserved stalk domain but divergent head domains (cH5/1 and cH8/1 HAs which share the H1 stalk domain have very different H5 and H8 head domains). **B** Anti-stalk antibodies (blue) generated in response to cHA vaccination bind and neutralize incoming viruses. Since these antibodies were induced by full length HAs they should bind efficiently to stalk epitopes on wild type full length HAs. Low levels of antibodies induced against the different head domains are most likely irrelevant to protection if the head domain of the incoming virus is not matched (red and green). If the head domains of the challenge virus and the cHA constructs match, these head antibodies might be beneficial for protection as well. **C and D** Antibodies induced by headless HA constructs may belong to three different categories: First, antibodies that bind stalk epitopes on headless HA vaccine constructs bind and neutralize wild type HA (blue). Second, antibodies that bind to surfaces on headless HAs are not exposed on wild type HA (yellow). These antibodies might not bind to wild type HA and might not be able to neutralize incoming virus. Third, antibodies that bind to stalk epitopes at angles may be unable to bind to stalk epitopes (due to steric hindrance) when a head domain is present (orange). These antibodies are unable to bind and neutralize incoming virus.

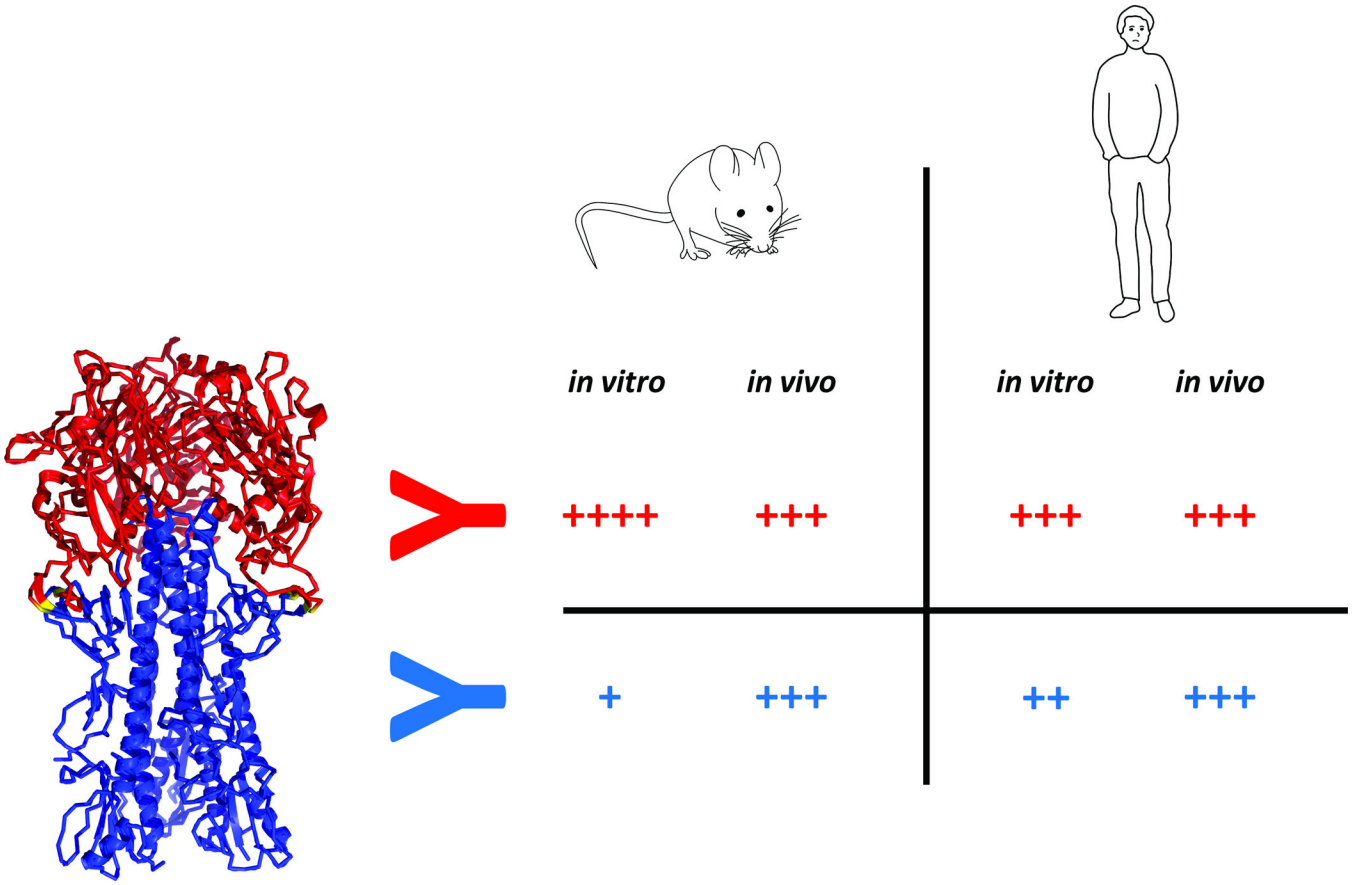

Figure 3. *In vitro* neutralizing potency and *in vivo* protective efficacy for murine and human anti-head and anti-stalk antibodies

Murine anti-head antibodies usually exhibit high potency *in vitro* as well as good protective efficacy *in vivo*. Their *in vitro* IC_{50} is 2–6 logs lower (better) than those of murine anti-stalk antibodies. However, the *in vivo* protective efficacy of murine head and stalk mAbs is similar. On the other hand human head and stalk antibodies behave similar in *in vitro* potency assays with anti-head mAbs performing slightly better. The *in vivo* protective efficacy of both types of human mAbs is similar as well. Anti-stalk antibodies gain *in vivo* potency through Fc-mediated immune mechanisms.

Table 1

Overview of HA stalk-based influenza vaccine approaches

Candidate	Development stage	Key points	References
Headless HA	pre-clinical	removal of globular head domain allows the immune system to focus on the stalk domain; must be expressed recombinantly and cannot be produced using traditional influenza vaccine production platforms	[15–24], reviewed in [13]
Chimeric HA (cHA)	pre-clinical, clinical phase in preparation	sequential presentation of the same stalk domain in combination with exotic head domains breaks the immunodominance of the head domain and refocuses the immune response to the stalk; can be produced using traditional influenza virus vaccine production platforms	[27,29–33,35,36]
Glycan shielding	pre-clinical	hyperglycosylation of the globular head domain shields it from the immune system	[58,59]
Prime-boost strategies	clinical	have been developed to increase the efficacy of seasonal, H5 and H7 influenza virus vaccines but have also been shown to broaden the immune response	[45,60,61,63,64]
Peptides	pre-clinical	allow the immune response to focus on the epitope of choice without distraction by the globular head domain; might not capture the right conformation of complex structural epitopes	[56,57]
VLP-based approaches	pre-clinical	present key epitopes on the surface of immunogenic VLPs, might not capture the right conformation of complex structural epitopes	[54,55]