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Influenza virus hemagglutinin stalk-based antibodies and vaccines

Florian Krammer[1] and **Peter Palese**[1,2]
One Gustave L. Levy Place, New York, 10029 NY, USA

[1]Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[2]Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Abstract

Antibodies against the conserved stalk domain of the hemagglutinin are currently being discussed as promising therapeutic tools against influenza virus infections. Due to the conservation of the stalk domain these antibodies are able to broadly neutralize a wide spectrum of influenza virus strains and subtypes. Broadly protective vaccine candidates based on the epitopes of these antibodies, e.g. chimeric and headless hemagglutinin structures, are currently under development and show promising results in animals models. These candidates could be developed into universal influenza virus vaccines that protect from infection with drifted seasonal as well as novel pandemic influenza virus strains therefore obviating the need for annual vaccination, and enhancing our pandemic preparedness.

Introduction

Seasonal influenza virus infections are a major public health concern and cause significant morbidity and mortality every year worldwide. In addition, novel influenza viruses are introduced into the human population occasionally. If there is no pre-existing immunity against these strains in humans they have the potential to cause severe pandemics. Human cases of avian H7N9 in China in early spring 2013 clearly demonstrated the threat by these zoonotic viruses [1]. Influenza virus vaccines are so far the best preventive countermeasure against influenza virus infections. These vaccines induce antibodies that block the binding of the hemagglutinin (HA), the major surface glycoprotein of the influenza virus, to sialylated host cell receptors [2]. By blocking this step the virus is unable to attach to cells and the virus life cycle is interrupted early on. Antibodies with this function have very potent neutralizing activity and can readily be measured in serum using the hemagglutination inhibition (HI) assay [3]. These antibodies are directed against the membrane distal part of the HA, the globular head domain (Figure 1A, B and D) [4-6], which has a high plasticity and is subject to constant antigenic drift driven by human herd immunity. Therefore, antibodies that target this domain (HI-active antibodies) are very strain specific and quickly lose efficacy against drifted strains. Due to this viral mechanism to escape human herd immunity influenza virus vaccines have to be reformulated almost annually based on surveillance data from laboratories in the Northern and Southern hemispheres [7]. Vaccine

Florian.Krammer@mssm.edu and Peter.Palese@mssm.edu

strain prediction based on surveillance is a complicated and error prone process and produces mismatches between circulating virus and vaccine strains from time to time which leads to a severe drop in efficacy of the seasonal influenza virus vaccines [8-12]. The fact that the vaccines need to contain three to four components (an H1N1, an H3N2 and one or two influenza B virus strains) adds to the complexity of the process [7]. Furthermore, seasonal vaccines usually lack efficacy against pandemic or potential pandemic viruses which have divergent globular head domains. In the case of a new pandemic virus, vaccines have to be manufactured rapidly. However, it usually takes several months from the identification of the right vaccine seed strain to the delivery of the vaccine for use in the population. In 2009 during the H1N1 pandemic the first batches of vaccine were delivered in the US after the first pandemic wave hit - and came therefore too late [13].

The membrane proximal stalk domain of the HA is, in contrast to the head domain, conserved among group 1, among group 2 and among influenza B viruses (Figure 1E). It is composed of the N- and C-terminal parts of HA1 and the N-terminal part of the HA2 subunit; the demarcation line between stalk and head domain is formed by two cysteine residues (amino acids 52 and 277, H3 numbering) that form a disulfide bond (Figure 1A, B and D) [14,15]. Functionally, the stalk domain mediates the fusion of viral and endosomal membranes once the virus is taken up into the cell; this function is crucial for the release of the viral genome into the cytosol [2]. During the process of fusion the stalk domain has to undergo extensive structural re-arrangements. These re-arrangements are essential for viral replication and mutations that interfere with this function are not tolerated. The plasticity of the stalk domain is therefore limited which explains its conservation and reveals a weak point of the virus that can be targeted by broadly-neutralizing antibodies, universal vaccine strategies and perhaps also by antiviral drugs.

Hemagglutinin stalk-reactive monoclonal antibodies

Antibodies against the stalk domain are generally not induced by vaccination with inactivated influenza virus vaccines and these types of antibodies are rare in humans [4,5]. The first antibody against the stalk domain, mouse monoclonal antibody (mAb) C179, shows a broad binding and neutralization profile against multiple group 1 HA expressing viruses and was isolated in 1993 [16] (Figure 1C). Recently, a number of mAbs against the stalk domain have been isolated from humans and mice and many of them show broad neutralizing activity against a variety of influenza virus strains and subtypes. The vast majority of stalk reactive antibodies (including CR6261, F10, FI6 and C179) share a common footprint on the stalk domain [17-21]. The epitope recognized by these antibodies is conformational [22] and involves HA1 as well as HA2 residues [17-21]. Most of these antibodies bind broadly and neutralize one (e.g. 6F12) or more (e.g. CR6261, F10, C179, KB2 or GG3) members of the group 1 HAs (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, Figure 1E) [17-21,23,24]. Two stalk-reactive antibodies, FI6 and 05-2G02, that cover both members of group 1 and group 2 HAs (H3, H4, H7, H10, H14, H15, Figure 1E) have been isolated as well [20,25]. In addition, mAb CR9114 is also able to broadly bind (but not neutralize) divergent influenza B virus HAs [26]. Many of these antibodies like F10, CR6261 and CR9114 are derived from the human germline $VH_{1\text{-}69}$ [17,19,26]. The fact that some of these antibodies make contacts only with their heavy chains might reflect the library based approach through which they were obtained [17-19].

The broadly-neutralizing group 2 antibody CR8020 has a distinct conformational epitope on the membrane-proximal part of the stalk domain. It almost exclusively makes contact with the short alpha helix of the HA2 subunit and is so far the only described antibody that binds to this epitope [27]. Yet another neutralizing epitope is located on the long alpha helix of the HA2 and is recognized by pan-H3 mAb 12D1 [28]. Although 12D1 recognizes its epitope

under denaturing conditions it is believed that the epitope is indeed microconformational [28]. In addition weakly or non-neutralizing epitopes in the stalk domain have been described [29-33]. So far only one stalk-reactive mAb that broadly neutralizes influenza B viruses, 5A7, has been identified [34].

In contrast to HI active antibodies which mainly work through inhibition of viral attachment to sialylated host cell receptors stalk reactive antibodies employ various mechanisms of direct and indirect neutralization. By binding to the stalk domain of the HA of incoming virions [35] many of these antibodies inhibit pH induced conformational changes of the HA in the endosome [17,23,27,28] (Figure 2). This mechanism prevents fusion of endosomal and viral membranes and the release of genomic material into the cytosol. HA on virus particles has to be cleaved into HA1 and HA2 subunits in order to yield infectious particles. The footprint of most stalk-reactive antibodies covers the protease cleavage site between HA1 and HA2 and antibody bound to the HA therefore prevents HA maturation [17,20,27]. This extracellular mechanism of action works for most influenza viruses. Strains that possess HAs with multibasic cleavage site however are cleaved intracellularly by furin-like proteases. In this case the HA is inaccessible to antibodies prior to cleavage [36]. Furthermore antibody-dependent cell-mediated cytotoxicity (ADCC) and complement mediated cytotoxicity have been shown to be involved as well [37-39]. Direct virus lysis by complement, as shown for HIV, can also not be excluded as a potential mechanism [40].

Stalk-reactive antibodies as therapeutic measures for influenza virus infections

Neuraminidase and M2-ion channel inhibitors are excellent therapeutics for treatment of influenza virus infections. However, resistant virus variants arise quickly in immuno-compromised patients and broadly-neutralizing stalk-reactive antibodies could be used as therapeutic treatment in these cases [41-47]. A number of stalk-reactive antibodies have been tested extensively in the mouse model and were able to show high efficacy even at low doses [23,27,48]. As an example, mouse mAb 6F12 was shown to be efficacious against H1N1 viruses spanning a period of almost one hundred years and was able to protect mice from morbidity when delivered even 5 days post infection at a 30 mg/kg dose [23]. Protective doses in mice can be as low as 1 mg/kg which is comparable to HI active anti-head mAbs [23,26,27,49]. It is of note that mAb CR9114, which has no *in vitro* neutralizing activity against influenza B viruses, is able to potently protect mice from *in vivo* challenge with influenza B virus [26]. As mentioned above this indicates that stalk-reactive antibodies employ alternative mechanisms of protection as well. MAbs CR6261 and 6F12 have also been evaluated in the ferret model of influenza disease and were both shown to efficiently inhibit viral replication [50,51]. Antibodies CR6261 and CR8020 are currently being tested in human clinical trials at doses of 2 to 50 mg/kg [52,53]. Broadly neutralizing stalk reactive antibodies might therefore become valuable additions to our therapeutic countermeasures against severe cases of influenza virus infection.

Induction of stalk-reactive antibodies by influenza viruses in nature

Stalk-reactive antibodies are not [4-6], or very rarely [18,54], found in individuals vaccinated with seasonal inactivated influenza virus vaccines. Similar findings were reported in the mouse model for which vaccination of naive animals with inactivated or DNA vaccine did not induce detectible levels of stalk reactive antibodies [6,55]. However, these antibodies are detected at low levels following natural infection with seasonal H1N1 and H3N2 influenza virus strains in humans as well as in mice [6,55,56]. Background levels of stalk-reactive antibodies against group 1 and group 2 HAs are found in many individuals, probably due to natural infection [6,56-58]. In stark contrast with these findings infection of

individuals with 2009 pandemic H1N1 virus induced high levels of stalk-reactive antibody responses [56,59]. We hypothesize that induction of stalk reactive antibodies is driven in these individuals by the exposure to a hemagglutinin which has a conserved stalk but a highly divergent H1 head domain (Figure 3). Individuals who were exposed to seasonal, pre-pandemic H1N1 virus mainly developed responses against the immunodominant globular head domain of the HA and only very low levels of B-cells with specificity for the immunosubdominant stalk domain were induced. After exposure to the 2009 pH1N1 virus, which has a divergent, roughly 30% different globular head domain, these B-cells with specificity for the conserved H1 stalk domain were boosted. This scenario could readily be reproduced in the mouse model [55]. The 2009 pandemic H1N1 strain completely replaced the pre-pandemic seasonal H1N1 strains circulating in the human population before 2009. Interestingly, the H2N2 pandemic virus from 1957 also replaced the H1N1 strains circulating prior to 1957. Both of these viruses express group 1 HAs with divergent head but conserved stalk domains. We therefore hypothesize that the increase of stalk reactive antibodies in a population who are sequentially exposed to two viruses with divergent heads but conserved stalks is a mechanism that drives elimination of the earlier strain [60]. The extinction of human H2N2 strains in 1968 after the introduction of H3N2 strains into the population might have been caused by a slightly different but related mechanism where antibodies against epitopes on the more conserved NA were boosted [60,61].

The described mechanism can also be applied to vaccines. Humans who received inactivated adjuvanted or non-adjuvanted pH1N1 vaccines also produced increased levels of stalk-reactive antibodies [25,48,62]. Furthermore, we could show that vaccinees who received the H1N1 A/New Jersey/76 (NJ76) swine flu vaccine had higher levels of stalk reactive antibodies compared to individuals who did not receive this vaccine [57]. Again, the NJ76 H1N1 strain expressed an H1 HA that had a conserved stalk domain but a very divergent globular head domain as compared to seasonal H1N1 viruses circulating in humans.

In conclusion low levels of stalk reactive antibodies, mostly induced by natural infection, are present in the human population. These antibodies can be boosted under special conditions when pandemic viruses with divergent, novel globular head domains but conserved stalk domains are introduced into the population. The resulting boost of stalk-reactive antibodies constitutes a mechanism that might drive elimination of pre-pandemic seasonal viruses that express HAs from the same HA group.

Stalk-based universal influenza virus vaccine candidates

Approaches to create broadly protective vaccines based on conserved parts of the HA by removal of the immunodominant head domain were first reported in 1983 [63] (Figure 4A and E). However, these vaccine constructs were prepared using acid treatment combined with a reducing agent and neutralizing conformational epitopes were most likely denatured which explains the absence of efficacy of these preparations. Later on recombinant DNA technology made it possible to express the immunosubdominant HA2 subunit of the HA in bacteria [64]. Vaccine candidates based on these constructs partially protected mice from challenge. However, most neutralizing epitopes in the stalk domain cover the HA1 as well as the HA2 part of the stalk. A stalk-only structure that covers the HA1 and the HA2 part of the stalk domain - a ‘headless’ HA - was expressed successfully by Steel and colleagues by taking advantage of the naturally occurring disulfide linkage between cysteines 52 and 277 (H3 numbering, Figure 4B and F)[15]. Replacement of the globular head domain with a glycine linker resulted in a headless HA protein that partially protected mice from viral challenge. However, this construct only expressed to low levels in mammalian cells and correct folding of the construct could not be confirmed. Another approach focused solely on the epitope of 12D1, the long alpha helix [28] (Figure 4C and G). Animals vaccinated with

this construct showed good homologous and partial heterologous protection demonstrating the breadth of stalk based vaccine approaches [65]. Furthermore, novel prime-boost strategies have been developed to focus the immune response on conserved regions of the HA [28,66,67]. However, DNA prime - protein boost strategies seem to be mostly limited to responses within one subtype [67]. Another strategy for the induction of broadly neutralizing stalk-reactive antibodies is the display of conserved stalk-epitopes on nanoparticles [68,69]. Similar to DNA prime - protein boost strategies, nanoparticle-based immunogens seem to induce mostly subtype-specific immunity [69].

Recently, we developed a novel approach that mimics the 2009 pH1N1 situation in nature. We constructed chimeric HA molecules (cHA) that combine H1 (group 1) or H3 (group 2) stalk domains in combination with ‘exotic’ head domains (e.g. H4, H5, H6, H7 or H9, Figure 4D and H) [14]. Sequential vaccination with these constructs, which always have the same stalk but divergent heads to which no cross-reactivity exists (measured by HI), boosts antibodies against conserved epitopes in the stalk domain [70]. This vaccination regimen induces high titers of stalk-reactive antibodies in mice and ferrets (Figure 5A) [51,70]. It is able to protect animals from heterologous (divergent H1/H3 strains) as well as from heterosubtypic challenge (challenge with members for the same HA group). Animals vaccinated with cHAs based on the H1 stalk were protected against lethal H5N1 and H6N1 challenges and developed broadly neutralizing antibodies against other group 1 HA expressing viruses (e.g. H2N2) as well [70]. Animals vaccinated with H3 stalk based constructs showed similar broad protection that included challenge with H7N1 and H7N9 strains. It was demonstrated that the protective mechanism is mainly based on broadly neutralizing antibodies but probably includes ADCC and complement as well. In contrast to HA2 or headless HA constructs, cHAs have a globular head domain (as defined by the amino acids between C52 and C277, H3 numbering) in place which guarantees the correct folding of the stalk domain. In fact, viruses expressing these cHAs grow to high titers in embryonated eggs demonstrating the full biological activity of these constructs [14]. Chimeric HA -based vaccines could therefore be produced using various production platforms ranging from novel recombinant technologies to traditional egg-derived inactivated vaccines. A human vaccine based on inactivated influenza viruses that express cHAs and relevant NAs (recent N1 or N2) could therefore boost cross-neutralizing antibodies against the stalk domain as well as the NA which is usually also immunosubdominant in the presence of an immunodominant HA head domain. Since most adult humans already have pre-existing humoral immunity to conserved epitopes in the stalk domain it is expected that one or two immunizations would be sufficient to boost these antibody levels to neutralizing titers (Figure 5B). Importantly, cross-neutralization between group 1 and group 2 HAs was not observed in preclinical studies and it is therefore likely that an effective universal influenza virus vaccine has to include a group 1, a group 2 and an influenza B HA immunogen [70].

Summary and outlook

Broadly reactive antibodies directed against the stalk domain of the HA have been isolated from mice and humans. These antibodies are able to neutralize divergent influenza virus strains and subtypes and are currently being evaluated in clinical trials for their potential use as anti-influenza therapeutics. Vaccines based on conserved epitopes in the stalk domain of the HA are currently in late stage pre-clinical development. Several promising candidates, like chimeric HA immunogens, have been reported recently and show good protection of animals from challenge with divergent viral strains and subtypes. A universal influenza virus vaccine that boosts stalk-reactive antibodies in the population to high, long lasting, neutralizing titers could potentially obviate the need for an annually reformulated vaccine and would substantially enhance our pandemic preparedness.

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References

1. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, et al. Human Infection with a Novel Avian-Origin Influenza A (H7N9) Virus. N Engl J Med. 2013
2. Palese, P.; Shaw, ML. Fields' virology. Lippincott Williams & Wilkins; Philadelphia: 2007.
3. Ohmit SE, Petrie JG, Cross RT, Johnson E, Monto AS. Influenza hemagglutination-inhibition antibody titer as a correlate of vaccine-induced protection. J Infect Dis. 2011; 204:1879–1885. [PubMed: 21998477]
4. Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng NY, Mays I, Garman L, Helms C, et al. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature. 2008; 453:667–671. [PubMed: 18449194]
5. Moody MA, Zhang R, Walter EB, Woods CW, Ginsburg GS, McClain MT, Denny TN, Chen X, Munshaw S, Marshall DJ, et al. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. PLoS One. 2011; 6:e25797. [PubMed: 22039424]
*6. Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, Palucka K, García-Sastre A, Palese P, Treanor JJ, et al. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J Virol. 2013; 87:4728–4737. [PubMed: 23408625] Demonstrates that infection but not vaccination with regular seasonal inactivated vaccines induces low levels of stalk-reactive antibodies.
7. Gerdil C. The annual production cycle for influenza vaccine. Vaccine. 2003; 21:1776–1779. [PubMed: 12686093]
8. de Jong JC, Beyer WE, Palache AM, Rimmelzwaan GF, Osterhaus AD. Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly. J Med Virol. 2000; 61:94–99. [PubMed: 10745239]
9. Klimov A, Simonsen L, Fukuda K, Cox N. Surveillance and impact of influenza in the United States. Vaccine. 1999; 17(Suppl 1):S42–46. [PubMed: 10471179]
10. Bridges CB, Thompson WW, Meltzer MI, Reeve GR, Talamonti WJ, Cox NJ, Lilac HA, Hall H, Klimov A, Fukuda K. Effectiveness and cost-benefit of influenza vaccination of healthy working adults: A randomized controlled trial. JAMA. 2000; 284:1655–1663. [PubMed: 11015795]
11. Nordin J, Mullooly J, Poblete S, Strikas R, Petrucci R, Wei F, Rush B, Safirstein B, Wheeler D, Nichol KL. Influenza vaccine effectiveness in preventing hospitalizations and deaths in persons 65 years or older in Minnesota, New York, and Oregon: data from 3 health plans. J Infect Dis. 2001; 184:665–670. [PubMed: 11517426]
12. Bhat N, Wright JG, Broder KR, Murray EL, Greenberg ME, Glover MJ, Likos AM, Posey DL, Klimov A, Lindstrom SE, et al. Influenza-associated deaths among children in the United States, 2003-2004. N Engl J Med. 2005; 353:2559–2567. [PubMed: 16354892]
13. http://www.virology.ws/2010/12/09/pandemic-influenza-vaccine-was-too-late-in-2009/
14. Hai R, Krammer F, Tan GS, Pica N, Eggink D, Maamary J, Margine I, Albrecht RA, Palese P. Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. J Virol. 2012; 86:5774–5781. [PubMed: 22398287]
15. Steel J, Lowen AC, Wang TT, Yondola M, Gao Q, Haye K, García-Sastre A, Palese P. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio. 2010; 1
16. Okuno Y, Isegawa Y, Sasao F, Ueda S. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J Virol. 1993; 67:2552–2558. [PubMed: 7682624]

17. Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA. Antibody recognition of a highly conserved influenza virus epitope. Science. 2009; 324:246–251. [PubMed: 19251591]

18. Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A, Cox F, van Deventer E, Guan Y, et al. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS ONE. 2008; 3:e3942. [PubMed: 19079604]

19. Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol. 2009; 16:265–273. [PubMed: 19234466]

20. Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science. 2011; 333:850–856. [PubMed: 21798894]

*21. Dreyfus C, Ekiert DC, Wilson IA. Structure of a classical broadly neutralizing stem antibody in complex with a pandemic h2 influenza virus hemagglutinin. J Virol. 2013; 87:7149–7154. [PubMed: 23552413] This study elucitated the structural basis for binding of the classical stalk-antibody C179 and showed that this mouse mAb is using the same footprint as many human anti-stalk mAbs.

22. Krammer F, Margine I, Tan GS, Pica N, Krause JC, Palese P. A carboxy-terminal trimerization domain stabilizes conformational epitopes on the stalk domain of soluble recombinant hemagglutinin substrates. PLoS One. 2012; 7:e43603. [PubMed: 22928001]

23. Tan GS, Krammer F, Eggink D, Kongchanagul A, Moran TM, Palese P. A pan-h1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. J Virol. 2012; 86:6179–6188. [PubMed: 22491456]

24. Desbien AL, Van Hoeven N, Reed SJ, Casey AC, Laurance JD, Baldwin SL, Duthie MS, Reed SG, Carter D. Development of a high density hemagglutinin protein microarray to determine the breadth of influenza antibody responses. Biotechniques. 2013; 54:345–348. [PubMed: 23750544]

*25. Li GM, Chiu C, Wrammert J, McCausland M, Andrews SF, Zheng NY, Lee JH, Huang M, Qu X, Edupuganti S, et al. Pandemic H1N1 influenza vaccine induces a recall response in humans that favors broadly cross-reactive memory B cells. Proc Natl Acad Sci U S A. 2012; 109:9047–9052. [PubMed: 22615367] Thourough analysis of the antibody response to the inactivated pandemic H1N1 vaccine on a single B-cell level. The authors show that the inactivated pH1N1, in contrast to seasonal H1N1 vaccines, is able to induce broadly neutralizing antibodies.

26. Dreyfus C, Laursen NS, Kwaks T, Zuijdgeest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, et al. Highly conserved protective epitopes on influenza B viruses. Science. 2012; 337:1343–1348. [PubMed: 22878502]

27. Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, et al. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science. 2011; 333:843–850. [PubMed: 21737702]

28. Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, Palese P. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. PLoS Pathog. 2010; 6:e1000796. [PubMed: 20195520]

29. Gocník M, Fislová T, Mucha V, Sládková T, Russ G, Kostolansky F, Varecková E. Antibodies induced by the HA2 glycopolypeptide of influenza virus haemagglutinin improve recovery from influenza A virus infection. J Gen Virol. 2008; 89:958–967. [PubMed: 18343837]

30. Stropkovská A, Mucha V, Fislová T, Gocník M, Kostolanský F, Varecková E. Broadly cross-reactive monoclonal antibodies against HA2 glycopeptide of Influenza A virus hemagglutinin of H3 subtype reduce replication of influenza A viruses of human and avian origin. Acta Virol. 2009; 53:15–20. [PubMed: 19301946]

31. Vareckova E, Wharton SA, Mucha V, Gocnik M, Kostolansky F. A monoclonal antibody specific to the HA2 glycoprotein of influenza A virus hemagglutinin that inhibits its fusion activity reduces replication of the virus. Acta Virol. 2003; 47:229–236. [PubMed: 15068378]

32. Hashem AM, Van Domselaar G, Li C, Wang J, She YM, Cyr TD, Sui J, He R, Marasco WA, Li X. Universal antibodies against the highly conserved influenza fusion peptide cross-neutralize several

subtypes of influenza A virus. Biochem Biophys Res Commun. 2010; 403:247–251. [PubMed: 21078301]

33. Prabhu N, Prabakaran M, Ho HT, Velumani S, Qiang J, Goutama M, Kwang J. Monoclonal antibodies against the fusion peptide of hemagglutinin protect mice from lethal influenza A virus H5N1 infection. J Virol. 2009; 83:2553–2562. [PubMed: 19109379]
34. Yasugi M, Kubota-Koketsu R, Yamashita A, Kawashita N, Du A, Sasaki T, Nishimura M, Misaki R, Kuhara M, Boonsathorn N, et al. Human monoclonal antibodies broadly neutralizing against influenza B virus. PLoS Pathog. 2013; 9:e1003150. [PubMed: 23408886]
**35. Harris AK, Meyerson JR, Matsuoka Y, Kuybeda O, Moran A, Bliss D, Das SR, Yewdell JW, Sapiro G, Subbarao K, et al. Structure and accessibility of HA trimers on intact 2009 H1N1 pandemic influenza virus to stem region-specific neutralizing antibodies. Proc Natl Acad Sci U S A. 2013; 110:4592–4597. [PubMed: 23460696] This elegant study demonstrates binding of stalk-reactive antibodies to HA on intact virus particles using cryoelectron tomographic methods.
36. Steel J, Lowen AC, Pena L, Angel M, Solórzano A, Albrecht R, Perez DR, García-Sastre A, Palese P. Live attenuated influenza viruses containing NS1 truncations as vaccine candidates against H5N1 highly pathogenic avian influenza. J Virol. 2009; 83:1742–1753. [PubMed: 19073731]
37. Terajima M, Cruz J, Co MD, Lee JH, Kaur K, Wrammert J, Wilson PC, Ennis FA. Complement-dependent lysis of influenza a virus-infected cells by broadly cross-reactive human monoclonal antibodies. J Virol. 2011; 85:13463–13467. [PubMed: 21994454]
38. Jegaskanda S, Weinfurter JT, Friedrich TC, Kent SJ. Antibody-dependent cellular cytotoxicity is associated with control of pandemic H1N1 influenza virus infection of macaques. J Virol. 2013; 87:5512–5522. [PubMed: 23468501]
39. Jegaskanda S, Job ER, Kramski M, Laurie K, Isitman G, de Rose R, Winnall WR, Stratov I, Brooks AG, Reading PC, et al. Cross-reactive influenza-specific antibody-dependent cellular cytotoxicity antibodies in the absence of neutralizing antibodies. J Immunol. 2013; 190:1837–1848. [PubMed: 23319732]
40. Huber M, Fischer M, Misselwitz B, Manrique A, Kuster H, Niederöst B, Weber R, von Wyl V, Günthard HF, Trkola A. Complement lysis activity in autologous plasma is associated with lower viral loads during the acute phase of HIV-1 infection. PLoS Med. 2006; 3:e441. [PubMed: 17121450]
41. van Kampen JJ, Bielefeld-Buss AJ, Ott A, Maaskant J, Faber HJ, Lutisan JG, Boucher CA. Case report: oseltamivir-induced resistant pandemic influenza A (H1N1) virus infection in a patient with AIDS and Pneumocystis jirovecii pneumonia. J Med Virol. 2013; 85:941–943. [PubMed: 23588718]
42. Hurt AC, Leang SK, Tiedemann K, Butler J, Mechinaud F, Kelso A, Downie P, Barr IG. Progressive emergence of an oseltamivir-resistant A(H3N2) virus over two courses of oseltamivir treatment in an immunocompromised paediatric patient. Influenza Other Respi Viruses. 2013
43. Shetty AK, Ross GA, Pranikoff T, Gubareva LV, Sechrist C, Guirand DM, Abramson J, Lin JJ. Oseltamivir-resistant 2009 H1N1 influenza pneumonia during therapy in a renal transplant recipient. Pediatr Transplant. 2012; 16:E153–157. [PubMed: 22764830]
44. Iioka F, Sada R, Maesako Y, Nakamura F, Ohno H. Outbreak of pandemic 2009 influenza A/H1N1 infection in the hematology ward: fatal clinical outcome of hematopoietic stem cell transplant recipients and emergence of the H275Y neuraminidase mutation. Int J Hematol. 2012; 96:364–369. [PubMed: 22773299]
45. DeVries A, Wotton J, Lees C, Boxrud D, Uyeki T, Lynfield R. Neuraminidase H275Y and hemagglutinin D222G mutations in a fatal case of 2009 pandemic influenza A (H1N1) virus infection. Influenza Other Respi Viruses. 2012; 6:e85–88. [PubMed: 22243670]
46. van der Vries E, Stittelaar KJ, van Amerongen G, Veldhuis Kroeze EJ, de Waal L, Fraaij PL, Meesters RJ, Luider TM, van der Nagel B, Koch B, et al. Prolonged influenza virus shedding and emergence of antiviral resistance in immunocompromised patients and ferrets. PLoS Pathog. 2013; 9:e1003343. [PubMed: 23717200]
47. Hu Y, Lu S, Song Z, Wang W, Hao P, Li J, Zhang X, Yen HL, Shi B, Li T, et al. Association between adverse clinical outcome in human disease caused by novel influenza A H7N9 virus and sustained viral shedding and emergence of antiviral resistance. Lancet. 2013

48. Thomson CA, Wang Y, Jackson LM, Olson M, Wang W, Liavonchanka A, Keleta L, Silva V, Diederich S, Jones RB, et al. Pandemic H1N1 Influenza Infection and Vaccination in Humans Induces Cross-Protective Antibodies that Target the Hemagglutinin Stem. Front Immunol. 2012; 3:87. [PubMed: 22586427]

49. Krause JC, Tsibane T, Tumpey TM, Huffman CJ, Basler CF, Crowe JE. A broadly neutralizing human monoclonal antibody that recognizes a conserved, novel epitope on the globular head of the influenza H1N1 virus hemagglutinin. J Virol. 2011; 85:10905–10908. [PubMed: 21849447]

50. Friesen RH, Koudstaal W, Koldijk MH, Weverling GJ, Brakenhoff JP, Lenting PJ, Stittelaar KJ, Osterhaus AD, Kompier R, Goudsmit J. New class of monoclonal antibodies against severe influenza: prophylactic and therapeutic efficacy in ferrets. PLoS One. 2010; 5:e9106. [PubMed: 20161706]

51. Krammer F, Hai R, Yondola M, Leyva-Grado V, Tan GS, Ryder A, Rose JK, Palese P, Garcia-Sastre A, Albrecht RA. Influenza hemagglutinin stalk-based immunity in ferrets: antibodies and vaccines. submitted.

52. http://clinicaltrials.gov/show/NCT01406418

53. http://clinicaltrials.gov/ct2/show/NCT01756950

54. Corti D, Suguitan AL Jr. Pinna D, Silacci C, Fernandez-Rodriguez BM, Vanzetta F, Santos C, Luke CJ, Torres-Velez FJ, Temperton NJ, et al. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J Clin Invest. 2010; 120:1663–1673. [PubMed: 20389023]

55. Krammer F, Pica N, Hai R, Tan GS, Palese P. Hemagglutinin Stalk-Reactive Antibodies Are Boosted following Sequential Infection with Seasonal and Pandemic H1N1 Influenza Virus in Mice. J Virol. 2012; 86:10302–10307. [PubMed: 22787225]

**56. Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, Tan GS, Krause JC, Moran T, Stein CR, et al. Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses. Proc Natl Acad Sci U S A. 2012; 109:2573–2578. [PubMed: 22308500] The authors quantitatively measure the polyclonal anti-stalk response following pH1N1 infection in humans using cHA structures.

*57. Miller MS, Tsibane T, Krammer F, Hai R, Rahmat S, Basler CF, Palese P. 1976 and 2009 H1N1 influenza virus vaccines boost anti-hemagglutinin stalk antibodies in humans. J Infect Dis. 2013; 207:98–105. [PubMed: 23087428] The study shows that inactivated vaccines containing divergent H1 HAs are able to induce stalk-reactive antibodies.

58. Miller MS, Gardner TJ, Krammer F, Aguado LC, Tortorella D, Basler CF, Palese P. Neutralizing antibodies against previously-encountered influenza virus strains increase over time: A longitudinal analysis. Sci Transl Med. in press.

**59. Wrammert J, Koutsonanos D, Li GM, Edupuganti S, Sui J, Morrissey M, McCausland M, Skountzou I, Hornig M, Lipkin WI, et al. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J Exp Med. 2011; 208:181–193. [PubMed: 21220454] Jens Wrammert and Patrick Wilson analyzed the B-cell response to pH1N1 infection on a single cell level using their single cell plasmablast expression-cloning strategy. They showed that B-cells with specificities in the stalk domain were induced by infection with pH1N1.

60. Palese P, Wang TT. Why do influenza virus subtypes die out? A hypothesis. MBio. 2011; 2

61. Kilbourne ED, Cerini CP, Khan MW, Mitchell JW, Ogra PL. Immunologic response to the influenza virus neuraminidase is influenced by prior experience with the associated viral hemagglutinin. I. Studies in human vaccinees. J Immunol. 1987; 138:3010–3013. [PubMed: 3571981]

62. Sangster MY, Baer J, Santiago FW, Fitzgerald T, Ilyushina NA, Sundararajan A, Henn AD, Krammer F, Yang H, Luke CJ, et al. The B cell response and hemagglutinin stalk-reactive antibody production in different age cohorts following 2009 H1N1 influenza vaccination. Clin Vaccine Immunol. 2013

63. Graves PN, Schulman JL, Young JF, Palese P. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. Virology. 1983; 126:106–116. [PubMed: 6189287]

64. Bommakanti G, Citron MP, Hepler RW, Callahan C, Heidecker GJ, Najar TA, Lu X, Joyce JG, Shiver JW, Casimiro DR, et al. Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. Proc Natl Acad Sci U S A. 2010; 107:13701–13706. [PubMed: 20615991]

65. Wang TT, Tan GS, Hai R, Pica N, Ngai L, Ekiert DC, Wilson IA, García-Sastre A, Moran TM, Palese P. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci U S A. 2010; 107:18979–18984. [PubMed: 20956293]

66. Wei CJ, Boyington JC, McTamney PM, Kong WP, Pearce MB, Xu L, Andersen H, Rao S, Tumpey TM, Yang ZY, et al. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. Science. 2010; 329:1060–1064. [PubMed: 20647428]

67. Wei CJ, Yassine HM, McTamney PM, Gall JG, Whittle JR, Boyington JC, Nabel GJ. Elicitation of broadly neutralizing influenza antibodies in animals with previous influenza exposure. Sci Transl Med. 2012; 4:147ra114.

68. Schneemann A, Speir JA, Tan GS, Khayat R, Ekiert DC, Matsuoka Y, Wilson IA. A virus-like particle that elicits cross-reactive antibodies to the conserved stem of influenza virus hemagglutinin. J Virol. 2012; 86:11686–11697. [PubMed: 22896619]

69. Kanekiyo M, Wei CJ, Yassine HM, McTamney PM, Boyington JC, Whittle JR, Rao SS, Kong WP, Wang L, Nabel GJ. Self-assembling influenza nanoparticle vaccines elicit broadly neutralizing H1N1 antibodies. Nature. 2013

**70. Krammer F, Pica N, Hai R, Margine I, Palese P. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. J Virol. 2013; 87:6542–6550. [PubMed: 23576508] This study demonstrates that cHA vaccines are able to protect mice from heterologous and heterosubtypic virus challenge.

Highlights

- Hemagglutinin stalk-reactive monoclonal antibodies are able to neutralize divergent influenza virus strains
- Stalk-reactive antibodies are currently being tested in clinical studies for their potential as anti-influenza therapeutics
- Vaccine constructs based on the conserved hemagglutinin stalk domain are able to protect mice from heterosubtypic challenge
- Stalk-based vaccines could be further developed into human universal influenza virus vaccines

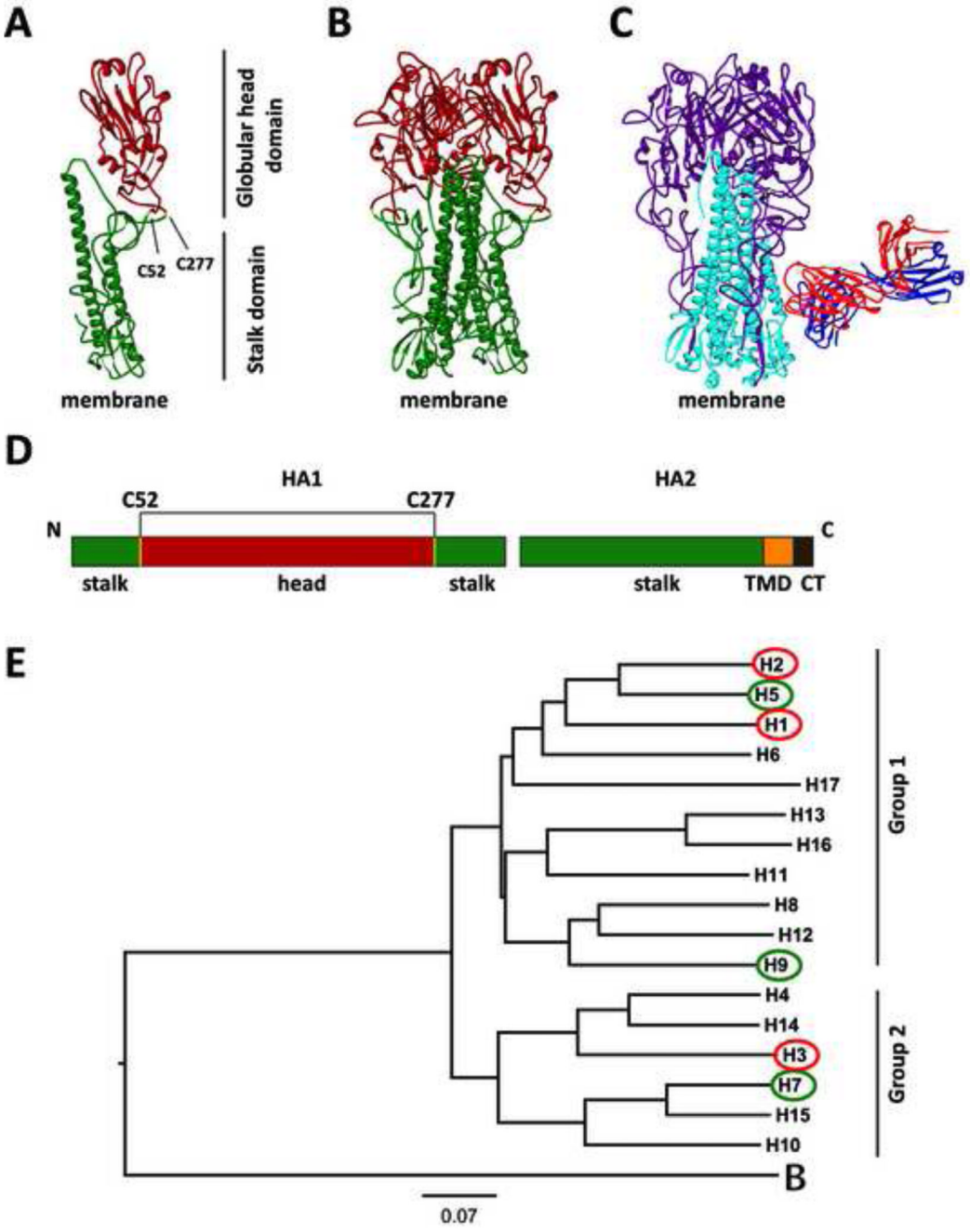

Figure 1. The structure of the influenza virus hemagglutinin (HA)

A The influenza virus HA can be divided into the highly divergent membrane distal globular head domain (red) and the conserved membrane proximal stalk domain (green). Cysteines 52 and 277 (H3 numbering) form a disulfide bond that serves as demarcation line between the two subdomains (PDB 1RU7). **B** Structure of homotrimeric HA molecule. Globular head domains are shown in red, stalk domains are shown in green. **C** Structure of an H2 HA in complex with an Fab fragment of the broadly neutralizing mAb C179 (PDB 4HLZ). The HA1 subunit is shown in purple, HA2 is shown in turquoise. Heavy (red) and light (blue) chain of the antibody make contacts with a conformational epitope on the HA stalk domain

that is formed by the HA1 and HA2 subunit (21). **D** Schematic of the head and stalk domains of the HA. The stalk domain (green) covers the C- and N-terminal part of the HA1 and most of the HA2 (excluding the transmembrane domain, TMD and the cytoplasmic tail, CT). The globular head domain (red) is located between cysteines 52 and 277 (H3 numbering) on the HA1 subunit. **E** Phylogenetic tree of influenza virus HAs. Influenza A virus HAs can be divided into group 1 and group 2 based on their sequence. Influenza A virus subtypes that currently circulate in humans or that have historically demonstrated their potential to do so are marked in red. Subtypes with pandemic potential are marked in green. The tree was built using ClustalW and was visualized in FigTree. The scale bar represents a 7% change in amino acid.

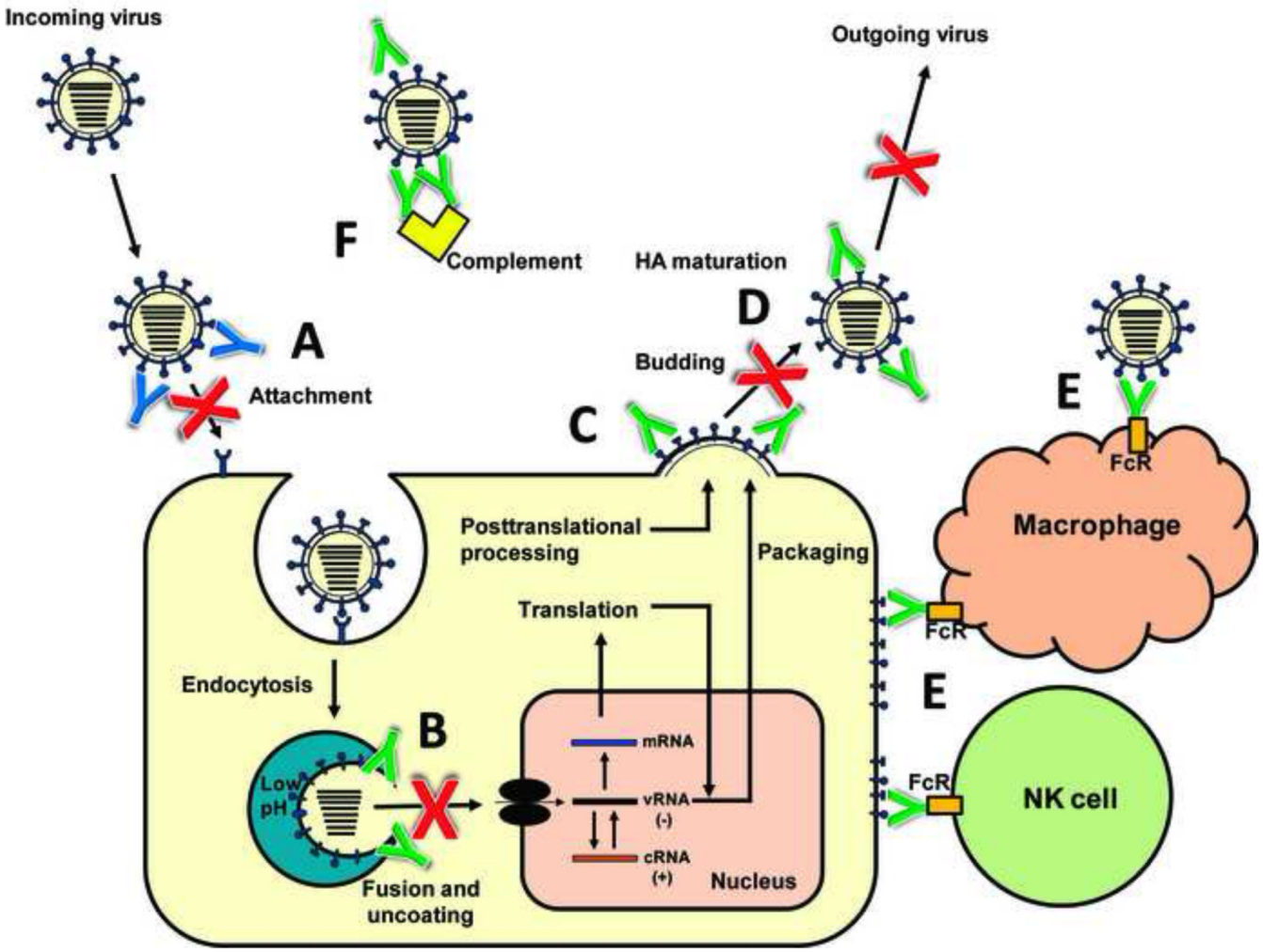

Figure 2. Alternative mechanisms of neutralization

A Classical HI active antibodies (in blue) neutralize by inhibiting attachment of the viral HA to sialylated host cell receptors and block entry at an early stage. **B** Stalk-reactive antibodies (green) bind to HA on the virus surface and may be taken up with the virus into the endosome. During acidification of the endosome they may prevent conformational change of the HA and inhibit release of the viral genome into the cytosol. **C** Broadly neutralizing antibodies may also inhibit viral egress. **D** Stalk-reactive antibodies may inhibit HA maturation by sterical hindrance of the interaction of host proteases with the HA cleavage site. **E** Stalk-reactive antibodies may also work through ADCC, infected cells as well as viruses are killed/cleared by macrophages and natural killer (NK) cells. **F** Stalk-reactive antibodies have been shown to trigger complement mediated lysis of infected cells and could potentially also help to clear influenza virus particles.

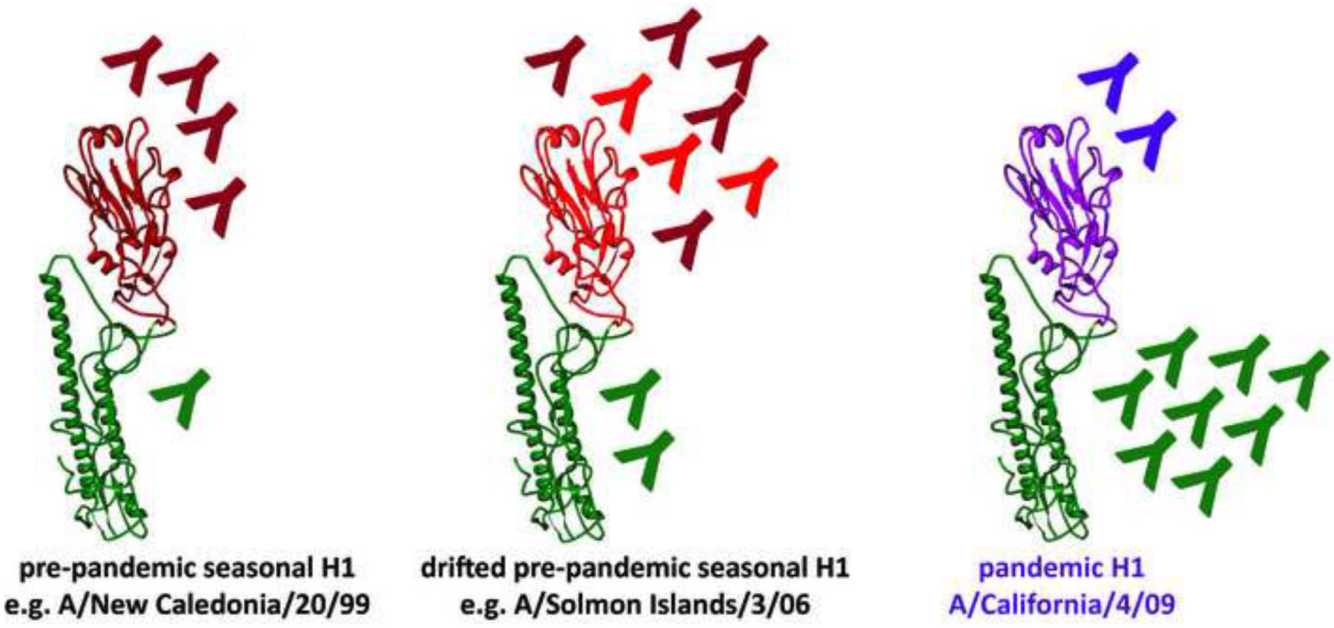

Figure 3. Boost of stalk-reactive antibodies in nature during the 2009 H1N1 pandemic

Individuals sequentially exposed (through vaccination or infection) to pre-pandemic seasonal H1N1 strains with slightly drifted globular head domains developed mainly an immune response against the globular head of the HA. Infection with pandemic H1N1 virus which features a very divergent H1 head domain (approx. 30% difference as compared to pre-pandemic seasonal strains) induced only a primary response against the immunodominant globular head domain but boosted immune responses directed against the conserved stalk domain. Structures are based on PDB 1RU7.

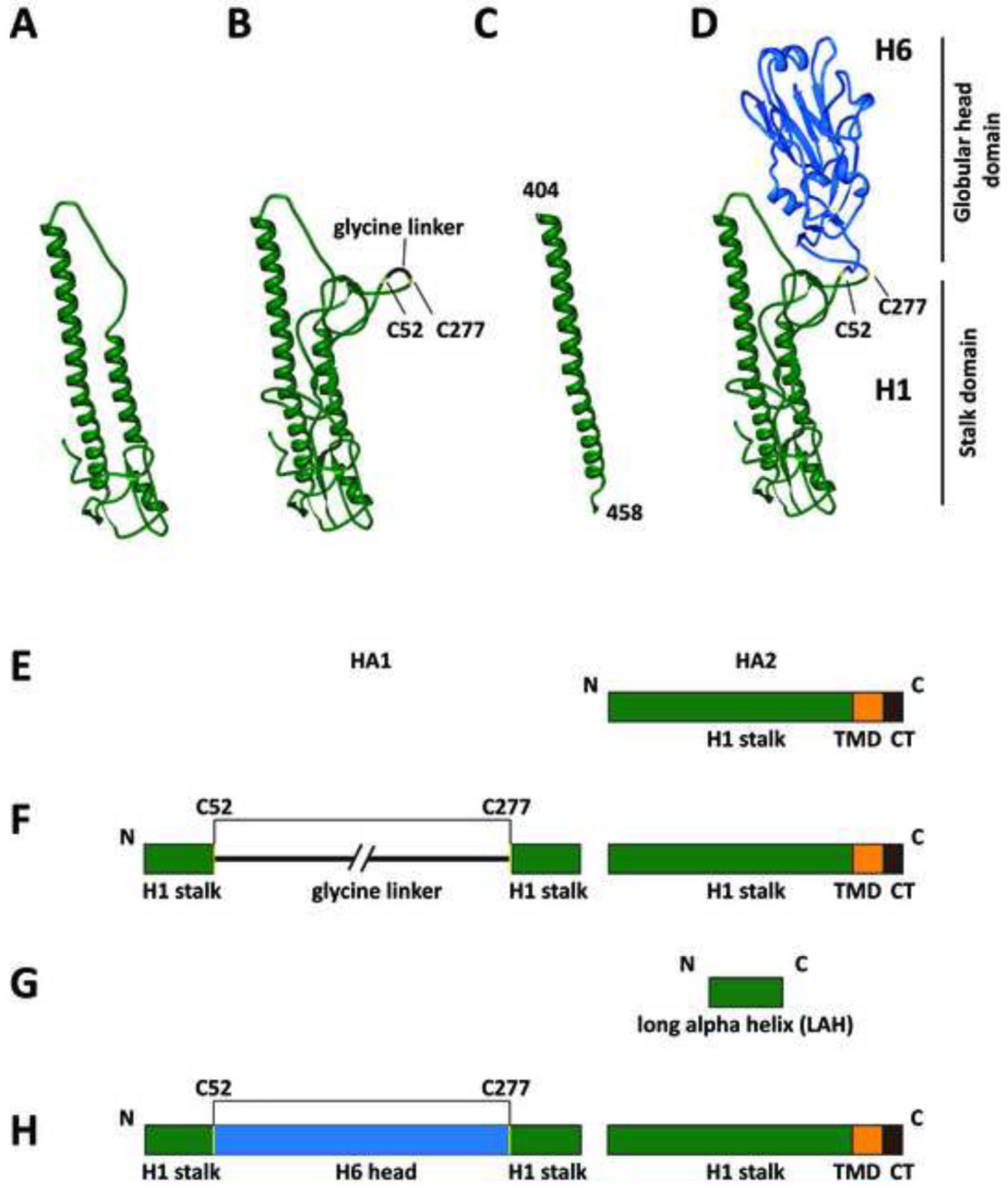

Figure 4. Design of stalk-based immunogens

A/E Vaccines based on the HA2 subunit only were the earliest candidate vaccines used to induce cross-reactive antibodies. **B/F** Headless HA constructs span the whole stalk domain including HA1 and HA2 parts of the stalk. The globular head domain located between cysteines 52 and 277 (H3 numbering) was replaced by a glycine linker. Structures shown in **B/F** and **C/G** are likely to be mis-folded if expressed on their own. **C/G** The long alpha helix (LAH) of the HA2 hosts the epitope of broadly neutralizing antibody 12D1 and was successfully used as vaccine construct. It spans amino acid 404 to 458 (H3 numbering) (28). **D/H** Chimeric HA molecules consist of either an H1 or an H3 stalk domain combined with 'exotic' globular head domains. Here, cH6/1 (H6 head on top of an H1 stalk) is shown as an

example. The H6 globular head domain (blue) is located between conserved cysteines 52 and 277 (H3 numbering). TMD is transmembrane domain; CT is cytoplasmic tail. Structures are based on PDB 1RU7.

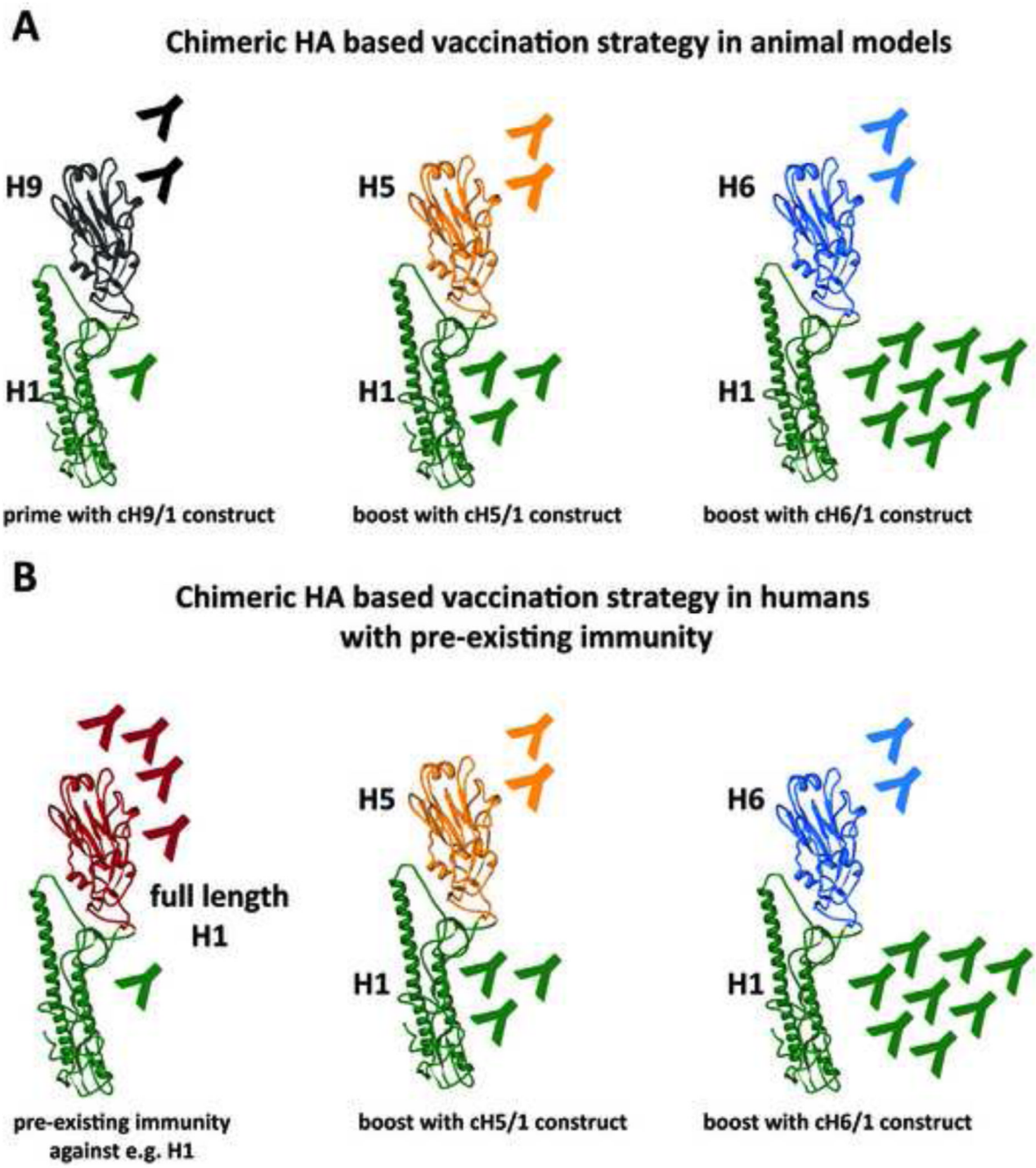

Figure 5. Vaccination strategies based on chimeric HAs

A Example for a cHA vaccination strategy in naive animals. Animals are primed with a cH9/1 construct (H9 head (black) on top of an H1 stalk (green)). The prime induces a strong primary response against the immunodominant globular head domain and an almost undetectable response against the immunosubdominant stalk domain. Animals are then boosted with a cH5/1 construct (H5 head (golden) on top of an H1 stalk domain (green)). Again, a primary response is developed against the divergent globular head but antibodies against the conserved stalk domain are boosted. A second boost with cH6/1 HA (H6 head (blue) on top of an H1 stalk domain (green)) induces a primary response against the

divergent H6 head domain but strongly boosts titers of antibodies directed to the conserved stalk domain. **B** Similar vaccination strategies could be employed for humans. It is likely that a prime is not necessary since most individuals have been exposed to human influenza viruses and have therefore a low but detectable pre-existing immunity directed toward the stalk domain. Individuals can be boosted once (e.g. with cH5/1 HA) or twice (e.g. with cH5/1 and cH6/1 HA) to induce high, broadly neutralizing antibody titers. Structures are based on PDB 1RU7.