JVI Journals.ASM.org

Induction of Broadly Reactive Anti-Hemagglutinin Stalk Antibodies by an H5N1 Vaccine in Humans

Raffael Nachbagauer,[a,h] Teddy John Wohlbold,[a,b] Ariana Hirsh,[a] Rong Hai,[a] Haakon Sjursen,[c,d] Peter Palese,[a,e] Rebecca J. Cox,[c,f,g] Florian Krammer[a]

Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA[a]; Graduate School of Biological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA[b]; The Influenza Centre, Department of Clinical Science, University of Bergen, Bergen, Norway[c]; Section for Infectious Diseases, Medical Department, Haukeland University Hospital, Bergen, Norway[d]; Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA[e]; Jebsen Centre for Influenza Vaccine Research, Department of Clinical Science, University of Bergen, Bergen, Norway[f]; Department of Research and Development, Haukeland University Hospital, Bergen, Norway[g]; Faculty of Life Sciences, University of Vienna, Vienna, Austria[h]

ABSTRACT

Influenza virus infections are a major public health concern and cause significant morbidity and mortality worldwide. Current vaccines are effective but strain specific due to their focus on the immunodominant globular head domain of the hemagglutinin (HA). It has been hypothesized that sequential exposure of humans to hemagglutinins with divergent globular head domains but conserved stalk domains could refocus the immune response to broadly neutralizing epitopes in the stalk. Humans have preexisting immunity against H1 (group 1 hemagglutinin), and vaccination with H5 HA (also group 1)—which has a divergent globular head domain but a similar stalk domain—represents one such sequential-exposure scenario. To test this hypothesis, we used novel reagents based on chimeric hemagglutinins to screen sera from an H5N1 clinical trial for induction of stalk-specific antibodies by quantitative enzyme-linked immunosorbent assay (ELISA) and neutralization assays. Importantly, we also investigated the biological activity of these antibodies in a passive transfer in a mouse challenge model. We found that the H5N1 vaccine induced high titers of stalk-reactive antibodies which were biologically active and protective in the passive-transfer experiment. The induced response showed exceptional breadth toward divergent group 1 hemagglutinins but did not extend to group 2 hemagglutinins. These data provide evidence for the hypothesis that sequential exposure to hemagglutinins with divergent globular head domains but conserved stalk domains can refocus the immune response toward the conserved stalk domain. Furthermore, the results support the concept of a chimeric hemagglutinin universal influenza virus vaccine strategy that is based on the same principle.

IMPORTANCE

Influenza virus vaccines have to be reformulated and readministered on an annual basis. The development of a universal influenza virus vaccine could abolish the need for this cumbersome and costly process and would also enhance our pandemic preparedness. This study addressed the following questions, which are essential for the development of a hemagglutinin stalk-based universal influenza virus vaccine. (i) Can stalk-reactive antibodies be boosted by vaccination with divergent HAs that share conserved epitopes? (ii) How long-lived are these vaccine-induced stalk-reactive antibody responses? (iii) What is the breadth of this reactivity? (iv) Are these antibodies functional and protective? Our results further strengthen the concept of induction of stalk-reactive antibodies by sequential exposure to hemagglutinin immunogens with conserved stalk and divergent head domains. A universal influenza virus vaccine based on the same principles seems possible and might have a significant impact on global human health.

Current influenza virus vaccines provide excellent protection against matched virus strains, but they are limited in efficacy against mismatched viruses. Immune responses induced by licensed inactivated influenza virus vaccines are focused toward the membrane-distal immunodominant globular head domain of the major surface glycoprotein of the virus, the hemagglutinin (HA) (1–3). This domain exhibits high structural plasticity and is strongly affected by antigenic drift. In contrast, the membrane-proximal HA stalk domain shows a high degree of conservation, but due to its immunosubdominant nature, conventional vaccines do not usually induce effective immune responses against this domain (1–3). However, antibodies directed against the stalk domain are known to be broadly neutralizing *in vitro* and broadly protective in passive-transfer challenge *in vivo* (mouse and ferret models) (4–10). Influenza virus HAs are phylogenetically divided into group 1 HAs (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 HAs (H3, H4, H7, H10, H14, and H15). The stalk domain shows conservation within these groups, and the binding pattern of broadly neutralizing antibodies—with some exceptions (11, 12)—usually resembles this phylogeny (4–7, 13–15). It has been hypothesized that exposure to HAs with divergent head domains and conserved stalk domains could refocus the

Received 21 July 2014 **Accepted** 26 August 2014
Published ahead of print 10 September 2014
Editor: D. S. Lyles
Address correspondence to Florian Krammer, florian.krammer@mssm.edu, or Rebecca J. Cox, rebecca.cox@gades.uib.no.

doi:10.1128/JVI.02133-14

immune response to the immunosubdominant conserved stalk domain of the HA by boosting antibodies to shared epitopes (16–22). A universal influenza virus vaccine based on this hypothesis using chimeric HAs (cHAs) is currently in late-stage preclinical development (10, 19, 20, 23). Since humans have low but detectable preexisting immunity to the conserved group 1 stalk domain (mainly from exposure to H1- and H2-expressing viruses), vaccination with H5N1 vaccines theoretically should boost stalk-reactive antibodies in individuals preexposed to influenza viruses. In the present study, we examined sera from an H5N1 clinical trial to test this hypothesis. We used assays based on chimeric HAs (24, 25) to quantitatively assess the induction of stalk-reactive antibodies upon H5N1 vaccination in humans. Furthermore, we characterized the breadth of these responses and assessed their longevity up to 12 months postvaccination. The humoral responses were then characterized for their functionality in *in vitro* neutralization assays and in passive-transfer challenge experiments with mice.

MATERIALS AND METHODS

Participants. Sixty healthy volunteers (aged 20 to 49 years; mean age, 31 years; 37% males) were recruited at the Haukeland University Hospital, Bergen, Norway, according to good clinical practice guidelines. None of the participants had received an H5N1 vaccine prior to the study. All study subjects provided written informed consent before their inclusion in the trial. The study was approved by the regional ethics committee (Regional Committee for Medical Research Ethics, Northern Norway [REK Nord]) and the Norwegian Medicines Agency (26).

Vaccine. The vaccine consisted of inactivated influenza virosomes from the vaccine strain RG14, which is a reassortant between A/Vietnam/1194/2004 (H5N1) and A/Puerto Rico/8/34 (H1N1) (PR8) produced by reverse genetics at the National Institute for Biological Standards and Control (NIBSC), United Kingdom. RG14 was grown in embryonated chicken eggs and inactivated with beta-propiolactone, and the surface glycoproteins and phospholipids were solubilized with the detergent octaethylene glycol monododecyl ether. HA and neuraminidase (NA) were purified, mixed with lecithin, and incorporated into the phospholipid bilayer by the stepwise removal of the detergent. HA content was measured and the presence of NA confirmed. The 3rd-generation ISCOM-like Matrix M adjuvant used in this study was prepared by Crucell/SBL vaccines, Sweden, under good manufacturing practices (26).

Study design. Participants were divided into four groups of 15 and received two intramuscular (i.m.) injections into the deltoid muscle at an interval of 21 (±1) days with the inactivated virosomal H5N1 vaccine alone (30 μg of HA) or 1.5, 7.5, or 30 μg of HA further adjuvanted with Matrix M (50 μg). All subjects provided blood samples on the day of vaccination and 21 days after the first vaccine dose. Fifty-nine serum samples were available 21 days after the second vaccination (day 42). For the 6-months and 12-months-postvaccination time points, 51 serum samples were provided for investigation. A more detailed description of patient cohorts, screening tests, vaccine, and study design was previously published (26).

ELISAs. Recombinant proteins as enzyme-linked immunosorbent assay (ELISA) substrates cH6/1 (A/mallard/Sweden/81/02 H6 head domain with an A/Puerto Rico/8/34 H1 stalk domain), H2 (A/mallard/Netherlands/5/99), H3 (A/Perth/16/09), H9 head only (A/guinea fowl/Hong Kong/WF10/99), H18 (A/flat-faced bat/Peru/033/10), and N1 NAs from A/Vietnam/1203/04 and A/California/04/09) were produced as described before (27, 28). Flat-bottom Immuno nonsterile 4 HBX 96-well plates (Thermo Scientific) were coated with 50 μl of protein diluted in ELISA coating buffer (pH 9.4) at a concentration of 2 μg/ml per well and refrigerated at 4°C overnight. Coating buffer was discarded and wells were blocked for 1 h at room temperature with 100 μl of blocking solution (phosphate-buffered saline [PBS] containing 0.1% Tween 20 [T-PBS], 3% goat serum [Gibco], and 0.5% milk powder). Another 100 μl was added to the first column of wells, as was 2 μl of human serum (starting concentration of 1:100). The samples were 2-fold serially diluted and incubated at room temperature for 2 h. The plates were washed 3 times, and 50 μl of blocking solution containing anti-human IgG (Fab specific)-peroxidase antibody (Sigma) at a concentration of 1:3,000 added. After 1 h of incubation at room temperature, the plates were washed 3 times and developed with 100 μl of SigmaFast *o*-phenylenediamine dihydrochloride (OPD; Sigma) per well. The developing process was stopped after 10 min with 3 M hydrochloric acid (HCl), and the reaction was read at an absorbance of 490 nm with a Synergy H1 hybrid multimode microplate reader (BioTek).

Treatment of sera with RDE. To prepare serum samples for microneutralization assays, they were treated with receptor-destroying enzyme (RDE) of *Vibrio cholerae* (Sigma). Reconstituted RDE was diluted 1:9 in calcium saline solution (1 g of calcium chloride, 9 g of sodium chloride, 1.203 g of boric acid, and 0.052 g of sodium tetraborate in 1,000 ml of distilled water). Serum samples were mixed 1:4 with diluted RDE and incubated overnight in a 37°C water bath. Three volumes (based on original serum volume) of 2.5% sodium citrate solution were added, and samples were heated at 56°C for 30 min. Two volumes of PBS were added to raise the dilution to 1:10.

Microneutralization assays. Ninety-six-well cell culture plates containing 100 μl of cell culture media with 1.5×10^7 to 1.8×10^7/ml of Madin-Darby canine kidney cells (MDCK cells) were incubated at 37°C overnight. RDE-treated serum samples were serially diluted in minimum essential medium, containing tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin at a concentration of 1:1,000 (T-MEM), and incubated with cH9/1N3 virus at a concentration of 1,000 PFU per ml at room temperature to allow antibody binding (24, 25). After 1 h, 100 μl of serum-virus mixture was transferred onto the plates containing MDCK cells and incubated for 1 h at 37°C. Cells were then washed and incubated with sera serially diluted in T-MEM without virus at 37°C. After 24 h, the cells were fixed with ice-cold 70% acetone and refrigerated at −20°C for at least 1 h. Cells were blocked with 100 μl of 3% hydrogen peroxide per well for 30 min, followed by 100 μl of 1% bovine serum albumin (BSA) in PBS for 30 min. They were then incubated at room temperature for 1 h with 1% BSA containing anti-influenza A virus nucleoprotein mouse IgG at a concentration of 1:3,000. Horseradish peroxidase (HRP)-linked anti-mouse IgG was used as a secondary antibody, diluted to the same concentration, and incubated for 1 h at room temperature. The plates were developed with 100 μl of SigmaFast OPD (Sigma) per well; the reaction was stopped after 30 min with 50 μl of 3 M HCl, and plates were read at an absorbance of 490 nm with a Synergy H1 hybrid multimode microplate reader (BioTek).

Passive serum transfer into mice. To assess the level of protection by the stalk-reactive antibodies *in vivo*, pre- and postvaccination serum samples of participants with an 8-fold or higher induction ($n = 27$) of stalk-reactive antibody titers after the second round of vaccination were tested in a passive transfer challenge experiment. Two groups of 10 BALB/c mice each were injected intraperitoneally (i.p.) with 250 μl of pooled pre- or postvaccination sera and challenged intranasally with a sublethal dose of cH9/1N3 virus 2 h later. Weight loss was observed, and lungs of 5 mice for each group were harvested 3 days and 6 days postchallenge. Mouse lungs were stored on ice in 600 μl of PBS, homogenized with a BeadBlaster 24 microtube homogenizer (Benchmark Scientific), and then centrifuged at 10,000 rpm at 4°C for 5 min. The supernatant was collected, aliquoted, and frozen to −80°C. All mouse experiments were approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee.

Plaque assays. Mouse lung supernatant was serially diluted in PBS, and 200 μl of each dilution was transferred onto MDCK cells on 12-well plates and incubated at 37°C for 40 min. Supernatant was removed and an overlay of MEM containing 2% Oxoid agar, TPCK-treated trypsin, and DEAE dextran was applied to the cells. After incubation at 37°C for 48 h,

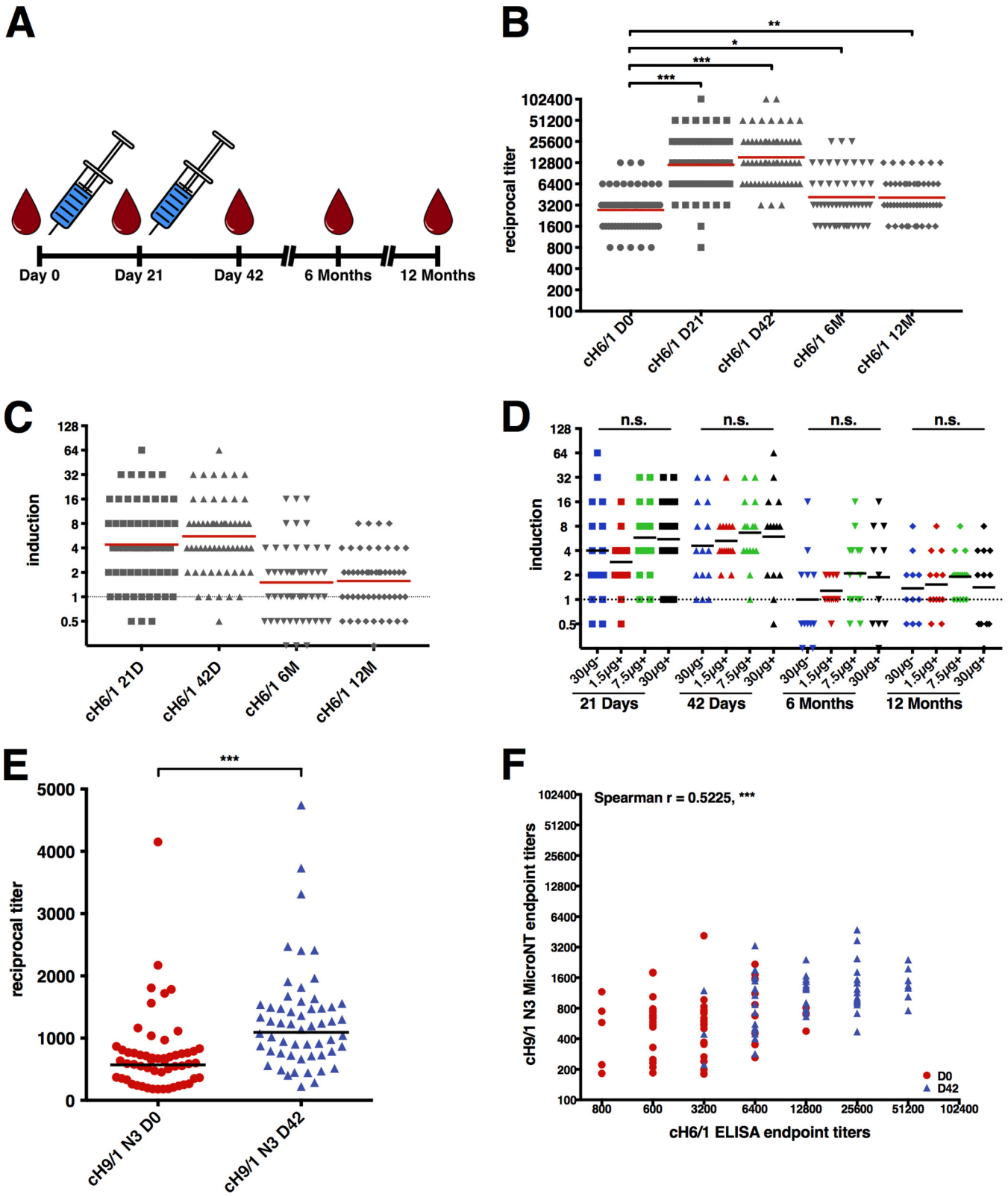

FIG 1 H5N1 vaccination induces stalk-reactive antibodies in humans. (A) Study design. Participants received the H5N1 vaccine intramuscularly on day 0 and day 21. Blood was drawn on days 0, 21, and 42 postpriming, as well as after 6 and 12 months. (B) Stalk-reactive antibodies were measured in a cH6/1 ELISA. Reciprocal geometric mean titers on day 0 were detected at 1:2,722 and showed a high, significant increase for day 21 (1:1,943; $P < 0.0001$) and day 42 (1:15,267; $P < 0.0001$). The levels of stalk-reactive antibodies declined after 6 months (1:4,143; $P = 0.032$) and remained at a similar level up to 12 months postvaccination (1:4,087; $P = 0.0051$), still significantly higher than baseline titers. (C) Induction of stalk-reactive antibodies over baseline. Stalk-reactive antibody titers showed a 4.4-fold induction over baseline after the first round of vaccination, with a further boost to 5.6-fold after the second round. After 6 months, the stalk-reactive

the cells were fixed with 4% formaldehyde and the overlay was removed. Immunostaining against H9 HA was performed, the resulting plaques were counted, and lung titers were calculated as PFU/ml.

Statistical analyses. Statistical significance was analyzed using GraphPad (GraphPad Software Inc.), and a P value of <0.05 was considered statistically significant. For paired samples, a paired t test was performed; otherwise, an unpaired Student t test was used. For group comparison, one-way analysis of variance (ANOVA) with a Tukey posttest was performed. Data are presented as geometric means.

RESULTS

H5N1 vaccination induces high titers of stalk-reactive antibodies. During the original trial (26, 29), healthy volunteers received two doses of a virosomal subunit H5N1 vaccine 21 days apart (Fig. 1A). The participants were divided into four groups: one group received nonadjuvanted virosomal vaccine containing 30 μg of HA, and the three other groups received 1.5, 7.5, or 30 μg of HA adjuvanted with 50 μg of Matrix M. Results from the four groups were initially analyzed separately (Fig. 1D), but since no statistically significant differences for induction and longevity of stalk-reactive antibodies were found, data from the groups were pooled for presentation. Samples were taken at day 0, day 21, day 42, 6 months, and 12 months postpriming and analyzed for the presence of stalk-reactive antibodies. To detect stalk-reactive antibodies, we used a cH6/1 construct consisting of an H6 head domain (to which humans are naive) on top of an H1 stalk domain (to which humans are known to have preexisting immunity). Since humans are naive to the H6 head domain (24, 30), reactivity measured with this substrate indicates reactivity to the H1 stalk. This reagent was used in an endpoint titer ELISA to quantitatively measure stalk-reactive antibodies. Day 0 geometric mean titers (GMT) of stalk-reactive antibodies were detected at 1:2,722 (Fig. 1B). This was not surprising since H1 and H2 infections—and in special cases also vaccination—can induce some level of antibodies reactive against the group 1 stalk in humans (16–18, 24, 31). Interestingly, titers of stalk-reactive antibody were boosted to 1:11,943 after H5N1 vaccination (Fig. 1B), an induction of more than 4.4-fold (Fig. 1C). The H5N1 booster vaccination increased the stalk titers minimally, to 1:15,267, to an induction of 5.6-fold over day 0 levels. This is in sharp contrast to H5-specific hemagglutination inhibition (HI) responses, which showed a strong increase after the second vaccination (26). However, levels of stalk-reactive antibodies decreased significantly only 6 months postvaccination, to 1:4,143, and stabilized at that level to 12 months postvaccination, representing a stable 1.6-fold induction over baseline. This pattern follows H5-specific serum IgG and microneutralization (MN) titers published previously (29). No statistically significant difference could be detected between peak induction or longevity between the four vaccine groups (Fig. 1D).

H5N1 vaccine induces stalk-reactive antibodies that cross-react to other group 1 but not group 2 HAs. The cH6/1 protein used to measure stalk-reactive antibodies includes an H1 stalk domain. H1-H5 stalk cross-reactive antibodies have been described in the literature (6, 13) and can be expected since humans have preexisting immunity to the H1 stalk and have now been exposed to the H5 stalk domain. However, it remained unclear how broadly the induced stalk-reactive antibodies would react to other members of the group 1 HAs and if there would be any cross-reactivity toward group 2 HAs. We therefore assessed the induction of antibodies against H2, H18, and H3 HAs on day 42. H2 is closely related to H1 and H5 and falls into the H1 clade of the group 1 HAs. H18 was recently discovered in Peruvian bats and forms a distinct clade of the group 1 HAs together with another HA discovered in bats, H17 (32). H3 is a group 2 HA and was used in this study to assess induction of cross-group stalk-reactive antibodies. As expected, H5N1 vaccination induced high titers of H2-reactive antibodies, rising from 1:1,119 on day 0 to 1:4,095 on day 42, a 3.6-fold induction (Fig. 2A and B). Anti-H2 titers also showed good correlation with anti-cH6/1 titers, an indication that the increased H2 reactivity is directed against the stalk domain (Fig. 2C). Titers of antibody against H18 rose from 1:791 on day 0 to 1:2,024 on day 42, which represents a 2.5-fold induction (Fig. 2A and B). However, titers of antibody against H3 rose only marginally, from 1:2,186 to 1:2,599, representing a 1.19-fold induction (Fig. 2A and B). It is questionable whether this increase in antibody titer is biologically relevant. Our data suggest that H5N1 vaccination is able to significantly induce titers against heterosubtypic group 1 HAs but is unable to induce titers against members of group 2 HAs. In addition to studying the induction of anti-HA stalk antibodies, we also assessed levels of antibody against the viral neuraminidase (NA). Titers of antibody against the homologous N1 NA from A/Vietnam/1203/04 were induced 1.5-fold on day 42 over day 0, and only a 1.1-fold induction against the N1 NA of A/California/04/09 was detected (Fig. 3).

Stalk-reactive antibodies are functional and protect in a passive-transfer challenge model. Antibodies detected by ELISA may or may not have biological functions and relevance *in vivo*. We therefore explored whether the detected stalk-reactive antibodies found in the sera of H5N1-vaccinated individuals have neutralizing activity *in vitro* and protective efficacy *in vivo*. To that end, we used a cH9/1N3 virus that was described previously (24). This virus can be used to specifically measure stalk-reactive antibodies since humans are naive to the H9 head domain. An N3 NA—to which humans are naive as well—was used to exclude the contribution of NA-reactive antibodies to neutralization or protection. As expected, no increase in cross-reactive anti-H9 head antibodies was detected between day 0 and day 42 (Fig. 4B). Subsequently, MN assays with day 0 and day 42 samples were performed using the cH9/1N3 virus. Microneutralization titers increased significantly upon H5N1 vaccination. Also, we could detect a clear correlation between cH6/1 reactivity and cH9/1N3 MN titers (Fig. 1E and F). To investigate if this response would also have biological relevance *in vivo* (Fig. 4A), we selected a subset

antibodies declined to a level of 1.5-fold over baseline and were retained at that level for up to 12 months (1.6-fold). (D) Induction of stalk-reactive antibodies for individual vaccine groups. There was no statistical significant difference in induction of stalk-reactive antibodies for the various HA contents of the vaccines, as well as adjuvant, for any time point (21 days, $P = 0.3391$; 42 days, $P = 0.6267$; 6 months, $P = 0.3068$; 12 months, $P = 0.9807$). (E) Biological activity of stalk-reactive antibodies was measured in a microneutralization assay with a cH9/1 N3 virus. Using an HA head domain and neuraminidase to which humans are naive ensured measurement of stalk-reactive antibodies only. Baseline titers were relatively high, at 1:569, but showed a significant increase 42 days postpriming, to 1:1,092 ($P < 0.0001$). (F) Correlation of stalk-reactive antibodies measured by ELISA and microneutralization assay. The levels of stalk-reactive antibodies measured by ELISA significantly correlated with their measured neutralizing ability *in vitro* (Spearman $r = 0.5225$; $P < 0.0001$). n.s., not significant. In Fig. 1 to 4, * indicates a P value of ≤ 0.05, ** indicates a P value of ≤ 0.01, and *** indicates a P value of ≤ 0.001.

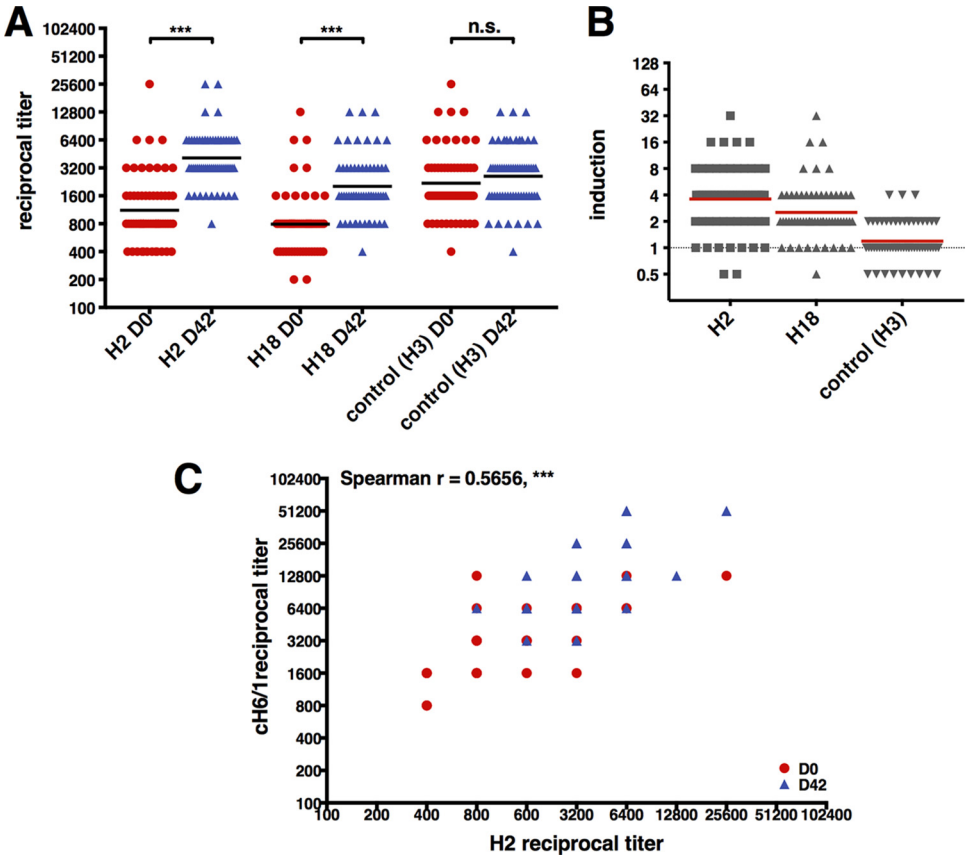

FIG 2 H5N1 vaccination induces cross-reactive group 1 HA antibodies in humans. (A) Titers of antibody against group 1 HAs H2 and H18 and group 2 HA H3 were measured on day 0 and day 42 postpriming. Titers of antibody against H2 increased from 1:1,118 to 1:4,095 ($P < 0.0001$) and titers of antibody against H18 from 1:791 to 1:2,024 ($P < 0.0001$). Titers of antibody against H3 remained at similar levels, 1:2,186 and 1:2,599 ($P = 0.66$). (B) Induction over baseline of antibodies against group 1 HAs H2 and H18 and group 2 HA H3 was calculated for day 42 postpriming. Antibodies against H2 show a 3.6-fold induction over baseline and induction of antibodies against H18 was 2.5-fold, confirming group cross-reactivity for group 1 HAs. Cross-group antibodies against H3 showed only a low induction, at 1.19-fold over baseline. (C) Correlation of stalk-reactive and H2 cross-reactive antibodies. Antibodies against the HA stalk domain and H2 showed a significant positive correlation (Spearman $r = 0.5656$; $P < 0.0001$). This indicates that the cross-reactivity is mostly caused by antibodies against shared epitopes in the stalk domain. All samples were analyzed, and overlapping results are presented as single points.

of sera, pooled day 0 and day 42 samples, and performed a passive-transfer challenge experiment with mice (Fig. 4A). We used the cH9/1N3 virus to measure protection by stalk-reactive antibodies only. Pooled sera from day 0 or day 42 (MN titers of selected sera shown in Fig. 4E) were transferred into mice via intraperitoneal injection, and mice were then challenged 2 h later intranasally with cH9/1N3 virus (Fig. 4A). The lungs of these animals were harvested on day 3 and day 6 postinfection, and weight was recorded on days 0, 3, and 6. Day 3 lung titers already showed a significant reduction in viral load, approximately 1 log, in the mice that received postvaccination sera compared to the animals that received prevaccination sera. These differences were even more pronounced on day 6, when mice that had received prevaccination sera still had lung titers of approximately 10^4 PFU/ml, whereas only 10^2 PFU/ml was detected in 2 of the mice that received postvaccination sera. Virus was undetectable in lungs of the three remaining mice (Fig. 4C). Weight loss followed a similar trend; animals that received prevaccination sera lost 3.7% of their initial weight by day 4, which continued until day 6 (4.6% weight loss) without signs of recovery (Fig. 4D). Mice that received postvaccination sera showed an average 1.6% weight loss on day 3 and essentially recovered their initial weight by day 6. These data indicate that the H5N1-induced antibodies are neutralizing and biologically active.

DISCUSSION

The isolation of stalk-reactive antibodies from human individuals in recent years has spurred the development of universal influenza virus vaccines against this conserved part of the viral HA. Given the constant drift of seasonal influenza virus strains and the threat from potential pandemic viruses like H2N2, H5N1, or H7N9, such a vaccine could have a significant impact on public health and pandemic preparedness. However, it has proven difficult to induce high levels of stalk-reactive antibodies due to the immunosubdominance of the stalk domain. Additionally, neutralizing epitopes in the stalk domain are almost exclusively conformational and relatively labile (6, 13, 27).

It has been hypothesized by us and others that sequential exposure to influenza viruses that have divergent HA head domains but conserved HA stalk domains could boost broadly neutralizing antistalk antibodies (16–18, 22, 24, 31, 33). This phenomenon was observed during the 2009 pandemic, when an H1 virus with a conserved stalk and a head domain that was very different from those of prepandemic seasonal H1 viruses was introduced into

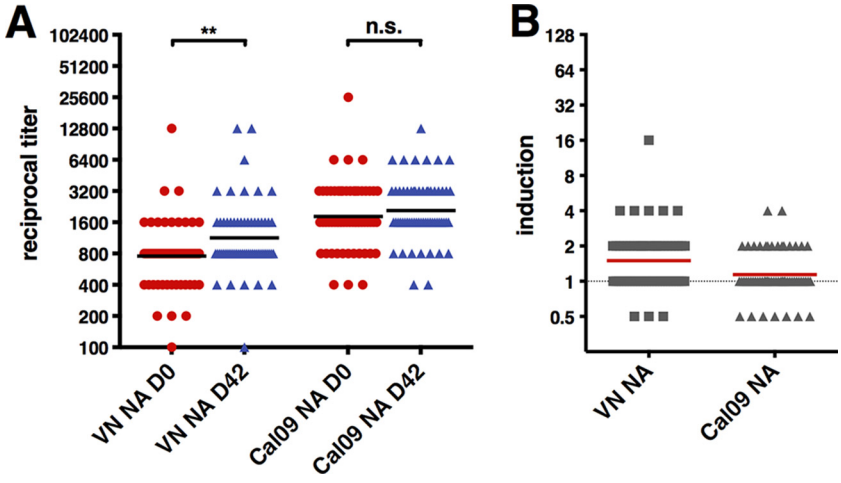

FIG 3 H5N1 vaccination elicits a weak antibody response against NA in humans. (A) Titers of antibody against homologous N1 (A/Vietnam/1203/2004 [VN]) and heterologous N1 (A/California/04/2009 [Cal09]) were measured on day 0 and day 42 postpriming. Titers of antibody against VN N1 increased slightly, from 1:755 to 1:1,131 ($P = 0.0031$), but the increase for Cal09 N1 from 1:1,817 to 1:2,072 was not significant ($P = 0.7231$). (B) Induction over baseline of antibodies against homologous N1 (VN) and heterologous N1 (Cal09) was calculated for day 42 postpriming. The induction for VN N1 was 1.5-fold over baseline, while induction for Cal09 N1 was only 1.1-fold.

the human population (17, 18, 22, 24, 33). Similar findings have also been reported for other cases of sequential exposure to influenza viruses with divergent HAs in humans (16, 31). Vaccination with H5 vaccines also constitutes a sequential-exposure scenario, since most adults have preexisting immunity against group 1 HAs from previous H1 or H2 infections or vaccination. Further evidence for the boosting of stalk-reactive antibodies comes from studies that report isolation of stalk-reactive antibodies from a survivor of H5N1 infection and from H5N1 vaccinees (34, 35). However, all previous reports were based on qualitative data only or data from studies that lacked matched pre- and postexposure samples. Our data clearly demonstrate, in a well-controlled and quantitative study, that high levels of stalk-reactive antibodies can be induced by H5N1 vaccination (Fig. 5A and B). This suggests that a sequential-exposure universal vaccine strategy (e.g., based on cHA inactivated split vaccine) could be successful (Fig. 5A and C).

We found that titers of stalk-reactive antibodies decline in a fashion similar to that of head-reactive antibodies. Antibody titers declined to almost baseline levels after only 6 months postvaccination. Although low levels of antibodies—and immunological memory—are associated with protection from mortality, it is questionable whether these low antibody titers have a protective effect against morbidity. Therefore, a strategy, possibly based on prime-boost regimens and/or more effective adjuvants (36–38), to induce long-term immunity may be needed for a successful stalk-based universal influenza virus vaccine. Another interesting question is how broadly reactive the antibody response induced by sequential vaccination would be. Most stalk-reactive antibodies that have been isolated react with either with group 1 or group 2 HAs, but two monoclonal antibodies that cross-react between group 1 and group 2 HAs have been reported (11, 12). Our data indicate that vaccination with H5N1 induces antibodies that react broadly against group 1 HAs but do not cross-react with group 2 HAs (e.g., H3), confirming qualitative results previously gathered using isolated monoclonal antibodies. This suggests that successful stalk-based universal vaccines against influenza A viruses would need either two components (group 1 and group 2) or an optimized stalk domain that could induce antibodies against both HA groups.

A sequential-vaccination regimen that enhances stalk immunity and breaks the immunodominance of the globular head domain could also render conserved epitopes in influenza virus NA more immunogenic (39). We tested this hypothesis by assessing binding to homologous and heterologous N1 NAs and found a weak but significant induction against the homologous NA. However, only the presence of NA in the vaccine was confirmed and not the concentration and structural integrity of the NA in the vaccine formulation. The lack of boosting of cross-reactive NA antibodies could have been caused by the lack of correctly folded NA in the vaccine, leaving this question open for future studies.

Recently, a phenomenon called vaccine-associated enhanced respiratory disease (VAERD) has been reported to occur in pigs vaccinated with M2e-NP antigens or mismatched inactivated virus upon challenge with H1 viruses (40, 41). One report also described a correlation between antibodies that bind to a linear epitope located near the fusion peptide—which is also located in the stalk domain—and VAERD, raising potential safety concerns about enhanced disease caused by high levels of stalk-reactive antibodies (42). We addressed this experimentally by performing a passive-transfer experiment with mice. However, mice that received postvaccination sera were better protected in terms of lung titers and weight loss than animals that received prevaccination sera with lower titers of stalk-reactive antibodies, and no exacerbated disease was observed.

In conclusion, we show that stalk-reactive antibodies are induced upon H5N1 vaccination. These data suggest that sequential exposure of humans to HAs with divergent heads and conserved stalks selectively boosts functional stalk-reactive antibodies. A universal influenza virus vaccine strategy based on the same prin-

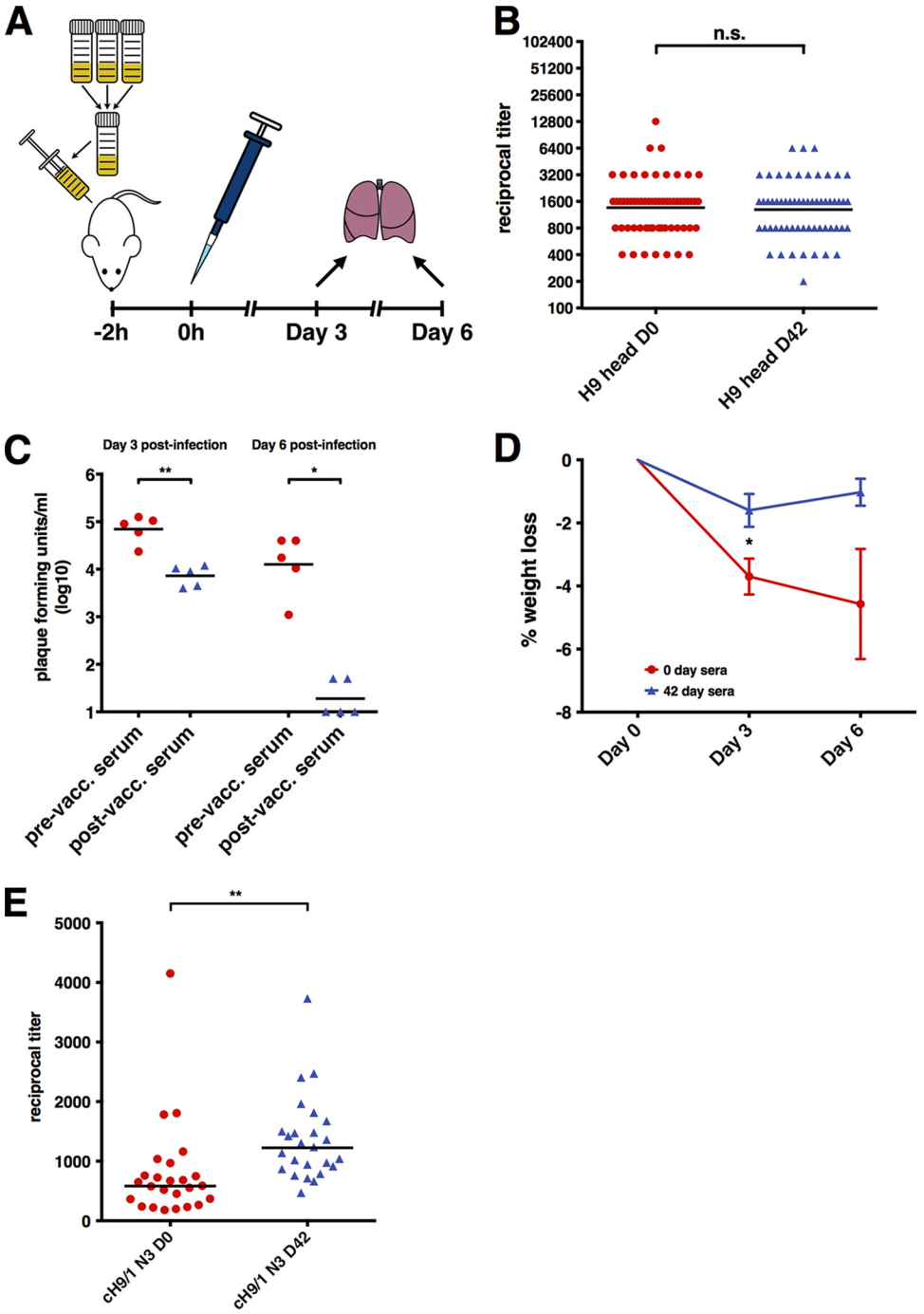

FIG 4 Protective activity of stalk-reactive antibodies. (A) All participants with an 8-fold or higher induction ($n = 27$) of stalk-reactive antibody titers 42 days postpriming were selected. Their day 0 and day 42 sera were pooled and intraperitoneally injected into mice. After 2 h, mice were challenged intranasally with cH9/1 N3 virus. Lungs of 5 mice for each group were harvested 3 days and 6 days after challenge. (B) An ELISA against the H9 head domain confirmed that there was no significant increase in antibodies against the H9 head domain 42 days postpriming (1:1,131 on day 0 versus 1:1,275 on day 42; $P = 0.4901$). (C) Virus titers in the lung were assessed in a plaque assay 3 days and 6 days postchallenge. Virus titers in the lungs of mice that received postvaccination sera were significantly lower on day 3 postchallenge (9.6-fold difference; $P = 0.0036$). On day 6 after virus challenge, 3 of the mice that received postvaccination sera had lung virus titers below the detection limit of 10 PFU per ml; the other 2 showed titers of 0.5×10^2, which was significantly lower than the mean titer of 1.3×10^4 for the mice that received prevaccination sera ($P = 0.0243$). (D) Mice that received postvaccination sera had significantly lower weight loss 3 days postchallenge (1.6% versus 3.7% weight loss; $P = 0.0144$) and even regained weight 6 days postchallenge, while mice that received prevaccination sera continued to lose weight (4.6% weight loss). (E) The neutralizing activity of stalk-reactive antibodies for the sera used in the passive-transfer experiment was assessed in a cH9/1 N3 microneutralization assay. The titer 42 days postpriming was significantly higher, at 1:1,225, than that on day 0, 1:582 ($P = 0.0022$).

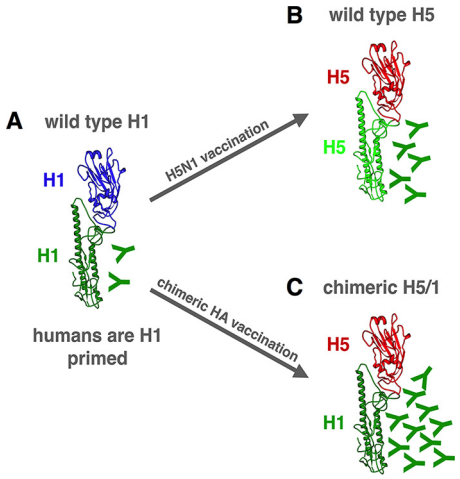

FIG 5 Model for induction of stalk-reactive antibodies by sequential exposure to HAs with conserved epitopes in the stalk but divergent globular head domains. Most humans had prior contact with H1 (A) and are therefore primed against the H1 stalk domain (dark green). The H5 (B) stalk domain (light green) shares conserved epitopes with the H1 stalk domain. This study shows that vaccination against H5N1 can boost antibodies against those shared epitopes. A vaccination strategy based on chimeric HAs (C) would combine an H1 stalk and an H5 head domain and might induce even higher titers of antibody against the HA stalk domain.

ciple seems feasible and could have an impact on public health and pandemic preparedness.

ACKNOWLEDGMENTS

We thank Randy Albrecht for advice on hemagglutination inhibition assays and Chen Wang for excellent technical assistance.

Florian Krammer was supported by an Erwin Schrödinger fellowship (J3232) from the Austrian Science Fond (FWF). This work was partially supported by Centers for Excellence for Influenza Research and Surveillance (CEIRS) grant HHSN266200700010C, NIH grant U19 AI109946, and an NIH adjuvant development contract (HHSN272200900032C). Rebecca J. Cox was supported by the EU FP7 Univax (601738), RCN Globvac project (220670), the K. G. Jebsen Centre for Influenza Vaccine Research, Helse Vest, and intramurally by the Influenza Centre, University of Bergen, through the Ministry of Health and Care Services, Norway.

REFERENCES

1. **Moody MA, Zhang R, Walter EB, Woods CW, Ginsburg GS, McClain MT, Denny TN, Chen X, Munshaw S, Marshall DJ, Whitesides JF, Drinker MS, Amos JD, Gurley TC, Eudailey JA, Foulger A, DeRosa KR, Parks R, Meyerhoff RR, Yu JS, Kozink DM, Barefoot BE, Ramsburg EA, Khurana S, Golding H, Vandergrift NA, Alam SM, Tomaras GD, Kepler TB, Kelsoe G, Liao HX, Haynes BF.** 2011. H3N2 influenza infection elicits more cross-reactive and less clonally expanded anti-hemagglutinin antibodies than influenza vaccination. PLoS One **6:**e25797. http://dx.doi.org/10.1371/journal.pone.0025797.
2. **Margine I, Hai R, Albrecht RA, Obermoser G, Harrod AC, Banchereau J, Palucka K, García-Sastre A, Palese P, Treanor JJ, Krammer F.** 2013. H3N2 influenza virus infection induces broadly reactive hemagglutinin stalk antibodies in humans and mice. J. Virol. **87:**4728–4737. http://dx.doi.org/10.1128/JVI.03509-12.
3. **Wrammert J, Smith K, Miller J, Langley WA, Kokko K, Larsen C, Zheng NY, Mays I, Garman L, Helms C, James J, Air GM, Capra JD, Ahmed R, Wilson PC.** 2008. Rapid cloning of high-affinity human monoclonal antibodies against influenza virus. Nature **453:**667–671. http://dx.doi.org/10.1038/nature06890.
4. **Tan GS, Krammer F, Eggink D, Kongchanagul A, Moran TM, Palese P.** 2012. A pan-h1 anti-hemagglutinin monoclonal antibody with potent broad-spectrum efficacy in vivo. J. Virol. **86:**6179–6188. http://dx.doi.org/10.1128/JVI.00469-12.
5. **Ekiert DC, Friesen RH, Bhabha G, Kwaks T, Jongeneelen M, Yu W, Ophorst C, Cox F, Korse HJ, Brandenburg B, Vogels R, Brakenhoff JP, Kompier R, Koldijk MH, Cornelissen LA, Poon LL, Peiris M, Koudstaal W, Wilson IA, Goudsmit J.** 2011. A highly conserved neutralizing epitope on group 2 influenza A viruses. Science **333:**843–850. http://dx.doi.org/10.1126/science.1204839.
6. **Sui J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, Santelli E, Stec B, Cadwell G, Ali M, Wan H, Murakami A, Yammanuru A, Han T, Cox NJ, Bankston LA, Donis RO, Liddington RC, Marasco WA.** 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol. **16:**265–273. http://dx.doi.org/10.1038/nsmb.1566.
7. **Wang TT, Tan GS, Hai R, Pica N, Petersen E, Moran TM, Palese P.** 2010. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. PLoS Pathog. **6:**e1000796. http://dx.doi.org/10.1371/journal.ppat.1000796.
8. **Throsby M, van den Brink E, Jongeneelen M, Poon LL, Alard P, Cornelissen L, Bakker A, Cox F, van Deventer E, Guan Y, Cinatl J, ter Meulen J, Lasters I, Carsetti R, Peiris M, de Kruif J, Goudsmit J.** 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One **3:**e3942. http://dx.doi.org/10.1371/journal.pone.0003942.
9. **Friesen RH, Koudstaal W, Koldijk MH, Weverling GJ, Brakenhoff JP, Lenting PJ, Stittelaar KJ, Osterhaus AD, Kompier R, Goudsmit J.** 2010. New class of monoclonal antibodies against severe influenza: prophylactic and therapeutic efficacy in ferrets. PLoS One **5:**e9106. http://dx.doi.org/10.1371/journal.pone.0009106.
10. **Krammer F, Hai R, Yondola M, Tan GS, Leyva-Grado VH, Ryder AB, Miller MS, Rose JK, Palese P, García-Sastre A, Albrecht RA.** 2014. Assessment of influenza virus hemagglutinin stalk-based immunity in ferrets. J. Virol. **88:**3432–3442. http://dx.doi.org/10.1128/JVI.03004-13.
11. **Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, Silacci C, Fernandez-Rodriguez BM, Agatic G, Bianchi S, Giacchetto-Sasselli I, Calder L, Sallusto F, Collins P, Haire LF, Temperton N, Langedijk JP, Skehel JJ, Lanzavecchia A.** 2011. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. Science **333:**850–856. http://dx.doi.org/10.1126/science.1205669.
12. **Dreyfus C, Laursen NS, Kwaks T, Zuijdgeest D, Khayat R, Ekiert DC, Lee JH, Metlagel Z, Bujny MV, Jongeneelen M, van der Vlugt R, Lamrani M, Korse HJ, Geelen E, Sahin Ö, Sieuwerts M, Brakenhoff JP, Vogels R, Li OT, Poon LL, Peiris M, Koudstaal W, Ward AB, Wilson IA, Goudsmit J, Friesen RH.** 2012. Highly conserved protective epitopes on influenza B viruses. Science **337:**1343–1348. http://dx.doi.org/10.1126/science.1222908.
13. **Ekiert DC, Bhabha G, Elsliger MA, Friesen RH, Jongeneelen M, Throsby M, Goudsmit J, Wilson IA.** 2009. Antibody recognition of a highly conserved influenza virus epitope. Science **324:**246–251. http://dx.doi.org/10.1126/science.1171491.
14. **Friesen RH, Lee PS, Stoop EJ, Hoffman RM, Ekiert DC, Bhabha G, Yu W, Juraszek J, Koudstaal W, Jongeneelen M, Korse HJ, Ophorst C, Brinkman-van der Linden EC, Throsby M, Kwakkenbos MJ, Bakker AQ, Beaumont T, Spits H, Kwaks T, Vogels R, Ward AB, Goudsmit J, Wilson IA.** 2014. A common solution to group 2 influenza virus neutralization. Proc. Natl. Acad. Sci. U. S. A. **111:**445–450. http://dx.doi.org/10.1073/pnas.1319058110.
15. **Okuno Y, Isegawa Y, Sasao F, Ueda S.** 1993. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J. Virol. **67:**2552–2558.
16. **Miller MS, Tsibane T, Krammer F, Hai R, Rahmat S, Basler CF, Palese P.** 2013. 1976 and 2009 H1N1 influenza virus vaccines boost anti-hemagglutinin stalk antibodies in humans. J. Infect. Dis. **207:**98–105. http://dx.doi.org/10.1093/infdis/jis652.
17. **Wrammert J, Koutsonanos D, Li GM, Edupuganti S, Sui J, Morrissey M, McCausland M, Skountzou I, Hornig M, Lipkin WI, Mehta A, Razavi B, Del Rio C, Zheng NY, Lee JH, Huang M, Ali Z, Kaur K, Andrews S, Amara RR, Wang Y, Das SR, O'Donnell CD, Yewdell JW, Subbarao K, Marasco WA, Mulligan MJ, Compans R, Ahmed R, Wilson PC.** 2011. Broadly cross-reactive antibodies dominate the human B cell

response against 2009 pandemic H1N1 influenza virus infection. J. Exp. Med. **208:**181–193. http://dx.doi.org/10.1084/jem.20101352.

18. **Li GM, Chiu C, Wrammert J, McCausland M, Andrews SF, Zheng NY, Lee JH, Huang M, Qu X, Edupuganti S, Mulligan M, Das SR, Yewdell JW, Mehta AK, Wilson PC, Ahmed R.** 2012. 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. **109:**9047–9052. http://dx.doi.org/10.1073/pnas.1118979109.
19. **Krammer F, Palese P.** 2013. Influenza virus hemagglutinin stalk-based antibodies and vaccines. Curr. Opin. Virol. **3:**521–530. http://dx.doi.org/10.1016/j.coviro.2013.07.007.
20. **Krammer F, Palese P.** 2014. Universal influenza virus vaccines: need for clinical trials. Nat. Immunol. **15:**3–5. http://dx.doi.org/10.1038/ni.2761.
21. **Krammer F, Jul-Larsen A, Margine I, Hirsh A, Sjursen H, Zambon M, Cox RJ.** 2014. An H7N1 influenza virus vaccine induces broadly reactive antibody responses against H7N9 in humans. Clin. Vaccine Immunol. **21:**1153–1163. http://dx.doi.org/10.1128/CVI.00272-14.
22. **Krammer F, Pica N, Hai R, Tan GS, Palese P.** 2012. Hemagglutinin stalk-reactive antibodies are boosted following sequential infection with seasonal and pandemic H1N1 influenza virus in mice. J. Virol. **86:**10302–10307. http://dx.doi.org/10.1128/JVI.01336-12.
23. **Krammer F, Pica N, Hai R, Margine I, Palese P.** 2013. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. J. Virol. **87:**6542–6550. http://dx.doi.org/10.1128/JVI.00641-13.
24. **Pica N, Hai R, Krammer F, Wang TT, Maamary J, Eggink D, Tan GS, Krause JC, Moran T, Stein CR, Banach D, Wrammert J, Belshe RB, García-Sastre A, Palese P.** 2012. 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. **109:**2573–2578. http://dx.doi.org/10.1073/pnas.1200039109.
25. **Hai R, Krammer F, Tan GS, Pica N, Eggink D, Maamary J, Margine I, Albrecht RA, Palese P.** 2012. Influenza viruses expressing chimeric hemagglutinins: globular head and stalk domains derived from different subtypes. J. Virol. **86:**5774–5781. http://dx.doi.org/10.1128/JVI.00137-12.
26. **Cox RJ, Pedersen G, Madhun AS, Svindland S, Saevik M, Breakwell L, Hoschler K, Willemsen M, Campitelli L, Nostbakken JK, Weverling GJ, Klap J, McCullough KC, Zambon M, Kompier R, Sjursen H.** 2011. Evaluation of a virosomal H5N1 vaccine formulated with Matrix M adjuvant in a phase I clinical trial. Vaccine **29:**8049–8059. http://dx.doi.org/10.1016/j.vaccine.2011.08.042.
27. **Krammer F, Margine I, Tan GS, Pica N, Krause JC, Palese P.** 2012. A carboxy-terminal trimerization domain stabilizes conformational epitopes on the stalk domain of soluble recombinant hemagglutinin substrates. PLoS One **7:**e43603. http://dx.doi.org/10.1371/journal.pone.0043603.
28. **Margine I, Palese P, Krammer F.** 2013. Expression of functional recombinant hemagglutinin and neuraminidase proteins from the novel H7N9 influenza virus using the baculovirus expression system. J. Vis. Exp. **2013**(81):e51112. http://dx.doi.org/10.3791/51112.
29. **Pedersen GK, Hoschler K, Oie Solbak SM, Bredholt G, Pathirana RD, Afsar A, Breakwell L, Nøstbakken JK, Raae AJ, Brokstad KA, Sjursen H, Zambon M, Cox RJ.** 17 June 2014. Serum IgG titres, but not avidity, correlates with neutralizing antibody response after H5N1 vaccination. Vaccine http://dx.doi.org/10.1016/j.vaccine.2014.06.009.
30. **Sangster MY, Baer J, Santiago FW, Fitzgerald T, Ilyushina NA, Sundararajan A, Henn AD, Krammer F, Yang H, Luke CJ, Zand MS, Wright PF, Treanor JJ, Topham DJ, Subbarao K.** 2013. B cell response and hemagglutinin stalk-reactive antibody production in different age cohorts following 2009 H1N1 influenza virus vaccination. Clin. Vaccine Immunol. **20:**867–876. http://dx.doi.org/10.1128/CVI.00735-12.
31. **Miller MS, Gardner TJ, Krammer F, Aguado LC, Tortorella D, Basler CF, Palese P.** 2013. Neutralizing antibodies against previously encountered influenza virus strains increase over time: a longitudinal analysis. Sci. Transl. Med. **5:**198ra107. http://dx.doi.org/10.1126/scitranslmed.3006637.
32. **Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO.** 2013. New World bats harbor diverse influenza A viruses. PLoS Pathog. **9:**e1003657. http://dx.doi.org/10.1371/journal.ppat.1003657.
33. **Thomson CA, Wang Y, Jackson LM, Olson M, Wang W, Liavonchanka A, Keleta L, Silva V, Diederich S, Jones RB, Gubbay J, Pasick J, Petric M, Jean F, Allen VG, Brown EG, Rini JM, Schrader JW.** 2012. Pandemic H1N1 influenza infection and vaccination in humans induces cross-protective antibodies that target the hemagglutinin stem. Front. Immunol. **3:**87. http://dx.doi.org/10.3389/fimmu.2012.00087.
34. **Kashyap AK, Steel J, Oner AF, Dillon MA, Swale RE, Wall KM, Perry KJ, Faynboym A, Ilhan M, Horowitz M, Horowitz L, Palese P, Bhatt RR, Lerner RA.** 2008. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc. Natl. Acad. Sci. U. S. A. **105:**5986–5991. http://dx.doi.org/10.1073/pnas.0801367105.
35. **Whittle JR, Wheatley AK, Wu L, Lingwood D, Kanekiyo M, Ma SS, Narpala SR, Yassine HM, Frank GM, Yewdell JW, Ledgerwood JE, Wei CJ, McDermott AB, Graham BS, Koup RA, Nabel GJ.** 2014. Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. J. Virol. **88:**4047–4057. http://dx.doi.org/10.1128/JVI.03422-13.
36. **Ledgerwood JE, Zephir K, Hu Z, Wei CJ, Chang L, Enama ME, Hendel CS, Sitar S, Bailer RT, Koup RA, Mascola JR, Nabel GJ, Graham BS, VRC 310 Study Team.** 2013. Prime-boost interval matters: a randomized phase 1 study to identify the minimum interval necessary to observe the H5 DNA influenza vaccine priming effect. J. Infect. Dis. **208:**418–422. http://dx.doi.org/10.1093/infdis/jit180.
37. **Talaat KR, Luke CJ, Khurana S, Manischewitz J, King LR, McMahon BA, Karron RA, Lewis KD, Qin J, Follmann DA, Golding H, Neuzil KM, Subbarao K.** 2014. A live attenuated influenza A(H5N1) vaccine induces long-term immunity in the absence of a primary antibody response. J. Infect. Dis. **209:**1860–1869. http://dx.doi.org/10.1093/infdis/jiu123.
38. **Goff PH, Eggink D, Seibert CW, Hai R, Martínez-Gil L, Krammer F, Palese P.** 2013. Adjuvants and immunization strategies to induce influenza virus hemagglutinin stalk antibodies. PLoS One **8:**e79194. http://dx.doi.org/10.1371/journal.pone.0079194.
39. **Wohlbold TJ, Krammer F.** 2014. In the shadow of hemagglutinin: a growing interest in influenza viral neuraminidase and its role as a vaccine antigen. Viruses **6:**2465–2494. http://dx.doi.org/10.3390/v6062465.
40. **Vincent AL, Lager KM, Janke BH, Gramer MR, Richt JA.** 2008. Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine. Vet. Microbiol. **126:**310–323. http://dx.doi.org/10.1016/j.vetmic.2007.07.011.
41. **Heinen PP, Rijsewijk FA, de Boer-Luijtze EA, Bianchi AT.** 2002. Vaccination of pigs with a DNA construct expressing an influenza virus M2-nucleoprotein fusion protein exacerbates disease after challenge with influenza A virus. J. Gen. Virol. **83:**1851–1859.
42. **Khurana S, Loving CL, Manischewitz J, King LR, Gauger PC, Henningson J, Vincent AL, Golding H.** 2013. Vaccine-induced anti-HA2 antibodies promote virus fusion and enhance influenza virus respiratory disease. Sci. Transl. Med. **5:**200ra114. http://dx.doi.org/10.1126/scitranslmed.3006366.