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Vaccination with drifted variants of avian H5 hemagglutinin protein elicits a broadened antibody response that is protective against challenge with homologous or drifted live H5 influenza virus

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Abstract

Substantial H5 influenza HA directed immunity is elicited after vaccination of human subjects who had been previously immunized with a drifted H5 HA variant. We sought to investigate the characteristics of H5 HA specific immune responses in more depth by developing an animal model of H5 HA vaccination using drift variants of recombinant H5 HA proteins. HA proteins derived from influenzas A/Vietnam/1203/04 (Clade 1) and A/Indonesia/05/05 (Clade 2.1) were chosen. The sequence of vaccination consisted of two doses of homologous protein, followed by one additional dose of the homologous or heterologous, drifted HA protein. Each dose of HA was combined with CpG as an adjuvant and was injected subcutaneously. All the animals exhibited a serum IgG antibody response that cross-reacted with both HAs in an ELISA. However, those animals that received the drifted variant exhibited higher reactivity to the heterologous HA. Competitive ELISA of serum from drift-variant recipients showed evidence of antibody focusing towards the drifted HA, suggesting modification of the response towards improved cross-reactivity, though development of neutralizing antibodies was limited. Nevertheless, animals were protected against live-virus challenge, and passive transfer of serum was sufficient to confer protection to otherwise naïve mice, indicating that both neutralizing and non-neutralizing antibodies offer some degree of protection. These findings suggest that pre-vaccination against H5 influenza has the potential to prime immunity against emerging drifted H5 strains, and could also lower the dose requirements of vaccination in the event of a pandemic.

Keywords

Influenza virus; vaccine; hemagglutinin; B cells; antibody; immunology

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1. Introduction

Highly pathogenic avian influenza viruses, of the H5N1 subtype, emerged as human pathogens in Hong Kong in 1997 [1–3]. These viruses continue to circulate in wild birds, and since 2003, H5N1 viruses have been responsible for yearly outbreaks in human populations in Asia, Africa, and Europe [4]. While the H5N1 viruses have currently shown limited human-to-human transmissibility, viral infection is characterized by a 60% mortality rate due to the induction of a fulminant viral pneumonia, leukopenia, hepercytokinemia, and systemic dissemination of the virus [5–10]. Since their emergence, H5N1 viruses have rapidly acquired a significant amount of mutations (antigenic drift), most notable in its hemagglutinin (HA) genes, resulting in their classification into various clades/subclades based on their phylogeny and antibody reactivity to prototype strains [10–16]. The World Health Organization has identified H5N1 viruses as a potential threat for a pandemic and various prototype viruses are selected yearly as potential vaccine candidates. Unfortunately, due to the rapid evolution occurring within viral surface proteins, HA in particular, the current predictive approach to formulating a vaccine that matches the circulating pandemic strain is challenging.

Various strategies have been proposed to generate vaccines that can either prevent or ameliorate the effects of viral infection in the event of an H5N1 pandemic. One such strategy is the pre-vaccination of the population with currently stockpiled H5N1 vaccines in an effort to confer partial immunity to drifted virus within the same or a different clade. One major concern regarding the pre-vaccination strategy is that vaccination with antigenically distinct variants will result in a misdirected antibody response primarily focused against the priming antigen [17–19]. Such a response could potentially be detrimental to the generation of a protective response against the new antigen. This phenomenon, termed “Original Antigenic Sin”, has been demonstrated in the context of live influenza infection, but its role in experimental influenza vaccines is still under investigation. Evidence supporting the idea of pre-vaccination is limited, but studies in humans suggest that immunization with baculovirus-expressed recombinant hemagglutinin (rHA) H5N1 vaccine (clade 0) primes the immune system, resulting in clinically significant serological responses following a single dose of a inactivated H5N1 vaccine containing a drifted (clade 1) virus [20]. Further studies, using the same cohort of subjects, have revealed that there is a population of memory B cells that are specific and cross-reactive to both H5 HA antigens and respond rapidly to vaccination with the drifted variant [21]. These studies show that pre-vaccination does not preclude the ability to generate new and/or cross-reactive cellular and antibody responses to the new drifted H5. Furthermore, the cellular and antibody responses in these subjects present the hallmarks of a boosted/recall response. Whether these findings are exclusive to the particular combination of drifted variants used for these studies needs to be determined. However, similar findings using B cell cloning strategies following vaccination of human subjects suggest that this phenomenon is not restricted to the H5 subtype [22]. Intriguing as these results might be, it is very challenging to determine the immune basis of these observations by solely studying human subjects. In addition, a few questions linger from these human studies regarding the sequence in which the vaccination is administered, the transferability of the findings to other combinations of drifted variant HA pairs, and the relationship between the H5-specific cellular and antibody response.

In this study we take advantage of a mouse model of vaccination, in which mice are immunized in a homologous or heterologous fashion, with baculovirus-derived recombinant H5 hemagglutinins (rH5) from representative avian influenza viruses from clade 2.1 (A/Indonesia/05/05) and clade 1 (A/Vietnam/1203/04) that share a 96% amino acid identity. With this system we have been able to study the cross-reactive antibody response to

heterologous vaccination with a different, drifted rH5 pair, and assess the extent protection induced by drift variant vaccination upon viral challenge.

2. Materials and Methods

2.1 Antigens

Baculovirus-derived, purified recombinant full-length hemagglutinins (HA0) of the H5 subtype from A/VietNam/1203/04 (H5VN04) and A/Indonesia/05/05 (H5IN05), were purchased from Protein Sciences (Protein Sciences Corporation, Meriden, CT) and/or obtained from the NIH Biodefense and emerging infections research resources (BEIR) repository (NR-10510, NR-10511). Full-length control hemagglutinins H3/Wisconsin/67/05 and H1/New Caledonia/20/99 were purchased from Protein Sciences (Protein Sciences Corporation, Meriden, CT).

2.2 Mice

Six to eight week-old male C57BL/6 (B6) mice were purchased from Charles River Laboratories and maintained in under pathogen free conditions at the University of Rochester vivarium facilities. Mice infected with H5N1 virus were housed in the BSL-3 facility under sealed negative pressure biocontainment units (BCU-2000).

2.3 Immunization and challenge

Mice, 10–12 weeks of age, were immunized subcutaneously at the base of the tail at day 0, followed by booster immunizations at the same site on days 14 and 28. Each mouse received a priming dose of 2μg of rHA followed by a booster dose, containing 1μg of the same rHA used for priming, at day 14. At day 28 a secondary booster immunization containing 1μg of homologous or heterologous rH5 HA was administered. A concentration of 10μg/mouse of CpG oligonucleotides (ODN-1826) was co-administered alongside the rHA as an adjuvant. All HA antigens were prepared in Phosphate Buffered Saline and administered in a final volume of 50uL/mouse. On days 50–56 after primary immunization mice were anesthetized with Avertin (2,2,2 Tribromoethanol) and inoculated intranasally with 30uL containing 25000 tissue culture infectious dose units (TCID50) of H5N1 (HA, NA) A/VietNam/1203/04 × A/PR/8/34 virus (Provided by Richard Webby at St. Jude Children's Research Hospital). Mice were monitored for weight loss and survival for a period of 12–14 days following infection. Challenge experiments were performed under BSL-3 conditions.

2.4 Blood collection

Blood was drawn at various time points by puncturing the submandibular vein. Blood was allowed to coagulate and serum was collected. Serum samples were stored at −80°C until needed.

2.5 ELISA

HA-specific immunoglobulin G (IgG) antibodies were detected by enzyme-linked immunosorbent assay (ELISA). 96 well flat-bottom plates (Nunc #422404) were coated with a concentration of 0.05μg per well of rH5 HA or control antigen. The plates were blocked with 1% PBS/BSA, serial dilutions of the serum were performed, and 50uL of each dilution were added in duplicate to the antigen-coated wells. Plates were incubated overnight at 4°C. Antigen specific IgG was detected by incubating the plates for 1 hour with 50uL of a goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Southern Biotech 1030-05) diluted according to manufacture's recommendations. The assay was developed by adding 75uL of TMB substrate (Biolegend Cat#421101) for 7–10 minutes and stopped by adding equal volume of 2N H2SO4. The optical density was read at 450nm. ELISA end point titers

are expressed as logarithm base 2 of the reciprocal of the highest dilution at which the optical density is above 4 standard deviations of the mean of a similarly diluted negative control.

2.6 Competitive

ELISA. To detect cross-reactive antibodies 96 well flat-bottom plates were coated as previously described with either H5VN04 or H5IN05 recombinant protein. The plates were blocked with 1% PBS/BSA and serum samples were diluted 1:25000 in 1% PBS/BSA. The competitor rHA antigen was prepared in PBS to a concentration of 10μg/ml. Serum samples and the competitor antigens were mixed in equal volumes (50ul) in a round bottom 96 well plate. The serum/competitor mix was incubated at 37°C for one hour. Following the incubation period, 50uL of each serum/competitor mix were added in duplicate to the antigen-coated wells. Plates were incubated overnight at 4°C. The remainder of the assay was performed using the antibodies and reagents described for the IgG ELISA.

2.7 Microneutralization assay

Neutralizing antibodies were detected in a microneutralization (MN) assay. Briefly, serum samples were inactivated at 56°C for 1 hour. Heat inactivated sera was serially diluted in incomplete Minimum Essential Medium (MEM) and incubated at 37°C with 100 TCID50 of H5N1 (HA, NA) A/VietNam/1203/04 × A/PR/834 (ΔH5xPR8) virus for 1 hour. A volume of 50uL of the serum/virus mix was transferred, in duplicate, into wells containing a confluent monolayer of Madin Darby Canine Kidney cells (MDCK) and the cells were incubated for 1 hour at 37°C. Unbound virus was removed by washing with incomplete media. 200uL of fresh incomplete media containing TPCK trypsin (0.5μg/ml) was added to each well and the plates were incubated at 37°C for 4 days. At the end of the incubation period the media was removed and the cells were fixed with of 1% Crystal violet in 10% Neutral Buffered Formalin. The neutralizing titer is expressed as the reciprocal of the highest dilution at which the sera provided complete protection from viral cytopathic effects in both (duplicate) wells. Microneutralization assays were performed under enhanced BSL-2 conditions.

2.8 Passive transfer of serum

Blood from immunized mice was collected 21 days after the last immunization. The serum was harvested, pooled, and stored at 4°C until use. A volume of 150uL of pooled serum was injected into the intraperitoneal cavity of age matched naïve mice one day prior to viral challenge with ΔH5xPR8.

3. Results

3.1 Development of an animal model of H5 drift variant immunization

The immunogenicity of avian H5 HA has been reported to be weak in comparison to other serotypes of influenza HA proteins [23]. The reasons for this are not clear, but recombinant, full-length, HA proteins (rHA) produced in baculovirus-infected insect cell cultures have been shown to be effective immunogens in both animal models and in human subjects [24–26]. We therefore chose to investigate the immunogenicity of H5 rHA proteins in mice. The quality of the rHA preparation can have an impact on the immune response elicited following vaccination [27]. To examine the integrity of the H5VN04 and/or H5IN05 protein preparations we tested the rHA stability by SDS-PAGE and biological activity by hemagglutination assay (data not shown). It is worth noting that no degradation in either of the rHA preparations throughout the duration of the experiments. Likewise, comparable ability to agglutinate chicken red blood cells was observed in both preparations suggesting

that the rHA proteins remained stable and biologically active throughout the duration of the experiments (data not shown). To test the immunogenicity of H5 rHA proteins initially, animals were immunized by a subcutaneous route with 2μg of purified rHA protein in the absence of any adjuvant. Vaccinated animals were sampled by collecting a small amount of blood from the submandibular vein at day 21 and the serum was tested for the presence of HA specific antibody by ELISA. No antibodies were detected, even after boosting at day 21 (data not shown). To compensate, 10 μg of CpG oligonucleotide was included as an adjuvant in the saline solution with the rHA for each animal, and an additional booster was administered again through the subcutaneous route (Table 1). The administration of CpG alongside the H5VN04 rHA allowed for the detection of specific IgG antibody 14 days after the first dose (Figure 1A). However, after boosting the same cohort of animals at day 14 with a second dose of the same rHA protein, a significant increase (18-fold average) in H5VN04 HA specific IgG antibodies was observed (Figure 1A). To further increase the HA specific antibody a third dose of rHA/CpG was administered 14 days after the second dose (28 days after priming immunization), and animals were bled at day 42 (14 days after the last boost). A modest increase (7-fold average) in HA-specific IgG was achieved upon administration of a third dose of protein. However, a third dose was necessary for the induction of antibodies with neutralizing activity against a virus containing the H5VN04 HA (Figure 3 and data not shown). It is worth noting that while H5 HA specific antibodies continue to increase over time in mice that were immunized just once, followed by CpG alone at 14 and 28 days, IgG titers (at day 42) were significantly lower than in mice vaccinated 3 times with antigen (Figure 1B). Similar results were achieved with the drifted rHA derived from H5 influenza A/Indonesia/5/05 (data not shown). These results reinforce the idea that, without adjuvant, H5 HA proteins are poorly immunogenic in H5-naïve mice; yet demonstrate that substantial antibody responses can be mounted when the response is adequately boosted.

3.2 The H5-specific serum IgG antibody response elicited by drift variant vaccination is comparable to the response elicited by homologous H5 vaccination

Previous reports from human studies have shown that prior immunization with an rH5 HA (A/Hong Kong/156/97) vaccine results in a “boosted” response to the drift variant VN04 upon re-vaccination with a single dose of A/VN/1203/04 split vaccine [20, 21]. To study how prior immunity to H5 HA can influence the antibody response elicited upon vaccination with rHA derived from a different H5 virus clade, an immunization schedule that included a drifted variant of rH5 HA was developed (Table 1). In this model, mice were vaccinated with two doses (day 0 and 14) of H5IN05 adjuvanted with CpG oligonucleotide. At day 28, mice received an equivalent dose of the drifted variant H5VN04. The choice to vaccinate in this order, H5IN05 followed by H5VN04, was dictated primarily in anticipation of live-virus challenge experiments with an H5N1 A/VietNam/1203/04 × A/PR/8/34 recombinant virus (ΔH5xPR8, see below) [28]. Parallel experiments in which the order was reversed (i.e. H5VN04 followed by H5IN05) produced comparable titers and the expected antigen specificity in the serological assays (data not shown).

Homologous vaccination of mice with either rH5 HA results in similar IgG titers against each immunizing antigen, suggesting that the two proteins are equally immunogenic (Figure 1B). Interestingly, serum ELISA titers in mice that received three doses of homologous protein were indistinguishable from those mice that received one dose of H5VN04 after priming with H5IN05 (Figure 1B). As expected, these H5VN04 specific IgG titers are significantly higher than those obtained from mice receiving only one dose of the protein 14 or 42 days prior to serum collection (Figure 1B and data not shown). The antibodies against H5 HA remain serotype-specific as the serum IgG response generated in either vaccination strategy demonstrated little or no cross-reactivity with H1 and H3 derived rHA proteins

(Figure 1C and 1D). Finally, the H5 specific responses elicited by homologous or heteorologous vaccination is comprised mainly of IgG2b, IgG2c, and IgG1 antibodies with very little to no IgM, IgA, or IgG3 (data not shown). These results suggest that prior vaccination with H5IN05 primes the humoral arm of the immune system to respond to closely-related, drifted HA proteins in a magnitude that is comparable to homologous vaccination.

3.3 A population of cross-reactive IgG antibodies is generated upon drift variant vaccination

Homologous vaccination, as well as drift variant vaccination, generates serum IgG antibodies that can react against both recombinant rH5 HAs (Figure 1C and 1D). However, the conventional ELISA assay does not reveal potential differences in the affinities of the antibodies for one HA or another. To determine the extent of cross reactivity between these antibodies we performed a competitive ELISA. In this assay, we selected one dilution of the immune sera that provided the most sensitivity to the concentration of competitor administered in the assay. Both the serum dilution and the concentration of competitor needed were determined empirically using a model antigen (data not shown). For this assay, the serum from vaccinated mice is first incubated with an excess of competitor rHA protein. Following this incubation the serum is transferred into a well coated with either rH5 HA (solid phase). In theory, H5 HA cross-reactive antibodies that bind the competitor protein are adsorbed resulting in a loss of reactivity to the rH5 HA in the well. In this assay, the serum IgG antibody elicited by homologous vaccination with either H5VN04 or H5IN05 showed very low cross-reactivity to the drifted variant when the corresponding immunizing HA is used as the solid phase antigen (Figure 2A and 2D). In both of these representative plots a decrease in optical density greater than 84% is observed only when the corresponding immunizing HA is used as both competitor and the solid phase antigen. In comparison, less than an 18% decrease in optical density is observed against the drifted and control rHAs. To account for variability among individual mice the data was normalized to BSA and expressed as percent signal reduction (Figure 2B, 2C, 2E, and 2F). Here, an 8–16% decrease in signal was observed against the drifted and control rHAs when the corresponding immunizing HA is used as both competitor and the solid phase antigen (Figure 2B and 2E). Interestingly, two observations stand out when using serum from a drift variant vaccination strategy. First, when H5VN04 is the solid phase antigen and H5IN05 is the competitor, we observe a 64–75% decrease in signal. The magnitude of this decrease is equivalent to that observed when using H5VN04 as the competitor (Figure 2C) suggesting that a population of cross-reactive antibodies is generated by drift variant vaccination. Second, when H5IN05 is used as the solid phase antigen, we observe a 70% decrease in signal when H5IN05 is the competitor (Figure 2F). However, when using H5VN04 as a competitor, the signal decrease is only 30% (Figure 2F). This suggests that only a subset of the total H5IN05 specific IgG generated upon drift variant vaccination can cross-react with the clade 1 H5VN04 variant. Finally, while drift variant vaccination did not significantly decrease the ability of H5IN05 to compete for binding to homologous protein in the ELISA (Figure 2F), it did increase the competition for H5VN04 by 25% (compare H5VN04 results in Figure 2E with that in 2F). Taken together these data suggest that drift variant vaccination can tune the IgG antibody response to increase reactivity against the new drifted variant of the same subtype.

3.4 Low incidence of neutralizing antibodies following drift variant vaccination

The efficiency of influenza vaccination in humans is measured by the ability of the vaccine to induce a neutralizing antibody titer equal or greater than 1:40 [29, 30]. We tested the ability of the antibodies, elicited by drift variant vaccination, to prevent the *in vitro* cytopathic effects of ΔH5xPR8 [28] using a microneutralization assay (Figure 3). Mice

given the three-dose homologous H5VN04 vaccine had the highest incidence of neutralizing activity (68%) with 15 out of 22 mice responding with a neutralizing titer of 1:40 or greater. On the other hand, only 6 out of 20 mice (30%) from the group that received the drift variant vaccine had relevant neutralizing activity. It is worth noting that no neutralizing antibodies against ΔH5xPR8 were detected following a single dose of H5VN04, two doses of H5VN04, or two doses of H5IN05 (Figure 3 and data not shown). Taken together these data suggest that while neutralizing antibodies can be raised by homologous vaccination, there is a low incidence of such antibodies generated upon rHA drift variant vaccination.

3.5 Drift variant vaccination reduces virus induced morbidity upon ΔH5xPR8 challenge

To test whether drift variant immunization elicits protection from viral challenge, we infected vaccinated mice with the ΔH5xPR8 strain of influenza virus [28]. While this virus is attenuated relative to the wild-type A/VN/1203/04 H5N1 influenza, infection causes substantial mortality and weight loss and in naïve C57BL/6 mice (Figure 4A and 4B). Mice receiving 3 immunizations, independent of vaccination strategy, survived the viral challenge. However, mice that received only one dose of H5VN04 HA and naïve controls continued to lose weight from day 3 onward, and were not significantly different statistically from one another. These two groups also had the highest mortalities of 50–60% (Figure 4A). On the other hand, no differences in weight loss were observed at any time point between mice vaccinated with 3 doses of H5VN04 and those vaccinated with the drift variant strategy. In addition, the drift variant strategy provided a significant reduction ($p<0.01$) in morbidity from days 4–9 or days 4–6 when compared to mice receiving only one dose of H5VN04 or naïve mice respectively (Figure 4C). These observations suggest that prior immunization with H5 HA can prime for a protective, HA-specific, response against a drifted variant of the virus following a single dose of vaccination with the drifted HA.

3.6 Antibodies elicited upon drift variant vaccination are sufficient to protect mice from virus-induced morbidity

Vaccination with rH5 HA proteins resulted in a low incidence of neutralizing antibodies when mice were vaccinated with the drifted HA proteins (Figure 3). To determine if there is a relationship between protection and virus neutralizing activity, we divided individual mice into separate groups based on the presence or absence of serum neutralizing antibodies, then challenged each group with ΔH5xPR8. Surprisingly, there were no consistent differences in morbidity between mice with MN antibody titers over 40 (MN>40) and those with low neutralizing activity (MN<40) (Figures 4C–E). The protection was most uniform in the drift variant group (Figure 4D), while two mice in the homologous H5VN04 group showed increased weight loss with one case of mortality (Figure 4C). All of the mice that received one dose of H5VN04 lost 15–30% of their starting weight (Figure 4E). These data suggest that both homologous and drift variant vaccination generates an H5 HA specific immune response that is capable of reducing morbidity upon viral challenge even in the absence of high neutralizing antibody titers.

The observation of substantial immune protection in the absence of neutralizing antibody titers suggested the possibility of other immune mechanisms operating to control infection. To control for the possible action of HA specific T cells, we performed passive transfer of immune serum to naïve mice, then challenged these mice with ΔH5xPR8. Immune serum was collected from individual mice and tested for the presence of neutralizing activity by MN assay. Serum with or without MN activity was then pooled for passive transfer. A portion of the serum from mice vaccinated with 3 doses of H5VN04 was diluted 1:8 to reduce MN activity below 1:40 (VN04-VN04-VN04 MN−). The MN titer of each resulting pool of sera is shown in figure 5A. Each serum pool was then transferred into naïve recipients, who were subsequently challenged with ΔH5xPR8 virus. As expected, mice

infused with MN+ serum from either drift variant or homologous H5VN04 vaccinated animals were protected from virus induced morbidity and mortality (Figure 5B and 5C). In contrast, recipients of MN− serum from donors vaccinated with 1 dose of H5VN04 showed intermediate weight loss and 60% succumbed to ΔH5xPR8 infection (Figure 5B and 5C). Likewise, severe morbidity and 100% mortality was observed in recipients of diluted serum (VN04-VN04-VN04 MN−). Interestingly, undiluted MN− serum from drift variant vaccinated mice was able to provide protection from morbidity and mortality in otherwise naïve mice (Figure 5B and 5C). Finally, passively transferred protection was H5 specific, as vaccinated mice were not protected from morbidity or mortality upon H1N1 A/PR/8/34 challenge (data not shown). Taken together these data suggests that serum antibodies elicited by drift variant vaccination are sufficient to reduce the morbidity and mortality induced by viral challenge with ΔH5xPR8. Furthermore, while this protection did not depended on neutralizing activity per se, the protective effect could be diluted, suggesting that a certain amount of HA-specific antibody is needed to confer protection.

4. Discussion

In this study we report that without adjuvant, H5 HA proteins are poorly immunogenic in H5 naïve C57BL/6 mice. In our system, substantial antibody responses can be mounted when the response is adequately boosted. The need for a booster shot is not unlike the recommendations for seasonal vaccination of infants receiving their first influenza vaccination or adults receiving a pandemic vaccination [31]. However, the immunogenicity of avian H5 HA in humans and other animal models has been reported to be weak in comparison to other serotypes of influenza HA proteins [23, 32, 33]. The reasons for this are not clear, but baculovirus-derived rHA have been shown to be effective immunogens in human subjects [24–26, 34]. These differences in immunogenicity between subtypes of rHAs in human subjects could reflect a past history of infection, or lack of thereof in the case of pandemic strains, with the various serotypes of influenza virus. However, the IgG titers generated following our homologous vaccination strategy are comparable to what has been previously reported in other adjuvanted vaccine-boost models with rH5 and rH3 [26, 35, 36]. Interestingly, the dose of rHA use in our system is between 3 and 20 fold lower than that administered by other groups. However, a direct and comprehensive comparison of the immunogenicity of baculovirus produced full-length rHAs of various subtypes in mice is not currently available. This makes it difficult to determine if rH5 HAs are intrinsically less immunogenic than other rHAs or if other factors such as virus-priming and/or previous vaccination can influence the immunogenicity of rHAs upon vaccination. Indeed, the data presented here suggests that prior vaccination with H5IN05 primes the humoral arm of the immune system to respond to closely related drifted HA proteins in a magnitude that is comparable to homologous vaccination. However, this result is in the context of vaccination with the drifted variant two weeks after boosting with H5IN05. The factors involved in this type of response could very well be time dependent, thus the length of time between priming with H5IN05 and the drifted variant administration merits further investigation. It is worth noting that human subjects retain the ability to respond to a drifted variant with “boost-like” antibody and cellular response years after priming [20, 21], with recent evidence that longer intervals between immunizations may enhance the response [37].

Evaluation of the antibody response in mice vaccinated with 3 doses of H5VN04 revealed antibody cross-reactivity to H5IN05, but not rH1 or rH3 HAs, in an ELISA based assay. However, very little cross-reactivity to H5IN05 was observed when testing the same serum in a competitive ELISA. This phenomenon can be replicated with serum from mice receiving a homologous vaccine containing H5IN05. One possible explanation is that the serum generated by H5VN04 vaccination is indeed cross-reactive to the drift variant HA, but it has a higher affinity to the immunizing antigen. Thus, it is possible that the antibodies

bound to H5IN05 dissociate in the presence of H5VN04 resulting in binding to the homologous protein rather that the drifted variant.

Administration of the drifted variant strategy elicited a subpopulation of H5IN05 specific IgG that cross-reacted with the clade 1 H5VN04 variant. More importantly, this increase in reactivity against the clade 1 variant was not at the expense of reactivity to H5IN05 (compare H5VN04 results in Figure 2E with that in 2F). However, whether this increased reactivity to the drifted translates to broadened antibody specificity against other 3rd party rH5 HAs and /or H5 avian virus is unknown. In addition, despite the high H5VN04 antibody titers, we observed low incidence of neutralizing antibodies against the ΔH5xPR8 virus generated following one dose of H5VN04 in mice previously immunized with H5IN05 (Figure 3). Indeed, other groups have reported low antibody neutralizing activity following administration of one dose of experimental H5N1 vaccines in mice [28, 36, 38]. Nevertheless, protection from viral challenge in the absence of a detectable neutralizing antibody response has been observed. [28, 38]. However, while the efficiency of influenza vaccination in humans is measured by the ability of the vaccine to induce a neutralizing antibody titer equal or greater than 1:40 [29, 30], it is not clear how appropriate this measurement is as a correlate for protection in animal models. In our studies in which immune serum was diluted below 1:40, protection was diminished, suggesting this correlate is sound. Nevertheless, previous vaccination with H5IN05 provides little to no advantage in the induction of neutralizing antibodies following one dose of the drifted H5VN04. Moreover, because our experimental system is currently limited to measuring MN activity to ΔH5xPR8 we do not know if this cross-clade antibody reactivity comes at the expense of the neutralizing antibodies to H5IN05. Indeed, recently published work [39] has shown that prime-boost vaccination with inactivated viruses, containing drifted H5 HAs, increases cross-clade neutralizing antibody reactivity at the expense of the magnitude of the neutralizing response to the priming virus. It is worth noting that, contrary to the observations made by Sabarth et al. [39], we observed minimal neutralizing activity against a virus containing the drifted variant H5VN04. Neuraminidase dependent enhancement of the HA-specific response has been recently reported by Bosch et al. [40]. It is possible that the different results on virus neutralization relate to the presence, or absence, of the viral neuraminidase in the vaccine preparation. We are currently investigating how these results relate to rH5 HA drift variant vaccination.

As previously stated, protection from viral challenge in the absence of neutralizing antibodies has been observed following vaccination [38]. Here we observe that both homologous and drift variant vaccination generates an H5 HA specific immune response that is capable of reducing morbidity upon viral challenge even in the absence of high neutralizing antibody titers. The observation of substantial immune protection in the absence of neutralizing antibody titers suggests the possibility of other immune mechanisms operating to control infection. More importantly, these mechanisms of control of infection potentially appear to play an important role early (>7 days) after challenge. It is possible that protection from morbidity is correlated to the total circulating H5VN04-specific antibody titers. Indeed, passive transfer of immune sera confirmed that the antibodies generated by drift variant vaccination are sufficient for protection, from virus induced morbidity and death, regardless of the antibody MN activity (Figure 5A and 5B). Interestingly, serum containing high MN activity failed to protect mice from morbidity and death when diluted 8-fold (Figure 5B and 5C open squares). A possible explanation for this result is that because the dilution of the MN activity also results in the dilution of the total non-neutralizing H5VN04 specific antibody it effectively eliminates the protective activity provided by these antibodies. Mechanisms for protection by non-neutralizing antibodies have been previously described for influenza virus [41–43]. However, the possibility of a threshold or balance between antibodies, with and without MN activity, which promotes protection is both novel

and intriguing. These data supports the notion that influenza vaccines that do not induce high titers of neutralizing antibodies can provide protection. However, the degree of protection offered by these vaccines may vary in comparison to those that induce neutralizing antibodies. Another possibility is that H5 HA specific memory T cell responses play a role in reducing virus induced morbidity effects in vaccinated mice. However, this route of vaccine administration is not associated with the induction of lung-resident memory T cells. Regardless, in spite of the poor incidence of neutralizing antibodies, prior vaccination may provide a clinical benefit.

These studies support the idea of pre-vaccination as a viable pandemic control strategy. However, one major concern to this strategy is that vaccination with antigenically distinct variants will result in a misdirected antibody response primarily focused against the priming antigen [17–19]. This phenomenon, termed “Original Antigenic Sin”, has been demonstrated in the context of live influenza infection, but its role in experimental influenza vaccines is still under investigation. Kim et al. [19] reported that sequential vaccination with inactivated virus resulted in a significant decreased in the development of protective immunity and recall responses to the virus containing the drifted HA with minimal effects of OAS. In addition, the group reported that sequential vaccination with DNA encoding drifted HAs induced significant responses directed to the priming antigen at the expense of the response to the drifted HA. However, *in vivo* protection was not accessed after DNA vaccination. In contrast, following our vaccination strategy we did not observe differences in the recall responses between mice receiving a homologous vaccine and those receiving a drift variant vaccine. The difference in results may be attributed to different vaccination strategies (DNA vs. rHA), the correlate of protection measured, the subtype of HA used (H1 vs. H5), and/or the antigenic distance between hemagglutinin pairs. We believe that our observation lends support to the idea that the Original Antigenic Sin may not apply to protein vaccination [44, 45]. However, it is currently unknown how vaccination with drifted variants affects the response to an infection with a 3rd party virus containing an HA related to those used for immunization.

This study further supports the observations reported in experimental human vaccine trials where B cell responses and serum HAI and MN activity [20, 21] suggest advantages to pre-pandemic vaccination. More importantly, the advantages of H5 pre-vaccination are not restricted to the H5 HA pair previously described [20, 21]. Pre-vaccination with stockpiled H5 vaccines can potentially induce baseline immunity to H5 that, in the event of a pandemic, could lower the dose requirement of vaccination while providing significant protection against the emerging drifted H5 strain.

Highlights

- H5VN04 and H5IN05 rHA are used in drifted variant vaccination strategy.
- Vaccine tunes reactivity to drifted variant without loss of reactivity to priming HA.
- Low incidence of neutralizing Ig generated to a virus containing the drifted HA.
- HA-specific cross-reactive, but not neutralizing, Ig are sufficient for protection.

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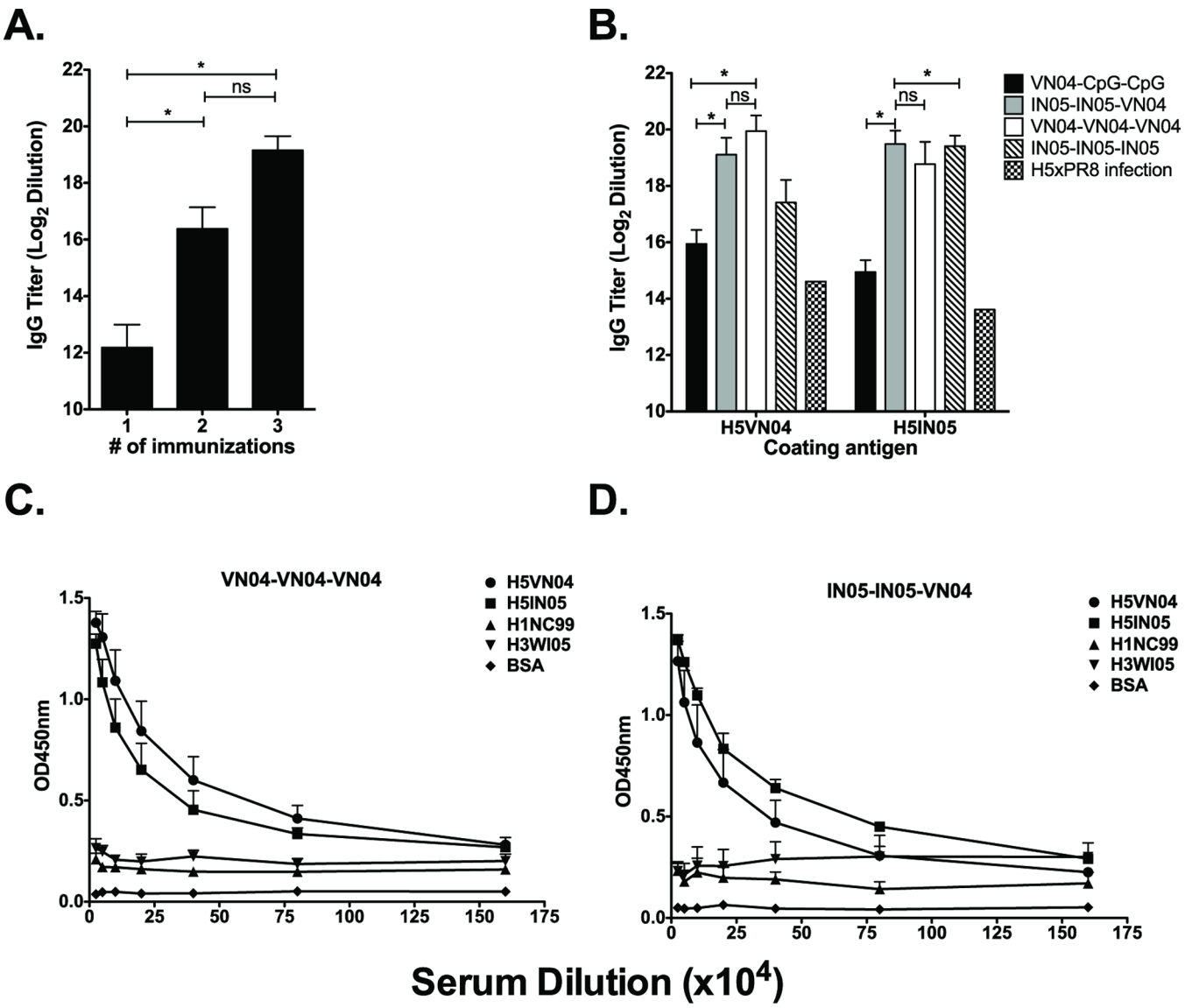

Figure 1. Vaccination with recombinant H5 hemagglutinin elicits a cross-reactive and subtype specific IgG response

Serum from immunized mice was harvested 14 days after the last vaccination and tested for binding to recombinant HA antigens in an IgG ELISA. (A) H5VN04 specific serum IgG titer from mice immunized with one, two, or three doses of recombinant H5VN04 (n=5/group). (B) Recombinant H5 HA specific serum IgG titers in mice (n=5–8/group) vaccinated with one dose of H5VN04 (black bars), three doses of H5VN04 (white bars), three doses of H5IN05 (hatched bars), or two doses of H5IN05 followed by one dose of the drift variant H5VN04 (grey bars). Checkered bars represent the IgG titer of ΔH5xPR8 immune sera obtained 22 days post live virus infection. Serum harvested from mice vaccinated with three doses of H5VN04 (C) or the two doses of H5IN05 followed by one dose of the drift variant H5VN04 (D) was tested for reactivity against a panel of recombinant hemagglutinin antigens. The antibody titer was determined as the reciprocal of the highest dilution at which the optical density was four standard deviations away from an equally diluted negative control. Log 2 transformation of the antibody titer is presented in A and B. Bars in graphs A and B represent the mean antibody titer. Mean optical density for is plotted for graphs C and

D (n=3). Error bars represent the standard error of the mean. Statistical differences obtained by paired (A) or unpaired (B) T test analysis with $p < 0.05$ (*).

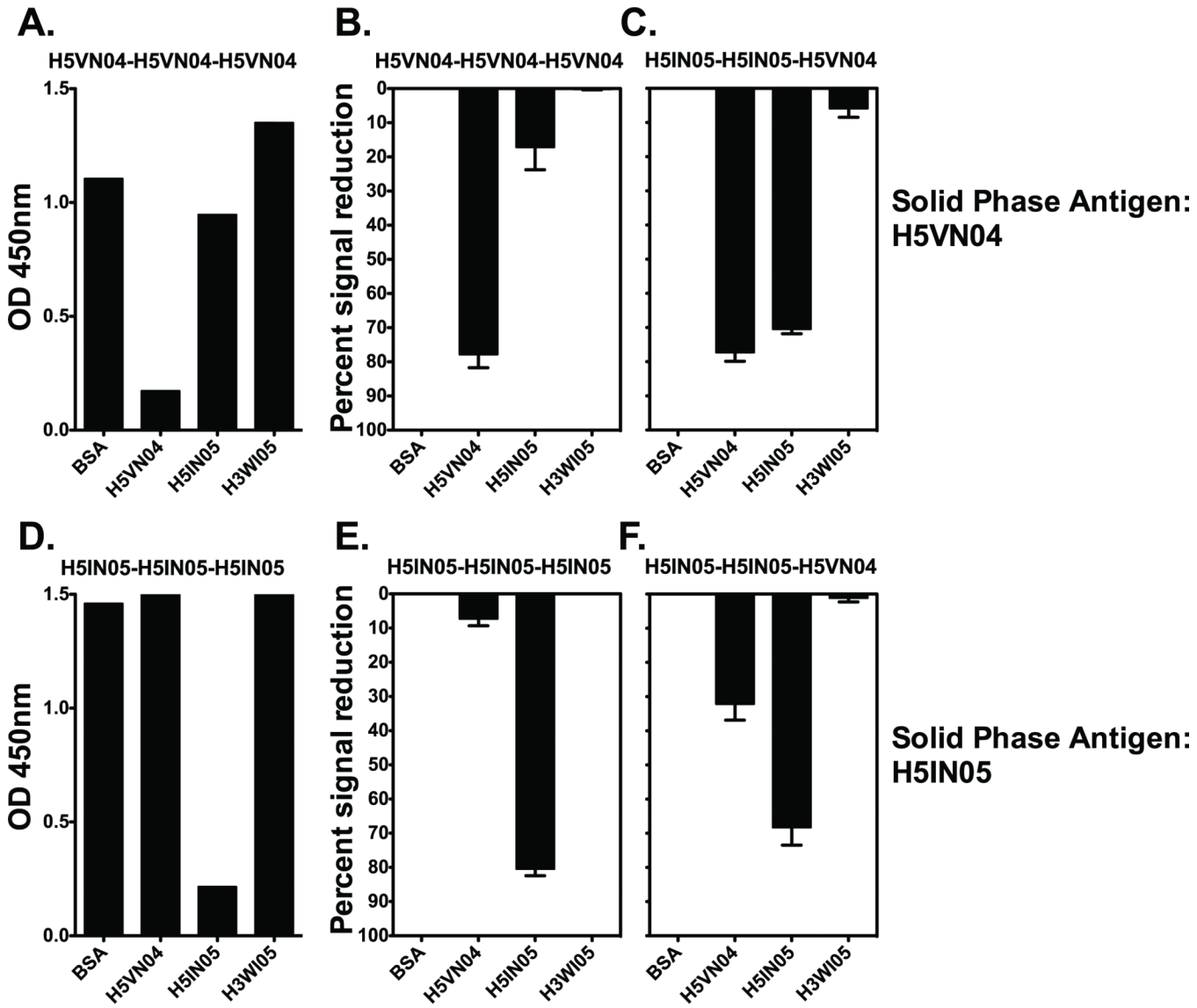

Figure 2. Drift variant vaccination increases antibody reactivity towards the drifted HA variant

Serum from immunized mice was harvested 14 days after the last vaccination and tested for binding to recombinant HA antigens in a competitive IgG ELISA. Serum from immunized mice was diluted 1:25000, incubated in the presence of 0.5μg/well of the competitor antigen (H5VN04, H5IN05, or H3/Wisconsin/05), and then transferred into an ELISA plate coated with 0.05μg/well of H5VN04 or H5IN05 recombinant antigen. The vaccination strategy is provided at the top of each plot and the solid phase antigen is indicated to the right of each row of panels. Representative plot of the changes in optical density following pre-incubation of H5VN04 (A) and H5IN05 (D) immune sera with rHA competitors. Data from 6–7 mice/group was normalized against BSA and is expressed as percent signal reduction (B, C, E, and F). Bar represents the arithmetic mean percent reduction of the optical density signal. Error bars indicate the standard error of the mean.

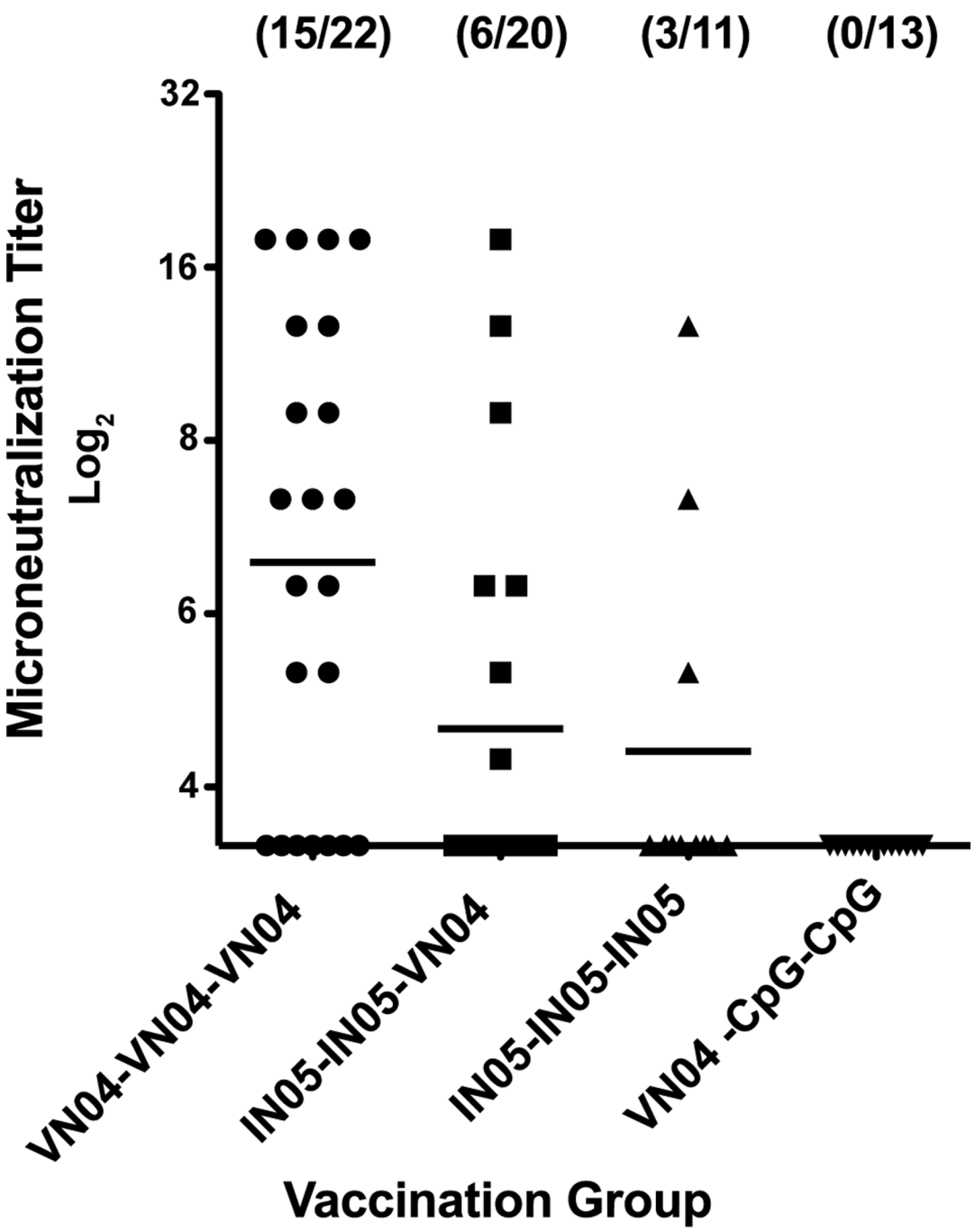

Figure 3. Low incidence of neutralizing antibodies are elicited upon drift variant vaccination with recombinant H5 hemagglutinins

Serum was collected, 14 days after the last immunization, from mice immunized with three doses of H5VN04 (circles), two doses of H5IN05 followed by one dose of H5VN04 (squares), three doses of H5IN05 (triangles), or mice that received one dose H5VN04 42 days prior serum collection (upside down triangle). The neutralizing activity of the serum was tested against 100 TCID50 of H5N1 A/Vietnam/1203/04 × A/Puerto Rico/8/34. The neutralizing titer was determined as the highest dilution at which the serum provided complete protection from cytopathic effects to duplicate wells. The number of mice that had

a microneutralization titer equal to or greater than 40 is shown in parenthesis. Line represents the geometric mean titer.

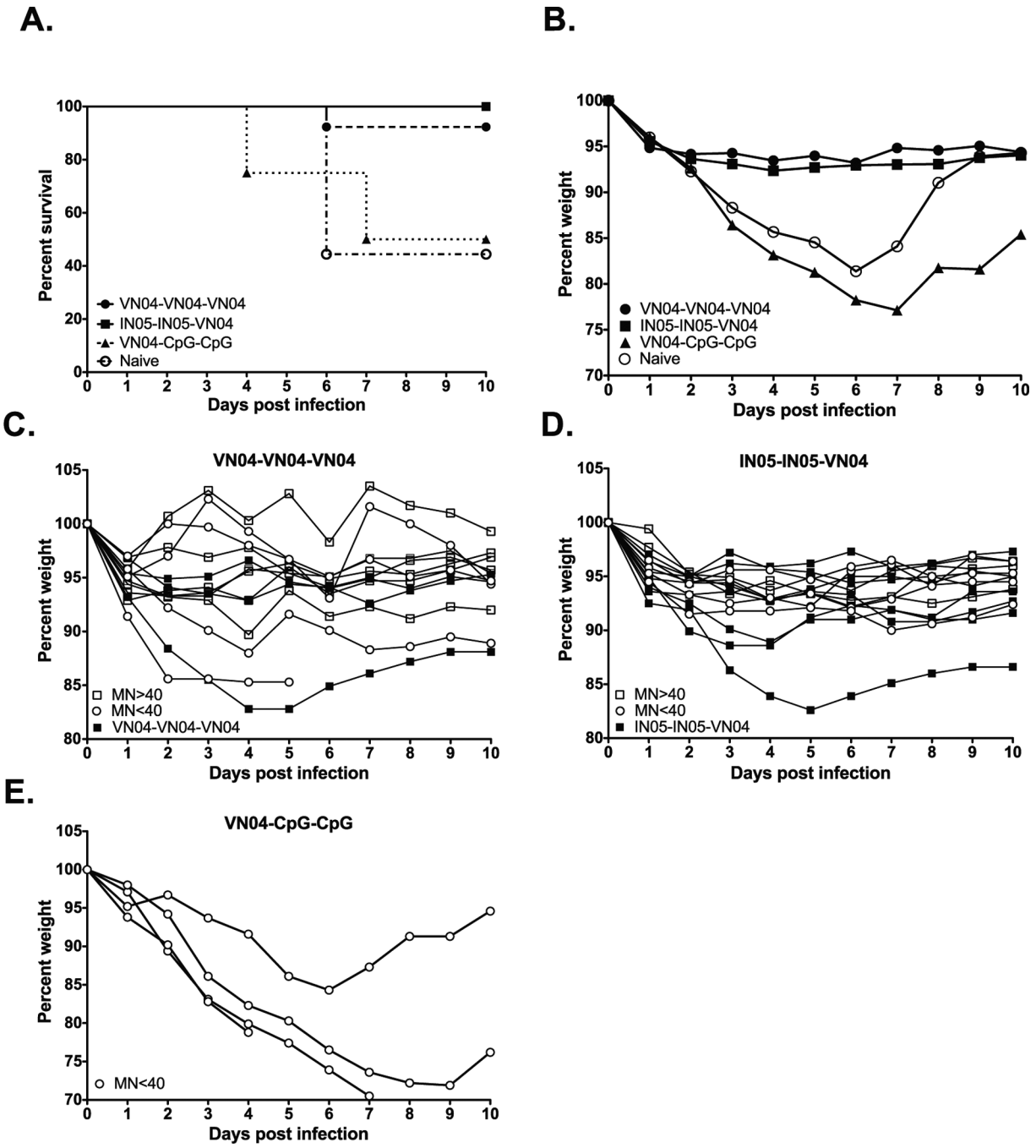

Figure 4. Recombinant H5 drift variant vaccination reduces virus induced morbidity upon challenge with ΔH5N1 A/Vietnam/1203/04 × A/Puerto Rico/8/34

Groups of 4–15 mice were immunized, as indicated, with recombinant H5 antigens and then challenged with 25,000 TCID50 units of ΔH5N1 × PR8 virus 28 days after the last immunization. Mice were monitored for survival (A) and weight loss (B) for a period of 10 days following infection. Weight loss curve of individual mice with available antibody neutralization data (C–E). For panel B 2-way ANOVA statistical analysis shows and interaction between vaccination and weight loss ($p<0.001$). Bonferroni adjustment for multiple comparisons shows statistical difference at days 4 through 9 and 4 through 6

between drift variant vaccinated mice and those that received one dose of H5VN04 or naïve mice respectively ($p<0.05$).

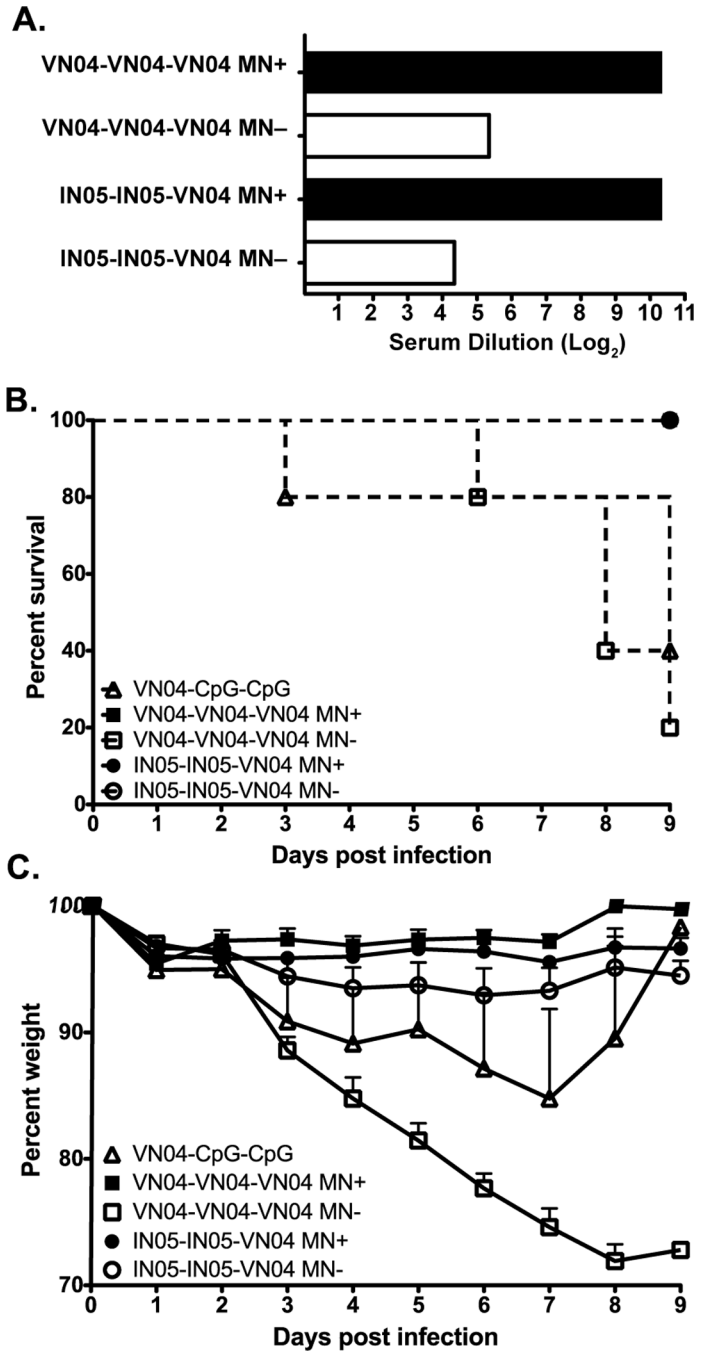

Figure 5. Passive transfer of serum from recombinant H5 drift variant vaccinated mice protects naïve mice from virus induced morbidity upon ΔH5N1 A/Vietnam/1203/04 × A/Puerto Rico/8/34 challenge

Groups of 8–10 donor mice were immunized, as indicated, with recombinant H5 antigens. Immune serum was harvested 21 days after the last immunization and screened for neutralization activity. Sera was pooled into MN+ and MN− pools. Pool neutralizing activity is shown in panel A. Serum was transferred into age matched naïve recipients (n=5/group) one day prior to infection. Recipient mice were infected with 25,000 TCID50 units of ΔH5N1xPR8 virus and mice were monitored for survival (B) and weight loss (C) for a

period of 9 days following infection. Symbols and error bars in figure C represent the mean percent weight of live mice and the standard error of the mean respectively.

Table 1

Recombinant H5 hemagglutinin immunization timeline

	Vaccination Sequence		
Group	**Primary immunization (Day 0)**	**Secondary immunization (Day 14)**	**Tertiary immunization (Day 28)**
G1	2μg H5VN04	1μg H5VN04	1μg H5VN04
G2	2μg H5IN05	1μg H5IN05	1μg H5VN04
G3	2μg H5VN04	10μg CpG	10μg CpG
G4	2μg H5IN05	1μg H5IN05	1μg H5IN05