RESEARCH ARTICLE

Vaccination with Adjuvanted Recombinant Neuraminidase Induces Broad Heterologous, but Not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice

Teddy John Wohlbold,[a,b] Raffael Nachbagauer,[a,c] Haoming Xu,[a,b] Gene S. Tan,[a] Ariana Hirsh,[a] Karl A. Brokstad,[d] Rebecca J. Cox,[e,f,g] Peter Palese,[a,h] Florian Krammer[a]

Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, New York, USA[a]; Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA[b]; Faculty of Life Sciences, University of Vienna, Vienna, Austria[c]; Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, Bergen, Norway[d]; The Influenza Centre, Department of Clinical Science, University of Bergen, Bergen, Norway[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]; Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York, USA[h]

ABSTRACT **In an attempt to assess the cross-protective potential of the influenza virus neuraminidase (NA) as a vaccine antigen, different subtypes of recombinant NA were expressed in a baculovirus system and used to vaccinate mice prior to lethal challenge with homologous, heterologous, or heterosubtypic viruses. Mice immunized with NA of subtype N2 were completely protected from morbidity and mortality in a homologous challenge and displayed significantly reduced viral lung titers. Heterologous challenge with a drifted strain resulted in morbidity but no mortality. Similar results were obtained for challenge experiments with N1 NA. Mice immunized with influenza B virus NA (from B/Yamagata/16/88) displayed no morbidity when sublethally infected with the homologous strain and, importantly, were completely protected from morbidity and mortality when lethally challenged with the prototype Victoria lineage strain or a more recent Victoria lineage isolate. Upon analyzing the NA content in 4 different inactivated-virus vaccine formulations from the 2013-2014 season via Western blot assay and enzyme-linked immunosorbent assay quantification, we found that the amount of NA does indeed vary across vaccine brands. We also measured hemagglutinin (HA) and NA endpoint titers in pre- and postvaccination human serum samples from individuals who received a trivalent inactivated seasonal influenza vaccine from the 2004-2005 season; the induction of NA titers was statistically less pronounced than the induction of HA titers. The demonstrated homologous and heterologous protective capacity of recombinant NA suggests that supplementing vaccine formulations with a standard amount of NA may offer increased protection against influenza virus infection.**

IMPORTANCE **Despite the existence of vaccine prophylaxis and antiviral therapeutics, the influenza virus continues to cause morbidity and mortality in the human population, emphasizing the continued need for research in the field. While the majority of influenza vaccine strategies target the viral hemagglutinin, the immunodominant antigen on the surface of the influenza virion, antibodies against the viral neuraminidase (NA) have been correlated with less severe disease and decreased viral shedding in humans. Nevertheless, the amount of NA is not standardized in current seasonal vaccines, and the exact breadth of NA-based protection is unknown. Greater insight into the cross-protective potential of influenza virus NA as a vaccine antigen may pave the way for the development of influenza vaccines of greater breadth and efficacy.**

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Address correspondence to Florian Krammer, florian.krammer@mssm.edu.

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Seasonal influenza virus infections cause significant morbidity and mortality worldwide (1). If well matched to currently circulating strains, influenza virus vaccines are efficient tools in protecting the human population from influenza virus infection. Although effective, these vaccines have a suboptimal efficacy (74 percent) in healthy adults for well-matched strains (2), and this value may drop sharply when the vaccine is mismatched (3). Furthermore, the seasonal vaccine is not protective against pandemic influenza viruses. Immune responses following vaccination with inactivated influenza virus (IIV) are predominantly raised to the viral hemagglutinin (HA), the major glycoprotein on the surface of the influenza virion. The majority of antibodies are directed against the immunodominant globular head domain of the molecule (4–7). These antibodies are highly potent in inhibiting virus replication and are often strain specific. Thus, the main focus of influenza virus vaccine development, production, and efficacy testing is on the HA. IIVs are standardized based on their HA content, and vaccine efficacy is measured based on the induction

of hemagglutination-inhibiting antibodies (8). The second influenza surface glycoprotein, the neuraminidase (NA), has enzymatic activity that is crucial for the virus and is the target of small-molecule NA inhibitors (9). While many studies propose the usefulness of NA as a vaccine antigen (10–19), the viral neuraminidase is mostly ignored in the context of influenza vaccine development, and the NA content of IIVs is not even measured.

Early epidemiological studies that were conducted in humans in the 1960s and 1970s demonstrated that higher neuraminidase inhibition (NI) titers were correlated with lower morbidity and decreased viral shedding (10, 20, 21). Recent studies have shown that NA-based immunity can have protective efficacy against influenza virus infection in animal models and humans (9, 16, 17, 20, 22–24). Here, we evaluated the breadth of influenza A and B virus NA-based immunogens and their protective efficacy in the mouse model. Furthermore, we compared the levels of induction of anti-NA immunity and anti-HA immunity after IIV vaccination in humans and analyzed the NA content of four 2013-2014 season IIVs from different manufacturers.

RESULTS

Expression of recombinant NA proteins. Influenza virus NA has been found to be immunosubdominant when administered in association with the influenza virus HA in animal models (30, 31, 32). We therefore chose to investigate the protective efficacy and breadth of divergent NAs using baculovirus-expressed antigens. These recombinant NAs (rNAs) include an N-terminal hexahistidine tag to facilitate purification and a tetramerization domain to guarantee optimal folding and are secreted into the cell supernatant, allowing posttranslational modification to occur in the endoplasmic reticulum and in the Golgi network (26, 27). All NAs were obtained at high purity and exhibited enzymatic activity (see Fig. S1 and S2 in the supplemental material). Their expression levels varied between approximately 0.1 and 5 mg/liter of culture.

Recombinant influenza virus NA immunogens protect mice from homologous virus challenge. To assess the protective efficacy of subtype N1, N2, and influenza B virus NAs, mice were vaccinated twice at a 3-week interval with N1 NA from PR8, N2 NA from HK68/X-31, or influenza B virus NA from Yam88 B (see Materials and Methods for descriptions of viruses). The vaccines were administered intramuscularly (i.m.) and intranasally (i.n.) [5 μg adjuvanted with 5 μg of poly(I · C) each] because the contribution of mucosal versus systemic immunity for NA-based protection was unclear. Four weeks postboost, the animals were challenged with 10 murine 50% lethal doses (mLD_{50}) of homologous virus or 1.1×10^6 PFU for Yam88 B. Animals that received PR8 N1 were fully protected from weight loss and mortality, comparable to the results for the positive-control animals, which received inactivated matched whole-virus vaccine (Fig. 1A and D), while control animals vaccinated with bovine serum albumin (BSA) or recombinant N2 (rN2) lost weight rapidly and succumbed to infection by day 8 or 9 postinfection, respectively. Similarly, animals vaccinated with HK68/X-31 N2 were completely protected from homologous lethal HK68/X-31 challenge, while control animals (BSA and rN1 vaccinated) lost weight and succumbed to infection by day 7 (Fig. 2A and C). N2 vaccination significantly reduced virus infection in the lungs of these mice on day 3 postinfection, and only one of five mice had detectable amounts of virus in the lungs on day 6 postinfection (Fig. 3B). N2 vaccination did not induce sterilizing immunity, as did two vaccinations with inactivated homologous virus, but it reduced lung titers 1,000-fold on day 3 and 100,000-fold on day 6 compared to the lung titers in the BSA control group. Finally, vaccination with Yam88 B recombinant NA completely protected mice against a nonlethal challenge with Yam88 B, while control animals (BSA and rN2 vaccinated) lost approximately 20% of their initial weight and had survival rates of only 80% (Fig. 4A and D), showing that influenza B virus NA is as protective as influenza A virus NA.

Protection is mediated by NA-reactive antibodies. To investigate the mechanism of protection, we measured anti-NA titers using enzyme-linked immunosorbent assays (ELISA) with purified whole virus as the substrate. In all three cases, we could detect high levels of reactivity against the homologous virus (Fig. 1G, 2E, and 4G). To assess the functionality of this antibody response, we determined NI titers against the respective homologous viruses using the enzyme-linked lectin assay (ELLA) and found a high level of activity in all three cases (Fig. 1J, 2G, and 4J). To confirm the role of antibodies as a contributing mechanism of protection, we performed a passive transfer challenge experiment. Sera from a positive-control group vaccinated twice with HK68/X-31 inactivated whole-virus vaccine, a group that received rN2, and a group that received BSA were transferred into three sets of naive mice, respectively. Two hours posttransfer, the animals were challenged with 5 mLD_{50} of HK68/X-31 virus. No difference in weight loss was observed between rN2-serum-treated and positive-control mice: both groups showed modest weight loss of approximately 10% of their initial weight and all mice in both groups survived the infection, while control mice lost weight rapidly and succumbed to infection on day 8 postchallenge (Fig. 3A).

Mucosal NA vaccination confers better protection than intramuscular NA vaccination. Mechanistically, NA antibodies may affect at least two important steps of the influenza virus life cycle. It is well established that the activity of NA is important for virus release from infected cells (9). However, there is also evidence that NA is necessary for the successful transport of incoming virus particles through mucins on the mucosa (9, 33). We were therefore interested to compare the efficacy of intramuscular versus intranasal vaccination, since the latter also induces mucosal antibodies. Mice were vaccinated twice with rN2 at a 3-week interval intranasally or intramuscularly [5 μg rN2 plus 5 μg of poly(I · C) per dose]. Control animals received BSA (i.n. and i.m.) or recombinant N1 (rN1) via i.n. or i.m. administration. At 4 weeks postvaccination, mice were challenged with 10 mLD_{50} of HK68/X-31. Although a small difference in weight loss could be observed on days 4 to 7 postvaccination, no statistically significant difference between the i.n. and i.m. routes could be established (Fig. 3C). All rN2-vaccinated animals (for both i.n. and i.m. vaccination) survived the infection, while all control animals succumbed to infection between days 6 and 7, regardless of the vaccination route. We then repeated the experiment with a higher challenge dose of 25 mLD_{50} and found that i.n. vaccination protected mice from weight loss significantly better than i.m. vaccination did (Fig. 3D). However, the systemic anti-N2 IgG antibody levels were similar in the three experimental groups, suggesting an important role of mucosal immunity—most likely mucosal IgA—in NA-based protection (Fig. 3E).

NA immunogens partially protect against heterologous but not against heterosubtypic influenza A virus challenge. Next, we wanted to assess the breadth of protection that NA-based immunity can afford. To look at the breadth of N1 immunity, we again

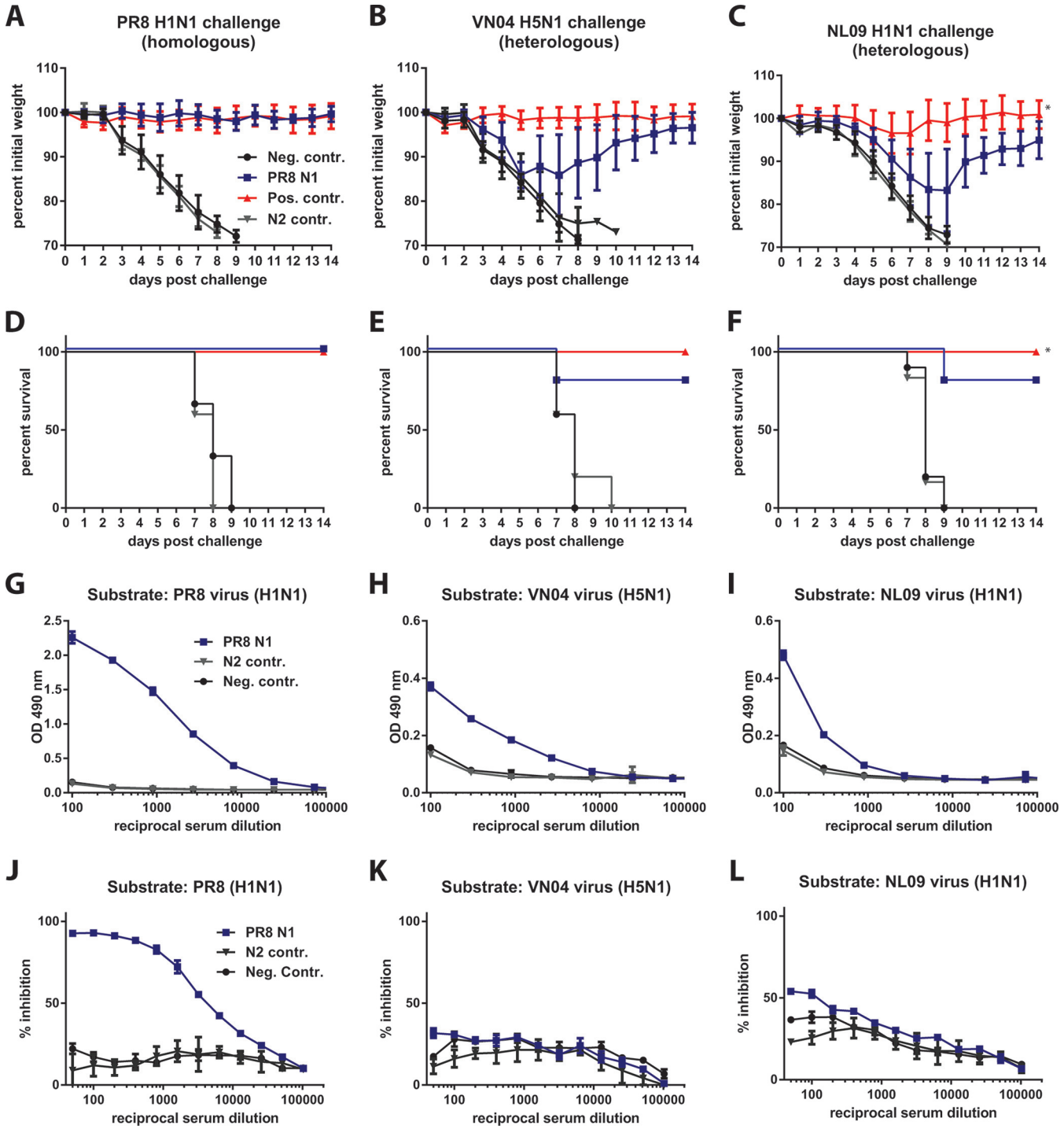

FIG 1 Vaccination with recombinant N1 protects mice from homologous and heterologous viral challenge. (A to C) Six- to 8-week-old naive BALB/c mice ($n = 5$ for PR8 N1 group and N2 control group; $n = 10$ for negative-control group and positive-control groups) were primed and boosted with 10 μg rNA from PR8 (5 μg delivered i.m. and 5 μg delivered i.n.) adjuvanted with poly(I · C). Negative-control mice were primed and boosted with 10 μg BSA (5 μg delivered i.m. and 5 μg delivered i.n.) adjuvanted with poly(I · C). Positive-control mice received a 1-μg i.m. prime and boost of a formalin-inactivated, unadjuvanted virus matching the challenge strain. Additionally, one experimental group was primed and boosted with rN2 in a fashion identical to the method used for the N1-vaccinated mice. Upon challenge, weight loss was monitored for 14 days postinfection as a measure of morbidity. Graphs plot the average amounts of weight loss as percentages of initial weight with standard deviation (SD). (D to F) Survival curves from the challenge experiments whose results are shown in panels A to C. (G to I) Pooled sera from individual mice (PR8 N1 vaccinated, rN2 vaccinated, or naive) in each experimental group were tested in triplicate for reactivity to purified virus via ELISA. (J to L) The same sera used in the experiment whose results are shown in panels G to I were tested in triplicate for NI activity against the respective challenge viruses. *, positive-control data shown in panels C and F were collected from the high-challenge-dose group (10 mLD_{50}). $n = 5$ mice per group unless otherwise stated.

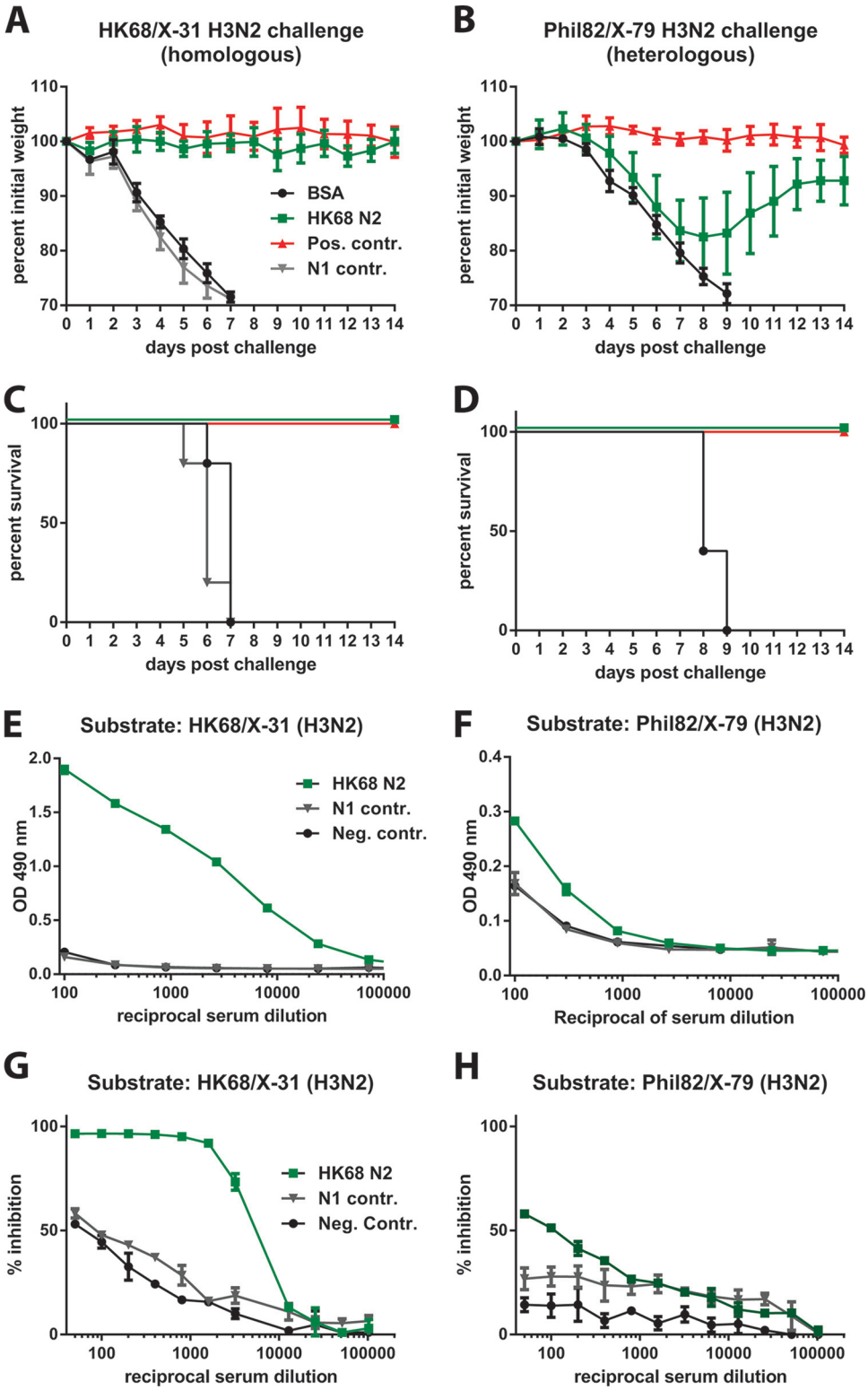

FIG 2 Vaccination with recombinant N2 protects mice from homologous and heterologous viral challenge. The experimental design for these challenge studies was identical to that detailed in the legend to Fig. 1, except that mice (n = 5 per group) were primed and boosted with rNA from HK68/X-31 (H3N2) and challenged with homologous H3N2 reassortant strain HK68/X-31 or the heterologous H3N2 strain Phil82/X-79. Control mice were primed and boosted with rNA from PR8 or BSA. (A to D) Weight loss and survival of mice challenged with HK68/X-31 (A and C) or Phil82/X-79 (B and D). (E to G) Pooled sera from individual mice (HK68/X-31 N2 vaccinated, rN1 vaccinated, or naive) in each experimental group were tested in triplicate both for reactivity to purified virus via ELISA (E and F) and for NI activity against HK68/X-31 (G) and Phil82/X-79 (H).

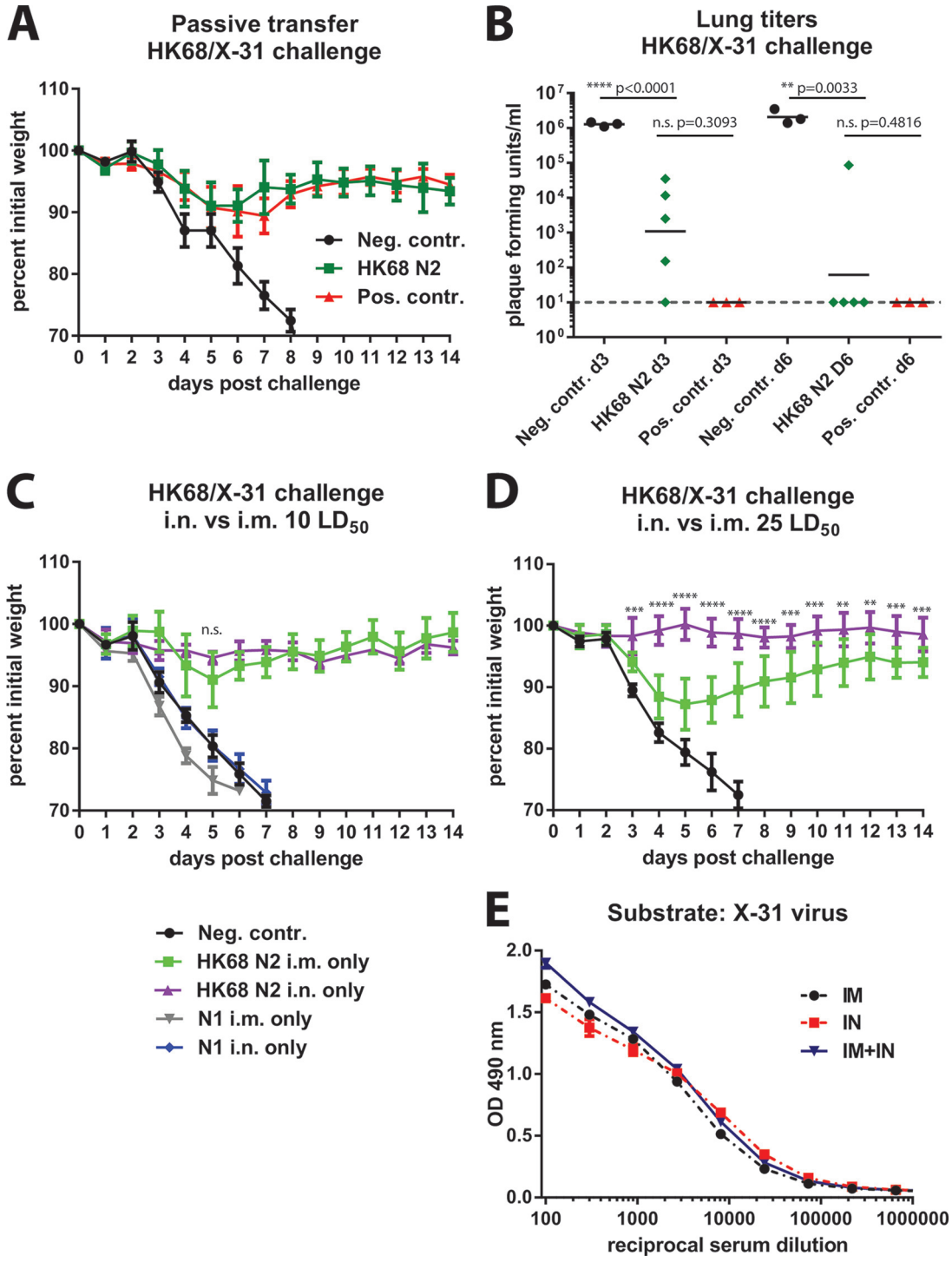

FIG 3 Passive transfer of sera from vaccinated mice and i.m. versus i.n. vaccination. To demonstrate that humoral immunity against NA is sufficient for protection, passive transfer experiments were performed. Sera from animals vaccinated with HK68/X-31 N2, inactivated whole HK68/X-31 virus, or BSA were transferred into naive mice, which were subsequently challenged with HK68/X-31 virus. (A) Weight loss postchallenge. All mice that received HK68/X-31 N2 or the inactivated whole-virus vaccine survived the challenge. (B) Lung titers of virus in animals vaccinated with HK68/X-31 N2, BSA, or inactivated whole HK68/X-31 virus on day 3 and day 6 postchallenge with HK68/X-31. (C and D) To assess whether the route of vaccine administration had an impact on protection, a challenge experiment identical to the one whose results are shown in Fig. 2A was performed, except that the mice in one group ($n = 10$) were primed and boosted with 10 μg N2 [adjuvanted with poly(I·C)] exclusively intramuscularly (i.m.), while those in the other ($n = 10$) were primed and boosted exclusively intranasally (i.n.). Initially, there was a slight but not very distinguishable difference in weight loss (C); however, upon repeating the experiment with a higher challenge dose (25 LD_{50}), a clear difference in the percentages of weight lost was seen, with the i.n.-vaccinated mice displaying significantly less weight loss than the i.m.-vaccinated mice (D). Survival was 100% in both groups. (E) Reactivities to HK68/X-31 virus were similar for mice that received HK68/X-31 N2 via the i.m. route, the i.n. route, or both at the same time (i.m.+i.n.). n.s., not significant; $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. $n = 5$ mice per group unless otherwise stated.

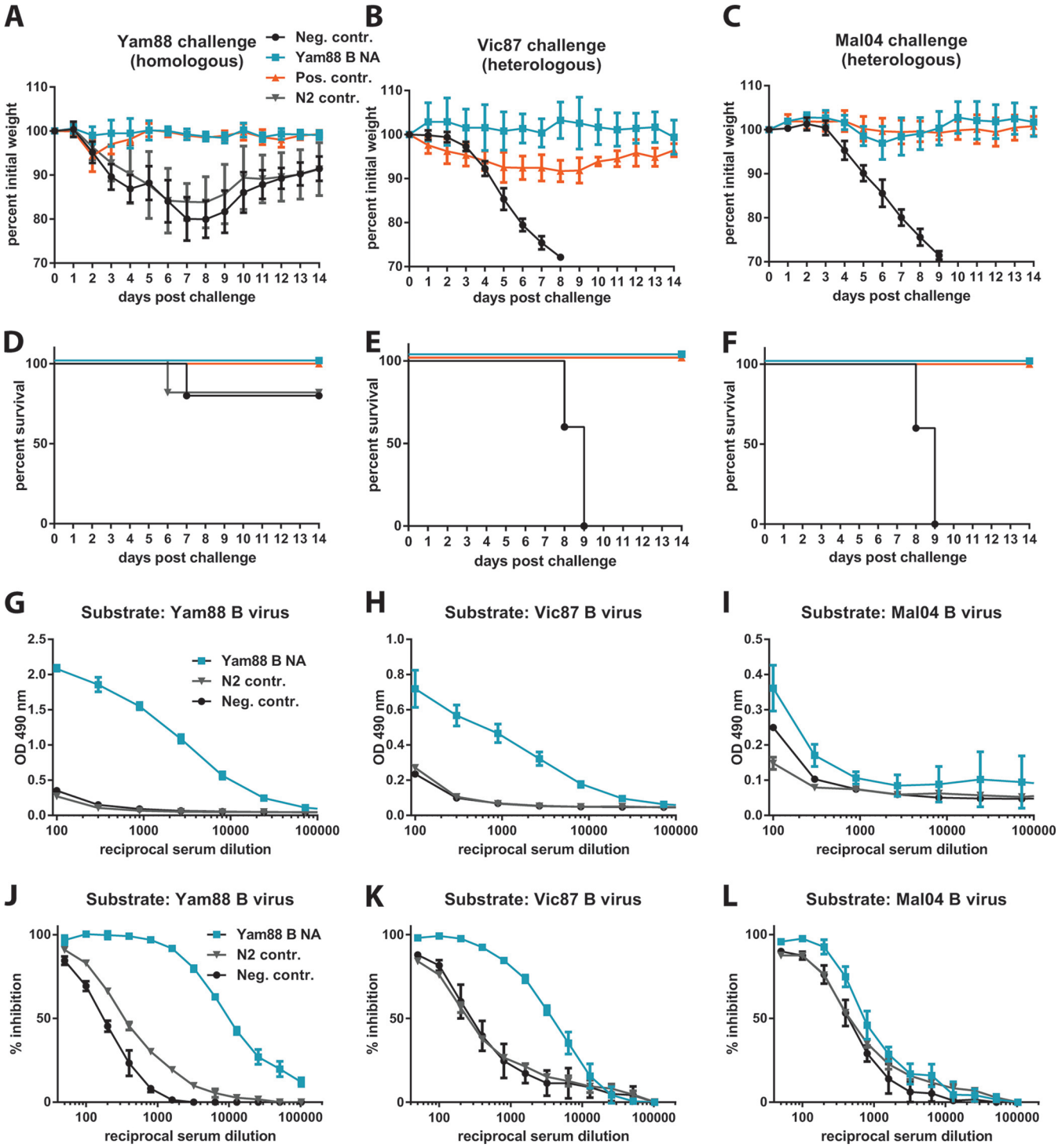

FIG 4 Vaccination with recombinant influenza B virus NA protects mice from homologous and heterologous viral challenge. The experimental design for these challenge studies was identical to those whose results are shown in Fig. 1 and 2, except that mice ($n = 5$ per group) were primed and boosted with rNA from Yam88 B and challenged with the homologous Yam88 B virus or the heterologous influenza B virus strains Vic87 and Mal04. The mice in the N2 control group were primed and boosted with rNA from HK68/X-31. (A to F) Weight loss and survival after homologous challenge with Yam88 B (A and D) or heterologous challenge with Vic87 (B and E) or Mal04 (C and F). (G to I) Seroreactivities of influenza B virus Yam88 B NA-vaccinated mice to Yam88 B (G), Vic87 (H), or Mal04 (I) virus. (J to L) The same sera used in the experiment whose results are shown in panels G to I were tested in triplicate for NI activity against the respective challenge viruses.

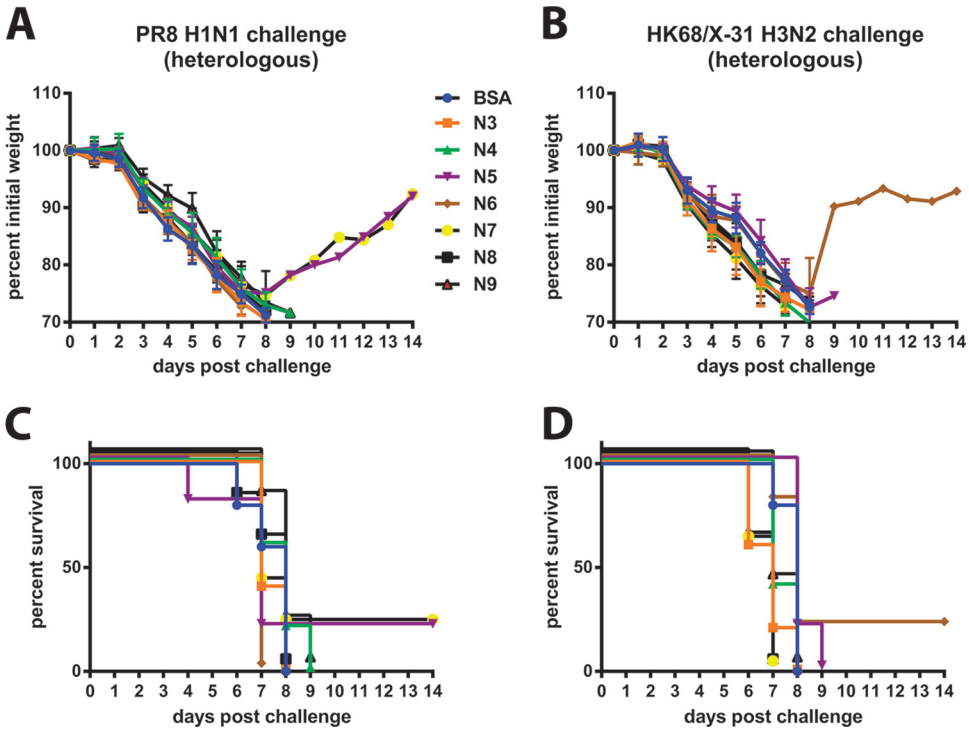

FIG 5 Vaccination with rNA does not induce heterosubtypic immunity in mice. To test the possibility of NA-induced heterosubtypic cross-protection, a sizeable challenge study was performed in which mice were separated into groups ($n = 5$) and primed and boosted with representative rNAs from subtypes N3 to N9. Similar to the experiment whose results are shown in Fig. 1, animals received identical primes and boosts of 10 μg rNA (5 μg delivered i.m. and 5 μg delivered i.n.) adjuvanted with poly(I · C). Negative-control mice were primed and boosted with 10 μg BSA (5 μg delivered i.m. and 5 μg delivered i.n.) adjuvanted with poly(I · C). No reduction in weight loss was observed upon lethal (5 LD_{50}) challenge with PR8 (A) or HK68/X-31 (B). (C and D) Survival curves from the challenge experiments whose results are shown in panels A and B. No appreciable protection from mortality was observed.

vaccinated animals with PR8 N1 but then challenged them with 5 mLD_{50} of either NL09 H1N1 (2009 pandemic strain) or VN04 H5N1. Both viruses carry N1 NAs that belong to a different N1 clade than PR8 N1, which falls into the human N1 clade (9). PR8 N1 was able to provide partial protection against weight loss (compared to the results for BSA and N2 control animals) and mortality (80% survival in both cases) (Fig. 1B, C, E, and F). However, when challenged with a higher dose of 10 mLD_{50} of NL09 virus, all PR8 N1-vaccinated mice succumbed to infection, showing the limit of cross-protection (data not shown). Specific reactivity to purified NL09 and VN04 virus particles could be detected by ELISA and might explain the observed cross-protection (Fig. 1H and I). However, only a low level of specific NI activity against NL09 could be detected and no activity above the background level was detected for VN04 (Fig. 1K and L). Cross-reactivity of N2 was tested using the heterologous Phil82/X-79 H3N2 strain that is separated from HK68/X-31 by 14 years of antigenic drift. Animals challenged with a 5 mLD_{50} dose of virus were completely protected from mortality but showed a body weight loss of approximately 80% (Fig. 2B and D). With a higher challenge dose of 10 mLD_{50}, survival dropped to 20% (data not shown). HK68/X-31 N2 sera showed low levels of specific cross-reactivity to Phil82/X-79 virus in ELISA and detectable but low levels of NI activity (Fig. 2F and H).

In the experiments described above, we used rN2-vaccinated mice as controls for challenge with viruses expressing N1 NAs and vice versa without observing protection in the controls. This indicated the absence of heterosubtypic immunity between N1 and N2. However, there are currently nine true influenza A virus NA subtypes known (those with demonstrated NA enzymatic activity), and it was unclear whether any of them would share protective epitopes with either N1 or N2 NA. To explore this, we vaccinated mice twice with rN3, rN4, rN5, rN6, rN7, rN8, or rN9 NA and challenged them with 5 mLD_{50} of either PR8 (H1N1) or HK68/X-31 (H3N2). All animals seroconverted to the respective NAs (see Fig. S3 in the supplemental material) but lost weight rapidly after infection with PR8 (Fig. 5A) or HK68/X-31 (Fig. 5B). Survival was 0% in most cases, but in the rN5 and rN7 PR8 challenge groups and the rN6 HK68/X-31 challenge group, survival was 20% (one animal). However, survival of a low percentage of animals is expected with a challenge dose of 5 mLD_{50}. Our conclusion from these experiments is that vaccination with influenza A virus NA does not induce heterosubtypic immunity.

Vaccination with influenza B virus NA induces broad protection against heterologous virus challenge. The genetic diversity of influenza B virus HAs and NAs is limited compared to the genetic diversity of influenza A virus HAs and NAs (9, 34). To assess the breadth of NA-based immunity against influenza B viruses, we vaccinated animals with NA from Yam88 B, the lineage-defining strain of the Yamagata lineage. We then challenged these animals with influenza B viruses Vic87 and Mal04, both of which belong to the antigenically distinct (based on HA) Victoria lineage. Interestingly, vaccination with Yam88 B NA protected against weight loss and mortality after challenge with the two heterologous strains (Fig. 4B, C, E, and F). NA cross-reactivity could be detected against both viruses, although the level of reactivity to

Vic87 was higher than the level of reactivity to Mal04 (Fig. 4H and I). Yam88 B NA vaccination induced robust NI titers against Vic87 (Fig. 4K). The NI activity against Mal04 was low but still detectable (Fig. 4L).

IIV does not efficiently induce N1 and N2 reactive antibody responses in humans. Next, we wanted to evaluate the response to HA and NA in the context of seasonal IIV vaccination in humans. Although earlier studies have indicated that the anti-NA response to IIV in humans is low, we wanted to assess this using a quantitative ELISA method based on recombinant HA and NA proteins. The H1N1 vaccine component of the vaccine was NC99, and immunity was assessed using homologous HA and NA reagents. Preexisting immunity against NC99 H1 HA was found to be relatively high at baseline, and the immunity increased greatly after vaccination (24-fold) (Fig. 6A and E). Preexisting antibody levels against the NC99 N1 were found to be low, and the antibody levels did not increase significantly after vaccination (1.1-fold) (Fig. 6B and E). The H3N2 component of the vaccine strains was A/Wyoming/3/03. However, due to the lack of homologous reagents, we used HA and NA proteins of a closely related H3N2 strain, A/Panama/2007/99. The H3 baseline titer was lower than the baseline titer of H1, and vaccination resulted in a 6.4-fold induction (Fig. 6C and E). The N2 baseline titer was higher than the one for N1, and the titer increased 2-fold upon vaccination (Fig. 6D and E). IIV induced a significantly stronger immune response against HA than against NA for both influenza A virus components of the vaccine, with a *P* value of 0.0003 for H1N1 and a *P* value of 0.0240 for H3N2 (Fig. 6E).

The N1 NA contents of inactivated influenza virus vaccines vary greatly. A possible reason for the low level of induction of anti-NA antibodies compared to the level of induction of anti-HA antibodies by IIV could be the amount of NA present in IIVs. We therefore quantified the N1 NA content in current IIVs. First, using the broadly N1-reactive monoclonal antibody (MAb) 4A5 (see Fig. S4 in the supplemental material), we performed a semiquantitative Western blot analysis of four 2013-2014 seasonal vaccines to measure their N1 contents. Three of four vaccines showed robust levels of NA, while one vaccine, Flucelvax (Novartis), only showed a very weak band (Fig. 7A). Interestingly, although the dominant band was running between 50 and 80 kDa, we also detected multiple and diverse higher-molecular-mass species in the three egg-derived vaccines (Fig. 7A). We then quantified the N1 NA content of the four tested vaccines using an ELISA-based assay and linear regression. The highest N1 NA content was measured in Fluzone (10.5 μg/vaccine dose), followed by Fluvirin (5.0 μg/vaccine dose) and FluLaval (4.4 μg/vaccine dose). The cell culture-derived Flucelvax appeared to have only minimal amounts of NA (0.02 μg/vaccine dose). Since we tested only one cell culture-derived vaccine (the only one on the market as of 2014-2015), it is unclear whether the low N1 content of this vaccine is caused by the virus production method or by steps downstream in the production process.

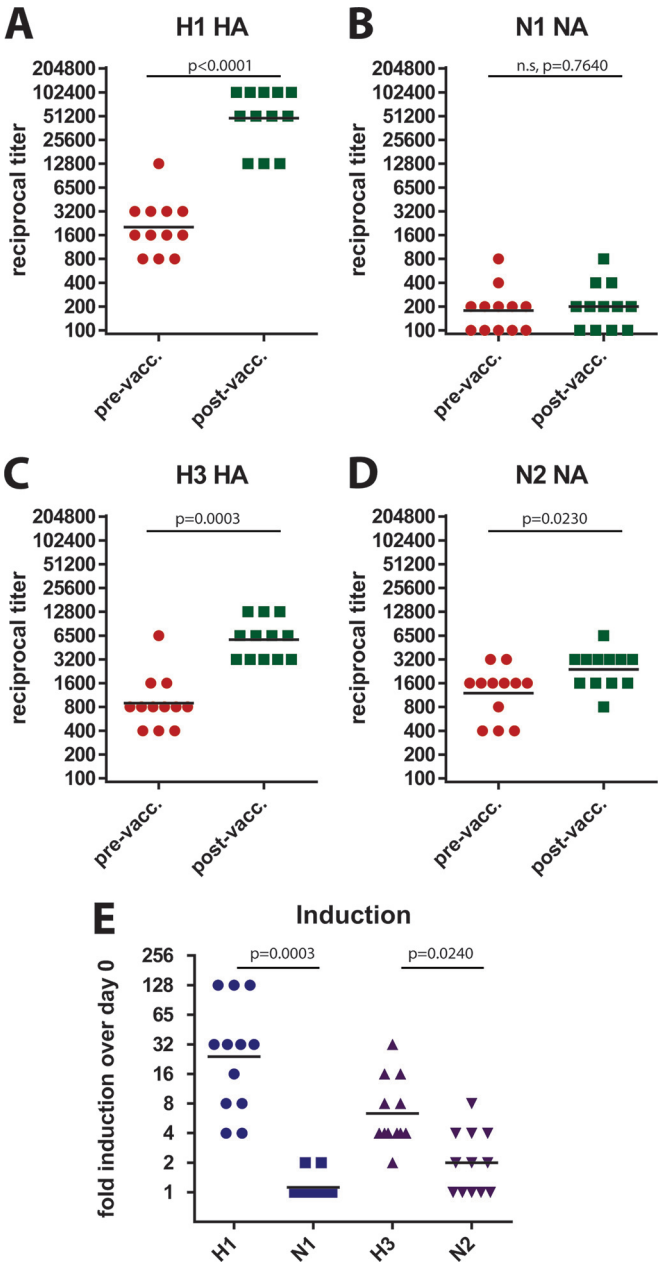

FIG 6 Seasonal IIV vaccination is inefficient at inducing NA reactive antibodies in humans. HA and NA reactivities of human pre- and postvaccination sera from 12 individuals who received the 2004-2005 inactivated seasonal vaccine were determined. (A and B) The geometric mean H1 titer was relatively high at baseline (~1,600) and was induced approximately 24-fold upon vaccination ($P < 0.0001$) (A), while the geometric mean N1 baseline titer was low (~200) and did not increase upon vaccination (B). (C and D) The geometric mean H3 baseline titer (~800) was lower than that of H1 and vaccination induced a 6.4-fold induction ($P = 0.0003$) (C), while the geometric mean N2 baseline titer was higher than that of N1 and increased 2-fold upon vaccination ($P = 0.0230$) (D). (E) IIV induced significantly higher endpoint titers against HA than against NA for both influenza A virus subtypes included in the vaccine ($P = 0.0003$ for H1N1, and $P = 0.0240$ for H3N2).

DISCUSSION

Inactivated influenza virus vaccines—both seasonal and pandemic—are standardized based on the amounts of the major surface glycoprotein HA that they include (8). Consequently, only an HA-based surrogate measure of protection, HA inhibition (HI) activity, is used to assess the efficacy of influenza virus vaccines (35). Importantly, IIV only induces a relatively narrow immune response, and protection is mostly limited to viral strains closely related to the vaccine strain (4–6). The IIV-induced immune response in humans against the NA is significantly weaker than the response against the HA (9, 22, 29, 30, 31, 36). We have shown

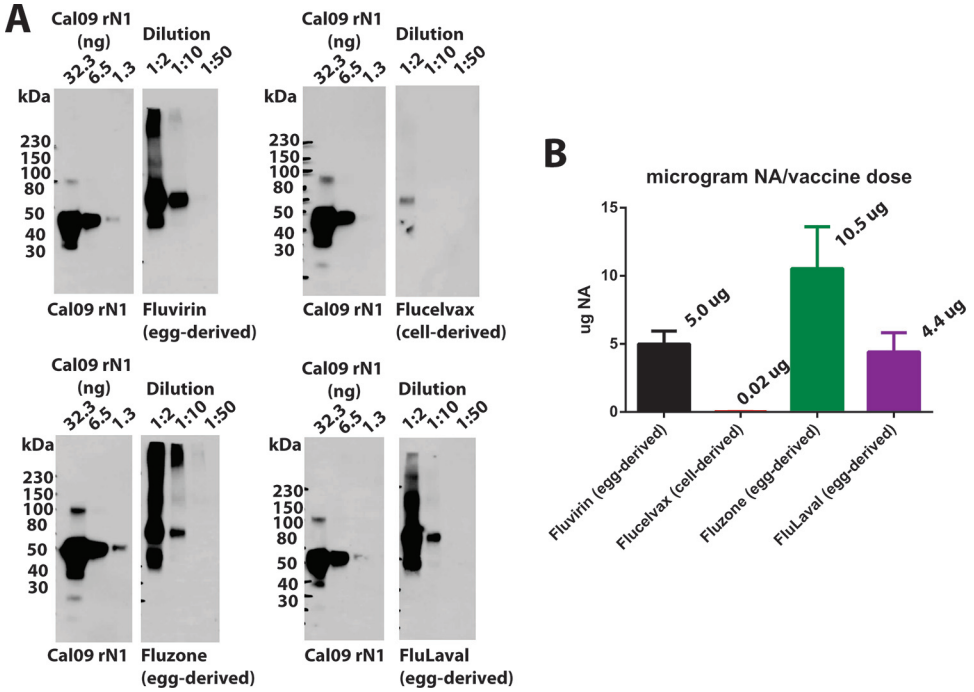

FIG 7 The amounts of Cal09 NA contained in seasonal IIVs from the 2013-2014 influenza season varied. (A) Five-fold serial dilutions of 4 IIVs recommended for the 2013-2014 influenza season were analyzed for N1 NA content via Western blot assay. Membranes were blotted with 4A5 (an MAb specific for N1 NA). Each panel represents a separately run Western blot of a unique vaccine brand. Dilutions of baculovirus-expressed Cal09 rN1 (left blot in each panel) of known concentrations were run alongside each vaccine sample on the same gel. Dilutions of vaccines and amounts of standard are displayed above the gels, and the names of the vaccines are displayed below. (B) Quantities of N1 NA per adult vaccine dose (0.5 ml) as measured by ELISA. Bar graphs show the mean values and standard deviation (SD).

here that in certain cases, no response against NA can be detected at all, despite a high level of induction of antibodies against the corresponding HA. There are several factors that could contribute to the low immunogenicity of NA compared to that of HA. First, NA seems to be inherently immunosubdominant to HA when both antigens are administered in conjunction (22, 30, 31). Second, HA is more abundant on the virus surface than NA (37), and third, the NA could be lost to some extent during the manufacturing of the vaccine. To investigate the third factor, we assessed the N1 NA content of four licensed IIVs from the 2013-2014 season, produced by three manufacturers. Interestingly, all egg-derived vaccines had robust amounts of NA, while the cell culture-derived preparation contained only minimal amounts of N1. However, since most licensed IIVs are derived from eggs (including the one used for measuring human HA and NA titers as described above [Fig. 6]), we dismiss a low NA content of vaccines as an explanation for the low immunogenicity.

It has been hypothesized that robust immune responses against NA could contribute to protection against both homologous and heterologous virus strains. To study the protective potential of NA, we decided to test recombinant-NA-based immunogens in mice. Purified recombinant NA was chosen due to the reasons outlined above. Also, we wanted to ensure that protection in these experiments was solely based on anti-NA immunity without interference from immunity to other influenza virus proteins. Interestingly, we found robust protection against homologous challenge, comparable to that conferred by vaccination with inactivated whole virus. Complete protection against morbidity and mortality was observed even at high challenge doses when the vaccine was given intranasally. Intramuscular vaccination still resulted in full protection against mortality at high challenge doses, but significant weight loss was observed, suggesting that mucosal immunity can play an important role in NA-based protection. In contrast to inactivated whole-virus vaccines, vaccination with NA antigens did not result in sterilizing immunity, but it reduced lung titers drastically, which is in line with the results of historic studies in humans and animal models (10, 20). However, it should be noted that two vaccinations with whole-virus vaccines were necessary to induce sterilizing immunity. Since passive transfer of sera from vaccinated mice protected naive mice from challenge, we conclude that humoral immunity is sufficient for protection, although a contribution of cellular immunity to NA-based protection cannot be ruled out.

Finally, we also assessed the breadth of NA-based protection. Previous studies have identified an epitope that is highly conserved among influenza A virus NAs (38). An antibody that recognized this epitope was also effective in inhibiting the influenza B virus neuraminidase (38, 39). However, the antibody has relatively low effective concentrations compared to those of specific anti-NA antibodies (15, 38, 39), and it is unknown whether similar antibodies against the same epitope can be induced by natural infection or vaccination with influenza virus vaccines. We found that vaccination with NAs N3 to N9 did not induce protective immunity against H1N1 or H3N2 challenge. Also, N1 antigens did not protect against challenge with N2-expressing viruses (and vice versa). However, we saw limited heterologous (within the subtype) cross-protection for influenza A viruses. N1 antigens from an early human isolate gave partial protection against challenge

with a pandemic H1N1 strain and an H5N1 strain, both of which carry avian-type N1 NAs that are phylogenetically distinct from the human N1 lineage (9). This finding is supported by reports of N1 cross-reactive monoclonal antibodies (15) and cross-protection against H5N1 induced by H1N1 exposure (13, 14, 16, 17, 40). We also found full cross-protection in terms of mortality for the N2 immunogen when mice were challenged with an H3N2 strain that had 14 years of drift. It is of note that this cross-protection was limited to lower challenge doses; no cross-protection was observed for either N1 or N2 at high challenge doses. Interestingly, cross-protection was solid for influenza B viruses; an NA immunogen from Yam88 B (Yamagata lineage) was able to fully protect against two Victoria lineage strains. Importantly, the influenza B virus NA has not diverged into two lineages, as has the influenza B virus HA, which may partially explain the good cross-reactivity.

In conclusion, NA-based immunity is able to provide robust protection against homologous influenza virus infection in mice. Cross-protection seems to be confined within the same subtype, with no intersubtypic protection displayed, as seen with HA stalk-reactive antibodies (29, 41). However, subtype-specific cross-reactive antibodies may have the potential to contribute to protection against drifted seasonal viruses in cases where the vaccine is mismatched, as in the current 2014-2015 season (42). Furthermore, strong N1- and N2-based immunity might be beneficial in the case of a new pandemic virus that may carry a heterologous N2 or N1 NA, such as H2N2 or H5N1. The current seasonal IIV is suboptimal in inducing robust immunity against NA. To achieve better induction of NA immunity, a number of strategies could be developed. NA could be rendered more immunogenic by presenting it in the context of a novel HA globular head domain or chimeric HA to which humans are naive (7, 22). Alternatively, the IIV could be supplemented with purified NA or purified NA could be given as an extra vaccine in addition to the IIV. Our data support the notion that NA-based immunity is an important part of the multifaceted immune response that protects against influenza disease. Utilizing the full potential of NA as a vaccine antigen may improve and broaden the protective efficacy of influenza virus vaccines, especially in the fight against influenza B virus infections.

MATERIALS AND METHODS

Viruses and cells. Madin Darby canine kidney (MDCK) cells were grown in complete Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with antibiotics (100 units/ml penicillin–100 μg/ml streptomycin [Pen-Strep]; Gibco) and 10% fetal bovine serum (FBS; HyClone). Sf9 insect cells were grown in TNM-FH insect medium (Gemini Bioproducts) supplemented with antibiotics (Pen-Strep) and 10% FBS, and High Five (BTI-TN-5B1-4 subclone; Vienna Institute of BioTechnology [41]) cells were grown in serum-free SFX-insect medium (HyClone) supplemented with antibiotics (Pen-Strep). Influenza viruses (A/Puerto Rico/8/34 [PR8; H1N1], HK68/X-31 [H3N2, PR8 internal genes and HA and NA from A/Hong Kong/1/68], A/Netherlands/602/09 [NL09; pandemic H1N1], Phil82/X-79 [H3N2, PR8 internal genes and HA and NA from A/Philippines/2/82], low-pathogenicity A/Vietnam/1203/04 [VN04; H5N1, PR8 internal genes and HA and NA from A/Vietnam/1203/04 with polybasic cleavage site of the HA deleted], B/Victoria/2/87 [Vic87], B/Yamagata/16/88 [Yam88 B], and B/Malaysia/2506/04 [Mal04]) were grown in 8- to 10-day-old embryonated chicken eggs, and titers were determined on MDCK cells in the presence of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. For ELISAs, influenza viruses were concentrated through a 30% buffered sucrose cushion by ultracentrifugation (Beckman L7-65 ultracentrifuge with SW-28 rotor at 25,000 rpm). Recombinant baculoviruses expressing neuraminidases were generated as described previously and grown in Sf9 cells (28).

Recombinant proteins. Recombinant neuraminidase proteins (PR8 N1, HK68/X-31 N2, A/Texas/36/91 N1, A/New Caledonia/20/99 N1, A/California/4/09 [Cal09] N1, A/Panama/2007/99 N2, Yam88 B NA, A/swine/Missouri/4296424/06 N3, A/mallard/Sweden/24/02 N4, A/mallard/Sweden/86/03 N5, A/mallard/Netherlands/1/99 N6, A/mallard/Interior Alaska/10BM01929/10 N7, A/mallard/Sweden/50/02 N8, and A/Anhui/1/13 N9) were expressed in High Five cells and purified from cell culture supernatants as described previously (26, 27). Briefly, cultures were infected with recombinant baculoviruses at a multiplicity of infection (MOI) of 10. Supernatants were then harvested by low-speed centrifugation 72 h postinfection and were purified via Ni-nitrilotriacetic acid (NTA) resin (Qiagen) using a published protocol (26).

Vaccination and challenge studies. Six- to 8-week-old female BALB/c mice were used for all vaccination and challenge studies. For standard challenge experiments, mice ($n = 5$ to 10) were anesthetized (0.15 mg/kg of body weight ketamine and 0.03 mg/kg xylazine intraperitoneally) and received recombinant NA adjuvanted with poly(I · C) [5 μg rNA and 5 μg poly(I · C) in 50 μl of PBS intranasally [i.n.] plus 5 μg rNA and 5 μg poly(I · C) in 50 μl of PBS intramuscularly (i.m.)], bovine serum albumin (BSA) adjuvanted with poly(I · C) [5 μg BSA and 5 μg poly(I · C) in 50 μl of PBS i.n. plus 5 μg BSA and 5 μg poly(I · C) in 50 μl of PBS i.m.; negative control], or matched inactivated whole- or split-virus vaccines (1 μg intramuscularly in 50 μl of PBS; positive control); in most cases, a mismatched rNA from a different subtype was used as an additional negative control [5 μg rNA and 5 μg poly(I · C) in 50 μl of PBS i.n. plus 5 μg rNA and 5 μg poly(I · C) in 50 μl of PBS i.m.]. A boost using the same formulations and routes was given at 3 weeks postprime. Animals used for i.n. versus i.m. experiments were vaccinated twice with 5 μg of rNA plus 5 μg of poly(I · C) either i.n. or i.m. at the same intervals and volumes as described above. At 4 weeks postboost, animals were anesthetized and intranasally challenged with 25 (i.n. vs i.m. experiment), 10 (homologous), or 5 (heterologous) murine 50% lethal doses (mLD_{50}) of virus in 50 μl of PBS. An exception was the Yam88 B experiment, where mice were challenged with a sublethal dose (1.1×10^6 PFU) of virus due to the low pathogenicity of the isolate. Weight was monitored for a period of 14 days.

Animals used for lung titer experiments were vaccinated via the i.n. and i.m. routes as described above, and lungs were harvested 3 and 6 days postchallenge. The lungs were then homogenized using a BeadBlaster 24 (Benchmark) homogenizer, and the virus lung titer was measured using a plaque assay in MDCK cells.

For the passive transfer experiments, animals (HK68/X-31 rN2, BSA, and positive-control groups) were anesthetized and terminally bled. Serum was harvested and transferred into naive mice (200 μl per mouse intraperitoneally; $n = 5$ per group). At 2 h posttransfer, the mice were challenged with 5 mLD_{50} of HK68/X-31 virus as described above. Weight was monitored for a period of 14 days.

All animal procedures were performed in accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee.

Human sera. Human sera were obtained from a clinical trial performed at the University of Bergen, Norway, with the 2004-2005 trivalent influenza vaccine Fluarix (GlaxoSmithKline) (A/Caledonia/20/99 [H1N1], A/Wyoming/3/03 [H3N2], and B/Jiangsu/10/03) (28). Serum samples were taken 14 days postvaccination. The trial was approved by the regional ethics committee (REK Vest, approval number 170-04) and the Norwegian Medicines Agency.

Mouse serum preparation. Sera collected in all vaccination studies were stored long term at −20°C until use. In all serological assays, serum samples from individual mice within an experimental group were pooled and inactivated by heating in a 56°C water bath for 1 h.

ELISA. For mouse serum ELISAs, plates (Immulon 4 HBX; Thermo Scientific) were coated overnight with 5 μg/ml (50 μl per well) of concentrated influenza virus in coating buffer (carbonate-bicarbonate buffer,

pH 9.4) at 4°C. Plates were then blocked using 3% milk in PBS containing 0.1% Tween 20 (TPBS) for 1 h at room temperature. Serum samples were diluted in steps of 1:3 starting with a 1:100 dilution in 1% TPBS. Plates with the serum samples were then incubated for 1 h at room temperature. After three washes with TPBS (100 μl/well for each wash), plates were incubated for another hour at room temperature with a horseradish peroxidase (HRP)-labeled anti-mouse antibody (1:3,000; GE Healthcare) and developed using SigmaFast OPD (*o*-phenylenediamine dihydrochloride; 100 μl per well [Sigma]) after another round of extensive washing. Plates were developed for 10 min, stopped with 3 M hydrochloric acid (HCl) (50 μl/well), and read at an optical density of 490 nm (OD_{490}) on a Synergy 4 (BioTek) plate reader.

The procedure for human ELISAs was similar, but the following modifications were made. Plates were coated with recombinant HA or NA (2 μg/ml, 50 μl per well), and blocking was performed in TPBS containing 3% goat serum and 0.5% milk (GM-TPBS). GM-TPBS was also used for making serum dilutions (steps of 1:2 starting with 1:100) and for diluting the HRP-labeled anti-human IgG secondary antibody (1:3,000; Sigma). Endpoint titers were calculated by using the value for the blank plus 3 times the standard deviation as the cutoff. The results are shown as fold induction, calculated by dividing postvaccination endpoint titers by prevaccination endpoint titers as described previously (33).

Enzyme-linked lectin assay (ELLA) to determine neuraminidase inhibition. In order to determine the ideal virus concentration to be used in the NI assay, NA assays were first performed for all virus stocks. In brief, flat-bottom nonsterile Immulon 4 HBX 96-well plates (Thermo Scientific) were coated (carbonate-bicarbonate coating buffer, pH 9.4) with 150 μl of fetuin (Sigma) at a concentration of 50 μg/μl and refrigerated at 4°C overnight. The coating buffer was discarded, and wells were blocked for 1 h at room temperature with 200 μl blocking solution (PBS containing 5% BSA). While plates were being blocked, virus stocks were serially diluted 1:2 in a separate sterile flat-bottom 96-well tissue culture plate (Sigma) using PBS containing 1% BSA. Dilutions were made horizontally across the plate, starting with undiluted stock and ensuring that the final volume in all wells was 150 μl. After blocking for 1 h, the plates were washed 6 times using TPBS (225 μl/well). After the last wash, plates were forcefully tapped on clean paper towels to ensure that no residual wash buffer remained (this technique was repeated for all subsequent wash steps). One hundred-microliter amounts of the viral dilutions were transferred in parallel to the fetuin-coated plates, after which the plates were incubated at 37°C for 2 h. The plates were again washed 6 times using TPBS (225 μl/well), and a secondary solution of peanut agglutinin (PNA) conjugated to HRP (PNA-HRP; Sigma) at a concentration of 5 μg/ml in PBS was added to the plates (100 μl/well). After a 1-h 45-min incubation in the dark, the plates were again washed 6 times using TBPS (225 μl/well) and developed with 100 μl SigmaFast OPD. The developing process was stopped after 7 min with 3 M HCl, and the reaction mixture was read at an absorbance of 490 nm with a Synergy H1 hybrid multimode microplate reader (BioTek). In order to determine the optimal concentration of virus to use for subsequent NI assays, ELISA data from the NA assay for each virus were plotted in GraphPad Prism 6 software and fit to a nonlinear curve. In this way, a 50% effective concentration (EC_{50})-like value could be obtained (in this case, the concentration of virus at which half the maximal OD reading was obtained). Two times this concentration (2× EC_{50}) was used for subsequent NI assays.

To perform NI assays, ELISA plates were coated and blocked in a fashion identical to the method used for the NA assay. While plates were blocking, mouse serum samples were serially diluted 1:2 in separate sterile flat-bottom 96-well tissue culture plates using PBS, starting with a 1:50 dilution and ensuring that the final volume in all wells was 75 μl. Virus stocks were diluted with PBS containing 1% BSA to the 2× EC_{50} concentration determined to be optimal. After virus was added to the antibody plates (75 μl/well), the plates were briefly tapped (for mixing) and incubated at room temperature for 1 h 40 min. Immediately before the incubation time expired, the blocked plates were washed 6 times using TPBS (225 μl/well). One hundred-microliter amounts of the virus/serum mixture were transferred in parallel to the fetuin-coated plates, after which the plates were incubated at 37°C for 2 h. The plates were again washed 6 times using TPBS (225 μl/well), and a secondary solution of PNA-HRP (Sigma) at a concentration of 5 μg/ml in PBS was added to the plates (100 μl/well). The rest of the NI assay protocol was identical to that of the NA assay. The values obtained from the plate reader were divided by the average value for virus-only control wells and then multiplied by a factor of 100 to obtain the NA activity. Percent inhibition was calculated by subtracting the NA activity from 100.

Western blot and quantitative ELISA analysis. Four different brands of FDA-licensed influenza vaccines intended for use in the 2013-2014 flu season were obtained from the Mount Sinai Hospital pharmacy, local pharmacies, or directly from the manufacturer. The trade names of the vaccines (with the respective lot number, H1N1 strain included, and manufacturer in parentheses) were as follows: Fluvirin (13472P, A/Christchurch/16/2010; Novartis Vaccines and Manufacturers), Flucelvax (161281, A/Brisbane/10/2010; Novartis Vaccines and Manufacturers), Fluzone (UH953AA, A/California/07/2009 X-179A; Sanofi Pasteur), and FluLaval (597FZ, A/California/07/2009; ID Biomedical Corporation of Quebec). All of the brands obtained are trivalent, egg-derived vaccines except for Flucelvax, which is produced in a suspension of MDCK cells. Using a small initial volume of each vaccine, 5-fold serial dilutions were prepared in PBS, mixed with an equal volume of 2× Laemmli buffer with 2% beta-mercaptoethanol (BME), and heated for 30 min at 100°C. Twelve microliters of each dilution was loaded on polyacrylamide gels (5-to-20% gradient; Bio-Rad). As a standard control, equivalent-volume dilutions of baculovirus-expressed, purified Cal09 rN1, with a known starting concentration of 0.672 mg/ml (as measured by Bradford protein assay; Bio-Rad) were loaded on the same gel, adjacent to the vaccine dilutions (each unique vaccine was run on a separate gel, however). After transferring for 40 min at 0.11 A using a semidry transfer apparatus (Owl Semi-dry Electroblotting System; Thermo Fisher), the blots were washed 3 times for 3 min in PBS (all subsequent wash steps were done in this way) and blocked with 3% milk in TBPS for 1 h at room temperature. The blocking solution was removed, a primary antibody solution of MAb 4A5 (1:3,000 in 1% milk TBPS) was added to the blots in enough volume so they were completely submerged, and the blots were incubated for 1 h at room temperature. MAb 4A5 is an antibody that broadly binds to N1 NA (Fig. S4). After removing the primary antibody solution and washing, the blots were incubated for 1 h at room temperature with an HRP-labeled anti-mouse antibody (1:6,000; GE Healthcare). The secondary solution was removed, the blots were washed, and developing solution was added (1 ml of enhanced luminol reagent plus 1 ml of oxidizing agent [Western Lightning ECL]; PerkinElmer). After ~30 s in the developing solution, the blots were developed on standard autoradiography film (HyBlot Cl; Denville Scientific) using a 1-min exposure time (SRX-101A; Konica Minolta).

In order to approximately quantify the amount of N1 NA contained in the vaccine formulations, flat-bottom nonsterile Immulon 4 HBX 96-well plates (Thermo Scientific) were coated with triplicate serial 1:2 dilutions of each vaccine sample in coating buffer (see above), starting with a 1:2 dilution and diluting horizontally across the plate. As a standard, wells were coated with baculovirus-expressed, purified Cal09 rN1 in an identical fashion, starting with a known concentration of 16 μg/ml. The plates were incubated at 4°C overnight. The general mouse ELISA protocol (as detailed above) was performed, except that the primary antibody used was MAb 4A5, added at a constant concentration of 3 μg/ml (in 3% milk TPBS, 100 μl/well). ELISA data were transferred to Microsoft Excel, the average of each triplicate reading was calculated, and points (dilution versus OD reading) were plotted in order to determine the portion of each sample curve that was most linear (using *R*-squared regression analysis). For the Cal09 rN1 curve, this best-fit linear equation was used to calculate the unknown N1 concentrations of the 4 vaccine formulations. The values were averaged and are reported in Fig. 7B.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02556-14/-/DCSupplemental.

Text S1, DOCX file, 0.02 MB.
Figure S1, TIF file, 27.9 MB.
Figure S2, TIF file, 15.7 MB.
Figure S3, TIF file, 47.4 MB.
Figure S4, TIF file, 0.05 MB.

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