Engineered Influenza Virus Virions Reveal the Contributions of Non-hemagglutinin Structural Proteins to Vaccine-Mediated Protection

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ABSTRACT The development of improved and universal anti-influenza vaccines would represent a major advance in the protection of human health. In order to facilitate the development of such vaccines, understanding how viral proteins can contribute to protection from disease is critical. Much of the previous work to address these questions relied on reductionist systems (i.e., vaccination with individual proteins or virus-like particles [VLPs] that contain only a few viral proteins); thus, we have an incomplete understanding of how immunity to different subsets of viral proteins contributes to protection. Here, we report the development of a platform in which a single viral protein can be deleted from an authentic viral particle that retains the remaining full complement of structural proteins and viral RNA. As a first study with this system, we chose to delete the major influenza A virus (IAV) antigen, the hemagglutinin (HA) protein, to evaluate how the other components of the viral particle contribute *en masse* to protection from influenza disease. Our results show that while anti-HA immunity plays a major role in protection from challenge with a vaccine-matched strain, the contributions from other structural proteins were the major drivers of protection against highly antigenically drifted, homosubtypic strains. This work highlights the importance of evaluating the inclusion of non-HA viral proteins in the development of broadly efficacious and long-lasting influenza vaccines.

IMPORTANCE Influenza virus vaccines currently afford short-term protection from viruses that are closely related to the vaccine strains. There is currently much effort to develop improved, next-generation influenza vaccines that elicit broader and longer-lasting protection. While the hemagglutinin protein is the major viral antigen, in this work, we developed an approach with which to evaluate the contributions of the nonhemagglutinin proteins to vaccine-mediated protection. Our results indicate that other structural proteins together may help to mediate broad antiviral protection and should be considered in the development of more universal influenza vaccines.

KEYWORDS influenza A virus, nonstructural proteins, universal vaccine

Infections with influenza viruses cause annual epidemics of respiratory disease and, as such, impose a large burden on human health (1). Influenza disease severity ranges from mild to severe, and it is estimated that 3 million to 5 million cases of severe illness and 290,000 to 650,000 respiratory deaths worldwide are the result of influenza virus infections (2). In the United States alone, there have been between 9 million and 35 million cases of illness and 140,000 to 710,000 hospitalizations annually since 2010 (3). This disease burden is in spite of FDA-approved antiviral inhibitors and annual vaccination campaigns (4, 5).

Despite suboptimal efficacy, the best prophylactic measure to prevent influenza remains vaccination (6). The seasonal influenza vaccines currently in use are predominately designed

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and formulated to induce antibodies (Abs) against hemagglutinin (HA). This is in no small part because the hemagglutination inhibition (HAI) titer of serum is a well-recognized correlate of protection from influenza virus infection (7). However, the antibodies elicited by current vaccines are typically directed against the immunodominant HA globular head domain, which is highly variable and plastic, and frequently provide only strain-specific protection (8, 9). Due to the lack of strong heterologous protection, new versions of influenza vaccines are developed each year because of viral antigenic drift (10). Furthermore, in addition to seasonal influenza, pandemic outbreaks are typically caused by antigenically distinct viruses against which seasonal vaccines are likely to provide limited protection (6).

To better combat seasonal influenza and prepare for pandemic influenza viruses of unknown antigenicity, novel vaccines able to induce cross-protective immunity against diverse influenza viruses are highly desirable. Theoretically, a “universal” influenza vaccine would elicit broadly protective immune responses by redirecting immune responses to more highly conserved viral epitopes (11). While the relatively conserved HA “stalk” domain has long been one such target (12–14), it is known that immunity against other viral structural proteins can also contribute to protection. For example, the neuraminidase (NA) is the second most abundant glycoprotein on the surface of virions and can evolve independently of the HA (15, 16), suggesting that anti-neuraminidase immunity may be able to afford protection even when the HA protein is highly drifted. Accordingly, serum neuraminidase inhibition (NI) or anti-NA antibodies are correlated with decreased susceptibility to heterologous influenza virus strains (17–19). The extracellular domain of the M2 protein (M2e) is also well conserved among different human influenza A virus (IAV) strains (20). Previous studies have demonstrated that M2e-containing virus-like particles (VLPs) or vectored M2e vaccines could induce broad cross-reactive immune responses and provide protection against heterologous and heterosubtypic challenges in mice (21, 22). Nucleoprotein (NP) and matrix protein 1 (M1) are internal proteins that are highly conserved among all influenza A subtypes. Vaccines containing NP alone or in combination with M1 have been reported to induce a cross-protective T-cell response against influenza viruses of different subtypes (23, 24).

Thus, while it is clear that non-hemagglutinin structural proteins can contribute to vaccine-mediated protection from influenza disease, the vast majority of studies illustrating these concepts have taken reductionist approaches and evaluated the antigens outside the context of the other viral proteins. We were interested in taking a different, “subtractive” approach, instead generating authentic influenza virus particles that contained all of the viral proteins but completely lacked the hemagglutinin protein. In this way, we could evaluate the contributions of all of the non-HA antigens to protection from viral challenge at the same time. In order to accomplish this, we genetically eliminated the HA open reading frame (ORF). By performing viral propagation on two different helper cell lines, we were able to produce IAV particles that lack the HA protein. We then used these “HA-negative” viruses as tools to probe the nature of the immunity elicited by HA-containing or HA-negative inactivated viral vaccination. We report that while HA-based immunity was a significant contributor to homologous protection, there was no apparent difference in protection against a heterologous viral strain. This work further supports the importance of including and standardizing the “other” structural proteins as components of universal influenza vaccine approaches.

RESULTS

Design and generation of “authentic” viral particles lacking the HA protein.

Our initial goal was to generate matched, inactivated viral particle-based vaccines that differed only by the presence of the HA protein. In order to accomplish this goal, we first took advantage of a well-established approach to delete the HA protein from segment 4 of the viral genome and replace it with an irrelevant protein (in this case, mCherry) flanked by the segment 4 packaging signals (25). Using the reverse-genetics system, plasmids corresponding to seven wild-type (WT) viral segments from A/Puerto Rico/8/1934 (PR8) and the segment 4 mCherry vector (along with an HA protein expression plasmid) were transfected into 293T cells. The resultant viral particles were subsequently propagated on MDCK (Madin-

FIG 1 Design and production of HA-negative IAV particles. (A) Diagram of the scheme to produce HA-negative IAV particles (right) relative to the propagation of WT IAV (left). (B) Immunofluorescence microscopy of HA-MDCK cells infected with segment 4 mCherry IAV at an MOI of 0.1. Bar = 100 μ m. (C) Immunofluorescence microscopy of HAtm-GFP MDCK cells infected with segment 4 mCherry IAV at an MOI of 10. Bar = 100 μ m. (D) Western blots of proteins from the indicated purified and concentrated viral particles. (E) Densitometric analysis of NA from Western blotting. Each dot represents a repeat. Error bars represent the standard deviations (SD). Statistical differences were determined using unpaired Student's *t* test and are denoted (*, $P < 0.05$).

Darby canine kidney) cells stably expressing the PR8 HA protein (Fig. 1A). These viral particles, despite not encoding a gene for HA, still harbor cell-expressed HA on the viral particle and efficiently propagate themselves when passaged on the HA-expressing cell line; we refer to this virus as Segment 4 mCherry IAV (Fig. 1B).

It has been reported that IAV budding is inefficient in the absence of the HA protein (26, 27). Therefore, in order to produce viral particles that efficiently bud without an HA protein, we generated a second MDCK cell line that expressed the transmembrane and cytoplasmic domains of HA fused to green fluorescent protein (GFP) in place of the normal HA ectodomain (Fig. 1A; see also Fig. S1 in the supplemental material). With this cell line, we were able to use segment 4 mCherry IAV to infect the HAtm-GFP MDCK cell line to produce viral particles that replace the HA ectodomain with GFP (Fig. 1A and C). After collection and concentration of the HA-negative virus particles (along with a corresponding WT virus propagated on MDCK cells), virion protein composition was analyzed via Western blotting. After normalization to the viral matrix (M1) protein, we evaluated HA levels. As expected, HA was detected in the WT viral particles but absent in the HA-negative IAV preparation; blots for GFP, however, showed the reciprocal trend (Fig. 1D). Interestingly, slightly more NA and moderately more M2 were present on the HA-negative viral particle, suggesting that virus lacking full-length HA (but still packaging an HA “replacement” protein) allows for an increased abundance of the other surface-exposed structural proteins in viral particles (Fig. 1D to F).

Vaccination with WT and HA-negative IAV particles leads to differential immune responses and protection against homologous viral challenge. We were next interested in evaluating the immune responses that would be generated after vaccination with either the WT or HA-negative viruses. As HA-negative virus particles are not

FIG 2 Serum reactivity against the vaccine-matched strain A/Puerto Rico/8/1934. Mice were vaccinated intramuscularly with BSA, inactivated WT PR8 IAV, or HA-negative PR8 IAV. Animals then received a single boost between 14 and 21 days later. Seven to 14 days after the boost, sera were collected and analyzed for antigen-reactive antibodies (BSA, WT IAV, and HA-negative groups, $n=5$; blank, $n \geq 2$). (A) Serum reactivity against intact, whole PR8 viral particles. OD450, optical density at 450 nm. (B) Area under the curve (AUC) analysis of the data in panel A. Arb. Units, arbitrary units. (C) Serum reactivity against the PR8 HA protein. (D) Area under the curve analysis of the data in panel C. (E) Serum hemagglutination inhibition (HAI) antibody response to PR8. ND (not detected) indicates that the samples were below the limit of detection (LOD). For statistical analysis of undetected samples, a value of one-half the LOD was used. (F) Serum reactivity against the PR8 NA protein. (G) Area under the curve analysis of the data in panel F. (H) Serum reactivity against the PR8 M2 protein. (I) Area under the curve analysis of the data in panel H. Statistical differences for panel E were determined using unpaired Student's t test, and those for panels B, D, G, and I were determined using one-way ANOVA followed by Tukey's *post hoc* analysis. For all panels, error bars represent the standard errors of the means (SEM) (*, $P < 0.05$; **, $P < 0.001$; ns, not significant).

"infectious" due to the lack of a receptor binding protein, we tested the viral particles in the context of inactivated vaccine formulations. C57BL/6 mice received an intramuscular prime and a single boost with formalin-inactivated viral preparations that were normalized via NA content or with bovine serum albumin (BSA) as a control. After the boost, immune sera were collected, and reactivity to the parental PR8 strain was evaluated via an enzyme-linked immunosorbent assay (ELISA). We chose to focus on serum IgG antibodies as our experimental readout as mucosal antibodies and CD8 T-cell responses are limited after inactivated influenza vaccination (28, 29). While both virus preparations were immunogenic, the overall reactivity to the WT virus-derived vaccine was higher than that to the HA-negative virus (Fig. 2A and

B). This difference was due primarily to high levels of anti-HA antibodies uniquely present in the WT vaccine group (Fig. 2C and D). Furthermore, as expected, serum from the WT-vaccinated animals had high HAI activity, while the serum from the HA-negative vaccine group had none (Fig. 2E).

We also tested for serum reactivity against the NA and M2 viral proteins. Although reactivity was above background levels, we were unable to detect a difference in reactivity against either antigen between the two groups (Fig. 2F to I). Thus, it appears that in the absence of HA and under these vaccination conditions, serum IgG responses against the other viral proteins are not appreciably boosted. The inherent immunogenicity and immune responses against all of the non-HA structural proteins together, however, are not insignificant and well above those in the BSA vaccine group (Fig. 2A and B).

We next investigated how the different immune profiles raised against these two vaccines would affect protection during homologous viral challenge. Mice again received a prime and a single boost of the PR8-based vaccine, followed by a lethal PR8 challenge (Fig. 3A). As a primary readout of protection from challenge, body weight and mortality were monitored for 14 days post-challenge. The control BSA group rapidly lost weight and reached the humane endpoint within 5 days of infection; however, all of the vaccinated mice ultimately survived the challenge (Fig. 3B and C). While the survival between the two viral vaccine groups was the same, there was a reproducible dip in body weight after infection of the HA-negative vaccine group (Fig. 3B).

In order to understand how the different vaccines were affecting viral burdens, we repeated the vaccination and challenge experiments and harvested lungs at 3 days post-infection. In contrast to the body weight and survival, there was a striking difference in this metric of vaccine protection: while the HA-negative vaccine significantly reduced viral titers (by ~ 2 orders of magnitude) compared to the control, the WT vaccine was much more effective and reduced viral titers by at least 5 orders of magnitude to below our limit of detection (LOD) (Fig. 3D). Despite the differences in viral titers, lung histopathological analysis at 4 days postinfection failed to reveal obvious differences between the WT IAV and HA-negative IAV groups (Fig. 3E). These data together demonstrate that while HA-negative IAV vaccines can provide significant protection against influenza disease, immunity against the HA protein contributes substantially to reducing viral titers.

The non-hemagglutinin structural proteins can be major drivers of protection from highly drifted, homosubtypic strains. Due to the relatively higher conservation of many of the non-HA structural proteins, we were interested in defining how the immunity induced by our HA-negative vaccine would compare to that induced by WT vaccines in the context of an antigenically distinct H1N1 virus. We therefore repeated our PR8-based vaccination scheme as described in the legend of Fig. 2 and assayed for reactivity against the A/California/04/09 (Cal09) virus. This is a prototype 2009 “swine” pandemic strain and, while still an H1N1 virus, is highly divergent from PR8, which was isolated in 1934. ELISAs against the intact Cal09 viral particle, as well as the HA and NA proteins individually, revealed trends similar to those from the PR8 ELISAs in Fig. 2, although the magnitudes of the changes were much lower (Fig. 4A to F). While total reactivity against the intact Cal09 viral particle and the HA protein trended higher in the WT vaccine group than in the HA-negative group, these differences were not reproducibly statistically significant (Fig. 4A to D). Furthermore, the magnitude of HA reactivity was in fact quite low and close to the background level for all groups (Fig. 4C and D). Cal09 NA reactivity was higher than HA reactivity and identical between the WT and HA-negative vaccine groups (Fig. 4E and F). As expected, none of the sera from any of the vaccinated groups revealed detectable HAI activity against Cal09 (Fig. 4G).

We next turned our attention to defining the protection that PR8-based HA-negative and WT IAV vaccines would provide against Cal09 challenge. Mice again received an inactivated prime and a single boost, followed by lethal infection with the Cal09 virus (Fig. 5A). As expected, all control BSA-vaccinated mice succumbed to infection. At this infectious dose of Cal09, all of the mice from both viral vaccine groups survived

FIG 3 Homologous viral challenge after WT or HA-negative IAV vaccination. (A) Diagram of the immunization scheme and time points for sample collection. (B and C) Mouse body weight (B) and survival (C) after intranasal infection with PR8 ($n=5$). In panel B, * indicates a P value of <0.001 comparing the HA-negative IAV and BSA treatment groups, and ** indicates a P value of <0.001 comparing the BSA group to both of the virally vaccinated groups. In panel C, ** indicates a P value of <0.001 between both viral vaccine groups and the BSA control group. Body weight changes and survival after viral challenge were analyzed by two-way ANOVA followed by Sidak's multiple-comparison test or a log rank (Mantel-Cox) test, respectively. (D) Virus titers in lung tissue after intranasal PR8 infection ($n=5$). Statistical differences were determined via one-way ANOVA followed by Tukey's *post hoc* analysis. ND indicates that the samples were below the limit of detection (LOD). For statistical analysis of undetected samples, a value of one-half the LOD was used. (E) Lung tissue histology after mice were intranasally infected with PR8 virus. For all panels, error bars represent the SEM. Statistical differences are denoted (**, $P < 0.001$; ns, not significant).

the challenge, but in contrast to the PR8 challenge, no obvious differences in body weight loss between the two groups were observed (Fig. 5B and C). Since the largest differences during the homologous challenge experiments were in the infectious viral titers from the lungs, we also analyzed lung titers from the Cal09 challenge experiments. While both vaccine groups had significantly less virus in the lungs than the control, there was no difference between the two viral vaccine groups (Fig. 5D). Hematoxylin and eosin (H&E)-stained lung sections showed significantly less immune cell infiltration in both viral vaccine groups than in the control, but as expected from

FIG 4 Reactivity of PR8-based vaccination-derived sera against the heterologous strain A/California/04/09. Mice were primed intramuscularly with BSA, WT PR8 IAV, or HA-negative PR8 IAV and received a single boost 2 to 3 weeks later (BSA, WT IAV, and HA-negative groups, $n=5$; blank, $n=3$). (A) Serum reactivity against intact whole Cal09 virus. (B) Area under the curve analysis of the data in panel A. (C) Serum reactivity against the Cal09 HA protein. (D) Area under the curve analysis of the data in panel C. (E) Serum reactivity against the Cal09 NA protein. (F) Area under the curve analysis of the data in panel E. (G) Serum hemagglutination inhibition (HAI) antibody response to Cal09. Statistical differences for panels B, D, and F were determined via one-way ANOVA followed by Tukey's *post hoc* analysis. For all panels, error bars represent the SEM (*, $P < 0.05$; **, $P < 0.001$; ns, not significant). ND indicates that the samples were below the limit of detection.

the viral titer data, we failed to observe any striking histological differences between the vaccine groups (Fig. 5E). These data together support the notion that as viral strains diverge, the non-HA structural proteins can play critical and efficacious roles in vaccine-mediated protection.

DISCUSSION

The development of improved influenza virus vaccines to protect public health is of the highest importance. In order to facilitate that goal, a better understanding of how different viral proteins contribute to immunity against both matched and antigenically drifted strains is needed. In this study, we generated "authentic" influenza virus particles deficient for the HA protein to understand how all of the other structural proteins together contribute to vaccine efficacy. We show that while anti-HA immunity plays a major role in reducing viral titers during homologous challenge, the contributions of HA become less important when evaluating protection from disease and even more so against highly drifted strains. In fact, we failed to detect any significant differences between WT and HA-negative vaccines in reducing viral titers or in mediating protection from a highly drifted, homosubtypic strain challenge.

These data highlight that particularly with respect to universal influenza vaccine development, the "other," non-HA structural proteins should be given serious attention. In most seasonal influenza vaccines, the formulation is based exclusively on HA content, and the contents of at least some of the other viral proteins have been shown to be highly variable (19). Our data suggest that while this is unlikely to affect protection against matched strains (and indeed, recombinant HA-only vaccines [30] are efficacious and FDA approved), ignoring the non-HA viral proteins could have significant

FIG 5 Heterologous viral challenge after WT or HA-negative IAV vaccination. (A) Diagram of the immunization scheme and time points for sample collection. The WT IAV and HA-negative IAV vaccines were PR8 based, the same as in Fig. 4. (B and C) Mouse body weight (B) and survival (C) after intranasal infection with Cal09 ($n=5$). In panel B, * indicates a P value of <0.05 comparing the WT IAV and BSA groups and also a P value of <0.001 comparing the HA-negative IAV and BSA treatment groups, and ** indicates a P value of <0.001 comparing the BSA group to both of the virally vaccinated groups. In panel C, ** indicates a P value of <0.001 between both vaccine groups and survival after viral challenge. Body weight changes and survival after viral challenge were analyzed by two-way ANOVA followed by Sidak's multiple-comparison test or a log rank (Mantel-Cox) test, respectively. (D) Virus titers in lung tissue after intranasal Cal09 infection ($n=5$). Statistical differences were determined via one-way ANOVA followed by Tukey's *post hoc* analysis. (E) Lung tissue histology after mice were intranasally infected with Cal09 virus. For all panels, error bars represent the SEM. Statistical differences are denoted (*, $P < 0.05$; **, $P < 0.001$; ns, not significant).

implications for the duration or breadth of protection. With that said, it is also important to avoid overgeneralization of the results of our study. We generated a single HA-negative vaccine in the PR8 viral background and evaluated immunity against one other strain, Cal09. At a minimum, our data show the magnitude of the protective effect that the non-HA structural proteins can have in mediating protection from drifted strains. However, it is also worth highlighting that our data are consistent with previous reports of "single-cycle" live attenuated viral vaccines, which, although genetically deleted for the HA protein, display HA on the incoming viral particle and perform one round of replication after vaccination (25, 31, 32). This approach elicits little HA-

directed immunity but is also associated with strong protection from viral challenge, in general agreement with our conclusions.

One major question that was not answered by our study was the definition of the relative contributions of each of the non-HA proteins to the protection phenotype. We believe that it is likely that anti-NA antibodies are major contributors, as vaccination with the NA protein alone has been reported to be sufficient to mediate protection from influenza disease (19, 33, 34). Furthermore, although we focused our analysis on serum antibodies against the surface-exposed viral proteins, T-cell responses against proteins like NP and M1 (35) may be modulated in the absence of the immunodominant HA protein and could have contributed to the observed protection. Future studies, however, will be required to resolve these questions.

It is also important to note that our HA-negative vaccine preparation was not truly “matched” to the WT comparison with respect to non-HA proteins. We know that at a minimum, the relative amount of NA and M2 in the HA-negative viral particle were altered. Although we normalized our vaccine formulation by NA activity and could not subsequently detect a difference in reactivity to these proteins in our assays, altered immune responses (antibody mediated or otherwise) to these or other structural proteins may have contributed to the protective effects that we observed during challenge. Additionally, our HA-negative IAV particles packaged GFP in place of HA. While we consider GFP an “irrelevant” protein with respect to influenza virus protective immunity, GFP itself may have some immunogenic properties (36), which should also be evaluated in the future.

In addition to its use as a tool to probe vaccine-mediated immunity in the absence of HA, our approach also has the potential to serve as a modular platform with which to package proteins onto an authentic viral particle. We have successfully packaged GFP, which in no meaningful way contributed to viral biology. Although not tested in this study, it is likely that a range of proteins can be fused to the transmembrane domain and cytoplasmic tail of HA to force incorporation into a viral particle. In contrast to genetically encoding them in a fully replication-competent virus, encoding the “foreign” proteins in the cell line negates the viral mechanisms to mutate or eliminate the protein. This would likely lead to high stability of the foreign protein on the viral particle. Finally, it has also been shown that anti-HA antibodies can interfere with neuraminidase activity via steric interference (37). Our HA-negative viral particles may be an attractive reagent to probe the effects of anti-NA antibodies without concerns of interference from HA antibodies.

In conclusion, many reports have evaluated the relative contributions of different IAV proteins to immunity when administered alone or in limited combinations. To our knowledge, no previous study has generated “complete” viral particles that lack only the HA protein. Using a combination of viral genetic manipulations and helper cell lines, we were able to produce inactivated IAV vaccines that allowed us to disentangle the antigenic contributions of HA from those of all of the other viral structural proteins. Our results suggest that, especially for vaccines designed to provide broad or long-lasting protection, the non-HA structural proteins should be carefully evaluated and may be important components of next-generation anti-influenza vaccines.

MATERIALS AND METHODS

Cell lines and viruses. Human embryonic kidney 293T cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum, HEPES, NaHCO₃, GlutaMAX, and penicillin-streptomycin. Madin-Darby canine kidney (MDCK) cells (ATCC) were cultured in minimal essential medium (MEM) supplemented with 5% fetal bovine serum, HEPES, NaHCO₃, GlutaMAX, and penicillin-streptomycin. The PR8 HA protein was introduced into an MDCK cell line via lentivirus delivery; cells were then grown under the same conditions as those for unmodified MDCK cells. All cell lines were grown at 37°C in 5% CO₂. The influenza A virus challenge strains A/Puerto Rico/8/1934 (PR8) and A/California/04/2009 (Cal09) were propagated in embryonated chicken eggs. As viral strains may acquire mutations during laboratory passaging, the strains used for challenge in this study were sequenced via Sanger sequencing. GenBank accession numbers for the A/Puerto Rico/8/1934 viral genes (with deviations noted in parentheses) are as follows: for PB2, [AF389115.1](#); for PB1, [CY148249.1](#) (G1557T, silent; C1773T, silent); for PA, [AF389117.1](#); for HA, [AF389118.1](#) (A651C, I207L); for NP, [AF389119.1](#); for NA,

AF389120.1; for MP, AF389121.1; and for NS, AF389122.1. GenBank accession numbers for the A/California/04/2009 (Cal09) viral genes (with deviations noted in parentheses) are as follows: for PB2, MN371615.1; for PB1, MN371613.1 (G498A, silent); for PA, MN371611.1 (G2022A,Q670H); for HA, MN371616.1 (C655A,A212E; A739G,Q240R; G1395A,V459M; T1487C, silent); for NP, MN371617.1 (A335G, D101G); for NA, MN371610.1; for MP, MN371612.1; and for NS, MN371614.1.

Experimental system for producing HA-negative viral particles. To generate HAtm-GFP MDCK cells, the HA transmembrane and cytoplasmic domains were fused to GFP. The gene fragment was synthesized (IDT) and cloned into lentivirus vector pLEX. Lentiviruses were packaged on 293T cells and used to transduce MDCK cells. The resultant transduced cell line was passaged in the presence of puromycin and maintained at low passage numbers to produce HA-negative viruses. Segment 4 mCherry IAV was designed and rescued based on the bicistronic pDZ rescue plasmid system. Viral sequences were based on the reverse-genetics rescue plasmids from the PR8 H1N1 background as previously described (38, 39). To remove the HA protein ORF from segment 4, the middle of the segment was deleted, and only the 5'-terminal 99 nucleotides (nt) and the 3'-terminal 150 nt (based on the nucleotide positions in the positive-sense RNA) were preserved to serve as packaging signals. Furthermore, to prevent inappropriate early translation, all ATGs in the 3' packaging signal were mutated to TTG. A consensus Kozak sequence and the mCherry gene (flanked by 3' EcoRV and 5' PmeI restriction sites) were inserted between the packaging signals to generate the final segment. Plasmids corresponding to seven WT PR8 viral segments along with segment 4 mCherry and the pLEX-HA plasmid were transfected into 293T cells using TransIT-LT1 (Mirus). The rescued viruses lacking the HA gene were then propagated and titers were determined on HA-MDCK cells.

Propagation of viral stocks and vaccine formulation. MDCK cells were infected at a multiplicity of infection (MOI) of 0.01 with wild-type PR8 to grow WT IAV. HAtm-GFP cells were infected at an MOI of 5 with segment 4 mCherry IAV to produce HA-negative IAV. Virus supernatants were layered on top of 30% sucrose-phosphate-buffered saline (PBS) and ultracentrifuged for 1 h at 27,500 rpm for concentration. For vaccine formulation, the concentrated viral particles were assayed for neuraminidase activity and then normalized. The protein concentrations of the normalized preparations were then quantified by a Bradford assay (40), and 10 μ g of the HA-negative viral preparation was administered in a given vaccination. The total amount of the WT control vaccine was allowed to fluctuate to match the amount of the NA in the HA-negative preparation, and usually slightly less total protein was used for WT vaccination. Viral preparations were inactivated by incubation with 0.02% formalin for 30 min and then dialyzed by using Slide-A-Lyzer cassettes (Thermo Scientific).

Western blotting. Equal amounts of protein were loaded into 4 to 20% acrylamide gels (Bio-Rad) and transferred to a nitrocellulose membrane. PBS with 5% (wt/vol) nonfat dried milk and 0.1% Tween 20 was used for blocking for 2 h at 4°C. Primary antibodies were then incubated with the membrane overnight at 4°C. Antibodies used were mouse anti-HA (PY102; a gift from Tom Moran at the Icahn School of Medicine at Mount Sinai), mouse anti-NA (4A5 [19]; a gift from Gene Tan at the J. Craig Venter Institute), mouse anti-M1/M2 (E10; a gift from Tom Moran at the Icahn School of Medicine at Mount Sinai), and mouse anti-GFP (catalog number 29555; Cell Signaling Technology). Membranes were washed five times in PBS with 0.1% Tween 20, and anti-mouse-horseradish peroxidase (HRP) or anti-rabbit-HRP secondary antibodies (GE Healthcare) were then incubated with the blots for 1 h. The membrane was then washed five times, and the Clarity or Clarity Max ECL substrate (Bio-Rad) was added before being exposed to film and developed. For densitometry analysis, quantification was performed with ImageJ (NIH), and values were normalized prior to statistical analysis.

Vaccination and animal challenge. Six- to ten-week-old C57BL/6 female mice were used for all experiments. For vaccination, the vaccine was administered intramuscularly at one injection site. After 2 to 4 weeks, mice were boosted in the same fashion and given another 2 to 3 weeks before challenge or the collection of serum. For infection, mice were administered 40 μ l of the virus (10,000 PFU for PR8 and 24,000 PFU for Cal09) intranasally after anesthesia with a ketamine-xylazine mixture. Mice were weighed daily and euthanized once their body weight reached <80% of the starting weight as a humane endpoint. Euthanasia was performed via CO₂ as the primary method, and a bilateral thoracotomy was performed as the secondary method. All procedures were approved by the Duke University IACUC.

ELISAs. For whole-virus ELISAs, virions were concentrated using a 30% sucrose cushion for 1 h at 25,700 rpm on the Sorvall TH-641 swinging-bucket rotor and then resuspended in PBS. For HA and NA ELISAs, PR8 HA protein was expressed by 293T cells and purified by immobilized metal affinity chromatography. PR8 NA and Cal09 HA and NA were obtained through BEI Resources (catalog numbers NR-19235, NR-51668, and NR-19234). Ninety-six-well plates were coated at 4°C with protein using a carbonate buffer overnight. For the PR8 M2 cell-based ELISA, the pLEX-M2 plasmid was transfected into 293T cells in suspension using TransIT-LT1 (Mirus), and cells were then seeded into 96-well plates and grown for 48 h. Cells were fixed with 4% paraformaldehyde (PFA)-PBS before the addition of the serum. Serum samples were then diluted and added to the wells. Bound Ab was detected by using sheep anti-mouse HRP-conjugated antibody (GE Healthcare). Color was developed by using the tetramethylbenzidine (TMB) substrate (Thermo Scientific), and reactions were stopped with 2 M sulfuric acid. Absorbance was measured at 450 nm on a plate reader. The area under the curve (total area) was calculated with Prism software (GraphPad) using the average of the blank samples as the background cutoff.

HAI assay. Serum was treated with receptor-destroying enzyme (RDE; Denka Seiken) at a 1:4 dilution at 37°C for 20 h, followed by inactivation at 56°C for 30 min and further dilution to 1:10 with PBS. Serum was 2-fold serially diluted in V-bottom microtiter plates. Virus adjusted to 4 HA units in 25 μ l was added to each well. The plates were incubated at room temperature for 15 min, followed by the addition of 50 μ l of 0.5% chicken (for PR8) or turkey (for Cal09) erythrocytes (Lampire Biologicals) in PBS. The reaction mixture

was then allowed to settle for 30 min at room temperature. Wells were examined visually for inhibition of HA. HAI titers were the reciprocal of the highest dilution of serum that completely prevented HA.

Plaque assay. Viral titers in the lungs were determined using a standard plaque assay protocol on MDCK cells. Virus was serially diluted, and after incubation with the cells for 1 h at 37°C, virus was removed and a 1% agar overlay containing *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-trypsin was applied. After incubation at 37°C for 48 to 72 h, assay mixtures were fixed in 4% PFA for 3 h. Serum from WT PR8- or WT Cal09-infected mice was diluted in antibody dilution buffer (5% [wt/vol] nonfat dried milk and 0.05% Tween 20 in PBS) and incubated on cells at 4°C for 12 h. Cells were then washed and incubated for 2 h in diluted sheep anti-mouse HRP-conjugated antibody in antibody dilution buffer. Assay mixtures were then washed with PBS and exposed to 0.5 ml of the True Blue peroxidase substrate (KPL) for 15 min. Plates were then washed with water and dried before the plaques were counted.

Microscopy. Fluorescence images were taken using HA-MDCK cells infected with segment 4 mCherry IAV at an MOI of 0.1 or HAtm-GFP MDCK cells infected with segment 4 mCherry IAV at an MOI of 10. At 48 h postinfection, cells were incubated with Hoechst 33342 stain (Life Technologies) to allow the staining of nuclei, and imaging was performed on the ZOE fluorescence cell imager (Bio-Rad). For H&E-stained slides, mouse lung tissues were fixed with 4% PFA-PBS at 4°C for more than 16 h. Samples were embedded in paraffin and sectioned after dehydration and wax immersion, and slides were then rehydrated and stained with H&E according to standard protocols. Microscopy was done on a Zeiss Axio Imager microscope. Images were then processed with ImageJ (NIH).

Statistical analysis. Data were analyzed using Prism software (GraphPad). Values below the limit of detection were assigned a value of one-half the LOD (LOD/2) in subsequent analyses. Unless otherwise noted, significance was determined by using Student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* analysis. Body weight changes and survival after viral challenge were analyzed by two-way ANOVA followed by Sidak's multiple-comparison test or a log rank (Mantel-Cox) test, respectively. Asterisks in all figures indicate significance (*, $P < 0.05$; **, $P < 0.001$).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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Duke University has filed for protection of intellectual property rights based on the work in this report.

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