VIROLOGY

Immunity to hemagglutinin and neuraminidase results in additive reductions in airborne transmission of influenza H1N1 virus in ferrets

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Currently, there is limited knowledge on the impact of immunity to hemagglutinin (HA) and/or neuraminidase (NA) on the transmission of influenza viruses. Therefore, using intramuscular vaccination, intranasal vaccination, or infection with reassortant viruses, we induced immunity to each antigen alone or both antigens combined in ferrets. We then assessed transmission of the 2009 pandemic H1N1 virus from these ferrets to naïve respiratory contacts. For all strategies used to induce immunity, combined immunity to HA and NA resulted in the largest reductions in transmission. Moreover, immunity to HA and NA conferred additive rather than synergistic reductions in transmission. No escape variants emerged in our transmission studies, and logistical regression showed that the probability of transmission was less than 50% when viral titers in donors were reduced to $10^{1.5}$ and 10^{2} median tissue culture infectious dose per ml on days 1 and 3 postinfection, respectively. These studies define the relationship between immunity to HA and NA on transmission and identify a threshold titer indicative of decreased transmission in ferrets.

INTRODUCTION

Each year, influenza A and B viruses cause epidemics, resulting in up to one billion human infections, with 3 to 5 million cases of severe illness and up to 500,000 deaths worldwide (*1*, *2*). Influenza viruses can be transmitted via fomites, direct contact, or airborne droplets. While all three modes of transmission contribute to the spread of influenza viruses, airborne or respiratory transmission is considered the major driver of epidemics (*3*, *4*). Influenza viruses express two major surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). The HA is responsible for attachment and entry into the host cell, while NA plays a critical role in budding by cleaving the cell surface receptor (i.e., sialic acids) to facilitate release of progeny virions from the cell (*5*).

To reduce the disease burden of influenza viruses, split-virion inactivated vaccines (IIV) have been developed and are formulated to induce antibodies against the HA. IIVs are administered via intramuscular (IM) injection and contain a standardized amount of HA (15 μg) per virus strain in the vaccine (*6*, *7*). The NA protein is also present in IIVs, but NA content is not measured or standardized, and both the quantity and quality of NA antigen vary by vaccine lot and manufacturer (*8*). While IIVs are widely used and reduce the severity of influenza virus disease, owing to their composition and route of administration, these vaccines have limited ability to prevent infection and onward viral transmission (*9*).

In response to vaccination or prior infection, a serum antibody titer toward the HA of ≥1:40 [determined via hemagglutination inhibition (HAI) assay] correlates with protection against severe disease (*10*, *11*). In animal and human studies, high NA antibody titers also correlate with reduced viral shedding and disease severity (*12*–*15*). While the impact of immunity toward HA and NA on disease severity is well established, the contribution of immunity to each antigen and both antigens combined on transmission has not been studied in depth.

The ferret model of influenza is the gold standard for evaluating preclinical vaccine efficacy and airborne transmission of influenza viruses (*16*–*19*). Therefore, using separate IM vaccination, intranasal (IN) vaccination, and infection-based strategies, we evaluated the role of immunity against HA and/or NA in limiting airborne transmission of influenza viruses using the ferret model. For each strategy, we generated ferrets with immunity (hereafter referred to as preimmune ferrets) toward HA, NA, or HA and NA. These preimmune ferrets were then challenged with the 2009 pandemic H1N1 (2009 H1N1) virus and used as donors (DRs) to immunologically naïve respiratory contacts (RCs) in transmission studies. For all strategies used to induce immunity, immunity toward HA or NA alone reduced viral replication in DR animals and transmission to varying degrees; however, combined immunity toward HA and NA conferred additive reductions in viral shedding and transmission that exceeded those induced by immunity to either antigen alone. Using whole-genome sequencing, we further show that transmission was not associated with the emergence of viral escape variants, and logistical regression analyses showed that reducing viral titers to below $10^{1.5}$ median tissue culture infectious dose ($TCID_{50}$)/ml early during infection reduces the probability of transmission to less than 50%. These studies define how immunity to HA and NA alters viral transmission and identify a threshold titer in DR ferrets below which the likelihood of transmission is greatly decreased.

RESULTS

IM and IN vaccination induced functional antibodies against HA and/or NA

To explore the relationship between route of vaccination and immunity to HA and/or NA on transmission, we developed IM and IN

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vaccine regimens that used the same antigen doses and vaccination timeline. As IIVs have limited immunogenicity, especially toward NA, and NA content and quality vary in IIVs, the goal of these studies was not to recapitulate immunity induced by IIVs but to determine the impact of robust immunity to HA and/or NA on viral transmission. Therefore, we vaccinated ferrets three times with adjuvanted vaccines containing 50 μg of each antigen. In our prior studies evaluating vaccination with HA stem or recombinant NA proteins, this vaccine dose and regimen induced a robust antibody response to the vaccine antigens (*20*, *21*). For IM vaccination, vaccines were formulated with Sigma Adjuvant System (SAS) adjuvant (formerly known as Ribi), and IN vaccines were prepared containing the LTA1 IN adjuvant. The SAS adjuvant is an oil-in-water adjuvant containing Monophosphoryl Lipid A (MPLA) and trehalose dicorynomycolate (*22*). The oil-in-water formulation creates an antigen depot when injected into muscle tissue, prolonging antigen release, while MPLA and trehalose dicorynomycolate stimulate Toll-like receptors 4 and 2, respectively (*23*, *24*). LTA1 is the A1 domain of heat-labile enterotoxin from *Escherichia coli* that lacks any nasal toxicity and is being advanced to clinical trials. The mechanism of action of LTA1 is still under investigation; however, enzymatic activity of the A1 domain is required to stimulate host immunity, and LTA1 induces both a CD4 T cell and B cell response when formulated with influenza antigens (*25*, *26*). Different adjuvants were used for the IM and IN routes, as IM adjuvants are either poorly immunogenic or have high toxicity when given IN (*27*, *28*). Groups ($n = 3$ or 4) of ferrets were IM or IN vaccinated three times, 28 days apart, with either a mock vaccine [adjuvant + phosphate-buffered saline (PBS)] or adjuvant mixed with recombinant HA (50 μg), NA (50 μg), or a combination of HA (50 μg) and NA (50 μg) protein. Serum was collected on days 28, 56, 70, and 82 post–primary vaccination, and immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs) were performed to evaluate the total amount of HA- and NA-binding antibodies. HAI and microneutralization assays were then performed to evaluate the functionality of HA antibodies, while enzyme-linked lectin assays (ELLAs) were used to measure the NA inhibitory (NAI) activity of serum antibodies.

After completing the IM vaccination regimen, on day 82 post–primary vaccination, ferrets vaccinated with HA or HA + NA had significantly higher H1-binding IgG and HAI antibody titers relative to mock- and NA-vaccinated animals ($P < 0.02$) (Fig. 1A and fig. S1A). All the HA- and HA + NA–vaccinated animals also developed a neutralizing antibody response (Fig. 1B), while one of three NA-vaccinated animals and none of three mock-vaccinated animals developed neutralizing antibodies. When antibodies toward N1 NA were evaluated, ferrets vaccinated IM with NA alone or HA + NA developed significantly higher titers of N1 NA-binding IgG antibodies, which also exhibit significant NAI activity relative to mock- or HA-vaccinated animals ($P < 0.02$) (Fig. 1, A and B, and fig. S1, A to C). As expected, animals vaccinated IM against NA alone did not develop antibodies toward HA, and animals vaccinated against HA alone did not developed antibodies toward NA. Moreover, none of the mock-vaccinated animals developed antibodies toward HA or NA (Fig. 1, A and B).

For IN vaccination, on day 82 post–primary vaccination, animals vaccinated with HA or HA + NA had significantly higher titers of H1-binding IgG antibodies relative to mock- and NA-vaccinated animals ($P < 0.02$) (Fig. 1, C and D, and fig. S1, D to F). Both the HA- and HA + NA–vaccinated groups also had significantly higher HAI antibody titers relative to the mock- and NA-vaccinated animals ($P < 0.02$). When neutralizing antibodies were evaluated, three of four HA-vaccinated and four of four HA + NA–vaccinated animals had a neutralizing antibody response; however, only the HA + NA–vaccinated animals had significantly higher titers of neutralizing antibodies relative to the mock- and NA-vaccinated animals ($P < 0.02$). None of the mock- or NA-vaccinated ferrets developed antibodies toward HA. Ferrets vaccinated IN with HA + NA had significantly higher titers of N1-binding and inhibitory antibodies ($P < 0.02$) relative to mock- and HA-vaccinated ferrets (Fig. 1, C and D, and fig. S1, D to F). Ferrets vaccinated IN with NA alone had N1-binding antibody titers that were in between but not significantly different from either the HA + NA–vaccinated animals and all other groups. However, the NA-vaccinated animals had significantly higher titers of NAI antibodies relative to mock- and HA-vaccinated animals, and these titers were not significantly different from those of HA + NA–vaccinated animals. While both N1-binding and NAI titers did not differ significantly between the NA and HA + NA–vaccinated groups, the HA + NA group did have increased titers relative to the NA-vaccinated animals. This suggests that incorporating HA into the vaccine may be enhancing the antibody response to NA, and this should be explored in future studies. As expected, IN mock- and HA-vaccinated animals did not develop antibodies toward NA (Fig. 1, C and D). In summary, both IM and IN vaccination with HA and/or NA induced antibodies against the respective antigens that could bind and inhibit the function of these glycoproteins.

IM vaccination with HA and NA reduced airborne transmission of the 2009 H1N1 virus to contact animals by 50%

To assess whether IM vaccination–induced immunity could reduce viral transmission, we performed airborne transmission experiments using IM vaccinated animals as DRs. Eighty-four days post–primary vaccination (28 days post–tertiary vaccination), IM vaccinated ferrets were challenged IN with 10^4 $TCID_{50}$ of A/California/07/2009 (H1N1pdm09; 2009 H1N1) virus. Twenty-four hours postinfection, IM vaccinated DRs were paired with naïve RCs in airborne transmission cages. Nasal wash samples were then collected every other day to evaluate the presence of virus in the nose, and 21 days postpairing of the DR and RC, blood was collected from the RCs to assess seroconversion. Contact animals were considered infected when virus was detected in the nasal wash on 2 or more days or upon seroconversion to the 2009 H1N1 virus. When an RC ferret had an HAI titer but did not shed virus, confirmatory microneutralization assays were performed. Transmission efficiency (TE) was defined as the percentage of contacts that became infected.

When IM mock-vaccinated DRs were challenged with the 2009 H1N1 virus, the animals became productively infected and transmitted the virus to three of three RC animals (100% TE) (Fig. 2A). These RCs shed infectious virus over multiple days and seroconverted (Table 1). Upon infection, all IM HA-vaccinated DRs shed virus for at least 1 day, and three of four RCs became infected (75% TE) (Fig. 2B). The one RC that did not become infected was paired with a DR that shed detectable virus (10^1 $TCID_{50}$/ml of nasal wash) for 1 day after pairing the animals. In the IM NA-vaccinated DRs, all animals became infected, and these animals transmitted the virus to three of three RCs (100% TE) (Fig. 2C). When evaluating airborne transmission from IM HA + NA–vaccinated DRs, all DR animals became infected; however, these animals shed low levels of virus ($\leq 10^{1.5}$ $TCID_{50}$/ml) (Fig. 2D). Virus was detected in the nose of two of four RCs (50%

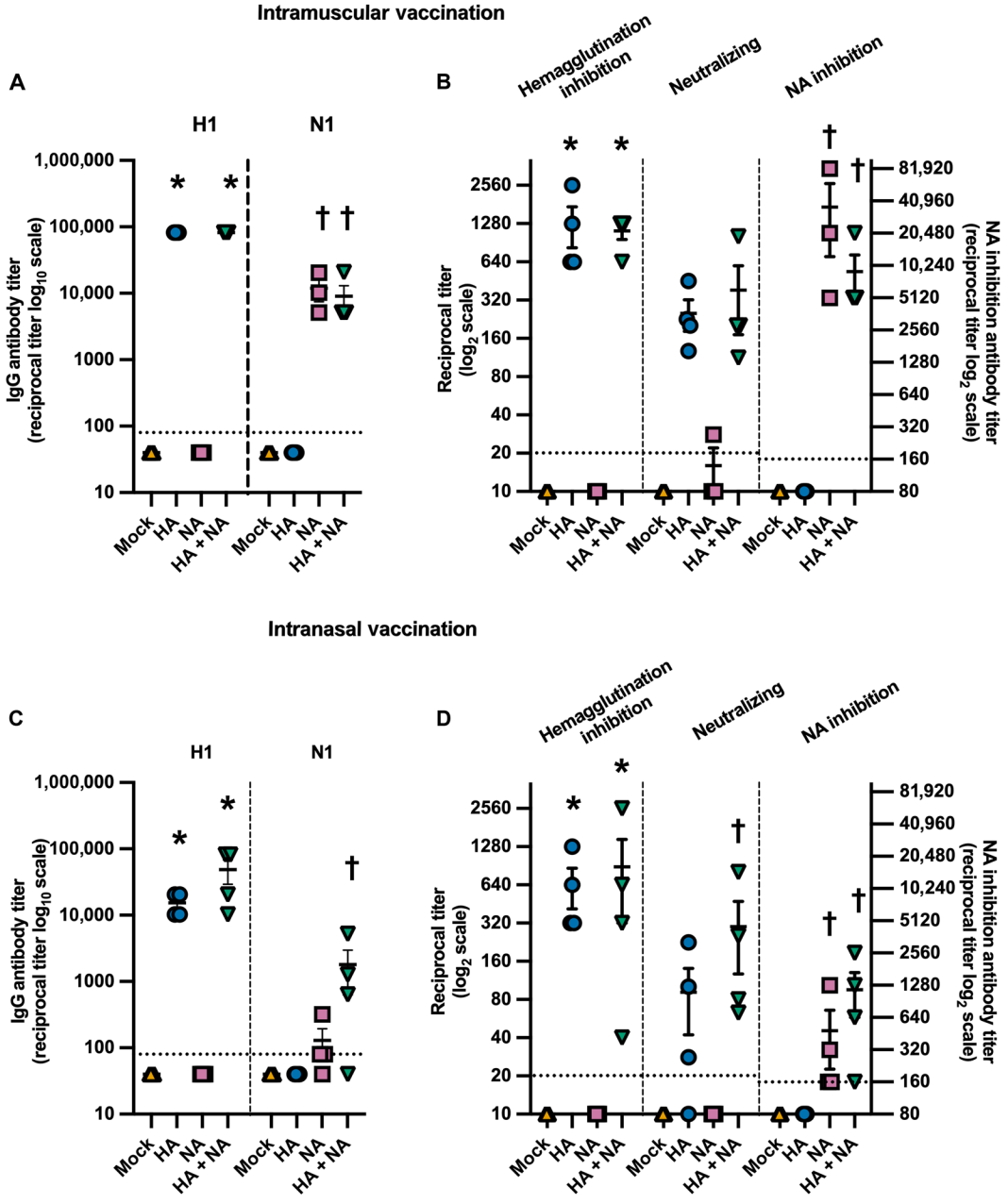

Fig. 1. Assessment of the antibody response in IM and IN vaccinated ferrets before 2009 H1N1 infection. Serum samples collected on day 82 post–primary vaccination from ferrets ($n = 3$ or 4 per group) given mock, HA, NA, or HA + NA vaccines via IM or IN routes were analyzed for antibodies to H1 HA and N1 NA. (**A**) H1 and N1 IgG binding antibody titers, and (**B**) HAI, neutralization, and NAI antibody titers in IM vaccinated ferrets. (**C**) H1 and N1 IgG binding antibody titers, and (**D**) HAI, neutralization, and NAI titers in IN vaccinated ferrets. Binding antibody titers were determined by ELISA. HAI and neutralization titers were determined via HAI assay and microneutralization assays, respectively. NAI titers were determined via ELLA. ELISA, HAI, and ELLA assays were performed in duplicate, and microneutralization assays were performed with four technical replicates. For all antibody analyses, individual values are displayed with horizontal line, and error bars showing mean ± SEM, respectively. Orange triangles, dark blue circles, pink squares, and green inverted triangles represent mock-, HA-, NA-, and IM HA + NA–vaccinated DRs, respectively. *Significantly different from mock- and NA-vaccinated DRs ($P < 0.02$). †Significantly different from mock- and HA-vaccinated DRs ($P < 0.02$) determined via Kruskal-Wallis test with post hoc two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli.

TE) with virus shedding over multiple days. In summary, IM vaccination against NA did not reduce transmission (100% TE), while vaccination against HA or HA + NA reduced transmission such that 75 and 50% of the RCs became infected, respectively.

To further evaluate the contribution of immunity to each antigen in limiting viral replication, we compared peak titers and total viral shedding between groups of IM vaccinated DRs (Fig. 2, E and F). Compared to IM mock-vaccinated DRs, IM HA + NA–vaccinated DRs had a significant decrease in peak titers ($P = 0.008$), while IM HA or NA-vaccinated DRs had peak titers between, but not significantly different from, the IM mock- and HA + NA–vaccinated DRs. When total viral shedding was compared using area under the curve

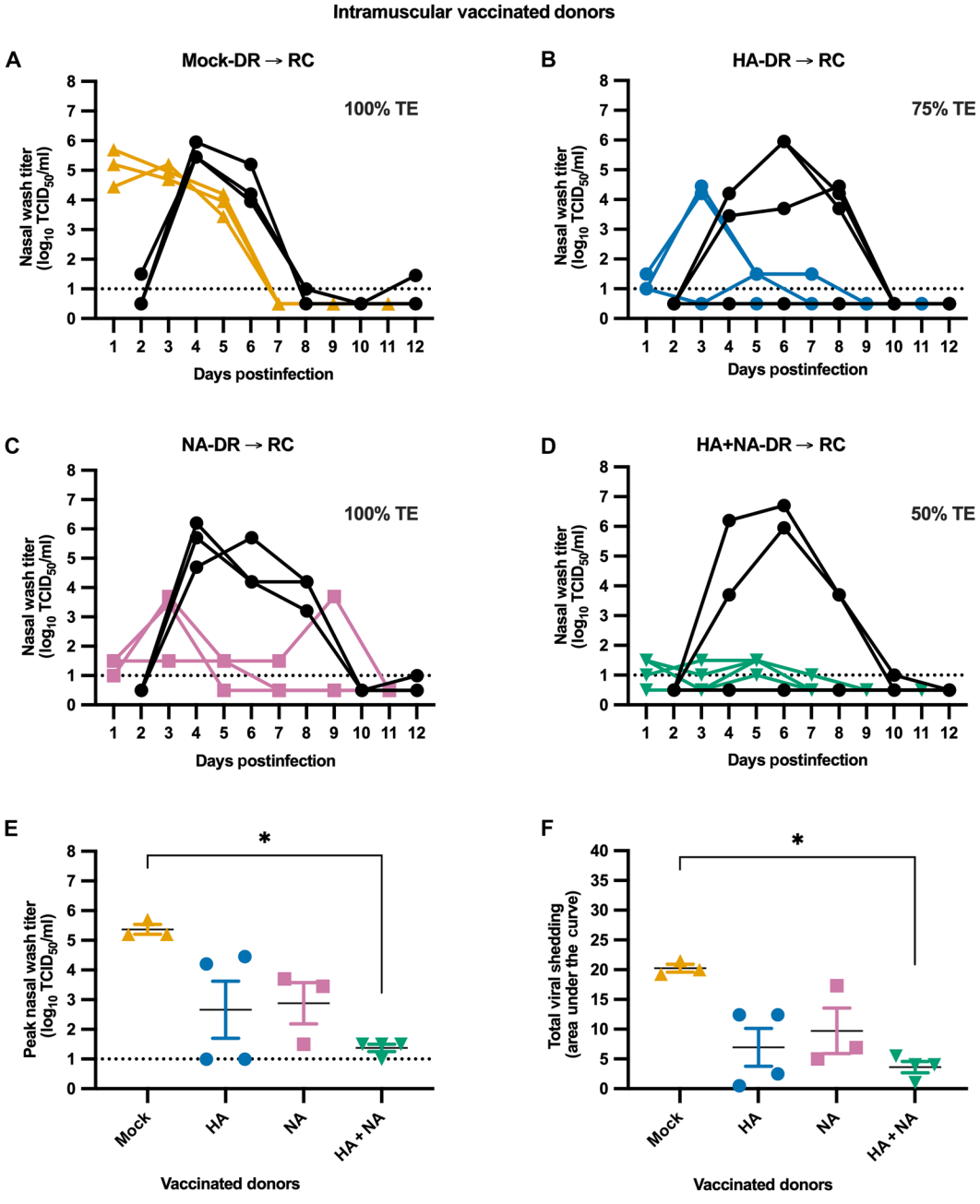

Fig. 2. Airborne transmission of the 2009 H1N1 virus from IM vaccinated preimmune DRs to immunologically naïve RC ferrets. IM mock-, HA-, NA-, and HA + NA–vaccinated ferrets ($n = 3$ or 4 per group) were IN inoculated with 1×10^4 $TCID_{50}$ of the 2009 H1N1 virus. Twenty-four hours later, each inoculated animal was introduced as the DR into a transmission cage with an immunologically naïve RC. Nasal washes were then collected every other day from each animal. (**A** to **D**) Viral shedding curves for IM mock-, HA-, NA-, or HA + NA–vaccinated DRs, respectively, and their paired RCs. DR shedding curves are shown in color, and RCs are shown with solid black lines. To determine whether vaccine-induced immunity altered viral shedding kinetics, analyses of viral titers were performed. (**E**) and (**F**) Peak nasal wash titers and total viral shedding by area under the curve (AUC) for IM vaccinated DRs, respectively. Orange, blue, pink, and green lines or symbols represent IM mock-, HA-, NA-, and HA + NA–vaccinated DRs, respectively. Nasal wash samples were titrated on Madin-Darby canine kidney cells (MDCK cells), and results are expressed as $\log_{10}$ $TCID_{50}$/ml of nasal wash. Dashed horizontal line denotes limit of detection. *Significant differences were determined by Kruskal-Wallis test with two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli post hoc ($P < 0.05$). For (E) and (F), horizontal line and error bars represent mean ± SEM, respectively, and unless significant differences are indicated, all other comparisons are not significant.

Table 1. Summary of infection status of RC ferrets for transmission experiments using IM vaccinated, IN vaccinated, or infection-induced preimmune DRs. Limit of detection is 20. Serum was collected 21 days after infection of the DR ferret.

Approach used to induce immunity	Group	Ferret	Shed infectious virus	Hemagglutination inhibition titer (reciprocal titer)	Percent contacts infected
IM vaccination	Mock	RC1	Yes	1,280	100%
		RC2	Yes	1,280	
		RC3	Yes	1,280	
	HA	RC1	Yes	2,560	75%
		RC2	Yes	1,280	
		RC3	Yes	1,280	
		RC4	No	<20	
	NA	RC1	Yes	1,280	100%
		RC2	Yes	1,280	
		RC3	Yes	1,280	
	HA + NA	RC1	No	<20	50%
		RC2	No	<20	
		RC3	Yes	640	
		RC4	Yes	2,560	
IN vaccination	Mock	RC1	Yes	2,560	100%
		RC2	Yes	320	
		RC3	Yes	1,280	
		RC4	Yes	640	
	HA	RC1	Yes	640	75%
		RC2	No	160*	
		RC3	Yes	640	
		RC4	No	<20	
	NA	RC1	Yes	640	75%
		RC2	No	<20	
		RC3	Yes	1,280	
		RC4	Yes	640	
	HA + NA	RC1	Yes	1,280	50%
		RC2	No	<20	
		RC3	Yes	2,560	
		RC4	No	<20	
Infection with reassortant viruses	1968 H3N2	RC1	Yes	<20	100%
		RC2	Yes	1,280	
		RC3	Yes	320	
		RC4	Yes	320	
	rsH1N2	RC1	Yes	640	75%
		RC2	No	<20	
		RC3	Yes	1,280	
		RC4	Yes	320	
	rsH3N1	RC1	Yes	320	50%
		RC2	No	<20	
		RC3	Yes	2,560	
		RC4	No	<20	
	rsH1N1	RC1	No	n/p†	25%
		RC2	No	<20	
		RC3	No	<20	
		RC4	Yes	2560	

(*Continued*)

(*Continued*)

Approach used to induce immunity	Group	Ferret	Shed infectious virus	Hemagglutination inhibition titer (reciprocal titer)	Percent contacts infected
Infection with reassortant viruses	2009 H1N1	RC1	No	<20	0%
		RC2	No	<20	
		RC3	No	<20	
		RC4	No	<20	

*Neutralization assay performed to confirm seroconversion—ferret had neutralizing titer of 1:20. †n/p, not performed. Serum sample was lost during processing of blood.

(AUC) analyses, we similarly observed that IM HA + NA–vaccinated DRs shed significantly less virus than IM mock-vaccinated animals ($P = 0.008$), and the IM HA and IM NA-vaccinated animals exhibited total viral shedding that was in between but not significantly different from either the IM mock- or IM HA + NA–vaccinated DRs (Fig. 2F). When differences in peak titers and total viral shedding were considered in the context of the transmission results, the 50% TE (i.e., 50% reduction in transmission) observed in the IM HA + NA–vaccinated DRs was associated with significant reductions in both peak titers and total viral shedding.

HA + NA immunity induced via IN vaccination reduced transmission by 50%

To evaluate whether immunity at the site of infection (i.e., the upper respiratory tract) could further reduce transmission, we challenged IN vaccinated animals with the 2009 H1N1 virus and similarly used these animals as DRs in transmission experiments. All IN mock-vaccinated DRs became infected and shed high viral titers. These animals transmitted the virus to four of four RCs (100% TE) (Fig. 3A). When IN HA-vaccinated DRs were challenged with the 2009 H1N1 virus, three of four DRs became infected and shed virus over several days. For the RCs, two of four animals shed infectious virus, while another RC did not shed virus but seroconverted (Fig. 3B and Table 1). Therefore, the IN HA-vaccinated DRs transmitted the virus to three of four RCs (75% TE). The IN HA-vaccinated DR that did not shed infectious virus did not transmit virus to its RC. For the IN NA-vaccinated DRs, all (four of four) animals became infected with the 2009 H1N1 virus, and these animals transmitted the virus to three of four RCs (75% TE) (Fig. 3C). The one IN NA-vaccinated DR that did not transmit to its RC shed infectious virus for 5 days (Fig. 3C). For the IN HA + NA–vaccinated DRs, all animals became infected (Fig. 3D). Two DRs had peak titers of $10^{1.5}$ $TCID_{50}$ (i.e., close to the limit of detection), while the remaining two DRs exhibited peak titers between 10^4 and 10^5 $TCID_{50}$. These latter DRs transmitted the virus to their paired RCs, and these RCs also seroconverted for a total of two of four RCs becoming infected (50% TE) (Fig. 3D and Table 1). When peak viral titers were compared between groups of IN vaccinated DRs, peak titers were not significantly different in animals vaccinated with HA or NA alone relative to mock-vaccinated animals (Fig. 3E); however, HA + NA–vaccinated DRs exhibited significant reductions in peak titers compared to mock-vaccinated animals ($P = 0.0077$). Analyses of total viral shedding showed that none of the IN vaccinated animals had significant reductions relative to the mock-vaccinated group. Our findings of reduced peak titers in the HA + NA–vaccinated animals are consistent with those for IM vaccination and indicate that vaccination against HA + NA via either route reduces peak titers, which reduces transmission to contacts by 50%.

Infection with reassortant viruses induced comparable antibody responses despite differences in viral replication

As vaccination by either the IM or IN routes reduced but did not abrogate transmission, we next sought to determine whether transmission could be further reduced by inducing immunity to HA and/or NA via infection. To selectively induce immunity via infection, we generated several recombinant viruses (Fig. 4A). These viruses included the recombinant wild-type 2009 H1N1 and A/Hong Kong/1/1968 (H3N2; 1968 H3N2) viruses and three H1N1-H3N2 reassortant viruses. The reassortant viruses contained six gene segments [i.e., segments I, II, III, V, VII, and VIII encoding polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), nucleoprotein (NP), matrix (M), and nonstructural (NS) proteins, respectively] from the 1968 H3N2 virus with different combinations of HA and NA gene segments (segments IV and VI, respectively). These viruses consisted of (i) rsH1N2 virus that contained the H1 from the 2009 H1N1 virus and the N2 from the 1968 H3N2 virus, (ii) rsH3N1 virus containing the H3 from the 1968 H3N2 virus and the N1 from the 2009 H1N1 virus, and (iii) rsH1N1 that contained the H1 and N1 from the 2009 H1N1 virus (Fig. 4A). To induce immunity, we infected ferrets ($n = 4$ per virus) with one of the reassortant viruses (rsH1N2, rsH3N1, and rsH1N1) or with the wild-type 2009 H1N1 or 1968 H3N2 viruses, the latter of which served as control groups. To verify the reassortant viruses replicated in ferrets, we collected nasal wash samples on day 1 postinfection and then every other day for 7 days. Nasal wash samples were then titrated on Madin-Darby canine kidney cells (MDCK cells) to evaluate viral titers (fig. S2, A to C). After 7 days of sampling, animals were allowed to recover for 84 days.

After inoculation with reassortant viruses, all animals became infected, and replicating virus was recovered in the nasal wash; however, the reassortant viruses exhibited different replication kinetics from the parental strains (fig. S2A). Animals infected with the 2009 H1N1 virus shed the highest titers on day 1, but after day 1 postinfection, animals infected with the 2009 H1N1, 1968 H3N2, and rsH3N1 viruses exhibited comparable shedding kinetics. Animals infected with the rsH1N2 and rsH1N1 viruses had reduced peak titers and shed less virus overall relative to the 2009 H1N1,

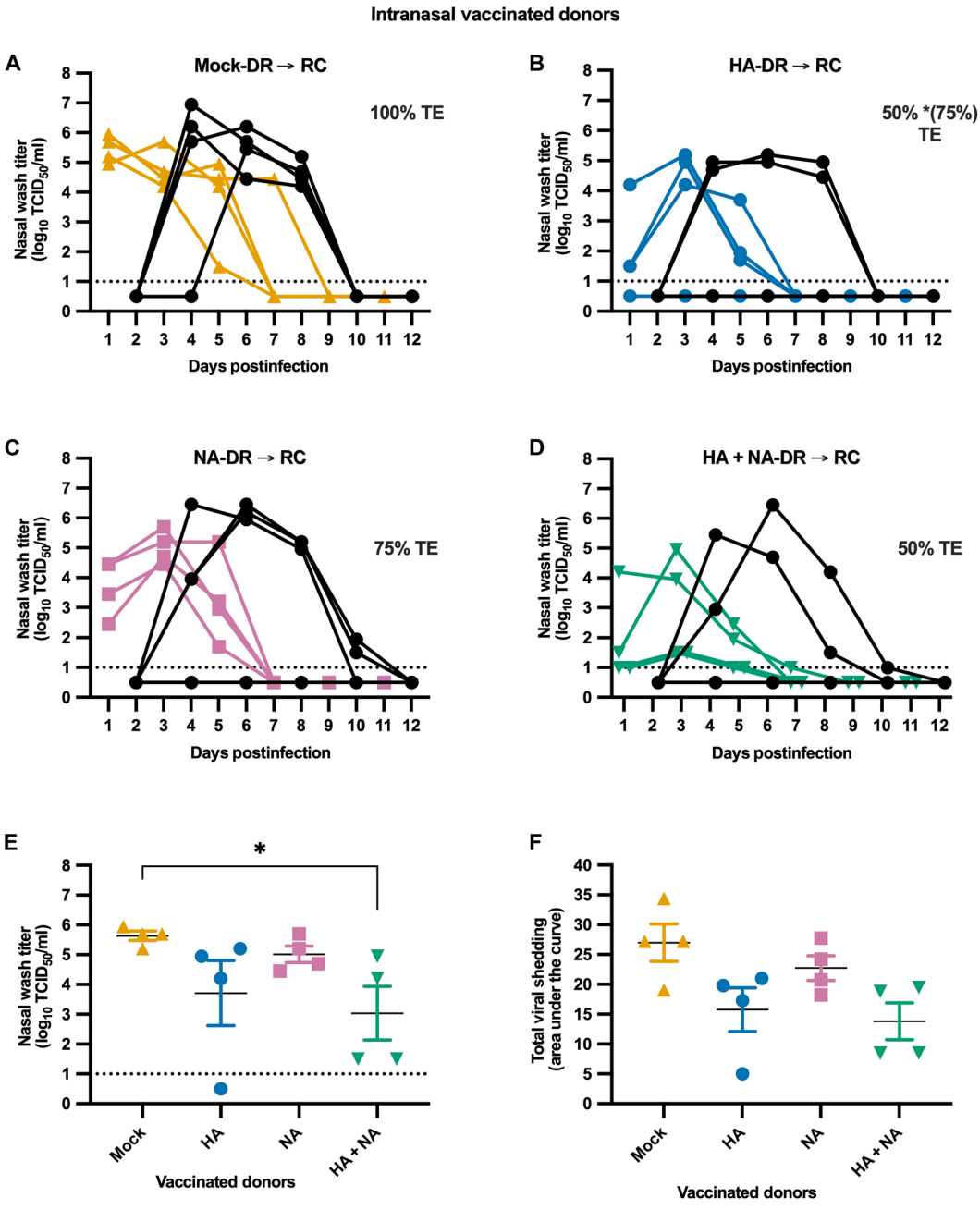

Fig. 3. Airborne transmission of the 2009 H1N1 virus from IN vaccinated preimmune DRs to immunologically naïve RC ferrets. IN mock-, HA-, NA-, and HA + NA–vaccinated ferrets ($n = 4$ per group) were IN inoculated with 1×10^4 $TCID_{50}$ of the 2009 H1N1 virus. Twenty-four hours later, each inoculated animal was introduced as the DR into a transmission cage with an immunologically naïve RC. Nasal washes were then collected every other day from each animal. (**A** to **D**) Viral shedding curves for IN mock-, HA-, NA-, or HA + NA–vaccinated DRs, respectively, and their paired RCs. DR shedding curves are shown in color, and RCs are shown with solid black lines. In (D), two DRs had superimposed shedding curves. These curves were interweaved to assist with visualizing the data. (**E**) and (**F**) Peak nasal wash titers and total viral shedding by AUC for IN vaccinated DRs, respectively. Orange, dark blue, pink, and green represent IN mock-, HA-, NA-, and HA + NA–vaccinated DRs, respectively. Nasal wash samples were titrated on MDCK cells, and results are expressed as $\log_{10}$ $TCID_{50}$/ml of nasal wash. Dashed horizontal line denotes limit of detection (1 $\log_{10}$ $TCID_{50}$/ml). *Significant differences were determined by Kruskal-Wallis test with two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli post hoc comparison ($P < 0.05$). For (E) and (F), horizontal line and error bars represent mean ± SEM, respectively, and unless significant differences are indicated, all other comparisons are not significant.

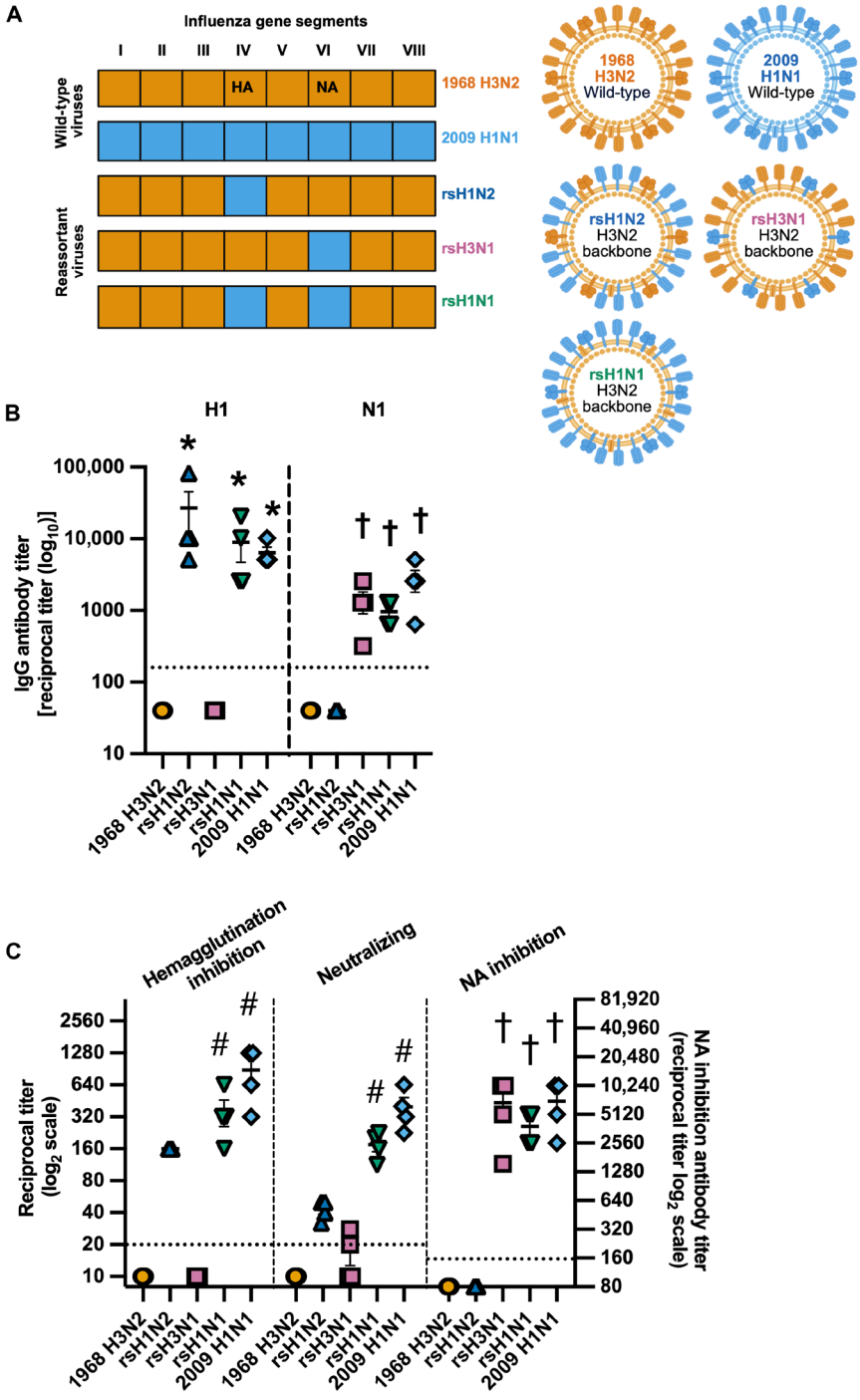

Fig. 4. Evaluation of antibody responses after primary infection with reassortant viruses to selectively induce immunity to HA, NA, or HA and NA. To induce immunity via infection, ferrets ($n = 4$ per group) were IN infected with 1×10^6 $TCID_{50}$ of reassortant or wild-type recombinant viruses. (**A**) Schematic showing the gene segment composition of reassortant viruses used to induce immunity to H1N1 HA and/or NA on the 1968 H3N2 virus backbone [created in BioRender; T.C.S. (2025); https://BioRender.com/hht98j9]. On day 82 post–primary infection with reassortant viruses, serum was collected from the ferrets, and the antibody response was evaluated. (**B**) displays binding IgG antibody titers against H1 HA and N1 NA, while (**C**) shows HAI titers, neutralization titers, and NA activity inhibiting titers. Binding antibody titers were determined by ELISA. HAI and neutralization titers were determined via HAI assay and microneutralization assays, respectively. NA inhibition titers were determined via ELLA. ELISA, HAI, and ELLA assays were performed in duplicate, and microneutralization assays were performed with four technical replicates. For all antibody analyses, individual values are displayed with horizontal line, and error bars showing mean ± SEM, respectively. Antibody responses in ferrets infected with rsH1N2, rsH3N1, and rsH1N1 are shown in dark blue, pink, and green, respectively. Antibody responses in ferrets infected with wild-type 1968 H3N2 and 2009 H1N1 viruses are shown in orange and light blue, respectively. Horizontal dotted black lines denote limit of detection. *Different from 1968 H3N2 and rsH3N1 DRs ($P < 0.02$). †Different from 1968 H3N2 and rsH1N2 DRs ($P < 0.042$). #Different from 1968 H3N2 and rsH3N1 DRs ($P < 0.05$), determined via Kruskal-Wallis test with post hoc two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. For (B) and (C), unless significant differences are shown, all other comparisons are not significant.

1968 H3N2, and rsH3N1 viruses (fig. S2, B and C). To determine whether differences in viral replication resulted in differences in the antibody response against HA or NA, we quantified levels of serum IgG against H1 and N1 by ELISA, and antibody functionality was further assessed using HAI assays, microneutralization assays, and ELLAs. Although viruses had different in vivo replication kinetics, on day 82 postinfection ferrets infected with viruses carrying the H1 HA: rsH1N2, rsH1N1, and 2009 H1N1 had significantly higher H1 HA IgG binding antibody titers compared to the 1968 H3N2– and rsH3N1-infected ferrets ($P < 0.02$) (Fig. 4B and fig. S2D). All ferrets infected with the rsH1N2, rsH1N1, and 2009 H1N1 viruses also had antibodies that exhibited HAI and neutralizing activity (Fig. 4C); however, only the rsH1N1- and 2009 H1N1–infected animals had significantly higher titers of HAI and neutralizing antibodies relative to the 1968 H3N2– and rsH3N1-infected animals ($P < 0.05$). The rsH1N2 animals had two- to eightfold lower titers of HAI and neutralizing antibodies relative to the rsH1N1- and 2009 H1N1–infected animals, and these titers were not significantly different from those of either the 1968 H3N2 and rsH3N1 or rsH1N1 and 2009 H1N1 groups (Fig. 4C). When the antibody response to NA was evaluated, animals infected with the rsH3N1 and rsH1N1 viruses developed titers of N1-binding and inhibitory antibodies that were similar to those of animals infected with the 2009 H1N1 virus (Fig. 4C and fig. S2, E and F). Moreover, both the N1-binding and inhibitory antibody titers were significantly higher in the rsH3N1-, rsH1N1-, and 2009 H1N1–infected animals compared to animals infected with the 1968 H3N2 and rsH1N2 viruses ($P < 0.042$). Collectively, our antibody analyses following primary infection of ferrets indicate that except for variations in HAI and neutralizing titers, these animals developed comparable antibody responses against the respective HA and NA antigens in the recombinant viruses.

Immunity induced by infection against HA and/or NA reduces transmission by up to 75%

To determine the effect of immunity against HA, NA, or HA and NA induced by prior infection on transmission, we challenged infection-induced preimmune ferrets with 10^4 $TCID_{50}$ of the 2009 H1N1 virus 84 days post–primary infection, and these animals were then used as DRs in airborne transmission studies to immunologically naïve RCs. DRs previously infected with the 1968 H3N2 virus (1968 H3N2–preimmune) served as a negative control, as these animals had no H1 or N1 immunity. All the 1968 H3N2–preimmune DRs challenged with the 2009 H1N1 virus became infected and shed infectious virus for 5 to 7 days (Fig. 5A and Table 1). These H3N2-preimmune DRs then transmitted the virus to four of four RC animals (100% TE) (Fig. 5A). Upon H1N1 challenge of the rsH1N2-preimmune DRs, all DR animals shed virus for 1 to 3 days (Fig. 5B), and three of four RCs became infected (75% TE). The one rsH1N2 DR that did not transmit to its RC shed low titers of infectious virus for 3 days (peak viral titer of $10^{1.7}$ $TCID_{50}$/ml). This ferret had the lowest peak titer among the rsH1N2-preimmune DRs. When rsH3N1-preimmune animals were challenged with the 2009 H1N1 virus, all ferrets became infected and shed infectious virus for 3 to 5 days. These rsH3N1-preimmune DRs then transmitted virus to two of four RCs (50% TE) (Fig. 5C). Upon H1N1 challenge of the rsH1N1-preimmune DRs, all animals became infected, but virus was only detected in each animal for a single day between days 1 to 5 postinfection (Fig. 5D). Viral titers in these animals ranged from 10^1 to $10^{4.2}$ $TCID_{50}$/ml, and the virus was transmitted to 1 RC animal (25% TE). The RC that became infected was paired with the rsH1N1-preimmune DR that had the highest peak titer ($10^{4.2}$ $TCID_{50}$/ml) among these DRs.

Last, we evaluated transmission when the 2009 H1N1–preimmune animals were used as DRs. In contrast to the other experimental groups, the 2009 H1N1–preimmune DRs did not shed virus upon challenge, and none of the exposed RC animals became infected (Fig. 5E) (0% TE). This demonstrates primary infection with the 2009 H1N1 virus provided complete protection from rechallenge with the homologous strain. Moreover, as the rsH1N2- and rsH3N1-preimmune DRs transmitted the virus to 75 and 50% of the RCs, respectively, while the rsH1N1-preimmune DRs transmitted the virus to 25% of RCs, these findings indicate that immunity to HA and NA confers additive reductions in transmission.

When we compared peak titers between groups, the 1968 H3N2– and rsH3N1-preimmune DRs had the highest peak titers, and the 2009 H1N1–preimmune DRs had significantly reduced titers relative to these groups ($P < 0.011$) (Fig. 5F). The rsH1N2- and rsH1N1-preimmune DRs had peak titers that were between but not significantly different from those of the 1968 H3N2– and rsH3N1-preimmune DRs and the 2009 H1N1–preimmune DRs. When total viral shedding was assessed in the preimmune DRs (Fig. 5G), the 1968 H3N2–preimmune animals shed the most virus, followed by rsH3N1-, rsH1N2-, rsH1N1-, and 2009 H1N1–preimmune animals. Both the rsH1N1 and 2009 H1N1 groups shed significantly less virus than the 1968 H3N2–preimmune group ($P < 0.007$), and the 2009 H1N1–preimmune DRs also shed significantly less virus than the rsH3N1-preimmune animals ($P < 0.01$). All other comparisons of total virus shedding were not significant (Fig. 5G). Collectively, the peak titer and AUC analyses show that immunity toward HA or NA alone induced via infection did not significantly decrease peak titers or total virus shedding; however, reductions in transmission were observed with rsH1N2- and rsH3N1-preimmune DRs transmitting to 75 of 50% of RCs, respectively. When animals had immunity to HA and NA, there were significant reductions in total virus shedding, and this resulted in additive reductions in transmission such that 25% of RCs paired with rsH1N1-preimmune DRs became infected.

Transmission from preimmune DRs to their contacts was not associated with the emergence of escape variants

To determine whether escape variants were selected during transmission of the 2009 H1N1 virus from preimmune DRs to their contacts, we performed deep sequencing on all nasal wash samples from DRs and RCs with a titer $>10^2$ $TCID_{50}$/ml. In parallel, we also sequenced the 2009 H1N1 virus stock used to infect the ferrets. Using whole-genome sequencing, all major (≥50%) and minor variants (5.0 to 49.99%) across all eight segments of the viral genome were identified.

Among the IM vaccinated DRs and their respective RCs, nonsynonymous major variants were identified in PB1 [Val^{609}→Ile (V609I)], PA [Ala^{598}→Thr (A598T)], HA [Glu^{171}→Lys (E171K), Gly^{172}→Glu (G172E), and Glu^{391}→Lys (E391K)], and NS1 [Glu^{196}→Lys (E196K)] (table S1). In PB1, the V609I substitution was identified in an RC paired with an HA-vaccinated DR, while PA A598T and NS1 E196K were identified in two mock-vaccinated DRs and an RC paired with an NA-vaccinated DR, respectively. The HA E391K substitution was similarly detected in a single RC that was paired with an HA + NA–vaccinated DR. The only nonsynonymous major variant (≥50%) that was detected in both DR and RCs was HA E171K. HA E171K was identified first as a minor variant that increased over time to become a major variant in four ferrets, including both DRs and RCs (Fig. 6A).

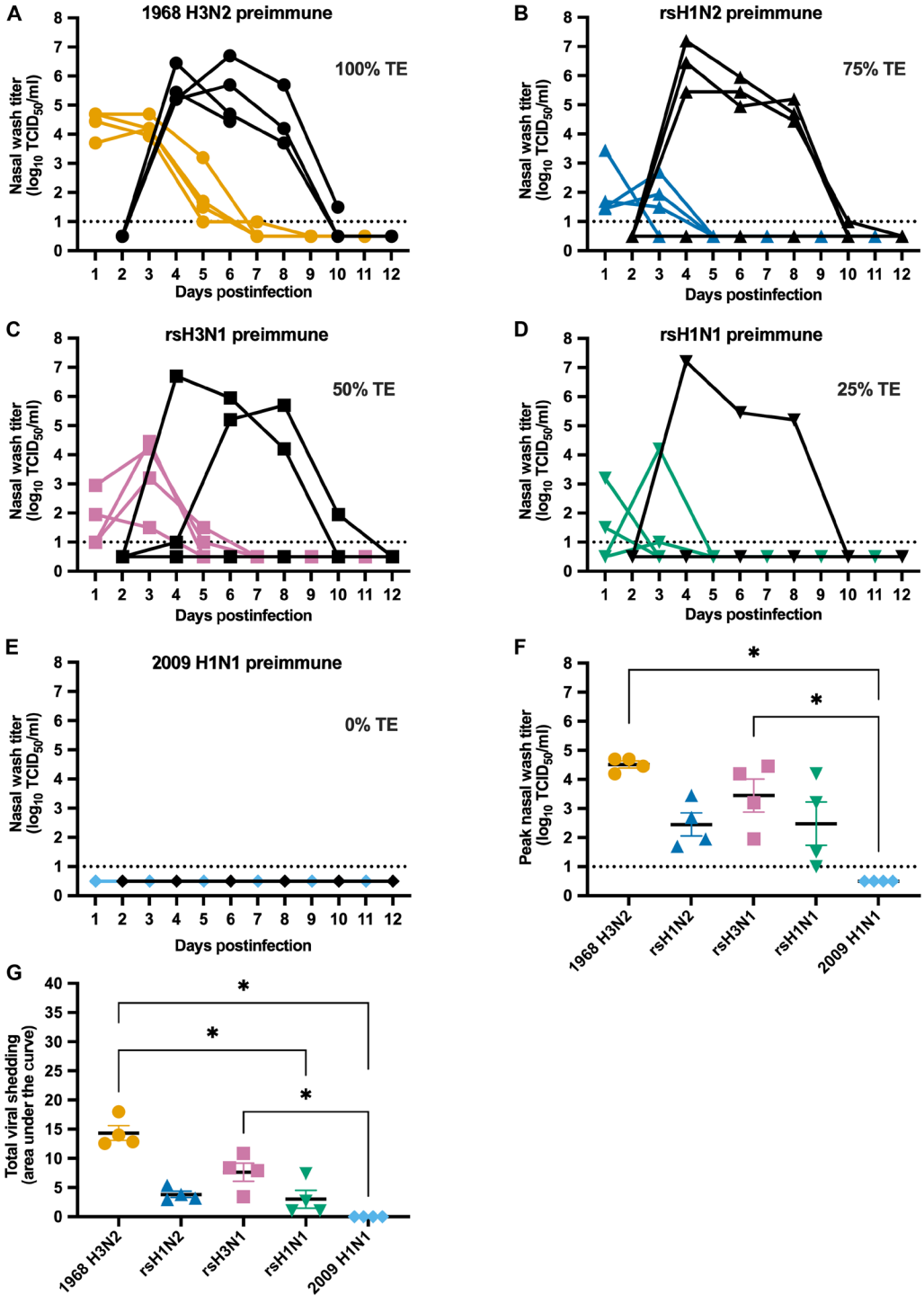

Fig. 5. Airborne transmission of the 2009 H1N1 virus from infection-induced preimmune DRs to immunologically naïve RC ferrets. 1968 H3N2–, rsH1N2-, rsH3N1-, rsH1N1-, and 2009 H1N1–preimmune ferrets were challenged with 1×10^4 $TCID_{50}$ of the 2009 H1N1 virus and used as DRs to immunologically naïve RCs in airborne transmission studies. (**A** to **E**) Viral shedding curves for 1968 H3N2–, rsH1N2-, rsH3N1-, rsH1N1-, and 2009 H1N1–preimmune DRs, respectively, and their paired RCs. Viral titers from DR animals are shown in solid-colored lines, and RC shedding curves are shown in black. 1968 H3N2-, rsH1N2-, rsH3N1-, rsH1N1-, and 2009 H1N1-preimmune DRs are displayed in orange, dark blue, pink, green, and light blue, respectively. Results are expressed as $\log_{10}$ $TCID_{50}$/ml of nasal wash. Dashed horizontal line denotes limit of detection. Analyses of viral titers were performed on (**F**) peak nasal wash titers and (**G**) total viral shedding (AUC). *Significantly different from indicated group ($P < 0.011$). Significant differences were determined by Kruskal-Wallis test with post hoc two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli for antibody analysis ($P < 0.05$). For (F) and (G), horizontal line and error bars represent mean ± SEM, respectively, and unless significant differences are indicated, all other comparisons are not significant. Two RC ferrets paired with 1968 H3N2 DRs developed a secondary infection after becoming infected and were removed from the study. Thus, data from one RC are displayed until day 6, while data from the second RC are displayed until day 10.

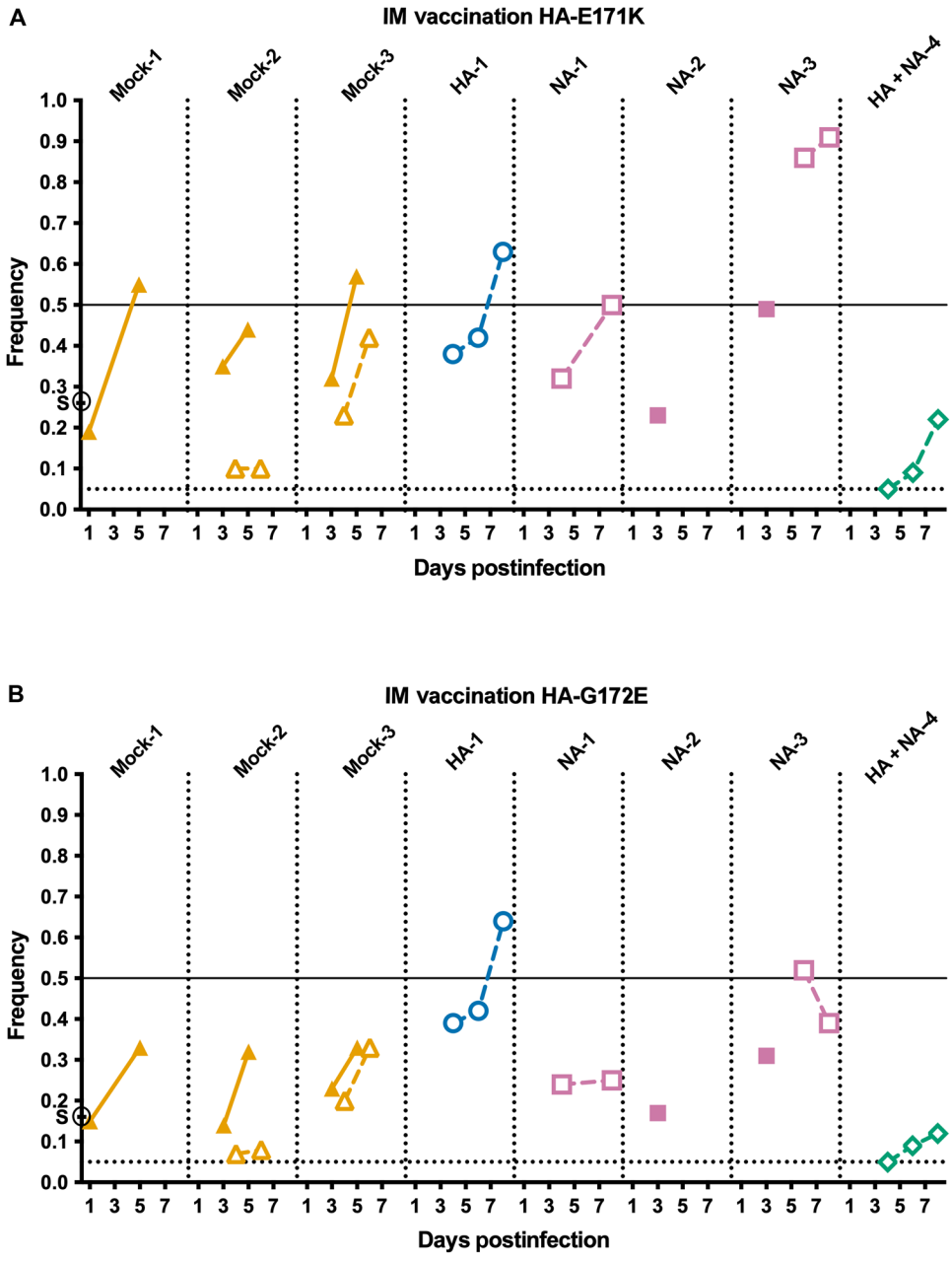

Fig. 6. Substitutions HA E171K and HA G172E identified during transmission of the 2009 H1N1 virus from IM vaccinated preimmune DRs to immunologically naïve RC ferrets. The relative frequency (*y* axis) of (**A**) HA E171K and (**B**) HA G172E present at ≥5% in at least one ferret nasal wash sample collected from transmission experiments with IM vaccinated ferrets. For all plots, data are grouped by vaccination status of the DR (across the top). The *x* axis represents days postinfection of the DR ferret. Variant frequencies for individual DR ferrets are shown with solid symbols with solid connecting lines, while individual RC ferrets are shown in open symbols with dashed connecting lines. Frequencies for mock-, HA-, NA-, and HA + NA–vaccinated DRs with their paired RC are shown in orange, dark blue, pink, and green, respectively. Dashed line indicates 0.05 frequency cutoff for sequence analysis. Solid line at 0.5 indicates 50% frequency above which nonsynonymous mutations are considered substitutions. Black circle and "S" on *y* axis denote frequency of the minor variant in the virus stock.

HA E171K was also detected only as a major variant in a single RC paired with an NA-vaccinated DR. This mutation was also present in six additional ferrets and the virus stock as a minor variant (Fig. 6A). HA G172E was identified as a major variant in one RC paired with an HA-vaccinated DR and a second RC paired with an NA-vaccinated DR. This mutation was also present as a minor variant in the virus stock and nine other ferrets including both DRs and RCs. All ferrets in which the HA E171K mutation was detected also had the HA G172E mutation (Fig. 6B). Six other nonsynonymous mutations were identified as minor variants in HA: Ile^{4}→Thr (I4T), Lys^{147}→Thr (K147T), Asn^{211}→Asp (N211D), Asp^{239}→Asn (D239N), Lys^{300}→Glu (K300E), and Lys^{521}→Arg (K521R) (table S1). All these nonsynonymous minor variants were identified in RCs except for HA N211D, which was identified in a single mock DR and an RC paired with an NA-vaccinated

DR. None of these variants were associated with transmission from DRs vaccinated against a specific antigen. No major or minor nonsynonymous variants were identified in the NA gene segment. Moreover, no substitutions were consistently identified and/or transmitted between IM vaccinated DRs with immunity to a specific viral antigen (i.e., HA, NA, or HA + NA), indicating that IM vaccination did not drive the emergence of escape variants in our experimental system.

Using the same approach, we sequenced nasal washes from IN vaccinated DRs and their RCs. Nonsynonymous major variants were identified in PB2 Asp^{253}→Asn (D253N), PA Arg^{262}→Met (R262M), HA Ser^{138}→Ile (S138I), E171K, G172E, and Lys^{177}→Asn (K177N), and NS1 Ser^{74}→Asn (S74N) (table S2). The PB2 D253N and PA R262M mutations were identified in individual RC animals paired with mock- and HA-vaccinated DRs, respectively. Similarly, the HA K177N and NS1 S74N mutations were present in two separate RCs paired with NA-vaccinated DRs, while the HA S138I mutation was present in a single RC paired with a mock-vaccinated DR. Consistent with our findings in IM vaccinated animals, HA E171K and G172E were identified as major variants in 13 and 9 ferrets, respectively, including both DRs with and without HA immunity and RC animals. Moreover, HA E171K was identified in an additional eight ferrets as a minor variant, and HA G172E was also detected as a minor variant in an additional 10 animals. When HA G172E was identified as a major or minor variant, it was always associated with HA E171K (Fig. 7, A and B).

Seven other nonsynonymous minor variants in HA consisting of Asn^{173}→Lys (N173K), Asn^{211}→Asp (N211D), Asp^{239}→Asn (D239N), Glu^{241}→Lys (E241K), Val^{267}→Ala (V267A), Arg^{450}→Lys (R450K), and Asp^{502}→Asn (D502N) (table S2) were identified in DRs or RCs in the transmission experiments with IN vaccinated DRs. HA D239N and HA E241K were detected in a single mock-vaccinated DR on day 7 postinfection, while the remaining minor variants were detected in individual DRs or RCs. As observed with IM vaccination, in the IN vaccinated DRs and their RCs, no nonsynonymous major or minor variants were identified in the NA gene segment. Moreover, no nonsynonymous major variants (substitutions) were consistently transmitted from DRs to RCs, and no variants were consistently identified in animals with immunity toward a specific viral antigen. Collectively, these findings indicate that IN vaccination did not result in the generation of escape variants.

When we sequenced nasal wash samples from DR and RCs in the transmission studies with infection-induced preimmune DRs, nonsynonymous major variants were identified in PB1 (V609I) and HA (E171K, G172E, and D239G) (table S3). PB1 V609I was identified in one RC paired with a 1968 H3N2–preimmune DR and a second RC paired with an rsH1N2-preimmune DR. HA E171K was identified as a nonsynonymous major variant in five animals, while HA G172E was found in three animals (Fig. 8, A and B). Both HA E171K and G172E were also identified as minor variants in 12 additional animals. HA E171K and G172E were identified in both DR and RC animals, and these variants were found across all the preimmune DR groups except for the 2009 H1N1–preimmune DRs, which did not shed virus. As observed for both IM and IN preimmune transmission studies, HA G172E was always associated with HA E171K. The HA D239G substitution was identified as a major variant in an RC paired with an rsH1N1-preimmune DR, and this change was also identified as a minor variant in a separate rsH1N1-preimmune DR.

Six other nonsynonymous minor variants were also identified in HA in at least one animal including Thr^{42}→Pro (T42P), Met^{274}→Thr (M274T), Met^{274}→Leu (M274L), Thr^{295}→Ala (T295A), Met^{433}→Leu (M433L), and Ile^{517}→Val (I517V) (table S3). These were unique to animals in transmission studies using DRs with preexisting immunity induced via infection. Consistent with our studies using IM or IN vaccination, we did not observe any nonsynonymous major variants that were consistently transmitted from DRs to RCs or that were associated with transmission from DRs with immunity to specific viral antigens, suggesting that immunity induced via infection did not drive the emergence of viral variants. In summary, throughout the transmission studies using vaccination or infection-induced preimmune DRs, no nonsynonymous major variants were consistently linked with transmission or immune status. However, the HA E171K and HA G172E substitutions were commonly found among all groups of ferrets and were present together in many animals across multiple sampling days.

Reductions in viral titer below $10^{1.5}$ $TCID_{50}$/ml of nasal wash are linked to a 50% reduction in the probability of transmission

As multiple modalities of immunity were evaluated in the context of transmission with consistent metrics of viral shedding, peak titers, and TE, we performed a comprehensive analysis using the aggregate dataset to determine whether reductions in viral titer were predictive of reductions in transmission. DR ferrets ($n = 50$) were categorized by immunity status and type of immunity: no immunity ($n = 11$ and 22%), immunity via vaccination ($n = 23$ and 46%), and immunity via prior infection ($n = 16$ and 32%). Among the 50 DRs, 32 (64%) transmitted the 2009 H1N1 virus to contacts, while 18 (36%) did not. Mean viral titers differed significantly by immunity status or type on days 1 ($P < 0.001$), 3 ($P < 0.0001$), and 5 ($P < 0.0001$), with no significant difference on days 7, 9, and 11 (Fig. 9A). On day 1, ferrets with no immunity had significantly higher mean viral titers than ferrets with immunity induced via vaccination or prior infection; however, viral titers were not significantly different between these later groups. On day 3, highest mean viral titer levels were seen among ferrets with no immunity, followed by those with immunity via vaccination, and then those with immunity via infection, and all groups differed significantly from each other. The same pattern was repeated on day 5 postinfection (Fig. 9A).

We then performed logistical regression and generated receiving operator characteristic (ROC) curves to define the relationship between reductions in viral load and transmission. This analysis showed that viral load on day 1 was significantly ($P = 0.0035$) associated with transmission, such that for each 1-log decrease (i.e., unit decrease) in mean titer on day 1, there was a 66% (95% confidence interval: 29 and 83%) decrease in the odds of transmission. The area under the ROC curve (AURC) value was 0.82, which suggests that viral load on day 1 is a useful predictor of whether the infected ferret will go on to transmit the virus. Viral load on day 3 was also significantly ($P = 0.0001$) associated with transmission. For each 1-log decrease in mean titer level on day 3, there was a 60% (95% confidence interval: 36 and 75%) decrease in the odds of transmission. The AURC value was 0.85, again demonstrating that viral load on day 3 was a strong predictor of transmission. Using the odds ratio conversion to probability of transmission, we plotted viral titer against probability of transmission, which demonstrated that viral titers below $10^{1.5}$ $TCID_{50}$/ml on day 1 and below 10^2 $TCID_{50}$/ml on day 3 were linked to less than 50% probability of transmission (Fig. 9B). Stated differently, when DR ferrets shed titers below these threshold values, the probability of not

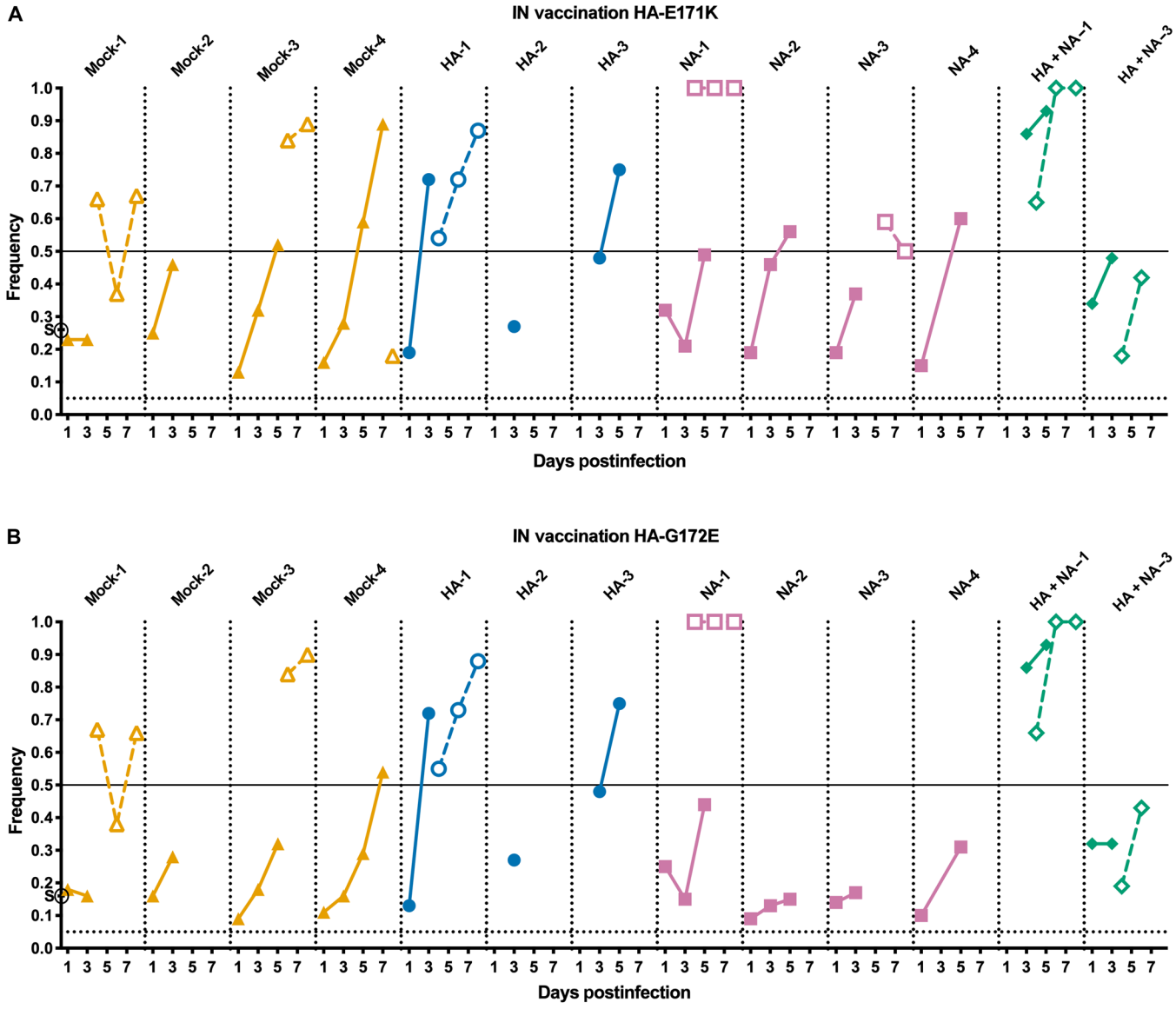

Fig. 7. Substitutions HA E171K and HA G172E identified during transmission of the 2009 H1N1 virus from IN vaccinated preimmune DRs to immunologically naïve RC ferrets. The relative frequency (*y* axis) of (**A**) HA E171K and (**B**) HA G172E present at ≥5% in at least one ferret nasal wash sample collected from transmission experiments with IN vaccinated ferrets. For all plots, data are grouped by vaccination status of the DR (across the top). The *x* axis represents days postinfection of the DR ferret. Variant frequencies for individual DR ferrets are shown in solid symbols with solid connecting lines, while individual RC ferrets are shown in open symbols with dashed connecting lines. Frequencies for mock-, HA-, NA-, and HA + NA–vaccinated DRs with their paired RC are shown in orange, dark blue, pink, and green, respectively. Dashed line indicates 0.05 frequency cutoff for sequence analysis. Solid line at 0.5 indicates 50% frequency above which nonsynonymous mutations are considered substitutions. Black circle and S on *y* axis denote frequency of the minor variant in the virus stock.

transmitting was greater than that of transmitting the virus, while at titers above these values, the probability of transmission was greater than the probability of not transmitting.

DISCUSSION

As airborne transmission of influenza viruses is a major driver of influenza epidemics, it is crucial to understand how vaccination and/or infection-induced immunity affects viral transmission. In this study, we evaluated whether HA and/or NA immunity induced via vaccination or infection could reduce airborne transmission of the 2009 H1N1 virus in ferrets. We used IM vaccination, IN vaccination, and infection-based approaches to induce immunity toward HA, NA, or a combination of HA and NA. We then challenged the preimmune ferrets with the 2009 H1N1 virus and used them as DRs in airborne transmission studies to immunologically naïve RCs. Most investigators perform transmission studies using a virus inoculation dose of 10^6 infectious units in the DR animals (*29*). As this virus dose may overcome vaccine-induced immunity, especially for immunity toward NA, which cannot prevent infection, we infected DRs with a 100-fold lower dose of 10^4 infectious units. We reasoned that this lower dose would permit the evaluation of smaller differences in immunity on viral transmission.

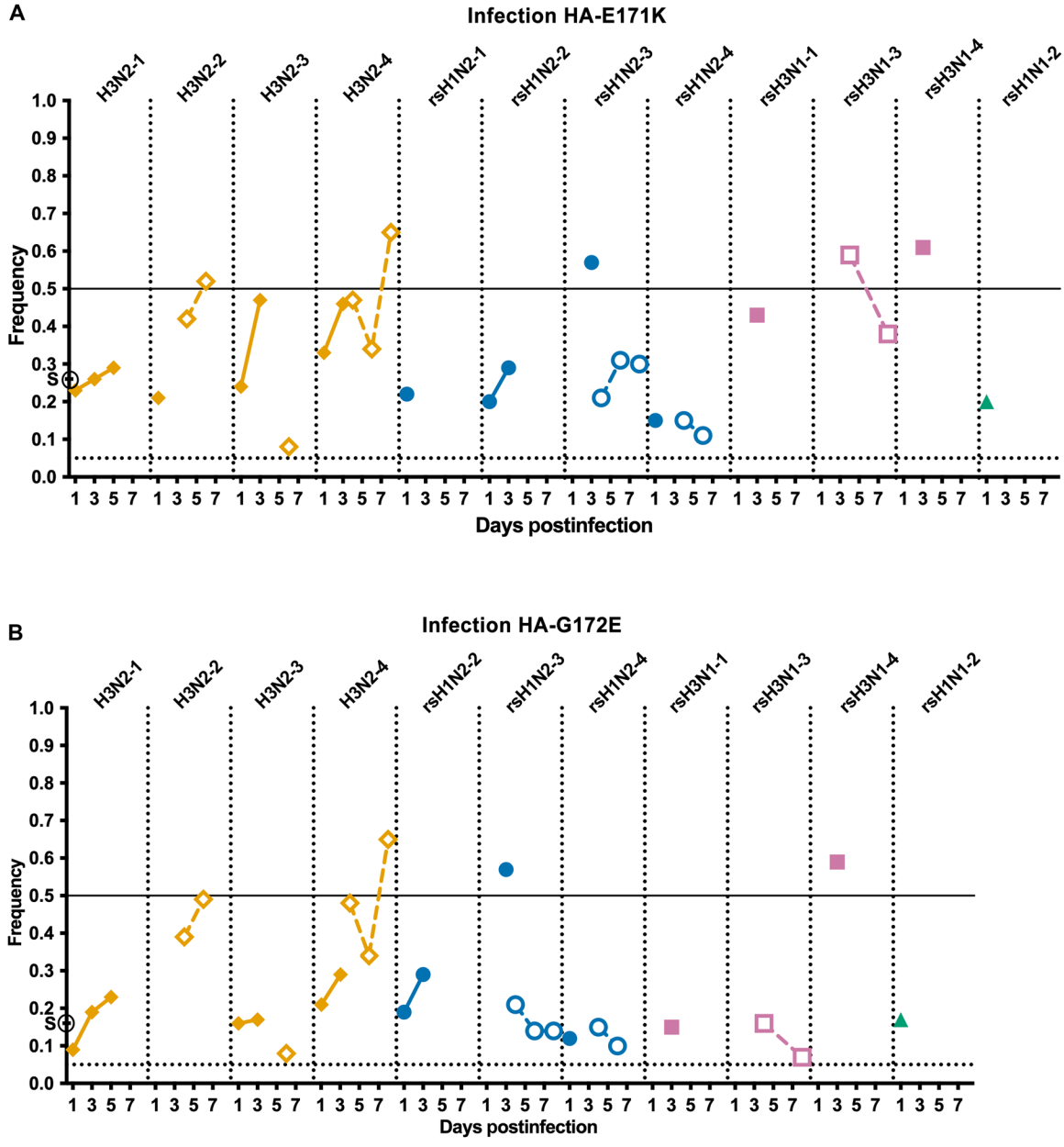

Fig. 8. Substitutions HA E171K and HA G172E identified during transmission of the 2009 H1N1 virus from infection-induced preimmune DRs to immunologically naïve RC ferrets. The relative frequency (*y* axis) of (**A**) HA E171K and (**B**) HA G172E present at ≥5% in at least one ferret nasal wash sample collected from transmission experiments with infection-induced preimmune DR ferrets. For all plots, data are grouped by immune status of the DR (across the top). The *x* axis represents days postinfection of the DR ferret. Variant frequencies for individual DR ferrets shown in solid symbols with solid connecting lines, while individual RC ferrets are shown in open symbols with dashed connecting lines. Frequencies for 1968 H3N2, rsH1N2, rsH3N1, and rsH1N1 preimmune DRs with their paired RCs are shown in orange, dark blue, pink, and green, respectively. No frequencies for H1N1-preimmune DRs and their RCs are reported, as infectious virus was not recovered from these animals. Dashed line indicates 0.05 frequency cutoff for sequence analysis. Solid line at 0.5 indicates 50% frequency above which nonsynonymous mutations are considered substitutions. Black circle and S on *y* axis denote frequency of the minor variant in the virus stock.

In transmission studies with IM vaccinated DRs, mock-vaccinated animals transmitted the virus to 100% of RCs. IM HA-vaccinated DRs transmitted the virus to 75% of RCs, while there was no reduction in transmission from IM NA-vaccinated DRs to contacts. IM HA+ NA– vaccinated DRs transmitted the virus to 50% of RCs, and these DRs also had significant reductions in peak viral titers and total viral shedding compared to mock-vaccinated DRs. When we evaluated transmission using IN vaccinated DRs, again, mock-vaccinated DRs transmitted the virus to 100% of RCs. The IN HA- and IN NA-vaccinated DRs transmitted the virus to 75% of their RCs, and peak titers and total viral shedding were not significantly reduced relative to mock-vaccinated animals. When IN HA + NA–vaccinated animals were used as DRs, 50% of the RCs became infected, and this was associated significantly with reductions in peak viral titers in HA + NA–vaccinated DRs.

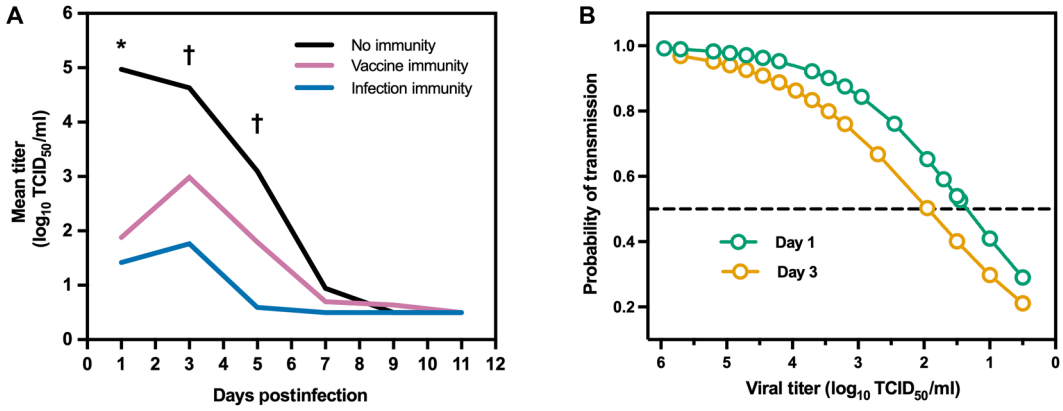

Fig. 9. Analysis of the relationship between reductions in viral titer in DR ferrets and airborne transmission to RCs. Using DR viral titer data and the TE across all experiments, we performed an aggregate analysis to assess the relationship between immune status, viral titer, and transmission. All viral titer data from DR ferrets were grouped categorically into immunity via infection, immunity via vaccination, or no immunity. Viral titers between these groups were then compared via one-way analysis of variance (ANOVA) with Tukey's post hoc test. Subsequently, logistical regression was performed, and receiving operator characteristic (ROC) curves were generated to define the relationship between reduction in viral load and transmission. (**A**) Viral titers plotted by immune status on each day postinfection. (**B**) Logistical regression curves defining the relationship between reductions in viral load on days 1 and 3, and probability of transmission to contact animals. *Significantly different from immunity via infection ($P < 0.001$). †All groups significantly different from each other ($P < 0.0001$).

Because neither IM nor IN vaccination could completely prevent transmission, we next sought to evaluate whether transmission could be further reduced when immunity was induced via infection. We infected animals with wild-type or reassortant H1N1-H3N2 viruses and allowed these animals to recover for 84 days. As with our vaccination studies, we then challenged these animals with the 2009 H1N1 virus and used them as DRs in transmission studies. H3N2-preimmune DRs became infected and transmitted the virus to 100% of their RCs, confirming that H3N2 immunity had no impact on transmission of the 2009 H1N1 virus. rsH1N2- and rsH3N1-preimmune DRs transmitted the virus to 75 and 50% of their contacts, respectively. The rsH1N1-preimmune DRs displayed further reductions in transmission with only 25% of their RCs becoming infected. When the 2009 H1N1–preimmune DRs were challenged with the 2009 H1N1 virus, no animals shed virus, and no RCs became infected. This indicates that prior infection with the 2009 H1N1 virus provided sterilizing immunity upon rechallenge, and our findings are consistent with those of other research groups, which have demonstrated prior infection protects against reinfection with a homologous virus in ferrets (*30*, *31*). When we assessed peak titers and total viral shedding, relative to the 1968 H3N2–preimmune DRs, the 2009 H1N1–preimmune animals had significant reductions in peak titers and viral shedding, while the rsH1N1-preimmune DRs only had significant reductions in total viral shedding. All other groups did not have significant decreases in peak titers or total virus shedding. These findings indicate again that combined immunity to HA and NA imparts the largest reductions in viral replication, which reduces transmission to contacts. Collectively, the findings with infection-induced preimmune animals are consistent with those of the IM and IN vaccination studies, as both the rsH1N1 and 2009 H1N1–preimmune DRs had the greatest reductions in transmission. Moreover, we consistently observed that for each strategy used to induce immunity, animals with immunity to HA and NA had reductions in transmission that reflected the sum of reductions in transmission for animals with immunity to HA and NA alone. As these reductions did not exceed the sum of reductions in transmission for animals with immunity to HA and NA alone, reductions in transmission were additive rather than synergistic.

To verify that differences in transmission were not the result of vaccine or immune escape variants, we performed whole-genome sequencing of viruses in the ferret nasal wash samples. This analysis revealed that no viral variants were linked with immune status among IM, IN, or infection-induced preimmune DRs and their RCs, indicating that preexisting immunity did not drive viral evolution in our studies. However, two nonsynonymous major variants or substitutions in the HA were consistently identified in DR and RC animals: HA E171K and HA G172E. Each of these mutations is in the Sa antigenic site and has previously been associated with tissue culture adaptation or immune escape, respectively (*32*–*36*). Upon reviewing the sequence of the HA reverse genetic plasmid used to generate the recombinant A/California/07/2009 (H1N1pdm09) virus, we found that our plasmid encodes HA 171K and 172G (154K and 155G in H3 numbering, respectively) (*37*). As HA 171E and 171K were present in the virus stock at variant frequencies of 0.74 and 0.26, respectively, it appears that during virus rescue and culture in MDCK cells, HA 171E was selected as a major variant, and HA 171K was retained as a minor variant. The HA G172E mutation was present in the virus stock at a variant frequency of 0.16, and thus, it appears to have been generated as minor variant during virus rescue (Figs. 6 to 8). In studies with live attenuated 2009 H1N1 vaccines, the HA 171E mutation has been shown to enhance viral replication in MDCK cells (*32*). The G172E mutation has also been linked to immune escape and shown to decrease the reactivity of postinfection ferret antiserum against the 2009 H1N1 virus (*33*, *34*); however, in our studies this substitution was not present at higher frequencies in animals with preexisting immunity to HA (i.e., rsH1N1, rsH1N2, IM and IN vaccinated HA, or HA + NA groups).

While our studies sought to specifically evaluate the individual and combined effect of immunity against HA and/or NA via vaccination or infection on airborne transmission, prior studies have evaluated whether preexisting immunity induced with recombinant vaccines or IIVs can prevent transmission in ferrets. When ferrets were given an adjuvanted IM vaccine consisting of recombinant 2009 H1N1 HA, all vaccinated animals became infected upon homologous virus challenge and transmitted the virus to immunologically naïve direct

contacts (*38*). Similarly, when ferrets were given an IM seasonal IIV and challenged with a homologous H3N2 virus, these animals transmitted the virus to 44% (four of nine) of immunologically naive RCs, suggesting that HA immunity alone can reduce but not block airborne transmission (*30*). These studies indicate that HA immunity plays a role in reducing viral replication and airborne transmission of influenza in ferrets, and this is consistent with the well-established role of anti-HA antibodies in reducing viral replication and disease. However, our studies build upon these studies and show HA and/or NA immunity alone is not sufficient to completely prevent airborne transmission in ferrets.

The incorporation of NA into influenza vaccines has become a major area of research, as NA is less prone to antigenic drift than HA, and immunity to NA has been shown to reduce viral shedding (*15*, *39*). Several studies have examined the role of NA immunity in the transmission of influenza B viruses in the guinea pig model. In these studies, guinea pigs given adjuvanted IM vaccines containing recombinant NA shed significantly less virus upon challenge with a homologous virus and transmitted the virus to 50% of naïve RCs. When NA immunity was induced via IN vaccination, vaccinated guinea pigs shed less virus, and respiratory transmission was completely blocked, indicating that a site-specific immune response to NA was sufficient to prevent transmission of influenza B viruses (*40*).

We previously evaluated the ability of IM vaccination against NA to disrupt a chain of airborne transmission in ferrets (*20*). In these studies, we used a sequential transmission model in which a naïve DR was infected with the 2009 H1N1 virus and paired with a primary RC ferret that was either mock or NA vaccinated. The primary RC animal was then sampled daily, and once confirmed to be infected, the primary RC ferret was used as the DR to an immunologically naïve RC. We found that IM NA vaccination did not prevent infection of the primary RC or subsequent transmission to a secondary naïve contact. Our current studies used the same adjuvanted IM vaccine formulation and regimen and yielded similar results with all IM NA-vaccinated animals becoming infected and transmitting the virus to their RCs. In addition, when we used an IN vaccination approach, NA-vaccinated ferrets all became infected and transmitted the virus to 75% of their contacts. Thus, the findings in guinea pigs and ferrets with influenza B and A viruses, respectively, yield contrasting results about the role of NA immunity in transmission. This may be due to inherent differences in the transmissibility of influenza A and B viruses or may be due to the enhanced breadth of the antibody response in guinea pigs compared to ferrets (*41*).

In the context of infection-induced immunity, our findings are consistent with prior studies that found reinfection of ferrets with the 2009 H1N1 virus 8 weeks after primary infection completely prevented transmission to cohoused ferrets (i.e., direct contact transmission) (*31*). The role of heterosubtypic immunity induced via infection on airborne transmission in ferrets has also previously been evaluated using the 2009 H1N1 virus and a seasonal H3N2 strain (sH3N2) [A/Perth/16/2009 (H3N2)] (*42*). In these studies, when transmission of the 2009 H1N1 virus was evaluated using DRs and RCs with preexisting immunity to the sH3N2 virus, two of three contacts became infected; however, when transmission of the sH3N2 virus was evaluated using DRs and RCs with immunity to the 2009 H1N1 virus, transmission was completely blocked (*42*). Our findings with the H3N2-preimmune DRs in which the 2009 H1N1 virus transmitted from these animals to 100% of their contacts are consistent with these studies. However, future studies are warranted to determine whether immunity toward the 2009 H1N1 virus induced by infection or vaccination has a similar impact on transmission of an H3N2 virus.

After completing our experimental studies, using our aggregate dataset encompassing 50 DR-RC pairs, we analyzed the relationship between reductions in viral titer and transmission. We found that reductions in viral titer were significantly associated with reductions in transmission on days 1, 3, and 5 postinfection ($P \leq 0.001$). This finding is expected; however, given the size of the dataset, we were able to extend this analysis to identify cut points or threshold titers below which the probability of transmission is less than 50% on days 1 and 3 postinfection ($<10^{1.5}$ or 10^{2} $TCID_{50}$ on days 1 and 3 postinfection, respectively). As stated in the results section, this analysis indicates that at titers below this threshold, the likelihood of not transmitting the virus is greater than the likelihood of transmission. Our analyses also showed that for every 1-log decrease in viral titer on day 1 or 3 postinfection, there were 66 and 60% reductions, respectively, in the odds of transmission. These thresholds have significant utility for preclinical development of transmission reducing vaccines, as they can be used as inclusion or exclusion criteria for further advancement of vaccine candidates. Moreover, our analysis indicates that reducing viral titers to low levels is sufficient, and sterilizing immunity is not required to prevent transmission.

While informative of how immunity to HA and/or NA can disrupt transmission of the 2009 H1N1 virus, our studies have several limitations. For both IM and IN vaccination and infection-induced immunity, due to a lack of ferret-specific reagents, our immune analyses focused on the serum IgG antibody response. Thus, we could not assess cellular or mucosal immunity in the ferrets. For both IM and IN vaccination against HA or NA, we vaccinated with a total of 50 μg of antigen, and when we vaccinated against HA + NA, the total amount of vaccine antigen was 100 μg per dose (50 μg of HA plus 50 μg of NA). It is possible that this higher antigen dose may have altered the immune response to the vaccine antigens; however, the antibody responses to HA or NA in animals vaccinated against each antigen alone did not differ significantly from those vaccinated against HA + NA (Fig. 1). In our transmission studies, we evaluated airborne transmission, and the DR and RC animals were paired for 14 days. This approach does not evaluate the contribution of direct contact transmission, and the prolonged exposure may not reflect transmission in humans. This is the standard approach in the field (*29*), and there is no consensus on the duration of exposure that would accurately represent human-to-human transmission.

Extensive research is currently underway to develop vaccines that induce broadly cross-reactive antibodies to HA and NA that can limit disease caused by seasonal and emerging influenza viruses (*43*). The addition of NA to IIVs has also been proposed as a strategy to enhance the efficacy of currently licensed vaccines (*15*, *44*, *45*). Our studies indicate that targeting HA and NA will also reduce transmission; however, additional strategies will have to be used to more closely recapitulate infection-induced immunity to maximize these reductions. These strategies could include the incorporation of adjuvants to enhance immunogenicity and/or cellular immunity, the incorporation of additional viral antigens to target other stages of the viral life cycle, and/or targeting the nasal mucosa to protect the site of infection.

Collectively, we demonstrate immunity toward HA or NA alone induced by vaccination or prior infection conferred variable reductions in transmission, while immunity to HA and NA combined consistently conferred the greatest reductions in transmission. Moreover,

using multiple approaches to induce immunity, we show that immunity to HA and NA results in additive rather than synergistic reductions in transmission. Throughout our studies, no escape variants readily emerged, and by performing logistical regression, we identified viral titer thresholds that can be used to facilitate the development of preclinical transmission reducing vaccines. These findings further emphasize the importance of developing vaccines that target both HA and NA, as this approach will likely reduce the severity of influenza virus infection and limit onward transmission.

MATERIALS AND METHODS

Cells and viruses

MDCK cells, London Line, FR-58, were obtained through the International Reagent Resource, Influenza Division, World Health Organization Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA. MDCK cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 mM Hepes buffer, and antibiotics and antimycotics. Human embryonic kidney 293 cells (American Type Culture Collection) were grown in Opti-MEM with 10% FBS plus antibiotics and antimycotics (Life Technologies) at 37°C in 5% CO_2. Recombinant influenza viruses A/Hong Kong/1/1968 (H3N2), rsH1N2, rsH3N1, rsH1N1, and A/California/07/2009 (H1N1pdm09) were generated by reverse genetics as previously described (*46*). After virus rescue, viruses were passaged once in MDCK cells to generate a virus stock. The $TCID_{50}$ per ml of the propagated viruses and nasal wash samples was determined on MDCK cells (*47*, *48*). For infection-induced immunity studies, viruses were concentrated via ultracentrifugation (Optima XE Centrifuge, Beckman Coulter) on a 30% sucrose cushion at ×80,000*g* for 2 hours. The supernatant was removed, and each pellet was resuspended in 1 ml of PBS.

Biosafety, biocontainment, and animal care and use

All experiments were conducted in US Department of Agriculture (USDA)–approved biosafety level 2 enhanced laboratories, which included both tissue culture rooms and animal vivariums. All procedures in which virus was cultured in MDCK cells were performed in a dedicated tissue culture room with restricted access and room-specific personal protective equipment (PPE). All personnel were enrolled in the Penn State Occupational Medicine program, and all animal studies were conducted in dedicated animal rooms with negative pressure airflow. For animal studies, personnel had dedicated clothing and PPE. This consisted of facility-dedicated shoes and scrubs, shoe covers, exposure gowns, multiple pairs of gloves, and powered air-purifying respirators. All experiments were conducted before 5 May 2025 and were reviewed and approved by the Penn State Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC). All experiments were conducted in full compliance with IBC Protocol No. 48971 and IACUC Protocol No. 201800250. All experiments were conducted in full compliance with all local, state, and federal regulations.

Ferrets

Equal numbers of 23-week-old male and female ferrets (Triple F Farms, Sayre, PA) were used for all experiments. Ferrets were spayed or neutered and descented by the supplier. Before experimentation, ferrets were confirmed to be seronegative by HAI assay against currently circulating influenza A viruses. For all transmission experiments, paired DR and RC ferrets were the same sex.

IM and IN vaccination of ferrets

Ferrets were vaccinated IM or IN three times, 28 days apart, with 50 μg of A/California/04/2009 H1 HA recombinant protein (Sino Biological) and/or 50 μg of enzymatically active A/California/07/2009 N1 NA recombinant protein (Sino Biological) mixed with adjuvant. For IM vaccination, we used an oil-in-water adjuvant, SAS (Sigma-Aldrich) (0.25 ml of adjuvant per vaccine), and for IN vaccination, LTA1 IN adjuvant (20 μg per vaccine) was formulated as part of the vaccine. LTA1 was provided by E.B.N., Tulane University, and produced as previously described (*49*). Mock-vaccinated animals were given adjuvant mixed with PBS. For both IM and IN vaccine regimens, a boost vaccination was given 28 days post–primary vaccination, and a second boost was given 28 days after the secondary vaccination (i.e., day 56). Following primary vaccination, blood collection was performed at regular intervals to obtain serum for antibody analyses.

IM vaccines were prepared by reconstituting recombinant protein in 0.250 ml of sterile PBS and mixing with an equal volume of SAS. For IM vaccination, ferrets received a total of 0.5 ml of vaccine, 0.25 ml per leg. IN vaccines were prepared by reconstituting 20 μg of LTA1 and recombinant protein(s) in a total volume of 0.2 ml. For IN vaccination, ferrets received a total volume of 0.2 ml of vaccine, 0.1 ml per nostril. Both IM and IN vaccines were administered to ferrets under sedation to minimize distress to the animals. Ferrets were sedated via IM injection with a cocktail of ketamine (30 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg). After vaccination, animals were given an injection of atipamezole to reverse the sedation. At 84 days post–primary vaccination, the vaccinated ferrets were used as DRs in respiratory transmission experiments.

Infection of ferrets to induce immunity

Ferrets ($n = 4$ per group) were sedated and IN inoculated with 1 ml of Opti-MEM containing 10^6 $TCID_{50}$ of concentrated recombinant viruses: 1968 H3N2, rsH3N1, rsH1N2, rsH1N1, or 2009 H1N1. After viral inoculation, nasal wash samples were collected every other day for 7 days, and serum was collected at 2- to 4-week intervals post–primary infection. At day 84 post–primary infection, ferrets were used as DRs in a respiratory transmission experiment.

Ferret transmission experiments

Transmission experiments were performed using custom-built cages (Allentown, NJ) that allowed two ferrets to be separated by an offset perforated divider such that ferrets share the same airspace but cannot have direct contact. To evaluate respiratory transmission, preimmune ferrets ($n = 3$ or 4 per group) were used as DRs. Animals were not randomized and could not be blinded. Sample size calculations were not performed, and the size of experimental groups was determined on the basis of the number of ferrets that could be housed within our vivarium. DR ferrets were anesthetized with a mixture of ketamine (30 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg) and inoculated IN with 1×10^4 $TCID_{50}$ of recombinant A/California/07/2009 (H1N1pdm09) virus in a 1-ml volume of Opti-MEM. After viral inoculation, atipamezole was administered to reverse the anesthesia. Twenty-four hours later, a nasal wash sample was collected from the DR ferrets. These DR ferrets were then introduced into one side of a transmission cage, and an

immunologically naive RC was housed on the other side of the perforated divider. To collect a nasal wash, DR ferrets were sedated with a mixture of ketamine (20 mg/kg), xylazine (2 mg/kg), and atropine (0.05 mg/kg), and 1 ml of PBS was instilled into the nasal passages. Sneezing was then induced by gently stimulating each nostril with a blunt tipped feeding needle, and the expelled liquid was collected in a petri dish. Nasal wash samples were processed by rinsing the dish with an additional 1 ml of PBS. The nasal wash sample was then divided into screw cap tubes and stored at −80°C. Nasal wash samples were collected on alternating days from the DR and RC ferrets for 12 to 14 days. For the duration of the experiment, ferrets were monitored for clinical illness and weight loss. Twenty-one days postinoculation of the DRs, ferrets were deeply anesthetized, and blood was collected via cardiac puncture before euthanasia with an overdose of sodium pentobarbital. Blood was then processed to isolate serum, and serum samples were stored at −80°C until use.

Enzyme-linked immunosorbent assay

To quantify anti-NA and anti-HA IgG binding antibodies in the serum of preimmune ferrets, 96-well MaxiSorp ELISA plates (Nunc) were coated overnight with 2 μg/ml (100 ng/50 μl) of recombinant H1 or N1 protein in sodium bicarbonate buffer (pH 9.4). Plates were blocked with PBST (0.05% Tween) containing 5% skim milk powder and 3% goat serum. Plates were washed with PBST and incubated for 2 hours with serial fourfold dilutions of heat-inactivated ferret serum diluted in PBST with 1% skim milk powder. Plates were washed again and incubated with anti-ferret horseradish peroxidase (HRP)–conjugated IgG (Alpha Diagnostic, catalog number 70511) diluted 1:1000 in PBST with 1% skim milk powder. Subsequently, after additional washes, *o*-phenylenediamine dihydrochloride (OPD) substrate solution (Sigma-Aldrich) was added to all wells. Plates were incubated in the dark for 10 min, and then 3 M HCl was added to each well to stop the reaction. Optical density was measured at 490 nm on a SpectraMax iD3 plate reader (Molecular Devices), and absorbance values greater than three SDs above the mean absorbance for day 0 serum were considered positive.

Enzyme-linked lectin assay

To quantify levels of NAI antibodies, MaxiSorp ELISA plates (Nunc) were coated with fetuin (25 μg/μl) in Dulbecco's Phosphate Buffered Saline (DPBS) and stored at 4°C overnight. On a separate dilution plate, heat inactivated ferret serum was serially diluted in PBST (starting dilution: 1:80 or 1:160) with 3% bovine serum albumin (BSA). Recombinant HA-A/turkey/Massachusetts/3740/1965:NA-A/California/07/2009:6-A/Puerto Rico/8/1934 (H6N1) virus was added to the plate at a dilution of 1:10,000 in PBST with 3% BSA. Fetuin-coated plates were washed three times with PBST (0.05% Tween), and 100 μl of virus-serum mixture was transferred from dilution plates to fetuin-coated plates and incubated at 37°C for 16 to 18 hours. Virus-only, no-serum, and no-virus controls were also included on the plate. Plates were washed six times, and peanut agglutinin conjugated to biotin (1:500) was added to each well. Plates were then incubated in the dark at room temperature for 2 hours, washed, and incubated with streptavidin conjugated to HRP (1:500) for an additional 2 hours in the dark. Last, OPD substrate solution was added to all wells, and the plates were incubated for 10 min at room temperature. The reaction was stopped with 1 N of sulfuric acid, and the optical density was measured at 490 nm on a SpectraMax iD3 plate reader (Molecular Devices). The inhibitory antibody titer was defined as the dilution at which absorbance was reduced by ≥50% of the no serum control.

HAI assay

To perform HAI assays, ferret sera was incubated overnight at 37°C with receptor-destroying enzyme (Hardy Diagnostics), followed by heat inactivation at 56°C for 30 min. In a V-bottom 96-well plate, PBS was added to all wells, and 1:10 diluted serum was added to the first dilution well. Serum was diluted twofold across the plate, and a standardized amount of virus (8 hemagglutination units/50 μl) was added to each well and incubated for 15 min. Turkey red blood cells (0.5% in sterile saline) (Lampire Biologicals) were then added to all wells, and plates were visually scored for hemagglutination after incubation for 35 to 40 min at room temperature. No-virus and virus-only wells were included as controls. The HAI titer was defined as the highest serum dilution that inhibited hemagglutination.

Microneutralization assay

To quantify neutralizing antibodies, heat-inactivated serum was serially diluted and mixed with $1 \times 10^{3.3}$ $TCID_{50}$ of recombinant A/California/07/2009 (H1N1pdm09) in an equal volume (*47*, *50*). The mixture was incubated for 1 hour at room temperature and then overlaid in quadruplicate on MDCK monolayers in 96-well plates. The virus-serum mixture was incubated on the cells for 1.5 hours at 37°C (5% CO_2) and then replaced with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) treated trypsin–supplemented Opti-MEM. Plates were further incubated at 37°C (5% CO_2) for 3 to 4 days and then scored for cytopathic effect. The neutralization titer was defined as the reciprocal of the serum dilution that completely neutralized virus infection.

Next-generation sequencing

Viral RNA was extracted from all nasal wash samples with titers ≥ 10^2 $TCID_{50}$/ml collected from DR and RC ferrets using the QIAmp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. RNA underwent reverse transcription polymerase chain reaction (RT-PCR) using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity (Invitrogen) and influenza universal primers. PCR clean-up was performed using AMPure XP beads (Beckman Coulter), and the concentration of samples was measured using Qubit dsDNA HS Assay Kit (Invitrogen). Samples were normalized, and libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) and IDT for Illumina Nextera DNA Unique Dual Index Set. Samples then underwent PCR clean-up using AMPure XP beads. Subsequently, libraries were pooled at equal molarities after the concentration was determined using Qubit, and fragment size was determined using TapeStation High Sensitivity. Size selection was performed using SPRIselect Beads (Beckman Coulter) on the pooled library. The library was normalized and diluted to a final concentration of 14 pM. Sequencing was performed on an Illumina MiSeq using the Illumina MiSeq Reagent Kit v3 (600 cycles). All samples were processed and sequenced in duplicate. After sequencing, raw sequences were trimmed, and PCR duplicates were removed (fastp v0.23.2) and aligned (BWA v0.7.17) first to the coding sequences of the H1N1 reference A/California/09/2007 (GenBank: CY121680.1). Variants were called, and consensus coding sequences were generated for each sample using timo v1, a variant caller available at https://github.com/GhedinSGS/timo. To further refine influenza sequences for each sample, reads were realigned to

the newly generated consensus sequences using BWA, variants were recalled, and a new consensus sequence was generated using timo. Consensus variants were identified at positions with ≥200× coverage and ≥50% variant frequency in a given sample. For minor variants, variants with a frequency between ≥2 and <50% in any replicate were first identified. Minor variants were further curated by requiring an average variant frequency of ≥5% across replicates. Positions where one of two replicates lacked sufficient sequencing depth to call a variant were flagged as having insufficient sequencing depth and were excluded from the analysis. Sequence data are available under BioProject ID: PRJNA1190981.

Statistical analyses and logistic regression models

Statistical tests to compare antibody titers, viral titers, and AUC in the preimmune DR ferrets were performed using Prism (GraphPad, v10.4). Shapiro-Wilk tests were used to test normality due to small *n* for all statistical analyses. Peak nasal wash titers and AUC datasets did not consistently have a normal distribution across IM vaccinated, IN vaccinated, and infection-induced preimmune DRs. Therefore, we used Kruskal-Wallis tests with post hoc two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli to determine statistical differences across all experiments. A P value of <0.05 was considered significant for all statistical tests.

To examine the complex relationship between immunity, viral load, and transmission, additional statistical analyses were performed. First, DR ferrets were categorized by immunity status and type (no immunity, immunity via vaccine, and immunity via infection), and viral load was examined across categorized immune status by day. We then compared mean titer levels across day (days 1, 3, 5, 7, 9, and 11) by immunity status using one-way analysis of variance (ANOVA) and Tukey's post hoc tests for pairwise comparisons. Next, we examined the relationship between viral load and transmission on a given day using logistic regression, with transmission (yes versus no) as the outcome and viral load (titer level) as the predictor. We examined this using viral load on days 1 and 3 in separate models. ROC curves were used to calculate the AURC, which provides an evaluation of the performance of viral load as a predictor of transmission. Last, we used the results from the logistic regression models to predict probability of transmission across continuous viral load. All analyses were completed using SAS 9.4.

Supplementary Materials

The PDF file includes:
Figs. S1 and S2
Legends for tables S1 to S4

Other Supplementary Material for this manuscript includes the following:
Tables S1 to S4

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