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## Receptor Specificity does not affect Replication or Virulence of the 2009 Pandemic H1N1 Influenza Virus in Mice and Ferrets

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### Abstract

Human influenza viruses predominantly bind 2,6 linked sialic acid (SA) while avian viruses bind 2,3 SA-containing complex glycans. Virulence and tissue tropism of influenza viruses have been ascribed to this binding preference. We generated 2009 pandemic H1N1 (pH1N1) viruses with either predominant 2,3 or 2,6 SA binding and evaluated these viruses in mice and ferrets. The 2,3 pH1N1 virus had similar virulence in mice and replicated to similar titers in the respiratory tract of mice and ferrets as the 2,6 and WT pH1N1 viruses. Immunohistochemical analysis determined that all viruses infected similar cell types in ferret lungs. There is increasing evidence that receptor specificity of influenza viruses is more complex than the binary model of 2,6 and 2,3 SA binding and our data suggest that influenza viruses use a wide range of SA moieties to infect host cells.

### Keywords

Influenza Virus; Tissue Tropism; Virulence; Receptor Specificity; Replication

### Introduction

Influenza A viruses pose a major public health burden and are responsible for thousands of deaths each year. Infection by influenza viruses is mediated via binding of the viral surface glycoprotein hemagglutinin (HA) to terminally attached 2,3 or 2,6-linked sialic acids (SA) on cell surface glycoproteins. Avian influenza viruses predominantly bind to glycan receptors terminating in 2,3-linked SA (henceforth referred to as 2,3 SA) while human-adapted viruses predominantly bind to glycan receptors terminating in 2,6-linked SA (henceforth referred to as 2,6 SA)<sup>1-4</sup>. Receptor-binding specificity is an important determinant of host-range restriction and transmission of influenza viruses<sup>4-7</sup>. The distribution of SA in the human respiratory tract and duck intestine are thought to dictate the specificity of viruses infecting these two species. In humans, the epithelium of the upper

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respiratory tract (nasal mucosa and nasopharynx) primarily expresses 2,6 SA, while the lower respiratory tract (lung) contains both 2,3 and 2,6 SA<sup>8,9</sup>. In contrast, avian species primarily express 2,3 SA in the cells lining the gut<sup>10</sup>. Ferrets, a well-established animal model for influenza, have an 2,3 and 2,6 SA distribution similar to humans, while mice predominantly express 2,3 SA and little 2,6 SA<sup>9,11,12</sup>. These observations have dominated the field and led to the paradigm that 1) 2,3 SA binding viruses are limited in their ability to replicate in humans and ferrets, and when they do so, they lead to severe lower respiratory tract infection and 2) that 2,3 SA binding viruses are more virulent and replicate more efficiently in mice than 2,6 SA binding viruses.

However, many recent studies provide evidence that this paradigm is an over-simplification. Many avian influenza viruses are able to bind both 2,6 and 2,3 SA<sup>13,14</sup> and a change in host range is likely influenced by association with complex, physiologically diverse glycans found on airway epithelial cells<sup>15</sup>. Additional glycan analyses found the presence of both 2,6 and 2,3 SA in the human and ferret respiratory tract and in different avian species<sup>12,16–18</sup>. Additionally, many influenza viruses with a preference for 2,3 SA, such as H5N1 viruses, have been recovered from nasal secretions of naturally infected humans and experimentally infected ferrets<sup>19,20</sup>.

In this study we assessed the role of receptor-binding preference of the viral HA on virulence and tissue tropism of the 2009 pandemic H1N1 (pH1N1) virus. The pH1N1 virus is known to predominantly bind to 2,6 SA and replicate well in the upper and lower respiratory tract of mice and ferrets<sup>21–25</sup>. We generated two mutant viruses by engineering four mutations in the viral HA gene to alter receptor-binding preference. One virus contained mutations designed to increase binding to 2,6 SA (2,6 pH1N1) and the second virus had mutations designed to switch binding preference from 2,6 to 2,3 SA (2,3 pH1N1). We found that the receptor specificity of the pH1N1 virus did not influence virulence in mice or viral replication in the respiratory tract of mice or ferrets. Additionally, we found that the WT, 2,6, and 2,3 pH1N1 viruses replicated in similar cell types in the lungs of ferrets.

## MATERIALS AND METHODS

### Ethics Statement

All animal experiments were done at the NIH, in compliance with the guidelines of the NIAID/NIH Institutional Animal Care and Use Committee (ACUC)

### Cells and Viruses

Madin-Darby canine kidney (MDCK) cells (obtained from ATCC) were maintained in minimum essential media (MEM) with 10% fetal bovine serum (FBS) and L-glutamine (Gibco). 293T cells (obtained from ATCC), were maintained in Dulbecco's MEM with 10% FBS.

The reverse genetics system for generating the 2009 pandemic H1N1 (pH1N1) virus (A/California/07/2009) was previously described<sup>26</sup>. Mutations were engineered using the Stratagene Site-Directed Mutagenesis kit per the manufacturer's protocol.

Recombinant viruses generated from reverse genetics plasmids were rescued in MDCK/293T cell co-culture and propagated for 2 passages in either specific pathogen free (SPF) embryonated eggs, for the 2,3 pH1N1 virus, or MDCK cells for the 2,6 pH1N1 virus. The 2,6 pH1N1 did not grow to high titers in eggs and was propagated in MDCK cells to prevent egg adaptation. The identity of viruses generated by reverse genetics was confirmed

by genomic sequencing. All experiments were performed using viruses passaged no more than 3 times in cells or eggs.

### Dose dependent glycan binding of wild-type (WT) and mutant pH1N1 viruses

The receptor specificity of the WT, 2,3 and 2,6 pH1N1 viruses were investigated using a selected panel of glycans comprised of both 2,3 and 2,6 sialylated glycans as previously described<sup>27</sup>. Briefly, the wells of streptavidin-coated high binding capacity 384-well plates (Pierce) were incubated with 50  $\mu$ l of 2.4  $\mu$ M biotinylated glycans overnight at 4°C. The glycans included were 3 SLN, 3 SLN-LN, 3 SLN-LN-LN, 6 SLN and 6 SLN-LN. LN corresponds to lactosamine (Gal 1-4GlcNAc) and 3 SLN and 6 SLN respectively correspond to Neu5Ac 2–3 and Neu5Ac 2–6 linked to LN. Glycans were obtained from the Consortium of Functional Glycomics ([www.functionalglycomics.org](http://www.functionalglycomics.org)). The viruses (quantified in HAU/50  $\mu$ l) were diluted to 250  $\mu$ l with 1X PBS + 1% BSA. 50  $\mu$ l of the diluted virus was added to each of the glycan-coated wells and incubated overnight at 4 °C. This was followed by three washes with 1X PBST (1X PBS + 0.1% Tween-20) and three washes with 1X PBS. The wells were blocked with 1X PBS + 1% BSA for 2 h at 4 °C followed by incubation with primary antibody (ferret anti-CA07/09 antisera; 1:200 diluted in 1X PBS + 1% BSA) for 5 h at 4 °C. This was followed by three washes with 1X PBST and three washes with 1X PBS. Finally the wells were incubated with the secondary antibody (goat anti-ferret HRP conjugated antibody from Rockland; 1:200 diluted in 1X PBS + 1% BSA). The wells were washed with 1X PBST and 1X PBS as before. The binding signals were determined based on the HRP activity using the Amplex Red Peroxidase Assay (Invitrogen) according to the manufacturer's instructions. Appropriate negative controls included were uncoated wells (without any glycans) to which just the virus, the antisera and the antibody were added and glycan-coated wells to which only the antisera and the antibody were added.

### Characterization of viruses in mice

Replication of the viruses in the upper and lower respiratory tract of 6–8 week old female BALB/c mice was determined as described<sup>28</sup>. Briefly, each mouse received  $10^5$  median tissue culture infectious dose (TCID<sub>50</sub>) of virus in 50  $\mu$ L intranasally (IN). Groups of four mice per virus were sacrificed on days 1, 3 and 7 post-infection. Nasal turbinates were collected by placing a clamp around the nose at the angle of the jaw and inverting 180° to expose the nasal cavity. Forceps were used to collect the exposed nasal bone and tissue. The entire lung was collected to measure viral titers in the lower respiratory tract. Tissues were homogenized and clarified supernatant was aliquoted and titered on MDCK cells. The 50% tissue culture infectious dose (TCID<sub>50</sub>) per gram of tissue was calculated by the Reed and Muench method<sup>29</sup>.

The median mouse lethal dose (MLD<sub>50</sub>) was determined by administration of serial 10-fold dilutions of the virus and observing weight loss and survival. In accordance with NIAID ACUC guidelines, mice were euthanized if they lost more than 25% of their initial body weight.

### Replication of viruses in ferrets

We evaluated the replication kinetics of the viruses in the respiratory tract of 8–10 week old ferrets as previously described<sup>25</sup>. All ferrets were screened prior to infection to ensure that they were naïve to seasonal influenza A and B, and to the viruses used in this study. Each ferret was inoculated IN with  $10^6$  TCID<sub>50</sub> of virus in 500  $\mu$ L. Tissues were harvested for pathology and to assess viral titers. Virus titers from tissue samples were determined on days 1, 3 and 5 post-infection as previously described<sup>30</sup> and expressed in TCID<sub>50</sub>/g of tissue. Briefly, tissues were weighed and Leibovitz's L-15 (L-15, Invitrogen) was added at 5%

(nasal turbinates and trachea) or 10% (lung) weight per volume (W/V). The nasopharynx was homogenized in 1 mL of L-15. Tissues were homogenized and clarified supernatant was aliquoted and titered on MDCK cells. The 50% tissue culture infectious dose (TCID<sub>50</sub>) per gram of tissue was calculated by the Reed and Muench method<sup>29</sup>.

### Immunohistochemistry

Ferret tissues were stained for influenza-specific antigens using antibodies derived against the whole influenza virus in goats. Paraffin-embedded microtome sections were incubated at 60°C for 30 min and then incubated in xylene and serial dilutions of ethanol ranging from 100%-0% in dH<sub>2</sub>O for 5 minutes (min) at room temperature, with a final step of 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. For antigen retrieval, the slides were incubated in sodium citrate buffer maintained at 95°C in a water bath for 30 min. Tissue sections were blocked in PBS-Tween with 1.5% rabbit serum and stained with 1:300 dilution of anti-influenza polyclonal antibody from Abcam (ab20841) and a secondary rabbit anti-goat biotin conjugated antibody (Vector Labs). Bound antibody was visualized using the Vectastain ABC system and DAB stain (Vector Labs) as per manufacturer's instructions. Slides were counterstained with hematoxylin (Vector Labs) and washed in Scott's Tap water substitute to enhance the blue stain. Permount was used per manufacturer's instructions to mount the slides. A trained veterinary pathologist analyzed all slides

### Statistics

All viral titer data from replication kinetic experiments are expressed as mean ± standard error and were analyzed using a Student's *t* test, two-way ANOVA, or Mann-Whitney U test in the graphing software, Prism (GraphPad). *p* values less than 0.05 are significant.

## Results

### Generation and rescue of α2,3 and α2,6 SA specific pH1N1 viruses

Multiple amino acids residues make up the receptor binding site (RBS) and control receptor specificity. Since, the HA gene segment of the pH1N1 virus evolved from the 1918 Spanish influenza H1N1 HA<sup>31,32</sup>, for this study we based changes in the pH1N1 RBS on those already characterized for the 1918 HA. Two amino acid changes (D187E and D222G) in the HA RBS of the 1918 H1N1 (A/South Carolina/1/18 or SC18) HA protein, referred to as the avianized or AV18 virus, were demonstrated to change receptor specificity from α<sub>2,6</sub> to α<sub>2,3</sub> SA<sup>6,33,34</sup>. Based on a detailed understanding of molecular contacts made by SC18 and AV18 respectively with α<sub>2,6</sub> and α<sub>2,3</sub> SA receptors, we designed mutations in pH1N1 to mimic the RBS of SC18 and AV18 (Table 1). The engineered mutations in the RBS of HA included residues 187, 216, 222 and 224 (H1 numbering), which make critical molecular contacts with the sialylated glycan receptors as depicted in the ribbon diagram in Figure 1A and B<sup>27,35,36</sup>. Residues 187 and 222, as stated above, determine receptor specificity of the 1918 HA, while residues 216 and 224 help stabilize the receptor-binding pocket and have been shown to enhance SA binding<sup>27</sup>. Two viruses were rescued; a α<sub>2,6</sub> pH1N1 virus that mimics SC18 at positions 216 and 224 and a α<sub>2,3</sub> pH1N1 virus that is similar to AV18 (Table 1). To construct the α<sub>2,6</sub> pH1N1 virus the HA segment was mutated at two sites, the Isoleucine (I) at 216 (codon ATA) to Alanine (A) (codon GCA) and Glutamic acid (E) at 224 (codon GAA) to A (Codon GCA) where the underlined nucleotide indicates the engineered change. The α<sub>2,3</sub> pH1N1 virus was created by combining the α<sub>2,6</sub> pH1N1 changes with the following two mutations Aspartic acid (D) at 222 (GAC) to a Glycine (G) (GGC) and D187 (GAC) to Glutamic acid (E) (GAG) (Table 1). The receptor specificity of the engineered pH1N1 viruses was determined by a dose-dependent glycan binding assay of the WT, α<sub>2,6</sub>, and α<sub>2,3</sub> pH1N1 viruses (Figure 1C–E). The WT and α<sub>2,6</sub> pH1N1 viruses revealed a strong preference for both long (6 SLN-LN) and short (6 SLN) α<sub>2,6</sub> SA (Figure

1C and D). In contrast, the 2,3 pH1N1 virus showed predominant binding to 2,3 SA with some minimal binding to 2,6 SA (Figure 1E). Previous studies using this type of glycan assay have shown that H5N1 viruses also have a low-level of 2,6 SA binding, similar to the 2,3 pH1N1 virus<sup>35</sup>.

A single change at residue 222 from D to G in pH1N1 has been observed in nature and was thought to lead to more severe disease in infected patients<sup>37,38</sup>. Interestingly, a single D222G substitution results in a virus with dual binding specificity that strongly associates with both 2,6 SA and 2,3 SA binding<sup>39,40</sup>. However, alteration of both D222 and D187 residues in the context of the other mutations at positions 216 and 224 results in switching the dominant binding preference of pH1N1 virus from 2,6 SA to 2,3 SA. Other investigators have also changed the receptor specificity of the pH1N1 virus by introducing a single change at position 226<sup>41</sup>, demonstrating that multiple amino acids in the RBS can influence receptor specificity.

### Characterization of WT, $\alpha$ 2,3 and $\alpha$ 2,6 pH1N1 viruses in mice

To assess whether receptor specificity of the pH1N1 virus alters the tissue tropism of the viruses, we tested their ability to replicate in the upper and lower respiratory tract of mice (Figure 2A and B). Replication of WT, 2,6, and 2,3 pH1N1 viruses was determined in the nasal turbinates and lungs of 4 individual animals on days 1, 3 and 7 post-infection. Despite the predominance of 2,3 SA in mice<sup>11</sup>, all three viruses replicated to high titer in both the nasal turbinates and lungs of all infected animals. Replication of WT and mutant pH1N1 viruses reached a peak on day 3 post-infection, with declining titers on day 7 as previously described for the WT pH1N1 virus<sup>42</sup> (Figure 2A and B). No significant difference was observed among the WT, 2,6, and 2,3 pH1N1 viruses. Thus, receptor specificity of the pH1N1 virus does not affect viral replication in the upper or lower respiratory tract of mice.

Previous studies have suggested that virulence of influenza viruses in mice correlates with receptor specificity<sup>43-45</sup>. We determined the MLD<sub>50</sub> for all three viruses by infecting mice (4 per group) with serial 10-fold dilutions of virus from a dose of 10<sup>6</sup> to 10<sup>3</sup>TCID<sub>50</sub>/50uL IN and following daily weights and survival. In accordance with our animal study protocol, mice that lost >25% of their original body weight were euthanized (Figure 2C and D). The MLD<sub>50</sub> was similar for all three viruses; 10<sup>5.5</sup>, 10<sup>4.8</sup> and 10<sup>4.9</sup> for WT pH1N1, 2,6 pH1N1 and 2,3 pH1N1, respectively (Figure 2E). These results demonstrate that a receptor preference for 2,3 SA does not alter the virulence of the pH1N1 virus in mice.

### Characterization of WT, $\alpha$ 2,3 and $\alpha$ 2,6 pH1N1 viruses in ferrets

Plant lectins, SNA-I and MALII that have been used extensively to characterize the distribution of 2,6 and 2,3 SA respectively in tissue sections from humans, ferrets, and mice<sup>5,8,10,11,16,35,46,47</sup>. Staining of ferret tracheal tissue have shown that airway epithelial cells predominantly express 2,6 SA while the submucosal glands express both 2,3 and 2,6 SA. Lectin staining of the large airways in the lung demonstrated that epithelial cells and the submucosal glands preferentially express 2,6 SA, while goblet cells and the alveolar interstitium express both 2,3 and 2,6 SA<sup>9,12</sup>.

Since ferrets have a differential distribution of SA in the respiratory tract; we evaluated viral replication kinetics in the nasal turbinates, nasopharynx, trachea and lungs of ferrets infected with WT pH1N1, 2,3 pH1N1, or 2,6 pH1N1 viruses (Figure 3). In the nasal turbinates, nasopharynx and trachea, all three viruses replicated to high titers that remained within a 10-fold range from day 1 to 5 post-infection (Figure 3A-C). Replication of the WT and mutant pH1N1 viruses was measured in the lower respiratory tract of ferrets by determining the viral titer from the right middle lobe of the ferret lung (Figure 3D). On day 1 post-infection,



the 2,3 pH1N1 and WT pH1N1 viruses replicated to high titers while the titer of 2,6 pH1N1 virus was much lower. However, by day 3 post-infection, replication of the 2,6 pH1N1 virus reached equivalent levels with WT and 2,3 pH1N1 viruses. Additionally, the peak titer for WT pH1N1 and 2,3 pH1N1 was on day 1 post-infection while it was day 5 post-infection for 2,6 pH1N1 infected ferrets (Figure 3D). The difference in replication kinetics in the ferret respiratory tissues is not related to receptor specificity, since the WT and 2,6 pH1N1 viruses have similar receptor specificities but differed in their kinetics. Additionally, WT and 2,3 pH1N1 viruses have distinct receptor preferences but had similar replication kinetics in the lungs of ferrets at all days tested. These data suggest that receptor specificity of pH1N1 virus does not greatly affect the replication in the ferret respiratory tract and replication kinetics can vary among virus strains.

We assessed the severity of disease, distribution and kinetics of pathological lesions in the lungs of ferrets (8 animals/virus). Ferrets infected with 2,6 pH1N1 virus showed the most severe pathological changes and while animals infected with WT pH1N1 had histopathological scores that were slightly less severe, the general degree of inflammation was similar between the two viruses. In contrast, ferrets that received the 2,3 pH1N1 virus displayed milder disease scores over the course of infection. The cellular composition of inflammatory lesions was similar among all three viruses and consisted predominantly of neutrophils and alveolar macrophages (days 1 and 3) that progressed to a more even admixture of neutrophils, lymphocytes and plasma cells by day 5 post-infection. The distribution of lesions differed among the three virus groups, ferrets infected with the 2,3 pH1N1 virus predominantly had inflammation associated with the alveolar interstitium on day 1 post-infection and the submucosal glands (SMG) by day 5 post-infection (Figure 4A and B). In contrast, animals infected with either 2,6 or WT pH1N1 viruses showed airway-centered disease with a moderate degree of bronchiolar epithelial hyperplasia (Figure 4C–F) on day 5 post-infection that likely reflects repair of epithelial cell damage. Bronchiolar hyperplasia was not observed in animals infected with 2,3 pH1N1 virus. Thus, infection with the 2,6 SA pH1N1 virus leads to more severe histopathological changes in the lungs than the 2,3 SA pH1N1 virus.

We attempted to identify the cell types infected by the WT and mutant pH1N1 viruses in ferret lungs on days 1 and 5 post-infection. With all three viruses, immunohistochemistry showed that goblet and ciliated cells were positive for viral antigens (Figure 5) and viral antigen was also found in the submucosal glands and alveolar interstitium (Figure 5). Submucosal glands and ciliated epithelial cells in the ferret lungs were previously thought to express only 2,6 SA<sup>12</sup>, yet the 2,3 pH1N1 virus replicated in these cell types on days 1 and 5. Therefore, our data demonstrate that receptor-binding preference does not significantly alter the tissue tropism of the pH1N1 virus in the ferret respiratory tract.

## Discussion

The previously held paradigm suggests that receptor specificity, based on association with 2,6 SA or 2,3 SA, determines replication and virulence of influenza viruses. It was believed that 2,6 SA binding viruses replicate more efficiently in the upper respiratory tract of ferrets while 2,3 SA binding viruses replicate more efficiently in mice and in the lower respiratory tract of ferrets<sup>8–11</sup>. A preference for 2,3 SA was also thought to increase virulence of influenza viruses for mice<sup>43–45,48</sup>. In this study we introduced four amino acid changes into the RBS of the 2009 pH1N1 HA, thereby changing the SA receptor-binding preference of the 2009 pH1N1 virus from 2,6 SA to 2,3 SA. Characterization of these engineered pH1N1 viruses revealed two important results. First, there was no difference in the virulence of the WT, 2,6, and 2,3 pH1N1 viruses in mice. Second, there was no difference in replication of the three viruses in the upper or lower respiratory tract of ferrets

and mice. This result is consistent with the previously published findings on the replication of 1918 pandemic H1N1 viruses, SC18 and AV18, in the ferret respiratory tract<sup>6</sup>.

Viruses with 2,3 SA specificity are expected to be more virulent in mice because the respiratory tract of mice bears 2,3 SA<sup>43,45,48</sup>. However, many properties likely affect virulence of influenza viruses in mice, such as the magnitude of replication, systemic spread of the virus, and affinity or specificity for host receptors. In our study, the 2,3 pH1N1 virus was as virulent as WT and 2,6 pH1N1 viruses, suggesting that virulence of the pH1N1 virus in mice is determined by factors other than receptor specificity. Our data support the hypothesis that viral replication may determine virulence in the mouse model, since the WT and 2,6 pH1N1 viruses replicated similarly to the 2,3 pH1N1 virus in the upper and lower respiratory tract of mice. Others have shown that some pH1N1 viruses that were more virulent in mice were derived as antibody escape mutants<sup>43,45</sup> and resulted in increased receptor avidity<sup>43</sup>. Since the pH1N1 virus does not produce a systemic infection in mice, viral replication and receptor avidity rather than binding specificity likely play a role in determining the virulence of this virus.

The differential distribution of SA in the human and ferret respiratory tract is thought to limit the replication of 2,3 SA dominant influenza viruses to the lower respiratory tract<sup>8,9,12,16,41</sup>. However, in this study the 2,3 pH1N1 virus, generated based on the RBS of the 1918 pandemic SC18 and AV18 viruses<sup>6</sup>, replicated as well as WT pH1N1 virus in the upper and lower respiratory tract of ferrets and all viruses replicated in the same cell types in the lungs of ferrets. In contrast, another recent study characterizing pH1N1 viruses that differed in receptor specificity found that an 2,3 SA binding pH1N1 virus (HA/226R) did not replicate well in the lungs of ferrets<sup>41</sup>. Taken together, these results suggest that the specific amino acid composition of the HA RBS, rather than 2,3 and 2,6 SA preference alone, affects viral replication in the lungs of ferrets and that influenza viruses likely associate with a wide range of SA species on cells in the respiratory tract. Recent advances in glycan array studies have demonstrated the complexity of SA association of influenza viruses<sup>13,17</sup>. One study analyzing human bronchus and lung tissues demonstrated a multitude of SA including both 2,6 and 2,3 linked SA in variable chain lengths<sup>17</sup> suggesting that multiple SA moieties are available for influenza viral entry. Therefore, physiological glycan diversity is more nuanced than characterization of terminal 2,6 and 2,3 SA linkages alone. This is also evident in the case of the recently emerged H7N9 influenza virus, where the binding of this viral HA to physiological receptors in the human respiratory tract could not be explained by its binding to a limited set of glycans with 2,6 and 2,3 SA linkages on prototypic glycan arrays<sup>15</sup>. Further characterization of the SA composition of ferret and mice respiratory tracts is needed to fully understand the replication of influenza viruses in these animal models.

We demonstrate that the 2,3 SA binding pH1N1 virus has similar virulence in mice and replication in mice and ferrets as the WT and 2,6 pH1N1 viruses. Previous studies have shown that association with 2,6 SA is required for respiratory droplet transmission of influenza viruses in ferrets<sup>5,6,41,49-51</sup>. Therefore, further characterization of the transmissibility of the 2,6 and 2,3 pH1N1 viruses generated in this study would be of interest.

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Committee on Dual Use Research of Concern (DURC). The committee concluded that the methods and results reported in our manuscript do not meet DURC criteria.

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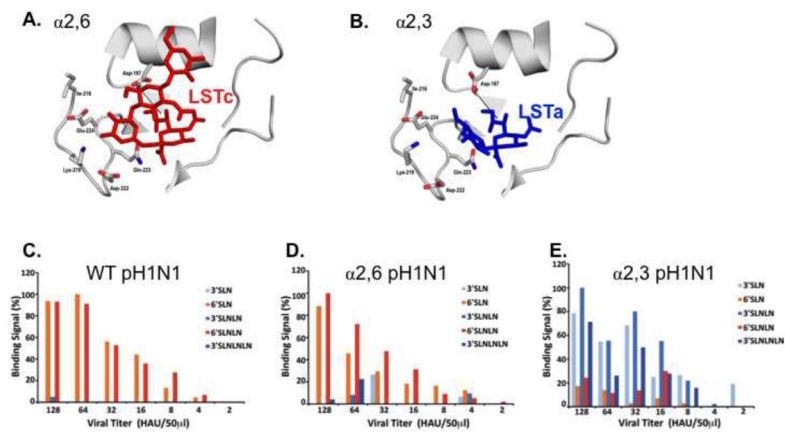
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### Highlights

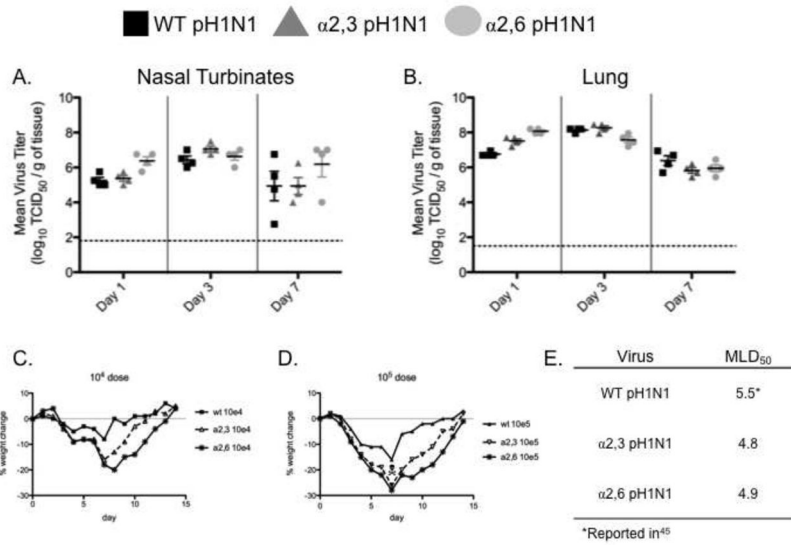
- We generated 2009 pH1N1 viruses with predominant 2,3 or 2,6 sialic acid binding.
- receptor specificity did not influence virulence in mice.
- receptor specificity did not influence virus titers in the respiratory tract of mice.
- receptor specificity did not influence virus titers in the ferret respiratory tract.
- 2,6 and 2,3 pH1N1 viruses replicated in similar cell types in the lungs of ferrets.



### Figure 1. Generation of 2,6 and 2,3 specific pH1N1 viruses

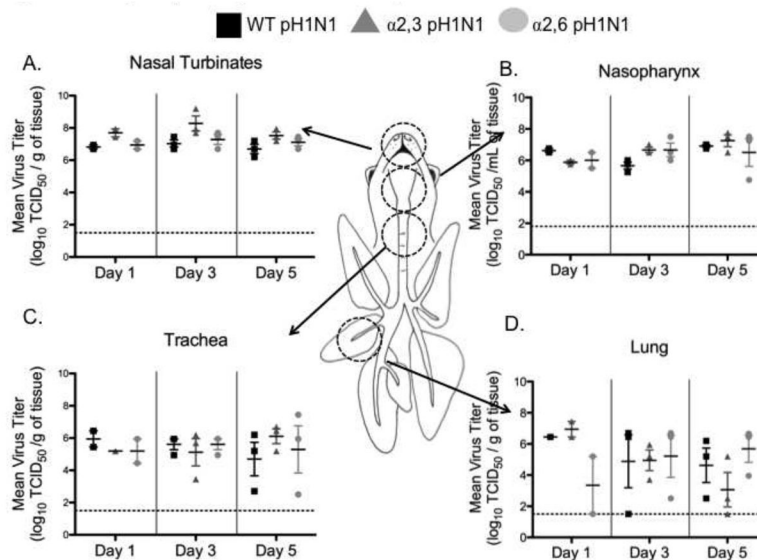
Ribbon diagrams of the pH1N1 HA receptor binding pocket highlight the critical amino acids in the receptor binding site of pH1N1 HA that bind with LSTc (2,6-linked lactoseries tetrasaccharide c) (A) and with LSTa (2,3-linked lactoseries tetrasaccharide a) (B). Amino acids at residues 187, 222, and 224 (H1 numbering) that make critical molecular contacts with the sialylated glycan receptor in the receptor-binding pocket of HA are shown. These residues were mutated to alter the receptor binding specificity of the pH1N1 HA (see main text for details). Receptor specificity of wild-type (WT) and mutant pH1N1 viruses was determined by dose-dependent glycan binding of inactivated whole viruses WT pH1N1 (C), 2,6 pH1N1 (D) and 2,3 pH1N1 (E). The glycans are indicated in the figure legend, orange colors represent 2,6 SA and blue colors represent 2,3 SA. Binding is expressed as percentage after normalization with the maximum binding signal





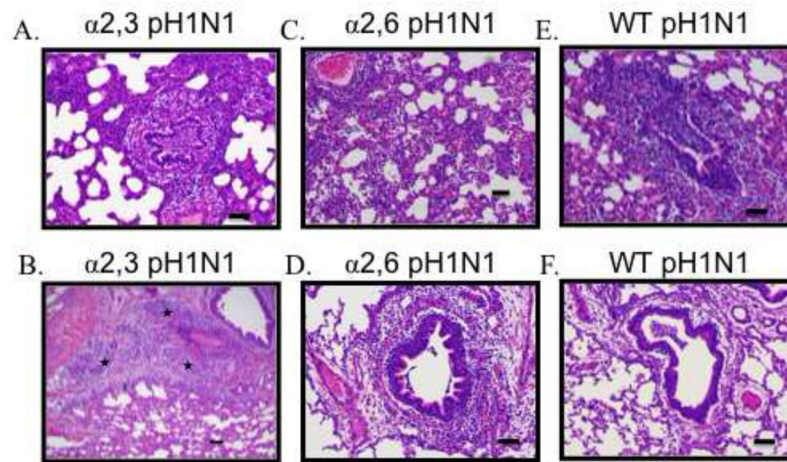
### Figure 2. Receptor specificity does not alter replication or virulence in mice

Viral titers in the nasal turbinates (A) and lung (B) were determined from mice infected with WT (■), α2,6 pH1N1 (●), and α2,3 pH1N1 (▲). Each point represents an individual animal, bars represent the mean titers among those animals, and the error bars represent the standard error between animals. Virulence of the WT and mutant pH1N1 viruses was measured in mice by inoculating animals with varying doses of virus ( $10^3$ ,  $10^4$ ,  $10^5$  TCID<sub>50</sub>/50 μL). Mice were weighed every day and animals were sacrificed if they lost 25% of their pre-study weight. All animals infected with  $10^4$  (C) and  $10^5$  (D) TCID<sub>50</sub>/50 μL lost weight, but only those in the  $10^5$  group were sacrificed as indicated by the (⊕) symbol. The calculated median mouse lethal dose (MLD<sub>50</sub>) for each virus was based upon the survival rate of mice in the  $10^4$  and  $10^5$  group (E).



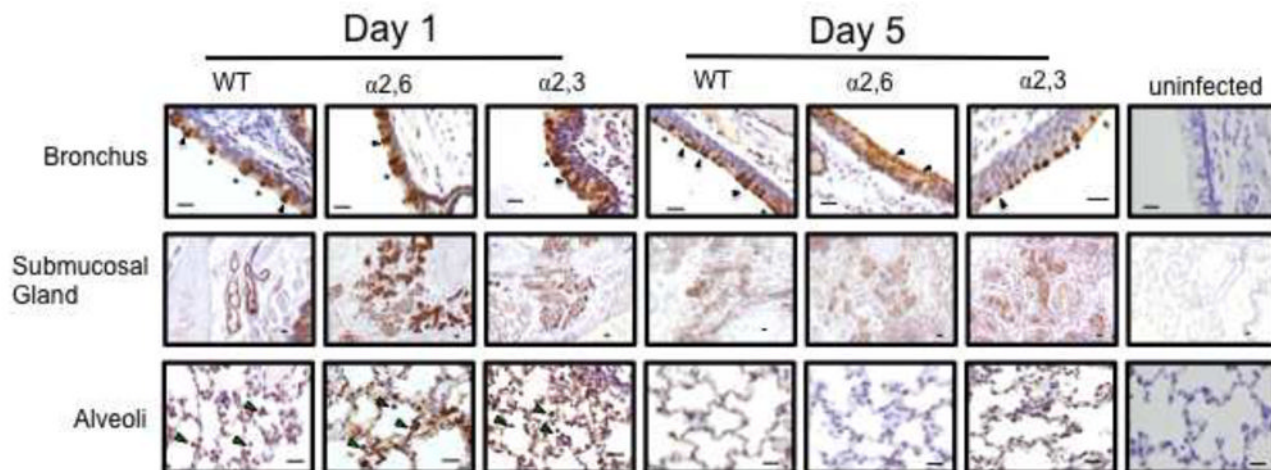
**Figure 3. Receptor specificity does not alter replication in ferrets**

Titers of the WT pH1N1 (■), 2,6 pH1N1 (●), and 2,3 pH1N1 (▲) from the regions of the ferret respiratory tract defined on the diagram with dashed circles, include the nasal turbinates (A), nasopharynx (B), trachea (C) and lung (D). Each point represents data from an individual animal. Mean and standard error are indicated.



**Figure 4. Histopathology of lung sections in ferrets during infection with WT and mutant pH1N1 viruses**

Lung lesions of  $\alpha 2,3$  pH1N1 infected ferrets were observed in the alveolar interstitium on day 1 post-infection (A) and in the submucosal glands (SMG) on day 5 post-infection (B). The star ( ) indicates SMGs demonstrating significant inflammation. Ferrets infected with WT or  $\alpha 2,6$  pH1N1 viruses displayed intense peribronchiolar inflammation on day 3 post-infection (C, E) and bronchiolar hyperplasia on day 5 post-infection (D, F). Note the marked thickening of the epithelial cell lining and degenerate cell debris in the lumen. A trained veterinary pathologist analyzed all slides. Scale bars represent 100  $\mu\text{m}$ .



**Figure 5. Localization of viral antigen in the lower respiratory tract of ferrets**

The animal with the highest viral titer in the lungs from each group sacrificed on days 1 and 5 post-infection was analyzed for the location of viral antigen. Lung sections were stained with polyclonal anti-influenza A antibody and counterstained with hematoxylin.

Representative images were captured for the bronchus, submucosal glands, and alveoli of a ferret infected with WT pH1N1 virus or the mutant viruses. The black arrow ( ) represents ciliated cells stained with influenza antigen, asterisk (\*) mark antigen positive goblet cells, and green arrows ( ► ) mark antigen positive inflammatory cells in the alveoli. A trained veterinary pathologist analyzed all slides. Scale bars represent 50  $\mu$ m.

**Table 1**

Mutations engineered in the 2009 pH1N1 virus based on 1918 HA sequence.

Pandemic	Virus	HA Amino Acid Position (H1 Numbering)			
		187	216	222	224
1918	SC18 ( 2,6)	D	A	D	A
	AV18 ( 2,3)	E	A	G	A
2009	WT pH1N1	D	I	D	E
	2,6 pH1N1	D	A	D	A
	2,3 pH1N1	E	A	G	A