Human H7N9 and H5N1 influenza viruses differ in induction of cytokines and tissue tropism

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Running Head: Unique properties of H5N1 and H7N9 influenza viruses

Key words: influenza, pathogenicity, emerging, immune response, aged mice, high-risk population

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Abstract

Since emerging in 2013, the avian-origin H7N9 influenza viruses have resulted in over 400 human infections leading to 115 deaths to date. Although the epidemiology differs from human highly pathogenic avian H5N1 influenza infections, there is a similar rapid progression to acute respiratory distress syndrome (ARDS). The aim of these studies was to compare the pathological and immunological characteristics of a panel of human H7N9 and H5N1 viruses in vitro and in vivo. Although there were similarities between particular H5N1 and H7N9 viruses, including association between lethal disease and spread to the alveolar spaces and kidney, there were also strain-specific differences. Both H5N1 and H7N9 viruses are capable of causing lethal infections, with mortality correlating most strongly with wider distribution of viral antigen in the lungs, rather than with traditional measures of viral titer and host responses. Strain-specific differences included hypercytokinemia in H5N1 infections that was not seen with the H7N9 infections regardless of lethality. Conversely, H7N9 viruses showed a greater tropism for respiratory epithelium covering nasal passages and NALT than H5N1 viruses, which may explain the enhanced transmission in ferret models. Overall these studies highlight some distinctive properties of H5N1 and H7N9 viruses in different in vitro and in vivo models.
Importance

The novel avian-origin H7N9 pandemic represents a serious threat to public health. The ability of H7N9 to cause serious lung pathology leading in some cases to the development of acute respiratory distress syndrome is of particular concern. Initial reports of H7N9 infection compared them to infections caused by highly pathogenic avian (HPAI) H5N1 viruses. Thus, it is of critical importance to understand the pathology and immunological response to infection with H7N9 as compared to HPAI H5N1 viruses. We compared these responses in both in vitro and in vivo models, and found that H5N1 and H7N9 infections exhibit distinct pathological, immunological and tissue tropism differences that could explain differences in clinical disease and viral transmission.
**Introduction**

Human infections with avian H7N9 influenza viruses were first detected in China in March 2013 (9). By June, the pandemic had spread to 9 provinces, and 132 human cases leading to 37 deaths were reported. The decrease in H7N9 cases over the summer likely resulted from a combination of control measures (e.g. closing of the live animal markets) and seasonal factors. However, in October 2013 reports of new human H7N9 infections began to increase and, as of March 2014, over 400 human infections leading to 115 deaths have been reported (17). Although the majority of human infections remain contained to mainland China, there have been imported cases confirmed in Taiwan, Hong Kong SAR and Malaysia (4, 12, 19). There is no evidence that sustained human-to-human transmission is occurring, although the H7N9 viruses possess several key amino acid changes often associated with mammalian replication and transmission, heightening public health concerns (2, 28, 30).

Patients infected with H7N9 viruses can exhibit severe and often lethal disease characterized by rapidly progressive pneumonia leading to acute respiratory distress syndrome (ARDS) and respiratory failure (12). Because of this, and its avian origin, the human H7N9 infections have been touted as an “H5N1-like” disease. A number of studies have demonstrated that high viral replication, lymphopenia, induction of a “cytokine storm”, replication in macrophages, and virus dissemination beyond the respiratory tract are associated with the increased severity of H5 disease in humans and animal models (5, 27). Whether these are also features of H7N9 infection warrants further study. This information will be critical to advance our understanding of pathogenicity of avian-origin viruses in mammals.

To fill this gap in knowledge, we compared the pathogenicity and immune response of a panel of H7N9 and H5N1 influenza viruses isolated from humans in the well-established murine...
model. Although many of the strains induced significant morbidity and mortality without the need for adaptation, we identified H5 and H7 viruses that were not highly lethal to mice. Overall, our studies demonstrate that although morbidity may be similar, there are distinct differences between H7 and H5 infections in the mouse model. H5N1 infections were distinguished by a hypercytokinemia phenotype, though this did not correlate with outcome. In contrast, H7N9 viruses showed an increased tropism for epithelium lining nasal passages and NALT. Finally, while both H5N1 and H7N9 viruses are capable of causing lethal infections in mice, mortality correlated most strongly with wider distribution of viral antigen in the lungs (as determined by nucleoprotein staining) rather than with traditional measures of viral titer and host responses. These studies suggest that although the outcome of infection may be similar, the underlying mechanisms and potential for aerosol spread differ between viral strains.

Materials and Methods

Ethics statement. All procedures were approved by the St. Jude Children’s Research Hospital Institutional Biosafety Committee (IBC) and animal care and use committee (ACUC) and were in compliance with the Guide for the Care and Use of Laboratory Animals. These guidelines were established by the Institute of Laboratory Animal Resources and approved by the Governing Board of the U.S. National Research Council.

Laboratory facilities. All experiments were conducted in a biosafety level 3 enhanced containment laboratory (20). Investigators were required to wear appropriate respirator equipment (RACAL, Health and Safety Inc., Frederick, MD). Mice were housed in HEPA-filtered, negative pressure, vented isolation containers.
Viruses. H5N1 and H7N9 viruses used in these studies included H5N1 A/Vietnam/1203/2004 (VN1203/H5, clade 1) and A/Hong Kong/213/2003 (HK213/H5, clade 1) (23) and H7N9 A/Shanghai/1/2013 (SH1/H7), A/Anhui/1/2013 (Anhui/H7), and A/Shanghai/2/2013 (9). Viruses were propagated in the allantoic cavity of 10-day-old specific pathogen-free embryonated chicken eggs at 37°C. Allantoic fluid was harvested, cleared by centrifugation, and stored at -80°C as described previously (3, 10). Viral titers were determined by TCID$_{50}$ analysis as previously described (10).

Cells and in vitro infections. MDCK cells were cultured in Eagle’s minimum essential medium (MEM, MediaTech, Manassas, VA) supplemented with 2 mM glutamine and 10% fetal bovine sera (FBS, Gemini BioProducts, West Sacramento, CA) and grown at 37°C under 5% CO$_2$. They were infected with a multiplicity of infection of 0.01 for 1 hour at 37°C, then washed three times to remove unbound virus and infected cells were cultured in appropriate media containing 0.075% BSA and 1 μg/ml TPCK-treated trypsin. Aliquots of culture supernatants were collected at 6, 24, 48, and 72 hour post-infection (hpi) and immediately stored at -80°C for determination of virus titers.

Animal experiments. Eight week-old C57Bl/6 mice (Jackson Laboratory, Bar Harbor, ME) were lightly anesthetized with isofluorane and inoculated intranasally with PBS or a predetermined TCID$_{50}$ unit of virus in 25 μl PBS. Mice were monitored daily for clinical signs of infection and weighed every 24 hpi (14). At days 3, 6 and 9 post-infection, mice (n = 9 mice per group) were euthanized and tissues were harvested and processed immediately for histopathology (n = 3) and flow cytometry (n = 6) or stored at -80°C for future analysis of viral titers and cytokine and chemokine expression. Mice losing greater than 30% body weight were humanely euthanized. Experiments were repeated at least twice.
Pathology. At 0, 3, 6, and 9 days post-infection (dpi), deeply anesthetized mice were perfused with 10% neutral buffered formalin, tissue collected and processed for H&E and immunohistochemistry for NP staining by the St. Jude Children’s Research Hospital Veterinary Pathology Core Facility as described (16). Blinded samples were scored by a veterinary pathologist.

Flow Cytometry. Bronchoalveolar lavage fluid (BALF) was collected on days 0, 3, 6, and 9 pi by flushing the lung twice with 1 ml PBS. BALF was centrifuged and the cell pellet was used for cell analysis. BALF supernatant was saved for cytokine analysis. Spleens were also collected at the same time point and were processed into single cell suspension for cell analysis. Anti-mouse CD11b, CD11c, Ly6C, Ly6G, NK1.1, CD3, CD4, CD8, B220 antibodies were used to stain the cells and the stained cells were analyzed on a FACS Calibur (BD).

Cytokines. BALF supernatant and sera were collected on days 0, 3, 6, and 9 pi for cytokine and chemokine analysis using the Milliplex Mouse 25-plex Cytokine Detection System™ kit (Millipore, Billerica, MA) according to the manufacturer’s protocol. Results were obtained on a Luminex 100 109 reader (Luminex Corp., Austin, TX) and data calculated using the calibration curve obtained within each experiment using recombinant proteins per manufacturer’s instructions.

Lectin staining. Nasal cavity tissues were prepared by the St. Jude Children’s Research Hospital Veterinary Pathology Core as described (16). Sections were de-paraffinized in citrosol and antigenic site retrieval was performed in heated sodium citrate buffer (10 mM sodium citrate pH 6.0 + 0.05% Tween). Tissues were blocked in 2% goat serum/PBS (Sigma, St. Louis, MO) for 1 hr at 37°C before overnight incubation with biotinylated *Maackia amurensis* lectin II (MAAII) (to detect α2,3-linked sialic acid receptors) or biotinylated *Sambucus nigra* agglutinin (SNA)
(to detect α2,6 linkages) (Vector Laboratories, Burlington, CA) at 10 µg/ml at 4°C. Tissues were washed three times in PBS/0.05% Tween (PBST) before incubating with streptavidin-conjugated Texas Red (Vector Labs) at 15 µg/ml for 1 hr at 37°C. After washing tissues three times in PBST slides were mounted with glycerol and visualized using a Nikon C2 confocal microscope (Nikon Instruments, Melville, NY).

**Statistical analysis.** Statistical analyses were performed using JMP Statistical Software (SAS Institute, Cary, NC, USA). Nonparametric data were analyzed using a Kruskal Wallis test ($\alpha = 0.05$). Data were analyzed by 2-way ANOVA with virus type and day post infection as main effects. Tukey’s HSD was used for post-hoc comparison and differences were considered significant at $p <0.05$.

**Results**

**Pathogenicity of H5N1 and H7N9 Viruses**

The highly pathogenic avian H5N1 viruses cause rapid and severe disease in mice without prior adaptation (7, 21). This also appears to be true for the few H7N9 viruses that have been analyzed to date (2). Thus, 8-week old C57Bl/6 mice were lightly anesthetized and intranasally inoculated with ten-fold dilutions of virus. Weight loss was monitored daily through 14 dpi and the 50% mouse lethal dose (MLD$_{50}$) was determined (Figure 1). VN1203/H5 virus caused significant weight loss within 4 dpi and 100% of the mice succumbed or had to be euthanized by 8 dpi. The MLD$_{50}$ value was $< 10^2$ TCID$_{50}$/ml. Consistent with previous studies (2, 13) (29), the Anhui/H7 and SH1/H7 viruses also caused significant weight loss and MLD$_{50}$ were calculated at $10^{3.5}$ TCID$_{50}$/ml for both viruses. Despite relatively similar MLD$_{50}$ values, mortality from VN1203/H5
virus consistently began 1-2 days before the H7N9 viruses, which correlates with the decreased
time to death observed in H5N1 human cases (6). We also identified two viruses that were
significantly less virulent in mice, HK213/H5 and SH2/H7. HK213/H5 infection resulted in up to
25% weight loss with $10^6$ TCID$_{50}$ units of virus and 50% mortality by 12 dpi (Figure 1). The
MLD$_{50}$ was calculated as $10^5$ TCID$_{50}$/ml. In contrast, the SH2/H7 virus induced minimal weight
loss (maximum 15%) and no mortality even at the highest dose tested: $10^5$ TCID$_{50}$ units. The
MLD$_{50}$ of SH2/H7 was thus > $10^5$ TCID$_{50}$/ml.

Histologically, four of the five groups of mice infected with equivalent MLD$_{50}$ doses had
similar lung pathology at 3 dpi, characterized by mild perivasculitis and focal bronchiolitis, with
little involvement of alveolar parenchyma (Figure 2). However, VN1203/H5 infection caused a
more severe and widespread lung pathology associated with necrosis and inflammation in the
bronchioles (Figure 2 arrows), alveolar necrosis and inflammation (Figure 2 arrowheads), and
evidence of interstitial pneumonia. At day 6 pi the Anhui/H7 and SH1/H7 viruses were also
associated with extensive alveolar and bronchiolar inflammation similar to the VN1203/H5
inoculated animals.

These differences in lesion severity were well-correlated with the extent of distribution of
viral antigen. In all groups, nucleoprotein (NP) staining at 3 dpi was restricted to bronchiolar
epithelium (Figure 2 arrows), with the exception of VN1203/H5 where minimal alveolar lesions
were evident and NP staining was spread extensively throughout the alveolar parenchyma
(Figure 2 arrowheads). By 6 dpi, the Anhui/H7 and SH1/H7 viruses also showed extensive
alveolar and bronchiolar inflammation and widespread NP staining in the alveolar parenchyma
similar to the VN1203/H5 inoculated animals (Figure 2). In contrast, viral antigen remained
more localized to bronchioles and adjacent alveoli with less extensive alveolar spread and
inflammation in the HK213/H5 and SH2/H7 inoculated animals. Histological lesions were only seen in the lungs and upper respiratory tract. No lesions were seen in the intestines, kidneys, or brain.

Replication efficiency of H5N1 and H7N9 viruses

In spite of these differences in mortality and NP staining, viral titers in the lungs were similar at 3 dpi amongst all groups except for the SH2/H7 virus, which had >3-log lower titers (Figure 3A). By 6 dpi, VN1203/H5, Anhui/H7, and SH1/H7 infected mice still had equivalent titers, while the non-lethal HK213/H5 and SH2/H7 viruses were both significantly lower. Thus, there is a clear divergence between viral titer measurements and the extent of virus spread in the lung.

To investigate replication efficiency amongst the viruses in vitro, MDCK cells were infected at a multiplicity of infection (MOI) of 0.01 and viral titers measured from 6 to 72 hpi by TCID\textsubscript{50}. VN1203/H5 virus replicated the fastest reaching a peak of $10^{8.1}$ TCID\textsubscript{50}/ml by 24 hpi (Figure 3B). While the HK213/H5 virus reached equivalent titers by 24 hpi, the titers of H7 viruses remained significantly lower, with the SH2/H7 virus peak titer being $10^{5.3}$ TCID\textsubscript{50}/ml at 24 hpi. These studies demonstrate that the H5 and H7 viruses differ in their replication kinetics in a strain independent fashion.

H5N1 viruses induce a stronger cytokine response in spite of similar lung immune cell infiltration

To assess the immune response, BALF and spleen were collected from mice infected with 1 MLD\textsubscript{50} virus. By 3 dpi all viruses induced depletion of alveolar macrophages and infiltration of leukocytes including inflammatory monocytes (tipDCs), neutrophils, and NK cells (Figure 4). By
6 dpi, both CD4+ and CD8+ T cell levels had increased in the lungs of infected mice. Except for HK213/H5, all viruses induced a high magnitude of infiltration of most types of leukocytes; HK213/H5 virus consistently induced the least infiltration of every cell type despite replicating to similar viral titers in the lung (Figure 3A). Of note, neutrophil levels were higher in the lungs of the H7-infected mice at 6 dpi as compared to H5-infected animals. There were no differences in these cells populations in the spleen (data not shown).

In contrast to the similar magnitude of cellular infiltration in the BALF, the two H5N1 viruses induced higher levels of BALF and plasma cytokines/chemokines than the H7N9 viruses by 6 dpi including IFN-γ, IP-10, MIP-1b, MCP-1, and TNF-α (Figure 5). Severe H5N1 has been associated with a “cytokine storm” with increased levels of both pro- and anti-inflammatory cytokines being detected in plasma and lung tissue of humans and animal models (18). This hyper-cytokinemia has been associated with the unique ability of H5N1 viruses to cause a viral hyper-induction of these factors compared to other influenza viruses (27). Thus, it is interesting to note that these cytokines were elevated regardless of the virulence of the H5 infection; HK213/H5 increased several cytokines/chemokines to similar levels as the lethal VN1203/H5 virus. Finally, only the non-lethal HK213/H5 and SH2/H7 infections led to increased BAL and plasma levels of IL-10 at 6 dpi (Figure 5).

**H7N9 Viruses Have a Distinct Tropism for the Nasal Passages and NALT**

Severe H5N1 infection is associated with systemic spread and replication in organs outside the respiratory tract including the brain and kidney (27). Thus, to compare systemic spread, 8-week old C57Bl/6 mice were infected with 1 MLD50 and viral titers determined in upper respiratory tract, brain, kidney, and intestine on days 3 and 6 pi (Figure 6). As expected, VN1203/H5 was
the only virus detected in the brain and kidney in 100\% of mice by 6 dpi; however, both Anhui/H7 and SH1/H7 were detected in the kidneys of \(-33\% (2/6)\) mice tested at 3 dpi (Figure 6A), which may correlate with the significant risk of renal failure in H7N9 infected individuals (26). All of the viruses except for SH2/H7 were detected in the intestines of mice 3 dpi reaching a titer of \(10^4\,\text{TCID}_{50}\) units/g tissue (Figure 6B), corroborating suggestions that these avian-origin viruses can possibly replicate in the mammalian gastrointestinal tract (27) although further studies directly assessing replication are needed.

NP staining was conducted on diverse tissues at 3 and 6 dpi. Unexpectedly, we found extensive NP staining in the nasal passages and nasal-associated lymphoid tissue (NALT) of the H7 but not H5-infected animals at 3 dpi (Figure 7). At 6 dpi, a small amount of NP staining was seen in the VN1203/H5 but not HK213/H5 inoculated animals. Viral titers on homogenized nasal turbinates correlated with the NP staining, indicating that H7N9 virus replicated to high titers in the nasal mucosa in contrast to the H5 viruses (Figure 8A). To correlate the observed viral tropism to expression of sialic acid receptors, we stained nasal tissue from uninfected mice for \(\alpha2,3\) and \(\alpha2,6\)-linked sialic acid receptors. Consistent with the mammalian respiratory tract (22), the more rostral respiratory epithelium lining nasal passages expressed primarily \(\alpha2,6\) sialic acids, while \(\alpha2,3\) sialic acids were not widely expressed until further back into the nasal passages (Figure 8B).

**Discussion**

Our *in vitro* and *in vivo* studies suggest that there are significant differences between avian H5N1 and H7N9 infections in mice including kinetics of viral replication, viral spread in the respiratory tract and host immune response and cytokine expression as summarized in Table
Since the infections were performed at an equivalent MLD\textsubscript{50} dose instead of equivalent viral particles, we did not observe major difference in viral replication between H5N1 and H7N9 viruses in the lung; however, the nasal tissues better supported replication of the H7N9 viruses. Positive viral staining in the nasal tissue of H7N9-infected mice correlated with high expression of α2,6-linked sialic acid receptors, indicating increased tropism for mammalian hosts. Sialic acid receptor expression in the nasal passages of C57Bl/6 mice reflected earlier studies done on BALB/c mice, which were found to express α2,6 sialic acid receptors in the upper respiratory tract while α2,3 receptors were not detected until the lower trachea and the lung (15). Indeed, H7N9 viruses can attach to human tissues from both lower and upper respiratory tract (24, 29). Replication in the upper respiratory tract may explain why H7N9 but not H5N1 viruses can transmit between ferrets (24, 29).

H5N1 viruses are noted for their ability to replicate systemically. As expected, VN1203/H5 was detected in the kidney by 6 dpi by TCID\textsubscript{50} assay. However, at 3 dpi we also detected Anhui/H7 and SH1/H7 in the kidney, although none of the H7N9 strains were detectable by 6 dpi. This may be related to the increased risk of renal failure in individuals infected with H7N9 (26). All of the H7N9 viruses tested except SH2/H7 were also detected by TCID\textsubscript{50} in the intestinal tract early in infection (3 dpi) although replication was not confirmed by NP staining (data not shown). In our study, the most striking predictor of a lethal outcome was the extent of viral antigen spread to alveoli. At 3 dpi, four of the five viruses tested were mostly restricted to the bronchial epithelium, while only VN1203/H5 demonstrated extensive alveolar spread, presumably compromising respiratory function. By 6 dpi, alveolar spread of Anhui/H7 and SH1/H7 was comparable to sections from animals infected with VN1203/H5, while HK213/H5 and SH2/H7, less pathogenic in the mouse model, still appeared to be mostly...
constrained to the bronchial epithelium. Furthermore, H5N1 was able to spread efficiently to non-respiratory tissues, such as the brain and kidney, while H7N9 was only transiently detected in those tissues, if at all. Highly pathogenic H5N1 viruses possess a multibasic cleavage site, able to utilize ubiquitous cellular proteases such as furin, which accounts for efficient spread throughout the alveolar space leading to systemic spread seen in H5N1 viruses (11). However, H7N9 viruses were able to spread efficiently throughout the alveoli at later points in infection.

Infection with H5N1 viruses led to a higher cytokine response. Measurement of cytokine levels in the BALF and the plasma revealed higher levels of most cytokines tested at 6 dpi, the peak of infection. Additionally, cytokine levels during H5N1 infection were increased as early as 3 dpi compared to H7N9 viruses. Of note, only the non-lethal viruses induced increased IL-10 levels 6 dpi; a cytokine typically thought to be anti-inflammatory. In spite of the differential cytokine response, no major differences in immune cell infiltration were evident between H5N1 and H7N9 viruses. Recent studies have shown that early activation of NF-κB and IL-17 mediated signaling pathways may substantially contribute to the development of severe disease (1), implying that the host cytokine/chemokine response may be just as crucial to disease outcome as viral load.

Taken together, these data indicate that the H7N9 viruses are not necessarily similar to H5N1 viruses; however, further studies are required to understand the differences in pathogenicity between H7N9 strains. Indeed, even with genetic similarities, SH2 infection was significantly different in all respects versus the Anhui/H7 and SH1/H7 strains. This finding is intriguing as SH2/H7 was isolated from a young adult while SH1/H7 and Anhui/H7 were isolated from older, high-risk subjects with severe infection (9, 25). Aside from the differences with H5N1, H7N9 infection still poses a significant threat to human health, especially with the
suggestion that these viruses have increased transmissibility versus H5N1 strains (8, 9). The reemergence of H7N9 highlights that early diagnosis of infected individuals and outbreak control measures such as closing of the poultry markets may not be sufficient to completely stall the outbreak of H7N9. Close monitoring of the population, especially high-risk groups, and generation of a vaccine capable of protecting against H7N9 infection is paramount in combating these novel viruses.

References


origin human influenza A(H7N9) can be transmitted between ferrets via respiratory

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affecting amino acid changes in the PA protein of H7N9 influenza A viruses. J Virol
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planned the project. V.A.M., E.A.K., T.C., P.F., S.D. and L.K. performed the experiments.
V.A.M., E.A.K., S.D., P.V., R.J.W., P.G.T., and S.S.C. analyzed the data. V.A.M., E.A.K.,
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Figure Legends

Figure 1: Determination of MLD$_{50}$ of H5N1 and H7N9 viruses. Mice were intranasally inoculated with PBS or the indicated dose of H5N1 (VN1203 or HK213) or H7N9 (Anhui, SH1, or SH2) viruses and monitored for morbidity and mortality. Data is presented as percent survival (left column) and percent of starting weight (right column). Error bars indicate SD. n = 10 mice/group. Experiments were repeated at least twice.

Figure 2: Lung pathology and viral spread during H5 and H7 infections. Mice infected with 1 MLD$_{50}$ of the indicated virus were sacrificed at 3 and 6 dpi. Lung sections were stained for inflammation by H&E stain and for the presence of influenza antigen by IHC. Widespread lung pathology associated with VN1203 infection is not present with the other viruses at 3 dpi, while Anhui and SH1 cause extensive pathology by 6 dpi. Magnification = 10X. Images are representative of n = 3 mice/group/time point.

Figure 3: Replication kinetics of H5N1 and H7N9 viruses. A) Mice infected with 1 MLD$_{50}$ of the indicated virus were sacrificed at 3 and 6 dpi and various tissues collected. Viral replication in the lungs was determined by TCID$_{50}$ assay. Data is presented as TCID$_{50}$/g tissue. n = 6 mice/group. B) MDCK cells were infected with the indicated virus at an MOI of 0.01. Viral titers were determined by TCID$_{50}$ assay at 6, 24, 48, and 72 hpi. Error bars indicate SD. n = 3 replicates/per virus and *p<0.05.

Figure 4: Immune cell response to H5N1 and H7N9 viruses. Mice infected with 1 MLD$_{50}$ were sacrificed at 3 and 6 dpi and BALF was isolated. Cells from the BALF were stained and
quantified by flow cytometry to determine the percentage of alveolar macrophages, tipDCs, neutrophils, NK cells, and CD4⁺ or CD8⁺ T cells. Data is presented as a percentage of the total number of cells isolated from the BALF of each animal. Error bars indicate SD. n = 6 mice/group. a - p < 0.05 H5 versus Anhui. b - p < 0.05 H5 versus SH1. c - p < 0.05 H5 versus SH2.

Figure 5: Cytokine response to H5N1 and H7N9 viruses. Cytokine response was measured by Luminex assay in the BALF (left column) and plasma (right column) of mice infected with 1 MLD₅₀ of the indicated virus. Error bars indicate SD. n = 6 mice/group. a - p < 0.05 H5 versus Anhui. b - p < 0.05 H5 versus SH1. c - p < 0.05 H5 versus SH2.

Figure 6: Systemic viral replication of H5N1 and H7N9 viruses. Mice infected with 1 MLD₅₀ of the indicated virus were sacrificed at 3 and 6 dpi. Viral replication was determined by TCID₅₀ assay in the kidney A) and the intestine B). Data is presented as TCID₅₀/g tissue. Error bars indicate SD. n = 6 mice/group.

Figure 7: Tropism of H5N1 and H7N9 viruses for the nasal tissue and NALT. Mice infected with 1 MLD₅₀ of the indicated virus were sacrificed at 3 and 6 dpi. Nasal tissues were stained for the presence of influenza antigen by IHC. Magnification = 10X (nasal passages) or 40X (NALT). Images are representative of n = 3 mice/group.

Figure 8: Virus replication and sialic acid distribution in the murine nasal tract. A) Mice infected with 1 MLD₅₀ of the indicated virus were sacrificed at 6 dpi. Viral replication in the nasal
passage was determined by TCID$_{50}$ assay. Error bars indicate SD. $n = 3$ mice/group. B) Sections of nasal tissue from uninfected mice were stained for $\alpha_{2,3}$ and $\alpha_{2,6}$ sialic acid receptors by immunofluorescence. Magnification = 10X. Sections are representative of 2 individual mice.
### Table 1. Summary of Results

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<th>Virus</th>
<th>Subtype</th>
<th>Phenotype</th>
<th>Lung</th>
<th>Kidney</th>
<th>Intestine</th>
<th>Nasal/Nalt</th>
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<td>Alveolar by 3 dpi</td>
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<td>Anhui</td>
<td>H7N9</td>
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*a* – as measured by immunohistochemistry and confirmed by titer analysis, *b* – measured by titer analysis.
Figure 1
Figure 3

A) Lung Virus Titer

Log TCID50/ml

Days Post Infection

0 2 4 6 8 10

VN1203 (H5N1)
HK213 (H5N1)
Anhui (H7N9)
SH1 (H7N9)
SH2 (H7N9)

B) MDCK Virus Titer

Log TCID50/ml

Hours Post Infection

0 6 24 48 72

VN1203 (H5N1)
HK213 (H5N1)
Anhui (H7N9)
SH1 (H7N9)
SH2 (H7N9)
Figure 4

- % Alveolar Macrophages
- % Neutrophils
- % TipDC
- % NK Cells
- % CD4+ T Cells
- % CD8+ T Cells

Days Post Infection

Uninfected
VN1203 (H5N1)
HK213 (H5N1)
Anhui (H7N9)
SH1 (H7N9)
SH2 (H7N9)
Figure 5
Figure 8