Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus

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Communicated by Roy Curtis III, Arizona State University, Tempe, AZ, January 5, 2009 (received for review August 28, 2008)

The mechanisms responsible for the virulence of the highly pathogenic avian influenza (HPAI) and of the 1918 pandemic influenza virus in humans remain poorly understood. To identify crucial components of the early host response during these infections by using both conventional and functional genomics tools, we studied 34 cynomolgus macaques (Macaca fascicularis) to compare a 2004 human H5N1 Vietnam isolate with 2 reassortant viruses possessing the 1918 hemagglutinin (HA) and neuraminidase (NA) surface proteins, known conveyors of virulence. One of the reassortants also contained the 1918 nonstructural (NS1) protein, an inhibitor of the host interferon (IFN) response. While both 1918 reassortant viruses also were highly pathogenic, the H5N1 virus was exceptional for the extent of tissue damage, cytokinemia, and interference with immune regulatory mechanisms, which may help explain the extreme virulence of HPAI viruses in humans.

Since 2003, the mortality for highly pathogenic avian influenza (HPAI) of the H5N1 virus subtype has been 63% of reported cases (www.who.int/csr/disease/avian_influenza/). Regardless of age or prior health, infected individuals have died within 10 days after onset of symptoms from a fast-progressing pneumonia, variably complicated by intestinal and CNS symptoms, often leading to respiratory distress syndrome and multiorgan failure (1–3). Mild infections or asymptomatic seroconversion in high-risk groups, such as health-care or poultry workers, has been rare (4). The extreme virulence and rapidly fatal clinical outcomes of human H5N1 virus infections are reminiscent of the 1918 pandemic virus, which reportedly caused up to 100 million fatalities (5).

Clinical and pathological features in H5N1- and 1918-infected humans and animal models (6–12) suggest that high levels of viral replication with early robust host responses play a key role in pneumonia severity and outcome. Recent nonhuman primate experiments comparing HPAI H5N1 and a reconstructed 1918 virus suggest many similarities in early host responses to these viruses. The goal of the present study, conducted in our well-characterized macaque and systems biology model of influenza (12–15), was to refine our understanding by using a 2004 human H5N1 Vietnam isolate and 2 1918 reassortant viruses possessing the 1918 hemagglutinin (HA) and neuraminidase (NA) surface proteins, based on the role surface glycoproteins play in the high virulence of the 1918 virus in the mouse model (6). One of the reassortants also contained the 1918 nonstructural (NS1) protein, an inhibitor of the host interferon (IFN) response (13, 15–22). This study revealed important similarities but also critical differences between the H5N1 and 1918-reassortant viruses, highlighting aspects of the host-pathogen interface caused by highly virulent influenza viruses.

Results

Differential Replication, Lung Tissue Tropism, and Inflammatory and Innate Immune Responses in Macaques Infected with H5N1 and 1918 Reassortant Influenza Viruses. To perform a systematic comparison of several highly pathogenic influenza viruses, we inoculated cynomolgus macaques (8 animals per group) with either influenza A/Vietnam/1203/2004 (referred to as H5N1), 1918HA/NA:A/Texas/36/91 (1918HANA), 1918HA/NA/NS:A/Texas/36/91 (1918HANANS), or the parental H1N1 A/Texas/36/91 (Texas) virus. Inoculation was via the tracheal, nasal, conjunctival, and tonsillar routes as described (13–15). On days 1, 2, 4, and 7 postinfection (PI), 2 animals per group were euthanized. One H5N1 animal died between days 6 and 7, because of extensive pulmonary damage. Despite having similar pathology, the remaining animal lived until its endpoint on day 7. Finally, 2 mock-infected animals were euthanized on day 7. Viral load was quantified by median tissue culture infectious dose (TCID50) on 4 lung lobes from each animal (Fig. 1) and tissue distribution of virus antigen indicative of actively replicating virus (Fig. S1A). All showed that the H5N1 virus replicated to a much higher extent and with a wider distribution within the lungs compared with the other viruses, particularly at the early time points (days


Conflict of interest statement: A.G.-S. owns patent positions for reverse genetics of influenza virus. Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Expression Array Manager at http://viromics.washington.edu/informatics/Project/Publications/ begin.Editorial www.pnas.org/cgi/content/full/ 0813234106/DCSupplemental.
and (ii) type I pneumocytes were identified as squamous cells with large, round nuclei and a vacuolated cytoplasm; type II pneumocytes were identified as large, rounded to cuboidal cells with large, round nuclei and a vacuolated cytoplasm; and (iii) type I pneumocytes were identified as squamous cells with small round to oval nuclei and mostly imperceptible cytoplasm. This analysis revealed a ratio of type I to type II in the reassortant virus-infected animals of 11: 1 or higher; in the H5N1-infected animals the ratio was 1:3 or higher. In addition, more macrophage-like cells and endothelial cells appeared to be productively infected in the H5N1-challenged animals compared to the reassortant virus-challenged animals. In the Texas-infected animals, where only few virus positive cells were detected on days 1–4 PI, the majority of virus-antigen positive cells were macrophages. While not approaching the levels of the H5N1 virus, the 1918HANA and 1918HANANS viruses replicated to higher titers than the Texas virus, confirming the role of the 1918 viral glycoproteins in enhancing viral replication in the nonhuman primate model (Fig. 1). As in prior studies (13–15), the Texas infection was only detectable by real-time PCR and viral antigen staining in lung tissue.

Viral replication, as determined by the TCID50 assay, remained not suitable for RNA isolation. In contrast, the 1918HANA and 1918HANANS viruses were productively infected in the upper respiratory tract of macaques. By immunolabeling, widespread H5N1 replication was also detected in tonsils on days 1–4 PI. Viral replication, as determined by the TCID50 assay, remained below the detection level (100 TCID50) in the upper respiratory tract of the other groups, with the exception of 1 animal in the Texas group on day 1. At several time points, the H5N1 virus was also found by a combination of viral culture and immunolabeling in tracheobronchial lymph nodes, but only to a small extent in macrophage- and dendritic cell-like cells in the retropharyngeal lymph nodes and spleen, and in 1 animal, killed on day 4, in cells of the leptomeninges. The presence of virus or viral antigens, however limited, in spleen and meninges suggests that viremia did occur, supported by detection of low levels of viral mRNA in blood samples of animals in the H5N1 group on days 1 and 2. Extrapulmonary spread of the virus through alternate routes, such as the gastrointestinal or olfactory tracts, did not appear to be a feature in these animals. This finding is consistent with the patterns observed in a number of human patients (1, 2, 23–27). In the reassortant 1918 and Texas virus groups, virus antigen was only found in lymph nodes and tonsils on day 1.

The H5N1 virus replicated to moderate levels in the nasopharynx through day 2 PI (2.24 × 102 to 2.8 × 107 TCID50) and in trachea through day 7 PI (1.31 × 105 to 2.8 × 104 TCID50), demonstrating that the H5N1 virus can be detected in the upper respiratory tract of macaques. By immunolabeling, widespread H5N1 replication was also detected in tonsils on days 1–4 PI. Viral replication, as determined by the TCID50 assay, remained below the detection level (100 TCID50) in the upper respiratory tract of the other groups, with the exception of 1 animal in the Texas group on day 1. At several time points, the H5N1 virus was also found by a combination of viral culture and immunolabeling in tracheobronchial lymph nodes, but only to a small extent in macrophage- and dendritic cell-like cells in the retropharyngeal lymph nodes and spleen, and in 1 animal, killed on day 4, in cells of the leptomeninges. The presence of virus or viral antigens, however limited, in spleen and meninges suggests that viremia did occur, supported by detection of low levels of viral mRNA in blood samples of animals in the H5N1 group on days 1 and 2. Extrapulmonary spread of the virus through alternate routes, such as the gastrointestinal or olfactory tracts, did not appear to be a feature in these animals. This finding is consistent with the patterns observed in a number of human patients (1, 2, 23–27). In the reassortant 1918 and Texas virus groups, virus antigen was only found in lymph nodes and tonsils on day 1.

The H5N1 infection resulted in very high transcriptional induction in affected lung tissue (Fig. 3) of type I IFNs, including several IFNα variants and IFNβ; IFN-induced genes, such as IFN-induced proteins with tetratricopeptide repeats (IFITs); IFN-inducible guanylate binding protein (GBP); and IFN-stimulated exonuclease (ISG20). Additionally, the H5N1 group exhibited dramatic induction of genes important during the acute

Fig. 1. Log (TCID50/mL) averaged from all lung lobes (4 out of 7, including left cranial, middle, caudal and accessory lobes) harvested. Detection limit of the plaque assay was 100 TCID50/mL. Note: Tissues from day 6 death were not suitable for viral isolation.

Fig. 2. Influenza antigen staining in lung tissues shows prolonged targeting of type II pneumocytes during the H5N1 infection (50×). In contrast, the middle insert illustrates targeting of type I pneumocytes in 1 of the 1918 recombinant groups (150×).

Fig. 3. Stronger IFN, inflammatory, and innate immune transcriptional induction in the H5N1 group. Heat maps were generated with genes of interest after performing a 1-way ANOVA (P ≤ 0.01, Benjamini-Hochberg FDR) with statistical cut-off criteria of a 5-fold change in at least 2 experiments (P ≤ 0.01). Up-regulation relative to the reference pool is in red, down-regulation in green, and lack or statistically nonsignificant regulation in the darker shades of these colors. *, this transcriptional profile is from a non-lesion area of the animal euthanized on day 7, since tissues from H5N1 day 6 death were not suitable for RNA isolation.
phase response, such as interleukin (IL)-1 and 6, tumor necrosis factor (TNF)-α, and several members of the complement pathway. Finally, genes coding for chemokines were also strongly and, in many cases, continuously induced in the H5N1 group. These genes included CXCL10, recently singled out as being highly expressed in H5N1-infected ferrets (28). While most of these genes were highly induced in the 1918 reassortant groups as well, particularly on days 1 and 2, expression in the H5N1-infected animals was often above the limit of detection of the array analysis software. Furthermore, in this group, a slight relative decline in induction on day 4 of some, but not all, of these genes, was followed by a rebound on day 7 to levels similar to that in early infection. The kinetics of this response were unique to the H5N1 group and likely due to a resurgence in viral replication in the animal still alive on day 7 and to the unremitting influx of immune cells, suggesting that the host response was unable to control the H5N1 infection.

Taken together, these data show that the H5N1 virus resulted in a more productive infection than the other viruses in both the lower and upper respiratory tracts of macaques, with evidence of limited viremic dissemination and concurrently strong and protracted induction of genes relevant to IFN, inflammatory, and innate immune responses.

H5N1 Virus in Macaques Resulted in More Severe Clinical Disease and Pathology than H1N1 Viruses Containing 1918 HA, NA and/or NS Genes. To closely monitor differences between experimental groups, animals were clinically scored twice daily (Table S1). Animals in the H5N1 group exhibited more severe clinical signs compared to those in the other groups within 24 h PI. Symptoms included anorexia, depression, coughing, diarrhea, a stress leukogram on day 1, and a death on day 6. A complete blood count revealed a thrombocytopenia from day 2 PI, worsening through day 7 when the platelet count reached a low of $139 \times 10^9/L$, a value comparable to that found in humans infected with H5N1 viruses (29). This thrombocytopenia is considered idiopathic despite a possible connection with viremia-induced damage to the vascular endothelium (30) and independent from occasional disseminated intravascular coagulation at the end-stage of the disease in humans. Animals in the H5N1 group also had dramatically higher levels of IL-6 in both serum and lung tissue and of TNF-α in lung tissue on days 1 and 2. While the H5N1 and 1918HANA virus groups had comparable lung levels of IFN-γ on day 1, the H5N1 group had much higher levels on days 2 and 7 (Fig. S2), the latter again corresponding to the resurgence of viral replication and the up-regulation of innate immune genes. IL-2, -4, -7, and -5 were also measured in serum and lung tissue by cytokine bead arrays, but failed to reveal significant differences among experimental groups. Trends with measured cytokines were corroborated by results obtained by microarray (Fig. 3) and real-time PCR analysis performed on lung samples with representative infection and pathology.

In both the H5N1 and 1918 reassortant virus groups, pathology findings showed that all lung lobes were affected by bronchopneumonia seen both grossly (consolidation and edema) and microscopically (bronchiolitis and alveolitis). However, pathology scores in the H5N1 group averaged 5.18 (SE 0.13) on a scale of 0 to 6, versus 3.2 (SE 0.17), 3.0 (SE 0.19), and 1.2 (SE 0.13) in the 1918HANA, 1918HANANS, and Texas group, respectively, over the 7 days of the study (Fig. S1B). A Mac387 stain specific for myeloid lineage cells, revealed extensive neutrophil and macrophage infiltration—a consistent feature with HPAI viruses in all models studied to date (31–33)—in the lungs of both the H5N1 and 1918 recombinant virus-infected animals (Fig. 4A). However, the degree of infiltration in the H5N1 animals far surpassed the others on day 7. The 1918HANANS infection resulted in more intense infiltration than the 1918HANA virus, but only on day 2. Consistent with human disease, H5N1 infection resulted in severe necrotizing bronchiolitis and alveolitis evident within 24 h PI (34). A similar but less severe inflammation was noted in the animals infected with the 1918HANANS group for Mac387 (Fig. 4B). No gross or histological lesions ascribable to the experimental infections were observed in any tissues outside the respiratory tract and draining lymph nodes. In summary, both H5N1 and 1918 recombinant viruses produced severe pathology, but in the case of the H5N1 virus, the degree of local inflammation, assessed by transcriptional induction, tissue cytokine secretion, and granulocytic infiltration, was substantially greater and more prolonged. The H5N1 virus also induced a more severe systemic reaction, suggested by high serum cytokine levels and clinical signs.

H5N1 Virus Caused Margination of Circulating T Lymphocytes and Reduction of Dendritic Cells in Lungs and Draining Lymph Nodes. To gain a better understanding of how these viruses affected other aspects of the host response, we conducted flow cytometric analysis of circulating lymphocytes and observed a dramatic depletion of CD4+ and CD8+ T cells through day 7, with a progressive and only partial recovery (Fig. S3 A and B). This effect was clearly most evident in the H5N1 group. This transient phenomenon is reportedly mainly due to vascular margination of T lymphocytes, induced by a direct interaction with circulating type I IFNs (35), although H5N1 viruses were shown to also cause T-cell apoptosis in multiple tissues in the mouse model (36).

Another event possibly impacting the adaptive response in the H5N1 group was a notable disappearance of activated (CD83+) dendritic cells in lungs and draining lymphoid tissue over the 7-day infection (Fig. 5A). In contrast, dendritic cells in the peripheral circulation decreased in absolute numbers in the H5N1 group compared to the others (Fig. S4), suggesting that
their absence in infected tissues was not the result of impaired recruitment or extravasation. Subsequent immunolabeling for activated caspase-3 suggested that dendritic cells underwent apoptosis in situ early in infection with H5N1 virus. This was determined by morphological examination of apoptotic cells (Fig. 5B), as well as by double CD83/activated caspase-3 staining, which highlighted activated dendritic cells undergoing apoptosis (Fig. 5B).

In summary, the H5N1 virus perturbed the cell-mediated antiviral response and, through increased or premature apoptosis of dendritic cells, mainly through morphology, due to the poor expression of the receptor type (Lack of affinity of avian influenza viruses for the most common sialic acids (the optimal receptor for H5N1 influenza virus), substantiating that a high viral inoculation load might overcome suboptimal receptor specificity for the H5N1 virus (38). The H5N1 virus also successfully spread to several extrarespiratory tissues, albeit to a limited extent.

The H5N1 infection resulted in very strong and protracted induction of genes relevant to inflammatory and innate immune responses. While reassortant 1918 influenza viruses also proved much more virulent in the nonhuman primate model than a contemporary human H1N1 virus, overall pathology and clinical course with the 1918HANA virus were less severe compared to the fully reconstructed 1918 virus (12). This was an unexpected finding, given the high virulence of the 1918HANA recombinant virus in mice. Also in contrast to the mouse model, the 1918 reassortant containing the 1918 HA, NA and NS1 genes was of equal virulence to 1918HANA virus in the macaque model, suggesting species-specificity of NS functionality (39).

**Virulence of HPAI Viruses May Be Closely Tied to Preferential Infection of Type II Pneumocytes.** Our data, when considered in light of other studies, suggests a connection between the ability of the H5N1 virus to infect type II pneumocytes and the extent of viral replication in the lower respiratory tract. These cells outnumber type I pneumocytes by 2-fold and have a faster metabolism (40) due to their role as stem cells and as source of surfactant with antimicrobial, immunomodulatory, and anti-inflammatory properties (41). These functions are in all likelihood compromised by infection, resulting in disrupted lung repair and increased vulnerability of remaining viable alveolar tissue. Indeed, while the 1918 recombinant viruses resulted in severe pathology, the amount of damage we observed in the H5N1 animals was extreme and unique in the lack of pneumocyte hypertrophy and hyperplasia, which are sign of repair, toward day 7. As early as 24 h PI, the H5N1 virus also destroyed bronchiolar epithelium, Clara cells, which act as stem cells and metabolize toxic substances at that level of the respiratory tract, and mucus-producing cells, which facilitate mechanical clearance.

The targeting of type II pneumocytes has been seen in human patients (34) who died 7 to 10 days after becoming symptomatic. It was also noted in other nonhuman primate studies with the fully reconstructed 1918 pandemic virus (12) and other H5N1 isolates, but only for the first 48 h PI, in contrast to the present study. This suggests that even subtle differences in cell tropism may account for differences in virulence among highly pathogenic influenza viruses in the nonhuman primate model and in humans.

**High Virulence of H5N1 Virus Is Associated with Increased Host Responses.** The H5N1 virus caused intense transcriptional induction and secretion of inflammatory cytokines, as evidenced by the lung microarray and bead array data, and as documented in human patients (23, 34). IL-6, recently suggested as a key mediator of acute lung injury in mice (42), was detected at high levels in serum. An increase in circulating serum cytokines is known to damage vascular endothelial cells, resulting in vascular fluid and protein leakage, and ultimately fibrin deposition into the alveolar space (40). This finding is particularly relevant to our H5N1 infection since the fibrin organization we observed constitutes the first step toward pulmonary fibrosis and permanently reduced physiologic reserve. Inflammatory proteins can be produced by almost any infected cells and by immune cells, including alveolar macrophages (43). They can also be produced in response to oxidation of surfactant phospholipids (42), an-
other consequence of infection. However, type II pneumocytes have a marked ability to secrete large amounts of cytokines, such as TNF-α, GM-CSF, MCP, and IL-8, in response to various insults (44, 45) and can be induced to secrete IL-1β, IL-6, RANTES, MCP-2, KC2, and MCP-1, in response to TNF-α (45, 46), the last being produced by alveolar macrophages during H5N1 infection. Therefore, it is possible that the much greater cytokine induction by the H5N1 virus was as much a consequence of the response of individual, infected cells as it was a consequence of the numbers of infected cells. This cytokine and innate response was still very much in effect on day 7 in lung tissue, in contrast to the response in animals infected with other viruses. This finding was consistent with gene expression in H5N1-infected ferrets (28), and in bronchial tissues of 1918-infected mice (12). This induction did not result in comparable protein production on day 7 for the cytokines we measured, suggesting either a delay between transcription and translation or repressive mechanisms at the translation stage.

H5N1 Infection May Disrupt Immunity Beyond the Innate Response. We observed a notable disappearance of dendritic cells in the lungs of H5N1-infected animals over the course of the study. During infection, these cells express several maturation markers including CD83, which we readily detected in both lung and draining lymph nodes throughout the infection in groups other than the H5N1 animals. Conventional dendritic cells (cDC), normally present in healthy lung tissue, are key antigen-presenting cells that start migrating to draining lymph nodes within 12 h PI while secreting chemokines that attract, among others, granulocytes, seen in abundance particularly in the H5N1 group. This subset of dendritic cells, as well as its equivalent in peripheral circulation, is susceptible to H5N1 virus infection and subsequent apoptosis (47), which could partially explain our results. Plasmacytoid dendritic cells (pDC), scarce at baseline, normally become the predominant population in lung tissue ~48 h PI, where they secrete large amounts of type I IFNs and T cell chemokines (48). Therefore, these cells may have significantly contributed to the powerful IFN response that we observed on the arrays 2 days PI. pDCs are presumed to be more resistant to comparable protein production on day 7 for the cytokines we infected macaques (12). This induction did not result in convention of the response of individual, infected cells as it was a consequence of the numbers of infected cells. This cytokine and innate response was still very much in effect on day 7 in lung tissue, in contrast to the response in animals infected with other viruses. This finding was consistent with gene expression in H5N1-infected ferrets (28), and in bronchial tissues of 1918-infected mice (12). This induction did not result in comparable protein production on day 7 for the cytokines we measured, suggesting either a delay between transcription and translation or repressive mechanisms at the translation stage.

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Viral Titer. Lung samples were frozen at ~80 °C, homogenized, and tested for viable virus by TCID$_{50}$ assays using a modification of the method reported by the WHO (53). Briefly, 90% confluent MDBK cells in 96-well plates were inoculated with 100 μL of serial 10-fold dilutions of each sample, and positive and negative control samples. Plates were incubated on day 2, 4, and 7 at 37 °C for determination of TCID$_{50}$/mL by the Reed-Muench method (54).

Cytokine Bead Array. Cytokines tested were: IL-2, IL-4, IL-5, IL-6, TNF-α, IFN-γ. One sample from the left middle lung lobe was analyzed for each animal. Frozen lung tissue was homogenized in 1 mL of T-PER Tissue Extraction Reagent (Pierce) containing protease inhibitor mixture (minitabs, Roche) and centrifuged at 750 × g for 10 min at 4 °C. Cytokines in lung levels in serum and urine were assessed using the Cytometric Bead Array (CBA) Nonhuman Primate Th1/Th2 assay kit (Becton Dickinson) according to the manufacturer’s instructions. Assays were read on an LSR II flow cytometer (BD Pharmingen) and analyzed using the BD Cytometric Bead Array Software (V 1.4).

Flow Cytometry. Whole blood was incubated at room temperature for 30 min in the dark with (i) CD3 (SP34–2), CD14 (M5E2), CD20 (2H7), CD56 (B159), CD45 (H130), CD8 (H15e), CD123 (7G3), CD1a (NA134-HLK Abd Serotec), and CD163 (3G8) monoclonal antibodies to identify plasmacytoid dendritic precursors; and (ii) CD20, CD14, CD4 (L200), CD8 (RA-TPA), CD56, CD45 (Milenyi Biotech), and CD3 monoclonal antibodies to define major leukocyte subsets. Lineage-positive leukocyte subsets were defined by using monoclonal antibodies to CD3, 20, 14, and 56 labeled with APC. All antibodies were from BD Pharmingen except where noted. Red blood cells were lysed using an amo-
nium chloride solution and the resultant leukocytes were washed twice with BD PharMingen Stain Buffer, and fixed with 4% paraformaldehyde. The fixed leukocytes were washed an additional time with BD PharMingen Perm/Wash Buffer, and stained with DAPI (Molecular Probes/Invitrogen). Events were initially selected based on DNA content with additional subsets isolated by their CD45 fluorescence and side scatter properties. A total of 30,000 gated events were collected with a FACSAria or LSR II flow cytometer using FACSDiva 5.0.1 software.

Macaque Oligonucleotide Arrays Analysis and Quantitative Real-Time PCR. Total RNA was extracted from tissues for macaque oligonucleotide arrays and quantitative real-time PCR (viral gene probe sequences available upon request) as previously described (13, 15, 55). Oligoarray analyses consisted of comparing array profiles of individual lung samples (with representative pathology and degree of infection) from infected animals to pooled equal masses of mRNA from all lung lobes of 7 reference cynomolgus macaques obtained through the tissue program of the University of Washington National Primate Research Center.

ACKNOWLEDGMENTS. This work was supported by Battelle Internal Research and Development funds and by National Institutes of Health Grants P30 AI42860, P01 AI85954, R01 AI051813, and UL1 TR000445 (to A.G.-S.); R24 RR16354–04, PS1 RR01666–45, and R01 AI22646–20A1 (to M.G.K.); K08 AI059106–02 (to Cambridge Bridge Researchologies); and R03 AI075919–01 (to H.B.–O.).

Fig. S1. (A) Lung viral antigen scoring. All scores are numbers of positive cells per “N” 40× objective fields; a minimum of 10 fields were counted for each specimen; when a lung appeared negative for influenza antigen, the entire specimen was scanned using a 20× objective to verify the finding. A 2-way ANOVA confirmed that the H5N1 group was statistically different from all of the others (P < 0.001). Pairwise comparisons (Student Newman–Keuls Method, P ≤ 0.05) revealed that the H5N1 group was different from all of the others at every endpoint, with the exception of day 7. (B) The pathological changes in the lungs were scored on a scale of 0 to 6, taking into account the following features of the inflammation: degree and type of leukocyte infiltration (granulocytes, monocyte-macrophages, and lymphocytes), vascular reactions (leukocyte margination and endothelial cell hypertrophy) and/or leakage (fibrin transudation, hyaline membrane formation, and erythrocyte extravasation or frank hemorrhage), cellular necrosis/apoptosis, and exfoliation. Finally, repair was judged by the degree and extent of pneumocyte hypertrophy and hyperplasia, and fibrin-organization. The scores were: 0 = no apparent changes; 1 = minimal changes (including background “noise”); 2 = mild inflammation and/or pneumocyte hypertrophy; 3 = moderate inflammation and/or pneumocyte hypertrophy; 4 = marked inflammation and/or pneumocyte hypertrophy; 5 = severe inflammation and/or pneumocyte hypertrophy affecting less than 50% of lung tissue examined; 6 = severe inflammation and/or pneumocyte hypertrophy affecting more than 50% of lung tissue examined. Multiple serial sections from each lung lobe were examined (5–25/lobe; 7 lobes). A 2-way ANOVA confirmed that the groups were statistically different from one another (P < 0.001), with the exception of the two 1918 reassortants. Pairwise comparisons (Student Newman–Keuls method, P ≤ 0.05) revealed that the H5N1 group was different from all the others at every endpoint, with the exception of day 1 with the 1918HANA group. On other days, this latter could not be differentiated from 1918 HANANS. The Texas group was different from all of the others but the control on every endpoint. Note: Day 6 death in H5N1 group is illustrated as day 7 for simplicity.
Fig. S2. Cytokine bead arrays demonstrate the dramatic elevation of serum and lung tissue IL-6, TNF-α, and IFN-γ in the H5N1 group as compared with 1918 reassortants and Texas groups. Note: No peripheral blood sample could be harvested at day 7 from the H5N1 animal that died on day 6 PI.
Fig. S3. Absolute average CD4⁺ and CD8⁺ cell counts, measured by FACS analysis, reveal dramatic and prolonged decrease of these cells in circulation after infection in all groups, but particularly in the H5N1 animals. Note: No peripheral blood sample could be harvested at day 7 from the H5N1 animal that died on day 6 PI.
Fig. S4. Absolute individual CD83$^+$ (Lin$^-$) cell counts, measured by FACS analysis, showed a moderate decrease in circulation in all groups. The data are shown as a percent of the baseline to allow the comparison of the progression of these counts between animals, which differed on day – 14. Note: No peripheral blood sample could be harvested at day 7 from the H5N1 animal that died on day 6 PI.
Table S1. Clinical scoring system used in this experiment

<table>
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<tr>
<th>Parameter</th>
<th>Degree of parameter</th>
<th>Possible score</th>
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<tbody>
<tr>
<td>Fever</td>
<td>Normal (&lt;102.2 °F)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Elevated temperature (102.2–104 °F)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>High temperature (&gt;104 °F)</td>
<td>5</td>
</tr>
<tr>
<td>Posture/attitude</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Piloerection of body hair</td>
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</tr>
<tr>
<td></td>
<td>Decreased activity, decreasing normal behavior, piloerection</td>
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<td></td>
<td>Found dead</td>
<td>15</td>
</tr>
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<td>Respiration</td>
<td>Normal</td>
<td>0</td>
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<td>Increased or decreased; mild cough and clear nasal discharge</td>
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<tr>
<td></td>
<td>Decreased</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No stools</td>
<td>3</td>
</tr>
<tr>
<td>Weight loss</td>
<td>None or &lt;5%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&gt;5%, &lt;10%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;10%</td>
<td>4</td>
</tr>
<tr>
<td>GI distress</td>
<td>Soft stools</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Vomiting</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Diarrhea</td>
<td>3</td>
</tr>
</tbody>
</table>

Animals were monitored and scored twice daily.