A Single-Amino-Acid Substitution in a Polymerase Protein of an H5N1 Influenza Virus Is Associated with Systemic Infection and Impaired T-Cell Activation in Mice^{∇†}

Jamie L. Fornek,^{1,‡} Laura Gillim-Ross,^{2,‡§} Celia Santos,² Victoria Carter,¹ Jerrold M. Ward,³ Lily I. Cheng,³ Sean Proll,¹ Michael G. Katze,^{1,‡*} and Kanta Subbarao^{2,‡}

Department of Microbiology, University of Washington, Box 358070, Seattle, Washington 98195¹; Laboratory of Infectious Diseases, NIAID, NIH, Bldg. 33, Room 3E13C_1, 33 North Dr., MSC 3203, Bethesda, Maryland 20892²; and Comparative Medicine Branch, NIAID, NIH, Rockville, Maryland 20852³

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The transmission of H5N1 influenza viruses from birds to humans poses a significant public health threat. A substitution of glutamic acid for lysine at position 627 of the PB2 protein of H5N1 viruses has been identified as a virulence determinant. We utilized the BALB/c mouse model of H5N1 infection to examine how this substitution affects virus-host interactions and leads to systemic infection. Mice infected with H5N1 viruses containing lysine at amino acid 627 in the PB2 protein exhibited an increased severity of lesions in the lung parenchyma and the spleen, increased apoptosis in the lungs, and a decrease in oxygen saturation. Gene expression profiling revealed that T-cell receptor activation was impaired at 2 days postinfection (dpi) in the lungs of mice infected with these viruses. The inflammatory response was highly activated in the lungs of mice infected with these viruses and was sustained at 4 dpi. In the spleen, immune-related processes including NK cell cytotoxicity and antigen presentation were highly activated by 2 dpi. These differences are not attributable solely to differences in viral replication in the lungs but to an inefficient immune response early in infection as well. The timing and magnitude of the immune response to highly pathogenic influenza viruses is critical in determining the outcome of infection. The disruption of these factors by a single-amino-acid substitution in a polymerase protein of an influenza virus is associated with severe disease and correlates with the spread of the virus to extrapulmonary sites.

In 1997, an H5N1 avian influenza virus was transmitted from birds to humans in Hong Kong, resulting in 18 human infections and six deaths (7, 28). Although the viruses isolated from clinical cases were closely related both genetically and antigenically, they were associated with a range of clinical illnesses, from relatively mild influenza-like illness to acute respiratory distress syndrome and death (30). The genetic determinants of virulence of the H5N1 viruses in humans still are very poorly understood. In the 1997 H5N1 outbreak in humans, a common feature observed among patients with a severe or fatal outcome was a low peripheral white blood cell count, or lymphopenia (30). In contrast, patients who did not display leucopenia were more likely to recover.

A similar phenomenon was observed in mice infected with two representative 1997 H5N1 viruses, A/Hong Kong/483/97 (HK483) and A/Hong Kong/486/97 (HK486) (29). The infection of mice with HK483, a virus that spreads systemically, was associated with progressive lymphopenia until the time of death, whereas the infection of mice with HK486, a virus that

does not spread systemically, was associated with a transient lymphopenia, with circulating lymphocyte counts returning to baseline by 6 days postinfection (dpi). Additionally, an increased number of apoptotic cells were detected in the lungs and spleen of HK483-infected mice compared to that for HK486-infected mice. Cytokine dysregulation, severe tissue pathology, and death also were characteristic of HK483 infection in mice but not HK486 infection.

The pathogenesis of influenza viruses has been demonstrated to be polygenic, with contributions from the HA, PB2, NS, and polymerase complex protein genes (5, 11, 14, 19, 22, 24). Although all 16 isolates from the 1997 outbreak of H5N1 infections in Hong Kong shared a multibasic amino acid motif in hemagglutinin (HA) and were highly pathogenic for chickens, the viruses differed in their pathogenicity in mice (10, 12, 19, 20). While 9 of the 16 human 1997 H5N1 isolates caused systemic, lethal disease in mice, 5 of the viruses did not spread systemically and were restricted to the respiratory tract. Of the remaining two isolates, one had an intermediate phenotype, and the other virus was unable to be amplified successfully for further use. Using the systemic HK483 virus with lysine (K) at amino acid position 627 in the PB2 gene, Hatta et al. demonstrated that a single-amino-acid residue at position 627 in the PB2 gene was a key molecular determinant for systemic spread and virulence in mice (14).

The amino acid at residue 627 of PB2 previously was demonstrated to be an important host range determinant of influenza A viruses (27). Shinya et al. demonstrated that the increased lethality and systemic spread of virus observed with

* Corresponding author. Mailing address: Department of Microbiology, University of Washington, Box 358070, Seattle, WA 98195. Phone: (206) 732-6136. Fax: (206) 732-6055. E-mail: honey@u.washington.edu.

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‡ These authors contributed equally to this work.

§ Present address: Colorado Department of Public Health and Environment, 8100 Lowry Blvd., Denver, CO 80230.

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HK483 and HK486 viruses was not due to a difference in virus tropism, because viruses could productively infect cells in the brains of mice when injected directly into this organ (25). The authors hypothesized that while the immune system prevents the spread of HK486, a single-amino-acid change at residue 627 of the PB2 gene is sufficient for the virus to overwhelm the host defense mechanisms that otherwise limit infection to the respiratory tract.

We evaluated the pathogenesis and modulation of virus-host interactions in BALB/c mice inoculated with HK483, HK486, and a recombinant HK486 virus containing an E627K substitution in the PB2 gene (HK486PB2 MT). Particular emphasis was placed on understanding how the presence of lysine at position 627 of the PB2 protein affects global virus-host interactions and is sufficient to allow the systemic spread of H5N1 influenza virus. We found that differences in gene expression patterns relating to the immune response in the lungs of mice infected with HK486PB2 MT and HK483 viruses compared to that of the HK486 virus correlated with the ability of these viruses to evade the immune response and spread to other organs.

MATERIALS AND METHODS

Viruses. Influenza viruses A/HK/486/97 (HK486), A/HK/483/97 (HK483), and A/HK/486 PB2-627K/97 (HK486PB2 MT) were generated by reverse genetics as previously described (5). Briefly, HK486PB2 MT was generated using plasmids carrying genes from HK486 with an amino acid substitution (E627K) in PB2. Virus stocks were propagated in the allantoic cavity of 9- to 11-day-old embryonated specific-pathogen-free hen's eggs (Charles River Laboratories, Wilmington, MA) at 37°C. The allantoic fluids were harvested at 24 to 48 h postinfection and tested for hemagglutinating activity using 0.5% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA). Infectious allantoic fluids were pooled, aliquoted, and stored at -80°C until use. Fifty percent tissue culture infectious dose (TCID₅₀) titers were determined by the serial titration of viruses in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) as previously described (17). Virus titers were calculated by the Reed and Muench method (23).

Biosafety level 3 containment. All experiments with live virus were conducted in biosafety level 3 containment laboratories approved for use by the U.S. Department of Agriculture and the Centers for Disease Control and Prevention.

Studies in mice. Four- to 6-week-old female BALB/c mice (Taconic Farms, Inc., Germantown, NY) were used in all mouse experiments. Mouse experiments were approved by the National Institutes of Health Animal Care and Use Committee and were conducted at the NIH. Lightly anesthetized mice were inoculated intranasally with 10⁵ TCID₅₀ of HK486, HK486PB2 MT, or HK483. Viruses were diluted in Leibovitz (L-15) medium (Invitrogen, Carlsbad, CA) to a final volume of 50 µl/inoculum. Mice in the mock-infected control group received L-15 medium alone.

Virus replication. To evaluate virus replication, four mice per group were sacrificed at 2 and 4 dpi, and lungs, brains, and spleens were harvested. Each organ was divided in half, with one half stored at -80°C for virus titration and the other half placed in 1 ml of solution D (4 M guanidine thiocyanate, 0.25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) to a final concentration of 10% (wt/vol), mechanically homogenized, and stored at -80°C for later RNA extraction. Virus titers in tissue homogenates were determined in MDCK cells as previously described (17) and were calculated by the Reed and Muench method (23). The statistical significance of differences in viral titers was determined using the nonparametric Mann-Whitney test.

Morbidity and mortality in mice. Groups of eight mice per virus and eight mock-infected mice were observed daily for 16 days for clinical signs of illness, including weight loss, ruffled fur, and hunching. In accordance with the animal study protocol, mice were euthanized if they lost ≥20% of their original body weight. The oxygen saturation levels of groups of eight mice per virus and eight mock-infected mice were measured daily using a pulse oximeter as previously described (26).

Pathology. Four mice per virus and four mock-infected mice were sacrificed at 2 and 4 dpi, and lungs, brain, and spleen were harvested. Lungs were inflated

with 10% neutral buffered formalin (NBF), and 10% NBF-fixed tissues were embedded in paraffin. Four 6-µm sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry (IHC) for influenza viral antigens was performed using goat polyclonal anti-influenza H5 (BEI Resources, ATCC) at a 1:2,000 dilution with a secondary rabbit anti-goat antibody at a dilution of 1:500 and the ABC Elite reagent (Vector Laboratories, Inc., Burlingame, CA), followed by 3,3'-diaminobenzidine (DAB) as the chromogen.

RNA isolation for microarray analysis. RNA was isolated at 2 and 4 dpi from the lungs and spleen of four mice per virus and four mock-infected mice by phenol-chloroform extraction, followed by isopropanol precipitation. RNA was resuspended in solution D and repurified using RNeasy column purification (Qiagen, Valencia, CA). Total RNA samples were treated with DNA-free DNase treatment and removal reagents (Ambion, Austin, TX). RNA concentration and purity were measured with an ND-1000 Bioanalyzer (NanoDrop, Wilmington, DE) and RNA Nano LabChip technology on the 2100 Bioanalyzer (Agilent Technologies, Germany).

Annexin V staining. To determine the percentage of apoptotic cells in the lungs and spleen of virus-infected mice, four mice per virus and four mock-infected mice were sacrificed at 2 and 4 dpi, and lungs and spleen were harvested and placed in 5 ml of complete RPMI (cRPMI) (RPMI 1640, 5% fetal bovine serum, 1× antibiotic/antimycotic, 2 mM L-glutamine) on ice. Single-cell suspensions of each organ were prepared. Briefly, each spleen was passed through a 70-µm cell strainer (BD Bioscience), washed twice with cRPMI, incubated with ACK buffer (Invitrogen) for 3 to 4 min at room temperature, and washed two more times with cRPMI. Lungs were minced with scissors and incubated with 2 mg/ml collagenase I (Sigma) for 45 min at 37°C before being passed through a 70-µm cell strainer. Cells were washed twice with Hank's balanced salt solution (HBSS) (Sigma) and incubated for 3 to 4 min at room temperature with ACK lysing buffer. The remaining cells were washed twice with HBSS. The single-cell suspensions of lungs and spleen were counted using the Guava Viacount assay on the Guava EasyCyte Mini system (Guava Technologies, Inc., Hayward, CA). As a positive control, cells from uninfected mice were treated with 100 nM staurosporine and were incubated at 37°C overnight to induce apoptosis. Cells were resuspended at 0.5 × 10⁶ cells per 100 µl in phosphate-buffered saline-1% bovine serum albumin (Invitrogen). Single-cell suspensions of lungs and spleen were analyzed for the presence of early apoptotic cells using the Guava Nexin kit (Guava Technologies, Inc.).

Microarray analysis. The microarray format, protocols for probe labeling, and array hybridization are described at <http://expression.viromics.washington.edu>. Lung samples from two representative samples of each experimental condition were run on single-channel Mouse 44K oligonucleotide gene expression arrays (Agilent Technologies, Germany). This analysis allows for the direct comparison of individual infected samples (e.g., HK486 versus HK483) or infected and mock-infected samples (e.g., mock infection versus HK486 infection). One-way, textbook analysis of variance ($P \leq 0.05$) was performed to annotate statistically significant gene expression between infection conditions at either day postinfection (e.g., HK486 versus HK483 at 2 dpi). Lung data are represented as the in silico pool of the mock-infected samples from 2 dpi (two mice) and 4 dpi (one mouse) compared to the in silico pool of two mice for each infected condition at each time point. The selection of genes for data analysis was based on a greater-than 99% probability of being differentially expressed ($P \leq 0.01$) and a change (n -fold) of 1.5 (log ratio = 0.18) or greater in at least one experiment.

The analysis of spleen samples was carried out in a single experiment comparing two mRNA samples on four replicate Mouse (V2) 22K oligonucleotide expression arrays (Agilent Technologies, Germany) using the dye label reverse technique. This allows for the calculation of mean ratios between expression levels of each gene in the analyzed sample pair, standard deviations, and P values for each experiment. The selection of genes for data analysis was based on a greater-than 99% probability of being differentially expressed ($P \leq 0.01$) and a change (n -fold) of 2.0 (log ratio = 0.3) or greater in at least one experiment. Spot quantitation, normalization, and the application of a platform-specific error model was performed using Agilent's Feature Extractor software, and all data then were entered into a local installation of the LabKey server (LabKey Software Foundation, Seattle, WA) and then uploaded into Rosetta Resolver system 6.0 (Rosetta Biosoftware, Seattle, WA) and Spotfire DecisionSite for Functional Genomics 8.1 (TIBCO Software, Inc., Palo Alto, CA). Primary data, in accordance with the proposed MIAME standards, is available at <http://viromics.washington.edu> (4). This website includes all of the raw scan data, the original feature extracted data, and quality control information for these experiments. Additionally, an experimental overview, design, and corresponding sample information are listed on this website.

Gene ontology analysis was performed using Entrez Gene (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>) and MetaCore software (GeneGo, Inc., St.

TABLE 1. Sequence differences between A/HK486/97 and A/HK483/97^a

Gene	No. of coding differences	Residues with coding differences
PB2	11	9, 199, 318, 355, 382, 477, 508, 627, 675, 683, 724
PB1	5	198, 317, 197, 442, 653
PA	9	227, 426, 499, 532, 557, 595, 636, 650, 670
HA	7	125, 156, 172, 242, 336, 384, 535
NP	3	284, 319, 423
NA	9	21, 29, 34, 39, 99, 182, 198, 204, 377
M1	2	15, 23
M2	2	64, 69
NS1	4	91, 101, 189, 197, 213
NS2/NEP	3	31, 40, 56

^a GenBank accession numbers for HK486 are the following: PB2, AAK49376; PB1, AAK49359; PA, AAK49341; HA, AAF74331; NP, AAK49275; NA, AAD16788; M1, AAK49243; M2, AAK49244; NS1, AAK49307; and NS2, AAK49308. Accession numbers for HK483 are the following: PB2, AAK49375; PB1, AAK49358; PA, AAK49340; HA, AAC32099; NP, AAK49274; NA, AAD16798; M1, AAK49241; M2, AAK49242; NS1, AAK49305; and NS2, AAK49306.

Joseph, MI). For genes whose expression was upregulated twofold or more (log ratio = 0.3) in each infected group, pathway analysis was performed using MetaCore (GeneGo, Inc., St. Joseph, MI). Information in this database was manually curated to include human protein-protein, protein-DNA, and protein-compound interactions, metabolic and signaling pathways, and the effects of bioactive markers. The database construction allows for the identification of affected pathways and networks from imported lists of genes, proteins, transcripts, or compounds (<http://www.genego.com>).

RESULTS

Systemic virus spread and increased virulence observed with a single-amino-acid substitution in the PB2 protein of H5N1 viruses. There are 55 coding differences between HK483 and HK486 viruses present throughout the genome (Table 1); however, an E-to-K substitution at aa 627 of the PB2 protein gene of HK483 is a virulence determinant in mice (14). To determine if differences in replicative capacity could explain the systemic spread of H5N1 viruses containing this mutation and to test the replicative capacity of the recombinant viruses used in this study, virus titers were measured in mice infected with 10^5 TCID₅₀ of HK486, HK486PB2 MT, and HK483 viruses. The kinetics of viral replication includes the magnitude (peak titer achieved) and temporal measures, including the day on which peak replication occurs and the duration of replication. It is not clear whether the magnitude and/or duration of pulmonary viral replication are determinants of systemic spread. Peak virus titers in the lungs of mice were $10^{5.5}$, $10^{7.1}$, and $10^{7.6}$ TCID₅₀/g in mice infected with HK486, HK486PB2 MT, and HK483, respectively (Fig. 1A). At 2 dpi, titers in the lungs of mice infected with HK486 were significantly lower than titers observed in mice infected with HK486PB2 MT and HK483 ($P < 0.05$); however, by 4 dpi, the observed differences were not statistically significant (Fig. 1A; also see Fig. S1A in the supplemental material). These findings were reproducible in replicate experiments (see Fig. S1A in the supplemental material) and indicate that although the peak titers differed, all three viruses replicated to high titers in the lung. There was no difference in the expression of viral HA and M1 RNA in the

lungs as measured by quantitative PCR (see Fig. S1B in the supplemental material), and no differences in viral antigen staining were observed in the lungs of the three infection groups (Fig. 2A, right).

The infection of mice with HK486 did not result in systemic spread, as virus was detected just above the limit of the assay in the brain and spleen of only one of four infected mice. In contrast, virus was detected in the brain and spleen of all mice infected with both HK483 and HK486PB2 MT, indicating the systemic spread of virus, which is consistent with previous reports (14, 19, 29). Peak virus titers ($10^{5.4}$ to $10^{6.3}$) were detected at 2 dpi in the spleen of mice infected with HK483 and HK486PB2 MT, with titers decreasing by approximately 1,000-fold by 4 dpi with no significant differences between the two viruses. Peak virus titers were detected at 4 dpi in the brain of HK483- and HK486PB2 MT-infected mice, although the titer achieved differed significantly between the two viruses.

Each of the H5N1 viruses caused significant morbidity and mortality in the mice; infection with HK483 and HK486PB2 MT was associated with 100% lethality by 4 and 5 dpi, respectively, while infection with a similar dose of HK486 was lethal for 75% of the inoculated mice by 8 dpi. (Fig. 1B). Additionally, a significant decrease in oxygen saturation levels (from 93% to a range of 80 to 83%) was observed in mice infected with HK483 and HK486PB2 MT at 4 dpi, corresponding with the time of death (Fig. 1C). While oxygen saturation levels decreased in HK486-infected mice, levels were significantly higher than those observed in HK483- and HK486PB2 MT-infected mice at 4 dpi. These data indicate that at an infectious dose of 10^5 TCID₅₀, all viruses used in this study were lethal; however, early death was associated with the systemic spread of infection.

A single-amino-acid change in the PB2 protein affects the lung and splenic lesions associated with H5N1 virus infection. The response to H5N1 infection in the lungs was evaluated by histopathological examination to determine if differences in the extent and/or nature of lung damage were associated with the systemic spread of HK483 and HK486PB2 MT viruses. At 2 dpi, all infected animals experienced moderate lung involvement with bronchial and bronchiolar necrosis (data not shown). Lung involvement was severe in all infected mice by 4 dpi (Fig. 2, left); severe bronchiolar necrosis and perivascular cuffing were observed in HK486-, HK486PB2 MT-, and HK483-infected mice. These data demonstrate that damage to the larger airways is similar in mice infected with each of the viruses and that severe lung lesions in the larger airways are not responsible for the extrapulmonary spread of HK486PB2 MT and HK483.

In contrast to the larger airways, there were differences in the alveolar lesions observed in the HK486-, HK486PB2 MT-, and HK483-infected mice. HK486PB2 MT- and HK483-infected mice demonstrated moderate to severe interstitial pulmonary inflammation at both 2 and 4 dpi (Fig. 2A, left, and data not shown). These mice also demonstrated increased hemorrhage, edema, and vasculitis. Taken together, these data demonstrate that there is a correlation between early death in HK486PB2 MT- and HK483-infected mice and alveolar disease. These findings correlate with the decrease in oxygen saturation observed during infection, which was especially notable at 4 dpi (Fig. 1C). Alveolar lesions were observed to a

FIG. 1. Replication and pathogenicity of H5N1 viruses in mice. Groups of four mice were inoculated intranasally with 10^5 TCID₅₀ of HK486, HK486PB2 MT, and HK483. (A) Lungs, brain, and spleen were harvested on 2 and 4 dpi, and virus titers were determined in MDCK cells. Each dot represents one animal, and black bars represent the mean viral titers for the four mice in each group. The limit of detection ($10^{1.8}$) is denoted by the dotted line. (B) Groups of eight mice were weighed daily for 16 dpi and were sacrificed if a weight loss of $\geq 20\%$ from the original weight was observed. (C) Oxygen saturation levels of groups of H5N1-infected mice were measured daily for 5 dpi using a pulse oximeter. Data points represent the means $\pm$ standard deviations for eight mice. Differences between HK486PB2 MT- and HK483-infected mice and HK486-infected mice were statistically significant at 4 dpi (*, $P < 0.005$).

lesser extent in HK486-infected mice, correlating with a significantly smaller decrease in oxygen saturation. Since both HK486PB2 MT and HK483 viruses spread to extrapulmonary sites, including the spleen and the brain, we

examined both organs for differences in the types or severity of the lesions. Differences in the nature or severity of the lesions were not observed among the brains from HK486PB2 MT-, HK483-, and HK486-infected mice (data not shown). In

contrast, prominent differences were found in splenic germinal center (GC) lesions between HK486-infected mice and HK486PB2 MT- and HK483-infected mice. As early as 2 dpi, HK486PB2 MT- and HK483-infected mice developed the hyperplasia of GCs and marked GC necrosis (Fig. 2C and D), while the GC architecture was intact in HK486-infected mice (Fig. 2B). Immunohistochemical staining revealed the presence of viral antigen-positive cells in the necrotic GCs of HK486PB2 MT- and HK483-infected mice but not in the HK486-infected mice (Fig. 2E and data not shown).

Increased numbers of apoptotic cells observed in the lungs of mice infected with HK486PB2 MT and HK483. Another potential cause for the increased virulence of HK486PB2 MT and HK483 viruses in mice is increased apoptosis in the lungs (29). Both flow cytometric analysis for annexin V staining and the microarray analysis of apoptosis-associated gene expression were performed. Lung cells isolated at 4 dpi from mice infected with each of the H5N1 viruses were stained with annexin V, a marker for the translocation of phosphatidyl serine to the outer cell surface membrane, which occurs in the early stages of apoptosis. A significant increase in the percentage of cells in the early stages of apoptosis was observed in the lungs of mice infected with HK483 and HK486PB2 MT (Fig. 3A). The percentage of early apoptotic cells in the lungs of HK486-infected mice did not differ significantly from that of mock-infected mice.

Pathway analysis using MetaCore software from GeneGo, Inc., was performed to examine gene expression profiles in the lungs of H5N1-infected mice. Figure 3B is a pathway depiction of the caspase cascade. Red and blue bars represent up- and downregulated genes, respectively. The extent of up- and downregulation of a particular gene is depicted by the amount of shading within a given bar. Bars 1, 2, and 3 represent lung samples taken at 4 dpi from HK486-, HK486PB2 MT-, and HK483-infected mice, respectively. Consistent with the observation described above, microarray analysis demonstrated the increased expression of genes in the caspase cascade in lungs of HK483- and HK486PB2 MT-infected mice relative that of to HK486-infected mice at 4 dpi (Fig. 3B, compare bars 2 and 3 to bar 1). Among these genes were *Tnf*, *Tnfrsf1a* (*Tnfr1*), *Tnfsf6*, *Ripk1*, *Bid*, *Casp7*, *Casp3*, and *Casp4*. *Tnfsf6* (*FasL*) mediates the apoptosis of lymphocytes (activation-induced death). *Bid* is cleaved by caspase 8 and translocates to the mitochondria, where it mediates apoptosis through the release of cytochrome *c*. Gene expression levels related to this pathway are presented in Table S1 in the supplemental material. While the activation of the cascade is a posttranslational process, the gene expression changes observed here indicate that the over-activation of certain members of this cascade leads to the enhanced cell death, potentially of lymphocytes, that we ob-

serve in the lungs of HK483- and HK486PB2 MT-infected mice at 4 dpi.

Sustained activation of immune and inflammatory processes in the lungs of H5N1-infected mice correlates with systemic spread of virus. To assess global differences in the host response to H5N1 influenza viruses that spread systemically or remain confined to the lungs, gene expression profiling was performed on the lungs of HK486-, HK486PB2 MT-, and HK483-infected mice. The analysis of differentially regulated processes in the lungs of H5N1 virus-infected mice revealed that there were notable differences in gene expression related to immune and inflammatory responses, particularly at 4 dpi (Table 2).

We focused our analysis on processes in which gene expression was demonstrated to be statistically different by analysis of variance in one of four comparisons performed (HK486 versus HK483 at 2 dpi, HK486 versus HK486PB2 MT at 2 dpi, HK486 versus HK483 at 4 dpi, or HK486 versus HK486PB2 MT at 4 dpi) (Table 2). At 4 dpi, inflammatory processes such as interferon (IFN) signaling, IFN- γ signaling, NK cell cytotoxicity, and the Kallikrein-kinin system still were strongly upregulated in HK483- and HK486PB2 MT-infected mice. In contrast, these responses appear to be resolving in HK486-infected mice. These results suggest that the sustained activation of inflammatory responses in the lungs of HK483- and HK486PB2 MT-infected mice facilitate the replication of these viruses in extrapulmonary sites.

Impaired TCR signaling in the lungs of HK486PB2 MT- and HK483-infected mice. The analysis of differentially regulated processes in the lungs of H5N1 virus-infected mice revealed that there were notable differences in gene expression related to T-cell receptor (TCR) signaling (Fig. 4A, Table 2). A distinct subset of genes related to TCR signaling is upregulated preferentially in the lungs of HK486-infected mice at 2 dpi. The further examination of this subset of genes revealed multiple components of the TCR complex. For example, *Cd3d*, *Cd3g*, and *Cd247* (*CD3 zeta*) were upregulated in the lungs of HK486-infected mice at this time point. Additionally, members of the CD8 coreceptor, *Cd8a* and *Cd8b1*, were upregulated.

Pathway analysis was performed using MetaCore software from GeneGo, Inc., to further elucidate the impaired TCR activation. Figure 4B is a depiction of the major histocompatibility complex class I (MHC I) processing and presentation pathway. Gene expression levels related to this pathway are presented in Table S2 in the supplemental material. Red and blue bars represent up- and downregulation, respectively. The extent of up- and downregulation of a particular gene is depicted by the amount of shading within a given bar. Numerical representations are given for individual samples. Bars 1, 2, and 3 represent lung samples taken at 2 dpi from HK486-,

FIG. 2. Lung and splenic lesions associated with H5N1 virus infection. (A) Histopathology of lungs from mice infected with HK486, HK486PB2 MT, and HK483 at 4 dpi were stained with H&E (left), and IHC was performed for H5N1 viral antigen (brown color on the right). Viral antigen is found in the bronchioles (B) and alveoli (A) of mice in each group except the mock infection group. More alveolar inflammation and viral antigen is present in the lungs of mice given HK486PB2 MT and HK483 strains. (B to E) Spleen of HK486-, HK486PB2 MT-, and HK483-infected mice at 2 dpi were stained with H&E (B to D), and IHC was performed for H5N1 viral antigen (E). Note the large pale GCs in panels C, D, and E but not in panel B and viral antigen (brown color) in panel E. The magnification for lung is $\times 200$ and for spleen is $\times 400$. The splenic section in panel E was taken from an experiment separate from those in panels B to D.

A

Lung

B

FIG. 3. Examination of early apoptosis and apoptosis-related gene expression in the lungs of H5N1-infected mice. (A) Lungs were isolated from mice infected with HK486, HK486PB2 MT, and HK483 at 4 dpi and were subjected to annexin V staining to determine the percentage of cells in early apoptosis. As a positive control, cells from uninfected mice were treated with 100 nM staurosporine overnight at 37°C. The means and standard errors for groups of four mice are presented. Statistical significance was determined using the unpaired *t* test. *, $P < 0.05$. (B) RNA was isolated from the lungs of mice infected with the H5N1 viruses mentioned in panel A and subjected to gene expression profiling. Represented is the expression of genes related to the caspase cascade as analyzed using MetaCore software (GeneGo, Inc.) at 4 dpi. Thresholds for inclusion in analysis were a change (*n*-fold) of 1.5 or greater (log ratio = 0.18) relative to levels for the mock infection group in at least one experiment at $P \leq 0.01$. Expression bars represent HK486 on day 4 (bar 1), HK486PB2 MT on day 4 (bar 2), and HK483 on day 4 (bar 3). Red bars and blue bars represent genes whose expression was up- or downregulated, respectively, by at least 1.5-fold (log ratio = 0.18) at $P \leq 0.01$ relative to results for the mock infection group. The extent of shading within each bar represents the magnitude of up- or downregulation. Red, green, and blue lines and hexagons represent negative, positive, and unspecified effects, respectively. Lettering within hexagons is as follows: B, binding; C, cleavage; +P, phosphorylation; and Tn, transport. TNF, tumor necrosis factor.

TABLE 2. Affected processes in the lungs of H5N1-infected mice related to inflammation^e

GeneGo process ^a	Day 2				Day 4				Total no. of objects ^d				
	HK486		HK486PB2 MT		HK483		HK486			HK486PB2 MT		HK483	
	<i>P</i> value ^b	No. of objects ^c	<i>P</i> value	No. of objects	<i>P</i> value	No. of objects	<i>P</i> value	No. of objects		<i>P</i> value	No. of objects	<i>P</i> value	No. of objects
Immune													
Antigen presentation	6.60E-09	72	1.39E-04	69	6.24E-03	67	2.41E-06	51	1.59E-04	74	6.40E-03	74	193
BCR pathway	4.70E-03	40	1.85E-02	44	1.56E-02	48	7.34E-01	17	5.49E-01	36	1.92E-01	46	137
Phagocytosis	2.46E-05	68	1.16E-02	67	2.33E-04	81	9.24E-03	43	1.32E-01	65	2.90E-02	78	217
Phagosome in antigen presentation	6.88E-05	74	2.72E-02	73	7.56E-03	83	5.01E-02	44	2.18E-02	80	7.07E-02	85	248
TCR signaling	8.26E-04	47	1.48E-01	43	6.59E-03	55	4.84E-01	22	5.09E-01	41	4.58E-01	47	154
Inflammation													
Amphoterin signaling	8.36E-04	38	8.06E-04	44	2.46E-07	57	1.04E-02	26	2.72E-02	41	3.19E-02	45	118
Complement system	1.79E-01	18	8.87E-02	23	5.78E-01	19	4.07E-02	16	8.48E-02	25	3.26E-01	24	73
Histamine signaling	3.44E-05	67	6.10E-03	68	1.46E-05	85	2.40E-04	49	5.17E-03	74	8.00E-04	86	215
IFN-γ signaling	7.51E-08	46	2.51E-05	46	1.90E-06	52	8.66E-06	33	2.97E-04	46	4.74E-03	46	110
IgE signaling	4.30E-04	44	2.10E-02	44	1.28E-03	53	3.95E-02	27	4.92E-01	37	8.64E-02	49	138
IL-10 antiinflammatory response	4.52E-04	31	6.13E-03	32	2.07E-04	39	1.66E-03	23	4.57E-04	38	9.61E-03	37	88
IL-12, IL-15, IL-18 signaling	1.57E-02	19	1.54E-02	22	2.36E-02	23	5.90E-02	13	1.25E-01	20	2.91E-01	20	59
IL-2 signaling	2.35E-03	33	1.49E-01	30	3.95E-02	36	8.75E-01	11	2.47E-01	31	2.95E-01	34	104
IL-4 signaling	2.21E-04	39	5.78E-03	40	1.07E-02	42	7.22E-02	22	6.71E-02	38	7.21E-02	42	115
IL-6 signaling	2.28E-04	40	3.52E-03	42	1.19E-03	47	2.11E-02	25	1.04E-01	38	1.15E-01	42	119
Inflammasome	1.17E-04	39	4.33E-05	46	2.18E-05	50	3.52E-05	32	3.47E-03	43	4.00E-03	47	112
Innate inflammatory response	1.55E-06	62	3.73E-07	73	2.07E-09	84	2.74E-08	53	8.37E-07	77	1.72E-06	83	179
IFN signaling	6.08E-20	65	1.08E-16	67	1.84E-16	70	6.88E-16	50	5.66E-12	63	1.55E-08	61	110
Jak-STAT pathway	3.46E-06	61	6.71E-05	66	2.26E-04	69	7.69E-03	37	2.04E-03	65	1.10E-02	68	179
Kallikrein-kinin system	4.45E-03	51	4.97E-02	54	6.33E-02	58	1.82E-02	36	6.21E-02	58	1.68E-01	61	183
MIF signaling	1.20E-02	39	1.05E-02	46	1.62E-05	60	1.32E-03	33	1.42E-04	57	1.00E-02	55	140
Neutrophil activation	1.77E-06	73	9.06E-04	74	4.74E-06	89	2.14E-02	42	8.17E-03	75	2.56E-02	80	222
NK cell cytotoxicity	2.57E-08	60	7.91E-04	55	7.64E-03	55	7.30E-03	33	5.99E-02	50	2.44E-03	63	155
Protein C signaling	6.51E-02	27	1.39E-02	35	1.05E-04	45	3.22E-03	25	3.37E-02	36	2.07E-01	35	103
TREM1 signaling	4.02E-05	49	2.69E-03	50	5.82E-06	63	4.24E-02	28	1.85E-02	50	8.74E-03	57	145

^a Functional process assigned to genes whose expression differed at by least 1.5-fold (log ratio = 0.18) and $P < 0.01$ in at least one experiment relative to results for the mock infection group. IgE, immunoglobulin E; IL-10, interleukin-10.

^b The P value was calculated by MetaCore GeneGo software for a given process based on the gene list and expression values indicated in footnote *a*.

^c Number of objects within a given GeneGo process affected in a particular sample. An object in MetaCore GeneGo may represent proteins, protein complexes or groups, RNA species, compounds, EC numbers (functions), or interactions. A gene is represented indirectly by the protein it encodes.

^d Total number of objects within a GeneGo process.

^e Boldface indicates processes in which gene expression was demonstrated to be statistically different by analysis of variance in at least one of four comparisons performed (HK486 versus HK483 on day 2, HK486 versus HK486PB2 MT on day 2, HK486 versus HK483 on day 4, or HK486 versus HK486PB2 MT on day 4).

A

B

HK486PB2 MT-, and HK483-infected mice, respectively. Bars 4, 5, and 6 represent lung samples taken at 4 dpi from HK486-, HK486PB2 MT-, and HK483-infected mice, respectively. As represented in Fig. 4B, MHC I processing and presentation is intact at 2 dpi in the lungs of HK486-, HK486PB2 MT-, and HK483-infected mice (bars 1, 2, and 3 in the upper portion of the pathway). In contrast, TCR activation is observed only in HK486-infected mice at this time point (bar 1 in the lower portion of the pathway). It is possible that the impaired or inefficient activation of TCR signaling and costimulation leads to inefficient antigen presentation by MHC I in HK483- and HK486PB2 MT-infected mice (Fig. 4B, bars 2 and 3). As a result, CD8 T cells in the lungs of these animals may become anergic or undergo apoptosis. HK483 and HK486PB2 MT viruses may evade the cell-mediated immune response by preventing the activation of CD8 T cells. The inability of CD8 T cells to control HK483 and HK486PB2 MT infection in the lungs may allow these viruses to replicate in extrapulmonary sites.

Enhanced activation of immune response pathways in the spleen of HK486PB2 MT- and HK483-infected mice. Since the increased severity of histopathologic lesions was observed in the spleen of HK486PB2 MT- and HK483-infected mice, gene expression profiling was performed on this tissue. Unsupervised clustering demonstrated that HK486-infected mice clustered with the HK486PB2 MT- and HK483-infected mice at 2 dpi (see Fig. S2 in the supplemental material). At this time point, the HK486PB2 MT-infected mice clustered with the HK483-infected mice; however, by 4 dpi they clustered with HK486-infected mice (see Fig. S2 in the supplemental material). Mice infected with HK483 had the most robust host response at 4 dpi and did not cluster with HK486- and HK486PB2 MT-infected mice. Gene expression profiling revealed striking differences in the kinetics of the early host response in the spleen of HK486PB2 MT- and HK483-infected mice. These differences may be linked to the increased virulence of these viruses.

Gene expression profiles in the spleen demonstrated that certain critical innate and early adaptive immune processes were differentially affected. Induced processes included NK cell cytotoxicity, antigen presentation, and interferon signaling (Fig. 5A to C).

Gene expression related to NK cell cytotoxicity was upregulated similarly in HK486PB2 MT- and HK483-infected mice at 2 dpi, while this response was not induced in the spleen of HK486-infected mice at this time point (Fig. 5A). At 4 dpi, the response still was upregulated in HK486PB2 MT-infected mice and now was upregulated in HK486-infected mice; however,

the response was most strongly induced in HK483-infected mice. Genes whose expression was preferentially upregulated in the spleen of HK486PB2 MT- and HK483-infected mice at both time points after infection included mediators of NK cell-induced killing, such as *Gzma* (granzyme A), *Gzmb* (granzyme B), and *Srgn* (serglycin). The expression of the NK inhibitory receptor *Klrd1* (CD94/NKG2A) also was preferentially upregulated in HK486PB2 MT- and HK483-infected mice at 4 dpi. *Cd2*, which is involved in NK cell- and cytotoxic T-cell-mediated cell destruction, was upregulated in HK486PB2 MT- and HK483-infected mice at 2 dpi and in HK486- and HK483-infected mice at 4 dpi.

Perhaps the most interesting differences in the spleen of H5N1-infected mice were related to antigen processing and presentation (Fig. 5B). *Tap1* and *Tap2*, which are necessary for the transport of antigenic peptides from the cytosol to the endoplasmic reticulum for loading onto MHC I, were upregulated in the spleens of HK486PB2 MT- and HK483-infected mice, but not HK486-infected mice, at both 2 and 4 dpi. Another component of the TAP complex, *Tapbp*, and *B2m* (β_2 -microglobulin), which associates with the MHC I α chain in the endoplasmic reticulum prior to antigenic peptide loading, were upregulated in HK486PB2 MT- and HK483-infected mice at both 2 and 4 dpi and in HK486-infected mice only at 4 dpi.

Certain genes related to MHC II presentation also were differentially expressed in the spleens of H5N1-infected mice. *Ctss* (cathepsin S), which degrades antigenic proteins into peptides for binding to MHC II molecules, was activated in HK486PB2 MT- and HK483-infected mice throughout the course of infection but only at 4 dpi in HK486-infected mice. *Ifi30*, which is proposed to play a role in MHC II processing, was upregulated only at 4 dpi in HK483-infected mice, and *Cd74*, which associates with newly formed MHC II, was upregulated in HK486- and HK483-infected mice at 4 dpi.

Antigen presentation to CD4 and CD8 T cells is required for an adaptive immune response to influenza infection. Therefore, we assessed whether gene expression changes related to T-cell activation were present in H5N1 virus-infected mice. *Cd3d*, the delta chain of one of the CD3 proteins that are associated with the TCR, was upregulated in the spleen of HK486PB2 MT- and HK483-infected mice at 2 dpi and in HK486- and HK483-infected mice at 4 dpi. Components of the CD8 coreceptor, *Cd8a* and *Cd8b1*, were upregulated in HK483-infected mice throughout infection, while *Cd8b1* was expressed in the spleen of HK486-infected mice at 4 dpi.

The IFN response was greatly upregulated in the spleen of HK486PB2 MT- and HK483-infected mice at both 2 and 4 dpi. For example, the expression of *Ifng*, *Ifit2*, *Gbp2*, and

FIG. 4. Impaired TCR activation in the lungs of HK483- and HK486PB2 MT-infected mice at 2 and 4 dpi. (A) Hierarchical clustering of selected genes related to TCR signaling in the lungs of HK486-, HK486PB2 MT-, and HK483-infected mice at 2 and 4 dpi based on analysis using MetaCore software (GeneGo, Inc.). Thresholds for inclusion in analysis were a change (n -fold) of 1.5-fold or greater (log ratio = 0.18) relative to results for the mock infection group in at least one experiment at $P \leq 0.01$. Green and red shading represent genes whose expression was down- or upregulated, respectively. (B) Antigen presentation by MHC I using MetaCore software (GeneGo, Inc.). Expression bars represent HK486 on days 2 (bar 1) and 4 (bar 4), HK486PB2 MT on days 2 (bar 2) and 4 (bar 5), and HK483 on days 2 (bar 3) and 4 (bar 6). Red bars and blue bars represent genes whose expression was up- or downregulated, respectively, by at least 1.5-fold (log ratio = 0.18) at $P \leq 0.01$ relative to results for the mock infection group. The extent of shading within each bar represents the magnitude of up- or downregulation. Red, green, and blue lines and hexagons represent negative, positive, and unspecified effects, respectively. Lettering within hexagons is as follows: B, binding, Tn, transport; and CS, complex subunit.

FIG. 5. Key processes related to the innate and early adaptive immune responses are preferentially upregulated in the spleen of HK483- and HK486-infected mice early during infection. The hierarchical clustering of selected genes related to (A) NK cell cytotoxicity, (B) antigen presentation, and (C) interferon signaling in the spleen of HK486-, HK486PB2 MT-, and HK483-infected mice at 2 and 4 dpi. Thresholds for inclusion in analysis were a change (*n*-fold) of twofold or greater (log ratio = 0.3) in at least one experiment at $P \leq 0.01$. Green and red shading represent genes whose expression was down- or upregulated, respectively. Graphs beneath each heat map represent genes whose expression was twofold (log ratio = 0.3) upregulated (UP) at $P \leq 0.01$ in an experimental group at a given time point postinfection based on analysis using MetaCore software (GeneGo).

Socs1 was upregulated in HK486PB2 MT- and HK483-infected mice by 2 dpi. This response was activated in HK486-infected mice by 4 dpi but never to the same extent as in HK486PB2 MT- and HK483-infected mice (Fig. 5C). These data suggest an enhanced activation of the inflammatory

response in the spleen of HK486PB2 MT- and HK483-infected mice. We speculate that the enhanced inflammatory response in an extrapulmonary site in HK483- and HK486PB2 MT-infected mice leads to the increased and early mortality observed in these mice.

DISCUSSION

The outcome of H5N1 virus infection *in vivo* is determined by the kinetics and magnitude of viral replication and the host's response to contain the infection. In this study, we have utilized conventional biologic techniques in conjunction with functional genomics to examine how a single-amino-acid substitution in the PB2 protein of a 1997 H5N1 influenza virus isolate leads to the spread of the virus from the lungs. Our results suggest that the kinetics and magnitude of replication correlates with a sustained inflammatory response and impaired TCR activation in the lungs of HK483- and HK486PB2 MT-infected mice. This results in an inability of HK483- and HK486PB2 MT-infected mice to contain the infection at the primary site of replication. As a result, the virus spreads to extrapulmonary sites, including the spleen. An enhanced immune response in the spleen of HK483- and HK486PB2 MT-infected mice leads to GC necrosis and is associated with increased mortality. What is remarkable is that an E-to-K substitution at aa 627 in the PB2 gene of a nonsystemic virus (HK486) leads to gene expression patterns that are similar to that in the lungs of HK483-infected mice and causes this mutant virus to behave in a similar fashion, although the genomes of the mutant virus and HK483 still differ by 54 aa.

All H5N1 viruses examined in this study replicated to high titers in the lungs of infected mice. Virus replication also was detected in the spleen and brain of HK486PB2 MT- and HK483-infected mice. Differences were observed in the pattern of mortality associated with the HK486, HK486PB2 MT, and HK483 viruses. Infection with either HK486PB2 MT or HK483 resulted in 100% mortality, with an average time to death of 5 and 4 dpi, respectively, while HK486-infected mice exhibited 75% mortality and a delay in the average time to death of 8 dpi. Our data suggest that it is not enhanced replication in the lungs of HK486PB2 MT- and HK483-infected mice that lead these viruses to disseminate.

A potential explanation for the extrapulmonary spread of virus in HK486PB2 MT- and HK483-infected mice is an increase in cell death (29). We observed an increase in the number of cells in early apoptosis in the lungs of HK486PB2 MT- and HK483-infected mice at 4 dpi. Genomic analysis revealed that the expression of genes related to caspase signaling and apoptosis was more highly upregulated in HK483- and HK486PB2 MT-infected mice at 4 dpi and that the increase in cell death potentially is linked to the apoptosis of lymphocytes. This observation is consistent with the progressive lymphopenia observed in HK483-infected mice (29). Taken together, our results suggest that the presence of a lysine at aa 627 of the PB2 protein of the 1997 H5N1 viruses may enhance virulence by driving host cells to apoptosis.

Our findings on the gene expression profiling of the lungs suggest that the sustained activation of the inflammatory response and the impaired activation of TCR signaling in the lungs of HK486PB2 MT- and HK483-infected mice compared to that of HK486-infected mice permit the dissemination of these viruses to other organs. Impaired TCR activation correlates with the increased viral replication observed in the lungs of HK486PB2 MT- and HK483-infected mice at 2 dpi.

A potential explanation for impaired TCR activation in HK483- and HK486PB2 MT-infected mice is impaired antigen

recognition resulting in the disruption of immunological synapse formation. The immunological synapse forms when the TCR complex comes into physical contact with antigen-presenting cells displaying MHC-associated peptides (9). This contact leads to the mobilization of the TCR complex, CD4 or CD8 coreceptors, and enzymes and adaptor proteins. The formation of the immunological synapse results in downstream signaling that is necessary for T-cell activation. Our data demonstrate that antigen processing and presentation by antigen-presenting cells is not impaired in HK483- and HK486PB2 MT-infected mice at 2 dpi compared to that of HK486-infected mice. In contrast, components of the TCR complex, the delta and gamma subunits of CD3 (Cd3d and Cd3g) and the TCR zeta chains (Cd247), along with components of the CD8 coreceptor (Cd8a and Cd8b1), are preferentially upregulated in the lungs of HK486-infected mice at 2 dpi. As a result, the expression of mediators of downstream signaling, such as Lat and Grap2, a member of the GRB2/Sem5/Drk family of adapter proteins, is absent or reduced in HK483- and HK486PB2 MT-infected mice.

The role of NK cells and the recognition of influenza by their receptors continues to be elucidated (8, 13, 15, 18). Mice lacking the NK cell-activating receptor *Ncr1* are more susceptible to influenza infection (13). Infection with the reconstructed 1918 virus led to the early activation of NK cell-related genes, and the aberrant regulation of NK cell-related transcriptional responses was observed in the lungs of mice infected with the reconstructed 1918 influenza virus (18). Gene expression profiles in the spleen revealed that there was an upregulation of NK cell responses in HK486PB2 MT- and HK483-infected mice at this time point. Recently, Achdout and colleagues have demonstrated that influenza binding to NK cell-inhibitory receptors leads to the reorganization of MHCI proteins on infected cells (1). It is possible that both HK486PB2 and HK483 viruses promote the expression of NK-inhibitory receptors on NK cells that results in the inefficient killing of infected cells by NK cells and also contribute to the prolonged reorganization of MHCI proteins. The reorganization of MHCI proteins on infected cells would not only hinder NK cell killing but also could have profound effects on antigen processing and presentation.

In the spleen, the upregulated expression of antigen presentation-related genes was observed at 2 dpi in HK486PB2 MT- and HK483-infected mice. Many of these genes were related to CD4⁺ and CD8⁺ T-cell activation. Of note, no difference was found in the percentages of CD4⁺ and CD8⁺ T cells between HK486- and HK483-infected mice (29), suggesting that the transcriptional changes that we observe between these two strains are not due to differences in T-cell numbers.

Interestingly, we observe GC hyperplasia and necrosis in the spleen of HK486PB2 MT- and HK483-infected mice as early as 2 dpi. This finding may be linked to the upregulation of antigen presentation and processing related genes at this time point. Even though there is an increase in antigen presentation, this does not mean that there is a productive and prolonged interaction between B cells and T cells in the GC. Recently, Allen and colleagues demonstrated that most B-T interactions in the GC are transient (3). One proposed model of B-T interactions in the GC suggests that even if there is adequate B-cell receptor (BCR) signaling, inadequate help from T cells will lead to

B-cell apoptosis. Another proposed model suggests that inadequate BCR signaling in the GC leads to B-cell apoptosis (2). The upregulation of genes related to MHCII processing and presentation in the spleen of HK486PB2 MT- and HK483-infected mice at 2 dpi suggests that there is adequate BCR signaling in the GC of these mice; however, it is possible that these B cells are not receiving adequate T-cell help, leading to GC necrosis.

Gene expression profiles in the spleen at 4 dpi demonstrated that HK486PB2 MT virus infection more closely resembled HK486 virus infection. HK483-infected mice exhibited the most profound upregulation of host response genes in the spleen at 4 dpi. Many coding differences are present between the genomes of HK486 and HK483 viruses. The differences in gene expression profiles in the spleen between HK486PB2 MT- and HK483-infected mice at 4 dpi may be a result of one or more of these differences. It is important to note that even though gene expression profiles differ between these two systemic infections at 4 dpi, the outcome of infection is still the same; there is an increase in virulence associated with both of these viruses.

The outcome of H5N1 virus infection is determined by the kinetics and magnitude of viral replication and the host response to contain the infection. Our results suggest that increased virus replication correlates with impaired TCR activation in response to H5N1 viruses containing a lysine at position 627 of the PB2 protein and allows the virus to spread to and replicate in extrapulmonary sites. To further assess an association of delayed TCR activation and systemic-spread of H5N1 viruses containing the PB2 mutation, future studies will be directed at understanding the effect of these viruses on mice with impaired or lacking T-cell responses. Distinct mutations present throughout the polymerase complex may be responsible for the virulence of H5N1 viruses (11, 16, 24) and infection with the pandemic 1918 influenza virus (6). Recently, Pappas et al., demonstrated that, in conjunction with HA and NA genes, the PB1 gene of the pandemic 1918 virus is necessary for the increased pathogenicity (22), further emphasizing that the polymerase genes are critical determinants of virulence. The molecular mechanism for the behavior of a virus with PB2 627K instead of PB2 627E is not known; it may be related to a cellular interacting protein (21). Future studies should focus on the mechanism(s) by which distinct mutations in genes of the polymerase complex lead to the enhanced pathogenicity of highly lethal influenza viruses. Such studies will be crucial in understanding the virulence of influenza viruses.

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