Local Innate Immune Responses and Influenza Virus Transmission and Virulence in Ferrets

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Host innate immunity is the first line of defense against invading pathogens, including influenza viruses. Ferrets are well recognized as the best model of influenza virus pathogenesis and transmission, but little is known about the innate immune response of ferrets after infection with this virus. The goal of this study was to investigate the contribution of localized host responses to influenza virus pathogenicity and transmissibility in this model by measuring the level of messenger RNA expression of 12 cytokines and chemokines in the upper and lower respiratory tracts of ferrets infected with H5N1, H1N1, or H3N2 influenza viruses that exhibit diverse virulence and transmissibility in ferrets. We found a strong temporal correlation between inflammatory mediators and the kinetics and frequency of transmission, clinical signs associated with transmission, peak virus shedding, and virulence. Our findings point to a link between localized innate immunity and influenza virus transmission and disease progression.

Influenza A viruses are a persistent global public health problem causing seasonal epidemics, occasional pandemics, and sporadic zoonotic infections. The disease burden of influenza viruses depends predominately on transmission efficiency and virulence in the human population. Seasonal influenza viruses cause a highly contagious disease and can be associated with >300 000 deaths annually worldwide, mostly among those aged ≥65 years [1]. The last 4 influenza pandemics have displayed a wide range of morbidity, mortality, and transmissibility [2–5]. Human influenza virus infections are typically characterized by respiratory tract symptoms, including rhinorrhea, congestion, sore throat, cough, and sneezing, in addition to systemic symptoms, including fever, chills, and muscle aches [6–8]. More severe disease involving the lower respiratory tract is often associated with age and underlying health conditions [1, 9]. Highly pathogenic avian influenza (HPAI) H5N1 viruses have caused hundreds of human infections in 15 countries with a case fatality rate of ~60%. Human infections are primarily a result of exposure to infected poultry, with no sustained transmission among humans [10]. Individuals infected with H5N1 viruses generally present with fever, headache, sore throat, dyspnea, and cough, with severe infections progressing to pneumonia, acute respiratory distress syndrome, and death [11].

The ability of influenza viruses to cause disease and spread easily among people depends on host and viral factors. With influenza virus infection, the host innate immune response is triggered through pathogen recognition receptors, such as RIG-I and TLR-7, which initiate the release of proinflammatory mediators [12, 13]. This early production of cytokines and chemokines at the site of infection has been shown to be the basis for many of the clinical and pathological manifestations of disease [14, 15]. During severe HPAI virus infections, a dysregulation of these proinflammatory cytokines, often called a cytokine storm, has been associated with pulmonary damage [15–17]. Although the host innate immune response’s
role in influenza disease progression has been studied to some extent, its role in influenza virus transmission remains poorly understood.

The ferret is widely recognized as an excellent model of influenza virus transmission and pathogenesis, but little is known about innate immune responses of ferrets, primarily due to the lack of ferret-specific immunological reagents. Recently, ferret genomic sequences have been reported, facilitating assessment of messenger RNA (mRNA) expression levels of a number of cytokines and chemokines [18, 19]. Although these studies have identified a role of select immune mediators in influenza virus pathogenesis [20–22], a comprehensive analysis of the local innate immune responses in upper respiratory tract (URT) and lower respiratory tract (LRT) tissues has not been performed. Additionally, direct comparison of viruses of multiple subtypes with differing transmission and virulence properties has not been undertaken. Therefore, we have selected an H3N2 human influenza virus (A/Panama/2007/99 [PN99]), a 2009 pandemic H1N1 virus (A/Mexico/4482/09 [MX82]) and 2 H5N1 HPAI viruses (A/Hong Kong/486/97 [HK97] and A/Thailand/16/04 [TH16]) and assessed for each the mRNA expression levels of a panel of cytokines and chemokines in ferret URT and LRT tissues after infection to characterize temporal relationships with disease progression and transmission. Our results establish a strong correlation between the frequency of URT clinical signs, peak virus shedding, the

**Figure 1.** Transmission and clinical signs observed in ferrets infected with 4 influenza viruses: an H3N2 human influenza virus (A/Panama/2007/99 [PN99]), a 2009 pandemic H1N1 virus (A/Mexico/4482/09 [MX82]) or an H5N1 HPAI virus (A/Hong Kong/486/97 [HK97] or A/Thailand/16/04 [TH16]). Kinetics and frequency of respiratory droplet transmission (A), sneezing (B), nasal discharge (C), lethargy (D), fever (E), and dyspnea (F) in ferrets (n = 2–9 per time point) are presented for the days shown after inoculation. Respiratory droplet transmission is defined here as detection of virus in nasal wash samples from contact animals housed in cages with perforated side walls adjacent to infected ferrets, so that virus is transmitted through the air in the absence of direct or indirect contact. All transmission data were published elsewhere [23, 29, 30]; clinical signs observed in the current study (at 0.5, 2, and 6 days after inoculation) are presented in the context of data from those studies. The relative inactivity index for each virus is listed parenthetically in panel D. Fever is defined as ≥1.5°C higher than baseline.
Figure 2. Detection of influenza virus in the respiratory tracts of ferrets infected with 4 influenza viruses: an H3N2 human influenza virus (A/Panama/2007/99 [PN99]), a 2009 pandemic H1N1 virus (A/Mexico/4482/09 [MX82]) or an H5N1 HPAI virus (A/Hong Kong/486/97 [HK97] or A/Thailand/16/04.
kinetics of transmission in ferrets, and the local expression of cytokine mRNAs that have been implicated in causing similar clinical signs in humans infected by highly transmissible viruses. Furthermore, increased expression of proinflammatory mediators and reduced expression of anti-inflammatory mediators in the LRT of ferrets correlates with severe disease and lethal outcome.

**MATERIALS AND METHODS**

**Viruses**

Virus stocks for the H5N1 and H3N2 viruses were propagated in the allantoic cavity of 10-day-old embryonated hens’ eggs, as described elsewhere [23]. The H1N1 virus stock was propagated in Madin-Darby canine kidney cells. All research with HPAI viruses was conducted under BSL3 containment, including enhancements required by the US Department of Agriculture and the Select Agent Program [24–26].

**Ferret Experiments**

Male Fitch ferrets aged 7–10 months old (Triple F Farms) and serologically negative for currently circulating influenza viruses were housed in cages within a Duo-Flo Bioclean mobile clean room (Lab Products) throughout each experiment. Baseline measurements and ketamine sedation of ferrets was performed as described elsewhere [27] before intranasal inoculation with 10⁶ 50% egg infectious dose (EID₅₀) or plaque-forming units of virus (6 ferrets per virus) in 1 mL of phosphate-buffered saline or phosphate-buffered saline alone for the mock-infected control animals. To target the timing of innate immune responses and transmission, 2 ferrets each were humanely killed at 0.5, 2 and 6 days after inoculation. Nasal wash and lung tissue specimens (harvested from each lobe and pooled) were collected from each ferret for virus titration, as described elsewhere [27]. Nasal turbinates and lung specimens (~30 mg excision in proximity to a bronchus) were collected and stabilized in RNAlater (Ambion). Serum samples were analyzed using VetScan Classic and Comprehensive Diagnostic Profile rotors (Abaxis). Ferrets were monitored for clinical signs of infection, and relative inactivity indexes were determined, as described elsewhere [27]. Animal research was conducted under the guidance of the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited animal facility.

**Quantification of mRNA**

RNA isolations were performed on ferret nasal turbinates and lung tissue samples with an RNeasy Mini Kit using the RNA stabilization and on-column DNase digestion protocols (Qiagen). Semiquantitative reverse-transcription polymerase chain reaction was performed as described elsewhere [18], using 0.5 µg of RNA and a Mx3005P ( Stratagene). Expression levels were normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and are reported as fold change compared with mock-infected animals. Primer sequences for interferon (IFN) α, IFN-γ, tumor necrosis factor (TNF) α, interleukin (IL) 6, IL-10, IL-12p40, GAPDH [18], and FluM1 [28] were published elsewhere. Primer sequences for the remaining genes are as follows: IFN-β, Forward 5’-GGTTGATCTCCAAACTCTCTCC-3’, Reverse 5’-CACCAGACCTTTCTCCACGACATAACTT-3’, Reverse 5’-GAGCTGCTGAGCAGCAGTCGCTTCCTG-3’; IL-8, Forward 5’-AACCACCTCCACGCCCTCTAC-3’, Reverse 5’-GGCACACCTCTTTTCATTGAC-3’; CXCL9, Forward 5’-GGTGTGTTCTTTTCTTGTGA-3’, Reverse 5’-GGTTGTTGTGTGTATG-3’; CXCL10, Forward 5’-CCTGGCTTAC CGGTTTCCT-3’, Reverse 5’-AGAGCGCCATGGTAAA3’; CXCL11, Forward 5’-CTTCTTTACACATCAGCTTCTC-3’, Reverse 5’-CTATAGCCATGCCCTTCACACTA-3’.

**Statistics**

Statistical significance was determined using area under the curve and 1-way analysis of variance with Bonferroni’s multiple-comparison posttest. The correlations between observational data (area under the curve) and peak mRNA expression (fold rise compared with values in mock-infected ferrets) were estimated by Pearson correlation coefficients.

**RESULTS**

**Transmission and Disease Observed in Influenza Virus–Infected Ferrets**

Work from this laboratory published elsewhere describes the respiratory droplet transmissibility of each of the viruses used in this study [23, 29, 30]. Typical of seasonal H3N2 viruses, PN99 transmitted efficiently (3 of 3 ferrets) as early as 2 days after contact (Figure 1A). Although the 2009 pandemic H1N1 virus, MX82, also transmitted by respiratory droplets, transmission was somewhat less efficient than for the seasonal virus, with only 2 of 3 contact ferrets shedding virus by 3 days after contact. No
virus shedding was detected in any of the contacts of animals infected by H5N1 viruses (TH16 or HK97), indicative of the inability of these viruses to transmit through respiratory droplets. In addition to the ferrets in these published studies, 6 ferrets each were intranasally inoculated with $10^6$ EID$_{50}$ or plaque-forming units of TH16, HK97, MX82, or PN99 virus and were observed for up to 6 days for clinical signs of disease. Sneezing was most prominent in PN99 virus–infected ferrets; frequency was 3–9 times greater for these ferrets compared with animals infected by any other virus ($P < .01$) (Figure 1B). Nasal discharge was observed more frequently among PN99 virus–infected animals up to 4 days after inoculation, although at later time points, TH16 and, to a lesser degree, HK97 virus, exhibited increased nasal discharge (Figure 1C). Although nasal discharge was observed in a majority of H5N1 virus–infected ferrets later in the course of infection, the exudates were opaque, viscous, and often associated with acute nasal obstruction, whereas rhinorrhea in PN99 virus–infected ferrets was characterized by clear, flowing discharge at early time points. Nasal discharge, congestion, and sneezing are often reported in humans experimentally infected with seasonal influenza viruses and peak 2–4 days after inoculation [8, 12, 13, 31–33]. Similar kinetics

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**Figure 3.** Innate immunity in upper respiratory tract tissues of ferrets infected with 4 influenza viruses: an H3N2 human influenza virus (A/Panama/2007/99 [PN99]), a 2009 pandemic H1N1 virus (A/Mexico/4482/09 [MX82]) or an H5N1 HPAI virus (A/Hong Kong/486/97 [HK97] or A/Thailand/16/04 [TH16]. Cytokine and chemokine messenger RNA expression in nasal turbinates of ferrets ($n = 2$ per time point) was measured by real-time reverse-transcription polymerase chain reaction and is presented as the mean fold change (± standard deviation) compared with values in mock-infected animals on the days shown after inoculation. Abbreviations: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.
of nasal symptoms were observed in ferrets infected with the seasonal influenza PN99 virus, which peaked 3–4 days after inoculation (Figure 1B and 1C). In contrast, we observed significantly reduced sneezing and less rhinorrhea early in infection in ferrets inoculated with H5N1 influenza viruses that do not transmit efficiently via respiratory droplets (P < .01) (Figure 1A–C).

Lethargy was observed in all H5N1 virus–infected ferrets by 3 days after inoculation and in 27% of PN99 virus–infected ferrets and 91% of MX82 virus–infected ferrets at 2 days after inoculation and was typically associated with fever (1.5°C above baseline), which peaked in all groups by 2 days after inoculation (Figure 1D and 1E). Severe lethargy was most evident in H5N1-infected ferrets (relative inactivity index, >2.0), whereas human influenza viruses induced more modest lethargy (Figure 1D). We observed an inverse correlation between transmission and both the frequency and severity of lethargy in ferrets (r < −0.9; P < .05) (Figure 1A and 1D). Dyspnea was pronounced in TH16 virus infection and ultimately required euthanasia, resulting in a 100% lethality rate by 7 days after inoculation for this virus (Figure 1F). Euthanasia was required for 35% of HK97 virus–infected ferrets because of the onset of neurological dysfunction by 7–13 days after inoculation, whereas MX82 virus had a 50% lethality rate due to excessive weight loss (mean maximum, 17.5%) requiring euthanasia by 8 days after inoculation; no ferrets died after PN99 virus infection (data not shown; [27, 29]). Serum chemistry analysis of ferrets infected by TH16 virus reflected significantly altered albumin, alkaline phosphatase, alanine aminotransferase, amylase, total bilirubin, calcium, sodium, and total protein levels by 6 days.
0.5 days after inoculation, indicative of acute dehydration and liver dysfunction (data not shown). Abnormal analyte levels of ferrets infected by any of the other viruses were sporadic and not significant. Together, these data highlight the diversity in transmissibility and virulence among the influenza viruses included in this study and provide a platform for investigating differences in innate immunity that are associated with transmission and disease severity.

**Influenza Virus Replication in the Ferret Respiratory Tract**

Virus titers were measured in ferret nasal wash and lung samples collected at 0.5, 2, and 6 days after inoculation with human or avian influenza virus. Nasal wash titers are presented in the context of virus shedding kinetics reported elsewhere from studies conducted in this laboratory (Figure 2A) [23, 27, 29]. In the current study, H5N1 virus infection resulted in mean peak virus titers in nasal wash samples of $10^{5.6}$ or $10^{5.7}$ EID$_{50}$/mL at 2 or 6 days after inoculation, respectively, as virus titers remain elevated throughout TH16 virus infection. In comparison, nasal wash virus titers were $\geq 2.5$ times higher for the 2009 pandemic H1N1 virus, MX82 ($10^{5.9}$ EID$_{50}$/mL), and 25 times higher for the seasonal influenza virus, PN99 ($10^{5.9}$ EID$_{50}$/mL), at 2 days after inoculation when respiratory droplet transmission is first observed with the latter virus (Figure 1A). Conversely, in the LRT, virus titers in TH16 virus–infected ferrets were greatest, significantly higher than either of the human influenza viruses ($P < .01$), and they remained elevated throughout infection (Figure 2B). Viral mRNA was measured in nasal turbinate and lung tissues of ferrets at the same time points after inoculation (Figure 2C and 2D). For the human influenza viruses, >200 times more viral mRNA was detected in the URT at 0.5 days after inoculation compared with the H5N1 viruses, and viral mRNA levels remained significantly greater at 2 days after inoculation ($P < .01$), but by 6 days after inoculation viral mRNA levels were more comparable among all groups (Figure 2C). At 0.5 days after inoculation, viral mRNA levels in lungs were greatest in TH16-infected animals ($\sim 200$-fold increase compared with mock–infected animals) (Figure 2D), and at 2 days after inoculation, H5N1 virus mRNA levels were significantly higher compared with the human influenza viruses (>75-fold; $P < .01$). By 6 days after inoculation, levels were reduced to $< 90$-fold for all viruses except TH16 virus, which remained elevated at levels nearly 800 times those in mock–infected animals. These comparative differences in virus replication in the URT versus the LRT for human and avian influenza viruses prompted us to examine the localized innate immune responses in the URT and LRT and to investigate their relationship with disease progression and transmission.

**URT Expression of Cytokine and Chemokine mRNA in Ferrets**

Expression of a panel of proinflammatory cytokine and chemokine mRNAs was measured in ferret nasal turbinates collected at 0.5, 2, and 6 days after inoculation with human or avian influenza virus (Figure 3). Twelve hours after inoculation with PN99 virus, mean peak levels of TNF-α and IL-6 mRNA in ferrets were significantly higher in nasal turbinates compared with any other virus group ($P < .01$) (Figure 3A and 3B). By 2 days after inoculation, PN99 and MX82 virus–infected ferrets expressed these cytokines at levels significantly greater than in either H5N1 virus–infected group; levels of mRNA in the PN99 virus group were at least twice that of MX82 virus ($P < .01$). In particular, levels of IL-6 mRNA at 2 days after inoculation were highest in ferrets infected by human influenza viruses, whereas H5N1 virus–infected ferrets exhibited levels that were
elevated or sustained through 6 days after inoculation. The URT peak expression levels of TNF-α and IL-6 correlated with virus shedding, sneezing, rhinorrhea, and transmission among the virus groups \( (r > 0.9; P < .05) \) (Figures 1–3, Table 1).

The induction of type I IFN (IFN-α and IFN-β) elicits one of the most effective defense mechanisms against influenza virus infection \([34]\). The most striking difference in cytokine mRNA levels in the URT among our ferret groups was with type I IFNs, which peaked in all groups at 2 days after inoculation (Figure 3C and 3D). In PN99 virus–infected ferrets, IFN-α was expressed at levels 9 times higher than in H5N1-infected ferrets and 2 times higher than in MX82 virus–infected ferrets, whereas IFN-β was expressed at levels 60 times higher than in H5N1-infected ferrets and 10 times higher than in MX82 virus–infected ferrets. The MX82 virus group also expressed significantly higher levels of IFN-α and IFN-β mRNA (at least 4- and 6-fold, respectively) than either H5N1 virus ferret group \( (P < .001) \). Thus, viruses that induced the greatest clinical signs and peak virus shedding in the URT also induced the highest levels of type I IFN mRNA expression \( (r > 0.9; P < .05) \) (Figure 1, Figure 3C and 3D; Table 1). Increased type II IFN (IFN-γ) mRNA levels were detected in nasal turbinates of all groups of infected ferrets, peaking at 2 days after inoculation (Figure 3E). However, levels of expression in TH16 virus–infected ferrets were 60-fold higher than in mock-infected ferrets, whereas levels in HK97, MX82, and PN99 virus groups were significantly higher, >120-fold those for mock-infected animals \( (P < .001) \). A strong inducer of IFN-γ, IL-12 mRNA was expressed at increased levels in all infected animals by 12 hours after inoculation, peaking by 2 days after inoculation. PN99 virus–infected ferrets expressed 5 times more IL-12 mRNA than any other group \( (P < .01) \) (Figure 3F).

IL-8 (CXCL8) is a neutrophil chemotactic cytokine that peaked at 6 days after inoculation in ferrets infected with H5N1 viruses, with the highest levels attained by TH16 virus; PN99 and MX82 virus–infected ferrets exhibited peak levels of this cytokine 2 days after inoculation (Figure 3G). Additional Th1 response chemokines in the CXC subfamily were elevated in URT tissues of all ferret groups, peaking 2 days after inoculation (Figure 3H–J). At this time point, CXCL9 (MIG) and CXCL11 (IP9) mRNAs were expressed in PN99 virus–infected ferret nasal turbinates at levels almost 900-fold those in mock-infected ferrets and ≥3 times those than in ferrets infected by any other virus (Figure 3H and 3J). CXCL10 (IP10) mRNA levels were elevated in all groups of ferrets ≥400-fold compared with mock infection (Figure 3I). These results demonstrate a gradient of CXCL9 and CXCL11 mRNA expression in the URT, with the highest levels achieved by PN99 virus–infected ferrets; there was more comparable elevated expression of CXCL10 mRNAs among all ferret groups.

Expression of anti-inflammatory cytokines such as IL-4 and IL-10 is important for disease resolution, preventing inflammation from going unchecked \([35]\). Similar to findings in human studies \([12]\), we observed no substantial changes in nasal turbinate levels of IL-4 mRNAs, but a gradual increase in IL-10 mRNAs was observed in all ferret groups (Figure 3K and 3L). However, the mean peak IL-10 expression in PN99 virus–infected ferrets was 25-fold that in mock-infected ferrets, compared with increases of 14–15-fold for MX82 and HK97 and only 4-fold in TH16 group, a significantly smaller difference \( (P < .01) \). We have demonstrated a correlation between the expression of inflammatory mediators in the URT of ferrets and the relative transmissibility of these influenza viruses; however, innate immunity in the LRT is probably a better indicator of severe disease.

**LRT Expression of Cytokine and Chemokine mRNA in Ferrets**

Severe disease and poor prognosis in H5N1 virus–infected patients has been associated with hypercytokinemia and hyperchemokinemia \([15, 36, 37]\). In lung tissues of H5N1 virus–infected ferrets, we detected significant increases in mRNA levels of TNF-α and IL-6 cytokines at 2 days after inoculation compared with animals infected by MX82 or PN99 viruses \( (P < .05) \) (Figure 4A and 4B). TNF-α and IL-6 were ≥8 and ≥54 times higher, respectively, than levels detected in ferrets infected by human influenza viruses. IFN-α/β mRNA levels were also elevated in H5N1 virus–infected ferrets compared with those infected with human influenza viruses, with 3–161-fold increases at 2 days after inoculation \( (P < .05) \) (Figure 4C and 4D). IFN-γ mRNA expression was significantly increased in lung tissues of both H5N1 virus ferret groups compared with human influenza viruses \( (P < .01) \) (Figure 4E), and IL-12 mRNA expression was highest at 0.5 days after inoculation in TH16 virus lung tissue but at later time points was more similar between ferret groups (Figure 4F).

Previous studies showed that increased expression of CXCL10 in lung tissues from H5N1 virus–infected ferrets was associated with disease severity \([20]\). We also found significantly higher levels of CXCL10 mRNA in lung tissue at 2 days after inoculation in H5N1 virus–infected ferrets compared with those infected by human influenza viruses \( (P < .01) \), which correlated with the presence of fever, dyspnea, and lethality among the virus groups \( (r > 0.9; P < .05) \) (Figure 4I). At this time point, levels of CXCL11 and CXCL9 mRNA were also significantly higher in H5N1 virus–infected ferrets compared with the human virus groups, whereas peak expression of CXCL9 was not significantly greater at other time points (Figure 4H and 4F). In humans, increased CXCL10 chemokine also correlated with severe disease and was highest in fatal cases, as was IL-8, which we found to be highest in H5N1-infected ferrets at 2 days after inoculation (Figure 4G) \([15]\). Overall, the more robust innate immune response in the lungs of H5N1-infected ferrets was consistent with virus titers measured: 1–2 logs higher compared with human influenza viruses (Figure 2B).
Despite elevated levels of proinflammatory cytokine and chemokine mRNAs in lung tissues of H5N1 virus–infected ferrets, no significant up-regulation of anti-inflammatory cytokines IL-4 or IL-10 was detected at any time point (Figure 4K and 4L), even though increased IL-10 mRNA levels were detected in nasal mucosa of HK97 but not TH16 virus (Figure 3L). The lack of anti-inflammatory regulation of H5N1 virus infections may be site specific; without the down-regulation of inflammation in the lungs, pulmonary tissue damage is certain, potentially preventing disease resolution [35, 38].

**DISCUSSION**

Host innate immunity plays a critical role during disease progression and resolution after influenza virus infection. We have examined the localized innate immune responses in the URT and LRT of ferrets after infection with 4 influenza viruses that
exhibit differential properties of virulence and transmission in both humans and ferrets. Our findings demonstrate similarities between the innate immune responses in the respiratory tracts of ferrets and humans after influenza virus infection and provide potential markers of innate immunity that may identify properties of disease severity and transmission. In human volunteers experimentally infected with seasonal influenza virus, URT expression of TNF-α, IL-6, and IFN-α correlated with virus shedding and nasal and systemic symptoms. We also observed a strong up-regulation of proinflammatory mediators (TNF-α, IL-6, IFN-α/β, IL-12, CXCL9, CXCL11) in the URT of ferrets at a point in infection when transmission, clinical signs associated with transmission, and peak virus shedding occur. Symptom onset in influenza virus–infected individuals has been shown to be associated with the timing of transmission to household contacts. Furthermore, the relative level of expression of proinflammatory cytokine mRNAs correlated with the relative ability of TH16, HK97, MX82 and PN99 viruses to transmit in our ferret model. Despite correlation with clinical signs associated with transmission, concurrent peak expression of IFN-α/β may also reduce virus replication. Exogenous treatment of guinea pigs or ferrets with type I IFN reduced virus shedding and transmission of human influenza viruses. Other immune mediators (IFN-γ, IL-8, CXCL10, IL-4, IL-10) were expressed in the URT in patterns seemingly unrelated to transmission and may be associated more with virus clearance or disease severity. Our assessment of LRT innate immunity revealed significant increases...
in peak expression of nearly all of the proinflammatory mediators tested for one or both of the H5N1 viruses compared with the human influenza viruses, and no significant increase was observed in either anti-inflammatory cytokine, supporting previous studies in ferrets, nonhuman primates, and humans that link dysregulation of innate immunity with severe disease after H5N1 virus infection [15, 20, 44, 45]. A more detailed time-course and, as more reagents become available, assessment of cytokine and chemokine protein expression levels will provide greater insight into the complexity of the host–virus interface and the innate immune response of ferrets to influenza virus infection.

Collectively, our findings demonstrate that the site of virus replication serves a critical role in the local expression of inflammatory mediators and the onset of clinical signs that influence transmission and disease severity. Viral factors, such as receptor specificity and infectivity, are known determinants of influenza virus transmission, but here we report host innate immunity profiles that correlate with clinical signs, such as rhinorrhea and sneezing, at time points that overlap with peak virus shedding and transmission of influenza virus in ferrets. Therefore, we conclude that this combination of events orchestrated during influenza virus infection may help facilitate efficient transmission of seasonal influenza viruses and that regulation of the host innate immune response in the LRT influences disease severity.

**Notes**

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