IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Age-Related Pathology Associated with H1N1 A/California/07/2009 Influenza Virus Infection

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Influenza virus infection causes a spectrum of diseases, ranging from mild upper respiratory tract infection to severe lower respiratory tract infection, that can lead to diffuse alveolar damage, interstitial and airspace inflammation, or acute respiratory failure. Mechanisms instructing disease severity are not completely understood, but host, viral, and bacterial factors influence disease outcome. With age being one host factor associated with a higher risk of severe influenza, we investigated regional pulmonary distribution and severity of pneumonia after 2009 H1N1 influenza virus infection in newly weaned, adult, and aged ferrets to better understand age-dependent susceptibility and pathology. Aged ferrets exhibited greater weight loss and higher rates of mortality than adult ferrets, whereas most newly weaned ferrets did not lose weight but had a lack of weight gain. Newly weaned ferrets exhibited minimal pneumonia, whereas adult and aged ferrets had a spectrum of pneumonia severity. Influenza virus—induced pneumonia peaked earliest in adult ferrets, whereas aged ferrets had delayed presentation. Bronchial severity differed among groups, but bronchial pathology was comparable among all cohorts. Alveolar infection was strikingly different among groups. Newly weaned ferrets had little alveolar cell infection. Adult and aged ferrets had alveolar infection, but aged ferrets were unable to clear infection. These different age-related pneumonia and infection patterns suggest therapeutic strategies to treat influenza should be tailored contingent on age. *(Am J Pathol 2019, 189: 2389—2399; https://doi.org/10.1016/j.ajpath.2019.08.017)*

Infants, young children, and individuals 65 years and older are more susceptible to developing severe disease caused by influenza A virus infection.[1–3] The developing lungs of children under 5 are vulnerable to obstruction of airflow and surfactant dysfunction induced by respiratory infection, resulting in approximately 20,000 flu-related hospitalizations in the United States since 2010.[2] In elderly populations, influenza virus infections are among the leading causes of morbidity and mortality, with an estimated 71% to 85% of seasonal influenza—related deaths occurring in elderly people.[3,4] This increased susceptibility to infectious diseases is thought to arise from less effective immune responses. Age-related changes include curtailed antibody production, qualitative antibody changes, diminished B-cell

activation, diminished number of naïve T cells and impairment of their induction, and contraction of the T-cell repertoire.[5–8] An increase in the basal levels of many inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-1β, and IL-6, is noted in the aging population.[9] During influenza virus infection, aged

individuals have an impaired memory T-cell response to conserved epitopes in influenza viral proteins.[10]

In contrast to seasonal influenza, pandemic outbreaks caused by transmission of a novel influenza virus subtype or strain can exhibit different age-dependent patterns of infection in a population. For example, in 2009, older children and adults between the ages of 5 and 59 years were disproportionately affected during the H1N1pdm09 virus pandemic.[11] Severe cases of H1N1pdm09 infection with A/California/07/2009 (CA/09)−like viruses were more frequent in these age groups and were associated with inflammation, leukocyte infiltration, and impaired gas exchange attributable to H1N1pdm-induced lung damage. Healthy infants had a lower proportion of deaths than observed in the prior seasonal influenza seasons.[12−14] Pediatric patients infected with H1N1pdm09 also experienced mild to moderate clinical disease.[15−17] Individuals ≥70 years of age had more diverse circulating antibody profiles against the internal genes and the HA1 receptor binding site as well as higher antibody binding affinity to the HA1 head of the CA/09-like viruses compared with antisera from younger individuals.[18] It is likely that this diverse antibody repertoire reflected exposures to previous H1N1-like viruses, including the 1918-like viruses, swine-origin H1N1 virus from 1976, and/or vaccinations against seasonal strains.[19−22] A similar outcome is observed in ferrets that were experimentally infected with multiple seasonal-like H1N1 viruses that represented different historical eras and elicited an antibody profile that bound and neutralized H1N1pdm09-like viruses.[23]

To better understand age-dependent susceptibility and pathology of CA/09-like viral infections and severity of pneumonia, a H1N1pdm09 virus (A/California/07/2009) was used to infect newly weaned (6 to 7 weeks of age), adult (6 to 12 months of age) and aged ferrets (5.5 to 7 years of age). The ferrets were observed for up to 14 days post infection (DPI). Ferrets are naturally susceptible to human influenza viruses and recapitulate clinical symptoms, viral pathogenesis, immune responses, and lung development in a similar manner as humans.[24,25] All age groups had subsets of animals with mild, moderate, and severe morbidity. However, >50% of aged ferrets had more severe disease with similar immune dysfunctions that are observed in elderly individuals. Although newly weaned ferrets were more resistant to disease and efficiently cleared viral infection, these animals did not follow normal growth and weight gain patterns as observed in noninfected ferrets.

Materials and Methods

Influenza Virus and Infection of Ferrets

Female Fitch ferrets (*Mustela putorius furo*) were obtained from Triple F Farms (Sayre, PA) and were seronegative to circulating influenza A (H1N1 and H3N2) and B viruses. Newly weaned ferrets were defined as 6 to 7 weeks of age. Adult ferrets were defined as 6 to 12 months of age. Aged ferrets were defined as 5.5 to 7 years of age. Ferrets were pair housed in stainless steel cages (Shor-Line, Kansas City, KS) that contained Sani-Chips laboratory animal bedding (P.J. Murphy Forest Products, Montville, NJ) and provided with food and fresh water *ad libitum*. To determine the inoculum dose for each age group, various doses were evaluated with the goal of achieving a mean of 20% weight reduction no earlier than 8 DPI. To achieve a mean of 20% weight loss no earlier than 8 DPI, adult and newly weaned ferrets were infected intranasally with H1N1pdm09 virus A/California/07/2009 [Influenza Reagents Resource (IRR), BEI Resources, the Centers for Disease Control and Prevention, Manassas, VA] at a dose of 10^6 plaque-forming units (PFU), whereas a dose of 10^5 PFU was used for aged ferrets. The animals were monitored daily for severity of clinical disease using weight loss. Disease symptoms, including elevated temperature, low activity level, sneezing, and nasal discharge, were noted if present (data not shown). Any animal reaching >20% weight loss was humanely euthanized. Ferrets were randomly assigned to be removed from the study at 1, 3, 5, 8, or 14 DPI unless their clinical conditions (eg, loss of >20% body weight) required a humane end point. Blood was collected from anesthetized ferrets via the anterior vena cava after infection. Serum was harvested and frozen at a means ± SD of −20°C ± 5°C. The University of Georgia Institutional Animal Care and Use Committee approved all experiments, which were conducted in accordance with the NIH's *Guide for the Care and Use of Laboratory Animals*,[26] The Animal Welfare Act, and the *Biosafety in Microbiological and Biomedical Laboratories* guide of the Centers for Disease Control and Prevention and the NIH.

Tissue

After serum was collected, necropsies were performed to collect lung tissue. Lungs were rinsed with cold phosphate-buffered saline via catheterized trachea to collect bronchoalveolar lavage fluid for another study. The right upper and lower lobes were removed, and each lobe was sectioned into quadrants. Sections were snap frozen. The left upper and lower lung lobes were formalin perfused. After fixation, tissue was paraffin embedded and 5-μm−thick sections were prepared for histopathologic analysis.

Viral Plaque Assay

Plaque assays were performed to determine viral burden in nasal washes and lung tissue.[27,28] Nasal washes were collected after infection, snap frozen, then stored at −80°C until use. Lung tissue supernatants were obtained from frozen lung pieces that were gently thawed on ice, forced through a cell strainer (70 μm) and syringe plunger in phosphate-buffered saline, then spun down (1342 × *g*, 5 minutes, 4°C) to collect supernatant. Nasal washes and lung supernatants were diluted in Iscove's modified Dulbecco's minimum essential medium.

Madin-Darby canine kidney cells were plated (5×10^5) in each well of a six-well plate. Samples were diluted (final dilution factors of 10^0 to 10^{-6}) and overlaid onto the cells in 100 μL of Dulbecco's modified Eagle's medium supplemented with penicillin-streptomycin and incubated for 1 hour. Samples were removed, cells were washed twice, and medium was replaced with 2 mL of L15 medium plus 0.8% agarose (Cambrex, East Rutherford, NJ) and incubated for 72 hours at 37°C with 5% carbon dioxide. Agarose was removed and discarded. The cells were fixed with 10% buffered formalin and then stained with 1% crystal violet for 15 minutes. After thorough washing in distilled water to remove excess crystal violet, the plates were dried, number of plaques was counted, and the number of PFU per milliliter was calculated.

Histopathologic Analysis

Tissue sections that contained trachea, lobe from the left upper lung, and lobe from the left lower lung were stained with hematoxylin and eosin. Sections were scored by two blinded readers (S.J.B. and C.A.W.) for percentage of lung involvement and bronchial and alveolar severity. Percentage of lung involvement was defined as the percentage of lung that had histologic pneumonia and was scored as follows: 0, ≤10%; 1, 10% to 25%; 2, 26% to 50%; and 3, ≥50%. Regional severity of bronchial (bronchi and bronchioles) and alveolar spaces was assessed by the following scoring guidelines: 0, normal; 1, mild pneumonia; 2, moderate pneumonia; and 3, severe pneumonia.[21,29]

In Situ Hybridization

Sense and antisense templates were generated from a 259-bp segment of influenza A virus matrix protein.[30] S-labeled riboprobes were synthesized using a Riboprobe *in vitro* transcription system (Promega, Madison, WI). Hybridization was performed on deparaffinized formalin-fixed, paraffin-embedded tissue sections of lung and trachea, as described previously.[31] The influenza riboprobe had no hybridization to noninfected tissue. The severity of influenza infection in trachea, bronchi and bronchioles, alveolar spaces, and submucosal glands was determined by scoring of influenza virus *in situ* hybridization (ISH) foci: 0, no definitive signal; 1, occasional focus; 2, focus in most fields; and 3, >1 focus per field.[21,29]

Pneumonia and Influenza Virus Infection Composite Scores

Pneumonia was evaluated by determination of the percentage of lung involved and bronchial and alveolar severity, whereas influenza infection severity was evaluated in the trachea, bronchiolar, alveolar spaces, and submucosal glands compartments as described above. Composite scores were derived by adding the scores for each lobe (upper and lower lobes) and each compartment. Pneumonia composite scores were scores for percentage of lung involvement. bronchial severity, and alveolar severity. Infection severity scores were scores for bronchial, alveolar, tracheal, submucosal infection.

Statistical Analysis

Differences in weight loss, sickness score, and viral titers were analyzed by two-way analysis of variance followed by Bonferroni's posttest for each group at multiple time points. Statistical significance was defined as $P < 0.05$. Statistical analyses were performed using GraphPad Prism software version 7.0a (GraphPad Software, San Diego, CA).

Data Availability

The data set pertaining to this study can be directly accessed at Synapse (*https://www.synapse.org//#!Synapse:syn18421089*). In addition, this work is part of an integrated data set that is openly accessible for further analysis at Synapse (*https://www.synapse.org//#!Synapse:syn2395480/wiki/63122*, both Synapse data sets were last accessed March 27, 2019). Data and metadata, including transcriptomics, viral genomics, microbiome, physiologic, virologic, immunologic, and outcome data, are available for each ferret.

Results

Aged Ferrets Exhibit Greater Weight Loss and Higher Rates of Mortality

Newly weaned, adult, and aged female ferrets were infected intranasally with CA/09 (10^5 to 10^6 PFU) and monitored daily for clinical illness and weight loss. Using weight loss to define severity of disease, ferrets were classified as having mild, moderate, or severe disease and morbidity associated with infection. Adult ferrets lost between 5% and 15% of original body weight by 8 DPI before beginning to recover (Figure 1A). Adult ferrets categorized as severe ($n = 18$) had >12% weight loss (12% to 20%), with a mean weight loss of 15%. Adult ferrets with moderate weight loss ($n = 28$) had a mean loss of approximately 10% of original body weight, whereas adult ferrets with mild disease ($n = 16$) lost weight at a slower rate, with peak weight loss at 5% to 8% of original body weight at 6 to 8 DPI.

Aged ferrets had greater weight loss (Figure 1B). Aged ferrets categorized as severe ($n = 33$) had 20% weight loss and had to be humanely sacrificed, with eight of the aged ferrets reaching clinical end points at 6 to 8 DPI. Aged ferrets in the moderate category ($n = 15$) lost 5% to 8% of their original weight, and aged ferrets categorized as mild ($n = 15$) lost <1% to 2% weight and had weight loss that was statistically similar to noninfected aged ferrets ($P < 0.0001$).

In contrast, newly weaned ferrets had a completely different pattern of weight loss and disease. All the newly

weaned ferrets appeared healthy and did not have signs of sneezing or lethargy. However, because these animals were actively growing and maturing, they were analyzed for lack of weight gain (Figure 1C). Noninfected, newly weaned ferrets ($n = 8$) increased their body weight by 30% during the 8 days of observation. Newly weaned animals categorized as severe ($n = 9$) lost a mean of 20% of their body weight by 6 DPI. Newly weaned ferrets that were categorized as moderate ($n = 40$) lost approximately 5% of their original body weight, and mild newly weaned ferrets ($n = 20$) had little weight loss or gained approximately 5% weight.

Influenza Virus Persists Longer in Nasal Washes of Aged Ferrets

Weight loss during CA/09-like virus infection is often correlated with viral titers in ferret nasal washes after infection.[32,33] Adult ferrets had a peak in nasal wash viral titers on 3 DPI at 1×10^5 PFU/mL, regardless of the severity of weight loss (Figure 1E). In contrast, the aged ferrets in the severe category had a mean viral titer in their nasal wash at 1×10^6 PFU/mL, which was 1 to 2 logs higher than ferrets in the mild and moderate category (Figure 1F). Unlike the adult ferrets, virus was detected in the nasal wash of aged ferrets at 1 DPI. The viral titer peaked at 3 DPI, but the virus persisted longer in aged ferrets compared with adult ferrets. Aged ferrets in the moderate and severe category still had detectable virus in their nasal washes at 8 DPI (Figure 1F). Newly weaned ferrets also had detectable viral titers in their nasal washes 1 DPI, and the titers were sustained at 1×10^5 to 1×10^6 pfu/mL between 3 and 5 DPI (Figure 1G). These titers peaked at 5 DPI before declining but did not reach baseline by 8 DPI.

Overall, the mean viral titers (Figure 1H) did not correlate with pneumonia severity or disease outcome. The mean weight loss (Figure 1D) for each group followed the same trend as pneumonia severity.

Newly Weaned Ferrets Show Mild Pneumonia and Aged Ferrets Exhibit Delayed Presentation

Evaluation of pneumonia was determined by assessing how much of the lung had pneumonia (percentage of lung involvement) and scoring regional lung compartment pneumonia severity in sets of ferrets that were sacrificed at 1, 3, 5, 8, and 14 DPI (Figure 2). The percentage of lung involvement had different kinetics in each cohort (Figures 2 and 3). The newly weaned ferrets had the least severe lung involvement, peaking between 5 and 8 DPI (Figure 3, A and D). The percentage of lung involvement in adult ferrets peaked at 5 DPI and then began to resolve (Figure 3, A and D). Both newly weaned and adult ferrets continued to present signs of pneumonia at 14 DPI. The aged ferrets had delayed development of pneumonia signs that was significantly different from adult ferrets at 3 ($P = 0.002$ for lower lobe), 5 ($P = 0.03$ for upper lobe and $P < 0.0001$ for lower lobe), and 8 ($P = 0.04$ for upper lobe) DPI, and the peak percentage of aged ferret lung involvement was observed at the time requiring euthanasia (8 DPI) (Figure 3, A and D).
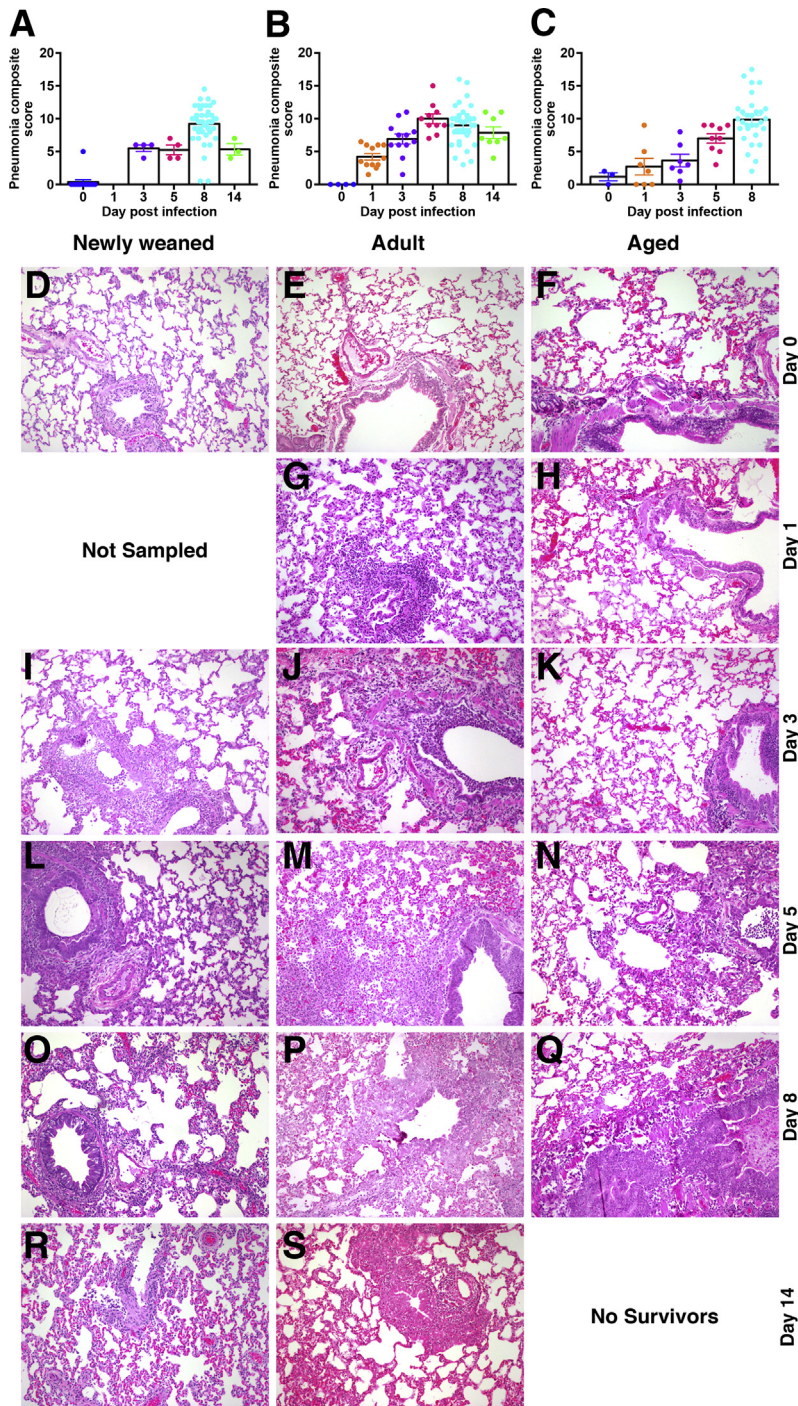
Bronchial Pneumonia Is a Feature Common to All Age Groups

Bronchial pneumonia severity was comparable among all cohorts, but the kinetics varied among the cohorts. Peak severity of bronchial pneumonia ranged from 3 to 8 DPI for newly weaned ferrets and 5 DPI for adults (Figure 3, B and
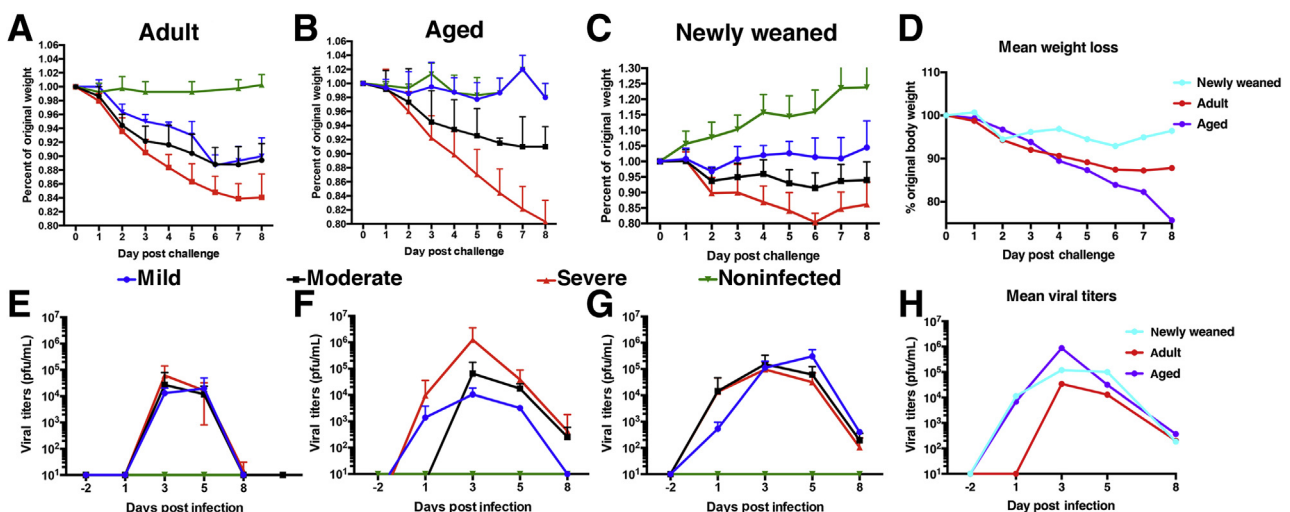
Figure 1 Weight loss and viral nasal wash titers after H1N1pdm09 virus infection. Naïve ferrets were infected with H1N1pdm09 virus (A/California/07/2009) and monitored for 2 weeks. **A–C:** Adult (**A**), aged (**B**), and newly weaned (**C**) ferrets were evaluated daily for weight loss. **D:** Mean weight loss for each group. **E–G:** Viral titers were determined from nasal washes collected at 1, 3, 5, and 8 days post infection. **H:** Mean viral titers for each group. Data are expressed as means ± SD virus titers.

Figure 2 Age-specific development of H1N1 influenza virus pneumonia. **A–C:** Pneumonia composite scores were assessed for 0, 1, 3, 5, 8, and 14 days post infection for each age group. **D–S:** Representative images from hematoxylin and eosin–stained sections are depicted for newly weaned (**D, I, L, O,** and **R**), adult (**E, G, J, M, P,** and **S**), and aged (**F, H, K, N,** and **Q**) ferrets during infection (0, 1, 3, 5, 8, and 14 days post infection). Data are expressed as mean scores (bars) and scores of individual animals (dots) (**A–C**). Original magnification, ×100 (**D–S**).

E). Aged ferrets had little bronchial involvement in the early infection period, but severity steadily increased through 8 DPI.

Alveolar Pneumonia Severity Is Strikingly Distinctive in the Age Groups

The presence of alveolar pneumonia differed among the age groups and correlated with survival and weight loss. Newly weaned ferrets had little to no alveolar pneumonia until 8 DPI (Figure 3, C and F), with statistically significant differences compared with adult ferrets at 3 ($P = 0.007$ for lower lobe) and 5 ($P < 0.0001$ for lower lobe) DPI and aged ferrets at 5 ($P = 0.02$ for upper lobe) and 8 ($P = 0.05$ for upper lobe) DPI. By 14 DPI, the mild alveolar pneumonia resolved. Alveolar pneumonia severity was prominent in both adult and aged groups, with adult severity peaking at 5 DPI. As with the bronchial pneumonia, development of
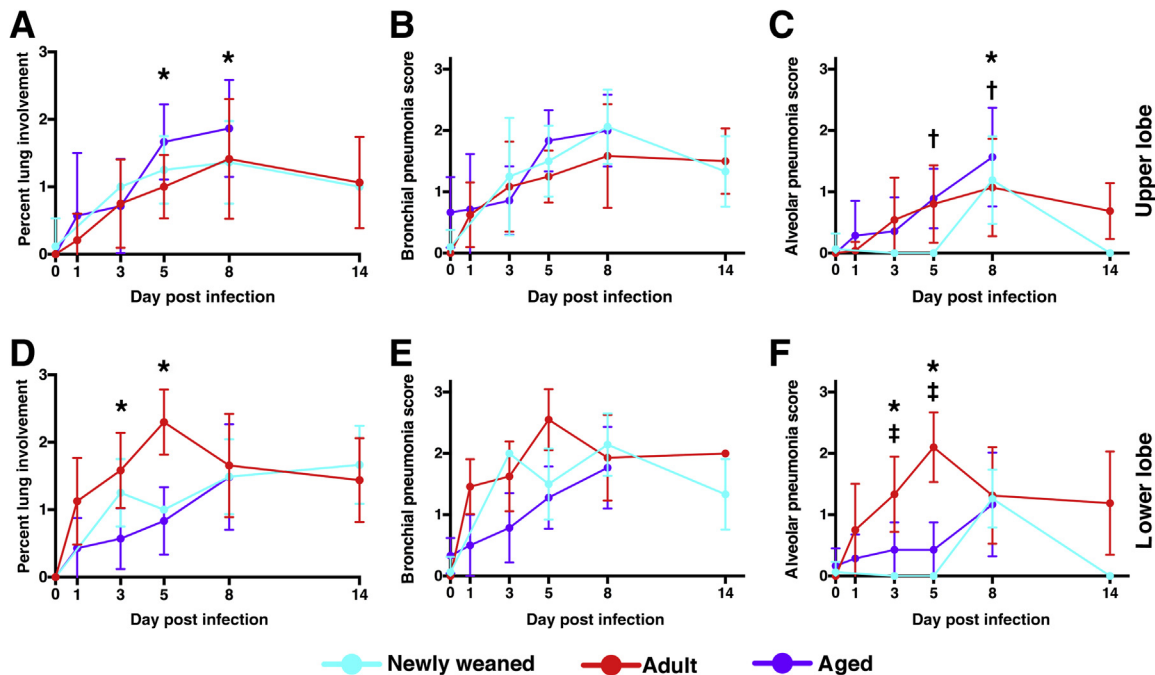
Figure 3 Comparison of global lung involvement and bronchial and alveolar pneumonia severity between each age group. **A** and **D:** The percentage of lung involvement was assessed in lung sections. Lung sections were scored as follows: 0, <10%; 1, 10% to 25%; 2, 26% to 50%; and 3, >50%. **B, C, E,** and **F:** Regional severity of pneumonia was assessed in the bronchi (**B** and **E**) and alveolar (**C** and **F**) spaces using the following scoring protocol: 0, normal; 1, mild pneumonia; 2, moderate pneumonia; and 3, severe pneumonia. Both the upper (**A**−**C**) and lower (**D**−**F**) left lung lobes were examined. $*P < 0.05$ adult versus aged; $^{†}P < 0.05$ newly weaned versus aged; $^{‡}P < 0.05$ newly weaned versus adult.

alveolar pneumonia in the aged ferrets was delayed and peaked at 8 DPI, with statistically significant differences from adults at 3 ($P = 0.004$ for lower lobe), 5 ($P < 0.0001$ for lower lobe), and 8 ($P = 0.04$ for upper lobe) DPI.

Newly Weaned Ferrets Show Remarkable Protection from Alveolar Cell Infection and Aged Ferrets Are Unable to Clear Infection

Regional influenza virus infection kinetics also varied among the age groups (Figures 4 and 5). To visualize viral replication, ISH for influenza A virus matrix protein transcripts was performed on sections that contained trachea and left upper and lower lung lobes for each time point (Figure 4). Regional infection severity for trachea, bronchi, submucosal glands, and alveoli were scored. As early as 1 DPI, influenza A virus infection was observed in each region for adult ferrets, whereas aged ferrets had minimal evidence of infection, mostly in the alveolar compartment. Bronchial influenza virus−infected cells peaked in the young ferrets at 3 DPI, in the adult ferrets at 1 to 5 DPI, and at 5 DPI in aged ferrets (Figure 5, A and C). Young and adult ferrets cleared influenza virus infection by 8 DPI; however, aged ferrets were unable to clear the virus and had significantly different influenza virus infection compared with adult ferrets at 5 ($P = 0.05$ for upper lobe) and 8 ($P < 0.0001$ for upper and lower lobes) DPI and newly weaned ferrets at 3 ($P = 0.008$ for lower lobe) and 8 ($P < 0.0001$ for upper and lower lobes) DPI. Although

kinetics differed, each age group supported robust bronchial cell infection. Little to no influenza virus was detected in alveolar cells of newly weaned ferrets during the infection (Figure 5, B and D). Adult and aged ferrets had peak alveolar infection at 5 DPI, but aged ferrets were unable to clear alveolar infection, with statistically significant differences from adult ferrets at 5 ($P = 0.04$ for upper lobe) and 8 ($P < 0.0001$ for upper and lower lobes) DPI and from newly weaned ferrets at 3 ($P = 0.05$ for upper and $P = 0.04$ lower lobe), 5 ($P = 0.004$ for upper and lower lobes), and 8 ($P < 0.0001$ for upper and lower lobes) DPI. CA/09 influenza A virus infection led to prominent submucosal gland infection in all age groups (Figure 6, A−D), but the kinetics differed, with aged ferrets having statistically significant differences from adult ferrets at 3 ($P = 0.007$) and 8 ($P < 0.0001$) DPI and newly weaned ferrets at 3 ($P = 0.0006$) and 8 ($P < 0.0001$) DPI. Trachea infection was most prominent in the newly weaned and aged ferrets, with each cohort having peak viral production at 5 DPI (Figure 6, E−H). Trachea infection was statistically different in aged ferrets compared with adult ferrets at 3 ($P = 0.009$) and 8 ($P < 0.0001$) DPI and newly weaned ferrets at 3 ($P = 0.0003$) and 8 ($P < 0.0001$) DPI.

Pneumonia Is Slow to Develop in Aged Ferrets but Viral Infection Is More Persistent

To visualize a measurement that encompasses the pneumonia severity or the infection severity during influenza
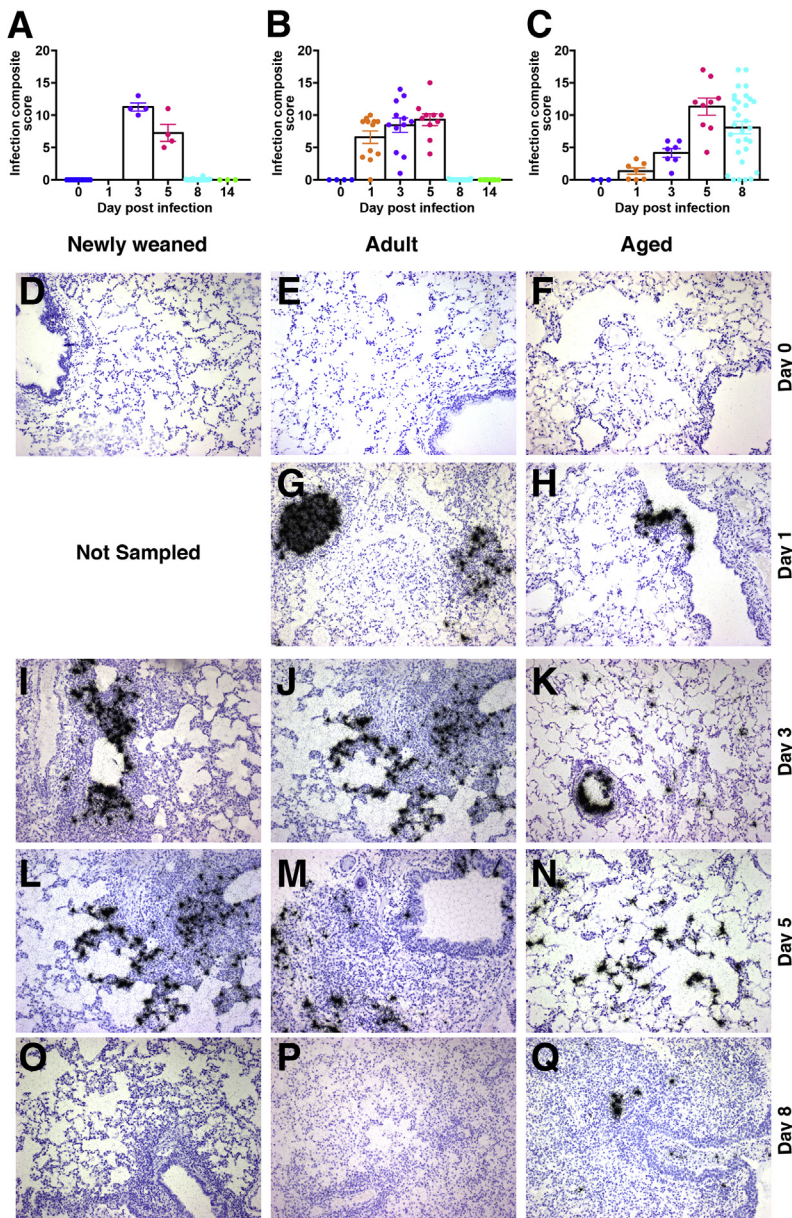
Figure 4 Age-specific H1N1 influenza virus lung infection. **A−C:** Infection composite scores were assessed for 0, 1, 3, 5, 8, and 14 days post infection for each age group. **D−Q:** Representative images of lung infection localized by influenza *in situ* hybridization are depicted for newly weaned (**D, I, L,** and **O**), adult (**E, G, J, M,** and **P**), and aged (**F, H, K, N,** and **Q**) ferrets during infection (0, 1, 3, 5, and 8 days post infection). Influenza matrix protein RNA transcripts appear as collections of black silver grains over cells counterstained with hematoxylin (blue). Data are expressed as mean scores (bars) and scores of individual animals (dots). Original magnification, ×100 (**D−Q**).

virus infection, a composite score was determined by adding the scores for each category (pneumonia or infection) from the upper and lower lobes at each time point. The pneumonia composite score indicates that pneumonia peaks later in the newly weaned ferrets compared with the mature adults, whereas pneumonia is slow to develop in aged ferrets (Figure 2, A−C). Adult ferrets were slower to resolve signs of pneumonia than newly weaned animals. There was individual ferret variability at most time points for all animals. Despite this variability in aged ferrets at 8 DPI, 100% of ferrets in this group required euthanasia. The hematoxylin and eosin images of pneumonia during infection reflect the mean composite score shown in Figure 2, A−C (Figure 2, D−S). Infection composite scores have little variability in newly weaned ferrets with peak infection at 3 DPI

(Figure 4). Adult ferrets have greater infection variability throughout the infection phase of the disease and cleared virus by 8 DPI (Figure 4). Aged ferrets also had considerable variability in infection. with a few ferrets having viral clearance by 8 DPI, but most aged ferrets had a range of viral infected cells (Figure 4).

Interlobular Variability in Pneumonia and Infection

Some animals in the adult (33%) and aged (18%) groups had marked variation in degree of pneumonia and infection present between the individual upper and lower lung lobes. Infection was more uniform in the newly weaned ferrets. In adults, the greater pathologic findings were usually observed in the lower lung lobe (Supplemental Figure S1, A−F), and
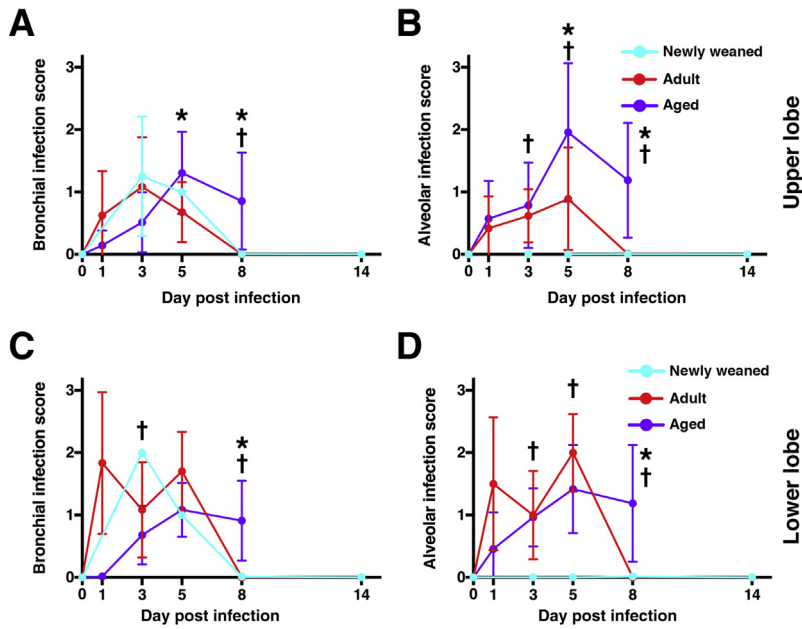
Figure 5 Comparison of regional lung infection severity between each age group. Graphs depict severity of influenza A virus infection in bronchial (**A** and **C**) and alveolar (**B** and **D**) spaces for the upper (**A** and **B**) and lower (**C** and **D**) lung lobes. The severity of influenza infection was determined by scoring of influenza virus *in situ* hybridization foci: 0, no definitive signal; 1, occasional focus; 2, focus in most fields; and 3, >1 focus per field. *$P < 0.05$ adult versus aged; †$P < 0.05$ newly weaned versus aged.

examples were found at 1, 3, and 5 DPI. Aged animals did not have disparate involvement until 5 DPI, reflecting the lagging disease (Supplemental Figure S1, G—J).

Discussion

Influenza virus infection in newly weaned, adult, and aged ferrets produces a range of severities in weight loss, disease symptoms, and virus-induced pathologic findings. Overall, there were age-related differences in the kinetics of CA/09 influenza virus lung infection and the onset and severity of pneumonia. CA/09 influenza virus infected the upper and lower respiratory tracts in all 3 age groups, as reported previously for adult ferrets.[33—37] In this article, we report that after infection with CA/09 influenza virus, newly weaned ferrets have a relatively mild clinical infection and pneumonia with no alveolar infection, whereas naïve aged ferrets >5.5 years old had delayed pneumonia development, less efficient clearance of virus infection, and a higher mortality rate.
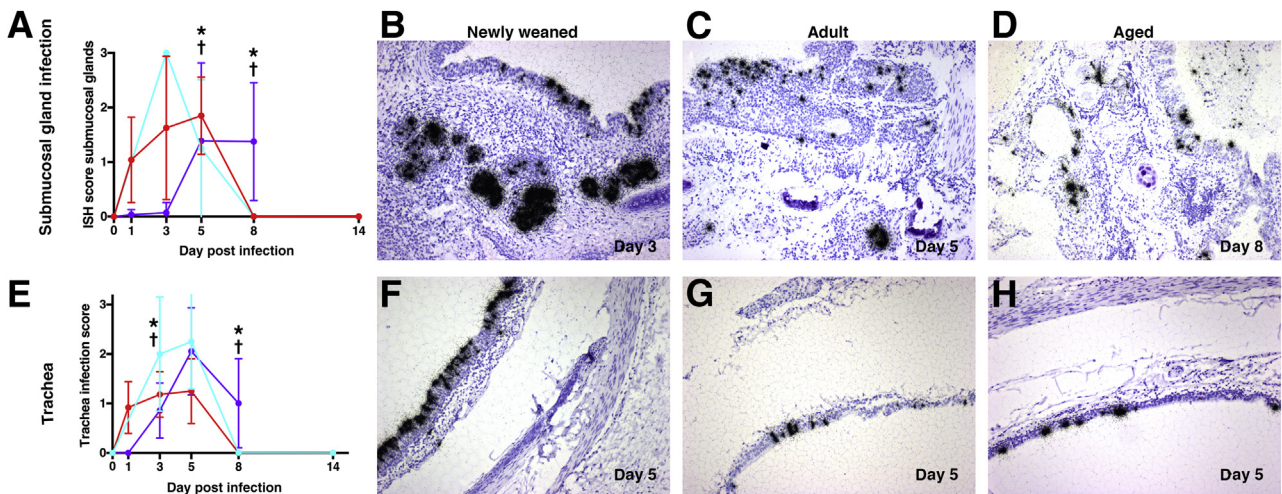
Figure 6 Comparison of submucosal gland (**A**—**D**) or trachea (**E**—**H**) infection severity between each age group. **A** and **E**: Submucosal gland (**A**) or trachea (**E**) infection severity. Severity of influenza infection was determined by scoring of influenza virus *in situ* hybridization (ISH) foci: 0, no definitive signal; 1, occasional focus; 2, focus in most fields; and 3, >1 focus per field. **B**—**D**: Representative images of submucosal gland infection of the lungs localized by influenza ISH are depicted for newly weaned (**B**) at 3 days post infection, adult (**C**) at 5 days post infection, and aged (**D**) ferrets at 8 days post infection. **F**—**H**: Representative images of trachea infection localized by influenza ISH are depicted for newly weaned (**F**), adult (**G**), and aged (**H**) ferrets at 5 days post infection. Influenza matrix protein RNA transcripts appear as collections of black silver grains over cells counterstained with hematoxylin (blue). Original magnification, ×100 (**B**—**D** and **F**—**H**). *$P < 0.05$ adult versus aged; †$P < 0.05$ newly weaned versus aged.

Newly weaned ferrets had remarkable resistance to alveolar infection despite abundant tracheal, bronchial, and submucosal gland infection. Infected newly weaned ferrets had a failure to thrive and gain weight compared with noninfected newly weaned ferrets, yet most had less weight loss and pneumonia than the adult and aged ferrets. Severe disease was observed in 9 of 69 infected newly weaned ferrets (13%) who lost ≥15% body weight by 6 DPI. In contrast, 33 of 63 aged ferrets (52%) had severe weight loss and disease. In addition to tracheal, bronchial, and submucosal cell infection, adult and aged ferret groups had significant alveolar infection.

Similar to CA/09 influenza virus infection in young children,[15−17] young ferrets had milder clinical illness than adult and aged ferrets. Compared with adult ferrets, newly weaned ferrets had similar viral clearance kinetics and similar pneumonia profiles but less weight loss and fever. These observations are comparable to previous observations of newly weaned and adult ferrets infected with A/Mexico/4108/2009 influenza virus, except that ferrets in the prior study cleared infection earlier and the newly weaned ferrets had earlier resolution of pneumonia.[36] These newly weaned ferrets also had fewer granulocyte infiltrates in the lung and formation of inducible bronchus-associated lymphoid tissue−like structures.[36] Inducible bronchus-associated lymphoid tissue is an ectopic lymphoid tissue formed during inflammation or infection and is located throughout the lung. Inducible bronchus-associated lymphoid tissue contributes to pulmonary immune responses by acquiring antigens from the airways, initiating local immune responses, and maintaining lung-resident memory cells.[30,38] We hypothesize that these immune mechanisms played an integrated role in reducing severity of influenza virus−induced disease and alveolar cell infection in the newly weaned ferrets.

Adult ferrets had the earliest peak in pneumonia, with a wide range of disease severity. All adult ferrets were able to clear infection by 8 DPI. For this age group, a cohort of male ferrets infected with CA/09 influenza virus was examined to determine sex-related differences in disease severity. Adult female and male ferrets had similar weight loss profiles. Pathologic scores for adult female and male ferrets had a broad, overlapping range of severity in the percentage of lung consolidation and bronchial and alveolar pneumonia with a predominance for bronchial pneumonia (Supplemental Figures S2 and S3). The finding that the severity of pneumonia and lung infection was similar between males and females suggests that sex differences do not overtly affect the parameters examined in this study. We are currently analyzing transcriptomic and genomic data to help determine the mechanisms behind the age group infection severity with a focus on innate immune-related mechanisms.

Interestingly, aged ferrets had a low frequency of infected cells at early time points after infection. Both pneumonia and infection progressed such that by 8 DPI, when the other groups had nearly complete viral clearance, aged ferrets still

had substantial influenza virus infection in all regional respiratory compartments. A recent study found that aged male ferrets (>4 years of age) sequentially infected with influenza A H1N1 viruses (Bris/59 then Mex/4108) had diminished antibody production and altered peripheral blood T-cell responses compared with adult ferrets (4 to 6 months of age).[39] Similar to the aged ferrets in this study, aged mice (72 to 76 weeks of age) had delayed responses, increased morbidity, accelerated weight loss, and slower recovery from influenza virus infection compared with adult mice (12 to 16 weeks of age).[40] Prior exposure of young mice to influenza-like particles that contained hemagglutinin and neuraminidase genes from the 1918 influenza virus at a young age conferred protection from CA/09 virus challenge when they were aged by preventing lower respiratory tract infection, whereas aged control mice succumbed to CA/09 infection by 7 DPI.[21] This finding suggests that inhibiting lower respiratory tract infection in aged animals limits severity of influenza virus−induced disease.

CA/09-like viruses are more likely to replicate in human and ferret lower respiratory tracts and are more likely to cause pathogenic effects in lung tissue compared with other H1N1 seasonal influenza strains.[41−44] Lower respiratory tract infection with influenza results in pneumonia and bronchiolitis and is a major cause of morbidity and mortality that disproportionately affects those >70 years of age and <5 years of age.[45] When CA/09-like virus induces severe lower respiratory tract infections, it can lead to acute lung injury or acute respiratory failure and carry a high mortality rate.[46] In this study, ferrets in all age groups had infection of the lower respiratory tract, potentially because of the intranasal infection route. Newly weaned and adult ferrets had comparable bronchial cell infection, yet the newly weaned ferrets had less weight loss. The striking difference in alveolar infection may contribute to the differences in disease outcomes. The CA/09-like strains that emerged and caused the 2009 H1N1 pandemic resulted in a primarily mild, self-limited illness, requiring hospitalization in <5% of infected individuals. However, as observed in the ferrets in this study, there were severe outcomes, including respiratory failure and death.[47] Both the adult and aged ferret groups had alveolar infection with a wide spectrum of severity, but the aged group had a lethal disease. Thus, the association between location of infection and disease severity attributable to influenza is complicated. Location of inflammation and kinetics of viral replication, viral clearance, and immune response likely factor into disease progression. Examination of immune responses within the local microenvironments of the respiratory tract of each age group would add insight to these dynamics.

Infant ferrets have differences in disease pathogenesis compared with newly weaned ferrets. Infant ferrets infected with CA/09-like viruses have more severe disease and decreased survival compared with adult mothers.[48] This finding may also recapitulate human disease in which CA/09-like virus−infected infants <6 months of age develop

high rates of hospitalization and death.[49–51] Influenza viruses that bind to $\alpha_{2\text{-}3}$-linked receptors are more likely to infect the lower respiratory tract because the proportion of $\alpha_{2\text{-}3}$-linked sialyl glycans is relatively greater than $\alpha_{2\text{-}6}$-linked sialyl glycans in both humans and ferrets.[52] Seasonal H1N1 influenza viruses bind exclusively to $\alpha_{2\text{-}6}$-linked sialyl sequences, whereas CA/09-like influenza viruses bind both $\alpha_{2\text{-}3}$-linked sialyl and $\alpha_{2\text{-}6}$-linked sialyl sequences.[53] Interrogation of possible differences in expression of sialyl glycans might aid in understanding why newly weaned ferrets are resistant to alveolar cell infection.[54]

This is the first report on the effects of CA/09-like H1N1 influenza viruses in extremely aged ferrets (5.5 to 7 years of age). The naïve aged ferret model appears to resemble humans with severe lower respiratory tract disease and pathologic findings. Despite the delay in lung infection and development of pneumonia, elderly individuals cannot clear influenza virus lung infection as quickly as adults or young people. A range of disease severity was observed with each age group, but there were age-related differences in infection kinetics. Each age group had striking submucosal infection with comparable bronchial pneumonia and infection severity. Newly weaned ferrets had a milder disease than the adult and aged ferrets, with a marked absence of alveolar cell infection. Overall, these ferret models can be used to test vaccines, therapeutics, and drug therapies and to better understand the human immune response to influenza virus infection associated with age.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2019.08.017*.

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