Compromised respiratory function in lethal influenza infection is characterized by the depletion of type I alveolar epithelial cells beyond threshold levels

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RECURRENT INFLUENZA EPIDEMICS and pandemics can be “mild,” with a relatively low incidence of hospitalizations and deaths, or “severe,” with a much higher prevalence of fatal events (as in the 1918 pandemic) (22). This difference in outcomes can be correlated with the distribution of pathology. Although both the upper and lower respiratory tracts (URT, LRT) are affected in most influenza cases, mild infections tend to be characterized by URT symptoms of nasal congestion and sinusitis whereas severe disease manifests with LRT injury and respiratory difficulty (7).

Airway pathology is characterized by inflammation, necrosis, and edema, with accompanying denudation of the bronchiolar and alveolar epithelia. Such damage can be mediated directly by virus invasion and the consequent lysis of respiratory tract cells, whereas further, more indirect compromise follows from immune cell activities, including neutrophils, macrophages, CD8+ T cells, and NK cells (1, 8, 17, 19). The pattern of virus distribution throughout the lung (e.g., proximal vs. proximal + distal airways) likely determines the nature and pattern of tissue damage (3), defining the overall pathogenicity profile.

The lung alveoli contain type I and type II pneumocytes. The cuboidal type II cells occupy roughly 5% of the alveolar surface, produce surfactant, and are thought to serve as progenitors for both type I and II pneumocytes (23). Type I cells cover most of the remaining surface area of alveoli. The thin, squamous type I cell population allows for the passage of oxygen and carbon dioxide between the alveolar space and the network of capillaries immediately opposing the shared basement membrane. These capillaries, which surround the alveolar sacs, are responsible for the delivery of oxygenated blood to and the removal of deoxygenated blood from the host.

Severe influenza virus strains A/PR/8/34 (H1N1), A/HK/483/97 (H5N1), and A/HK/54/98 (H1N1) replicate productively in differentiated human primary alveolar cells (4, 28), and PR/8 infection of human alveolar cells induces their apoptosis (12). Type II pneumocytes are thought to be preferential targets, although infection, virus replication, and cell death of both categories of pneumocyte have been reported (4, 5, 8, 26). Alveolar cell death is frequently observed in autopsies of patients with severe respiratory infections, such as H5N1 influenza and SARS (11, 20, 24). Although significant influenza-associated epithelial cell death will result in clinical impairment, the extent of alveolar cell loss may have the largest impact on the overall health and recovery ability of the host.

Although reports have shown that severe influenza is associated with increased alveolar damage, the “whole animal” physiological impact of mild vs. severe influenza virus-induced airway epithelial cell depletion has not been well documented. Importantly, it is not definitively known whether severe infections are the result of qualitative vs. quantitative differences in the extent of virus spread and tissue damage. We have used a spectrum of physiological and pathological assessments to probe this key question.

MATERIALS AND METHODS

Mice and infections. Female 11- to 14-wk-old C57BL/6J (B6) mice (Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Prior to virus inoculation, mice were anesthetized by intraperitoneal injection of 2,2,2-tribromoethanol. Sedated mice were given 30 μl PBS intranasally containing 4 × 105 EID50 (egg infectious dose 50) A/Puerto Rico/8/34 (PR/8) and 1 × 106 EID50 A/Aichi/02/68 (HA, NA) × A/Puerto Rico/8/34 (x31). Both strains are mouse adapted and
grow only in the murine respiratory tract. All procedures were done according to an institutional approved IACUC protocol following guidelines established by the Institute of Laboratory Animal Research and euthanasia following evidence (body index score) of severe morbidity, based on clinical observation and measurement of substantial weight loss.

**Arterial blood gas measurements.** Left ventricle cardiac puncture for arterial blood collection was performed under sedation with an isoflurane-100% oxygen mix, and samples were analyzed immediately for blood gases by handheld diagnostics (VetScan i-STAT 1 Analyzer, Abaxis, Union City, CA).

**Unrestrained whole-body plethysmography with gas analysis.** Respiratory and gas measurements were acquired by using an animal plethysmograph with gas analysis capabilities (Buxco, Wilmington, NC). Briefly, unanesthetized animals were placed in chambers that measure slight changes in pressure due to respiratory behaviors. Uninfected mice and relatively healthy mice were acclimated to chamber environments for an hour before data acquisition, whereas mice that were clearly ill (and much less mobile) were acclimated for 20 min. No measures involved forced actions. For individual mice, respiratory measurements were taken every 2 s for 6 min, and gas measurements every 2 s in 30-s intervals.

The plethysmographic measurements were breath frequency (counts/min), expiratory time (s), tidal volume (ml, calculated), and minute volume (ml/min, calculated). Gas measurements determined the volume of oxygen consumed (VO_{2}) and the volume of carbon dioxide (VCO_{2}) produced (both ml/min). Metabolic rate was calculated from VCO_{2} and VO_{2}, VCO_{2}, V_{CO_{2}}, minute volume, and tidal volume values are used for direct comparison of animal groups in our experiments; however, because of technique, absolute values of these parameters may be different in others’ hands. Data analysis was performed by one-way ANOVA with Tukey’s correction.

**Histopathological evaluation.** Following euthanasia by CO_{2} inhalation, mouse lungs underwent gravity inflation (reservoir placed 23 cm above the animal) via tracheal infusion with 10% neutral-buffered formalin solution (vol/vol). After removal, lungs were immediately fixed in the same formalin solution for a minimum of 24 h before embedding, sectioning, and staining for conventional histopathology (hematoxylin and eosin (H&E), viral influenza virus (US Biological, Swampscott, MA), podoplanin (T1a, gp38, and aggrus) (eBioscience, San Diego, CA), and surfactant C (Santa Cruz Biotechnology, Santa Cruz, CA). One slide including all lung lobes per mouse was used for evaluations.

The podoplanin- and surfactant C-stained sections were blinded for pathology evaluation and the loss of type I pneumocytes was quantified by capturing digital images of whole lung sections stained for podoplanin using an Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA), then manually outlining entire fields together with areas with noticeably decreased (or absent) staining for podoplanin. The percentage of lung field with reduced type I pneumocyte coverage was calculated by use of Aperio’s ImageScope software.

Five types of pulmonary lesions were scored histologically for H&E sections on a 1–5 scale, as follows: 0 = no lesions; 1 = minimal, focal to multifocal, inconspicuous; 2 = mild, multifocal, prominent; 3 = moderate, multifocal, prominent; 4 = marked, multifocal or coalescing, lobar; 5 = severe, extensive, and diffuse, with multilobar consolidation.

**Detecting virus.** Tissue sections were stained for influenza virus as described above. Whole lungs were homogenized in infection medium (double-distilled H_{2}O, 0.3% BSA, 0.45% NaHCO_{3}, 1× MEM), and added to confluent monolayers of Madin-Darby canine kidney (MDCK) cells in 10-fold dilutions (10^{-1} to 10^{-6}) for 1 h. The supernatant fluid was then removed and the cultures were overlaid with agar containing 1 μg/ml trypsin (TPCK treated, Sigma-Aldrich, St. Louis, MO). After 37°C incubation for 72 h, agar plugs were removed, cell layers were stained with crystal violet, and plaques were counted for titer calculation.

**RESULTS**

Significant illness, measured by percent weight loss, occurred following intranasal challenge with both the “mild” x31 and “severe” PR/8 virus strains (Fig. 1A). Following x31 infection, mice lost more weight initially than PR8-infected animals, but they lost less weight overall and recovered completely by day (d) 10. At the virus doses used, all the x31 mice survived whereas there was a 60% morbidity/mortality rate following PR/8 infection (Fig. 1B).

Respiration patterns for nonlethal and lethal infections. We tracked the respiration of individual x31- and PR/8-infected mice for 14 days using unrestrained whole body plethysmography with gas analysis. Here, we determined breaths per minute (BPM), tidal volume, minute volume, and expiratory time on d2–d15 in at least four mice per influenza strain. Breath waveforms of uninfected vs. d9 mild and severe infections showed virus-strain related alterations in respiration (Fig. 2A). On d2, the x31 mice were distinctly different from the uninfected and PR/8 mice, as shown by decreased BPM, increased tidal volume, and increased expiratory time (Fig. 2, B, C, and E). The two groups of infected mice began to converge on d3, and were well matched for most measures by d6. BPM was normal for both viruses by d9, and expiratory time was no longer elevated in either infection by d8 (Fig. 2, B and E).

![Fig. 1. Comparison of weight loss and necessary euthanasia in PR/8- vs. x31-infected mice after infection. A: weight loss as a percentage of original weight. B: percent mice not reaching guidelines for euthanasia (severely moribund). x31, n = 27; PR/8, n = 16 mice. Means ± SE shown.](http://ajplung.physiology.org/)[Downloaded from by 10.20.32.246 on June 21, 2017](http://ajplung.physiology.org/)
Inspiratory time changed only at later times after x31 infection (data not shown), whereas tidal and minute volumes were significantly decreased in the PR/8 mice, beginning on d10 and d6, respectively (Fig. 2, C and D). Surprisingly, these two volume measures increased above uninfected mouse levels in the x31-infected group. Enhanced Pause and Pause, although considered controversial, are thought to be influenced by changes in respiratory patterns (2, 22). These patterns were similar to those of expiratory time (Fig. 2 E and data not shown).

Compromised arterial blood oxygenation in lethal infection. Oxygen levels were determined for arterial blood over the course of the disease. Partial pressure of oxygen (ppO2, mmHg) is a measure of O2 in arterial blood and is indicative of the effectiveness of gas exchange. Analysis of arterial blood oxygenation showed similarly decreased levels on d3 and d6 after either infection, although these were not statistically significant from the values for uninfected mice (Fig. 3A). However, ppO2 levels clearly diverged, with x31-infected mice returning to normal by d12, whereas PR/8-infected mice exhibited substantially lower levels beginning d9. This ppO2 nadir overlaps with the time PR/8-infected mice succumb to disease (Fig. 1B). Although not statistically significant, the ppO2 levels of PR/8 survivors were still lower than normal on d30, suggesting persistent pulmonary damage and compromised function. Oxygen saturation levels, which represent the percentage of occupied oxygen binding sites on hemoglobin compared with the total number of possible binding sites, reflected ppO2 throughout in these groups of mice (Fig. 3C) although arterial blood carbon dioxide remained constant (Fig. 3B).

Poor gas exchange in lethal infection begins by day 6 after infection. Given the decreased blood oxygenation seen after d6 in the PR/8 mice, we investigated the kinetics of respiratory

Fig. 2. PR/8-infected mice have more severe respiratory functions than x31-infected mice. Respiratory functions of mice days 2–15 after infection. A: box flow waveform of uninfected vs. day (d) 9 postinfection mice; 0.8 s acquisition (ml/s). B: breaths per minute. C: tidal volume (ml). D: minute volume (ml/min). E: expiratory time (s). Four to 6 mice per time point until d13. d13–15 PR/8, n = 2; x31, n = 3. SE shown. *P < 0.05, **P < 0.005, ***P < 0.0005. Asterisks by bars: significance between uninfected (uninf.) and specified group on that day. Significance between x31 and PR/8 groups on specific days (not shown on graph): breaths per minute (B) d2 P < 0.0005, d12 P < 0.05; tidal volume (C) d2 P < 0.005, d6 P < 0.05; d7, d8, d9 P < 0.005; d10, d11 P < 0.0005; d12 P < 0.05; minute volume (D) d5 P < 0.05, d6 P < 0.005; d7 P < 0.05; d8 P < 0.005; d9 P < 0.05; d10, d11, d12 P < 0.0005; d13 P < 0.005; d14 P < 0.05; expiratory time (E) d2 P < 0.0005, d3, d4, d11 P < 0.05.
gas exchange by quantifying $\dot{V}_O_2$ and $\dot{V}_C0_2$ in discrete time units and calculating the MR, or energy expenditure due to metabolism. As with other respiratory measures, the $\dot{V}_O_2$, $\dot{V}_C0_2$, and MR responses to the two viruses were similar from d3–d6 (Fig. 4, A–C), with the x31 group experiencing only minor shifts in these parameters before returning to normal by d9. However, PR/8-infected mice exhibited drastically decreased tidal volume, $\dot{V}_O_2$, $\dot{V}_C0_2$, and MR by d9 through d12 (Figs. 2C and 4A–C). By d15, however, the PR/8 survivors regained normal levels for all these measures, despite their reduced ppO2 levels.

Lung damage is more severe in lethal infection after day 9 and the virus persists longer. We hypothesized that lethal infection caused increased tissue damage and thus functional deficit. Whole lungs were removed on d3, d6, d9, d12, and d15 after infection and H&E-stained sections were analyzed for five categories of lung lesions: alveolitis (necrosis and inflammation), bronchiolitis (necrosis, inflammation, and plugging of the bronchioles), peribronchiolitis (edema and inflammation), perivasculitis (margination and cuffing), and interstitial pneumonitis (septal thickening and inflammation). The x31 mice had slightly higher levels of bronchiolitis on d3 after infection caused slightly elevated levels of bronchiolitis on d3–d6 (Fig. 4, B–E). Blinded sections were also scored (scale 0–5) for virus staining. Comparable levels of influenza were detected in bronchioles and alveoli on d3 and d6 for x31 and PR/8, coinciding with the lung virus titer (plaque assay) results (Fig. 6, A–C). By both methods, x31 was controlled by d9 whereas PR/8 virus could be found until d12.

Lethal infection is associated with at least 10% type I pneumocyte loss. To pinpoint the amount of type I cell loss that associates with a negative impact on physiological functions in vivo, we quantified lung sections for type I (podoplanin/T1a/gp38/aggrus) and type II (surfactant C) pneumocytes on d3, d6, and d10 after x31 or PR/8 infection (Table 1, Fig. 7). Podoplanin/T1a is a known marker for type I pneumocytes and has been documented in lung injury models to demonstrate loss of these cells (6, 14, 15). PR/8 induced a progressive loss of type I pneumocytes, with coverage of alveolar septa declining from 95% (d3), to 91% (d6), to 60% on d10 (Table 1, Fig. 7, E, G, and H). By d10, most alveoli in affected areas of PR/8-infected lungs had markedly reduced coverage by type I and type II...
pneumocytes (Table 1, Fig. 7, E and F, and data not shown). This loss was accompanied by increased amounts of intra-alveolar and septal collagen, thickened alveolar septa, and associated intra-alveolar edema. Conversely, type I pneumocyte coverage of alveolar septa for the x31 group was 100% (d3), 99% (d6), and 97% (d10), with any type II pneumocyte loss reflecting the profile for type I cells in being limited to restricted foci (Table 1, Figs. 7, C and D, and data not shown). In addition, the d10 x31-infected lungs showed evidence of more advanced bronchiolar and alveolar repair, characterized by an absence of denuded bronchiolar airways and the presence of alveolar epithelial hyperplasia around some terminal airways.

DISCUSSION

Severe influenza virus infections, including those caused by the avian H5N1 and historical pandemic strains, result in substantial respiratory incapacitation and have been shown to induce significant alveolar epithelial cell death in patients, animal models, and in vitro cell cultures (4, 5, 8, 11, 12, 20, 22, 24, 26, 28). In the present analysis, analyzing in vivophysio-
logical responses in mice following infection with two influenza viruses that cause mild vs. severe disease has allowed us to link altered respiratory function to the extent of type I pneumocyte depletion assessed by subsequent histopathology.

The extent and severity of infection and inflammation did not diverge appreciably at early time points (d3 and d6), although they differed thereafter for the more (PR/8) and less (x31) virulent strains. Curiously, although x31 virus titers and deep lung location scores were highest early, and there was significantly increased alveolar inflammation, histological analysis on d6 showed only small foci of type I cell depletion. In contrast, despite comparable lung titers and alveolar inflammatory profiles, d6 PR/8 lungs showed type I cell loss in excess of that seen at any time point for the x31-infected respiratory tract. Many of the PR/8-infected mice generally succumbed by d9–d12, whereas the clinical status of the x31 set was greatly improved. Evidence of morbidity in the PR/8 group corresponded to the extent of alveolar damage, with functional measures (tidal volume, minute volume, V\(\dot{O}_2\), V\(\dot{CO}_2\), and arterial blood oxygen) reaching a nadir then showing improvement in the d15 survivors, likely reflecting the removal (by euthanasia) of severely affected mice.

### Table 1. Assessment of type I and type II alveolar epithelial cell presence in lungs of x31- and PR/8-infected mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day After Infection</th>
<th>Type I AEC</th>
<th>AEC I % Lung Field with Decreased or Absent Podoplanin</th>
<th>Type II AEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td></td>
<td>Diffusely positive</td>
<td>0</td>
<td>Uniform distribution</td>
</tr>
<tr>
<td>PR/8</td>
<td>3</td>
<td>Diffusely positive</td>
<td>0</td>
<td>Multifocal mild reduction</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Small foci with reduced positivity</td>
<td>1.2 (\pm) 0.2</td>
<td>Type II reduced in affected areas</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Small multiple foci negative</td>
<td>2.6 (\pm) 0.7</td>
<td>Type II reduced in small areas only</td>
</tr>
<tr>
<td>x31</td>
<td>3</td>
<td>Diffusely positive</td>
<td>0</td>
<td>Multifocal mild reduction</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Small foci with reduced positivity</td>
<td>1.2 (\pm) 0.2</td>
<td>Type II reduced in affected areas</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Small multiple foci negative</td>
<td>2.6 (\pm) 0.7</td>
<td>Type II reduced in small areas only</td>
</tr>
<tr>
<td>PR/8</td>
<td>3</td>
<td>Multifocal patches with reduced positivity</td>
<td>4.9 (\pm) 0.7</td>
<td>Multifocal moderate reduction</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Multifocal patches with reduced positivity</td>
<td>9.1 (\pm) 0.8</td>
<td>Type II reduced in affected areas</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Extensive areas negative</td>
<td>41 (\pm) 2.6</td>
<td>Type II reduced in affected areas</td>
</tr>
</tbody>
</table>

Whole lung tissues removed from mice on noted days after infection were sectioned and stained with podoplanin (type I alveolar epithelial cells (AEC)) or surfactant C (type II AEC). A pathologist blindly graded alveolar areas of lung tissues for intensity and localization of staining. Means \(\pm\) SE are shown for 3–4 mice per virus examined per day.

![Fig. 7. Increased depletion of type I and type II alveolar epithelial cells (AEC) in PR/8-infected vs. x31-infected mice. Whole lung tissue from uninfected or d10 postinfection mice were stained with podoplanin (type I AEC) or surfactant C (type II AEC). Representative images of uninfected mouse lung podoplanin staining (A), uninfected mouse lung surfactant C staining (B), d10 podoplanin staining in x31-infected mouse lungs (C), d10 surfactant C staining in x31-infected mouse lungs (D), d10 podoplanin staining in PR/8-infected mouse lungs (E), and d10 surfactant C staining in PR/8-infected mouse lungs (F). Whole lungs from mice d10 after PR/8 (G) or x31 (H) infection were removed, sectioned, and stained for podoplanin. Representative whole lobe section shown.](http://ajplung.physiology.org/doi/10.1152/ajplung.00343.2012/fig7)
Analysis of the data set from d9–d12 time reveals two curious findings. First, despite the absence of virus at d9, the x31 mice had high alveolitis scores indicative of continued inflammation. However, this had little effect on respiration or gas exchange and did not lead to the severe loss of type I pneumocytes. Second, although virus titration assays detected only very low levels of replicating virus in d9 PR/8-infected lungs, there was widespread virus staining in both the alveolar and bronchiolar compartments. This persistence of PR/8 virus appears to be a major distinguishing factor between the two infections, suggesting that cells with virus protein in their cytoplasm are indeed damaged and contribute to a downward spiral of ever diminished gas exchange and decreased arterial blood oxygenation with the net result being major functional compromise. Even so, increased alveolar epithelial cell loss (>x31) was noted in PR/8 lungs from d3, indicating that the damage cycle establishes early and that the effect of d9 virus persistence may be additive rather than a primary determinant of pathology. Such phenomenon is suggestive of differences between the two viruses’ effects on host tolerance, a concept long identified in plant biology but only recently introduced to animal infectious disease (16). Our data indicate that despite the equivalent capacities of the two viruses to replicate in lungs (titration on MDCK cells) and to invade both alveolar (and bronchiolar) epithelial cells, PR/8 infection results in more rapid and diffuse type I alveolar cell loss than is caused by the x31 virus.

Because it is unclear whether type I pneumocytes can be directly infected by influenza and support viral replication, the cause of type I pneumocyte loss in our hands is unknown (4, 8, 10, 26). The large surface area of type I cells lends them to increased susceptibility to environmental insults, which may result from direct virus-induced or “bystander” damage (25). This characteristic of type I cells may explain their quick demise during influenza infection. In addition, type I cell loss may reflect the lack of type II pneumocyte secretory products that are normally protective and limit type I cell apoptosis (13, 18, 27). Furthermore, as type II cells are the precursors of the type I cells, loss of the former could compromise the rapid repair of the alveolar epithelial lining and favor the development of interstitial and intra-alveolar fibrosis.

Coincident with the increased loss of type I pneumocytes, the PR/8 lungs show a reduced capacity to take in oxygen and expel carbon dioxide. It is likely that the higher mortality in these mice is due to the basic lack of proper respiration. Besides epithelial cell depletion, another possible contributor to poor respiration is edema. This condition generally occurs as a result of decreased barrier function. Therefore, despite the direct effect edema has on respiration, it can be attributable to the epithelial cell loss.

An overlay of time progression of \( V_{O_2} \) and \( V_{CO_2} \) (Figs. 4, A and B), arterial blood oxygenation (Fig. 3A), and animal survival (Fig. 1B) illustrates the coincidence of gas exchange nadirs with peak concession to disease. By adding in type I and type II staining scores to this time overlay, we can observe the degree of alveolar cell loss that corresponds to negative impact on systemic respiration. Combining measurements of d6–d9 \( V_{O_2}, V_{CO_2} \), tidal volume, and arterial blood oxygenation with alveolar cell staining, we see that mice lose the ability to respire properly coincident with a 10–40% depletion of type I cells. We can also consider the converse situation: how much alveolar cell loss can an organism experience and still retain adequate lung function? Assuming that d6 is the last time point when PR/8-infected mice respire and oxygenate their blood well, the data suggest that ~10% loss of type I cells in multifocal patches, and comparable type II loss in affected areas, is survivable.

By extending studies out to 30 days after infection, we observed long-term damage and recovery in mice after acute influenza virus infection. Our findings are in accord with previous work showing extended lung impairment by histopathology and mean breath volume despite early virus clearance (9). Overall, alveolar cell loss can simply be too great to allow functional recovery in mice exposed to a virulent influenza virus.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C.J.S., P.V., P.C.D., and P.G.T. conception and design of research; C.J.S., P.V., P.C.D., and P.G.T. interpreted results of experiments; C.J.S. and P.V. prepared figures; C.J.S. drafted manuscript; C.J.S., P.V., P.C.D., and P.G.T. edited and revised manuscript; C.J.S., P.C.D., and P.G.T. approved final version of manuscript; P.V. analyzed data.

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