Chapter 20

Intranasal Influenza Infection of Mice and Methods to Evaluate Progression and Outcome

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Abstract

In vivo influenza infection models are critical for understanding viral dynamics and host responses during infection. Mouse models are extremely useful for infection studies requiring a high number of test animals. The vast array of gene knockout mice available is particularly helpful in investigating a particular gene's contributions to infection. Thus, more in vivo scientific experimentation of influenza has been done on mice than any other animal model. Here, we describe the technique of intranasal inoculation of mice and methods for assessing the severity of disease and humane endpoints, and discuss data acquired from infection of female C57BL/6J mice.

Key words Intranasal inoculation, influenza, 2,2,2-Tribromoethanol, Body weight loss, Animal euthanasia guidelines, Lung damage

1 Introduction

Influenza has dramatically impacted human populations. The 1918–1919 "Spanish" influenza epidemic resulted in roughly 50 million deaths worldwide [1, 2]. Subsequent pandemics in 1957 (H2N2 "Asian influenza") and 1968 (H3N2 "Hong Kong influenza") caused a reported 1.5–2 million and 1 million deaths, respectively [3–5]. The recent 2009 H1N1 pandemic, believed to cause relatively mild disease, has been found to have a global infection rate between 11 and 21 % and a case fatality rate of 0.03 % [6, 7]. Normal "seasonal" waves of influenza regularly result in over 30,000 deaths and 200,000 hospitalizations in the United States alone [8]. The continuing high number of severe cases demonstrates the need for further study on influenza pathogenesis. The host response to influenza, and the interplay of virus–host dynamics, is not particularly well understood and more work is needed.

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The advantages of using mice for in vivo studies are manifold. In comparison to other animal models used for influenza studies, breeding is quick and generally produces many offspring. As mice are an exceptionally well-studied species, their behavior in response to disease is well documented.

The genetic background of the host plays a critical role in the determination of susceptibility to a wide range of influenza isolates [9–11]. Mouse strains considered highly susceptible to influenza include A/J and DBA/2J [11, 12]. Strains considered moderately to minimally susceptible include Balb/C and C57BL/6J. Curiously, wild mice are resistant to some strains of influenza. This trait is attributed to the ability to synthesize Mx protein, an inducer of an antiviral state [13, 14]. Most inbred strains of mice have a functionally deleted Mx gene.

Influenza studies examining immunological memory responses frequently use a primary/secondary infection method, in which two different strains of virus are administered using the same or two different routes (for example, intranasally and intraperitoneally), separated by a specified period of time. Coinfection of influenza with bacteria or parasites generally follows a more complex administration protocol. Here, we describe a method for administration and evaluation of a primary influenza infection in C57BL/6 mice.

2 Materials

2.1 Infection

1. Virus diluted in 1× phosphate-buffered saline (PBS). Ensure that it is kept at 4 °C throughout preparation and administration. Select a virus concentration appropriate to the intention of the study (*see* **Note 1**).
2. 2,2,2-Tribromoethanol (Avertin) (*see* **Notes 2** and **3**).
3. Sterile 1 ml syringes and needles.
4. Positive displacement pipet.
5. Pistons.
6. Piston sheaths.

2.2 Noninvasive Evaluation: Weight Loss and Regain

1. Scale capable of weighing mice that range from 10 to 50 g.
2. Sided, flat-bottomed container that can fit on the weighing platform of the scale and can hold an active mouse without allowing for escape.

2.3 Invasive Evaluation: Plaque Assay on Madin–Darby Canine Kidney Cells

1. Confluent MDCK cells in 6-well plates (*see* **Note 4**) [15].
2. 37 °C incubator.
3. 37 °C water bath.
4. 65 °C water bath.

5. Vortex.
6. 6-Well plates.
7. Growth medium for MDCK cells (total volume 1 l): 100 ml of 10× MEM, 50 ml of fetal calf serum, 30 ml of 7.5 % sodium bicarbonate, 10 ml of penicillin/streptomycin (10,000 U/ml; 10,000 μg/ml), 10 ml of L-glutamine—200 mM (100×), 10 ml of MEM vitamin solution (100×), and 790 ml of ddH_2O.
8. Infection medium (total volume 1 l): 100 ml of 10× MEM, 40 ml of 7.5 % bovine serum albumin, 30 ml of 7.5 % sodium bicarbonate, 10 ml of penicillin/streptomycin (10,000 U/ml; 10,000 μg/ml), 10 ml of L-glutamine—200 mM (100×), 10 ml of MEM vitamin solution (100×), and 800 ml of ddH_2O.
9. 2× MEM medium (total 1 l): 200 ml of 10× MEM, 80 ml of 7.5 % bovine serum albumin, 60 ml of 7.5 % sodium bicarbonate, 20 ml of penicillin/streptomycin (10,000 U/ml; 10,000 μg/ml), 20 ml of L-glutamine—200 mM (100×), 20 ml of MEM vitamin solution (100×), and 600 ml of ddH_2O.
10. Tissue homogenizer.
11. Round-bottom polypropylene tubes able to hold minimum 2 ml volume (preferably 5 ml).
12. 1× PBS.
13. 200 μl and 1 ml pipettors.
14. TPCK trypsin (TPCK: L-(tosylamido-2-phenyl)ethyl chloromethyl ketone).
15. 1.8 % agar for overlay: 9 g Bacto-Agar, 500 ml sterile ddH_2O. Completely dissolve by heating, then autoclave.
16. Capped flask for mixing.
17. Tool, such as a spatula, for removing solidified agar overlay from 6-well plates.
18. Crystal violet staining reagent (1 l): 270 ml of 37 % formaldehyde solution, 730 ml of distilled H_2O, 1 g of crystal violet.

2.4 Invasive Evaluation: Lung Histology

1. Surgical tools (e.g., scissors, scalpel).
2. Syringe capable of holding 3 ml of fluid.
3. Thin catheter, able to fit in mouse trachea.
4. 10 % neutral buffered formalin solution.
5. Paraffin.
6. Device for sectioning tissues to 4 μm thickness.
7. Positively charged glass slides.
8. Hematoxylin and eosin stains.
9. Collagen stains Masson's Trichrome or Sirius Red, if desired.

3 Methods

3.1 Virus Dilution Method

1. Rapidly thaw aliquots of virus at 37 °C (*see* **Note 5**). Put the virus aliquots on ice immediately upon thawing.
2. Dilute the virus tenfold in ice-cold 1× PBS in polypropylene tubes and keep the virus on ice (*see* **Note 6**).
3. Keep the last dilution on ice throughout the duration of mouse infections.

3.2 Infection Method

1. Administer 2,2,2-tribromoethanol by intraperitoneal injection. Each animal should receive 250 mg/kg (*see* **Notes 7** and **8**).
2. Hold mice such that the head is tipped back and the nose is pointed upwards. Ensure that no pressure is exerted on the soft areas needed for nasal respiration (i.e., the nose and throat area) (Fig. 1).
3. Carefully expel virus through a positive displacement sheath/tip and droplets are put directly on the nostril openings. The mouse should inhale these droplets quickly. The speed of administration should match that of the mouse's inspiration of droplets.
4. Once the mouse has been inoculated, gently place the animal in an environment with mild heat and monitor until it regains consciousness (*see* **Note 9**).
5. If the experiment entails tracking the progress of infection of individual mice, then each mouse should be individually marked for identification (*see* **Note 10**).

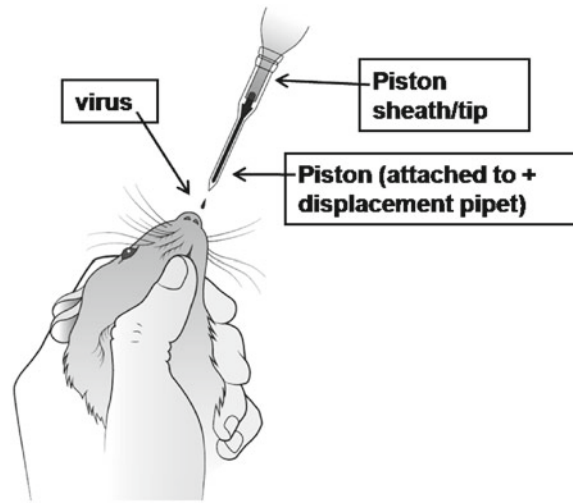

Fig. 1 Schematic illustrating the intranasal inoculation of virus to an anesthetized mouse

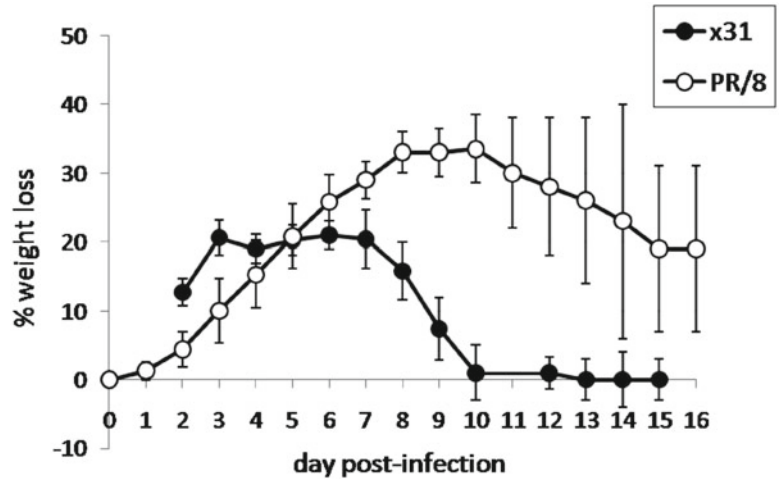

Fig. 2 Percent body weight loss kinetics of a nonlethal dose of ×31 (1×10^6 EID_{50}) to a dose of PR/8 that induces roughly half of C57BL/6 mice to meet our institutional guidelines for animal euthanasia (4×10^3 EID_{50}). ×31, $n = 20$ mice; PR/8, $n = 17$ mice. Eight days after infection, PR/8 infected mice are gradually euthanized. Standard deviation shown

3.3 Evaluation of Weight Loss and Regain

1. Monitor weight loss and recovery throughout the duration of the model (*see* **Note 11**). To evaluate weight, gently pick up the mouse by the base of the tail and transfer the animal to the container on the scale. Once a steady reading can be made, which is generally when the mouse has stopped moving around, record the weight. Ensure that a weight measurement has been taken for each mouse before the infection. This initial weight is necessary for the calculation of percent weight loss.
2. Calculate the percent weight change for each animal. Divide the difference of pre- versus postinfection weights and divide this difference by the pre-infection weight to obtain percent body weight change. Alternatively, divide postinfection body weight by baseline (pre-infection) body weight for each mouse to obtain the percent of original body weight. For example, weight loss curves over time of mice given a nonlethal dose of mouse-adapted influenza strain ×31 or a lethal dose of mouse-adapted strain PR/8 are shown in Fig. 2 (*see* **Notes 12–15**).

3.4 Viral Titer by Plaque Assay on MDCK Cells

1. One day before performing the plaque assay, seed an appropriate number of 6-well plates (one plate per six dilutions) with roughly 1.5×10^6 MDCK cells/well using 2 ml of infection medium/well. Keep overnight at 37 °C until use.
2. Homogenize each thawed lung in 2 ml of infection medium, and keep at 4 °C.
3. Prepare six tubes (select a size able to comfortably accommodate a 2 ml volume) for a tenfold serial dilution of each lung by adding 1.8 ml of infection medium/tube (*see* **Note 16**).

4. Set up the six tenfold dilutions of each virus. Briefly, add 200 μl of homogenized lung to the first tube and vortex. Continue this process for the remaining tubes.
5. Wash and prepare the MDCK-confluent plates. Briefly, aspirate the infection medium for each plate, and quickly replace with 3 ml of PBS. This is the first wash. Do three total washes per plate. Keep the cells on the last PBS wash until immediately before adding lung dilutions.
6. Add lung dilutions to the plates. Aspirate the last PBS wash, and then quickly add 1 ml of lung dilutions. Start by adding the lowest dilution to the last well of the 6-well plate, which allows for the addition of all dilutions without changing tips. Do not let the wells dry out.
7. Repeat this process for each plate.
8. Place the plates at 4 °C for 10 min.
9. Move the plates to 37 °C, and keep at this temperature for 50 additional minutes.
10. While the plates are incubating at 37 °C, prepare the agar overlay. Estimate 10 ml of 2× MEM per 6-well plate. Add enough 2× MEM for all plates to a capped flask and place in a 37 °C water bath. Next, add TPCK trypsin. You will need 20 μg per 10 ml of 2× MEM. The final step is to melt 1.8 % agar in a microwave. Once the agar is melted, place it in a 65 °C water bath to maintain its liquid state. You will need 10 ml of agar per 6-well plate.
11. After the plate incubation, remove 2× MEM and agar from the baths. Add TPCK trypsin to the 2× MEM then agar. For each 6-well plate, you will have 10 ml of 2× MEM, 10 μg of TPCK trypsin (1 μg/ml final concentration), and 10 ml of 1.8 % agar. Mix well without shaking.
12. Aspirate the liquid one plate at a time. Add 3 ml of 2× MEM/agar/TPCK trypsin mixture to each well, and repeat for each plate.
13. Allow the mixture to solidify on the plates for about 20 min.
14. Incubate at 37 °C for 72 h.
15. After incubation, carefully remove the solidified agar with a spatula. Do not scratch the bottom of the wells.
16. Add enough crystal violet to the wells to cover the bottom. Allow roughly 15 min for staining to occur.
17. Remove the crystal violet solution by a distilled water rinse.
18. Let the plates dry.
19. Count plaques and calculate the titer, keeping in mind the sequential tenfold dilutions of the lungs (*see* **Note 17**).

3.5 Lung Histology

1. Euthanize mouse on selected day after infection. Figures 5 and 6 show examples of lung histology on days 3 and 14 after infection, respectively (*see* **Notes 18** and **19**).
2. Gently inflate the lungs with 10 % neutral buffered formalin solution. This technique can be done via pushing the fluid through a syringe and an attached thin catheter inserted in a small hole in the trachea.
3. Surgically remove the whole lungs.
4. Immerse the infused tissue in the formalin for a minimum of 48 h.
5. Fix, paraffin embed, section (Figs. 5 and 6, tissues were sectioned at 4 μm), and mount the sections on positively charged glass slides.
6. Stain with hematoxylin and eosin (H&E). If visualization of collagen deposition is desired, stains such as Masson's Trichrome and Sirius Red allow for its detection. Figures 5 and 6 show examples of H&E and Sirius Red staining (*see* **Note 19**).
7. Evaluate lung histology.
8. Correlate the histologic evaluations with weight loss (*see* **Note 20**).

4 Notes

1. For infections to determine susceptibility, it is recommended to select a virus dose in the range of the LD_{50} of the particular virus stock. Figures 2, 3, and 4 show PR/8 doses ranging from 2×10^3 EID_{50} to 8×10^3 EID_{50} (50 % egg-infective dose).

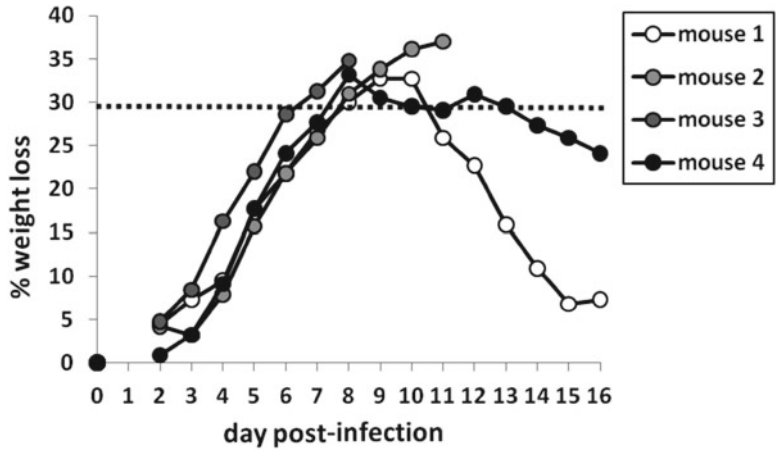

Fig. 3 Weight loss over time of individual C57BL/6 mice infected with 4×10^3 EID_{50} of mouse-adapted PR/8

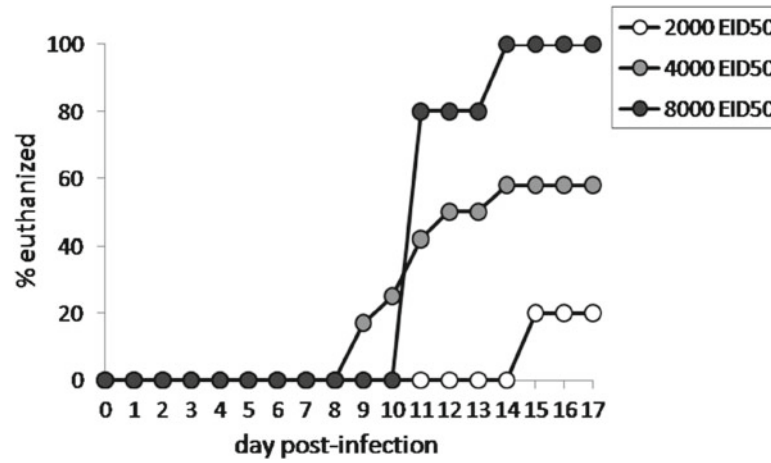

Fig. 4 Percentage of C57BL/6 mice meeting guidelines for euthanasia after PR/8 infection. 2×10^3 EID_{50}, $n = 5$ mice; 4×10^3 EID_{50}, $n = 12$ mice; 8×10^3 EID_{50}, $n = 5$ mice. All mice except one (in the 4×10^3 EID_{50} group) lost at least 30 % of their original body weight

2. Use of this anesthetic must be included on one's animal protocol, and must be approved by the institution's Institutional Animal Care and Use Committee (IACUC).
3. Protocols for the preparation of 2,2,2-tribromoethanol are widely available from numerous sources [16, 17]. Briefly, 0.5 g of 2,2,2-tribromoethanol is dissolved with heat and stirring per ml of 2-methyl-2-butanol. Distilled water is added to a final volume of 40 ml. This method results in a 12.5 mg/ml concentration. Sterile filter through a 0.2 μm filter. Protect from light and store at 4 °C.
4. The Madin–Darby canine kidney (MDCK) cell line is a highly utilized culture system for studying virus growth.
5. Aliquots of virus are generally kept at no more than 500 μl. This practice allows for rapid, thorough thawing.
6. We have found virus to be most stable at 4 °C. In our hands, maintaining consistent practices of keeping freshly thawed virus aliquots, and all virus dilutions, at ice-cold temperatures, gives reproducible in vivo (and in vitro) results.
7. 2,2,2-Tribromoethanol sedation will give approximately a 10-min window of time for inoculation. Isoflurane sedation, not described here but frequently used, provides about 10 s. A ketamine/xylazine injectable cocktail is another often used method of anesthesia of mice.
8. 250 mg/kg animal is equal to 0.4 ml of prepared 2,2,2-tribromoethanol solution per 20 g mouse.
9. Proper dosing with 2,2,2-tribromoethanol should lead to sedation within several minutes. The mouse should regain consciousness and the ability to right itself within 30–90 min.

10. Individual marking of mice may be done by a variety of methods, including but not restricted to metallic ear tag, ear notching, designated toe removal, tail marking, or tattooing. Most methods of uniquely identifying mice require proper animal protocol and IACUC approval.
11. The "gold standard" noninvasive method for evaluating morbidity in mice from influenza infection is the measurement of percentage weight loss. The technique is easily done as described in this protocol. Despite the widespread use of measuring mouse weight loss as a proxy for severity of infection, the method tells us little regarding physiological changes in the mouse. Body weight loss is in part a result of anorexia and lack of fluid intake, which presumably is a result of the mice feeling poor [18, 19].
12. Virus designations: PR/8: A/Puerto Rico/8/34 (PR8), H1N1. ×31: A/Aichi/02/68 (HA, NA)×A/Puerto Rico/8/34, H3N2.
13. Figure 2 demonstrates two clearly different weight loss curves as a result of infection with two different strains of influenza. Incidentally, the two strains of influenza only differ in their hemagglutinin (HA) and neuraminidase (NA). The nonlethal infection, ×31, induces a faster initial weight loss than the lethal PR/8 infection. Such patterns are presumably due to physiological responses to the higher viral load this group of mice received. However, weight loss of any individual mouse does not breech more than 25 % of the original weight. Mice infected with this dose of PR/8 exhibit a more gradual weight loss, yet ultimately reach, and in some cases surpass, 30 % loss of original body weight. Behaviors of mice at this point of infection, which we consider peak illness, include hunching, lack of grooming, and anorexia. Mice that become moribund as defined by our institutional protocols and body index scoring are euthanized. In this particular infection, about half of the mice reached this state. It is important to point out that about half the mice that reach the 30 % mark do not become moribund. In fact, they recover their body weights, demonstrating that body weight alone is a poor predictor of lethal outcome. The percent body weight loss of four example mice from the experiment shown in Fig. 2 are plotted individually in Fig. 3. Note the weight loss patterns of these four mice. Mouse 3 lost weight at an accelerated rate and became quickly moribund, while mice 1, 2, and 4 lost weight at comparable rates. However, only mouse 2 reached a state of illness requiring euthanasia. The other two recovered their body weights, albeit at different rates (Fig. 3).
14. The infectious dose generally dictates whether or not mice will reach a stage of illness requiring euthanasia. Comparison of percentage of mice meeting our institutional guidelines for

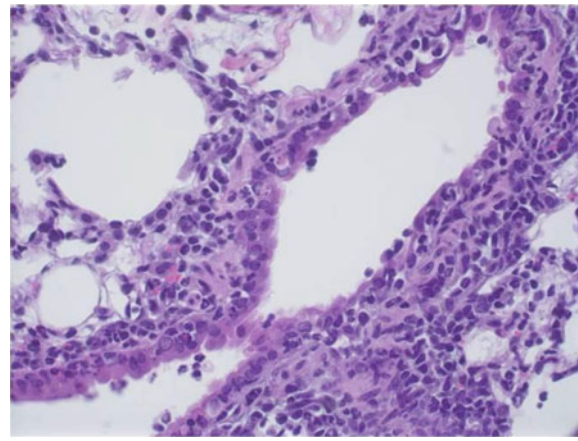

Fig. 5 C57BL/6 mouse lung histology 3 days after PR/8 infection. H&E stain shown at 400× magnification

sacrifice by PR/8 dose demonstrates this concept (Fig. 4). With the exception of one mouse that lost 27 %, all mice shown lost a minimum of 30 % of their original body weight. Here, 4/5 of mice receiving 2×10^3 EID_{50}, 5/12 mice in the 4×10^3 group, and 0/5 mice in the 8×10^3 EID_{50} group survived. Thus, care should be taken in infectious dose selection.

15. Tracking weight loss continues as a common measure of influenza progression. Guidelines for how much weight loss is considered acceptable (frequently including animal behavior) for continuing an in vivo animal infection experiment are often strictly set by an investigator's institution. It is becoming increasingly common to record humane endpoint euthanasia as "death by infection," but this should only be done if the humane endpoint serves as a relevant proxy for expected mortality. As shown, mice can lose at least 30 % of their original body weights and still recover, indicating that weight loss alone is not an appropriate proxy for death.
16. We have found 5 ml polypropylene tubes to work best for this purpose.
17. Infectious virus will form plaques, which when enumerated allows for the calculation of virus titer in the lungs. Units are recorded as plaque-forming units (PFU).
18. Extent and nature of cellular infiltration, tissue damage, and repair will vary by viral strain, initial viral load, and characteristics of the host animal.
19. Three days after the 4×10^3 EID_{50} PR/8 infection, the lungs exhibit focal necrosis of airway epithelium and a mild inflammatory response characterized by the presence of neutrophils, lymphocytes, and macrophages (Fig. 5). Figure 6 demonstrates

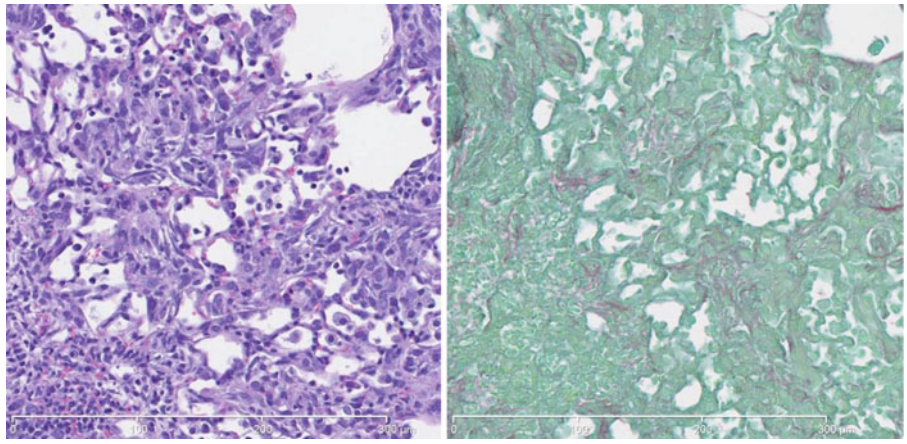

Fig. 6 C57BL/6 mouse lung histology 14 days after PR/8 infection. *Left*, H&E; *right*, Sirius Red

the type and degree of inflammation and damage seen in mouse lungs by day 14 after 4×10^3 EID_{50} PR/8 infection. At this later time, pneumonia was present in mice. Areas of active inflammation consisting of neutrophils, eosinophils, lymphocytes, and macrophages are present in the distal airways and the adjacent interstitium. Also in these locations, inflammation primarily composed of mononuclear cells is present. There is deposition of eosinophilic material here as well. Stains for collagen deposition, such as Masson's Trichrome and Sirius Red (Fig. 6, right), at this time point reveal a thin layer of collagen lining the alveolar spaces.

20. Though both weight loss and lung pathology are frequently used techniques for influenza disease evaluation, it is not well established how indicative the former is of the latter. Recently, a number of groups have published alternate methods for evaluation of the in vivo progress of influenza infection that correlate to lung pathology. These techniques, including pulse oximetry and plethysmography, appear promising in terms of prediction of susceptibility to infection [20, 21].

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