Chapter 8

In Vivo Infection Model of Severe Influenza A Virus

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Abstract

The lung is constantly exposed to both environmental and microbial challenge. As a "contained" organ, it also constitutes an excellent "self-contained" tissue to examine inflammatory responses and cellular infiltration into a diseased organ. Influenza A virus (IAV) causes both mild and severe inflammation that is strain specific following infection of the lung epithelium that spreads to other cells of the lung environment. Here, we describe a method of intranasal inoculation of the lung with IAV that can be used as a preclinical model of infection. Mice can be monitored for clinical signs of infection and tissue and lung fluid collected for further analysis to dissect the immunological consequences of IAV infection. Importantly, this method can be modified to introduce other pathogens, therapies and environmental stimuli to examine immune responses in the lung.

Key words Intranasal, Influenza A virus, Anesthetic, Clinical signs of disease, PR8, HKx31, Bronchoalveolar lavage (BAL)

1 Introduction

Influenza A viruses (IAV) are a major cause of morbidity and severe illness globally. The emergence of highly pathogenic H5N1 and H7N9 avian IAV strains predominately across Asia has caused sporadic infections in humans with high mortality. The mouse model of IAV infection provides a convenient system to examine virus replication, disease pathogenesis and the inflammatory response elicited following intranasal infection of mice with IAV. Mice are not naturally infected with IAV, and intranasal inoculation with human seasonal strains leads to replication of the virus in the upper and lower respiratory tract but it generally does not result in severe disease [1, 2]. Highly Pathogenic Avian Influenza (HPAI) H5N1, H7N9, H9N2, and H4N8 viruses, and the 1918 H1N1 pandemic virus are capable of inducing disease in mice without the requirement of prior adaptation [3, 4].

The sequential passage of human isolates through mouse lung allows for the selection of virus mutants that show increased replication efficiency in vivo resulting in the induction of disease similar

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to that observed in humans [5, 6]. The mouse adapted A/PR/8/34 (PR8) strain has been widely used in studies examining disease pathogenesis and the immune responses elicited following IAV infection of mice. PR8 was adapted to mice by >300 sequential passages in mouse lung [7] and is therefore highly virulent for mice, resulting in rapid weight loss and high mortality follow inoculation with as low as 50 plaque-forming units (PFU) [1, 5]. During the process of adaption, it was likely that PR8 acquired mutations associated with increased replication efficiency in the respiratory tract of mice and/or evasion of innate host responses. For example, PR8 is largely poor in its ability to infect macrophages, which are permissive to viral replication [1, 8]. HKx31 (H3N2) is a high-yielding reassortant of PR8 that expresses the hemagglutinin (HA) and neuraminidase (NA) of A/Aichi/2/68 (H3N2) from the Hong Kong pandemic of 1968. Unlike the mouse-adapted PR8 strain, HKx31 is similar to many pathogenic IAV strains such as H5N1 and H7N9 in its ability to infect macrophages [9, 10].

IAV of mice is characterized by the early production of proinflammatory cytokines (e.g., IL-6, MCP-1, TNFα, KC, and IL-1β), as well accumulation of inflammatory cells in the alveoli, including neutrophils, macrophages and lymphocytes, with subsequent recruitment of T cells and the development of humoral responses [2, 5]. Viral clearance is generally achieved 7–10 days after sublethal infection. Severe IAV infections of mice (i.e., following inoculation with mouse-adapted or HPAI viruses) are associated with a number of pathological features such as high viral loads, greater numbers of inflammatory infiltrate and elevated levels of inflammatory mediators [1, 2]. Vascular leak and pulmonary edema are hallmark pathological features of acute respiratory Distress Syndrome (ARDS) and have been observed during severe IAV infection of mice and humans [1, 11].

While the adaptive immune response plays an important role in the clearance of IAV and eventual resolution of infection, increasing evidence demonstrates the innate immune response to IAV infection plays a crucial role in the pathophysiology of disease. Innate immune recognition receptor such as Toll-like receptor (TLR)-2, TLR3, TLR4, and TLR7 [12, 13], have all been shown to play important roles in recognizing IAV infection and initiating inflammation as a response to infection. Increasing evidence from our and other laboratories also identifies a critical role for the nucleotide-binding domain and leucine-rich-repeat-containing (NLR) family of pattern-recognition molecules in inducing inflammation and contributing the pathophysiology and disease outcome during IAV challenge.

The NLRP3 inflammasome is an oligomeric innate immune intracellular signaling complex that senses many pathogen-, host-, and environmental-derived factors [14]. Inflammasome-induced cytokine release requires two signals: (1) upregulation of

components of the NLRP3 inflammasome and synthesis of pro-IL-1β through activation of the prototypic inflammatory transcription factor NF-κB; and (2) inflammasome formation that results in IL-1β maturation and secretion. Following activation, NLRP3 binds to the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC). ASC further recruits the enzyme caspase-1 to form the inflammasome complex initiating autocatalytic cleavage of caspase-1. The NLRP3 inflammasome is now recognized as a major route by which the innate immune system recognizes and responds during IAV infection [14]. To date, IAV single-stranded RNA (ssRNA) and proton flux via the IAV-encoded matrix-2 (M2) ion channel have been shown to activate the NLRP3 inflammasome [15, 16], which is important in the development of adaptive immune responses to IAV. Studies utilizing mice lacking components of the NLRP3 inflammasome have demonstrated its importance in eliciting rapid protective responses following infection with PR8 [15, 17]. Recently, we identified that the PB1-F2 protein from the PR8 and H7N9 strains of IAV induced NLRP3 inflammasome activation in mice via intranasal inoculation, contributing to severe disease pathophysiology [18, 19]. Importantly, intranasal delivery of a small molecule inhibitor of NLRP3 provided protection from PR8- and HKx31-induced lethality, commensurate with reduced lung inflammatory cytokine expression such as IL-6, MCP-1, TNFα, and IL-1β reduced inflammatory cellular infiltrates [5].

The intranasal inoculation method therefore provides an excellent animal model to (1) challenge mice with pulmonary focused pathogen models such as IAV, and (2) a means to therapeutically deliver intervention strategies that may mimic intranasal drug delivery similar to that possible for humans.

2 Materials

2.1 Intranasal Inoculation of Mice with Influenza Virus

1. Biological safety cabinet.
2. Influenza virus stock: In a BSL2 facility, influenza viruses are routinely grown in 10-day-old embryonated hen's eggs and allanotic fluid aliquots and stored at −80 °C for several years (*see* **Note 1**).
3. Preparation of influenza virus inoculum: Remove aliquot of influenza virus from −80 °C. Thaw at room temperature and perform the following in a biological safety cabinet. Dilute influenza stock in sterile phosphate buffered saline (PBS) to desired plaque-forming units (PFU) in 50 μL, e.g., 50 PFU in 50 μL or 1000 PFU per mL (*see* **Note 2**).
4. Isoflurane.

5. Anesthetic machine or glass jar with screw lid containing cotton wool or tissues.
6. Steel mesh/grid that can be placed inside the glass jar on top of the tissues/tissues, so that mice cannot come into direct contact with the anesthetic.
7. P200 pipette and P200 pipette tips.
8. Male or female C57Bl/6 mice at 6–8 weeks of age.

2.2 Monitoring of Mice Following Influenza Infection

1. Monitoring sheet (*see* **Note 3**).
2. Weigh scale.
3. Small open lid container to place on weigh scale.

2.3 Euthanasia of Mice with Pentobarbitone

1. Pentobarbitone.
2. 1 mL syringe.
3. 29-gauge needle.
4. Dissection scissors and tweezers.

2.4 Collection of Bronchoalveolar Lavage (BAL)

1. 80% w/v ethanol.
2. Sterile phosphate-buffered saline (PBS).
3. 3 mL syringe.
4. 18-gauge needle.
5. 23-gauge needle that has been blunted by removing the bevel/tip with scissors.
6. 10 or 15 mL tubes.

2.5 Harvesting of Lung Tissue and Sera

1. 1.5 mL Eppendorf tubes.
2. Box containing wet ice.
3. Container containing liquid nitrogen.
4. 1 mL syringe.
5. 29-gauge needle.
6. Heparin-coated Eppendorf tube or commercial serum collection tube.

3 Methods

3.1 Intranasal Inoculation of Mice with Influenza Virus

1. Place the mouse cage into a biological safety cabinet.
2. Place a glass jar containing a screw lid into the biological safety cabinet.
3. Insert cotton wool or tissues into the bottom of the jar (*see* **Note 4**).

Fig. 1 Intranasal inoculation of mice with influenza virus. Holding the anesthetized mouse upright and pipetting the virus inoculum onto the nares

4. Place 100 μL of isoflurane onto the cotton wool or tissues and cover with a steel mesh/grid.
5. Place the mouse into jar containing the isoflurane.
6. Monitor the mouse carefully until it is anaesthetized and ensure that breathing is no longer rapid, nor there is any movement (*see* **Note 5**).
7. Remove the mouse from the jar or anesthetic machine and hold it upright (Fig. 1).
8. Slowly place 50 μL of the virus inoculum evenly over the two nares using a pipette.
9. Keep the mouse upright until the inoculum is completely inhaled.
10. Return the mouse to a cage and monitor until it regains consciousness and appears fully recovered from the effects of the anesthesia, i.e., alert and moving around freely.

3.2 Monitoring of Mice Following Influenza Infection

1. Following influenza virus infection, mice should be euthanized as per below and monitored in accordance with ethical approval.

2. For monitoring, each mouse needs to be easily identified, i.e., ear clipping, tattoos, or marking the tail.
3. Mice should be weighed daily by placing the mouse in an open lid container that is on top of a weighing scale.
4. Record the mouse weights on a monitoring sheet.
5. Score the mice daily for physical signs of disease. 0 = no visible signs of disease; 1 = slight ruffling of fur; 2 = ruffled fur, reduced mobility and 3 = ruffled fur, reduced mobility, rapid breathing (*see* **Note 6**).

3.3 Euthanasia of Mice with Pentobarbitone

1. Remove mouse from its cage.
2. Restrain the mouse by the scruff.
3. Inject >10 mg/kg of pentobarbitone into the peritoneal cavity (*see* **Note 7**).
4. Monitor the mouse and ensure euthanasia by measuring reflexes, i.e., pinching of the foot. Once mouse is euthanized, the contents of the lung can be examined for cellular infiltrates, cytokines, chemokines, and other cellular factors by extracting bronchoalveolar lavage as described below, or, the organ can be excised and examined by histological sectioning as described below.

3.4 Collection of BAL

1. Euthanize the mouse as per above.
2. Spray the mouse's coat with 80% ethanol ensuring the coat is wet to confidently "sterilize" the area.
3. If blood is required to be collected, this can be done prior to BAL (see below).
4. Using dissection scissors, expose the trachea of the mouse.
5. Carefully cut away the tissue that sounds the trachea (Fig. 2a).
6. Hold the mouse upright and carefully make a small incision in the trachea by inserting a 18-gauge needle into the trachea.
7. Fill a 3 mL syringe containing a blunted 23-gauge needle, with 1 mL of PBS.
8. While holding the mouse upright, carefully place the trip of the blunted needle into the trachea of the mouse (Fig. 2b).
9. Inject the 1 mL of PBS into the lung and immediately draw back on the syringe to recover the PBS (approx. 60–80% recovery).
10. Place the BAL into a 10 or 15 mL tube on ice.
11. Repeat **steps 6–9** twice to flush the lung a total of three times.
12. Store the tube containing the BAL on ice.
13. Spin the tubes containing BAL at 500 × *g*, 4 °C for 5 min.

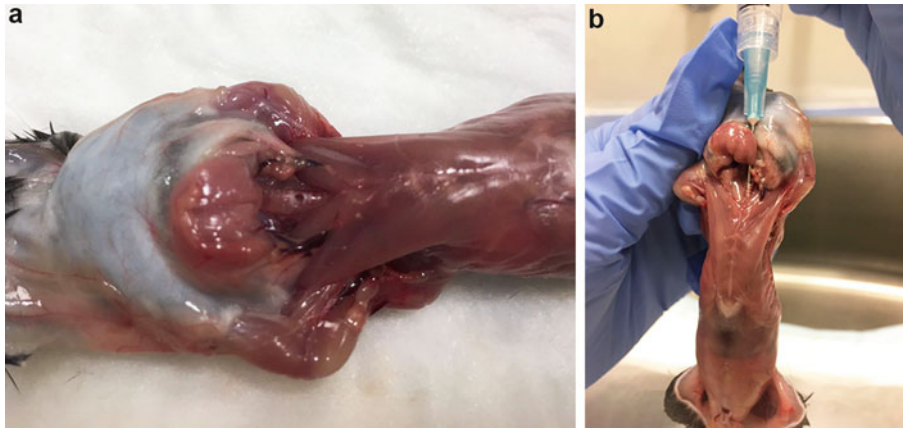

Fig. 2 Collection of BAL from mice. (**a**) Exposing and creating small incision in the trachea. (**b**) Inserting the blunted needle into the trachea and flushing the lungs with 1 mL of PBS

14. Remove the supernatant, aliquot in Eppendorf tubes and store at −80 °C for analysis, e.g., levels of proinflammatory cytokines via ELISA or cytokine bead array according to manufacturer's instructions. BAL can also be assayed for inflammasome activation by examining ASC speck formation by flow cytometry [20] (*see* accompanying Chapter 14).
15. The cell pellet can then be immediately utilized for flow cytometry analysis of cellular infiltrate (e.g., neutrophils, macrophages, T cells [1, 5]).

3.5 Harvesting of Lung Tissue and Sera

1. Euthanize the mouse as per above.
2. For collection of sera, harvest the blood directly from the heart by performing a cardiac puncture using a 1 mL syringe and 29-gauge needle. Place the blood in a heparin-coated Eppendorf tube or a commercial serum collection tube. Approximately 500 μL can be collected on average.
3. BAL can then be performed as per above without significantly impacting analysis of viral loads in the lung tissue.
4. Remove the lung tissue from the mouse.
5. Place the lung tissue in an Eppendorf tube.
6. Snap freeze the Eppendorf in liquid nitrogen.
7. Store sera and lung tissues at −80 °C for analysis, e.g., analysis of viral loads, cytokine levels, mRNA or protein expression.

4 Notes

1. The titer of the influenza stock should be determined by standard plaque assay on MDCK cells as previously described [21].

2. In place of 50 μL of viral stock or PBS as mock infection, this can be replaced with either inflammasome activators such as silica or nigericin, drugs, small molecule inhibitors, and antibodies dissolved or suspended in a total volume of 50 μL. Other pathogens such as viruses or bacteria can also be substituted in place of IAV at this step also.
3. Monitoring sheet should describe the ethical requirements for scoring of clinical signs of disease and weight loss.
4. An anesthetic machine if available can be used in replace of **steps 3–5**.
5. Do not leave mouse in the exposed to isoflurane for too long as this may result in lethality. If in doubt, remove the mouse and check if it is anaesthetized. If it is still breathing rapidly or shows signs of movement, reexpose to isoflurane in the jar. If the mouse is not anaesthetized enough or too deeply, the mouse will not inhale the virus inoculum or PBS through its nostrils.
6. Monitoring signs of clinical disease is a crucial ethical aspect of responsible monitoring of mice and animal welfare. All animal experimentation should be conducted in accordance with local ethics committee requirements which may vary. Always check with your institutional ethics and biosafety committee to obtain the relevant documentation and information relating to ethical handling and experimentation on animals and their scoring matrix and level of clinical signs of disease before ethical euthanasia of animals is required.
7. As an alternative to >10 mg/kg injection of pentobarbitone, mice can be euthanized by CO_2 asphyxiation. Cervical dislocation as a method of euthanasia is not recommended, however, as it can cause bleeding around the lung.

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References

1. Tate MD, Pickett DL, van Rooijen N, Brooks AG, Reading PC (2010) Critical role of airway macrophages in modulating disease severity during influenza virus infection of mice. J Virol 84:7569–7580
2. Tate MD, Schilter HC, Brooks AG, Reading PC (2011) Responses of mouse airway epithelial cells and alveolar macrophages to virulent and avirulent strains of influenza A virus. Viral Immunol 24:77–88

3. Lai C, Wang K, Zhao Z, Zhang L, Gu H, Yang P et al (2017) C-C motif chemokine ligand 2 (CCL2) mediates acute lung injury induced by lethal influenza H7N9 virus. Front Microbiol 8:587
4. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM (2008) H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog 4:e1000115
5. Tate MD, Ong JD, Dowling JK, McAuley JL, Robertson AB, Latz E et al (2016) Reassessing the role of the NLRP3 inflammasome during pathogenic influenza A virus infection via temporal inhibition. Sci Rep 6:27912
6. Sweet C, Smith H (1980) Pathogenicity of influenza virus. Microbiol Rev 44:303–330
7. Taylor RM (1941) Experimental infection with influenza a virus in mice: the increase in intrapulmonary virus after inoculation and the influence of various factors thereon. J Exp Med 73:43–55
8. Londrigan SL, Tate MD, Brooks AG, Reading PC (2012) Cell-surface receptors on macrophages and dendritic cells for attachment and entry of influenza virus. J Leukoc Biol 92:97–106
9. Gu J, Xie Z, Gao Z, Liu J, Korteweg C, Ye J et al (2007) H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 370:1137–1145
10. Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL et al (2007) Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat Med 13:147–149
11. Short KR, Kroeze EJ, Fouchier RA, Kuiken T (2014) Pathogenesis of influenza-induced acute respiratory distress syndrome. Lancet Infect Dis 14:57–69
12. Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM et al (2013) The TLR4 antagonist Eritoran protects mice from lethal influenza infection. Nature 497:498–502
13. Ichinohe T (2010) Respective roles of TLR, RIG-I and NLRP3 in influenza virus infection and immunity: impact on vaccine design. Expert Rev Vaccines 9:1315–1324
14. Iwasaki A, Pillai PS (2014) Innate immunity to influenza virus infection. Nat Rev Immunol 14:315–328
15. Thomas PG, Dash P, Aldridge JR Jr, Ellebedy AH, Reynolds C, Funk AJ et al (2009) The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. Immunity 30:566–575
16. Ichinohe T, Pang IK, Iwasaki A (2010) Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nat Immunol 11:404–410
17. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ et al (2009) The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity 30:556–565
18. McAuley JL, Tate MD, MacKenzie-Kludas CJ, Pinar A, Zeng W, Stutz A et al (2013) Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. PLoS Pathog 9: e1003392
19. Pinar A, Dowling JK, Bitto NJ, Robertson AA, Latz E, Stewart CR et al (2017) PB1-F2 peptide derived from avian influenza A virus H7N9 induces inflammation via activation of the NLRP3 inflammasome. J Biol Chem 292:826–836
20. Sester DP, Zamoshnikova A, Thygesen SJ, Vajjhala PR, Cridland SO, Schroder K et al (2016) Assessment of inflammasome formation by flow cytometry. Curr Protoc Immunol 114:14.40.11–14.40.29
21. Anders EM, Hartley CA, Reading PC, Ezekowitz RA (1994) Complement-dependent neutralization of influenza virus by a serum mannose-binding lectin. J Gen Virol 75:615–622