Review

Animal models for influenza virus pathogenesis, transmission, and immunology

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ABSTRACT

In humans, infection with an influenza A or B virus manifests typically as an acute and self-limited upper respiratory tract illness characterized by fever, cough, sore throat, and malaise. However, influenza can present along a broad spectrum of disease, ranging from sub-clinical or even asymptomatic infection to a severe primary viral pneumonia requiring advanced medical supportive care. Disease severity depends upon the virulence of the influenza virus strain and the immune competence and previous influenza exposures of the patient. Animal models are used in influenza research not only to elucidate the viral and host factors that affect influenza disease outcomes in and spread among susceptible hosts, but also to evaluate interventions designed to prevent or reduce influenza morbidity and mortality in man. This review will focus on the three animal models currently used most frequently in influenza virus research – mice, ferrets, and guinea pigs – and discuss the advantages and disadvantages of each.

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1. Influenza in the human host

1.1. Pathogenesis of influenza viruses in humans

Within one to two days of infection with an influenza A or B virus, influenza disease most commonly manifests with the sudden onset of characteristic respiratory and systemic symptoms (Treanor, 2010). Respiratory symptoms, such as dry cough, pharyngitis, and nasal congestion and discharge, are often similar to those observed in other viral upper respiratory tract infections (URTIs). The systemic symptoms of influenza, including fever and chills, headache, myalgia, lethargy, and anorexia, develop early in the course of disease. Fever generally ranges from 100 °F to 104 °F (38 °C to 40 °C), but may be as high as 106 °F (41 °C), with peak temperatures on the first day of symptoms and decreasing over three to eight days thereafter (Treanor, 2010). The prominent presence of systemic symptoms is often said to differentiate influenza from other viral URTIs. However, considerable syndromic overlap exists among these viral illnesses, particularly in the elderly; thus, the phrase "influenza-like illness" (ILI) is often employed to describe clinically indistinguishable viral URTIs (Widmer et al., 2012; Woolpert et al., 2012; Haas et al., 2013). In typical uncomplicated influenza, systemic symptoms generally resolve earlier than respiratory symptoms like cough and sore throat, which may persist for several days to a week after systemic symptoms abate (Treanor, 2010).

Pulmonary complications of influenza virus infection include primary viral pneumonia and secondary bacterial pneumonia. Clinically, primary influenza viral pneumonia initially manifests like a typical uncomplicated URTI, but the acute infection rapidly progresses to the lower respiratory tract, accompanied by signs and symptoms of pneumonic involvement like cough, dyspnea, and hypoxemia. In contrast, secondary bacterial pneumonia occurs subsequent to a typical influenza URTI. After an initial clinical improvement lasting four to 14 days, recrudescence of fever, dyspnea, and cough with sputum signals the onset of bacterial pneumonia, particularly caused by staphylococcal or streptococcal species (Treanor, 2010).

In immunocompetent persons, epidemic (often called "seasonal") influenza is most often uncomplicated, remaining confined to the upper respiratory tract. Though primary viral pneumonia occurs rarely overall, women in late pregnancy or the early post-partum period, the elderly, and those with comorbid cardiovascular or lung disease are at a higher risk of developing this complication (Treanor, 2010; Mertz et al., 2013). Influenza pandemics, while relatively infrequent, usually result in higher morbidity and mortality than seasonal epidemics. Pandemic influenza viruses arise from reassortment, the creation of a genetically and antigenically new virus by "mixing-and-matching" viral genes from human and/or animal influenza viruses. These "antigenic shift" events introduce an immunologically novel influenza virus into the human population, which has no pre-existing immunity to it. During recent pandemics, including those of 1918, 1957, 1968, and 2009, younger people have been disproportionately affected by lower respiratory tract disease requiring hospitalization, relative to inter-pandemic years (Murata et al., 2007; Lapinsky, 2010; Treanor, 2010). Theories to explain the unusual morbidity and mortality of pandemic influenza among the young include an immunopathology specific to this age group (such as an antibody-dependent enhancement of disease in persons with particular, previous exposures to other seasonal influenza virus strains) and, conversely, immunoprotection in older adults (for example their exposure, many decades before, to influenza viruses that induced cross-protective immune responses that are not present in those who had yet to be born at that time) (Taubenberger and Morens, 2006).

The kinetic course of influenza virus replication in and then eradication from the human respiratory tract is often inferred from influenza challenge studies, in which human volunteers were experimentally inoculated with influenza viruses and then observed for symptomatic and virological measures of disease. A meta-analysis of human challenge studies (Carrat et al., 2008) found that, on average, viral shedding in nasal secretions begins within the first 24 h after inoculation, peaks on day 2, and ends by day 8 or 9 post-infection. Overall, only 66% of experimentally inoculated subjects developed disease; however, viral shedding could be detected even in asymptomatic persons. Average symptom scores peaked at three days post-infection (dpi), indicating that viral shedding precedes the development of disease by approximately one day (Carrat et al., 2008). The findings of this meta-analysis were echoed in a recent human challenge study (Y. Huang et al., 2011), in which 17 healthy volunteers were experimentally inoculated with influenza A/Wisconsin/67/2005 [H3N2], but only 9 (53%) developed symptomatic influenza. Infectious virus could be isolated from half of the asymptomatic volunteers, although viral shedding from symptomatic subjects was of greater magnitude and longer duration. Similar to the meta-analysis findings, in this study symptoms appeared, on average, between 1 and 2 days post-inoculation (range, 22 to 60 h). Changes in messenger RNA (mRNA) levels in peripheral blood mononuclear cells (PBMCs) were quantified by microarray every 8 to 24 h throughout the course of the study, in order to describe for each subject a specific gene expression signature in response to influenza virus infection. Interestingly, symptomatic and asymptomatic subjects displayed characteristic yet

dissimilar molecular transcriptional responses. These data suggest that, in humans, the absence of influenza symptoms does not signal an immunologically "passive" state, but rather that transcriptionally different but equally "active" host immune response programs are stimulated by influenza virus infection in symptomatic and asymptomatic hosts (Y. Huang et al., 2011).

1.2. Transmission of influenza viruses among humans

There is still much that is unknown about the transmission of influenza viruses among humans. There are two main modes of transmission, *contact* and *airborne*, by which influenza and other respiratory viruses are thought to spread from person to person (IOM (Institute of Medicine), 2011; Pica and Bouvier, 2012). Contact transmission occurs by two routes, direct and indirect. In *direct contact transmission*, a susceptible person's hand becomes directly contaminated with infectious virus – perhaps by shaking the hand of an infected person, or by wiping a sick child's nose – and then the susceptible person inoculates himself by transferring infectious virus from his hand to his nose. *Indirect contact transmission* occurs when infectious virus is transferred from an inanimate object, or *fomite*, (such as a doorknob, telephone, or computer keyboard touched by an infected person) to the hand of a susceptible person, who then inoculates her own nasal mucosa by rubbing or touching her nose. In both cases of contact transmission, however, a contaminated hand or other body part plays a role in bringing infectious virus to the respiratory mucosa. In contrast, airborne transmission occurs when infectious virus inoculates the respiratory tract directly from the air, without a contaminated hand or other physical intermediate mediating its transport to the respiratory mucosa. It can occur by two modes: In *droplet spray transmission*, an infected person coughs or sneezes, expelling respiratory droplets, containing contagious virus particles, which impact directly on the nasal mucosa of a susceptible person. *Aerosol* (also called *droplet nuclei*) *transmission* occurs when water- and virus-laden respiratory droplets that are exhaled by an infected person desiccate, becoming light enough to remain suspended in the air for minutes to hours; these infectious aerosols can then be inhaled into the respiratory tract of a susceptible person to initiate infection (Pica and Bouvier, 2012).

Early research in human subjects demonstrated that airborne influenza virus, inhaled as an aerosol, was more infectious than virus applied via liquid droplets into the nose (Alford et al., 1966). These experiments suggest that humans can be infected by both airborne- and contact-based transmission modes, but that contact modes may require a higher infectious dose. Observational studies of influenza outbreaks (Buxton Bridges et al., 2003) imply that both contact and airborne routes can play a role in the human-to-human transmission of influenza viruses. Recent reviews of the literature (Brankston et al., 2007; Tellier, 2009) have reached conflicting conclusions regarding the relative importance of airborne, droplet, and contact-based spread among humans, and uncertainty remains on this issue, having significant implications for infection control and public health planning (IOM (Institute of Medicine), 2011).

In infectious disease epidemiology, the *secondary attack rate*, a percentage reflecting the number of new cases of disease arising among all the contacts exposed to an index case, is a measure of pathogen infectiousness within a population. During the 2009 H1N1 pandemic, observational studies estimated secondary attack rates ranging between 4% and 51% among household contacts of index cases with influenza, with an average of 10–20% (France et al., 2010; Morgan et al., 2010; Cauchemez et al., 2009; Yang et al., 2009; Carcione et al., 2011; Glatman-Freedman et al., 2012). Although the method of case ascertainment, clinical or virological, affected secondary attack rate estimates, the presence of children within households did as well, with higher rates of infection in children than in adults (Glatman-Freedman et al., 2012). For 2009 pandemic influenza, the *serial interval*, the time that elapses between the infection of an index case and subsequent transmission of disease to a secondary contact, has been estimated in most studies to fall in the range of two and a half to three days (France et al., 2010; Morgan et al., 2010; Cauchemez et al., 2009; Yang et al., 2009; Donnelly et al., 2011). In these studies, the majority of secondary transmission events occurred just before or just after the onset of influenza symptoms in the index case (Cauchemez et al., 2009). Altogether, these data indicate that influenza viruses have an incubation period of one to two days in humans, with secondary transmission to a subsequent host occurring within another one to two days.

Specific host factors that greatly enhance the efficiency with which certain influenza virus-infected persons transmit disease to subsequent, susceptible hosts – so-called "superspreaders" (Lloyd-Smith et al., 2005; Stein, 2011) – are unknown. Children, who have no or minimal prior exposure to and thus immunity against influenza viruses, and immunocompromised individuals shed influenza viruses to higher titers for a longer duration than immunocompetent adults and, according to epidemiological data, appear to be good transmitters (Hall et al., 1979; Frank et al., 1981; Hall, 1981; Weinstock et al., 2003; Sato et al., 2005; Glezen, 2006). Among healthy adults, the amount of respiratory particles exhaled while coughing or breathing can vary greatly – by orders of magnitude – from person to person, suggesting that some individuals may indeed shed infectious virus much more efficiently than others (Lindsley et al., 2012; Milton et al., 2013).

It remains uncertain whether expulsive respiratory events, such as coughing or sneezing by infected persons, are required for maximally efficient transmission of influenza viruses among humans. In a study of healthy adults, a similar amount of airborne respiratory droplets, on average, were produced by counting aloud from 1 to 100 as by coughing 20 times (Xie et al., 2009). Whether the same would be true in influenza virus-infected people is unknown, although influenza virus infection does enhance the generation of cough aerosols (Lindsley et al., 2012). In a small qualitative study, influenza virus could be detected in the exhalations of infected persons during normal tidal breathing or talking but not during coughing (Stelzer-Braid et al., 2009). More recent quantitative studies have assessed the production of respiratory particles by influenza patients while breathing or coughing, but not both maneuvers side-by-side (Lindsley et al., 2010, 2012; Milton et al., 2013). Thus, the transmission of influenza viruses among humans is

likely affected, in small or large part, both by host physiology and by host immune competence overall, and by immune control of influenza viruses in particular.

1.3. Immunology of influenza infection in humans

The immunology of influenza in humans is too large a topic to summarize completely here. However, broad areas of understanding of the innate and adaptive immune responses in the human host will be outlined, with references made to comprehensive reviews.

In humans and other mammals, the initial protective response to influenza virus deposition on the mucosa of the respiratory tract is both physiological and immunological. Nonspecific mucoproteins on the mucosal surface adhere to the virus particle and aid in its clearance by the mucociliary apparatus. Secretory IgA immunoglobulins, made in response to infection by previous influenza virus strains, are also present on mucosal surfaces and may confer some degree of cross-protection upon reinfection with an antigenically drifted influenza strain (Treanor, 2010).

Once virus entry into a host cell is accomplished, the nonspecific innate immune response is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Hale et al., 2010). The 5′ triphosphate group on genomic viral RNA (vRNA) is thought to be the main influenza virus PAMP recognized by the cytoplasmic retinoic acid-inducible gene-I-like (RIG-I) PRR. RIG-I activation results in the expression of more than 300 antiviral interferon-stimulated genes (ISGs) through Type I interferon (IFN) signaling pathways. However, influenza virus encodes a protein, nonstructural 1 (NS1), that interferes with the IFN-induced upregulation of innate immunity by inhibiting RIG-I signaling, among other mechanisms (Hale et al., 2010).

Innate immune cells, such as natural killer (NK) cells, alveolar macrophages, and dendritic cells (DCs) play critical roles not only in the initial control of viral replication but also in the elaboration and regulation of influenza virus-specific adaptive immunity (McGill et al., 2009). However, the nonspecific innate immune response, while effective, can be locally destructive if not appropriately contained. Certain influenza viruses, such as highly pathogenic avian influenza (HPAI) A/H5N1 strains, can trigger an overly inflammatory innate immune response, often called "cytokine storm" (de Jong et al., 2006; Peiris et al., 2009). Certain persons seem particularly susceptible to developing cytokine-driven clinical syndromes like sepsis and acute lung injury/acute respiratory distress syndrome (ALI/ARDS), suggesting a potential genetic basis for the immune dysregulation that is thought to be a major factor in severe human influenza (Oshansky and Thomas, 2012; Tisoncik et al., 2012). The role of local inflammation in influenza pathogenesis in humans is not entirely clear, however (Oshansky and Thomas, 2012). Mouse studies have demonstrated that the local inflammatory milieu in the influenza virus-infected respiratory tract can differ greatly from the cytokine profile elaborated in PBMCs. Human cytokine responses to influenza have been mainly assayed in PBMCs, not in the respiratory tract, and the majority of human studies have been observational, which can provide correlations between peripheral cytokine profiles and clinical outcomes in influenza disease but cannot show causality or

provide mechanistic details. Thus, while the pathogenesis of severe influenza is thought to result from a dysregulated innate immune response in both mice and men, confirmatory data in humans are scarce (Oshansky and Thomas, 2012).

In mammals, pathogen-specific adaptive immunity includes both humoral and cellular components (Oshansky and Thomas, 2012). In humans, B-cell production of influenza virus-specific antibodies, particularly those directed at the surface glycoproteins hemagglutinin (HA; the receptor-binding protein) and neuraminidase (NA; the receptor-destroying enzyme) has been shown to confer protection from influenza virus infection or pathogenicity. Non-neutralizing antibodies, such as those directed at the highly conserved matrix 2 (M2) protein and nucleoprotein (NP), can aid in the immune response without providing sterilizing immunity, although the mechanisms in humans require further elucidation (Oshansky and Thomas, 2012). In a process called antibody-dependent cellular cytotoxicity (ADCC), the variable region of an influenza virus-specific antibody binds to viral proteins expressed on the surface of an infected host cell, while its Fc region interacts with CD16, an Fc receptor on the surface of NK cells; the subsequent NK cell activation initiates lysis of the infected cell (Hashimoto et al., 1983a, 1983b; van de Sandt et al., 2012; Jegaskanda et al., 2013). Humanized mouse monoclonal antibodies directed against the immunodominant but genetically labile HA "head" region, where receptor binding occurs, are less potent inducers of human NK cell activation, and thus ADCC, than are neutralizing antibodies directed against the HA "stalk" region, which is less immunogenic but more highly conserved among influenza A subtypes (DiLillo et al., 2014). In mice, antibodies can also act as opsonins, enhancing the ability of phagocytes to engulf and present antigens to T lymphocytes, though this mechanism has yet to be convincingly demonstrated in human influenza (Oshansky and Thomas, 2012; Bodewes et al., 2013).

The cellular immune response to influenza virus infection includes activation of virus-specific T lymphocytes (van de Sandt et al., 2012). CD4+ T cells coordinate and regulate the immune response against influenza virus infection by recognizing viral peptide epitopes bound to MHC class II molecules on antigen-presenting cells (APCs). The concurrent cytokine milieu directs effector CD4+ T cell differentiation into T helper 1 (Th1) or T helper 2 (Th2) cells. Th1 cells produce IFN-γ and IL-2, which stimulate cytotoxic T lymphocyte (CTL) responses that eradicate virus-infected cells and promote viral clearance. Th2 cells secrete cytokines such as IL-4, IL-5, and IL-13, which are involved in the activation and differentiation of B cells to produce and refine virus-specific antibody responses. CD8+ T cells recognize viral epitopes bound to MHC class I molecules on APCs that have migrated from the site of infection into the draining lymph nodes. Activated CD8+ T cells then migrate back to the site of infection to identify and lyse influenza virus-infected cells, thereby curtailing viral replication (van de Sandt et al., 2012). Although only minimal indirect evidence exists for the role of CTLs in the human immune response against influenza viruses, activation of T lymphocytes appears to induce the formation of memory T cell pools in both mice and humans, which enable a more potent and rapid adaptive immune response in future influenza virus infections (Oshansky and Thomas, 2012; van de Sandt et al., 2012).

2. Animal models of influenza

In vitro and in silico models cannot adequately simulate the physiological and immunological complexity of the human host. Thus, animal models are necessary to elucidate common mammalian factors that affect influenza virus pathogenesis and inter-host transmissibility, as well as to perform pre-clinical assessment of the efficacy of preventive and therapeutic interventions like vaccines and antivirals. Many animal models have been used in the past to research various aspects of mammalian influenza, including mice, cotton rats, Syrian hamsters, guinea pigs, ferrets, dogs, cats, domestic swine, and non-human primates such as rhesus, pigtailed, and cynomolgus macaques and, more recently, marmosets (Barnard, 2009; Tripp and Tompkins, 2009; Bouvier and Lowen, 2010; Eichelberger and Green, 2011; Moncla et al., 2013). In this review, the advantages and disadvantages of the mouse, ferret, and guinea pig models will be discussed, with particular attention to the fidelity with which they model human influenza disease, virus transmission, and immunological responses.

2.1. Mice (Mus musculus)

Mice have many advantages as a model for influenza virus research, including their relatively low cost, ready availability, small size, and ease of handling and housing. Many inbred strains and outbred stocks of mice are commercially available, with their susceptibility to influenza virus infection varying according to their genetic background, the influenza virus strain, and the virus inoculum. In addition, numerous transgenic, knockout, and knock-in strains of inbred mice allow specific immune effectors to be studied in the context of influenza virus infections. Mouse-specific immunological reagents are widely available, and the ability to deplete specific immune cell populations has demonstrated the importance of macrophages, DCs, NK cells, and cytotoxic T and B lymphocytes in the murine immune response to influenza virus infection (Srivastava et al., 2009; Bouvier and Lowen, 2010). The main drawback to the mouse model is the need to use mouse-adapted viruses in order to achieve productive infection and clinically apparent signs of disease. Additionally, murine influenza is a primarily lower respiratory tract infection that is physiologically dissimilar from typical uncomplicated influenza in humans.

2.1.1. Pathogenesis of influenza viruses in mice

The clinical signs of influenza virus infection in mice are somewhat different from those of typical human influenza (Barnard, 2009; Belser et al., 2009; Tripp and Tompkins, 2009; Bouvier and Lowen, 2010). Upon infection with certain influenza virus strains, mice display marked anorexia and demonstrate behaviors consistent with physical discomfort or lethargy, such as huddling, hunching, and fur ruffling from lack of grooming and dehydration. Unlike humans, though, mice have been reported to become hypothermic upon influenza virus infection, rather than mounting a febrile response (Bouvier and Lowen, 2010). Importantly, influenza in mice characteristically manifests as a primary viral pneumonia, which is demonstrated clinically by labored breathing and cyanosis and post-mortem by severe pulmonary histopathology (Tripp and Tompkins, 2009; Bouvier and Lowen, 2010; Fukushi et al., 2011). Thus, mouse studies are not particularly reflective of the typical disease course of human influenza.

In addition, the vast majority of primary human influenza virus isolates are not infectious or pathogenic in the BALB/c and C57BL/6 inbred mouse strains, which are the most commonly employed in influenza virus research (Bouvier and Lowen, 2010). Thus, a few mouse-adapted virus strains are frequently used in the mouse model, including two influenza A/H1N1 isolates from the 1930s and an influenza B virus isolated in 1940: A/Puerto Rico/8/1934 (PR/8), A/WSN/1933 (WSN), and B/Lee/1940. Studies requiring an influenza A/H3N2-subtype virus are often carried out with X-31, a 6:2 genetic reassortant encoding the HA and NA genes of an A/H3N2 virus isolated in 1968, in a backbone comprising the six internal genes of PR/8 (Bouvier and Lowen, 2010). These antiquated mouse-adapted viruses are not necessarily genetically or antigenically representative of influenza viruses currently circulating among humans. The WSN strain in particular causes disease manifestations not typically seen in human influenza, such as a highly pathogenic neurotropism attributable to the plasminogen-binding activity of its NA protein (Goto et al., 2001). Primary human virus isolates typically have to be serially passaged through mice before they will productively infect and cause disease in this species (Tripp and Tompkins, 2009); however, notable exceptions include the 1918 H1N1 pandemic strain; most highly pathogenic avian influenza (HPAI) A/H5N1 viruses and selected low pathogenicity avian influenza (LPAI) subtypes; some H7 subtype viruses, including the novel influenza A/H7N9 virus that emerged in China in 2013; and the 2009 pandemic influenza A/H1N1 ("H1N1pdm09") strain (Bouvier and Lowen, 2010; Hai et al., 2013).

Less commonly used mouse strains have been shown to exhibit enhanced pathogenesis compared to BALB/c and C57BL/6 mice. Upon infection with PR/8, DBA/2 mice demonstrated a greater susceptibility to infection, more rapid weight loss and death, higher cytokine production, and more severe lung histopathology than C57BL/6 mice (Srivastava et al., 2009). DBA/2 mice were also more susceptible than C57BL/6 mice to infection with a range of human and swine influenza virus strains but produced comparable humoral immune responses (Pica et al., 2011).

As demonstrated in humans (Alford et al., 1966), the infectious dose required to produce clinical signs and cause death in mice depends on the route of inoculation. With the mouse-adapted reassortant X-31, the median mouse lethal dose (MLD_{50}) in BALB/c mice was more than 10-fold higher when virus was delivered intranasally (IN) rather than by aerosol (Smith et al., 2011a). Although similar viral titers in nasal washes and lung homogenates were obtained from mice inoculated by both routes, the aerosol-inoculated mice exhibited more severe and extensive lung histopathology, which appeared to correlate with elevated IL-6 and activated neutrophils in bronchoalveolar lavage (BAL) fluid (Smith et al., 2011a). Similar results were seen with aerosolized PR/8, which had an MLD_{50} in BALB/c mice of 8.7 plaque-forming units (pfu) per mouse, compared to 51.6 pfu, or approximately 6-fold higher, for virus instilled intranasally (Bowen et al., 2012).

2.1.2. Transmission of influenza viruses in mice

The influenza virus was first isolated by Wilson Smith, Christopher H. Andrewes, and Patrick P. Laidlaw in 1933 (Smith et al., 1933). In 1940, Monroe D. Eaton published a series of experiments (Eaton, 1940) in which he investigated the murine transmission of mouse-adapted influenza viruses, including PR/8 and the "WS" strain, an influenza virus isolated by Wilson Smith in 1933 – reportedly from his own throat washings while ill with influenza (Evans, 1966) – and an ancestor of the still commonly used laboratory strain WSN, its neurotropic variant ("Wilson Smith Neurotropic") (Reeve et al., 1980).

Eaton's experiments employed Swiss mice (Eaton, 1940), an outbred stock derived from nine albino mice imported from Lausanne in 1926 and maintained at the Rockefeller Institute (now University) in New York City. Over the years, the descendants of the original "Swiss mice" were dispersed widely to researchers and commercial breeders around the world, becoming the founding stock of many of the in- and outbred mouse lineages used in scientific research today (Chia et al., 2005). Eaton's experiments comprised eight intranasally inoculated Swiss mice, co-housed in large glass jars with eight uninfected contact mice. Inoculated mice usually died within a week, but contact mice rarely succumbed to infection. Instead, after 10 to 11 days, they were necropsied for evidence of lung consolidations characteristic of influenza pneumonia, which would imply that infection via transmission from the inoculated mice had occurred. Eaton reported highly efficient transmission of PR/8 and WS viruses in his model; depending on the experimental conditions, as many as 88% or 100% of contact mice (for PR/8 and the WS strain, respectively) would display typical influenza lung pathology. Transmission of these influenza virus strains was also observed among Swiss mice obtained from seven different breeders. In general, Eaton found that mice inoculated with higher doses of PR/8 or WS viruses were more likely to transmit to naïve contact mice; that peak transmission efficiency was achieved when contact mice were exposed to inoculated mice between 24 and 48 h post-inoculation for at least 72 h of contact time; and that older mice were more susceptible to infection than newly weaned pups (Eaton, 1940).

For more than 20 years, other researchers were unable to duplicate Eaton's findings, despite using the same influenza viruses and Swiss mouse strains (Schulman and Kilbourne, 1963a). Finally, in the 1960s, Schulman and Kilbourne succeeded in establishing a murine transmission model with CFW ("Carworth Farms White") (Schulman and Kilbourne, 1963b; Schulman and Kilbourne, 1963a) or MF-1 ("Manor Farms 1") (Schulman, 1967a, 1967b) mice, both commercially bred stocks derived from the Rockefeller Swiss mice (Institute of Laboratory Animal Resources, 1966; Lyle and Jutila, 1968; Chia et al., 2005). Schulman studied influenza virus transmission between infected and susceptible mice not only when they were physically commingled in the same cage, as Eaton had done, but also when infected and susceptible mice were separated on either side of a 3/4-inch (1.9 cm) thick wire-mesh partition, through which air could flow but no direct contact could occur (Schulman and Kilbourne, 1962, 1963a). Similar to Eaton's method, transmission to naïve mice could only be confirmed after necropsy. Schulman, however, verified that viable influenza virus could be re-isolated from the lungs of the exposed mice, by inoculating lung homogenates into embryonated hen's eggs (Schulman and Kilbourne, 1963a).

Contrary to Eaton's findings, Schulman reported relatively poor transmissibility of PR/8 and WSN, as well as the mouse-adapted A/H1N1 strains A/CAM/1946 and A/FM/1/1947, with transmission occurring only 5 to 25% of the time among his mice (Schulman and Kilbourne, 1963a; Schulman, 1968). The mouse-adapted influenza B/Lee/40 virus also transmitted poorly in this model, with only 10% efficiency (Schulman, 1967a). However, many influenza A/H2N2 isolates, which had begun to circulate in the human population after the 1957 "Asian flu" pandemic, transmitted among mice with comparative efficiency, including the mouse-adapted strains A/Japan/305/1957 (62.5% transmission rate), A/Rockville/1957 (40%), A/Ann Arbor/1960 (55%), and A/Bethesda/10/1963 (35%). Even the human H2N2 isolate A/Rockefeller Institute/5/1957, which had not been previously mouse-adapted, transmitted with 30% efficiency among mice (Schulman, 1968). In this mouse transmission model, Schulman made many prescient observations regarding the mammalian transmissibility of influenza viruses, some of which confirmed Eaton's earlier findings and some of which have been subsequently observed in other animal models. For example, rapid airflow and high humidity reduced the efficiency of airborne influenza virus transmission among mice (Schulman and Kilbourne, 1962); transmission occurred most efficiently when inoculated and susceptible animals were together during the time period of 24 to 48 h after inoculation (Schulman and Kilbourne, 1963a); older mice were more susceptible to being infected by transmission than were younger mice (Schulman and Kilbourne, 1963b); transmission efficiency in mice depended more on the inoculated animals being "good transmitters" than on the exposed animals being particularly susceptible to infection, even though "good" and "bad transmitters" demonstrated equivalent viral loads, infection kinetics, and lung pathology (Schulman and Kilbourne, 1963b); transmission efficiency was higher in the winter than in the summer, even when transmission experiments were conducted in temperature- and humidity-controlled environments (Schulman and Kilbourne, 1963b); and a virus that transmitted well could be sampled from the air surrounding infected mice at higher titers than could a virus that transmitted poorly, even though the two strains demonstrated identical lung titers and pathology in the infected mice (Schulman, 1967a).

The experiences of Eaton and Schulman suggest that murine transmission of influenza viruses is inefficient overall and may only be possible with specific mouse strains and mouse-adapted virus isolates. Transmission efficiency among mice may also be enhanced by laboratory-specific experimental configurations that are difficult to duplicate exactly in other environments. In a recent assessment of the murine transmissibility of five influenza A viruses, including the mouse-adapted laboratory strain WSN; the human seasonal influenza isolates A/Hong Kong/8/1968 [H3N2] and A/Texas/36/1991 [H1N1]; and the highly pathogenic viruses A/Vietnam/1203/2004 [H5N1] and the 1918 pandemic influenza A strain, reconstructed from vRNA preserved in human pathological specimens (Tumpey et al., 2005a), Lowen and colleagues were unable to detect any of these viruses in nasal or lung tissues of BALB/c contact mice, and none of the contact mice were seropositive for the virus to which they

were exposed (Lowen et al., 2006). These results suggest that as-yet unknown factors affect the transmission of influenza viruses in this species.

2.1.3. Immunology of influenza infection in mice

The commercial availability of mouse-specific immuno-logical reagents such as antibodies and recombinant cytokine and chemokine proteins, along with the ability to genetically manipulate this species with relative ease, has made mice the main model in which the immunology of influenza has been studied (Barnard, 2009; Tripp and Tompkins, 2009; Bouvier and Lowen, 2010).

The use of enzyme-linked immunosorbant assay (ELISA) has elucidated the cytokine and chemokine milieu induced by influenza virus expression in the mouse model. Induced levels have been shown to differ according to the strain of mouse, as demonstrated by PR/8 infection of inbred DBA/2J and C57BL/6 mice (Srivastava et al., 2009). The cytokines and chemokines IL-1α, IL-5, IL-6, IL-12, G-CSF, CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL1 (KC), CXCL2 (MIP-2), CXCL9 (MIG), and CXCL10 (IP-10) were found at higher levels in the BAL fluid of DBA/2 compared to C57BL/6 mice, correlating with the DBA/2 strain's increased susceptibility to infection and to lung pathology. As in humans (de Jong et al., 2006; Peiris et al., 2009; Tisoncik et al., 2012), it has been proposed that an overly inflammatory innate immune response results in immune-mediated lung pathology in mice, and the DBA/J strain appears particularly susceptible to cytokine storm (Srivastava et al., 2009).

ELISA has also been used to assess the immunopathology associated with particular viral genes and genetic polymor-phisms (Conenello et al., 2007). PB1-F2, an accessory protein that is expressed from an alternate reading frame in the PB1 gene in some but not all influenza A viruses, has been associated with greater pathogenicity in mice (Gocnikova and Russ, 2007). The 1918 pandemic influenza strain A/Brevig Mission/1918 [H1N1] encodes an N66S polymorphism in the PB1-F2 protein, which has also been observed in HPAI H5N1 strains. When BALB/c mice were inoculated with either the 1918 pandemic strain or a point mutant in which the serine at position 66 was reverted back to the more typical asparagine residue, the 1918 S66N mutant was less infectious and pathogenic than the wild-type 1918 virus. The pro-inflammatory cytokines IL-1α, IFN-γ, and TNF-α were detected at significantly lower levels in the 1918 S66N mutant-infected mice (Conenello et al., 2007), suggesting that this polymorphism plays a role in inducing high cytokine levels in mice infected with the 1918 pandemic strain.

In BALB/c mice, a pre-2009 seasonal influenza A/H1N1 ("sH1N1") isolate, engineered to express the HA gene of the 1918 pandemic virus, induced higher lung levels of cytokines – including CCL2 (MCP-1), CCL4 (MIP-1β), CCL20 (MIP-3α), CXCL2 (MIP-2), IL-1β, IL-6, IL-12p40, IL-18 and G-CSF – than did the seasonal virus itself (Kobasa et al., 2004). The authors suggest that the 1918 HA protein is responsible for stimulating this particular cytokine profile, which induces macrophage activation and neutrophil chemotaxis that may subsequently lead to acute lung injury (Kobasa et al., 2004). In BALB/c mice, a human seasonal influenza A/H1N1 virus encoding the 1918 HA and NA proteins induced a severe inflammatory pathology, consisting primarily of alveolar macrophages and neutrophils (Tumpey et al., 2005b). Mice infected with the 1918 HA/

NA-expressing virus displayed significantly higher lung levels of the pro-inflammatory markers IFN-γ, TNF-α, CCL3 (MIP-1α), and CXCL2 (MIP-2) than did mice infected with the seasonal virus itself. Compared to control mice, mice depleted of alveolar macrophages and neutrophils prior to infection demonstrated significantly lower levels of these cytokines and IFN-α, as well as higher viral titers in the lungs and viral spread to the brain (Tumpey et al., 2005b). Together, these data suggest that the HA and perhaps NA proteins of the 1918 influenza virus induce high levels of pro-inflammatory cytokines in the mouse lung, with alveolar macrophages and neutrophils playing a critical role in their production. While this inflammatory response is important for controlling viral replication and extrapulmonary spread, it also results in immune-mediated acute lung injury in the infected mouse.

Functional genomics has also provided a comprehensive overview of the effect of influenza infection on global host mRNA levels (Fornek et al., 2007). BALB/c mice were infected with the mouse-adapted WSN strain, or two WSN-based reassortants, one expressing the 1918 influenza HA and NA, and the other expressing the HA and NA of the recent seasonal human influenza isolate A/New Caledonia/20/99 [H1N1] (Kash et al., 2004). Both WSN and the 1918-HA/NA: WSN reassortant induced severe histopathological changes in infected mouse lungs, while the New Caledonia-HA/NA:WSN reassortant was relatively attenuated in the mouse model. Gene expression profiling performed on lungs harvested from infected mice revealed that, at early time points, mice infected with the virulent WSN and the 1918-HA/NA:WSN reassortant viruses demonstrated increased activation of genes involved in inflammation, lymphocyte activation, and stress response, relative to the gene expression in the lungs of mice infected with the New Caledonia-HA/NA:WSN recombinant virus (Kash et al., 2004). Similar studies with reassortants between the 1918 influenza virus and the recent human influenza isolate A/Texas/36/1991 [H1N1] showed variable histopathology in the lungs, with the 1918 virus causing the most severe damage and Texas/91 the least, with the various reassortants causing an intermediate phenotype (Kash et al., 2006). This differential pathology was mirrored by the expression of immune-related genes; the 1918 virus again induced the most significant and earliest expression of immune-related genes, followed in timing and magnitude by the reassortants and then by Texas/91 itself. Many of the genes that were significantly upregulated in 1918-infected mouse lungs were involved in inflammatory responses and cell death pathways. Thus, the rapidity and enhanced virulence of 1918 influenza virus infection in mice correlated with an increased expression of inflammatory response genes (Kash et al., 2006). These studies and others demonstrate that, in in-fluenza virus infections, innate immune gene expression cor-relates with the severity of pulmonary pathology, implicating the regulation of the immune response in disease outcomes in influenza.

Many host immune responses to influenza virus infection, too numerous to summarize in a single review, have been elucidated in the mouse model, including the interactions of innate and adaptive immune systems (Kreijtz et al., 2011), immune cell signaling and trafficking (Moltedo et al., 2009; Mount and Belz, 2010; Moltedo et al., 2011), antigen presen-tation (Mount and Belz, 2010; Eisenlohr et al., 2011), the

immune response to vaccines (van der Laan et al., 2008; Steel, 2011), the immune senescence of aging (Katz et al., 2004; Jiang et al., 2011), and the development of memory recall responses (Kedzierska et al., 2006; Mount and Belz, 2010). Many of the observations made in influenza virus-infected mice have led to the discovery of parallel phenomena in human immunology, but some still await confirmation of their significance in human influenza (Oshansky and Thomas, 2012).

2.2. Ferrets (Mustela putorius furo)

The influenza virus was first isolated by Smith, Andrewes, and Laidlaw in 1933 by filtering the throat washings of an influenza patient – reported to be Andrewes himself (Evans, 1966) – through a membrane impermeable to bacteria and then inoculating the sterile filtrate into the nares of two ferrets (Smith et al., 1933). Smith and colleagues had tried previously to transfer the agent of influenza to numerous animal species – guinea pigs, mice, rabbits, hamsters, hedgehogs, and monkeys – by the intracerebral, intratesticular, and intraperitoneal routes, but none had developed disease. Thus, their breakthrough resulted from two fortuitous choices — not only to infect the ferret, a species capable of manifesting an influenza-like illness upon infection with a human influenza isolate, but also to inoculate the filtered virus directly onto the susceptible mucosal epithelium of the respiratory tract (Evans, 1966). Smith and colleagues reported that, by 3 dpi, both inoculated ferrets became symptomatic with an influenza-like illness characterized by fever, malaise, anorexia, sneezing, yawning, and nasal discharge and congestion for a period of 3 to 10 days, "after which the ferret again becomes perfectly normal" (Smith et al., 1933). A surge of influenza research in the ferret model soon followed, along with the discovery, shortly thereafter, that disease in mice could be achieved with mouse-adapted influenza strains (Shope, 1934; Francis and Magill, 1935; Shope, 1935; Francis and Stuart-Harris, 1938; Stuart-Harris and Francis, 1938). Since then, the ferret, along with the mouse, has played a major role in our understanding of influenza virus virulence, pathogenesis, species tropism, and transmission.

Other strengths of the ferret model include its susceptibility to infection with unadapted human influenza virus isolates, its efficiency in transmitting influenza virus to others of its species, and its manifestation of clinical signs of disease akin to human influenza. Compared to the mouse and guinea pig models, though, the ferret model is disadvantaged by relatively limited commercial availability, more complex husbandry requirements, and greater expense, which can make adequately powered experiments difficult to perform. Unlike mice, there are few ferret-specific immunological reagents, and the ferret genome is not yet fully annotated (Belser et al., 2009; Tripp and Tompkins, 2009; Bouvier and Lowen, 2010).

2.2.1. Pathogenesis of influenza viruses in ferrets

Ferrets are naturally susceptible to a number of influenza A subtypes isolated from humans, birds, and swine, as well as influenza B viruses (Bouvier and Lowen, 2010; S.S. Huang et al., 2011; Pushko et al., 2011). Inoculation of ferrets with human isolates of seasonal influenza A/H1N1, A/H3N2, and B viruses generally results in an upper respiratory tract infection featuring fever, nasal congestion, malaise, and anorexia, similar to uncomplicated influenza in humans. Sneezing is a prominent clinical sign often observed in influenza virus-infected ferrets (Tripp and Tompkins, 2009), but less so in people. A dry cough is more typical of human influenza (Call et al., 2005; Treanor, 2010), while sneezing occurs frequently with common cold viruses like rhino- and coronaviruses (Turner, 2010). As in complicated influenza in humans, both the reconstructed 1918 pandemic influenza virus and HPAI A/H5N1 strains cause more severe disease and associated histopathology in the ferret lower respiratory tract. This differential disease pattern may, in part, be attributable to receptor distribution in the human and ferret respiratory tracts (Shinya et al., 2006; van Riel et al., 2006, 2007; Bouvier and Lowen, 2010).

In ferrets, influenza virus infection of the central nervous system (CNS) is particularly common with HPAI H5N1 viruses. It can also be observed with human seasonal influenza virus isolates, although their neurovirulence may be strain- and dose-dependent (Zitzow et al., 2002; Kwon et al., 2010; van den Brand et al., 2012). In contrast, influenza-associated encephalitis or encephalopathy is rarely reported in human influenza, and evidence of virus replication in the human CNS is limited (Studahl, 2003; Gambotto et al., 2007; Fonseca and Lavoie, 2014). A study comparing two HPAI H5N1 strains with varying lethality in ferrets (Plourde et al., 2012) found no significant difference in weight loss, viral titers, or respiratory tract pathology. However, all 10 ferrets challenged with A/Vietnam/1203/2004 died, after displaying prominent neurological signs such as seizure, torticollis, and paralysis. Post-mortem, the challenge virus was recovered at high titers from the olfactory bulb, cerebral cortex, cerebellum and brain stem, and histopathological lesions were widespread throughout these anatomical regions. Of ferrets challenged with A/Hong Kong/483/1997, only 20% (2 of 10) died, no neurological signs were observed, no viable virus was detected in the CNS, and histopathological lesions were seen only in the olfactory system. Thus, the lethality of HPAI H5N1 infection in the ferret model may be due, at least in part, to viral replication in the CNS and subsequent neurological dysfunction, rather than to respiratory tract pathology alone (Plourde et al., 2012). If so, this marks an important difference from human H5N1 influenza, in which most patients die of progressive respiratory failure, complicated by ARDS and multi-organ dysfunction (Gambotto et al., 2007).

2.2.2. Transmission of influenza viruses in ferrets

Unlike mice, ferrets are readily infected with a broad variety of unadapted human influenza virus isolates and are capable of transmitting disease to others of their species, whether housed in the same cage (most often called a "contact transmission model") or separated by a physical barrier that allows airflow from infected to exposed ferret but precludes direct or indirect contact between them (variably described as a "respiratory droplet," "aerosol," or "airborne transmission model"). Exposed ferrets can be monitored for transmitted infection by the onset of typical clinical signs (fever, nasal discharge, sneezing, and lethargy) and by the presence of influenza virus in nasal lavage specimens or eluted from throat or nose swabs. Transmission to exposed ferrets can also be confirmed after two to three weeks by assessing for seroconversion, the demonstration of

strain-specific anti-HA antibodies in the serum of infected ferrets (Matsuoka et al., 2009; Belser et al., 2011).

In the 1930s, it was observed that an influenza-virus naïve ferret, placed in the same cage as an experimentally inoculated ferret, would develop a similar influenza-like illness (Smith et al., 1933). Other early influenza researchers noted that ferrets occasionally arrived from the breeder already immune to experimental inoculation with laboratory strains of influenza virus. Serum from these animals was shown to contain antibodies capable of neutralizing PR/8 and another contemporary human influenza strain, suggesting that some members of the ferret colony had experienced prior infection with influenza virus, either through ferret-to-ferret or perhaps human-to-ferret transmission (Francis and Magill, 1935). Ferret-to-human transmission was suspected in the case of a lab worker tending to infected ferrets at the Rockefeller Institute (Francis, 1934), as well as in the case of Dr. C. H. Stuart-Harris, an influenza researcher who was examining recently inoculated ferrets, including one that sneezed on him (Smith and Stuart-Harris, 1936). Both men developed influenza-like illness, and their nasal washes, inoculated intranasally into previously uninfected ferrets, induced typical ferret influenza disease.

In 1940, Andrewes and Glover conducted a series of experiments to investigate the "aerial transmission" of influenza A virus among ferrets separated over various distances (Andrewes and Glover, 1941). They observed that co-caged ferrets readily transmitted disease one to another, but that ferrets in side-by-side solid metal cages did not. When infected and naïve ferrets were placed in open wire-mesh cages, however, disease transmission was frequently observed when cages were up to 5 ft (1.5 m) apart, even when exposed ferrets were placed several feet above infected ferrets, to preclude droplet spray transmission associated with sneezing. When windows in the animal room were opened to improve ventilation, ferret-to-ferret transmission was abolished. Transmission experiments were also conducted with infected and susceptible ferrets in separate cages at either end of straight, S-shaped, and U-shaped ducts 100 in^2 (644 cm^2) in cross-section and ranging between 5 and 9 ft (1.5 and 2.7 m) long. Air was pulled through the ducts with a fan, situated so that air currents passed from the infected ferrets' cage and through the susceptible ferrets' cage prior to being exhausted from the duct. Transmission of infection to the susceptible ferrets occurred at a range of air speeds between 3 and 14 linear feet per minute (59 and 275 liters per minute), even around the 180° bend in the U-shaped duct. Andrewes and Glover interpreted these findings to be most consistent with ferret-to-ferret transmission occurring primarily via aerosols (also called droplet nuclei) that were small enough to float upwards through still air or to be pulled around the air duct corners without impacting on its walls (Andrewes and Glover, 1941).

Influenza virus transmissibility by air between ferrets has been positively associated with the amount of infectious particles exhaled by the infected (virus-donor) ferret. Using reassortants between human H1N1pdm09 and swine H1N1 isolates, Lakdawala et al. (2011) found a positive correlation between inter-ferret transmission efficiency and the amount of vRNA-containing particles (measured by quantitative RT-PCR) collected from the air surrounding the ferrets' cages.

Higher NA enzymatic activity and a filamentous morphology were also characteristic of the more transmissible viruses. With pre-2009 seasonal sH1N1 and 2009 pandemic H1N1pdm09 strains of varying pathogenicity in ferrets, Koster et al. (2012) evaluated airborne transmission between infected and susceptible ferrets in airflow-controlled chambers, quantifying the size, number, and vRNA content of airborne respiratory particles. They found that high vRNA levels in the air did not predict efficient transmissibility for a given strain; interestingly, the more days that passed between donor ferret inoculation and its pairing with a susceptible recipient ferret, the less efficient transmission between them became, even though airborne vRNA levels remained relatively constant over the first 5 dpi. Even though infected ferrets were maintaining high viral particle output post-inoculation, the infectiousness of those particles appeared to be declining; thus, vRNA content in air samples may not be an accurate surrogate marker for infectious airborne virus. The strains causing overt disease also displayed the least efficient transmission, despite high vRNA levels in the air surrounding infected ferrets. This finding suggests that a more robust inflammatory response may reduce the viability of exhaled virus, without adversely affecting the quantity of vRNA-containing particles shed from the respiratory tract. Gustin et al. (2013) found that ferrets infected with highly transmissible human influenza viruses both exhaled and sneezed out more respiratory particles overall than those infected with poorly transmissible avian strains. In ferrets infected with transmissible human isolates, infectious virus particles in the respirable range (<5 μm) could be recovered in greater amounts from their exhaled breath (3–11 pfu) and sneezes (up to 8 pfu) than from the exhaled breath (0–6 pfu) or sneezes (up to 4 pfu) of ferrets infected with avian strains. Though the authors report statistical significance of these results, it is important to note that the majority of ferrets in both human and avian virus groups exhaled or sneezed no detectable infectious virus at all. The low recoverability rate of live virus particles in this system introduces the possibility of significant error; according to Poisson's distribution, when rare events are expected (e.g., 10 or fewer pfu per assay), there is a greater than 5% probability that 4 fewer to 4 more pfu will be observed in any given replicate (Gumbel, 1941). Capturing infectious virus-containing particles from exhaled breath remains a technical hurdle to a fuller understanding of the aerobiology of influenza viruses (Milton et al., 2013).

The ferret model has also been used to assess the pandemic potential of avian and swine influenza strains, or their reassortants with circulating human viruses. These include HPAI H5N1 viruses (Maines et al., 2006; Herfst et al., 2012; Imai et al., 2012); avian H7 (Belser et al., 2008) and H9 (Wan et al., 2008; Sorrell et al., 2009; Kimble et al., 2011) strains, including the recently emerged H7N9 virus (Belser et al., 2013a; Richard et al., 2013; Watanabe et al., 2013; Xu et al., 2014; Zhang et al., 2013; Zhu et al., 2013); and swine-origin H1N1 (Itoh et al., 2009; Maines et al., 2009; Munster et al., 2009), H1N2 (Pascua et al., 2012), and H3N2 (Pearce et al., 2012; Houser et al., 2013) strains. The ferret model has also been used to assess the mammalian transmissibility of oseltamivir-resistant human H1N1 (Herlocher et al., 2004; Yen et al., 2005; Duan et al., 2010; Hurt et al., 2010; Kiso

et al., 2010; Abed et al., 2011) and H3N2 (Herlocher et al., 2002; Yen et al., 2005; Memoli et al., 2010) isolates. These studies, as a whole, have shown that the prevalence of drug resistance among human isolates is proportional to their ferret transmissibility. Oseltamivir resistance is rarely seen in human influenza A/H3N2 isolates, which have demonstrated moderate to severe impairment in ferret transmissibility when encoding resistance mutations. However, human seasonal influenza A/sH1N1 viruses from 2008 to 2009, which were virtually 100% oseltamivir-resistant, transmitted highly efficiently in the ferret model.

Some of these studies, however, point out a disadvantage of the ferret model. Experiments that purport to show a difference between two or more influenza virus isolates are often conducted with only 2 to 4 ferret pairs per group, due in part to the cost, size, and husbandry requirements of ferrets. This sample size lacks sufficient statistical power to demonstrate a small difference that truly exists, leading to a Type II (i.e., "false negative") error (Cohen, 2012; Nishiura et al., 2013). It has been argued that pooling the data from several small but similar experimental groups increases power (Belser et al., 2013b). However, the degree of similarity among the groups to be combined must be explicitly considered, as heterogeneity compromises the reliability of conclusions drawn from pooled data. In general, a well-designed experiment with a sample size adequate to demonstrate a significant difference will give more precise and reliable results than a post-hoc meta-analysis of several smaller and variably similar experimental groups (Walker et al., 2008).

2.2.3. Immunology of influenza infection in ferrets

Because ferrets demonstrate a similar disease entity as human influenza, their immune response to influenza virus infection may be more relevant to that of symptomatic humans than is that of mice. However, immunological studies in ferrets have been limited by the lack of readily available reagents, such as recombinant ferret cytokines for use in ELISA or ferret-specific antibodies that recognize immune cell-surface markers (Belser et al., 2009; Tripp and Tompkins, 2009; Bouvier and Lowen, 2010). In some cases, reagents specific to other species, such as mink, dogs, and even humans, have shown sufficient cross-reactivity with ferret immune mediators (Rutigliano et al., 2008; Martel and Aasted, 2009). A commercially available canine microarray assay has been used to analyze differential immune gene expression after infection of ferrets with either low-pathogenicity human seasonal isolates or moderate-to-highly pathogenic influenza viruses (an avian H5N1 strain and a human H1N1pdm09 isolate) (Cameron et al., 2008; Rowe et al., 2010). In general, interferon response genes were expressed earlier and to higher levels in ferrets infected with the higher-pathogenicity strains, in particular a robust upregulation of CXCL10 (IP-10) that has also been seen in fatal human cases of H5N1 influenza (Peiris et al., 2009).

More commonly, expression levels of mRNA, quantified by qRT-PCR, have been measured as a surrogate marker for protein levels of immune mediators. As demonstrated by qRT-PCR of the ferret transcriptome, a strongly pro-inflammatory cytokine profile, particularly early in the course of infection, has been associated with more severe influenza disease in ferrets (Kang et al., 2011; Maines et al., 2011), similar to that observed in mice. Upon infection with

human and avian-origin influenza A strains, ferrets displayed nasal wash titers and symptom severity that were positively correlated with expression levels of TNF-α, IL-6, IFN-α, and IFN-β mRNA in the nasal turbinates. Higher levels of TNF-α and IL-6 were also significantly associated with more efficient airborne transmissibility, which ranged from 67 to 100% for the human isolates to 0% for the H5N1 strains (Maines et al., 2011). Also similar to mice, depletion of alveolar macrophages resulted in worsening of influenza disease in ferrets infected with an H1N1pdm09 isolate, including higher viral loads and greater inflammatory cell infiltrates (Kim et al., 2013). However in some of these studies, higher viral loads in respiratory tissues have been observed with the more pathogenic influenza virus infections, and thus the pro-inflammatory response may result from enhanced viral replication, rather than from particular attributes of the virus itself.

As in mice, some virus strain-specific differences in ferret pathogenesis can be attributed to certain genetic polymorphisms. For instance, introducing the open reading frame of the PB1-F2 accessory protein, as encoded by the 1918 influenza virus, into the seasonal influenza isolate A/USSR/90/1977 [H1N1] prevented the upregulation of mRNA transcripts for the pro-inflammatory cytokines IL-1β, IL-6, and IL-8 in ferret PBMC-derived macrophages infected ex vivo (Meunier and von Messling, 2012). USSR/77 itself, which encodes a truncated PB1-F2 protein that lacks the C-terminal domain that appears to mediate its inflammatory, antiviral, and pro-apoptotic effects in mice, induced only low-level, late expression of these genes, while USSR/77 with a complete knockout of the PB1-F2 open reading frame stimulated significantly higher cytokine mRNA expression. However, unlike in mice, expression of the 1918 PB1-F2, encoding the N66S polymorphism, resulted in a slight but non-significant enhancement in pathogenicity and equivalent viral replication in the lungs of infected ferrets (Meunier and von Messling, 2012).

A recent study (Huang et al., 2012) evaluated influenza A/H1N1pdm09 virus infection in newly weaned kits (5 to 8 weeks old). Kits displayed significantly attenuated disease, compared to adult ferrets (4 to 6 months old), despite similar viral loads in the respiratory tract. Their immune response, however, was quite different. In both peripheral blood and lungs, the kits mounted a predominantly mononuclear cell (lymphocyte and monocyte) response, and lung pathology was relatively mild, including bronchiolar desquamation and debris plugging. In contrast, the adults displayed higher granulocyte (neutrophil, eosinophil, and basophil) counts in the peripheral blood and more granulocytic infiltrate in the lungs, which was associated with bronchiolitis and alveolitis. Kits also demonstrated significant, early upregulation of mRNA encoding the pro-inflammatory chemokines CXCL9 (MIG) and CXCL10 (IP-10), as well as the regulatory cytokine IL-10; at 3 dpi, CXCL9, IL-10, and TGF-β1 mRNA levels were significantly higher in the lungs of kits than in those of adults. Thus, ferret kits mounted a distinctly different immune response, paralleling clinical outcomes in the 2009 influenza pandemic, in which children generally manifested milder influenza disease than adults (Huang et al., 2012).

Like mice, the ferret model has been widely used in the pre-clinical development of candidate influenza vaccines against a variety of human and avian-origin influenza strains.

Though the studies are too numerous to detail here, many review articles have addressed this topic (Subbarao and Luke, 2007; van der Laan et al., 2008; Tripp and Tompkins, 2009; Bodewes et al., 2010; D'Aoust et al., 2010), and recent publications feature innovative new approaches to antigen design and presentation to the immune system (Hamouda et al., 2011; Petsch et al., 2012; Wei et al., 2012; Kanekiyo et al., 2013; Scallan et al., 2013; Krammer et al., 2014).

2.3. Guinea pig (Cavia porcellus)

In recent years, the guinea pig most commonly used in influenza virus research has been the albino Hartley strain, an outbred stock that is easily obtained from commercial breeders. Parti-colored Strain 2 and Strain 13 guinea pigs – two of many inbred lineages that the geneticist Sewall Wright created while studying coat color, polydactyly, and other inherited traits (Wright, 1934; Wright and Chase, 1936) – are still maintained in private university, government, and military colonies but are not as readily available (Banks, 1989). The guinea pig genome, sequenced to 7x coverage, was derived from a female Strain 2 guinea pig (Di Palma et al., 2008).

Strengths of the guinea pig model include its susceptibility to infection with unadapted human influenza virus isolates, its efficiency in transmitting influenza virus to others of its species, and its commercial availability, small size, ease of handling and housing, and low cost, relative to the ferret model. Disadvantages include the lack of clinically overt signs of influenza disease and a paucity of immunological reagents (Bouvier and Lowen, 2010). However, the guinea pig immune system, to the extent that it has been characterized, appears to share many genetic and phenotypic features in common with that of humans (McMurray, 2001; Padilla-Carlin et al., 2008). Additionally, the anatomy and physiology of the guinea pig lung resemble that of humans, and the guinea pig is a well-characterized model for non-infectious respiratory diseases such as asthma, chronic obstructive pulmonary disease, and allergy and anaphylaxis in humans (Canning and Chou, 2008).

2.3.1. Pathogenesis of influenza viruses in guinea pigs

Guinea pigs have been occasionally used in the study of influenza pathogenesis for over fifty years (Janssen et al., 1963; Wetherbee, 1973; Fehlmann et al., 1974; Phair et al., 1979; Azoulay-Dupuis et al., 1984). Guinea pigs are readily infected by human, avian, and swine influenza isolates, without prior adaptation (Bouvier and Lowen, 2010). Of the human and avian isolates tested to date, the median infectious dose required to initiate infection in the guinea pig by intranasal inoculation ($GPID_{50}$) is on the order of 1 to 100 pfu per animal, with a geometric mean of approximately 10 pfu (Lowen et al., 2006; Bouvier et al., 2008; Steel et al., 2009; Gabbard et al., 2013). These published $GPID_{50}$ values are comparable to the median ferret infectious dose (FID_{50}) that has been reported for several influenza virus strains administered intranasally (Toms et al., 1977; Gustin et al., 2011; Roberts et al., 2011). The median intranasal infectious dose for humans (HID_{50}) is in the range of 100 to 500 median tissue culture infectious doses ($TCID_{50}$) (Tellier, 2009), which is a different method of virus titration but should be within one order of magnitude of the corresponding pfu value (Watanabe et al., 2012). The HID_{50} for influenza virus delivered by aerosol, rather than by intranasal inoculation, has been estimated to be on the order of 1 $TCID_{50}$, or more than 100 times less than the intranasal HID_{50} (Tellier, 2009), while the FID_{50} for both aerosol and intranasal inoculation routes appears to be roughly equivalent (Gustin et al., 2011; MacInnes et al., 2011). Experimental aerosol inoculation of guinea pigs has been performed, but the delivered dose and thus the $GPID_{50}$ were not explicitly calculated (Mubareka et al., 2009).

Upon intranasal inoculation of influenza virus, replication is mainly confined to the upper respiratory tract, with naso-pharyngeal titers being highest on day 2 or 4 post-inoculation and clearance of virus by 8-10 dpi. Viral replication can occur in the lungs, but it is typically at lower levels than is seen in the nasopharynx (Lowen et al., 2006; Gabbard et al., 2013; Seibert et al., 2013). Despite productive infection, however, influenza viruses do not typically cause overt signs of disease in guinea pigs, as they do in ferrets (Bouvier and Lowen, 2010). Signs such as ruffled fur, listlessness, and anorexia are either absent or so subtle as to not be readily apparent in guinea pigs. Increased nasal mucus can be appreciated in infected animals (Tang and Chong, 2009; Bouvier and Lowen, 2010), but in our experience this sign is not consistently obvious and would be difficult to follow clinically, although it can be appreciated histopathologically (Tang and Chong, 2009).

One group has reported sneezing in H1N1pdm09-infected guinea pigs (Sun et al., 2010), but in our experience it is not regularly observed; we have witnessed a single guinea pig sneeze, over the course of five years working with this species. However, it is not known if the efficient transmission of influenza viruses among humans requires expulsive events like coughing or sneezing, as opposed to the less forceful but more continuous exhalation of respiratory droplets during tidal breathing and talking. Akin to humans, guinea pigs are social animals that communicate through sound, making a variety of high- and low-frequency vocalizations by altering the tension of the glottal folds and causing them to vibrate by forcing air through them (Berryman, 1976); a similar, though more powerfully expulsive physiology underlies the cough mechanism (Zayas et al., 2012).

The virus content of respiratory emissions has been studied in guinea pigs to a lesser extent than in humans (Stelzer-Braid et al., 2009; Xie et al., 2009; Lindsley et al., 2010, 2012; Milton et al., 2013) or in ferrets (Lakdawala et al., 2011; Koster et al., 2012; Gustin et al., 2013). However, Mubareka et al. (2009) found that an influenza A virus isolate that transmitted efficiently by air between guinea pigs could be sampled from the air surrounding infected guinea pigs with higher infectious titers than could a virus that transmitted poorly, despite both isolates demonstrating similar peak nasopharyngeal virus titers. These results echo those of Schulman and Kilbourne in the mouse transmission model (Schulman, 1967a) and suggest that respiratory lavage or tissue virus titers may not accurately reflect the amount of infectious virus actually being released into the air during respiration. Similar air-sampling experiments in people with influenza have also found a lack of strong correlation between nasopharyngeal and exhaled viral loads (Milton et al., 2013).

The lethality of influenza virus infection in guinea pigs and ferrets is markedly different, even though similar viral loads can be isolated from respiratory tract tissues. Guinea pigs infected with 10^6 egg infectious doses (EID_{50}) of an HPAI H5N1 virus exhibited only mild listlessness and then recovered (Kwon et al., 2009), while ferrets inoculated with the same dose of the same H5N1 virus experienced severe disease and died by 5 to 7 dpi (Govorkova et al., 2005). Another HPAI H5N1 virus, inoculated into ferrets and guinea pigs at similar doses, achieved comparable nasal wash and lung viral loads. However, the ferrets' mean maximal weight loss was 16%, and 3 of 3 ferrets died or were euthanized by day 7. In contrast, all guinea pigs survived, with an average peak weight loss of 7% (Maines et al., 2005; Van Hoeven et al., 2009). No viable virus could be isolated from the non-respiratory tissues of guinea pigs infected with a panel of HPAI H5N1 viruses (Gao et al., 2009), suggesting that systemic spread is absent or at least undetectable. The lack of neurovirulence of H5N1 viruses in the guinea pig may, in part, account for the very different morbidity of avian-origin strains in this species, relative to that which can be seen in ferrets (Plourde et al., 2012).

It is important to note, however, that people exhibit a range of symptoms and signs when infected with influenza viruses, and specific immunological "signatures" correlate with the degree of symptomatology (Zaas et al., 2009; Y. Huang et al., 2011); guinea pigs and ferrets appear to model the two ends of the symptom spectrum, with human influenza in between. However, respiratory tract pathology in the influenza virus-infected guinea pig does correlate with the clinical severity of human infection, with the most striking histopathological damage in the guinea pig being caused by influenza strains known to be especially virulent in humans, such as the 1918 pandemic influenza virus and avian-origin H5N1 and H7N9 strains (Kwon et al., 2009; Van Hoeven et al., 2009; Gabbard et al., 2013).

2.3.2. Transmission of influenza viruses in guinea pigs

Despite differences in host symptomatology, similarly designed transmission experiments in the guinea pig and ferret models have, in general, arrived at similar conclusions (Bouvier and Lowen, 2010; Govorkova, 2013). However, the major contribution of the guinea pig model to the field of influenza virus transmission is its opening of new areas of investigation that would be difficult to pursue in the larger and more expensive ferret model.

The relatively small size of guinea pigs enabled Lowen et al. to study the effects of relative humidity (RH) and temperature on influenza virus transmission, using commercially available, 30 ft^3 (850 l) environmentally controlled chambers, each large enough to house four guinea pig transmission pairs. At various combinations of temperature (5, 20, or 30 °C) and RH (20, 35, 50, 65, and 80%), the airborne transmission efficiency of A/Panama/2007/1999 [H3N2] (Pan/99) has been assessed in 8 to 12 guinea pig pairs per environmental condition (Lowen et al., 2007, 2008; Steel et al., 2011). Overall, the efficiency of transmission by airborne routes increased as temperature decreased. The same general trend towards increased transmission efficiency was observed with decreasing humidity; however, at 20 °C, transmission rates followed a bimodal pattern, being least efficient at both high (80%) and mid-range (50%) RHs, more efficient at moderate–high (65%) RH, and

most efficient at low (20–35%) RH (Fig. 1). The same inverse correlation between transmission efficiency and ambient temperature has since been demonstrated in influenza A/sH1N1, A/H1N1pdm09, and influenza B viruses (Steel et al., 2011; Bouvier et al., 2012; Pica et al., 2012). These laboratory experiments suggest that wintertime weather conditions may play a role in the seasonality of influenza epidemics in temperate climates.

The relatively economical guinea pig model also allows for many experimental replicates to be performed, if needed, to tease out small effects on transmission efficiency. For example, prior to 2008, the vast majority of in vitro and in vivo data suggested that oseltamivir resistance mutations in the influenza virus NA came at some cost to viral fitness, leading many at that time to conclude that most oseltamivir-resistant viruses were "unlikely to be of clinical consequence," as noted in a recent review (Govorkova, 2013). However, in the winter of 2007–2008, coincident with the circulation of a new antigenic drift variant characterized by A/Brisbane/59/2007 [H1N1], a sudden increase in the prevalence of oseltamivir resistance among seasonal influenza A/sH1N1 virus isolates was noted. Within 5 months, 25% of European sH1N1 isolates encoded the NA-H274Y oseltamivir-resistance mutation; by 2009, it was found in 96% of sH1N1 isolates worldwide (Bouvier et al., 2012). Enhanced transmission efficiency among humans was one of only a few hypotheses that could account for the unprecedented, exponential increase in prevalence of the NA-H274Y mutation in sH1N1 viruses (Chao et al., 2012). With paired oseltamivir-sensitive and -resistant Brisbane/59-like clinical isolates from the New York State Department of Health, it was shown that the oseltamivir-resistant isolate transmitted more efficiently among guinea pigs than its oseltamivir-sensitive counterpart. With various point mutants of and reassortants between the oseltamivir-sensitive and -resistant isolates, the enhanced transmissibility of oseltamivir-resistant Brisbane/59-like viruses could be attributed to expression of oseltamivir-resistant NA, specifically the residues H275Y and/or D354G, which were characteristic of the resistant viruses (Bouvier et al., 2012). Because guinea pigs are less expensive than ferrets to purchase and maintain, these experiments assessed four different recombinant viruses, in 8 guinea pig pairs per virus, in order to reveal a small but statistically significant fitness advantage conferred primarily by the oseltamivir-resistant Brisbane/59-like NA alone.

2.3.3. Immunology of influenza infection in guinea pigs

As with ferrets, there is a relative paucity of data on the immune response to influenza virus infection in the guinea pig, in part due to a lack of species-specific immunological reagents. However, as in the ferret model, alterations in expression levels of immune-related mRNAs, quantified by qRT-PCR, have been used as a surrogate marker for the up- and downregulation of immune effector proteins.

While investigating the effect of temperature and humidity on the transmission efficiency of the human influenza A isolate Pan/99, Lowen and colleagues noted that infected guinea pigs housed at lower temperature (5 °C) shed virus longer and to higher titers than infected guinea pigs held at higher temperature (20 °C). To assess whether the more robust viral replication in guinea pigs housed at 5 °C resulted
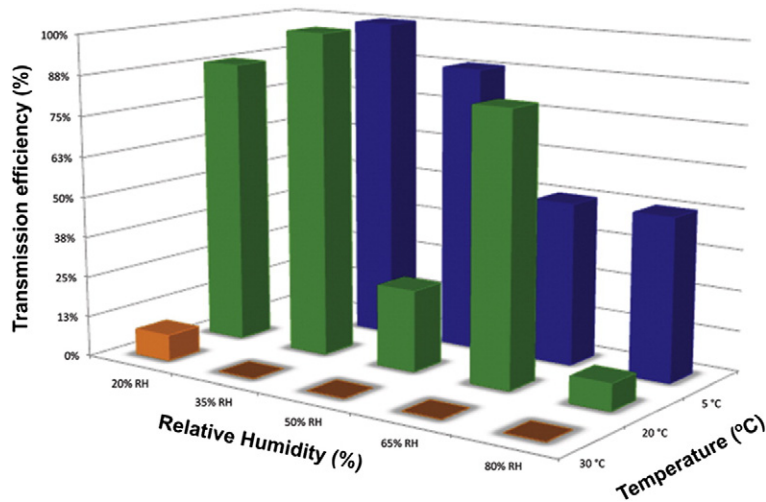
Fig. 1. Transmission efficiency of influenza A/Panama/2007/1999 [H3N2], as a function of temperature and relative humidity, in the guinea pig model. Drawn from data presented in Lowen et al. (2007, 2008) and Steel et al. (2011).

from a cold-induced impairment in host innate immune defenses, they quantified by qRT-PCR the mRNA expression of innate immune effectors in the nasal turbinates of Pan/99-infected guinea pigs housed at either 5 or 20 °C. In both groups of guinea pigs, expression of Mx1, TLR3, MDA5, IRF7, STAT1, IL-1β, CCL2 (MCP1), CCL5 (RANTES), and CCL7 (MCP3) mRNAs were upregulated, while expression of TNF-α, TBK1, IRF5, and IFN-γ mRNAs were not. They found that CCL5 mRNA expression was greater in guinea pigs at 5 °C, while peak levels of IL-1β and MDA5 were higher in guinea pigs at 20 °C. Altogether, it appeared as though innate immune signaling was not significantly compromised in guinea pigs housed at 5 °C, suggesting a different mechanism may be responsible for their higher nasopharyngeal viral loads, compared to animals at 20 °C (Lowen et al., 2007).

The innate immune response, particularly the presence of Type I IFN, appears to affect influenza virus replication in and transmission among guinea pigs. Similar to that which had been shown in ferrets (Kugel et al., 2009), daily intranasal treatment of influenza virus-infected guinea pigs with recombinant human Type I IFN reduced influenza virus growth in the respiratory tracts of inoculated animals (Van Hoeven et al., 2009; Steel et al., 2010). In addition, IFN-treated, influenza virus-infected guinea pigs failed to transmit the virus to untreated, co-caged guinea pigs. The converse experiment – exposing IFN-treated guinea pigs to untreated, influenza virus-infected animals – also resulted in no virus transmission to the treated guinea pigs (Steel et al., 2010).

The ability to block influenza virus transmission among guinea pigs by vaccination was shown in a series of elegant experiments by Lowen, Steel, et al. (2009). Adaptive immunity in guinea pigs was induced with an inactivated vaccine, a live-attenuated vaccine, or a live virus infection, followed by challenge with either a homologous or heterologous influenza A/H3N2 virus. Virus challenge was delivered by two routes, either by direct intranasal inoculation of virus, or by exposure to an acutely infected guinea pig in the same cage. Prior infection with live virus was fully protective

against both homologous and heterologous challenge by direct intranasal inoculation and by exposure to an infected cage-mate. A live-attenuated virus vaccine provided sterilizing immunity against homologous challenge by both routes, but was less effective against heterologous challenge. Finally, the inactivated virus vaccine failed to provide sterilizing immunity against homologous or heterologous challenge by either route. Thus, different vaccine constructs appear to elicit different levels of protection against influenza virus infection, with live-attenuated virus vaccines inducing a protective response that is more similar to natural infection than an inactivated vaccine.

In an attempt to dissect the differential protection conferred by the vaccine constructs used by Lowen, Steel, et al. (2009), Seibert et al. (2013) studied the protection conferred by different immunoglobulin isotypes against airborne transmission of influenza virus. They passively immunized guinea pigs by intramuscular administration of a neutralizing mouse monoclonal IgG$_{2b}$ antibody directed against the HA protein of A/California/04/2009 [H1N1pdm09] (Cal/09). Although high serum antibody titers were achieved, the immunized animals were not protected from infection by transmission of Cal/09 from inoculated partner animals. In contrast, a single intranasal (IN) administration of this antibody, at a 10,000-fold lower dose, was sufficient to protect immunized guinea pigs from infection by transmission, suggesting that neutralizing antibody at the respiratory mucosa can provide sterilizing immunity against infection by airborne influenza virus. To provide further evidence for this hypothesis, they isotype-switched this antibody by cloning its variable region into the murine IgA heavy chain gene and expressing it with murine κ and J chains. This chimeric IgA antibody was administered intramuscularly to guinea pigs at two doses. The lower dose did not prevent immunized guinea pigs from becoming infected with Cal/09 by transmission, but 7 of 8 guinea pigs immunized with a 5-fold higher dose were protected. Further supporting a role for mucosal immunity in preventing infection by airborne routes, the chimeric IgA antibody was detectable by ELISA only in the nasal washes of

guinea pigs immunized with the higher, protective dose. Thus, sufficient quantities of a mucosal neutralizing antibody can prevent infection with airborne influenza virus in the guinea pig model, while serum antibody cannot.

3. The influenza virus transmission model in ferrets and guinea pigs

To assess virus and host factors that influence the transmissibility of influenza viruses, there are many important techniques involving the growth and titration of stock viruses; the inoculation of animals and collection of samples; and the analysis of samples for evidence of infection in susceptible, virus-exposed animals. Many detailed protocols for these basic procedures are available (Klimov et al., 2012; Kroeze et al., 2012; Smee and Barnard, 2013).

Experimental animals are most commonly inoculated with influenza viruses by the intranasal route, under anesthesia. Multiple weight-based anesthesia regimens can be used, and each institution's veterinary staff will be able to suggest a preferred method. The volume of the virus inoculum used depends on the animal species. Currently, guinea pigs are most often inoculated intranasally with virus suspended in a total volume of 300 μl of phosphate-buffered saline (PBS), divided evenly between each nostril (Lowen et al., 2006; Bouvier et al., 2008; Mubareka et al., 2009; Van Hoeven et al., 2009; Sun et al., 2010; Long et al., 2011). However, over the years a range of inoculum volumes – 200 μl (Wetherbee, 1973; Azoulay-Dupuis et al., 1984), 500 μl (Phair et al., 1979), and 1 ml (Kwon et al., 2009; Bushnell et al., 2010) – have been used to infect guinea pigs. There is less consistency among different laboratories regarding the total volume of intranasal inoculation in ferrets: 200 μl (Matsuoka et al., 2009; Smith et al., 2011b), 300 μl (van den Brand et al., 2012), 500 μl (Bodewes et al., 2011; Lakdawala et al., 2011; Herfst et al., 2012; Kroeze et al., 2012), and 1 ml (Herlocher et al., 2001; Duan et al., 2010; Gustin et al., 2011) volumes have been recently reported. Ferrets are also sometimes inoculated intratracheally with a larger volume (3 ml), which appears to enhance disease in this species (Herfst et al., 2012; Kroeze et al., 2012; van den Brand et al., 2012). Additionally, aerosol inoculation – by inhalation of influenza virus that has been nebulized into an airborne mist – has been described for ferrets (Lednicky et al., 2010; Tuttle et al., 2010; Gustin et al., 2011) and for guinea pigs (Mubareka et al., 2009). Usually, inoculated animals are kept separate from the susceptible animals for 24 h, to insure that the liquid inoculum dissipates prior to contact with the exposed animals.

Contact transmission studies are usually performed with a virus-inoculated animal and a susceptible animal housed in the same cage, so that virus spread can occur by all or any mode of transmission: direct contact, indirect contact, droplet spray, and short-range aerosol (Bouvier and Lowen, 2010). Some laboratories place more than one inoculated animal and/or more than one susceptible animal in the common cage (Herlocher et al., 2004; Duan et al., 2010). In this case, the cage must be large enough to accommodate all animals comfortably. National and international laws and guidelines differ considerably, but, depending on weight, guinea pigs require approximately 200 to 900 cm² of floor space per animal, with the minimum cage size for a single animal ranging between 387 and 2500 cm² by 21 to 23 cm high. Ferret cages should be, at

minimum, 2250 to 6000 cm² by 50 cm high, depending on the size of the animal, plus 1500 to 6000 cm² per each additional ferret (Animals (Scientific Procedures) Act, 1986; Animal Research Review Panel, 2006; The European Parliament and the Council of the European Union, 2010; National Research Council of the National Academies, 2011; Home Office of the Government of the United Kingdom, 2013).

Airborne transmission experiments are conducted with inoculated and susceptible animals physically separated by an air-permeable barrier that precludes direct and indirect contact transmission of influenza virus while allowing airborne routes. Cage configurations vary among laboratories; for instance, animals may be placed in separate cages that have at least one air-permeable wall, such as wire mesh (Lowen et al., 2006) or perforated Plexiglas (Maines et al., 2009); the cages of inoculated and susceptible animals are then placed side-by-side, with air-permeable walls directly opposed, so that air flows freely between them. Alternatively, one large cage can be divided by an air-permeable wire mesh (Herfst et al., 2012) or perforated Plexiglas (Seibert et al., 2010) barrier, on either side of which inoculated and susceptible animals are housed. Most experimental systems currently in use do not allow the differentiation between droplet spray and short-range aerosol transmission, as both could conceivably occur over the short distances between the animals.

In the conduct of airborne transmission experiments in animal models, little methodological standardization exists among laboratories (Nishiura et al., 2013), which may be a consideration if groups come to differing conclusions, or if one group is unable to replicate the findings of another. The speed and directionality of airflow between inoculated and susceptible animals is likely an important variable that is poorly studied and often not controlled in airborne transmission modeling. Similarly, ambient temperature and humidity fluctuates seasonally in temperate climates, even in buildings with environmental control systems, and transmission efficiency may depend on the time of year. Finally, different inoculating doses of virus are often used (Nishiura et al., 2013), which has been shown to impact transmissibility or pathogenicity in ferrets and in guinea pigs (Tang and Chong, 2009; Herfst et al., 2012).

Nasal washes or swabs are usually collected every other day, under anesthesia, for viral load titration (Kroeze et al., 2012). The isolation of viable influenza virus from the nasal specimen of a susceptible animal exposed to an inoculated animal is indicative of virus transmission between them. Nasal washes are performed by instilling PBS into the nares of an anesthetized animal (1 ml for guinea pigs (Bouvier et al., 2008) and 1–2 ml for ferrets (Lednicky et al., 2010; Herfst et al., 2012), divided over both nostrils). In guinea pigs, the PBS is instilled slowly, one nostril at a time, allowing the animal's inhalations to help draw it into the nasal cavity. During the nasal wash procedure, guinea pigs can be held over a sterile Petri dish to allow lavage fluid to drain from the nares by gravity (Bouvier et al., 2008). In ferrets, the PBS is instilled dropwise into the nostril until it induces sneezing, which can be caught in a sterile Petri dish (Lednicky et al., 2010; Herfst et al., 2012). If the ferret does not sneeze, then drainage by gravity can be captured in a Petri dish, similar to the guinea pig procedure (Kroeze et al., 2012). The fluid is then collected from the Petri dish and placed in a centrifuge tube on ice. Nasal and throat swabs can also be collected from ferrets and

placed in transport medium, using similar equipment and technique as diagnostic swab collection from humans (Kroeze et al., 2012). However, the nares of guinea pigs are too small for most commercially available swabs. Nasal specimens are centrifuged at low speed for 5 min to pellet nasal epithelial cells or other inhaled debris, and then the supernatant is collected into a clean vial and stored at −70 to −80 °C until titration (Bouvier et al., 2008; Kroeze et al., 2012).

4. Conclusions

Many mammalian animal models have been used in influenza virus research, including cotton rats, Syrian hamsters, cats, dogs, domestic pigs, and non-human primates. In this review, we have discussed the three models currently used most frequently: mice, ferrets, and guinea pigs.

Symptoms of influenza virus infection in humans are most closely mimicked by the ferret, in which influenza virus disease is manifested by fever, nasal discharge, lethargy, weakness, anorexia, and sneezing. Like humans, infection with highly pathogenic avian influenza viruses can induce ARDS and multi-organ system dysfunction in ferrets. Mice and guinea pigs are less overtly symptomatic; thus, the use of antivirals or vaccines to prevent or reduce clinical illness is difficult to study in rodent models. Also, the guinea pig has proven to be more resistant to lethal influenza virus infection than humans, and thus appears less useful to model the cytokine storm that results in immune-mediated morbidity and mortality with highly pathogenic strains such as avian-origin H5N1 and H7N9 viruses.

Ferrets and guinea pigs are readily susceptible to infection with human influenza virus isolates, with no prior requirement for species adaptation. Depending on the virulence of the influenza virus strain, they manifest upper and lower respiratory tract viral replication and pathology in proportion to that seen in human influenza disease. Commonly used inbred mouse strains are resistant to infection with most primary human virus isolates, except at very high doses; thus, mouse experiments are generally performed with a small handful of well-characterized but outdated mouse-adapted strains. However, the conveniences of the mouse model – small size, low cost, ease of handling, and availability of reagents – make them an ideal initial species in which to perform preliminary pre-clinical studies, such as assessing drug and vaccine efficacy, even though their modeling of human influenza physiology is inexact.

Efficient inter-host transmission of influenza viruses has been shown repeatedly, under a variety of experimental conditions, in both ferrets and guinea pigs. Past experiments suggest that mouse-to-mouse transmission is possible, but it appears to require a precise but not fully understood combination of factors, such as virus strain, mouse breed, and perhaps also laboratory conditions, to maximize efficiency. Thus, to study interventions intended to prevent the spread of influenza viruses among susceptible hosts, the ferret and guinea pig models are most often employed. The rodents do, however, have the benefit of lower cost and less complex husbandry requirements, so that statistically robust data can be obtained at less expense.

The mouse has elucidated many immune response mechanisms that are beginning to find parallels in human influenza.

The immunology of influenza in the ferret and guinea pig models is relatively poorly understood, due to the paucity of species-specific reagents. However, some progress has been made in the ferret model, using alternative modalities such as qRT-PCR and cross-reactive antibodies, and guinea pig-specific ELISA reagents are becoming increasingly available, due to its utility in modeling allergy responses and non-infectious respiratory disease.

In sum, influenza researchers have a variety of animal models in which to study various aspects of disease caused by these important human pathogens. It is incumbent upon the researcher not only to select the most appropriate model in which to investigate the experimental question, but also to understand the limitations of that model when interpreting data and conveying conclusions drawn from animal experiments.

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