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A model of respiratory syncytial virus (RSV) infection of infants in newborn lambs

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Abstract

Many animal models have been established for respiratory syncytial virus (RSV) infection of infants with the purpose of studying the pathogenesis, immunological response, and pharmaceutical testing and the objective of finding novel therapies and preventive measures. This review centers on a neonatal lamb model of RSV infection that has similarities to RSV infection of infants. It includes a comprehensive description of anatomical and immunological similarities between ovine and human lungs along with comparison of pulmonary changes and immune responses with RSV infection. These features make the newborn lamb an effective model for investigating key aspects of RSV infection in infants. The importance of RSV lamb model application in preclinical therapeutic trials and current updates on new studies with the RSV-infected neonatal lamb are also highlighted.

Keywords Respiratory syncytial virus · Neonatal lamb model · Antiviral therapy · Animal model · Infants

Introduction

Human respiratory syncytial virus (RSV) is a common cause of respiratory infection in infants and children worldwide (Hall et al. 2013). RSV is a ubiquitous virus that targets the respiratory system causing rhinitis, bronchiolitis, pneumonia, and occasionally otitis media. Children younger than 5 years of age and individuals over the age of 65 have a higher risk of severe infection. One surveillance study determined that > 57,000 children under the age of 5 years were hospitalized

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annually due to RSV-associated acute respiratory illness (ARI) (Hall et al. 2009). While infection of RSV in immunocompetent adults causes a common cold and mild upper respiratory tract symptoms, the disease outcome can be severe and fatal in preterm infants and children younger than 1 year of age (Hall et al. 2013; Rossi et al. 2007; Sommer et al. 2011).

Currently, there is a limited choice of therapeutic compound and no available vaccine for RSV infection. Numerous studies are conducted in search of preventive methods and treatment options from RSV. Development of such therapeutic compounds and vaccines requires extensive laboratory testing, animal trials in the preclinical stage, and multiple phases of clinical trials. Establishing a suitable animal model that mimics the RSV infection in human is challenging due to the high degree of specificity of the RSV to its natural host and lack of virulence in other species (Table 1). Experimental infection of RSV in mice and cotton rat has been well established and became a widely use animal model for RSV. These rodent models are employed in many types of RSV studies including the understanding of the viral pathogenesis as well as preventive and treatment trials. In addition to these rodent models, experimental infection of RSV in larger mammals has been carried out in non-human primates (NHPs) and ruminants.

In the past decade, an experimental lamb model for RSV infection was developed and currently a fully established animal model for RSV. This model has now been increasingly

Table 1 Features of RSV infection in human infant compared with lamb, cotton rat, and mice models

Pulmonary cellular immune response to RSV infection	Human infants	Neonatal lamb	Cotton rat	Mice
Alveolar macrophages	+++, increase in lung parenchyma and alveolar spaces (Johnson et al. 2007)	++ CD1$^+$ cells (DCs, B cell and monocytes) (Sow et al. 2011b)	++ (Grieves et al. 2015)	+++ (Dakhama et al. 2005)
Dendritic cells (DCs)	+, pDC and mDC increase (Gill et al. 2005)		ND	pDC and mDC increased (Beyer et al. 2004)
Neutrophils	++/+++ (varied upon RSV strain) (Everard et al. 1994)	+++ (Derscheid et al. 2014b; Larios Mora et al. 2015; Sow et al. 2011b)	+ (Grieves et al. 2015)	+ (Dakhama et al. 2005)
Eosinophils	+/− (Everard et al. 1994)	− (Derscheid et al. 2013a, b)	++ (Grieves et al. 2015)	+/− (Dakhama et al. 2005)
NK cells	+/− (Larranaga et al. 2009)	ND	ND	+++ (Hussell and Openshaw 1998)
CD4$^+$ T cell	++, Th2 > Th1 (Bendelja et al. 2000)	++ (Larios Mora et al. 2015; Sow et al. 2011b)	+++ (predominant cellular component) (Grieves et al. 2015)	Neonatal: + Th2 > Th1 (Ripple et al. 2010) Adult: ++, Th1 > Th2 (Tripp et al. 2001)
CD8$^+$ T cell	+++ (Heidema et al. 2007)			Neonatal: + Adult: +++ (Tregoning et al. 2008)
B cells and antibody	Variable production (IgA > IgG > IgM) (Reed et al. 2009)	ND	Neonatal: Neutralizing antibody detection > 6 DPI Adult: Neutralizing antibody detection > 5 DPI (Prince et al. 1978)	Neonatal: IgG2a > IgG1 > IgGa > IgE (Ripple et al. 2010) Adult: IgG2a > IgG2b > IgG1 > IgE (Dakhama et al. 2005)

ND no data

used for therapeutic and immunomodulatory trials with promising outcome such as follows: a small molecule fusion inhibitors (Roymans et al. 2017), a small molecule replication inhibitor (Sitthicharoenchai, et al. 2018), an immunotherapy compound (Larios Mora et al. 2018), VEGF (Meyerholz et al. 2007), and potassium iodine administration (Derscheid et al. 2014a). This review will briefly describe different types of animal models for RSV with comparison with the unique characteristic of the lamb model. In addition, we will provide a general knowledge of the RSV lamb model and current update of the model application.

Components and features of pulmonary airway in lambs

Animal models are considered the bridge between in vitro studies and human clinical trials. Developing animal models for RSV infections is challenging due to the high degree of specificity of the RSV to its natural host and lack of virulence in other species (Bossert and Conzelmann, 2002; Schlender

et al. 2003). The ideal animal model should replicate key features of the disease in humans, including anatomical structure, immunologic responses, clinical signs, and respiratory tract lesions to RSV infection. The age-related severity outcome of RSV infection is an additional factor to consider when choosing the proper animal model. Nevertheless, many concerns and limitations are unavoidable with animal studies including animal husbandry, handling, housing, costs, and ethical issues. The familiarity and appropriate understanding of strengths and weaknesses for each animal model is crucial for constructing research experiments, performing laboratory tests, and interpretation of the findings.

The timeframe of alveologenesis during fetal development differs among certain animal species and human. Alveologenesis in rodents occur after parturition while ovine and human alveolar development begins prenatally (Alcorn et al. 1981; Schittny 2017). This development difference makes neonatal rodent models less favorable as a representative for infant lung. Only 2% of all rodent model-based RSV studies have been conducted with infant mice (< 7 days old) (Cormier, et al. 2010) and even fewer with infant cotton rats

(Prince et al. 1978). However, with the ability to manipulate gene expression and abundance of molecular tools available, the use of neonatal mice for immunopathological studies remains to be the appropriate choice. In addition to the ovine lung development, the lung structure, cellular components in airways, immunological responses, and bronchiolar lesions of lambs are analogous to human infants (Ackermann 2014). Both human infants and lambs have comparable lung size, dichotomous branching pattern of airways, amount and distribution of submucosal glands in the airways, and percentage of club cells lining the respiratory bronchioles (20–30%) (Barth et al. 1994; Derscheid and Ackermann 2012; Plopper 1983). These features have an effect on the host susceptibility to the RSV infection, the distribution of the virus in the lung, and the characteristics of lesions (Derscheid and Ackermann 2012). Furthermore, the larger size of the animal provides easier access to the trachea for canalization, ability to collect multiple repeated blood samples, performing surgical trials, and measuring respiratory parameters that are limited when utilizing mouse or rat models. In rodents, the percentage of club cells lining in respiratory bronchioles is higher (50–60%) (Pack et al. 1981). The variation in number of these club cells that function in production of secretory defense protein (CC10 or CC16) and their role as progenitor cells for regeneration process of the conducting airways can contribute to the difference in the outcome to RSV infection (Wang et al. 2003). Unlike older children and immunocompetent adults where RSV infection often results in mild upper respiratory tract infection, the lower respiratory changes of bronchiolitis are the key pathological features in infants that lead to the impairment of airflow movement into the alveoli for gas exchange. The inflammation and exudate within RSV-infected bronchioles can obstruct the bronchiolar lumen resulting in airway dilation, atelectasis, and emphysema which has been reported in human infants (Newman and Yunis 1995). These pathological changes are associated with the absence or minimal collateral ventilation in newborns which is a feature present in many species including ovine and rodents (Terry et al. 1987; Van Meir 1991). Thus, it is important to consider these specific features of infant lungs when selecting the appropriate animal model for RSV research.
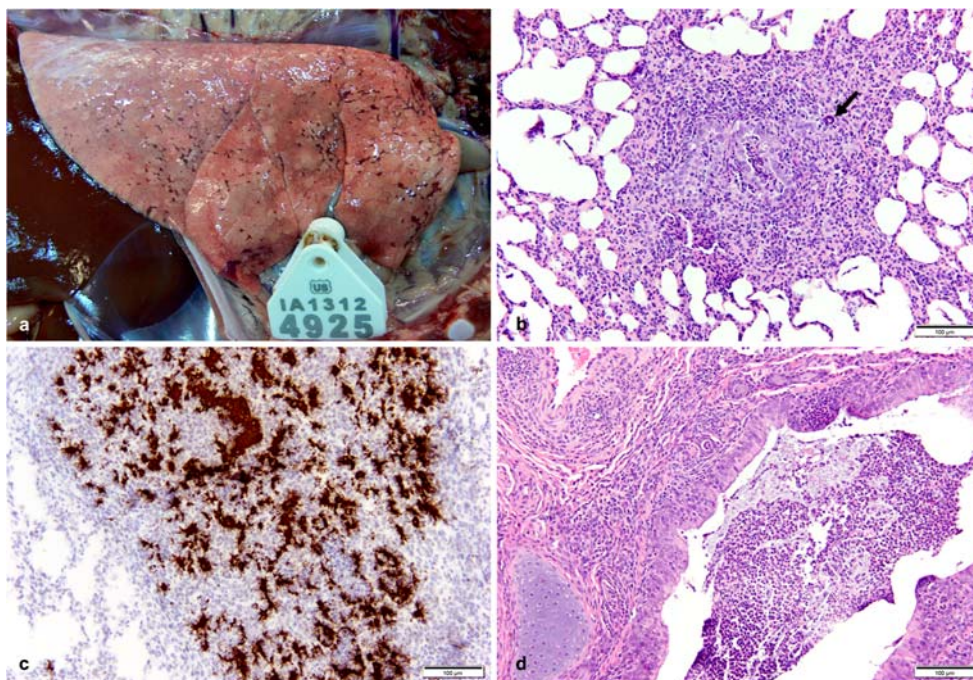
Lamb model of RSV infection

There are several pathological features of RSV-infected lambs that mimic the infection in human infants including development of acute lower respiratory tract infection, changes in the infected lungs, and the observed clinical symptoms. The information regarding the lesions of acute RSV infection in human are limited due to modern treatment and rare lung biopsy samples. Published data on the subject were from retrospective study of autopsy specimens before 1950s when severe

and fatal acute RSV infection was first identified (Johnson et al. 2007). The changes in the infant lungs with acute RSV infection include necrotizing bronchitis and bronchiolitis, interstitial pneumonia, and diffuse alveolar damage. Syncytial cells appeared in the alveoli and bronchioles with occasional presence of eosinophilic intracytoplasmic viral inclusions (Pritt and Aubry 2017). Pulmonary lesions of RSV-infected lamb reflects these changes including bronchitis, bronchiolitis, and alveolar inflammation characterized by bronchiolar epithelial cell damage/necrosis, syncytial cell formation, intraluminal accumulation of cell debris, mucin and neutrophils, macrophages, and mild adventitial infiltration by lymphocytes and plasma cells (Fig. 1a, b) (Derscheid et al. 2014b; Derscheid et al. 2013b; Johnson et al. 2007; Larios Mora et al. 2015; Lehmkuhl and Cutlip 1979). In both human and lambs, the RSV viral antigen was detected in bronchial and bronchiolar epithelial cells as well as infection in type II pneumocytes (Fig. 1c) (Johnson et al. 2007; Larios Mora et al. 2015). Increased bronchiolar secretion does not appear to be a feature with RSV-infected lamb, as it had been described in human infants and some mice model (Stokes et al. 2011). However, this can be related to certain strains of RSV that stimulate more airway secretion or host genetic that are more susceptible to airway secretory production which remains to be elucidated (Drajac et al. 2017; Lukacs et al. 2010).

There is variation in the degree of viral replication between different strains of RSV in lambs. The RSV replicates well in neonatal lamb respiratory tract airways with a peak of viral replication at day 6 after intratracheal inoculation with RSV A2 strain that then declines with time (Olivier et al. 2009; Sow et al. 2011b). Another study using RSV Memphis 37 strain demonstrated peak viral replication at day 3 and replication persisted until day 6 post-viral nebulization (Larios Mora et al. 2015). Given the higher replication and rapid peak of virus observed with Memphis 37 strain nebulization, we have based most of the recent studies with lamb model using this strain of virus and nebulization method of inoculation in contrast to the rodent model where intranasal infection of A2 and long strains are commonly used. As in human infants, lambs have variable clinical signs associated with RSV infection. Clinical symptoms vary from mild systemic signs such as fever, reluctant to move, and reduce milk consumption to respiratory symptoms such as coughing, wheezing, and increased expiratory efforts. Signs of infection appear as early as 2 days post-infection and apparent until day 6 post-infection (Derscheid et al. 2013b; Larios Mora et al. 2015; Olivier et al. 2009). Enhanced RSV disease severity was demonstrated in preterm lambs compared with newborn lambs and in lambs vaccinated with formalin-inactivated RSV vaccine (Derscheid et al. 2013a). Also, lambs are susceptible to at least three strains of RSV (Memphis 37, A2, and Long strains) (Derscheid and Ackermann 2012; Derscheid et al. 2014b) as well as bovine respiratory syncytial virus (bRSV) (Meehan et al. 1994), ovine, and human

Fig. 1 Lung from RSV-infected lamb at day 6 post-infection. Multifocal lung consolidation appeared throughout the pulmonary parenchyma. **a** Bronchiolitis with neutrophilic inflammation and lymphoplasmacytic peribronchiolar infiltrates with presence of multinucleated syncytial cell (arrow), H&E stain. **b** Viral RNA indicated by BROWN chromogenic stain is demonstrated in the bronchiolar epithelial cells and type II pneumocytes, RNA in situ hybridization. **c** Lung from lamb coinfected with RSV and *Streptococcus pneumoniae*. There is marked neutrophilic bronchitis with presence of mucinous exudate in the airway lumen (**d**)

parainfluenza viruses (Grubor et al. 2004). Thus, the lamb model of RSV infection can be used for modeling RSV infection in newborn infants, preterm infants, and also vaccine, therapeutic, pathogenesis, and potentially asthma studies.

Pulmonary immune response in RSV-infected lambs

In contrast to the mild respiratory symptoms with RSV infection in immunocompetent adults, infants and children less than 6 months of age can develop severe RSV-associated acute lower respiratory tract infection. Many factors contribute to the degree of severity including viral virulence, host genetics, environmental factors, and host immune response (DeVincenzo et al. 2005; El Saleeby et al. 2011; El Saleeby and Devincenzo 2011). In rodents, major concerns on immunological differences are the balance of blood leukocytes, toll-like receptors (TLRs) expression, different immunoglobulin isotypes, and lack of defensin expression in neutrophils. Similar to rodent species (75–90%), sheep (41–83%) has a higher proportion of circulating lymphocytes compared with human (30–50%). Ten types of TLRs (TLR1-10) are identified in sheep with 84–97% amino acid homology to human TLRs nucleotide sequences (Menzies and Ingham 2006). The degree of TLRs expression in sheep has been previously characterized in the gut-associated lymphoid tissue, but this has not been conducted in ovine pulmonary tissues. In contrast to sheep and human, rodents have a different set of TLRs with lack of TLR10 expression and additional TLR11, TLR12, and TLR13 (Beutler, 2009). The five isotypes of immunoglobulin

are analogous in mammalian species. However, there are variation of IgG and IgA subtypes which have been well characterized in human, mice, and rats. In sheep, two subclasses of IgG (IgG1, IgG2) and IgA (IgA1, IgA2) have been identified (Bird, et al., 1995).

Many components of innate immunity response to respiratory tract infection have been studied in the lamb model including the presence and response of pulmonary dendritic cells (DCs) (Fach et al. 2007), expression of sheep ß-defensin-1 (SBD-1), and surfactant protein (SP-A and SP-D) (Grubor et al. 2004; Kawashima et al. 2006), as well as cytokine and chemokine levels (Larios Mora et al. 2015; Sow et al. 2012; Sow et al. 2011a). There is reduction of SP-A and SP-D in infants with severe RSV infection measured in bronchoalveolar lavage (BALF) (Kerr and Paton 1999). Similar significant decreased SP-D mRNA expression was demonstrated with BRSV-infected lamb bronchiolar epithelial cells, although the expression of SP-A did not significantly change (Kawashima et al. 2006). Interestingly, there is increased SBD-1, SP-A, and SP-D mRNA levels with concurrent decreased of parainfluenza-3 virus replicating. These results suggest that there might be a direct or indirect RSV-dependent factor regulating the lung production of these antimicrobial molecules. The cytokine and chemokine expression profile has been evaluated with BRSV, human RSV A2, and Memphis 37 strain infections in lambs. Increased expression of CCL2 or monocyte chemotactic protein-1 (MCP-1) was demonstrated with BRSV and RSV infections in lambs (Kawashima et al. 2006). The chemokine CCL2 (MCP-1) is responsible for chemoattraction of cellular inflammatory components in the lung which, similar to lambs, is increased in

infants with severe RSV bronchiolitis. Other chemokines responsible for the recruiting cells into the lung in response to the infection including CXCL10 (IP-10), CCL3 (MIP-1α), and CCL5 (RANTES) can be increased in infants with severe RSV infection (McNamara et al. 2005). At day 6 post-infection, both CXCL10 (IP-10) and CCL3 (MIP-1α) in lambs were infected with Memphis 37 and A2 strains of RSV (Larios Mora et al. 2015; Sow et al. 2012; Sow et al. 2011a). Interestingly, CCL5 (RANTES) in RSV-infected lambs did not appear to significantly increase at 6 dpi (Derscheid and Ackermann 2012). The lack of RANTES (CCL5) expression could be due to a host defect in production or direct viral blockage, although further study is needed in order to clarify the mechanism of this atypical response. The expression of T cell regulatory ligand, PD-L1 (CD274), was also elevated in RSV-infected neonatal lamb at 6 dpi from previously reported data (Sow et al. 2011b). This elevation of PD-L1 (CD274) level may play a role in the inactivation of cytotoxic T cell response against RSV infection which was observed in human and mouse studies (Telcian et al. 2011; Yao et al. 2015). Other immunological factors that mimic the human include the presence of dendritic cells (DCs) response to RSV (Derscheid and Ackermann 2012), genetic expression of IL-8 (rodents lack IL-8 gene) (Ackermann et al. 2004; Olivier et al. 2009; Redondo et al. 2011), and presence of Duox/LPO system in the airways (Gerson et al. 2000; Salathe et al. 1995; Salathe et al. 1997).

There is a limited number of studies on the adaptive immune response in RSV lamb model. This is due to the more substantial role of innate immunity in response to the viral infection and the short duration of viral persistent in the host. However, there are long-term impact of RSV infection such as increased risk for the development of asthma that requires extensive study of the adaptive immunity in the model. The balance of different types of CD4+ T cell response is an important component in asthmatic development. Studies of RSV association with asthma are mostly conducted in mouse model and has been reviewed elsewhere. Recent finding demonstrated that binding of complement molecules C5a-C5aR can regulate the T cell activation and differentiation in the pathogenesis of RSV-associated asthma development. Previous study has demonstrated the increased of PD-1 cytokine expression in preterm lamb (Sow et al. 2011a). PD-1 functions as a regulator of T cell activation which suggests that there may be changes of lymphocytic response in RSV-infected ovine lung (Table 2). The detailed characterization of lymphocytic subtypes in RSV-infected lamb is currently unknown and would be interesting to see if alteration of T cell subsets exist between various factors such as age of infection (neonatal vs adult), RSV strains, stage of infection, and in response to subsequent inflammatory stimuli in the lamb model.

Miscellaneous unique features and limitations of lamb model

While immunoglobulin is passed transplacentally to the fetus in humans, maternal immunoglobulin transfer in sheep only occurs by ingestion of colostrum. Therefore, lambs deprived of colostrum have zero maternal antibodies and thereby no antibodies directed specifically to RSV. This feature is advantageous for efficacy and vaccination studies in lambs infected with RSV as it eliminates the question regarding passive antibody inhibition of RSV at stages of RSV infection, replication, and release. Lambs are easy to handle and restraint and have large, accessible blood vessels for sampling or placement of an intravascular drug delivery system including dwelling catheters. The application of lamb model has been further used for numerous studies on asthma development and cardiovascular conditions, and thus, there is extensive rigor for such data in the literature (Milani-Nejad and Janssen 2014; Scheerlinck et al. 2008).

There are some limitations with lambs compared with other models for RSV including the sources of lamb provider, the experimental housing for large animal, and the necessary husbandry care. In some areas of the world, there are limited sources of large-scale sheep-breeding facilities to produce and customize the lamb for experimental use. A middle size to large housing facility is needed with lamb studies compared with rodents, although not as specialized and extensive as non-human primates. Colostrum-deprived lambs also require close monitoring and specialized attention/care with much experience/expertise. Another concern with using ovine experimental model is the limited commercial molecular kits that would require customized experimental assays, although key types of information are routine and proteomics as well as genetic sequencing assays are readily available.

Other animal models for RSV

RSV animal models can be divided into two main groups, i.e., heterologous or cognate host-virus models. RSV can infect and replicate in heterologous host-virus models such as chimpanzees (Belshe et al. 1977; Whitehead et al. 1999), baboons (Papin et al. 2013), sheep (Larios Mora et al. 2015; Olivier et al. 2009; Sow et al. 2011b), cotton rats (Boukhvalova et al. 2018; Prince et al. 1978), ferrets (Stittelaar et al. 2016), and mice (Graham et al. 1988; Openshaw 2013; Taylor et al. 1984), while related Orthopneumoviruses can be used as cognate host-virus models, such as murine pneumonia virus in mice model (Cook et al. 1998) and bovine respiratory syncytial virus (BRSV) in calves (Blodörn et al. 2015; Valarcher et al. 2003).

Non-human primates (NHPs) are excellent animal model for human diseases in regard to the similarities in anatomy, physiology, genetic, and immune response. In chimpanzees,

Table 2 Cellular immune response in RSV-infected lung

RSV infection	Human infants	Neonatal lamb	Cotton rat	Mice
Infective dose/route of infection		10^8 pfu M37 aerosol (Larios Mora et al. 2015) 10^8 pfu A2 intratracheal (Sow et al. 2011b)	10^5–10^6 pfu intranasal (Boukhvalova et al. 2018)	10^4–10^7 pfu intranasal (Taylor et al. 1984)
Virus replication and localization	Localized in nasal, bronchial and bronchiolar mucosal epithelium, rarely pneumocytes (Johnson et al. 2007)	Semi-permissive M37 peak pulmonary viral load at 3 dpi (Larios Mora et al. 2015) A2 peak pulmonary viral load at 6 dpi (Sow et al. 2011b) Localized in bronchial and bronchiolar epithelium, rarely pneumocytes (Larios Mora et al. 2015)	Semi-permissive Peak pulmonary viral load at 4 dpi Localized in nasal, bronchial, and bronchiolar mucosal epithelium, rarely pneumocytes (Prince et al. 1999; Prince et al. 1978)	Low to semi-permissive Peak pulmonary viral load at 4–5 dpi Viral infection primarily targets pneumocytes (Graham et al. 1988; Taylor et al. 1984)
Clinical symptoms	Mild to severe acute respiratory disease syndrome (Hall et al. 2013)	Mild to severe respiratory symptoms (Derscheid et al. 2014a, b; Derscheid and Ackermann 2012; Larios Mora et al. 2015)	No clinical symptoms (Prince et al. 1999; Prince et al. 1978)	No clinical symptoms (Graham et al. 1988; Taylor et al. 1984)
Lung microscopic changes	Severe necrotizing bronchitis and bronchiolitis, interstitial pneumonia, alveolitis, syncytial formation (Johnson et al. 2007)	Moderate to severe necrotizing bronchitis, bronchiolitis, lymphoplasmacytic peribronchiolitis, syncytial formation (Derscheid and Ackermann 2012; Larios Mora et al. 2015)	Mild bronchitis, bronchiolitis, lymphoplasmacytic peribronchiolitis, high dose causes interstitial pneumonitis and alveolitis, ± syncytial formation, pulmonary eosinophilia (Grieves et al. 2015; Prince et al. 1986)	Mild to moderate bronchiolitis (Graham et al. 1988; Taylor et al. 1984)
FI-RSV-enhanced respiratory disease	Yes (Kapikian et al. 1969; Openshaw et al. 2001)	Yes (Derscheid et al. 2013a)	Yes (Prince et al. 1999, 1978)	Yes (Knudson et al. 2015)

RSV is highly permissive and can be naturally infected (Blount et al. 1956). The virus is able to replicate in the nasal sinuses and upper respiratory tract epithelium with induction of clinical symptoms similar to that found in human RSV-associated upper respiratory tract infection (Belshe et al. 1977; Whitehead et al. 1999). Advanced vaccine studies have benefited from this animal model due to the close similarity of immune response between humans and chimpanzees (Hancock, et al. 2000). However, chimpanzees rarely develop lower respiratory tract infection that would represent the severe form of the disease reported in infants and elderly individuals. Other NHPs have been experimentally infected with RSV including owl monkey and rhesus macaques, many of which are less permissive to RSV infection (McArthur-Vaughan and Gershwin 2002; Prince et al. 1979). Experimental infection of RSV in infant baboon achieved clinical symptoms and pulmonary changes similar to human infants and recently been use in vaccine studies (Papin et al. 2013; Welliver et al. 2017). Even with the natural occurrence and development of clinical symptoms with RSV infection in NHPs, several limitations regarding the concerns with the substantial economic, ethical, and emotional burden diminished the use of these animals for RSV studies.

Rodent models are widely used in biomedical studies including for RSV infection. Mice models have the advantages for transgenic studies and the vast availability of molecular tools. However, there are limitations with using the mice model for RSV in respect to the variability between different strains of mice, low-permissiveness of the virus, and the lack to minimal clinical symptoms associated with infection. A cognate host-virus model using murine pneumonia virus infection in mice which resembles RSV infection in human has been proposed. Murine pneumonia virus targets bronchiolar epithelium and leads to severe disease with marked respiratory disease correlates positively with the viral inoculum (Bonville et al. 2006; Rosenberg et al. 2005). The critical disadvantages of rodents as a model for RSV disease are the difference in lung anatomy, histology, and immune response between human and rodents that subsequently question the translations of studies performed in these models to human. One of the most widely used rodent model for RSV is the cotton rat (*Sigmodon hispidus*). Since the establishment of the cotton rat model for RSV in the 1970s (Prince et al. 1978; Prince et al. 1999), this animal model has been utilized in many vaccine, therapeutic, and pathogenesis studies that contributed to the current advancement and greater understanding of RSV infection.

Cotton rats are relatively small and are highly permissive for RSV replication. It is considered the standard model for testing RSV therapeutics. A throughout review of cotton rat model for RSV has been published elsewhere and beyond the scope of this article (Boukhvalova and Blanco 2013; Boukhvalova et al. 2018). However, several aspects of model comparison between the neonatal lamb and cotton rat will be described in later sections of this review.

Another cognate host-virus model for RSV is the BRSV infection in cattle. The lung anatomy and histology of cattle and human are in many ways analogous, i.e., the presence of pharyngeal and nasopharyngeal tonsils, the presence of ciliated pseudostratified epithelium and submucosal glands, and similar innate and adaptive immune response to human (Taylor 2013). Natural infection of BRSV induces severe upper and lower respiratory tract infection in cattle and often presented with secondary bacterial infection. Young calves less than 6–10 months are most susceptible to clinical disease. In experimental setting, the clinical signs of BRSV infection can be easily assessed in cattle including pyrexia, tachypnea, dyspnea, lung sound, coughing, and ocular and nasal discharge making this a useful model for evaluating clinical symptoms. Many vaccine disease protection, immune stimulation, and safety trials have been conducted for BRSV infection in cattle for not only the purpose of disease prevention and control in the animal, but also relating the findings to human RSV vaccine development. Furthermore, special housing and handling are required when utilizing the BRSV calve model due to the larger size of the animal. Experimental inoculation of human RSV in cattle failed to establish infection and pathological changes, thus limited the use of this model for heterologous model studies.

Application of lamb model for RSV

RSV antiviral drug tests in lamb model

The lamb model of RSV infection has been used for preclinical efficacy testing of many newly developed antiviral drug against RSV including small molecule fusion protein inhibitors and non-fusion protein inhibitors. For these studies, neonatal colostrum-deprived, 2–3 days old lambs were inoculated with RSV virus by nebulization and housed for 6 days post-infection. Between studies, there were variations in route of administration, concentration of the antiviral treatment, timepoint of treatment, and the amount of the treatment given to determine the most suitable therapeutic conditions. Small molecule fusion protein inhibitors prevent the conformation transformation of fusion protein required for cell entry, and both JNJ-53718678 and JNJ-49214698 fusion inhibitors have been tested in lambs. Oral administration of these compounds 24 h post-infection at appropriate dose has shown promising

results by demonstrating stabilized plasma compound level, reduced lung lesions, and decreased the viral load. In addition, the prophylactic administration of JNJ-49214698 in lamb model had significant reduction in viral load, lesions, and lack of clinical signs indicating the potential for future use in patients with high risk of severe RSV infection such as premature infants, children with congenital heart and lung diseases, immunosuppressed individuals, and children born during RSV season with high risk of exposure (Roymans et al. 2017).

Possible cross-resistance mutation of the RSV virus has been identified in experimental settings (Yan et al. 2014) and with fusion inhibitors for other viruses (Reeves et al. 2005). Moreover, the effective treatment window reported in vitro with fusion inhibitors is limited in time with a potential loss of the antiviral effect once the virus has entered the cells, and the ability of blocking the entry of the virus in neighboring cells. Also, considering the threat associated to the emergence of antiviral resistance (all fusion inhibitors published so far share the same binding pocket), an alternative mechanism is desirable. RSV replication inhibitors that inhibit post-entry pathway of viral replication have a wider effective treatment window timeframe that is up to 3 days post-infection when tested in the HuAEC model (Mirabelli et al. 2018). Efficacy of replication inhibitor in neonatal lambs infected with RSV was evaluated for antiviral efficacy and also for its impact on the severity of RSV infection including changes in clinical parameters and degree of pulmonary lesions. In dose-dependent manner, the small molecule replication inhibitor prevented increased respiratory efforts and reduced RSV viral titer, RSV RNA in the lung and BALF (Sitthicharoenchai et al. 2018).

RSV immunotherapeutic compounds in lamb model

There are numerous approaches to inhibit viral infection in lung through activation/enhancement of innate or adaptive immune systems, and some of these conceptually could reduce infection by a various type of viruses, including RSV. For example, vascular endothelial growth factor (VEGF) has many physiologic activities including upregulation of surfactant protein A (SP-A) by lung epithelial cells. SP-A is a collectin (collagenous lectin) that can bind RSV and also activate macrophages. In two separate studies, prophylactic administration of VEGF reduced RSV disease severity in lambs (Meyerholz et al. 2007; Olivier et al. 2011). Although VEGF can upregulate SP-A, the precise mechanism(s) but which VEGF reduced RSV disease severity has not been determined since VEGF can also induce vascular leakage, induce monocyte infiltration into lung (Meyerholz et al. 2006), and affect other immunologic parameters. Also, these studies demonstrate anti-RSV activity when VEGF is delivered prophylactically and therapeutic delivery of VEGF for treatment of RSV could be less effective than prophylactic treatment due to the time needed for upregulation of anti-RSV substances. High

levels of VEGF in lambs induce extensive monocyte infiltration (Meyerholz et al. 2006) and therefore is a limitation and side effect of VEGF delivery.

The Duox-lactoperoxidase system is an innate immune defense system that also has potential to reduce viral infection through production of oxidative radicals in the airway lumen that can kill or inactivate viruses or other pathogens. This system includes dual functioning oxidases (Duox) produced by epithelial cells, lactoperoxidase produced by airway submucosal glands, and cyanide present at low levels in the airway mucosa. The Duox produces hydrogen peroxide that converts cyanide to thiocyanate in the presence of lactoperoxidase. Potassium iodide (KI) can replace cyanide in this reaction to produce a hypoiodite compound that has potent antimicrobial activity to the level of bleach in vitro. One study in lambs demonstrated that prophylactic administration of KI reduced RSV disease severity (Derscheid et al. 2014a). Sheep, humans, and a several other species have submucosal glands that produce the lactoperoxidase needed for this reaction to occur. Some species (rodents) lack submucosal glands (e.g., rodents) in airways.

Administration of antibodies can also have anti-RSV activity through passive immunity. Nanobodies are small antibodies derived from the heavy chain portion of camelid immunoglobulin and have been tested for both prophylactic and therapeutic treatment of RSV in lambs (Larios Mora et al. 2018). Nanobody ALX-0171 was delivered by aerosol (mesh nebulizers) and had good efficacy against RSV in lambs when deliver prophylactically and also therapeutically at various doses and nebulization times. This compound lacked toxicity or any other side effects. ALX-1071 is a trimeric nanobody that binds the antigenic site of F protein and neutralizes RSV activity. There are numerous other monoclonal antibodies against RSV antigens that have therapeutic potential and assessment for efficacy in lambs. Yet, many other approaches modulate immune responses to treat viral/RSV infection prophylactically or therapeutically. Some have been tested in lambs, but those data are yet under study and/or proprietary.

Interactions of concurrent bacterial infection with RSV in lamb model

Bacterial superinfection is one of the major concerns with primary viral-associated bronchiolitis. Up to 40% of children hospitalized with RSV infection have been reported with concurrent bacterial infection which increases the severity of the respiratory symptoms and results in longer time of intensive intervention. Common secondary bacterial pneumonia in human are caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Haemophilus influenza* (DeLeo and Musser 2010; Madhi et al. 2004; Thorburn et al. 2006). Experimental coinfection of RSV with *Streptococcus pneumoniae* (*Spn*) has been

performed in lambs and demonstrated enhanced disease severity with combined RSV-*Spn* infection similar to human. Lambs infected with RSV followed by *Spn* inoculation had increased tissue damage, interalveolar wall thickness, and neutrophil infiltration in the airways with higher RSV viral titer in comparison with lambs infected with only RSV (Fig. 1d) (Alnajjar et al. 2018). The establishment of viral bacterial coinfection in neonatal lambs designates the potential of the model for investigating pathogenesis of respiratory pathogen interaction and treatments on RSV cases with secondary bacterial infection.

Formalin-inactivated RSV vaccination enhances RSV infection severity in lambs

Vaccination of infants with a formalin-inactivated vaccine was associated with enhanced RSV disease severity upon subsequent RSV infection (Kapikian et al. 1969; Openshaw et al. 2001). In several animal models of infant RSV infection, including newborn lambs, this phenomenon has been replicated (Derscheid et al. 2013a). According to (Taylor 2017) a detailed review on RSV vaccination animal model, chimpanzees are considered the best animal model to fit RSV vaccine studies due to the similarity in immune response and high susceptibility to the RSV; also, calves are the best cognate host-virus model that fit vaccine studies. Several other animal models were used to conduct vaccine studies, and each had different levels of response and somehow similar enhanced RSV disease following FI-RSV vaccine (Taylor 2017). Calf model of RSV uses bRSV to produce the infection and had a conflicting result in regard to the FI-RSV vaccine-enhanced disease (Gershwin et al. 1998; Kalina et al. 2004). Cotton rat is another great model for RSV and FI-RSV-enhanced disease (Prince et al. 2001), but the transition of the data to human is questionable. Lambs have similar success as an RSV mode as chimpanzees, except for the need to higher inoculation dose to produce infection. However, the ability to use lambs with or without maternal immunity through colostrum deprivation is a unique characteristic and beneficial for immunity and vaccine studies. Lambs have been utilized to study the effect of RSV maternal immunity, and according to these studies, lambs born to vaccinated ewes had 50-fold higher viral neutralizing antibody, 70% reduction in viral titer, and a significant reduction in disease pathology when compared with lambs born to nonvaccinated ewes (Garg et al. 2016). FI-RSV vaccination-enhanced disease was observed in lambs through the extensive peribronchiolar cellular accumulation, but the vaccinated lambs had less lesion associated with the RSV infection in comparison with the non-vaccinated lambs (Sow et al. 2011a, b) In conclusion, lambs serve as a unique RSV model to study immunity and vaccination giving all the unique characteristics that make lambs and chimpanzees as the leading model for RSV immunity and vaccination.

Other models of human respiratory disease in newborn lambs

Newborn lambs have also been infected with ovine parainfluenza virus resulting in similar lesions and findings as human strains of RSV infection (Grubor et al. 2004). Parainfluenza and RSV can alter cyclooxygenase expression (Radi et al. 2010). Alcohol consumption during gestation can predispose infants to preterm birth, and preterm birth is associated with more severe infections with RSV. A model of in utero exposure of lambs to maternal alcohol was developed demonstrating reductions in lungs of preterm lambs of hypoxia-inducible factor (HIF), vascular endothelial growth factor (VEGF), and surfactant protein A (SpA) (Lazic et al. 2007). Finally, lambs can be depleted of mast cells with administration of capsaisin (Ramírez-Romero et al. 2000) to study the effects of these cells on inflammatory responses. Lambs have also been used by others for studies of asthma and various types of pulmonary infections.

Conclusion

There is a need for safe and effective therapeutic and vaccination regimens against RSV, and these require assessment in vivo model prior to human clinical trials. Neonatal lambs have several anatomic, developmental, physiologic, and immunologic features similar to human infants. Neonatal lambs are also susceptible to human strains of RSV, development pulmonary lesions identical to human infants, and can be deprived of maternal immunoglobulins containing anti-RSV antibodies. Lambs infected with human strains of RSV have been used successfully to test efficacy of various small molecule RSV replication and fusion inhibitors, anti-RSV antibodies, immunomodulators, oxidative enhancement, and vaccination studies. The establishment of this model has contributed to many advancements of RSV studies, and further utilization of this model can extend the knowledge base to the path of developing the appropriate treatment and prevention of RSV infection.

Compliance with ethical standards

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