2014

Lamb Model of Respiratory Syncytial Virus–Associated Lung Disease: Insights to Pathogenesis and Novel Treatments

Mark R. Ackermann
Iowa State University, mackerma@iastate.edu

Follow this and additional works at: http://lib.dr.iastate.edu/vpath_pubs

Part of the Veterinary Infectious Diseases Commons, and the Veterinary Pathology and Pathobiology Commons

The complete bibliographic information for this item can be found at http://lib.dr.iastate.edu/vpath_pubs/66. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.
Lamb Model of Respiratory Syncytial Virus–Associated Lung Disease: Insights to Pathogenesis and Novel Treatments

Abstract
Preterm birth is a risk factor for respiratory syncytial virus (RSV) bronchiolitis and hospitalization. The pathogenesis underlying this is not fully understood, and in vivo studies are needed to better clarify essential cellular features and molecular mechanisms. Such studies include analysis of lung tissue from affected human infants and various animal models. The preterm and newborn lamb lung has developmental, structural, cellular, physiologic, and immunologic features similar to that of human infants. Also, the lamb lung is susceptible to various strains of RSV that infect infants and cause similar bronchiolar lesions. Studies in lambs suggest that viral replication in airways (especially bronchioles) is extensive by 4 days after infection, along with bronchiolitis characterized by degeneration and necrosis of epithelial cells, syncytial cell formation, neutrophil infiltration, epithelial cell hypertrophy and hyperplasia, and innate and adaptive immune responses. RSV bronchiolitis greatly affects airflow and gaseous exchange. RSV disease severity is increased in preterm lambs compared with full-term lambs; similar to human infants. The lamb is conducive to experimental assessment of novel, mechanistic therapeutic interventions such as delivery of vascular endothelial growth factor and enhancement of airway epithelial oxidative responses, Club (Clara) cell protein 10, and synthesized compounds such as nanobodies. In contrast, exposure of the fetal ovine lung in vivo to ethanol, a risk factor for preterm birth, reduces pulmonary alveolar development and surfactant protein A expression. Because the formalin-inactivated RSV vaccination enhances some inflammatory responses to RSV infection in lambs, this model has the potential to assess mechanisms of formalin-inactivated RSV enhanced disease as well as newly developed vaccines.

Keywords
bronchiolitis, infants, lambs, pneumonia, preterm, respiratory syncytial virus (RSV)

Disciplines
Veterinary Infectious Diseases | Veterinary Pathology and Pathobiology

Comments
This is a manuscript of an article from ILAR Journal 55 (2014): 4, doi:10.1093/ilar/ilu003. Posted with permission.

This article is available at Iowa State University Digital Repository: http://lib.dr.iastate.edu/vpath_pubs/66
Lamb model of respiratory syncytial virus-associated lung disease: Insights to pathogenesis and novel treatments

Mark R. Ackermann, DVM, PhD, DACVP

Mark R. Ackermann, DVM, PhD, DACVP is a professor in the Department of Veterinary Pathology and Acting Chair, Department of Veterinary Clinical Sciences, at the College of Veterinary Medicine, Iowa State University in Ames, Iowa, USA.

Address correspondence to: Mark R. Ackermann, DVM, Ph.D., Diplomate ACVP, Department of Veterinary Pathology, 2738 College of Veterinary Medicine, Iowa State University, 1600 Christiansen Drive, Ames, Iowa, USA, 50011-1250. Telephone: 515-294-3647; FAX 515-294-5423; E-mail: mackerma@iastate.edu
Abstract. Preterm birth is a risk factor for respiratory syncytial virus (RSV) bronchiolitis and hospitalization. The pathogenesis underlying this is not fully understood and in vivo studies are needed to better clarify essential cellular features and molecular mechanisms. Such studies include analysis of lung tissue from affected human infants and various animal models. The preterm and newborn lamb lung has developmental, structural, cellular, physiologic and immunologic features consistent similar to human infants. Also, the lamb lung is susceptible to various strains of RSV that infect infants and cause similar bronchiolar lesions. Studies in lambs suggest that viral replication in airways (especially bronchioles) is extensive by four days post-infection along with bronchiolitis characterized by degeneration and necrosis of epithelial cells, syncytial cell formation, neutrophil infiltration, epithelial cell hypertrophy and hyperplasia, innate and adaptive immune responses. RSV bronchiolitis greatly affects airflow and gaseous exchange. RSV disease severity is increased in preterm lambs compared to full term lambs; similar to human infants. The lamb is conducive to experimental assessment of novel, mechanistic therapeutic interventions such as delivery of vascular endothelial growth factor (VEGF), enhancement of airway epithelial oxidative responses, Clara cell protein 10 (CC10), and synthesized compounds such as nanobodies (ALX-0171). In contrast, exposure of the fetal ovine lung in vivo to ethanol, a risk factor for preterm birth, reduces pulmonary alveolar development and surfactant protein A expression. And because the formalin inactivated RSV (FI-RSV) vaccination enhances some inflammatory responses to RSV infection in lambs, this model has potential to assess mechanisms of FI-RSV enhanced disease as newly developed vaccines.

Key words: bronchiolitis; infants; lambs; preterm; pneumonia; respiratory syncytial virus (RSV)
Introduction. Respiratory syncytial virus (RSV) is a major cause of acute lower respiratory infection in infants and young children worldwide, and a leading cause of infantile bronchiolitis that can result in hospitalization and occasionally death (Aujard and Faurox 2002; Blanken et al. 2013; Collins and Graham 2008; Collins and Melero 2011; Hon et al. 2012; Welliver et al. 2010). Such RSV infections can be worsened by secondary bacterial pathogens (Stark et al. 2006; van den Bergh et al. 2012). In industrialized countries, RSV accounts for up to 70% of hospitalized bronchiolitis cases (Aujard and Faurox 2002; Collins and Melero 2011; Garcia et al. 2010; Hall et al. 2013; Nair et al. 2010; Sommer et al. 2012). However, the mechanistic basis by which lung immaturity contributes to increased RSV disease severity is not fully understood. Key elements affecting susceptibility of the newborn lung to RSV may include bronchiolar epithelial cell immaturity or alterations in the types of cells lining bronchioles such as non-ciliated cells (including Clara cells) and type II cells. The ovine lung has much similarity to lung in human infants in terms of alveologenesis, airway branching patterns, percentage of Clara cells, and the presence of submucosal glands. This allows studies in lambs demonstrating effects of RSV on airway epithelial cell degeneration and necrosis, inflammatory responses, innate (e.g.; surfactant proteins, antimicrobial peptides, cytokines, chemokines, dendritic cells, etc.) and adaptive (e.g.; Th1, Th2, etc.) immune responses. Also, the lamb model has been used to assess the effects of drugs such as ethanol which can alter bronchiolar epithelial cell differentiation/maturation in utero and alter innate and adaptive immune responses.

Although Palivizumab (Synagis; MedImmune) has potent protective properties and Ribavirin has been used in some clinical situations, there are no fully satisfactory vaccines or therapeutic regimens (Abed and Bovin 2006; Anderson 2013; Empey et al. 2010; Hon et al. 2012). Thus, there is a need to develop new therapeutic approaches that could be used to prevent or treat RSV infections and these need to be tested in relevant animal models. Several therapies that have reduced severity of RSV infection have been tested in lambs and include: delivery of
vascular endothelial growth factor (VEGF), enhancement of airway oxidative responses, Clara cell protein 10 (CC10) and nanobody (ALX-0171).

**RSV disease in humans.** RSV is an enveloped, negative strand RNA virus of the family *Paramyxviridae*, subfamily *Pneumovirinae* and genus *Pneumovirus* first discovered in 1952 as a lower respiratory tract pathogen of children in their first year of life. RSV most commonly causes mild respiratory tract disease; however, severe bronchiolitis and pneumonia can occur in 25-50% of children resulting in ICU admission, supplemental oxygen, mechanical ventilation, impaired learning, and death (Aujard and Faurox 2002; Collins and Melero 2011; Espinosa et al. 2013; Garcia et al. 2010; Hall et al. 2013; Nair et al. 2010; Sommer et al. 2012). World-wide, RSV is estimated to cause 199,000 deaths annually in children less than five years of age (Garcia et al. 2010; Hall et al. 2013; Nair et al. 2010). Re-infections can occur especially if RSV-specific neutralizing antibody titers are low. In the 1960’s a formalin-inactivated RSV (FI-RSV) vaccine was associated with enhanced RSV disease severity and two deaths in infants that were subsequently infected with RSV (Castilow et al. 2007; Collins and Graham 2008; Collins and Melero 2011). Today, a licensed RSV vaccine is not available. *Streptococcus pneumoniae* and *Haemophilus influenza* are both common respiratory bacterial pathogens that can complicate primary RSV and other pulmonary viral infections (Stark et al. 2006; van den Bergh et al. 2012).

**Therapies, vaccine strategies.** Numerous therapeutic compounds against RSV have been and are being developed and assessed. Of, these, ribavirin and Palivizumab have been used in hospital settings. Ribavirin has toxicity issues and Palivizumab is not used universally due to set criteria as well as availability and cost issues (Abed and Bovin 2006; Empey et al. 2010; Hon et al. 2012; Wu et al. 2008). Many other innovative and promising therapies are under development and testing and include: antibodies and small molecules against the various RSV envelope proteins (F, G, SH), nucleocapsid proteins (N, P, L), nucleocapsid-associated proteins
(M2-1, M2-2), matrix protein (M1), and nonstructural proteins (NS1, NS2) (Empey et al. 2010). These therapies inhibit aspects of RSV replication such as fusion and entry, attachment, virus transcription, assembly and cytokine/immune responses.

The FI-RSV vaccine-related deaths of the 1960’s greatly affected RSV vaccine development. Since that event, the mechanistic basis underlying FI-RSV enhanced response has been studied extensively (Castillow et al. 2007; Collins and Melero 2011; Delgado et al. 2009). During this same time, numerous new approaches to RSV vaccination have been developed with close attention and care to avoid vaccine-enhanced responses. Vaccine approaches include live virus, vectored (replicating or nonreplicating) and subunit vaccines (Empey et al. 2010) that utilize attenuated or inactivated RSV, RSV protein(s) adjuvanted or incorporated into micro/nanoparticles, epitope scaffolds, virosomes, virus-like particles, virus or bacteria or plant-based vectors, prime boost vaccination with heterologous vectors (Rudraraju et al. 2013). The variety of approaches both enhance the understanding of effective responses to RSV and increase the likelihood that a safe and effective vaccine (or vaccines) will be developed.

Difficulties in developing an RSV vaccine include safety issues that are incumbent for any vaccine and the avoidance of vaccine-induced enhanced disease with subsequent RSV infection. Recently, a novel approach has focused on RSV fusion F glycoprotein antigenic site Ø, a metastable site specific to the prefusion state which is potent in inducing RSV-neutralizing antibodies (McLellan et al. 2013). Immunization with site Ø-stabilized variants of RSV F in mice and macaques induced RSV-specific neutralizing activity that protected against RSF infection. This approach is a break-through for RSV and also a conceptual platform for development of vaccines against other viruses.

**Animal models of RSV infection.** A number of animal models of RSV infection have been developed. As reviewed by Bem, RSV models include Heterologous host-virus models in which an animal is infected by human strains of RSV and cognate host-virus models in which an
animal is infected by a virus closely related to human RSV (Bem et al. 2011). Heterologous host-virus models include: Chimpanzees, sheep, Cotton rat, and mice; all of which can be infected with human strains of RSV. Cognate host-virus models include cattle which can be infected with bovine strains of RSV (bRSV) and mice infected with pneumonia virus of mice (PVM), respectively (Bem et al. 2011, Gerschwin 2012; Sacco et al. 2013). In addition to those above, other animals models of human RSV infection include other types of sub-human primates (baboons, African green and rhesus monkeys), ferrets, and Chinchillas (Bem et al. 2011; Eyles et al. 2013). Of the models above, each has some type of advantage and disadvantage depending on the clinical, immunological, cellular or pathogenic aspect of RSV disease being investigated. Other considerations for all animal models include costs, availability, and ethical considerations.

**Lamb model of RSV infection and insights into RSV pathogenesis.** Despite the numerous animal models of RSV infection, our laboratory and others have shown that ovine respiratory tract, particularly that of lambs, has features well suited for comparison human infants in terms of development, structure, susceptibility to human RSV strains, lesion development, immune response and clinical features (Derscheid and Ackermann 2012). In addition, the ovine lung is susceptible to respiratory bacterial pathogens that can occur secondary to an initial RSV infection as in humans. Alveolar development in humans and lambs occurs prenatally, in contrast to the post-natal alveolar development that occurs in rodents (Alcorn et al. 1981; Derscheid and Ackermann 2012; Flecknoe et al. 2003; McGowan 2004; Plopper et al. 1983). Lamb and infant lung have include similarities in airway branching patterns, relative numbers of Clara cells, type II cell development and the presence of airway submucosal glands which express lactoperoxidase (LPO). Airway branching in lambs and infants is less truncated than rodents (Scheerlinck et al. 2008). Numbers of Clara cells in bronchioles of lambs and infants are 18-22% whereas airways of mice are roughly 50% Clara cells (Barth et al. 1994; Derscheid
and Ackermann 2013; McGowan 2004; Plopper et al. 1983). We have shown that Clara cells and type II cells progressively differentiate with gestational age and have increased surfactant protein A (SP-A) and SP-D expression with maturation; both SP-A and SP-D bind and aggregate RSV and reduced expression may underlie susceptibility to RSV infection in infants born preterm (Derscheid and Ackermann 2013; Meyerholz et al. 2006). Submucosal glands are present in airways of lambs and infants, but absent or at insufficient amounts in rodents to produce LPO levels required for an increasing recognized epithelial oxidative system (Derscheid and Ackermann 2012; Derscheid and Ackermann 2013; Derscheid et al. 2013; Gerson et al. 2000; Mestas and Hughes 2004; Pack et al. 1980; Widdicombe et al. 2001; Wijkstrom-Frei et al. 2003).

**RSV pathogenesis and bronchiolitis.** The similarity of lung RSV lesions in sheep and ruminants to human RSV pathology has been well-documented by us and others (Derscheid and Ackermann 2012; Johnson et al. 2007; Lehmkuhl and Cutlip 1979; Lehmkuhl and Cutlip 1979). Lambs are susceptible to bovine and human strains of RSV (Derscheid and Ackermann 2012; Derscheid et al. 2013; Meehan et al. 1984; Meyerholz et al. 2007; Meyerholz et al. 2004; Meyerholz et al. 2004; Olivier et al. 2009; Olivier et al. 2011). We have shown that human RSV A2 strain infects lambs and others have shown that the Long strain of RSV infects lambs (Fig. 1) (Derscheid and Ackermann 2012; Olivier et al. 2009; Olivier et al. 2011). More recently, we have shown that RSV Memphis Strain 37 infects lambs robustly which has compelling comparative relevance as the Memphis Strain 37 is used in human clinical trials (Derscheid and Ackermann 2012; Derscheid et al. 2013; Derscheid et al. 2013). A pre-term lamb model of RSV infection has been developed which has features such as enhanced bronchiolitis, neutrophil infiltration, syncytial cell formation, myeloperoxidase production, and enhanced RSV replication levels all of which are similar to that seen in preterm human infants (Meyerholz et al. 2004; Sow et al. 2010). This model is significant since lambs can be born preterm (90% gestation) and
survive for such studies whereas rodents do not survive preterm birth. Whilst 90% gestation is not consistent with premature birth in human infants; studies increasing show that even preterm birth of infants at 95% gestation (37-38 weeks; e.g., “early term”) have a degree of clinical alterations. Finally, lambs (and other ruminants as well as swine) receive maternal immunoglobulin only through the ingestion of colostrum at birth. This biological feature allows lambs to be deprived of colostrum and thereby lack maternal antibodies to RSV and other pathogens thereby eliminating any confounding questions of the effects of maternal antibody on clearance of RSV or other pathogens (lambs do not receive maternal antibodies transplacentally). This feature is especially useful for vaccination studies and recently it has been shown that lambs receiving formalin-inactivated RSV (FI-RSV) vaccine produced responses which enhanced disease with subsequent RSV infection (Derscheid et al. 2014). Clinical parameters in lambs mirror those of human infants in terms of temperature (which is variable in both lambs and infants) and enhanced expiratory effort. Dendritic cell activity as well as immune and inflammatory responses in lambs also have consistencies with RSV infection in human infants as reviewed previously (Derscheid and Ackermann 2012; Derscheid and Ackermann 2013; Fach et al. 2007; Fach et al. 2010; Fach et al. 2007; Sow et al. 2012; Sow et al. 2009; Sow et al. 2010; Sow et al. 2011).

Because of the similarities between lambs and infants outlined above, studies in lambs provide insight to RSV pathogenesis that cannot be assessed or inferred in studies from human infants, especially preterm/premature infants due to ethical considerations. Many RSV studies of human RSV infection focus on nasal or bronchoalveolar wash fluids or ciliated cells isolated from the upper respiratory tract or continuous cells lines originally generated from the upper respiratory tract (Guo-Parke et al. 2013; Pickles 2013; Villenave et al. 2013). These fluids and cells are more readily available than bronchiolar fluids and cells. In lambs and from reports of human infants with severe RSV disease, lesions are especially intense in the distal bronchi,
bronchioles and terminal airways/alveoli. Clinically, this is significant as infants often do not become hospitalized or die from RSV rhinitis, tracheitis or upper bronchitis; severe RSV is more often associated with RSV-induced bronchiolitis. This is due, at least in part, to: 1) the tropism of RSV to the bronchiolar epithelium and 2) the narrow lumen of the bronchioles and distal airway, 3) low airflow pressure at this level and 4) alterations in airway dilation due to inflammation (Tayyari et al. 2011). In lambs and human infants with severe RSV, RSV-infected bronchiolar epithelial cells undergo apoptosis/necrosis and subsequent cell debris from the degenerate/necrotic epithelial cells along with infiltrating neutrophils and macrophages, mucin, and seroproteinaceous fluid partially occlude the lumen of these airways. In addition, bronchiolar epithelial cells adjacent to the RSV-infected epithelial cells undergo hyperplasia in order to repair the areas damaged by RSV-induced degeneration and necrosis and this hyperplasia further narrows airway lumens. In addition to having a narrow and partially occluded lumen, the bronchiole has a relatively weak level of airflow compared to the upper respiratory tract and thus it is difficult to expel intraluminal exudates by coughing and enhanced expiration. Also, peribronchiolar infiltrates of lymphocytes and plasma cells present in the airway adventitia have physical effect on alter airway dilation. Moreover, the cytokines, chemokines and other inflammatory mediators present further affect lung physiology at this location. Thus, the bronchiole is, in a way, an unfortunate location for RSV to replicate as it is an essential conduit for airflow through a narrow lumen with poor airflow pressures. Thus gaseous exchange in the alveoli immediately adjacent is profoundly affected. In preterm infants and preterm/newborn lambs, many of the above are exacerbated by increased level of RSV replication that occurs in both infants and lambs. The increased RSV replication that occurs triggers enhanced bronchiolar lesions and exudate accumulation which obstructs airflow.

Why does RSV replicate at higher levels preterm and in newborns? This poorly understood but considerations include the cell types present in bronchiole (Clara cells and other non-ciliated
cells), the extent of maturation of these cells at preterm and in newborns, RSV receptor expression, and extent/intensity of innate and adaptive immunity responses in this location. The bronchiole is a difficult microenvironment to study as the cells are difficult to isolated and grow in culture and the airflow, blood flow, and other physiologic conditions are difficult to replicate *in vitro*. Thus, *in vivo* models are essential for shedding new light on the mechanistic basis of RSV bronchiolitis and lesion development. For example, using laser capture microdissection bronchiolar epithelial cells from preterm and term lambs had significant differences in SP-A and other innate immune responses in both preterm and term lambs with and without RSV infection (Gallup et al. 2005; Kawashima et al. 2006; Meyerholz et al. 2006; Sow et al. 2009). And in recent studies in RSV-infected lambs, we have determined that syncytial cell formation occurs by three days post RSV inoculation and epithelial hyperplasia is present at four days post inoculation; viral titers appear to peak at day four but bronchiolar lesions develop a bit furtherer and peak at five and six days post RSV infection when RSV titers are beginning to decrease. By days 8 and 14, RSV titers are minimal and lesions are resolving. A more in-depth understanding of the kinetics of RSV bronchiolitis is needed in animal models to identify features not readily accessible in human infants.

**Assessment of therapies in lambs.**

**Vascular endothelial growth factor (VEGF).** VEGF is essential for fetal lung development and maturation and also has numerous other activities including: endothelial cell proliferation, enhancement of SP-A by lung epithelial cells, and monocyte chemotaxis (Brown et al. 2001; Meyerholz et al. 2006; Voelkel et al. 2006). Because of these activities, we developed the hypothesis that VEGF may reduce RSV disease severity in newborn lung. We discovered that pre-treatment of lambs with vascular endothelial growth factor (VEGF) protects lambs from RSV infection (Meyerholz et al. 2007; Olivier et al. 2011) (Fig. 2). VEGF reduced disease severity against both a bovine strain of RSV and a human strain (hRSV A2) of RSV. The mechanistic
basis by which VEGF protects is not known but could be related to VEGF effects on epithelial cell differentiation, maturation, and/or expression of SP-A. Alternatively or in addition, VEGF protection may be mediated by recruitment of alveolar macrophages. Low doses of VEGF do not alter the number of alveolar macrophages within the ovine lung; however, higher doses induce infiltration of a relatively uniform population of alveolar macrophages into alveoli and distal airways (Meyerholz et al. 2006). VEGF can affect many additional inflammatory and immune responses that could mediate protection against RSV infection.

**Dual functioning oxidase (Duox)/lactoperoxidase (LPO) defense.** Accumulating data suggest that an oxidative host defense system contributes to airway sterility (Bae et al. 2010; Banfi 2007; Fischer 2009; Lorentzen et al. 2011; Moskwa et al. 2007). We and others have shown that the oxidative system kills bacteria by producing OSCN⁻ in a lactoperoxidase (LPO)-catalyzed reaction: H₂O₂+SCN⁻ → OSCN⁻ (Banfi 2007; Derscheid et al. 2013; Fischer et al. 2011; Fischer 2009). OSCN⁻ production requires three processes working in concert: 1) LPO secreted by submucosal glands, 2) H₂O₂ generated by dual oxidases (Duox) of airway epithelia, and 3) SCN⁻ secretion (Banfi 2007; Belding et al. 1970; Conner et al. 2007; Conner et al. 2002; Dohan et al. 2003; Fischer et al. 2007; Forteza et al. 2005; Fragoso et al. 2004; Furtmueller et al. 2002; Harper et al. 2005). The mechanism(s) by which OSCN⁻ eliminates microbes is not known, but OSCN⁻ can oxidize thiol groups in surface proteins (Fischer et al. 2011; Klebanoff 1967). Importantly, OSCN⁻ is not toxic to eukaryotic cells. LPO has high affinity for SCN⁻ and also I⁻ (Fragoso et al. 2004; Furtmueller et al. 2002). LPO can catalyze the oxidation of I⁻ to OI⁻ in the presence of H₂O₂ (Furtmueller et al. 2002; Gattas et al. 2009). Although I⁻ is not a physiological component of the airway surface liquid (ASL), when present, I⁻ allows HOI generation by the LPO/Duox enzymes. There is some evidence that OSCN⁻ and OI⁻ have slightly different spectrums of antimicrobial activity. For example, while OSCN⁻ lacks activity against RSV *in vitro*, OI⁻ has anti-RSV activity *in vitro* and *in vivo* (Derscheid and Ackermann
2012; Fischer et al. 2011). The lung also has other oxidative systems with antimicrobial activity including the phagocytic NADPH oxidase (Phox), MPO, and nitric oxide synthase. Of these, MPO, like LPO, can convert KI and NaSCN to a halide in the presence of H$_2$O$_2$ and neutrophils expressing MPO enter lung airways during RSV infection (Nauseef 2013); however, the amount of MPO in ASL and extent to which MPO in ASL contributes to halide formation in ASL are not known.

The Duox/LPO defense system has activity against both bacteria and multiple respiratory viruses, including RSV and adenovirus (Ad) in vitro (Banfi 2007; Fischer et al. 2011; Gerson et al. 2000). To test the potential antiviral activity of this system in vivo, large animal models of viral infections are essential because mouse airways lack sufficient LPO and rat airways contain only few LPO-secreting cells. The ovine respiratory tract is similar to that of humans in terms of LPO and Duox expression and other physiologic and immunologic features. After in vitro studies and pilot work in lambs, we developed the hypothesis that the antimicrobial activity of Duox/LPO can be optimized in vivo through the supplementation of KI or NaSCN to treat RSV and possibly a number of other respiratory infections. Amelioration of respiratory viral infections by enhanced oxidative responses is significant because the therapy is relatively inexpensive, available, and easily distributed and administered.

The following study assessed the extent to which KI administration (for generation of HOI) reduces RSV disease in vivo using lambs (Derscheid et al. 2013). NaSCN was not assessed because it’s oxidative product, OSCN$, lacks anti-RSV activity in vitro (Fischer et al. 2011). The study included four groups of lambs: 1) Control group (no KI and no RSV); 2) M37 group (no KI and challenged with nebulized M37 strain of RSV); 3) KI + M37 (KI administered daily with nebulized M37 strain of RSV); and 4) dapsone plus KI and M37 strain of RSV). KI administration significantly reduced RSV disease severity, RSV gross lesions, RSV mRNA levels, RSV viral antigen distribution in lung and RSV viral titers (Fig. 3). This experimental design was repeated
in older lambs (3-week-old lambs) with similar significance (not shown). One group also received dapsone and the lack of anti-RSV activity in this group that received dapsone, as reflected by a markedly increased RSV lung lesions, RNA levels, RSV antigen and RSV titers (not shown), confirmed/validated that the anti-RSV effect(s) of KI is mediated by oxidative responses and not by KI itself. This is because dapsone inhibits LPO activity, and without LPO activity, O1' (the oxidative product of KI in the Duox/LPO reaction) cannot be formed (Bozeman et al. 1992; Derscheid et al. 2013). The findings of anti-RSV activity mediated by the Duox/LPO oxidative defense system are consistent with those in sheep by Gerson in which dapsone administration significantly enhanced colonization of *Pasteurella haemolytica* (renamed to *Mannheimia haemolytica*) a Gram-negative pathogen of ruminant respiratory tract (Gerson et al. 2000). *P. haemolytica* (*M. haemolytica*) bears much phenotypic and genotypic similarity to *Haemophilus sp.*, including *H. influenzae*. In contrast to the anti-RSV activity mediated by the Duox/LPO system, RSV can inhibit the anti-oxidant enzymes of lung epithelia (Hosakote et al. 2012).

**Clara cell protein (CC10).** Clara cells are non-ciliated bronchiolar epithelial cells that biometabolize xenobiotics, secrete immunomodulatory substances, and are progenitor cells for Clara and type II cells (Wang et al. 2010). The Clara cell proliferation pool is vulnerable to exhaustion, especially in neonates and chronic smokers (chronic toxin exposure) (Barth et al. 1994; Derscheid and Ackermann 2013). With Clara cell injury or loss, a proinflammatory environment can form due to the loss of immunomodulatory secretions by Clara cells (Wang et al. 2003). Clara cells secrete large amounts of Clara cell secretory protein (CCSP), also known as CC10 and also known as CC16, secretoglobin, and uteroglobin. CC10 is increased in bronchoalveolar lavage fluid and serum during acute injury such as smoke inhalation or application of pneumotoxicants (naphthalene, 4-ipomeanol (4-IM), chloroethylene). In contrast, CC10 is decreased in chronic or dysplastic airway dysfunction (asthma, chronic obstructive
pulmonary disease, or bronchopulmonary dysplasia (BPD) (Barth et al. 1994; Elizur et al. 2007; Wang et al. 2003). With RSV infection, CC10-deficient mice have increased inflammatory responses and RSV persistence. With CC10 expression there is a reduction of inflammatory responses and RSV persistence (Derscheid and Ackermann 2013). CC10 may not only have a protective effect on Clara cells, but also stimulate development of Clara cells. Clara (and type II) cells also produce substances with known anti-RSV activity including: SP-A, SP-D (which bind and opsonize RSV), beta-defensins, beta-galactoside-binding protein, and RSV receptors such as retinoic acid inducible gene-1 (RIG-I) which triggers epithelial responses as well as inflammatory/immunomodulatory substances (Derscheid and Ackermann 2013; Elizur et al. 2007). RSV infection enhanced production of secretoglobin (CC10) family proteins in nasal mucus secretions from calves infected with bovine RSV (bRSV) (Sacco et al. 2013).

The extent of CC10 expression in developing fetal lamb lung was measured by qPCR at 50, 75, 90% gestation, full term and adults. CC10 levels were low preterm and increased with age after birth (Fig. 4); these findings are consistent with the degree of epithelial cell maturation as determined with periodic acid-Schiff (PAS) stain which detects carbohydrate-like molecules (e.g., glycogen). Epithelial cells with increased PAS staining were present during gestation and were less mature and produced less SP-A and SP-D than term and adult lambs. The expression of CD208+ (type II cells) and CD208- cells (Clara cells) were reduced preterm which is also consistent with immaturity (Meyerholz et al. 2006).

**CC10 reduces RSV M37 severity.** In a pilot study, we have assessed the effects of recombinant human CC10 (rhCC10) in preventing RSV M37 pneumonia. Briefly, two lambs received rhCC10 intravenously (1.5 mg/kg) twice daily one day prior to RSV inoculation and each day thereafter until lungs were collected on day 6 post RSV inoculation (6 ml, 10^7 PFU/ml via nebulizer); another group of lambs (n = 6) received RSV alone and no rhCC10; a third group lacked rhCC10 and were nebulized with media (control). Lambs receiving rhCC10 had no side
effects from inoculation. rhCC10-treated lambs had reduced gross lesions and decreased RSV mRNA levels by RT-qPCR (Fig. 5).

**ALX-0171.** Ablynx NV, has developed a therapeutic protein based on a *camelid* variable heavy chain only (V_{H/H}) immunoglobin backbone with strong binding affinity to the F (fusion) protein of the RSV virus, thereby inhibiting RSV fusion to target cells. Briefly, ALX-0171 is composed of V_{H/H} chains linked to form a trivalent binding structure with a 2,000 fold increase in potency compared to monovalent structures. ALX-0171 is delivered through nebulization and it is highly stable. ALX-0171 has demonstrated antiviral efficacy in both *in vitro* and *in vivo* studies, the latter being performed in the cotton rat therapeutic model for RSV. In order to further assess the extent to which ALX-0171 may treat RSV infections *in vivo*, ALX-0171 is currently being assessed in neonatal lambs infected with a human RSV strain (hRSV M37). The lamb studies demonstrate the capacity of the lamb model to characterize delivery, distribution and efficacy of a nebulized drug formulated to treat RSV infection in infants.

**RSV Vaccination in lambs.** Recently, we have shown that the newborn lamb develops enhanced RSV lesions following vaccination with a formalin-inactivated RSV (FI-RSV) vaccine (Derscheid et al. 2014). This work was completed in lambs deprived of colostrum which eliminated the potential influence of maternal antibodies. This study sets the stage for additional work in lambs in regards to understanding FI-RSV pathogenesis and for assessment of vaccines.

**Refining RSV Models.** Experimental RSV infection in lambs and other models requires constant assessment and validation of basic methods. As indicated, lambs are susceptible to various human (Long, A2, Memphis 37 strain) and bovine strains (bRSV) of RSV as well as ovine parainfluenzavirus 3 (ovine PI-3) and a human strain of parainfluenzavirus 3 (PIV-3) (Derscheid and Ackermann 2012). All of these are enveloped viruses and must be
administered with suitable physiologic conditions. RSV and PI-3, PIV-3 can be delivered by intranasal inoculation with a syringe or atomizer, intrabronchially with a fiberoptic bronchoscope, intratracheally with a syringe and needle and with whisper jet nebulizers (Derscheid and Ackermann 2012; Derscheid et al. 2013; Meyerholz et al. 2007; Olivier et al. 2009). For nebulization, we have shown that the addition of sucrose at 15-20% reduces RSV loss during nebulization and enhances RSV disease severity (manuscript under review). Work by others demonstrated that RSV grown in Vero cells have truncated G glycoprotein which altered infectivity in vitro. Similarly, our recent work in lambs demonstrated that RSV grown in Hep2 cells have increased virulence compared to RSV grown in Vero cells (Derscheid et al. 2013).

**Effects of ethanol on fetal lung development and innate immunity.** Maternal ethanol consumption is a risk factor for preterm birth and the enhanced severity of RSV in preterm infants is well known (Albertson and Gronbaek 2004; Aujard and Fauroux 2002; Garcia et al. 2010; Sommer et al. 2012). It is possible that ethanol increases RSV disease severity beyond that of preterm birth alone due to Clara cell damage or alteration in development/function. If true, other toxins or unknown substances reaching the fetal lung in utero may affect Clara cells and thus, susceptibility to RSV infection (Lazic et al. 2010; Lazic et al. 2011; Lazic et al. 2007). Clara cells biometabolize toxins and other substances knowingly or unknowingly consumed by pregnant mothers (e.g., ethanol, nicotine) (Lazic et al. 2010; Lazic et al. 2011; Lazic et al. 2007). Ethanol and other toxins can injure Clara cells and nearby type II cells resulting in reduced innate immunity and increased susceptibility for RSV infection (Derscheid and Ackermann 2012; Derscheid and Ackermann 2013). Despite the fact that ethanol has detrimental effects on the developing fetus, one out of 29 pregnant women consumes alcohol during pregnancy and another survey found that 5 to 10 out of 1000 pregnant women consume 7 or more drinks per week (Ethen et al. 2009; Jaddoe et al. 2007). While ethanol has effects on the nervous system and liver, the detrimental effects of ethanol on developing fetal lung are of increasing concern.
Ethanol decreases surfactant synthesis and pro-inflammatory cytokines and impairs response to bacterial challenge. Clara cells produce cytochrome CYP2E1 which biometabolize ethanol to acetaldehyde and malondialdehyde (MDA). These form protein adducts, invoke inflammatory responses, and reduce repair of bronchiolar epithelium.

The effects of ethanol on prenatal lamb lung development has been developed by delivering ethanol to gestating ewes resulting in blood levels consistent with a moderate drinker, which is the most common form of ethanol consumption by pregnant women (Ethen et al. 2009; Jaddoe et al. 2007; Lazic et al. 2011; Lazic et al. 2007; Sozo et al. 2009). Our work shows that ethanol-exposed lambs have reduced epithelial maturation, ciliary function, innate immune gene expression of surfactant protein A and D (SP-A, SP-D), TNF alpha, IL-10, and monocyte chemotactic protein (MCP-1) by lung epithelia along with striking reductions in hypoxia-inducible factor (HIF) 1α and 2α, vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFR) (Lazic et al. 2011; Lazic et al. 2007). SP-A and SP-D have antimicrobial activity against RSV and enhance macrophage activation (Lazic et al. 2011; Lazic et al. 2007). Loss of these innate immune and inflammatory responses increases susceptibility to infection (Derscheid and Ackermann 2013; Krishnan et al. 2003; Starner et al. 2005; Stokes et al. 2013). Ethanol-induced changes in lung development and immunity in lambs have been confirmed by the Harding laboratory and sheep are used for other types of ethanol studies (Sozo et al. 2009). The precise effect of ethanol on fetal Clara cells is not known but being assessed (below).

Maternal ethanol consumption alters transcriptomes of developing ovine lung (Derscheid and Ackermann 2012). RNA expression levels in RNA from lung of four lambs were determined by Next-Generation Sequencing (NGS) with Illumina NGS on GAIIx (1 x 40 cycles/bases single read) and HiSeq 2000 (2 x100 cycles/bases, paired-end). Two lambs were preterm and two were term; of these, one preterm and one term lamb were exposed to ethanol in utero (maternal consumption) and the other two lambs were not exposed to ethanol at any time. Data analysis
was with SCS/RTA, TopHat workflow, and Cufflinks, Cuffcompare and Cuffdiff. Transcripts with increased expression in preterm lung exposed to ethanol included genes related to: 1) Inflammation, immunity and growth factors: lysozyme, Secreted frizzled-related protein 2, SFRP2 which affects Wnt signaling, Interleukin 12 A, CXCL10, insulin-like growth factor-1, IGF-1, Fos, IGF binding protein 5; early growth response protein 1, EGR1, CD28, CD4; 2) Metabolism/stress: leptin receptor, LEPR, serotonin transporter, SERT; 3) Cell proliferation inhibition: CDKN1C, p57, a Kip2 cell proliferation inhibitor and 4) Angiogenesis/vascularization: SERPINF1 (PEDF). Genes down-regulated by ethanol but up-regulated by RSV include: IL-8, TNFα, ICAM-1.

**Conclusions.** The various animal models of RSV infection contribute to the broad understanding of the RSV pathogenesis. The lamb model has several features consistent with RSV in infants which allows insight into epithelial cell infection, pathogenesis, development of bronchiolitis and its effect on airflow and gaseous exchange, inflammatory responses, innate and adaptive immune responses. The ability to study lambs born preterm is one of several unique features of the lamb model. Also, the lamb model is conducive to therapeutic interventions; especially those that are mechanistic and at a fundamental level. While VEGF and CC10 can enhance a wide-variety of immunomodulators that mediate resistance to RSV infection, ALX-0171 is a very precise inhibitor of RSV. Enhancement of oxidative defense (Duox/LPO) through KI administration invokes oxidative radical defense against RSV and potentially other viruses and microbial agents. Lambs develop enhanced RSV disease if vaccinated with formalin-inactivated RSV (FI-RSV) which opens up the model to studies of FI-RSV enhanced disease and assessment of vaccines. The ability to deprive lambs of maternal antibodies is advantageous feature of the lamb model with is particularly useful in assessment of vaccines. The findings that maternal ethanol consumption alters development and innate
immunity of the lung *in utero* may explain, at least in part, why ethanol is a risk factor for preterm birth and also predispose the infant lung to increased RSV disease severity.

**Acknowledgements.** The author acknowledges and thanks all of those who have contributed to the work and funding agencies that include The National Institutes of Health NIAID R56AI09100, MedImmune, Inc. Gilead, Inc., Ablynx NV.
REFERENCES


FIGURE LEGENDS

Figure 1: Respiratory syncytial virus strain A2 causes bronchiolitis in lambs (Olivier et al. 2009).  
A. Lung from a control lamb not infected with RSV that contains a bronchiole and alveoli.  
B. Lung from a lamb 6 days post-inoculation with RSV with a bronchiole (outlined) containing epithelial cells admixed with neutrophils (*).  
Alveoli around the bronchiole are collapsed with accumulation of degenerate neutrophils and areas of necrosis (arrow).  
C and D. Immunohistochemical detection of RSV antigen in lung from a lamb 6 days post-inoculation with RSV, in which RSV viral antigen is present within the bronchi, epithelial cells lining the bronchi, and the syncytial cells in areas of alveolar consolidation.  
Bars = 25 μm.

Figure 2: Pretreatment with exogenous rhVEGF decreases histologic consolidation score of lambs infected with hRSV A2 strain (Olivier et al. 2011).  
Lambs received either media followed by RSV A2 (no VEGF) or rhVEGF followed by RSV A2 and were assessed for clinical signs, viral lesions and mRNA levels.  
At six days post RSV A2 infection VEGF-treated lambs had reduced histologic consolidation scores (an overall score of bronchiolitis and lung inflammation) indicating a decreased in alveolar consolidation and inflammatory cell infiltration (*p<0.05).  
Exogenous rhVEGF treatment also reduced RSV mRNA levels as 4 and six days post RSV inoculation (not shown).  
Previous studies have demonstrated the VEGF treatment also reduces the severity bovine RSV (bRSV) in newborn lambs (not shown).

Figure 3: Respiratory syncytial virus Memphis 37 strain causes bronchiolitis in lambs that is reduced with enhanced airway oxidative responses.  
A. Lung from a lamb infected with RSV M37 strain four days post inoculation in which there is erosion of the epithelium with accumulation of cell debris in the airway lumen and formation of a syncytial cell.  
B. Lung from a lamb 6 days post-inoculation with RSV M37 strain in which viral antigen is detected by immunohistochemistry and viral antigen is present with epithelial cells, macrophages and in cell
debris of the airway lumen. There is moderate thickening of the epithelium due to hyperplasia. 

C. Enhancement of the airway oxidative response (Duox/LPO oxidative system) by administration of potassium iodide (KI) reduces RSV M37 mRNA levels (as well as viral titers, antigen and lesions; not shown) in RSV M37-inoculated lambs treated with KI (M37 + KI) compared to lambs inoculated with RSV M37 but not receiving KI (M37). Inhibition of the oxidative defense system with dapsone (DAP) in a group of lambs resulted in significantly enhanced RSV M37 RNA levels (DAP + M37 + KI) despite KI treatment (Derscheid et al. 2013).

Figure. 4. **CC10 reduces RSV gross lesions and RSV mRNA levels.** Two lambs received rhCC10 intravenously (1.5 mg/kg) twice daily one day prior to RSV inoculation (6 ml, 107 PFU/mL via nebulizer) and each day thereafter; another group of lambs (n = 6) received RSV alone and no rhCC10; a third group lacked rhCC10 and were nebulized with media (control). rhCC10-treated lambs had reduced gross lesions and decreased RSV mRNA levels by qPCR.

Figure. 5. **Maternal ethanol consumption reduces lung maturation through hyxopoxia inducible factor (HIF) and it’s downstream gene vascular endothelial growth factor (VEGF) (Lazic et al. 2011).** **Fig. 5A.** Relative mRNA expression of hypoxia inducible factor-1α, 2α and 3α (HIF-1α, HIF-2α and HIF-3α respectively) in the neonatal ovine lung. Exposure to ethanol *in utero* reduced mRNA expression of HIF-1α and HIF-2α in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. Such changes are not observed in the lungs of full-term (FT) lambs exposed to ethanol *in utero*. No significant changes are seen with HIF-3α mRNA expression levels in both, PT and FT lambs. (*p<0.1, significance was established at 90% confidence interval). **Fig. 5B.** Relative mRNA expression
of vascular endothelial growth factor (panVEGF) in the neonatal ovine lung. Exposure to ethanol *in utero* reduces mRNA expression of panVEGF in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. Such changes are not observed in the lungs of full-term (FT) lambs exposed to ethanol *in utero*. (*p<0.1, significance was established at 90% confidence interval). **Fig. 5C.** Relative mRNA expression of vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and VEGFR-2 respectively) in the neonatal ovine lung. Exposure to ethanol *in utero* reduces mRNA expression of VEGFR-1 and VEGFR-2 in the lungs of pre-term (PT) lambs when compared to the control lambs of the same age group. Such changes are not observed in the lungs of full-term (FT) lambs exposed to ethanol *in utero*. (*p<0.1, significance was established at 90% confidence interval). **Fig. 5D.** Periodic acid-Schiff (PAS) stain for glycogen granules in the type II pneumocytes (ATII). Glycogen granules in the ATII are more abundant in the pre-term lambs exposed to ethanol *in utero* (B) when compared to the control lambs of the same age group (A). Glycogen content in the ATII is similar in the full-term lambs exposed to ethanol *in utero* (D) and the control lambs of the same age group (C).
Figure 1.
Figure 2.
Fig. 3.

Lesions: Control vs. CC10-M37 vs. M37

Control
CC10-M37
M37

Avg. % of lung lesions/lobe

Relative M37 mRNA

Control
CC10-M37
Just M37

RSV M37 RT-qPCR
Fig. 4.
Figure 5.