Delivery of ALX-0171 by inhalation greatly reduces respiratory syncytial virus disease in newborn lambs

Alejandro Larios Mora, Laurent Detalle, Jack M. Gallup, Albert Van Geelen, Thomas Stohr, Linde Duprez, and Mark R. Ackermann

College of Veterinary Medicine, Department of Veterinary Pathology, Iowa State University, Ames, IA, USA; Ablynx NV, Zwijnaarde, Belgium

ABSTRACT
Respiratory syncytial virus (RSV) is a common cause of acute lower respiratory disease in infants and young children worldwide. Currently, treatment is supportive and no vaccines are available. The use of newborn lambs to model hRSV infection in human infants may provide a valuable tool to assess safety and efficacy of new antiviral drugs and vaccines. ALX-0171 is a trivalent Nanobody targeting the hRSV fusion (F) protein and its therapeutic potential was evaluated in newborn lambs infected with a human strain of RSV followed by daily ALX-0171 nebulization for 3 or 5 consecutive days.

Colostrum-deprived newborn lambs were infected with hRSV-M37 before being treated by daily nebulization with either ALX-0171 or placebo. Two different treatment regimens were examined: day 1–3 or day 3–5 post-infection. Lambs were monitored daily for general well-being and clinical parameters. Respiratory tissues and bronchoalveolar lavage fluid were collected at day 6 post-inoculation for the quantification of viral lesions, lung viral titers, viral antigen and lung histopathology.

Administration by inhalation of ALX-0171 was well-tolerated in these hRSV-infected newborn lambs. Robust antiviral effects and positive effects on hRSV-induced lung lesions and reduction in symptoms of illness were noted. These effects were still apparent when treatment start was delayed and coincided with peak viral loads (day 3 post-infection) and at a time point when signs of RSV disease were apparent. The latter design is expected to have high translational value for planned clinical trials. These results are indicative of the therapeutic potential of ALX-0171 in infants.

INTRODUCTION
Human respiratory syncytial virus (hRSV) is classified in the genus Orthopneumovirus of the newly created Pneumoviridae family within the order Mononegavirales, standing apart from the original Paramyxoviridae family member.

As such, it encodes two major surface glycoproteins termed G-protein and F-protein. These two glycoproteins play a crucial role in viral replication as they are responsible for viral binding to the target cell and virus-cell membrane fusion, respectively. In addition, hRSV has a linear single stranded, non-segmented RNA molecule of negative polarity of ~15 kb.

As a respiratory virus, hRSV may present as an upper respiratory tract infection (including rhinitis, otitis media and pharyngitis), or as a lower respiratory tract infection (including acute bronchiolitis or pneumonia) in vulnerable populations such as infants, the elderly and the immunocompromised.

Further, due to concerns for potential teratogenicity and minimal evidence of benefit, it is not recommended for routine use in infants but may be considered for use in select patients with documented, potentially life-threatening RSV infection. In the prevention of RSV infections, palivizumab (Synagis, MedImmune), a humanized monoclonal antibody (IgG) against the F-protein of RSV, has been approved for use in high risk infants; however, due to high costs associated with palivizumab prophylaxis, a limited effect on RSV hospitalizations on a population basis, a lack of measurable effect on mortality and a minimal effect on subsequent wheezing, use
of palivizumab is restricted to certain high-risk pediatric populations.7,8

There is thus a need to develop new therapeutic treatment options for RSV infection. Different animal models for RSV infection, including non-human primates, calves, lambs, mice, guinea pigs, ferrets, hamsters and cotton rats have been described.5,10 The cotton rat (Sigmodon hispidus) RSV infection model has been successfully used in the development of both RSV-IgIV (RespiGam®, MedImmune) and palivizumab and findings in the cotton rat for those agents translated well to the clinical setting.11,12 The cotton rat model also serves as the primary model for the determination of vaccine safety because these animals develop vaccine-associated worsening of pulmonary disease upon subsequent infection with RSV that reflects what is seen in humans and non-human primates.13 However, although susceptible to RSV infection, cotton rats do not exhibit any clinical signs of upper and lower respiratory tract disease or any age-related susceptibility to hRSV, in contrast to what is seen for human infants.10

Similarly to humans, lambs exhibit several key features of RSV-vaccine enhanced disease;14 anatomically, the respiratory tract of sheep and humans share many structural features, such as the size of the nasal cavity and Airways,15 as well as lung development where alveolarization starts pre-term.16 When experimentally infected with either bovine (bRSV) or human (hRSV) strains of RSV, neonatal lambs develop mild clinical symptoms including fever, tachypnea or increased expiratory effort (wheeze) and malaise, as well as mild to moderate gross and histologic lesions.17

ALX-0171 is a novel inhaled biotherapeutic in development for the treatment of RSV infections in infants.18 Local pulmonary administration of ALX-0171 was considered optimal for this indication as it enables targeted delivery straight to the site of infection, with a potentially more rapid onset of action while using lower doses compared to systemic administration.19 Pre-clinical evaluation of ALX-0171 using a face-mask, which is highly relevant for the intended clinical use, would thus require a larger animal species than rodents. In addition to the pathophysiologic similarities of RSV disease with human infants, lambs also have advantages in terms of drug delivery by inhalation. Indeed, drug deposition in the lung is affected by the respiratory rates and breathing volumes, both parameters being dependent on body size, and lung anatomy, which are similar to infants. Other important parameters that affect drug deposition is the breathing maneuver and the aerosol aerodynamic diameter.20-22 Lambs breathe through the nasal meatus, although oral inhalation is possible when the nasal airways are obstructed, which is also the case for human infants.23 For the reasons stated above, the neonatal lamb model was selected for the evaluation of ALX-0171 efficacy and safety in a neonatal setting.

Results
Pharmacokinetics of ALX-0171 in neonatal lambs

Four independent studies were performed in hRSV-infected neonatal lambs (see study design section). In all of these four studies, blood samples were taken at selected time points following the first dose and all the subsequent doses for pharmacokinetic (PK) purposes. On Day 6, bronchoalveolar lavage fluid (BALF) sampling was performed post-mortem for PK analysis in the lung compartment. Once daily administration of ALX-0171 via inhalation for 5 (Figure 1) or 3 (Figure 2) consecutive days resulted in high concentrations (>10 times the in vitro IC50 on RSV/B 1853718) of ALX-0171 in lung epithelial lining fluid (ELF). A dose-dependent increase in ELF concentrations was seen on day 6. All the assessed lambs had high ALX-0171 concentrations with the notable exception of lamb number 10 from the 1 mg/kg ALX-0171-treated group. This lamb had ALX-0171 levels that were below the quantification limit. The reason for this is unknown, but an inadequate BALF retrieval cannot be excluded. This particular lamb, however, had been adequately exposed to ALX-0171 as shown by the blood concentrations. Plasma ALX-0171 concentrations were roughly 3 log lower than those observed in the lung compartment following pulmonary delivery to neonatal lambs (Figure 1 and Figure 2). The systemic PK were linear with dose and time and the clearance was dependent on the animal weight. Although no intravenous data are available from neonatal lambs for ALX-0171, it is likely that absorption from lung to blood is the driving force of the ALX-0171 PK in lamb.

![Figure 1](image_url)

*Figure 1.* Pharmacokinetic profiles from study 1. (a) Mean plasma concentration-time profiles of ALX-0171 and (b) ALX-0171 concentrations in epithelial lung lining fluid (ELF) after five consecutive daily administrations by inhalation to neonatal lambs. ALX-0171 concentrations in ELF were derived from concentrations measured in BALF, which was sampled postmortem, after normalization for dilution based on the Urea correction method (values were red blood cell corrected). Bronchoalveolar lavage fluid (BALF) was sampled 24 hours after the last dose. Results are expressed as mean ± standard error for plasma curves and as individual lamb results with mean indicated as horizontal line for ELF. The hatched line represents the lower limit of quantification (LLOQ) of the assay. ALX-0171 levels were below quantification limit for lamb N°10 from the 1 mg/kg dose group.
Effect of daily ALX-0171 administration to neonatal lambs when started on day 1 post-infection

To assess the therapeutic efficacy of ALX-0171 when administered by inhalation, thirteen lambs (twelve lambs for analysis) were inoculated with hRSV on day 0. The day after infection (day 1), the lambs were randomized to either the placebo group or to one of the three ALX-0171 dose groups (Table 1) and were treated daily by inhalation for 5 consecutive days. Lambs underwent daily physical examinations and body weights, heart rates, rectal temperatures, respiratory distress and hRSV infection-related symptoms were recorded. On day 6, the animals were euthanized and lung lavage samples and lung tissues were obtained for analysis of viral load in lung, histopathology and immunohistochemical analysis.

hRSV inoculation by inhalation resulted in robust infection of all the analyzed lambs as confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) performed on BALF and lung tissue (Table 2). In the placebo-treated lambs, hRSV infection induced gross and microscopic lung lesions. Gross lung lesions were typical of experimental hRSV infection and consisted of locally extensive dark plum-red foci of pulmonary consolidation on each lung lobe, which ranged from 8.3% to 13.3% (mean percent consolidation per lung of 11.7%). Microscopic examination of lung tissue revealed the presence of locally extensive alveolar consolidation with mild epithelial necrosis in multifocal bronchioles, syncytial cell formation, infiltrates of neutrophils into the bronchiolar lumens, peribronchiolar infiltrates of lymphocytes and plasma cells; multifocal bronchioles had mild to moderate hyperplasia of epithelia. hRSV antigen, assessed by immunohistochemistry, was present in areas with lesions and was localized to epithelial cells lining the bronchi, bronchioles and the alveoli, as well as the cytoplasm of occasional macrophages. These gross and microscopic lesions and hRSV antigen distribution were consistent with those observed previously in a study assessing hRSV kinetics in lambs.24

Treatment of lambs with ALX-0171 at all three doses resulted in significant reductions in viral RNA copy numbers that ranged from 1.44 to 1.82 Log10 viral RNA copies/mL in BALF (p < 0.0001) and between 0.83 to 1.88 Log10 viral RNA copies/mg in lung tissue (p = 0.035 and p = 0.003, respectively) depending on the dose (Table 2). All treated lambs had undetectable infectious virus. However, during staining of the...
foci, a monoclonal antibody was used (Mouse anti-Fusion protein Meridian MAb to hRSV Fusion Protein, Cat. No. C87610M 1 mg/mL, Clone: RSV 3216 [B016]) instead of the intended polyclonal antibody (Goat polyclonal Ab to hRSV [all antigens]). The monoclonal antibody tool was shown to compete with ALX-0171 binding and was not used in subsequent assays and studies. Therefore, reductions in infectious virus in the ALX-0171-treated lambs of study 1 are not reported in Table 1.

Consistent with the reductions in viral loads, gross lung lesions were absent in all ALX-0171-treated lambs (Figure 3a) and microscopic alveolar consolidation scores were significantly reduced ($p < 0.0001$) following treatment (Figure 3b). Only in lamb number 10, from the 1 mg/kg ALX-0171-treated group, was there observable alveolar consolidation (score of 0.5). The mean number of bronchi/bronchioles and alveoli per field in which viral antigen was detected was also significantly reduced ($p < 0.0001$ and $p = 0.007$, respectively) (Figure 3c). Lamb 10 had the highest viral antigen expression, although levels remained well below those seen in the placebo-treated lambs. Two lung lobes from this lamb showed some consolidated areas. Grossly and microscopically, this lamb also had lesions in these lobes that were not consistent with hRSV infection but, rather, with bacterial infection. This lamb was not excluded from analysis because: 1) the bacterial cause was not confirmed, 2) systemic PK profiles indicated that this lamb had been adequately exposed, 3) no other findings were seen, and 4) only regions (a minority of the surface area) of the lung had these lesions.

Each clinical sign was individually scored, which may not be the best measure for the severity of disease because single clinical signs do not correlate well with the degree of dyspnoea and airway narrowing in acute wheeze in human infants. In infants with acute respiratory infections, clinical scoring usually relies on a combination of clinical symptoms and signs (feeding intolerance, medical intervention, respiratory difficulty, respiratory frequency, apnoea, general condition, fever). For this reason, an exploratory composite clinical score was calculated post-hoc for each lamb in the treatment and post-treatment period based on the scoring of the individual clinical signs. The parameters included in the clinical severity score were body weight evolution, blood oxygenation levels, relative change in respiratory rates, presence or absence and severity of expiratory effort and wheeze. Each of these parameters were either scored 0 or 1 for body weight evolution and blood oxygenation saturation; 0-3 for expiratory efforts, wheeze and relative change in respiratory rates from baseline based on fixed criteria (see Figure 3d and supplemental Table S1) with a possible maximal clinical severity score of 11. The hRSV-infected, placebo-treated lambs displayed increased clinical severity scores throughout the treatment and post-treatment period (i.e., assessments performed on day 2 to day 6) (Figure 3d) with 3 of 3 lambs having a score of ≥2. These increased clinical severity scores were mainly driven by labored breathing as expiratory effort was apparent on day 6 post-infection in 2 of 3 lambs (maximum expiratory effort score of 2), but was absent on other days (except on day 0 where 1 lamb displayed expiratory effort). Wheeze was absent in all placebo-treated lambs throughout the study. Respiratory rates in the placebo-treated lambs increased on day 3 post-infection and reached a peak on day 4 with a mean of 66 breaths/min (% increase of 18.4% versus day 0). In the ALX-0171-treated lambs, 6 of 9 (67%) lambs had a clinical severity score of 0 and 3 lambs had a score of 1. Neither expiratory effort nor wheeze were detected at any time during the study in the ALX-0171-treated lambs, whilst mean respiratory rates remained constant throughout the study where the mean

| Study | ALX-0171 target inhaled dose | Viral culture (log10 FFU/mL ± SEM) | Reduction versus placebo (log10 FFU/mL ± SEM) | RT-qPCR (log10 viral RNA copies/mL ± SEM) | Reduction versus placebo (log10 viral RNA copies/mL ± SEM) | Viral load (BALF) | Viral load (lung tissue) |
|-------|-----------------------------|---------------------------------|-----------------------------------------------|-----------------------------------------|-----------------------------------------------|------------------|---------------------|
| 1     | Placebo                     | 4.49 ± 0.43                     | NA                                            | —                                       | —                                             | 6.02 ± 0.13      | —                    |
|       | 0.3 mg/kg UD                | 4.57 ± 0.16                     | 1.82***                                       | 4.82 ± 0.17                             | 1.21***                                       | —                | —                    |
|       | 1 mg/kg UD                  | 4.95 ± 0.09                     | 1.44***                                       | 5.19 ± 0.36                             | 0.83***                                       | —                | —                    |
|       | 3 mg/kg UD                  | 4.60 ± 0.06                     | 1.79***                                       | 4.14 ± 0.16                             | 1.88***                                       | —                | —                    |
| 2     | Placebo                     | 4.83 ± 0.04                     | —                                             | 7.15 ± 0.20                             | 7.63 ± 0.07                                   | —                | —                    |
|       | 0.3 mg/kg UD                | 4.13                           | 1.25*                                         | 5.79 ± 0.21                             | 1.84***                                       | —                | —                    |
|       | 3 mg/kg UD                  | 7.01 ± 0.18                     | 0.14                                          | 7.09 ± 0.11                             | 0.54**                                        | —                | —                    |
| 3     | Placebo                     | 4.98 ± 0.41                     | 7.26 ± 0.30                                   | 5.47 ± 0.21                             | 5.00 ± 0.24                                   | 0.47             | —                    |
|       | 0.3 mg/kg UD                | 4.11                           | 0.55                                          | 7.08 ± 0.31                             | —                                             | —                | —                    |
|       | 0.04 mg/kg UD               | 2.40 ± 1.70                     | 1.84                                          | 7.27 ± 0.23                             | -0.19                                         | —                | —                    |
|       | 0.06 mg/kg UD               | 3.84                           | -0.24                                         | 6.93 ± 0.34                             | 0.15                                          | —                | —                    |
|       | 0.3 mg/kg BQL               | 3.84                           | 0.54                                          | 6.71 ± 0.38                             | 0.37                                          | —                | —                    |

Table 2. Lung viral loads in hRSV-infected neonatal lambs for the four performed studies. **Inhaled dose is defined as the total nebulised drug reaching the nose of the lamb. UD: Undetected foci. BQL: Below quantification limit (1 of 2 lambs had detectable foci), i.e., <5 FFU/mL.**
respiratory rates on day 4 in the 3 mg/kg-dose group was 56 breaths/min (% increase of 5.7% versus day 0). In the 3 lambs with a clinical severity score of 1, the relative body weight change between day 0 and day 6 ranged from -2% to 17%. When considering mean body weight evolution of all lambs throughout the study duration, there were no significant differences between the groups. For the clinical severity score a significant effect was noted (p = 0.0084).

**ALX-0171 treatment delayed to start on day 3 post-infection effectively reduces infection-related changes**

Following the first study, 2 additional studies were performed to evaluate the therapeutic activity of ALX-0171 when administered close to the viral peak and onset of symptoms. In the first of these additional studies (i.e., study 2), two dose levels from study 1 were used. Based on the results of study 2, the lowest tested dose was selected for study 3, where appropriate mock-infected control groups were included to evaluate safety and efficacy of ALX-0171. Clinical parameters in study 2 were assessed as in study 1. However, for study 3, the general health status of the lambs was additionally scored daily and is referred to as malaise score. This additional evaluation was included because of the observed variability in clinical parameters observed in study 1 and 2, which seemed to be associated with a more intense sampling regimen (e.g., multiple blood draws, holding the animals during respiratory rate auscultations). The procedure to observe the lambs was adapted in study 3, which involved observing the lambs for increased periods of time without picking them up to make the behavioral clinical assessments and respiratory rate assessments and by reducing the blood sampling to only 3 over the 6 days.

hRSV-infected, placebo-treated lambs from studies 2 and 3 had similar lung viral loads to placebo-treated lambs from study 1, whereas mock-infected, placebo-treated lambs from study 3 had no detectable virus as expected. Consistent with
findings from study 1, hRSV infection resulted in the presence of gross and microscopic lung lesions that was paralleled by lung epithelial cell viral protein expression (Figure 4, Figure 5 and Figure 6). ALX-0171 treatment initiated on day 3 post-infection, reduced infectious virus titers by more than 4 Log10 focus forming units (FFU)/mL at both tested doses. The effect on viral RNA copy numbers was less pronounced as the mean reductions were 1.25 Log10 and 0.14 Log10 in BALF (p = 0.019 and p = 0.6, respectively) and 1.84 Log10 and 0.54 Log10 in lung tissue (p < 0.0001 and p = 0.017, respectively) in study 2 and 0.55 Log10 in BALF (p = 0.2) and 0.47 Log10 in lung tissue (p = 0.2) in study 3. Despite these more modest reductions in viral RNA copy numbers, ALX-0171 treatment significantly reduced microscopic alveolar consolidation score in both study 2 and 3 (p ≤ 0.0001 for combined ALX-0171-treated groups versus placebo) as well as for most of the individually scored microscopic lesions (Figure 4b, Figure 5b, Figure 6b and supplemental Figure 1). Reductions in the number of bronchi/bronchioles and alveoli expressing viral protein were also noted in hRSV-infected, ALX-0171-treated groups from both studies, albeit non-significantly for study 2 (p ≤ 0.002 for study 3 for hRSV-infected ALX-0171-treated group versus RSV-infected placebo-treated group) (Figure 4c and Figure 5c).

Gross lung examination in treated lambs confirmed the antiviral effect of ALX-0171-treatment as mean percent lung consolidation was 40.7% for the placebo-treated lambs compared to 0% and 7.6% for the 0.3 mg/kg and 3 mg/kg ALX-0171-treated lambs in study 2 (Figure 4a). In study 3, the mean percent lung consolidation was 39% for the hRSV-infected, placebo-treated lambs compared to 4.9% in the hRSV-infected, ALX-0171-treated lambs (Figure 5a and Figure 6a).

Clinically, the hRSV-infected placebo-treated lambs in study 2 all had a clinical severity score of ≥ 5. This increased clinical severity score was driven by labored breathing as expiratory effort was evident in all 3 lambs (expiratory effort score of 1) on at least one occasion during the treatment and post-treatment period (i.e., assessments performed on day 4 to day 6) and in 1 lamb on day 0. In contrast to study 1, wheeze was also present on day 0 (in 1 lamb), day 3 (in 1 lamb), day 4 (in 1 lamb), day 5 (in all 3 lambs) and day 6 (in 2 lambs) with a wheeze score of 2 on all occasions. Two of three placebo-treated lambs had blood oxygen saturation levels ≤ 92% on 2 of the 3 days post-treatment initiation which dropped to 90% in one lamb. Respiratory rates, although relatively variable throughout the study duration, were also increased in all placebo-treated lambs with a maximum observed respiratory rate of 84 breaths/

![Figure 4.](image-url) Gross and microscopic evaluation of lungs from hRSV-infected lambs treated with either placebo or ALX-0171 from study 2. (a) Viral-related lung gross lesions were scored and percentage parenchymal involvement was estimated for each lung lobe. Mean percentage averages per lobe were calculated. Legend: Rt Cr = Right cranial lobe; Rt Mid = Right middle lobe; Rt Cd = Right caudal lobe; Acc = Accessory lobe; Lt Cr = Left cranial lobe; Lt Mid = Left middle lobe; Lt Cd = Left caudal lobe. (b) Microscopic (histopathologic) alveolar consolidation score was determined as percent area of RSV lesions followed by conversion to an integer-based consolidation scale as described in materials and methods section. (c) hRSV antigen expression in lung tissue was determined by counting the number of affected bronchi/bronchioles or alveoli per field. (d) Lamb clinical severity scores. Clinical severity scores were calculated as described in Figure 3. Results are expressed as mean ± standard error for panels (a) and (c); group averages with individual lamb scores are indicated by bullet points for panel (b) and median with range for panel (d).
min (55.6% increase) on day 4. The mean relative change in respiratory rates on day 6 compared to day 0 was 23.3% ± 3.1% (mean ± se). In the ALX-0171-treated lambs, clinical severity scores were reduced compared to the placebo-treated lambs (Figure 4d) (p = 0.0214) with 1 lamb scoring 0, 3 lambs scoring 1 and 1 lamb scoring 2. Expiratory effort and wheeze were only present in 1 lamb in the 0.3 mg/kg ALX-0171 dose group on day 3 (expiratory effort and wheeze score of 1 and 2, respectively) and wheeze was present in 1 lamb in the 3 mg/kg ALX-0171 dose group on day 6 (wheeze score of 1). The mean relative change in respiratory rates was -10.6% ± 10.6% and -6.2% ± 9.4% for 0.3 mg/kg and 3 mg/kg ALX-0171-treated lambs on day 6, although this was not significantly different to the placebo-treated lambs.

In study 3, the hRSV-infected placebo-treated lambs and the mock-infected placebo-treated lambs all had a clinical severity score between 0 and 3 due to variable increases in respiratory rates. No noticeable expiratory effort and wheeze was apparent in these lambs. All hRSV-infected placebo-treated lambs on day 2 post-infection demonstrated unremarkable/normal behavior, energy level, perambulatory activity, and were alert and interactive. By the next day, 4 of 5 hRSV-infected lambs clearly exhibited a decline in their general health status (Figure 7). On day 4 and 5 all the hRSV-infected, placebo-treated lambs displayed some degree of reduced general health status as they were more lethargic in their movements, less prone to interact with each other and, when standing or laying down, drooped their heads and ears downward and appeared to be in a generally weakened and/or lethargic state. Blood oxygen saturation was ≤ 92% in 3 of 5 lambs on day 4 or 5. In contrast, none of the mock-infected lambs treated with placebo displayed an altered general health status throughout the study duration. Clinical severity scores in hRSV-infected ALX-0171-treated lambs were similar to the mock-infected, ALX-0171-treated lambs. The only noticeable finding in the hRSV-infected, ALX-0171-treated lambs was variably increased respiratory rates and malaise, which occurred on day 3 just prior treatment initiation. Blood oxygen saturation of only one lamb of this group was ≤ 92% on day 4. None of the mock-infected, ALX-0171-treated lambs displayed an altered general health status over the study duration. In the ALX-0171-treated lambs, a decline in the general health status of 3 of 6 lambs was apparent on day 3 only, which was the first treatment day. On the next and all subsequent days, all the ALX-0171-treated lambs displayed normal behavior and general health status. Clinical severity scores were not significantly different between the groups (p = 0.251).
Dose-response exploration of ALX-0171 on antiviral effects when ALX-0171 treatment is delayed to start on day 3 post-infection

From the results of the previous 3 studies, ALX-0171 administration by inhalation to neonatal lambs was shown to be highly effective. However, no consistent differences were observed between the studied doses. A fourth study was therefore performed to explore the dose of 0.3 mg/kg as anchor dose and 2 additional lower inhaled doses of 0.08 mg/kg and 0.04 mg/kg for which only a partial viral load response was expected. Due
to the low number of lambs used per group and due to the rich blood sampling, only terminal endpoints were assessed in this study. As in the 3 previous studies, hRSV infection in neonatal lambs resulted in robust viral replication in the lungs of vehicle-treated lambs with mean viral load titers on day 6 post-infection of 4.54 Log10 FFU/mL versus 4.49 and 4.83 Log10 FFU/mL on the same day for the previous studies. Viral load was reduced by 2.14 log10 FFU/mL in the 0.04 mg/kg ALX-0171 dose group. In contrast, ALX-0171 treatment at doses of 0.08 mg/kg and 0.3 mg/kg reduced the infectious virus titers to below the limit of quantification. Viral RNA copy numbers, however, were only moderately reduced following ALX-0171 treatment at the 0.3 mg/kg dose (0.54 Log10 RNA copies/mL BALF and 0.37 Log10 RNA copies/mg lung tissue) (Table 2). Regarding the gross lung viral lesions and the histopathological changes on Day 6, the lambs treated with the 0.04 mg/kg ALX-0171 dose generally had the highest scores and the lambs treated with the 0.3 mg/kg ALX-0171 dose generally had the lowest scores overall (Figure 8a–c).

Correlations between the different readouts
As described above, ALX-0171 treatment had a positive effect on viral replication. This viral replication and effects thereof were independently assessed using different readouts (viral titers, viral antigen in lung tissue and lung viral lesions). To better define the relationship between these different variables, the presence of a correlation between these different readouts was assessed for all individual lambs. There were significant positive correlations between the different variables, meaning that, when a lamb scored high on one variable, it also scored high on the others (Figures 9a–f). Additionally, most of the placebo-treated lambs scored higher than the ALX-0171-treated lambs, confirming the positive effects of ALX-0171 treatment.

Discussion
Currently, the only US Food and Drug Administration-approved antiviral treatment for RSV is ribavirin, but, because of conflicting evidence of its effectiveness, it is no longer recommended, with the only remaining treatment modality being supportive care.26,27 For the prevention of RSV-infections, palivizumab administration is only recommended for high risk-infants.8 The development of new approaches and therapeutic modalities are thus needed. This study assessed a novel therapeutic, ALX-0171, in neonatal lambs as a model for hRSV infection in infants.

In vivo efficacy of ALX-0171 has been previously tested in the cotton rat (Sigmodon hispidus).18 Although semi-permissive to hRSV infection, cotton rat studies have proven useful as both RSVIg (RespiGam®) and palivizumab (Synagis®) were advanced to clinical trials solely based on cotton rat data.11 However, as the intended route of administration for ALX-0171 is by inhalation, adequate drug deposition in the lungs is a prerequisite for efficacy. Drug deposition in the lung is largely dependent on three main factors that must be considered when extrapolating the results of preclinical species to humans. Such deposition is strongly affected by: 1) breathing patterns (respiratory frequency and breathing volume), which is dependent on body size; 2) airway anatomy/geometry as function of gender, age and disease status; and 3) by the aerosol aerodynamic diameter, which is dependent on the administration device characteristics.20-22 For small animal species such as rodents, direct instillation of drug to the lungs is the principal lung-dosing method, which offers the advantage of enabling accurate dosing. However, this method can result in substantial variations in lung-regional deposition as well as variations in systemic drug profiles.28 In addition, drugs tend to be less potent using this method compared to an aerosol inhalation method likely due to the altered lung distribution patterns.29 Whole-body exposure is also feasible in rodents, but the size of the inhaled particles cannot be controlled and therefore the exact quantity of drug deposited in the lungs is difficult to determine making extrapolations from rodent data to humans challenging.21 In contrast, and because of their size, the use of large animals offers the opportunity to better study PK, in conjunction with formulation or device efficiency.26 The respiratory system of lambs and human infants share many anatomical, physiological and developmental features that increase the translational value of studies performed in lambs for inhaled drugs.15,20 Colostrum-deprived (lacking maternal antibody and thus any anti-RSV antibodies) neonatal lambs are highly relevant for the study of RSV infection due to their natural susceptibility to ovine, bovine and human strains of RSV30-32 and to the similarities in disease pathogenesis to that of human infants.17,33 This lamb hRSV-infection model therefore constitutes a valuable tool for use in pre-clinical studies of vaccines or therapeutics.

Our previous work has demonstrated that hRSV-infection with Memphis 37 strain in neonatal lambs results in robust viral replication in the lungs that peaks around day 3 post-infection before subsiding by day 8. The viral replication was paralleled by an increase in hRSV lung viral antigen expression, gross and histopathologic lesions and appearance of respiratory distress.17,24,32

Figure 7. Scoring of general health status of hRSV-infected lambs treated with either placebo or ALX-0171. General health status was assessed daily until day 5 post-infection using the following scoring criteria: 0 – No clinical signs; 1 – Reluctant to move; 2 – Reluctant to move, head down, depressed, not interested in eating; 3 – Down, unwilling to get up or difficulty standing, not eating; 4 – Down and should be euthanized, probably cannot eat. Results are shown for each individual lamb and means are shown as bars.

![Figure 7](image-url)
In the studies described here, the administration of nebulized ALX-0171 resulted in systemic exposures that were indicative of absorption dependent PK. Overall, no unexpected retention of ALX-0171 in studied tissues or organs was observed. No difference in ALX-0171 deposition was noted between hRSV-infected and uninfected lambs, although subtle differences may have been masked by the observed variability. Local lung ALX-0171 concentrations attained were adequate for reducing clinical or illness score (malaise), gross and microscopic lung lesions, viral titers, viral antigen, and viral RNA levels, even when administered around the peak of viral replication.

Previous studies have shown that RSV infects ciliated and bronchiolar airway epithelial cells in the respiratory tract, as well as type II pneumocytes. Consistent with these findings, viral antigen expression was present on these cell types in the hRSV-infected lambs, with a significant correlation between hRSV expression in alveoli and bronchioles (Figure 8a). ALX-0171 greatly reduced the viral antigen expression both in the bronchioles and alveoli, which is indicative of a decreased number of infected epithelial cells. This decreased viral antigen expression was correlated with the decreased gross viral lesions (Figure 9b–c) and decreased viral titers (Figure 9d–e). There was also a significant correlation between infectious virus titers and gross viral lesions (Figure 9f). Thus, measuring infectious virus titers in BALF could provide a reliable surrogate endpoint to monitor disease progression, at least in lambs. Similarly, viral loads were consistently correlated with increases in multiple different disease measurements (symptoms, physical examination, and amount of nasal mucus) in a human challenge study and would seem to predict disease severity in previously healthy infants. ALX-0171 was less effective at reducing hRSV RNA copy numbers in lung tissue and BALF (as determined by RT-qPCR) than infectious virus titers, as expected, since ALX-0171 binds virus but does not directly inhibit transcription. Similarly, motavizumab, when given therapeutically to infants hospitalized with RSV disease, resulted in a ≥2 Log10 PFU/mL decrease in infectious virus between day 0 and day 1. This reduction ranged from 0.3 to 1 Log10 PFUe/mL for viral transcripts over the same period. In our studies, infectious virus was reduced by >4 Log10 FFU/mL on day 6 in the ALX-0171-treated lambs compared to the placebo-treated lambs. On the same day, viral RNA titers were reduced by 0.14 to 1.88 Log10 viral RNA copies/mL or mg in both BALF and lung tissue. The likely explanation of the difference between the two measures is that, on the one hand, RT-qPCR not only

Figure 8. Gross and microscopic findings of lungs from mock-infected and hRSV-infected lambs treated with either placebo or ALX-0171 from study 4. (a) Viral-related lung gross lesions were scored and percentage parenchymal involvement was estimated for each lung lobe. Mean percentage averages per lobe were calculated. Legend: Rt Cr = Right cranial lobe; Rt Mid = Right middle lobe; Rt Cd = Right caudal lobe; Acc = Accessory lobe; Lt Cr = Left cranial lobe; Lt Mid = Left caudal lobe; Lt Cd = Left caudal lobe. (b) Microscopic (histopathologic) alveolar consolidation score was determined as percent area of hRSV lesions followed by conversion to an integer-based consolidation scale as described in materials and methods section. (c) hRSV antigen expression in lung tissue was determined by counting the number of affected bronchi/bronchioles or alveoli per field. Results are expressed as mean ± standard error for panels (a) and (c); group averages with individual lamb scores are indicated by bullet points for panel (b).
quantifies fully replication-competent viruses but also complete viral particles unable to replicate, partially assembled virions, and whole and fragmented viral genome, on which ALX-0171 has no effect. Consequently, the natural rate of viral load decline is less steep when using a RT-qPCR method than when using quantitative culture, which is likely to confound antiviral efficacy determination of test compounds targeting RSV.

The culture assay, on the other hand, quantifies fully infectious particles, but it is conceivable that viral particles that are neutralized by ALX-0171, but that are still present in the respiratory tract, would not be quantified in the culture assay whereas they would be quantified by RT-qPCR. The culture assay would thus reflect the fact that either less virus is present in a sample or that any virus still present is effectively neutralized. The positive effects of ALX-0171 on the other viral-related endpoints (viral antigen expression, gross lesions, histopathology) further substantiates the robust anti-viral effect of ALX-0171 in neonatal lambs.

Clinically, in the hRSV-infected neonatal lambs, a decline in respiratory function and general health status was observed in

Figure 9. Correlation between independently scored readouts in hRSV-infected neonatal lambs. Correlation coefficient was calculated by Spearman’s rank correlation test for the indicated parameters. Mock-infected lambs are excluded from the analysis. Black dots show the ALX-0171-treated lambs; red dots show the placebo-treated lambs. Dotted lines represent the 95% confidence bands. QL = Quantification limit; IHC = Immunohistochemistry.
the herein described studies, with observable expiratory effort and wheeze in some lambs and increased respiratory rates. However, these observations were not consistently present in all lambs, as occurs in human infants, thus making the analysis of ALX-0171 effects on altering individual clinical signs difficult. The natural individual susceptibility of hRSV infection in the lambs may explain part of this observation. Similarly, most clinicians recognize bronchiolitis as a "constellation of clinical signs and symptoms occurring in children younger than 2 years, including a viral upper respiratory tract prodrome followed by increased respiratory effort and wheezing"\(^2\) with a high heterogeneity in disease severity likely due to a combination of host and viral factors.\(^4\) Study procedures may also explain part of the variability seen in the clinical parameters as the procedures for clinical assessments were adapted for study 3, allowing for increased observation times and more robust scoring of lamb behavioral changes.

One important challenge in the performance of these studies was the minimization of any cross-contamination between not only hRSV-infected and uninfected lambs, but also of ALX-0171- and placebo-treated lambs, making full blinding of the evaluation of individual clinical parameters difficult. The need to objectify as much as possible the effects of ALX-0171 on these clinical parameters and their observed heterogeneity led to the development of an exploratory composite clinical severity score. Although scoring systems to quantify respiratory distress have been developed for infants, none have been sufficiently validated to allow meaningful clinical use in infants.\(^4,6,7\) Most of these scores are calculated by summing points of each assessed parameter such as respiratory rates, chest wall recessions, general condition, feeding, wheezing and oxygen saturation,\(^4,6,8,9\) where respiratory rate, oxygen need, and presence of retractions were considered the most useful in predicting emergency department disposition.\(^4\)

In the absence of validated clinical scores for lambs, we decided to include respiratory rates, expiratory efforts and wheeze as well as blood oxygen saturation and body weight gain as these parameters were considered meaningful based on currently available scoring systems for infants (see supplemental data Table S1). All these parameters have also been shown to be important in hRSV-disease both in animal models and infants.\(^2,5,26,32,48-53\) This analysis showed that ALX-0171 treatment reduced the clinical severity and had a positive effect on general health status. However, in the different studies performed the maximal clinical scores reached were variable, with placebo-treated lambs from study 2 reaching scores of 5 or 6 of a theoretical maximal clinical severity score of 11, which is reflective of moderate disease. Similarly, infants admitted to hospital with moderate disease severity had clinical scores of half the maximal scale, while severe disease was considered as being scores of \(\geq 14\) of \(20^{25}\) or \(\geq 4\) of \(5^{49}\). Thus, variability of clinical scores in lambs parallels that of infants and this likely reflects differences in hRSV infection severity between lambs. All the inoculated lambs become infected with hRSV and this likely explains part of this observation. Similarly, most lambs, as occurs in human infants, thus making the analysis of ALX-0171 was noted on all established markers, although the clinical and quantitative expression of hRSV infection differed somewhat in the different studies. Viral replication parameters were consistent with associated gross and microscopic lesions and viral antigen distribution. That is, in lambs not treated with ALX-0171, the high hRSV titers and high levels of hRSV RNA were associated with increased severity of gross and microscopic lesions and viral antigen distribution. Following ALX-0171 treatment, reduced hRSV parameters were associated with reduced lesions and viral antigen expression. There was a trend to dose dependency seen with the 0.3 mg/kg dose already providing full efficacy. ALX-0171 administered therapeutically may thus have the potential to be effective in the context of a developed hRSV infection in infants. Inhaled ALX-0171 is currently being evaluated in Phase 2 studies in infants and young children hospitalized for RSV lower respiratory tract infection (clinicaltrials.gov numbers NCT02979431 and NCT03418571).

**Materials and methods**

**Experimental design**

Four independent studies were performed in which a total of 53 lambs were randomly assigned based on weight and sex to three or four groups depending on the study (Table 1). In study 1, thirteen lambs were assigned to four groups (groups 1, 2, 3 and 4). Three lambs were in group 1 (hRSV-infected placebo-treated), four lambs were in group 2 (hRSV-infected 0.3 mg/kg ALX-
0171-treated), three lambs were in group 3 (RSV infected 1 mg/kg ALX-0171-treated) and three lambs were in group 4 (hRSV-infected 3 mg/kg ALX-0171-treated). In study 2, 10 lambs were assigned to three groups (groups 1, 2 and 3). Three lambs were in group 1 (hRSV-infected placebo-treated), four lambs were in group 2 (hRSV-infected 0.3 mg/kg ALX-0171-treated) and three lambs were in group 3 (hRSV-infected 3 mg/kg ALX-0171-treated). In study 3, 22 lambs were assigned to four groups (groups 1, 2, 3 and 4). Five lambs were in group 1 (uninfected placebo-treated), six lambs were in group 2 (uninfected 0.3 mg/kg ALX-0171-treated), five lambs were in group 3 (hRSV-infected placebo-treated) and six lambs were in group 4 (hRSV-infected 0.3 mg/kg ALX-0171-treated) (see Table 1). In study 4, two lambs were assigned to each of 4 groups. Group 1 lambs were hRSV-infected and placebo-treated, whereas group 2, 3 and 4 lambs were hRSV-infected and treated with ALX-0171 at 0.04 mg/kg, 0.08 mg/kg and 0.3 mg/kg, respectively. On day 0, all lambs in study 1, study 2, study 3 (except group 1 and 2) and study 4 were infected with hRSV Memphis 37 strain by the inhalation route, whereas uninfected control lambs (study 3 – groups 1 and 2) received nebulized cell-conditioned media as described below. ALX-0171 treatment started either on Day 1 (study 1) or Day 3 (study 2, 3 and 4) post-infection and was repeated daily until Day 5 post-infection. In total, four lambs were excluded from the studies on various days post-infection because of secondary bacterial infections. One lamb was from study 1 group 4, two lambs were from study 2 group 2 and one lamb was from study 3 group 2. On day 6 post-infection, all other lambs were euthanized with an intravenous injection of sodium pentobarbital (Beuthanasia®, Schering-Plough Animal Health Corporation) overdose (1 mL/5 kg). During necropsy, tissue samples were collected from each lung lobe of all animals in the same manner, with uniform sampling of each lobe.

**Animals**

Male and female Suffolk, Polypay or Dorsett cross colorstrum-deprived neonatal lambs (1-3 days of age) were obtained from local farms (Lester, Iowa, USA). Lambs were fed lamb milk replacer (Milk Products Inc., Chilton, WI, USA) that lacked supplemental iodide as of birth and were given Naxcel (Ceftiofur sodium, Pfizer) 1–2 mg/kg subcutaneously once daily to reduce/prevent secondary bacterial infections. The animals had not been subjected to other experiments before the study. All efforts were made to minimize animal discomfort and limit the number of animals used. Study protocols were approved by the Institutional Animal Care and Use Committee (approval #12-12-7470-O and 8-15-8064-O) and were performed in accordance with the animal welfare bylaws of the Iowa State University, which are in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) regulations. The studies were also approved by the Institutional Biosafety Committee (approval #10-D/I-0020-A and 15-I-0024-A/H) at Iowa State University.

**Compounds**

ALX-0171 is a trimeric Nanobody consisting of three hRSV-targeting subunits linked by two glycine-serine linkers, and was produced using a Pichia pastoris X-33 expression system. ALX-0171 formulation buffer, which consists of NaCl as osmolality agent and phosphate as buffer component, was used as a placebo. The formulation components and their concentration were selected based on their compatibility with pulmonary administration.

**Infection of lambs**

PARI LC Sprint™ nebulizers (PARI Respiratory Equipment, Inc., Lancaster, PA, USA) were used to administer virus or cell-conditioned media (media from HEp-2 cells lacking hRSV) to each lamb. They were attached to a conical mask fitted with a round rubber diaphragm with a pre-cut center hole through which the nose and mouth of the lamb was inserted (MidWest Veterinary Supply, Inc., Burnsville, MN). Three 2 mL aliquots of virus-containing media or control media were administered to each animal over the course of 23 minutes at 4 L/min at 16 PSI (Philips Respironics Air Compressor, Andover, MA, USA), resulting in the total inhalation of about 6 mL by each lamb. Identical viral inoculum doses were used for each lamb (hRSV M37 strain at 1.27-1.48 × 10⁷ FFU/mL in media with 20% w/v sucrose).

**Drug administration**

An Aeroneb® Solo System (Aerogen Ltd, Galway, Ireland), consisting of the Aeroneb® Solo mesh nebulizer and the Aeroneb® Pro-X controller were used in accordance with the instruction manual as provided by the manufacturer. The estimated particle sizes obtained with these meshes were in the range of 3.27 ± 0.13 μm (MMAD). The assembly and operation of the Aeroneb® Solo System was done according to the nebulizer instruction manual. The nebulizer and the T-piece were inserted into the breathing circuit. Air was supplied to the system at an airflow speed of 2 L/min using a compressed air canister that was attached directly to the nebulizer T-piece.

Prior to dosing, the nebulizer reservoir was filled with following volumes of different concentrations of ALX-0171 or ALX-0171 formulation buffer (placebo): either 4 mL (3 mg/kg target inhaled dose), 1.3 mL (1 mg/kg target inhaled dose) 0.4 mL (0.3 mg/kg target inhaled dose), 0.2 mL (0.08 mg/kg target inhaled dose) or 0.1 mL (0.04 mg/kg target inhaled dose). A cone mask (Cat # 05305, A.M. Bickford, Inc, US) was attached to the nebulizer T-piece and was placed over the lamb’s nose, mandible and maxilla. The nebulizer was turned on at a constant nebulization mode and the cone mask was firmly held in place during the duration of the nebulization. Once the dose had been nebulized (i.e., when the nebulizer reservoir was empty), the face mask was removed and the nebulizer switched off. The lamb was then returned to its cage and general health (alertness, responsiveness, ability to stand and move) was monitored for 10 minutes.

**Calculation of inhaled dose**

The total dose emitted by the nebulizer was determined per administration by weighing the nebulizer before and after the administration and deducting the nebulized volume. The
nebulized volume of ALX-0171 was then multiplied by the concentration of ALX-0171 to determine the delivered dose. The inhaled dose per lamb (i.e., the dose reaching the tip of the snout) was estimated to be 11% of the delivered dose based on experiments in which a similar nebulizer setup as the one that was used in these studies was connected to a breathing simulator programmed for neonatal breathing. The inhaled fraction was defined as the fraction that was found back on the inhalation filter in relation to the total nebulized dose. The body-weight adjusted inhaled doses were expressed as mg/kg and were calculated as the inhaled dose on each specific day divided by the measured body weight on the corresponding dosing day.

**Preparation of hRSV Memphis 37 virus stock**

Memphis 37 (M37) hRSV is a wild type RSV-A, first isolated from a 4 month-old infant and used in human clinical studies. HEp-2 cells were infected in 300 cm² flasks at 0.5-1 multiplicity of infection (MOI) at a confluence of 80-90% by applying 4-5 mL of hRSV stock (Memphis 37 hRSV that was used originated from Meridian LifeScience, Memphis, TN, USA). Usually within 48-60 hours, abundant syncytia appeared and close to 100% cytopathic effect was reached (as ascertained using a large rubber policeman (sterile plastic tool commonly used for cell scraping). The collected cell solutions were transferred to polypropylene 15 mL conical centrifuge tubes (~1 tube per 300 cm² flask), vortexed for 10 seconds at high speed and centrifuged at 2500 rpm (~1260 x g) for 10 minutes. Supernatants were then collected from each tube and kept in separate tubes, while each resulting pellet was resuspended in 5 mL of phosphate-buffered saline (PBS) pH 7.4. Each cell pellet sample was then sonicated for 5 × 2-second pulses with 5 seconds in between (to avoid overheating samples) using a tip-style sonicator (Sonic Dismembrator Model 500, Fisher-Scientific) with a pre-sterilized tip (tip was pre-soaked in 70% ethanol for 10 minutes followed by air-drying). Sonicated samples were then centrifuged again at 2500 rpm (~1260 x g) for 10 minutes and the resulting supernatants were added to each of the corresponding supernatants collected initially. The samples were then diluted with a solution of 60% sucrose, 10% fetal bovine serum (FBS) in PBS to obtain 20% w/v total sucrose concentration, which was shown to be beneficial in preserving virulence post freeze-thaw and post-nebulization. The titer of virus was subsequently determined, after one freeze-thaw cycle at -80°C, in a small portion of the resulting stocks that were aliquoted into cryovials for this purpose using the infectious FFU assay. The remainder of the stock was frozen/stored at -80°C until needed for use in the lambs. The Memphis 37 hRSV strain used in this study was passaged 6 times on Vero cells, then twice on HEp-2 cells. Sucrose was added to 20% and the virus stock was frozen at −80°C and titered for infectivity on HEp-2 cells as has been characterized previously in this model.

**Monitoring of clinical parameters**

Lambs were monitored daily for body weight, rectal temperature, heart rate, respiratory rates (by auscultation) and percent blood oxygenation measurements. Oxygenation levels of arterial blood were assessed by a pulse oximeter (PalmSAT 2500A VET, Nonin Medical, Inc Plymouth, MN, USA). The probe of the oximeter was manually secured at the root of the tail (a naturally hairless site), nearest the anus. The femoral artery was then palpated to measure the pulse rate and was compared with the pulse rate displayed on the oximeter. The Sp0₂ values were considered for the analysis only if the two pulse rates were within 10% of each other as described. Increased expiratory effort (forced expiration) was scored daily, as were animal “wheeze” scores, using criteria previously reported.

**Blood collection**

Approximately 1-1.5 mL of blood (at least 500 μL of plasma) was drawn from the external jugular vein with a syringe, and placed in spray-coated K₂EDTA vacutainer tubes. The fur at the sampling site was shaved and extensively washed prior to blood withdrawal to avoid any contamination of deposited ALX-0171. The blood samples were then centrifuged at 1,600 x g for 10 min at 4°C in order to obtain plasma. Samples were stored at ≤ -60°C prior to analysis.

**Collection of bronchoalveolar lavage fluid**

Following euthanasia, the lungs of each lamb were removed and each left and right lung was separated and weighed. BALF was collected and processed from the right lung lobe as previously described.

**Gross lesions evaluation and scoring**

Following euthanasia, the thorax was opened and the heart and esophagus were removed from the lungs. The percentage parenchymal involvement of gross hRSV lesions was scored for each individual lung lobe. The percentage of a specific lobe tissue that was affected by hRSV in relation to the overall lobe tissue being scored was estimated. Mean percentage averages per lobe were calculated for each day of necropsy.

**Histologic lung evaluation and scoring**

A histologic score was determined by evaluating percent involvement. This consolidation score is an overall score based on the percentage of lung involvement in areas with hRSV lesions. Alveolar consolidation was defined by reduced expansion of alveolar lumen due to alveolar septal infiltration of neutrophils, lymphocytes, plasma cells, and type II cell hypertrophy along with intraluminal accumulation of neutrophils, macrophages, and small amounts of cell debris. The score was defined by converting the observed percentage ranges to a simple integer-based consolidation scale: 0% consolidation = 0, 1-9% consolidation = 1, 10-39% consolidation = 2, 40-69% consolidation = 3, 70-100% consolidation = 4. Group averages were calculated for the alveolar consolidation score. In addition to the alveolar consolidation score, bronchiolitis, neutrophil infiltration, peribronchial and perivascular infiltration of lymphocytes, syncytial cell formation, and epithelial alterations were also individually scored as previously published.
Immunohistochemistry for viral antigen detection

Immunohistochemistry for the detection of hRSV antigen was performed on 5 μm-thick formalin-fixed paraffin-embedded lamb lung tissue sections taken from the right and left cranial, left middle, and left caudal lung lobes of each animal in accordance with methods published previously.24 20 unique 10X fields on each slide (containing two lung sections each) were assessed for hRSV antigen staining by counting positively-stained cells within bronchioles and alveoli. The mean number of stained bronchi/bronchioles and alveoli per field were counted.

Reverse transcription polymerase chain reaction assessment of hRSV RNA expression levels in lamb lung tissue

For each animal, tissue samples from right and left cranial, left middle and left caudal lung lobes (0.3-0.4 g of each lobe) were homogenized for total RNA isolation in TRIzol (Invitrogen), assessed for quantity and purity by spectrometry, and then RT-qPCR was performed using a One-Step Fast RT-qPCR kit master mix (Quanta, BioScience, Gaithersburg, MD) in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, CA) with PREXCEL-Q for all set up calculations as previously described.32 62 Primer and probe sequences for all targets were designed with ABI Primer Express 2.0, and have been used previously.32 62 All samples were diluted to achieve a final RT-qPCR concentration of 0.784 ng/μL. Thermocycling conditions were 5 minutes at 50°C; 30 seconds at 95°C; and 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Samples and standards were assessed in duplicate, and each target gene quantification cycle (Cq) value was converted to a relative quantity (Qr) based on each target’s standard curve using: Qr = EAMP(b-Cq), wherein “b” and “EAMP” are the y-intercept and exponential PCR amplification value, respectively. EAMP values were obtained from the slope (m) of each target standard curve by: EAMP = 10(-1/m), and all Q, values interpolated from standard curves were normalized to total lung RNA per RT-qPCR. No-RT control reactions gave either no signal or generated Cq values greater than 13 cycles later than those in the corresponding RT-qPCR target reactions.

Reverse transcription polymerase chain reaction assessment for hRSV RNA in bronchoalveolar lavage fluid

Viral RNA was quantified by RT-qPCR in BALF obtained from the right caudal lung lobe of each animal at necropsy as previously described.24 RT-qPCR for hRSV was then carried out as described above.

Focus forming unit assay

BALF viral titers were measured for each lamb by flushing the excised right caudal lung lobe with 5 mL of cold modified Iscove’s media (42.5% Iscove’s modified Dulbecco’s medium, 7.5% glycerol, 1% heat-inactivated FBS, 49% DMEM, and 50 μg/mL kanamycin sulfate) after which 1 mL of the resulting BAL fluid was placed on ice and spun down for 5 minutes in a centrifuge at 3000 x g to pellet large debris. Approximately 800-850 μL of each supernatant was collected and then spun through 850 μL-capacity 0.45 μm Costar SPIN-X filter at 15,600 x g for 5 minutes before being used in the standard infectious FFU. For this, 200 μL of serially-diluted BALF samples were applied to HEp-2 cells (ATCC, CCL-23) grown to 70% confluence in 12-well culture plates (cat n° 07-200-81; Fisher Scientific, Hanover, IL) in DMEM media (Mediatech, Inc., Manassas, VA) supplemented to 10% with heat-inactivated FBS (Atlanta Biologicals, Atlanta, GA) and 50 μg/mL kanamycin sulfate (Invitrogen). Each sample was analyzed undiluted and at four additional serial-dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 in duplicate. Following a 48 hour incubation at 37°C, 5% CO2, the cells were fixed with cold 60% acetone/40% methanol solution for 1 minute. Overnight primary antibody (Goat polyclonal Ab to hRSV [all antigens] Cat n° AB1128, EMD/Millipore/Chemicon, Billerica, MA) incubation was then followed by washing and secondary antibody (Alexa Fluor 488 F(ab)2 fragment of rabbit anti-Goat IgG (H+L), cat n° A21222; Molecular Probes/Life Technologies) incubation for 30 minutes. Plates were rinsed and inspected for the presence of fluorescing foci of infection using the FITC/GFP filter on an inverted fluorescence microscope (Olympus CKX41, Center Valley, PA, USA). Five or more fluorescing cells were counted as single focal events. An average of 40 counts in a 1:10- diluted (duplicate) sample indicated an original BALF sample “titer” of 2,000 [40 counts x dilution of 10 × 1,000 μL/mL]/200 μL assessed = 2,000 infectious FFU/mL. The quantification limit of the assay was set at 5 FFU/mL (0.7 Log10 FFU/ML).

Quantification of urea in BALF and in plasma

Quantification of urea in both plasma and BALF was done using the QuantiChrom® Urea Assay Kit (BioAssay Systems) according to the manufacturers instructions. The method was validated specifically for lamb BALF and plasma. During urea sample analysis, the calibrator inter-curve precision did not exceed 5.4% and the intercurve accuracy was between -3.8% and 3.0%.The inter-assay precision of the QC samples did not exceed 2.5% and the inter-assay relative error was between -0.6% and 2.1% for plasma and BALF combined.

Quantification of ALX-0171 in BALF and plasma

Plasma samples from hRSV-infected lambs were treated by UV-irradiation for 1 hour in a biosafety cabinet prior to analysis in the ELISA. During qualification of the assay, it was demonstrated that this treatment did not affect the accuracy of ALX-0171 determination in plasma. For BALF samples, ALX-0171 quantification was performed in a biosafety cabinet. Qualified ELISA and/or MSD methods were used for the quantification of ALX-0171 in lamb plasma and BALF. In brief for the ELISA methods, an anti-Nanobody Nanobody (Ablynx) for plasma or a Nanobody-specific mouse monoclonal antibody generated against ALX-0171 (Ablynx) for BALF were coated overnight on a 96-well Maxisorp plate (Nunc). Samples were applied on the coated plate and ALX-0171, present in the sample, was detected with a biotinylated monoclonal antibody.
generated against ALX-0171. This mAb was then detected by horseradish peroxidase (HRP)-labeled streptavidin (Thermo Scientific). Bound streptavidin-HRP was revealed by adding 100 μl/well of soluble high-sensitivity tetramethylbenzidine (s(HS)TMB; SDT Reagents) for 15 min, followed by 1 N HCl. The absorbance was read at 450 nm using a spectrophotometer, and the reference wavelength was 620 nm. The sensitivity of the ELISA methods were 21.5 ng/mL and 10.7 ng/mL in plasma and BALF, respectively. During ALX-0171 sample analysis, the calibrator inter-curve precision did not exceed 8.8% and the intercurve accuracy was between -4.8% and 9.9%. The interassay precision of the QC samples did not exceed 14.2% and the inter-assay relative error was between -10.7% and 6.6% for plasma and BALF combined.

For study 4, a more sensitive method was used. In brief, streptavidin GOLD plates (MSD Mesoscale) were blocked with SuperBlock T20 (Thermo Scientific) and a biotinylated Nano-body-specific mAb was applied on the plates as capturing reagent. Samples were applied on the plate and ALX-0171, present in the sample, was detected with a sulfo-tagged Nano-body-specific mAb. After addition of 1/2 diluted Read Buffer, electricity was applied to the plate electrodes by the MSD instrument leading to light emission by the sulfo label of the mAb. Light intensity was subsequently measured by the MSD instrument. The sensitivity of the MSD method was 1.0 ng/mL. During ALX-0171 sample analysis, the calibrator inter-curve precision did not exceed 4.5% and the intercurve accuracy was between -4.9% and 5.4%. The inter-assay precision of the QC samples did not exceed 12.0% and the inter-assay relative error was between -14.8% and 13.4% for plasma.

Calculation of ALX-0171 concentration in lung Epithelial Lining Fluid (ELF)

The ALX-0171 concentration in the epithelial lining fluid at necropsy was calculated based on the ALX-0171 concentration measured in BALF and following normalization by the Urea method. The urea concentration in BALF was first corrected for possible contamination with urea from blood using the method described previously. The concentration of ALX-0171 in epithelial lining fluid was calculated as described in different studies using the following formula: \[ \text{ELF}_{\text{ALX-0171}} = \text{BALF}_{\text{ALX-0171}} \times \frac{[\text{Urea}_{\text{Plasma}}]}{[\text{Urea}_{\text{BALF Corr}}]} \]. Where: \( \text{ELF}_{\text{ALX-0171}} \) is the ALX-0171 concentration in epithelial lining fluid, \( \text{BALF}_{\text{ALX-0171}} \) is the ALX-0171 concentration in recovered BALF, \( [\text{Urea}_{\text{Plasma}}] \) is the concentration of urea in plasma and \( [\text{Urea}_{\text{BALF Corr}}] \) is the corrected urea concentration in BALF.

Statistical analysis

All results were analyzed and interpreted using methods appropriate for the type of response analyzed. The lung measurements (viral RNA copies/mL BALF and mg of tissue) were analyzed with an ANOVA type model and the correction for multiple testing was performed using the Hommel procedure. The histopathological responses were summed per animal and per response and were analyzed with a Poisson model. The gross lesions and the immunohistochemistry scores were analyzed with a negative binomial model whereas the continuous responses were analyzed with a mixed model. All statistical analyses were performed using SAS 9.4. Interpretation of outcome (e.g., statement on significance including significance level as a p value) are given in the text or in the caption of figures. For clinical severity scores, a Kruskal-Wallis test was used but, due to this exploratory endpoint, it was decided to perform an exploratory analysis at a 5% significance level. Statistical analysis was performed with GraphPad Prism (version 7) software.

Abbreviations

BALF Bronchoalveolar lavage fluid
BQL Below quantification limit
DMEM Dulbecco’s Modified Eagle Medium
ELF Epithelial lining fluid
FBS Fetal bovine serum
FFU Focus Forming Units
(h)RSV (Human) Respiratory Syncytial Virus
IC90 Inhibitory Concentration 90%
LLOQ Lower limit of quantification
MMAD Mass Median Aerodynamic Diameter
PBS Phosphate-buffered Saline
PK Pharmacokinetics
RNA Ribonucleic acid
Rpm Rotations per minute
RT-qPCR Reverse transcription quantitative polymerase chain reaction

Disclosure of Potential conflicts of interest

L.De. and L.Du. are current employees of Ablynx; all employees own stock/stock options of Ablynx. T.S. was an employee of Ablynx at the time of data generation. A.L.M., J.M.G., A.V.G., M.R.A. undertook this work as part of a research contract with Ablynx NV.

Acknowledgments

We thank Diane Gerjets, Toni Christofferson, and Jennifer Groeltz-Thrush for their technical histology assistance and expertise, as well as Diane McDonald, Kathleen Mullin, Michelle J. Tye, Alan D. Elsberry, and Dale Hinderaker of the Laboratory Animal Resources (LAR) and the Livestock Infectious Disease Isolation Facility (LIDIF). We are also thankful to Wouter Willems and Ariella Van de Sompeel for performing the statistical analysis.

Funding

The work was funded by Ablynx NV and by the “Agentschap voor Innovatie door Wetenschap en Techniek (IWT)” – Belgium – grant N° 130562.

ORCID

Jack M. Gallup http://orcid.org/0000-0003-4675-0731
Mark R. Ackermann http://orcid.org/0000-0002-3522-5395

References

1. Afonso CL, Amarasinghe GK, Bányai K, Bao Y, Basler CF, Bavari S, Bejerman N, Blasdell KR, Briand FX, Briese T, et al. Taxonomy of the order Mononegavirales: update 2016. Arch Virol. 2016;161(8): 2351–60. doi:10.1007/s00705-016-2880-1. PMID:27216929.
2. Everard M. Diagnosis, admission, discharge. Paediatr Respir Rev. 2009;10(Suppl 1):18–20. doi:10.1016/S1526-0542(09)70099-0. PMID:19651395.

3. Favreux B. Special populations. Paediatr Respir Rev. 2009;10(Suppl 1):21–2. doi:10.1016/S1526-0542(09)70010-7. PMID:19651396.

4. Shi T, McAllister DA, O'Brien KL, Simoes EAF, Madhi SA, Gessner BD, Polack FP, Tsiodra O, Acacio S, Aguayo C, et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study. Lancet. 2017;390(10098):946–58. doi:10.1016/S0140-6736(17)30938-8. PMID:28689664.

5. Falsey AR, Hennessey PA, Formenta MA, Cox C, Walsh EE. Respiratory viral infections in elderly and high-risk adults. The N Engl J Med. 2005;352(17):1749–59. doi:10.1056/NEJMoa043951. PMID:15858184.

6. Drysdale SB, Green CA, Sande CJ. Best practice in the prevention and management of paediatric respiratory syncytial virus infection. Ther Adv Infect Dis. 2016;3(2):63–71. doi:10.1177/2049936116630243. PMID:27034777.

7. Resch B. Respiratory Syncytial Virus Infection in High-Risk Infants – an Update on Palivizumab Prophylaxis. Open Microbiol J. 2014;8:71–7. doi:10.2174/1874288081408010071. PMID:25132870.

8. Updated guidance for palivizumab prophylaxis among infants and young children at increased risk of hospitalization for respiratory syncytial virus infection. Pediatrics. 2014;134(2):415–20. doi:10.1542/peds.2014-1665. PMID:25070315.

9. Byrd LG, Prince GA. Animal models of respiratory syncytial virus infection. Clin Infect Dis. 1997;25(6):1363–8. doi:10.1086/516152. PMID:9431379.

10. Bem RA, Domachowske JB, Rosenberg HF. Animal models of respiratory syncytial virus infection. Am J Physiol Lung Cell Mol Physiol. 2011;301(2):L148–9. doi:10.1152/ajplung.00065.2011. PMID:21571908.

11. Niewiesk S, Prince GA. Diversifying animal models: the use of sheep models: from asthma to vaccines. Trends Biotechnol. 2008;26(5):259–66. doi:10.1016/j.tibtech.2008.02.002. PMID:18353472.

12. Scherlirck JP, Snibson KJ, Bowles VM, Sutton P. Biomedical applications of sheep models: from asthma to vaccines. Trends Biotechnol. 2008;26(5):259–66. doi:10.1016/j.tibtech.2008.02.002. PMID:18353472.

13. Derscheid RJ, van Geelen A, Davis MS, Stohr T, Gallup JM, Ackermann MR. Kinetics of Respiratory Syncytial Virus (RSV) memphis strain 37 (M37) infection in the respiratory tract of newborn lambs as an RSV infection model for human infants. PLoS One. 2015;10(12):e0143580. doi:10.1371/journal.pone.0143580. PMID:26641081.

14. Boukhvalova MS, Prince GA, Blanco JC. The cotton rat model of infectious diseases. Lab Anim. 2011;45(10):1479–85. doi:10.1258/labmed.2011.011071. PMID:22299825.

15. Berthiaume L, Joncas J, Boulay G, Pavilanis V. Serological evidence of respiratory viral infections. Biologicals. 2009;37(3):152–9. doi:10.1016/j.biologicals.2009.02.017. PMID:19394861.

16. Cooper AE, Ferguson D, Grime K. Optimisation of DMPK by the inhalation of biotherapeutics for lung diseases. 2016.

17. Derscheid RJ, van Geelen A, Davis MS, Stohr T, Gallup JM, Ackermann MR. Kinetics of Respiratory Syncytial Virus (RSV) membrane strain 37 (M37) infection in the respiratory tract of newborn lambs as an RSV infection model for human infants. PLoS One. 2015;10(12):e0143580. doi:10.1371/journal.pone.0143580. PMID:26641081.

18. Villenave R, Thavagnanam S, Sarlang S, Parker J, Douglas I, Skibinski G, Heaney LG, McKaigue JP, Coyle PV, Shields MD, et al. In vitro modeling of respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target of infection in vivo. Proc Natl Acad Sci U S A. 2012;109(13):5040–5. doi:10.1073/pnas.1110203109. PMID:22411804.

19. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol. 2002;76(11):5655–66. doi:10.1128/JVI.76.11.5655-5666.2002. PMID:11991994.

20. Zicari D, Murphy P, Carson JL, Noel TL, Zhang L, Pickles RJ. The effect of respiratory syncytial virus on chemokine release by differentiated airway epithelium. Exp Lung Res. 2004;30(1):43–57. doi:10.1080/01902140490252812. PMID:14967603.

21. Tyrrell DA, Mika-Johnson M, Phillips G, Douglas WH, Chapple PJ. Infection of cultured human type II pneumocytes with certain respiratory viruses. Infect Immun. 1979;26(2):621–9. PMID:232693.
38. Peebles RS Jr, Graham BS. Pathogenesis of respiratory syncytial virus infection in the murine model. Proc Am Thoracic Soc. 2005;2(2):110–5. doi:10.1513/pats.200501-002AW.

39. DeVincenzo JP, Wilkinson T, Vaishnaw A, Cehelsky J, Meyers R, Nochur S, Harrison L, Meeking P, Mann A, Moane E, et al. Viral load drives disease in humans experimentally infected with respiratory syncytial virus. Am J Respir Crit Care Med. 2010;182(10):1305–14. doi:10.1164/rccm.201002-0221OC. PMID:20622030.

40. DeVincenzo JP, El Saleeby CM, Busj AJ. Respiratory syncytial virus load predicts disease severity in previously healthy infants. J Infect Dis. 2005;191(11):1861–8. doi:10.1086/430008. PMID:15871119.

41. Buckingham SC, Busj AJ, DeVincenzo JP. Nasal quantity of respiratory syncytial virus correlates with disease severity in hospitalized infants. Pediatr Infect Dis J. 2000;19(2):113–7. doi:10.1097/00006454-200002000-00006. PMID:10693996.

42. Lagos R, DeVincenzo JP, Muñoz A, Hultquist M, Suzich J, Connor EM, Losonsky GA. Safety and antiviral activity of motavizumab, a respiratory syncytial virus (RSV)-specific humanized monoclonal antibody, when administered to RSV-infected children. Pediatr Infect Dis J. 2009;28(9):835–7. doi:10.1097/INF.0b013e3181a165ef. PMID:19663278.

43. Perkins SM, Webb DL, Torrance SA, El Saleeby C, Harrison LM, Atken JA, Patel A, DeVincenzo JP. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. J Clin Microbiol. 2005;43(5):2356–62. doi:10.1128/JCM.43.5.2356-2362.2005. PMID:15872266.

44. Murray P, Laxton T, Alton C, Conibear B, Sargent T. Measurement of viral load in bronchiolitis. Hosp Pediatr. 2012;2(4):202–6. doi:10.1542/hpeds.2012-0013. PMID:24313026.

45. Behkof J, Reimink R, Brand PL. Systematic review: insufficient validation of clinical scores for the assessment of acute dyspnoea in wheezing children. Paediatr Respir Rev. 2014;15(1):98–112. PMID:24120749.

46. Oymar K, Skjerven HO, Mikalsen IB. Acute bronchiolitis in infants, a multicentre, randomized, controlled trial of dexamethasone for bronchiolitis. N Engl J Med. 2007;357(4):331–9. doi:10.1056/NEJMoa071255. PMID:17652648.

47. Goralski JL, Davis SD. Breathing easier: addressing the challenges of aerosolizing medications to infants and preschoolers. Respir Med. 2014;108(8):1069–74. doi:10.1016/j.rmed.2014.06.004. PMID:25012949.

48. Kim YI, DeVincenzo JP, Jones BG, Rudraraju R, Harrison L, Meyers R, Cehelsky J, Alvarez R, Hurwitz JL. Respiratory syncytial virus human experimental infection model: provenance, production, and sequence of low-passaged memphis-37 challenge virus. PLoS One. 2014;9(11):e131300. doi:10.1371/journal.pone.0131130. PMID:25415360.

49. DeVincenzo JP, Whitley RJ, Mackman RL, Scaglioni-Weinlich C, Harrison L, Farrell E, McBride S, Lambkin-Williams R, Jordan R, Xin Y, et al. Oral GS-5806 activity in a respiratory syncytial virus challenge study. N Engl J Med. 2014;371(8):711–22. doi:10.1056/NEJMc1401184. PMID:25140957.

50. DeVincenzo J., Lambkin-Williams R, Wilkinson T, Cehelsky J, Nochur S, Walsh E, Meyers R, Gollob J, Vaishnaw A. A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. Proc Natl Acad Sci U S A. 2010;107(19):8800–5. doi:10.1073/pnas.0912186107. PMID:20412463.

51. DeVincenzo JP, McClure MW, Symons JA, Fathi H, Westland C, Chanda S, Lambkin-Williams R, Smith P, Zhang Q, Beigelman L, et al. Activity of oral ALS-008176 in a respiratory syncytial virus challenge study. N Engl J Med. 2015;373(21):2048–58. doi:10.1056/NEJMoa1413275. PMID:26580997.

52. Grosz DD, van Geelen A, Gallup JM, Hostetter SJ, Dersch RJ, Ackermann MR.Sucrose stabilization of Respiratory Syncytial Virus (RSV) during nebulization and experimental infection. BMC Research Notes. 2014;7:158. doi:10.1186/1756-0500-7-158. PMID:24620844.

53. Torres JA, Jackson PG, Taylor PM. Measurement of arterial oxygen- haemoglobin saturation in newborn lambs by pulse oximetry. Vet Rec. 1998;142(5):107–9. doi:10.1136/vetrec.142.5.107. PMID:9501385.

54. Dersch RJ, van Geelen A, McGill JL, Gallup JM, Cihlar T, Sacco RE, Ackermann MR. Human respiratory syncytial virus Memphis 37 grown in Hep-2 cells causes more severe disease in lambs than virus grown in Vero cells. Viruses. 2013;5(11):2881–97. doi:10.3390/v5112881. PMID:24284879.

55. Rennard SI, Basset G, Lecossier D, O’Donnell KM, Pinkston P, Martin PG, Crystal RG. Estimation of volume of epithelial lining fluid recov- ered by lavage using urea as marker of dilution. J Appl Physiol. 1986;60(2):532–8. doi:10.1152/jappl.1986.60.2.532. PMID:3512059.

56. Conte JE Jr, Golden JA, Duncan S, McKenna E, Zurlinden E. Intrapulmonary pharmacokinetics of clarithromycin and of erythromycin. Antimicrob Agents Chemother. 1995;39(2):334–8. doi:10.1128/AAC.39.2.334. PMID:7726492.

57. Furui H, Saisho Y, Yoshikawa T, Shimada J. Intrapulmonary pharmacokinetics of S-013420, a novel bicyclolide antibacterial, in healthy Japanese subjects. Antimicrob Agents Chemother. 1995;39(2):334–8. doi:10.1128/AAC.39.2.334. PMID:7726492.