Supplementary methods

Animals

This study was conducted under legislation of the Dutch Central Authority for Scientific procedures on Animals and after approval of the Body of Animal Welfare of Wageningen University and Research. Twelve 4-week old, colostrum-deprived dairy calves were transported to and raised in an isolated environment after birth until the start of the study. All calves were tested free of antibodies against bRSV (Priocheck ELISA bRSV, Thermo Scientific, Rockford, IL, USA). In addition, after the study we tested a selection of broncho-alveolar lavage samples by PCR to exclude co-infection with bovine respiratory pathogenic bacteria, including: *Pasteurella multocida*, *Histophilus somni*, *Mannheimia heamolytica*, *Arcanobacterium pyogenes* and *Mycoplasma* species.

Virus

Intranasal inoculation was performed on day zero using an air-jet nebulizer as described before (1). The viral inoculum contained 3.6 $\log_{10}$ $TCID_{50}$ of bRSV (Odijk strain, seventh *in vivo* passage) in a 2 mL volume. The Odijk isolate (subtype A) was obtained during a field outbreak in Odijk, The Netherlands (2, 3) and induces in our experience severe disease in calves.

Experimental design

Calves were randomized into the dornase alfa (treatment) or normal saline (control) group, with six calves per group. Starting on day 5 after viral inoculation at the onset of LRTD symptoms, thereby mimicking a therapeutic approach, the experimental group received twice daily dornase alfa (Pulmozyme® 1 mg/mL, Hoffmann-La Roche, Basel, Switzerland) and the control group received twice daily 0.9% NaCl. The treatments were performed in the morning (intratracheal) and afternoon (intranasal). During the morning session, the calves received either 5 mL dornase alfa in 15 mL 0.9%NaCl (experimental group) or 20 mL 0.9%NaCl (control group) by direct intratracheal instillation. The instillation was performed after a broncho-alveolar lavage (BAL, see below). For deposition into both lungs, the intratracheal BAL catheter was repositioned just above the carina. The required length of the catheter

was determined beforehand by a bronchoscopy on study day -13. In the afternoon, 5 mL dornase alfa (experimental group) or 5 mL 0.9% NaCl (control group) was aerosolized using a portable nebulizer (AT-Neb, Atlantean Corp., Chubay City, Taiwan) and mask.

Monitoring and Sampling

The animals were followed for a maximum of 9 days after viral inoculation or until reaching the pre-defined humane endpoint (see below). Daily clinical observations were performed by a veterinarian blinded for the treatment groups, according to the scoring system outlined in supplemental table 1. Animals were sacrificed if they reached the predetermined endpoint (single severity score of 4 for general illness or for LRTD, or four subsequent scores of 3 for general illness and LRTD).

Because of rapid health deterioration of more than half of the animals from day 7 after viral inoculation (see supplemental results), daily sampling of blood and BAL was completed for all calves of both treatment groups from day -2 up to day 7. Arterial blood pCO_2, pO_2 and haemoglobin saturation values were measured on a blood gas analyser (GEM3000, Instrumentation Laboratories, Bedford, USA) after puncture of the auricular artery. BAL was performed according to the method described by Fogarty *et al.* with 50 mL D-PBS (Gibco, Grand Island, NY, USA) (4). BAL total cell counts were performed on the Coulter Counter (Beckman Coulter, Brea, USA). Cells were centrifuged to a slide in the Shandon cytospin 3 (Thermo Scientific), stained with a modified Wrights staining and a total of 400 cells were evaluated by microscope for leukocyte differential counts. DNA concentration in BAL was measured by UV-spectrophotometer (NanoDrop 2000, Thermo Scientific).

Supplemental table 1: Clinical scoring system

Score	General Illness	Upper Respiratory Tract Disease	Lower Respiratory Tract Disease
0 (absent)	Bright, alert Normal appetite Normal behaviour	No nasal discharge No coughing No ocular discharge	Normal respiration RR < 50 (breathings per minute)
1 (mild)	Reduced responsiveness Decreased appetite Otherwise normal behaviour	Nasal or ocular discharge Intermittent watery – mucus Occasional spontaneous dry cough – induced cough (unproductive) present	Increased respiration RR 51-70 (breathings per minute)
2 (moderate)	Dull Decreased appetite Retreats	Increased nasal or ocular discharge Persistent mucoid – mucopurulent discharge Frequent spontaneous productive cough – induced productive cough present	Abnormal respiration obvious abdominal breathing RR 71-100 (breathings per minute)
3 (severe)	Lethargic Anorexia Stays down	Severe nasal or ocular discharge Persistent purulent – haemorrhagic discharge Frequent spontaneous productive cough – induced productive cough present, prolonged when induced	Dyspnoeic severe abdominal breathing e.g. stretched neck and / or accessory breathing sounds RR >100 (breathings per minute)
4 (severe)	Soporific (non-responsive) Anorexia Unable to stand without assistance		Asphyxia e.g. mouth breathing / frothing

Lung tissue sample collection

Lung tissue was stored in 10% neutral buffered formalin. Blinded histological evaluation (of the lung samples) was performed independently by two veterinary pathologist (and discordant results were re-evaluated with multiheaded microscope to reach consensus), using a pathology score from 0 (minimal) to 4 (very severe) in the following categories: bronchitis/-iolitis, peribronchitis, interstitial pneumonia and alveolitis, adapted from described before (5).

Histopathology and immunohistochemistry

Upon reaching the humane end point or on day 9 after virus inoculation, calves were euthanized with an overdose of pentobarbital followed by exsanguination. Lung tissue was collected from five designated sites, as indicated in supplemental fig. 1 and described before (5). To detect NETs and airway obstruction in the respiratory tract, lung sections were stained with rabbit anti-human citrullinated anti-Histone H3 (CitH3, Abcam, Cambridge, UK), anti-PAD4 (Abcam) or mouse anti-cytokeratin Pan ab1 (Thermo Scientific) as described before (6). Lung sections were dewaxed and rehydrated, followed by antigen retrieval. The sections were subsequently incubated with the primary antibody and detected with HRP conjugated anti rabbit-Ig polymer (Immunologic, Duiven, The Netherlands). HRP activity was visualized with Novared (Vector Laboratories, Peterborough, UK). All sections were mounted with glycerol and scanned using the Philips Ultra Fast Scanner 1.6RA (Philips, Eindhoven, The Netherlands). Negative controls (including both lung tissue sections from non-infected calves and from bRSV infected calves in which the specific primary antibody had been omitted) were always included. Immunostaining of whole lung tissue sections was analysed using a colour deconvolution plugin (ImageJ) followed by quantitative thresholding. CitH3-positive pixels within the airways were counted and divided by total amount of tissue pixels to calculate the percentage of positive NET staining in each specimen. To quantitate the degree of histopathological airway obstruction, the percentage of open, closed or partially closed (small) airways was evaluated by visual counting of the whole lung tissue section using the counter plug-in in ImageJ.

Viral load measurement

All BAL samples were tested by reverse transcription (RT)-PCR to determine the viral load as described before (6). From 200µl BAL sample, total nucleic acid was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Hoffmann-La Roche). The conserved bRSV N-gene was detected with a primer/taqman probe mix by realtime RT-PCR using the QuantiFast Multiplex Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All assays included reverse transcription of RNA into cDNA and were run on the Applied Biosystems 7500 under optimized cycling conditions (40 cycles).

Statistics

Statistical analysis was performed using Graphpad Prism 6 (Graphpad Software Inc, La Jolla, CA, USA). Results are presented as mean with SD, or as mean with individual values. Results between groups were compared using the Mann–Whitney U–test or unpaired student t-test where appropriate, based on normality. Results between groups in time with multiple measurements were compared using mixed 2-way ANOVA. $P < 0.05$ was considered statistically significant.

References

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