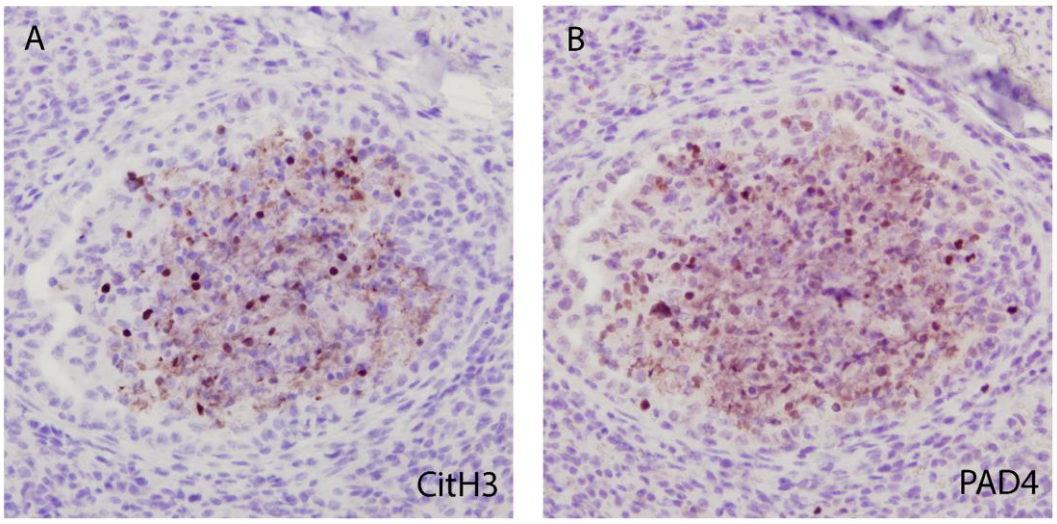
Supplemental results

Severe bRSV-LRTD is associated with PAD4 activation and NETs formation in the airways

First, we confirmed our previous findings (1) of widespread presence of NETs in mucus plugs residing in the lumen of (small) airways in severe bRSV-LRTD in calves (supplemental fig. 1A). To further establish our animal model of NETosis, we additionally evaluated local PAD4 expression, demonstrating both intra- and extracellular PAD4 immunostaining within the NETs-positive mucus plugs (supplemental fig. 1B). This is consistent with the current understanding of the localisation of PAD4 during NETosis in inflammatory processes (2).

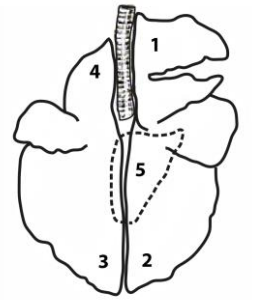


Supplemental fig. 1: Intra-luminal airway neutrophil extracellular traps formation.

Representative images of immunohistochemistry stainings for citrullinated histone H3 (CitH3, **A**) and protein arginine deiminase type 4 (PAD4, **B**) in lung tissue sections of a calf with severe bovine respiratory syncytial virus (bRSV)-induced lower respiratory tract disease, magnification 200×.

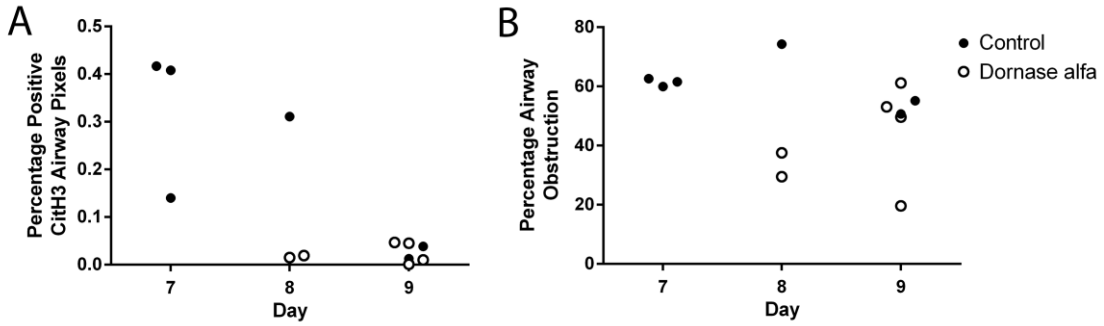
Local dornase alfa treatment reduces the amount of NETs in the respiratory tract

Next, we investigated whether local treatment with dornase alfa degrades the NETs in the airways during the course of severe bRSV-LRTD. To assess the spatial distribution of NETs throughout the lungs, we collected left and right cranial and caudal, and central area lung tissue samples (supplemental fig. 2). Overall, there was a significant reduction of NETs in the dornase alfa treated animals, as compared to the control group (fig. 1A-D, Avg: P = 0.02). In the lung areas which showed most NETs in control animals (right/left cranial and central tissue samples), NETs were almost completely destroyed by dornase alfa treatment. Importantly, the large difference in NETs content between the two treatment groups was not related to the timing of autopsy at peak disease on either day 7 or 8 (supplemental fig. 3A).



Supplemental fig. 2: Lung tissue sampling locations

Dorsal overview of the five pre-determined lung tissue locations sampled for histology, including cranial right (1), cranial left (4), caudal right (2), caudal left (3) and central areas (5).



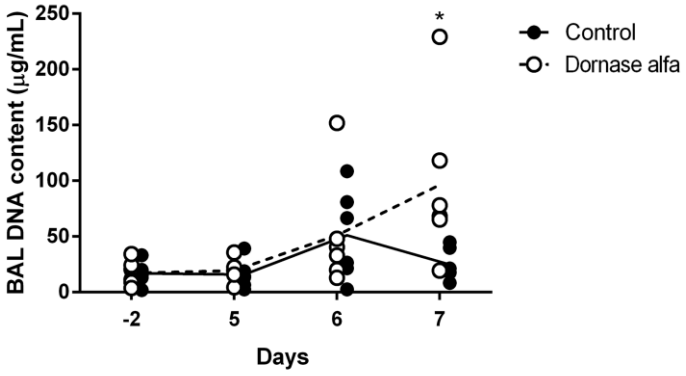
Supplemental fig. 3: Neutrophil extracellular trap formation at different time-points.

(A) Percentages of positive citrullinated histone H3 (CitH3) airway pixels (average of the 5 locations) in control animals (black circles, N = 6) and dornase alfa treated animals (white circles, N = 6) on the day

of sacrifice. Importantly, at peak disease (day 7-8), when reaching the humane endpoint, dornase alfa treated animals showed very limited positive staining, as compared to marked positive staining in control animals. **(B)** Percentage of partial or complete airway obstruction in control animals (black circles, N = 6) and dornase alfa treated animals (white circles, N = 6) on the day of sacrifice.

NETs lysis by dornase alfa treatment reduces histopathological airway obstruction

NETs are web-like 'sticky' structures that may hold together mucus plugs within the airways. To determine if degradation of NETs indeed leads to less airway obstruction, we counted the number of open or partially/completely obstructed airways within all lung tissue sections. On average, the percentage of obstructed airways in the dornase alfa group was 31% lower as compared to normal saline group (41.8 ± 6.4% versus 60.7 ± 3.3% respectively, fig. 1E, avg: *P* = 0.03), with a highest improvement of 51% in the left cranial area (36.3 ± 8.4% versus 74.5 ± 8.4%, fig. 1E, *P* = 0.03). In line with the hypothesis that NETs are immobilized within airway mucus plugs, the DNA content in BAL at peak disease was increased by dornase alfa treatment, (supplemental fig. 4, *P* = 0.03), indicative of active DNA-rich mucusplug lysis.

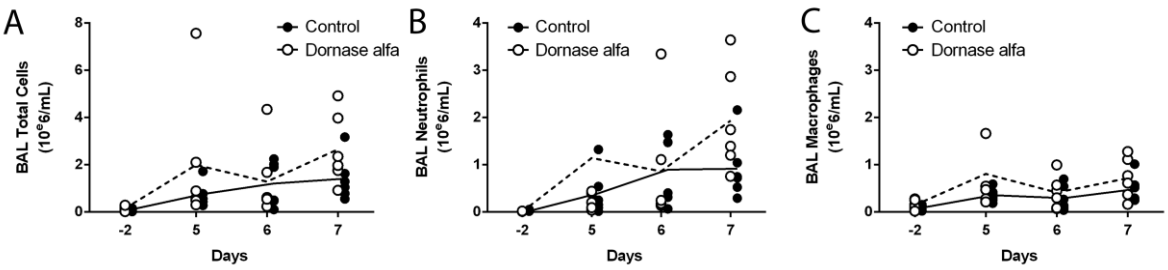


Supplemental fig. 4: Broncho-alveolar lavage DNA content.

DNA concentration (µg/mL) in broncho-alveolar lavage (BAL) in control calves (N = 6, black circles) and dornase alfa treated calves (N = 6, white circles). Data are expressed as mean + individual values, * P = 0.03.

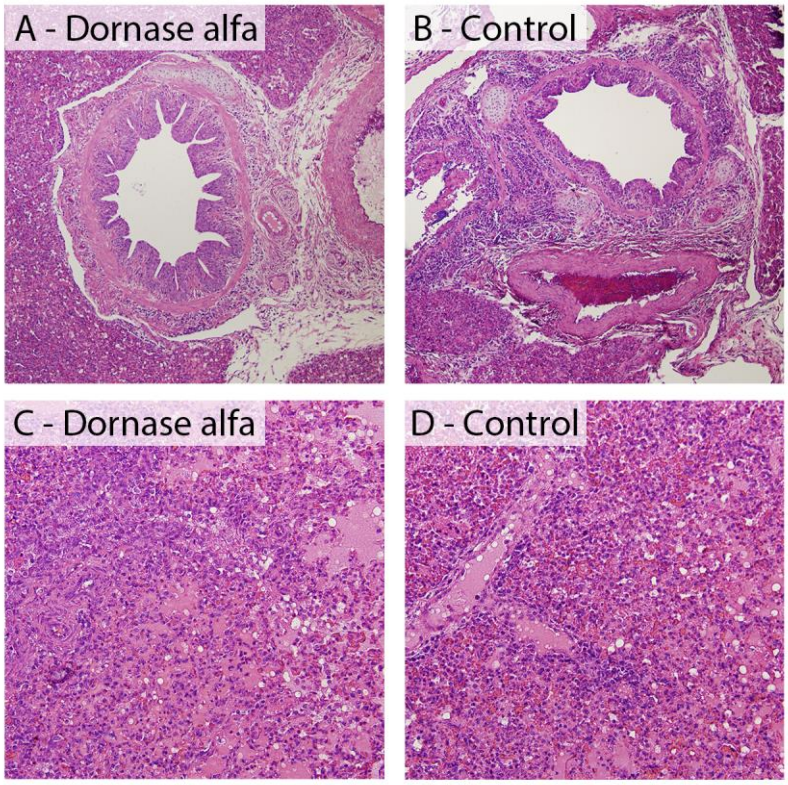
Local dornase alfa treatment does not affect bRSV-induced lung inflammation

To detect any influence of dornase alfa treatment on the lung inflammatory response to bRSV, we analysed BAL cells and the degree of cellular infiltration in airway and alveolar pathology samples. There were no significant differences in the total BAL cell counts or the number of neutrophils and macrophages between both groups (supplemental fig. 5A-C). Lymphocytes were near absent in both groups (data not shown). Although lung pathology scores revealed slightly less peri-bronchiolar and interstitial cellular infiltrates in the dornase alfa treated group, as compared to the control group (supplemental fig. 6A-B, supplemental table 2), the animals in both groups had evidence of widespread intra-alveolar changes (supplemental fig. 6C-D), indicating a strong lung inflammatory response to bRSV infection as described before (3).



Supplemental fig. 5: The lung inflammatory response.

Total white blood cells **(A)**, neutrophils **(B)** and macrophages **(C)** in broncho-alveolar lavage (BAL) during the course of severe bovine respiratory syncytial virus (bRSV)-induced lower respiratory tract disease in control animals (N = 6, black circles) and dornase alfa treated animals (N = 6, white circles). Differences not significant, data are shown as mean + individual data points.



Supplemental fig. 6: Lung pathology

Representative images of haematoxylin and eosin stained lung tissue sections (location 1, see supplemental fig. 2) during severe bovine respiratory syncytial virus (bRSV)-induced lower respiratory tract disease. Bronchiolitis in dornase alfa treated **(A)** and control animals **(B)**, magnification 40×. Alveoli showing diffuse alveolar injury consisting of cellular alveolitis, alveolar wall thickening, capillary congestion and haemorrhage with intra-luminal deposition of protein-rich oedema, present in dornase alfa treated **(C)** and control animals **(D)**, magnification 100×.

Supplemental table 2. Histopathology scoring of lung sections.

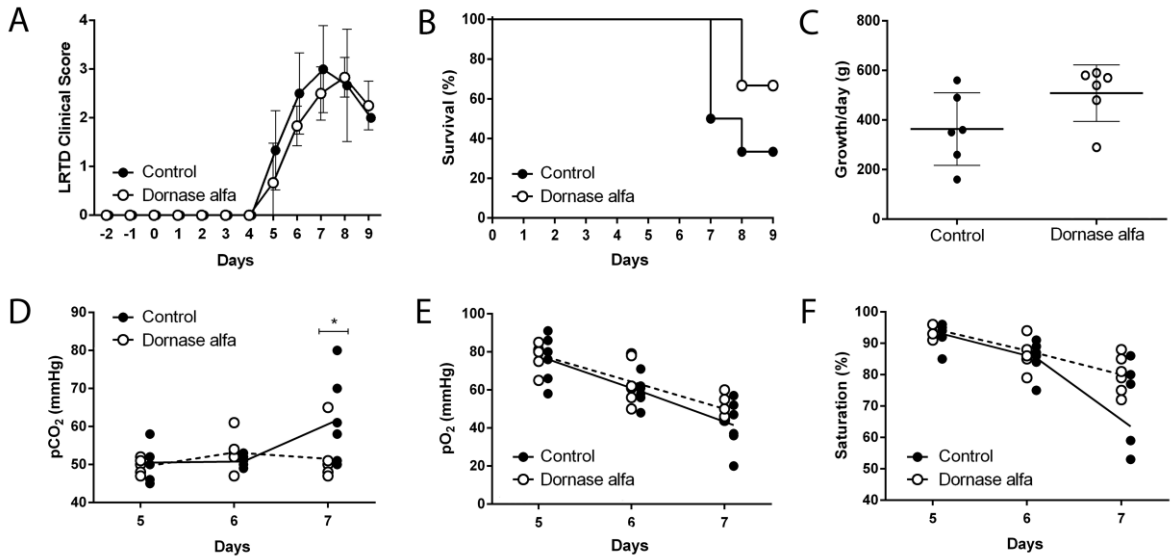
Group	Calf ID	Section Day	(endo)-Bronchi (oli)tis	Peribronchi (oli)tis/ -vasculitis	Interstitial pneumonia	Alveolitis	Sum score
Control	**9685**	**7**	2.5	1.5	2.6	2.6	**9.2**
	9687	**9**	3.5	1.7	3.5	3.1	**11.8**
	9689	**8**	3.7	2	3.5	3.4	**12.6**
	9690	**7**	2.5	1.3	2.5	2.4	**8.7**
	9691	**7**	2.7	1.6	3.2	2.7	**10.2**
	9695	**9**	3.1	1.6	3.6	3.0	**11.1**
Avg.			**3**	**1.6**	**3.2**	**2.9**	**10.6**
DNase	**9688**	**9**	3.1	1.5	2.9	3.1	**10.6**
	9692	**8**	2.8	1.2	2.4	2.5	**8.8**
	9693	**9**	1.6	0.9	2.0	1.6	**6.1**
	9694	**9**	3.3	1.2	3.0	3.1	**10.5**
	9696	**8**	3.1	1.0	1.8	2.6	**8.4**
	9700	**9**	1.6	1.0	2.1	1.3	**6.0**
Avg.			**2.6**	**1.1 ***	**2.4 ***	**2.3**	**8.4**

Nb. Individual values are the average of the five lung locations sampled, scored by two blinded pathologists. * P = 0.01

Local dornase alfa treatment partially improves clinical airway obstruction

BRSV infection induced severe LRTD in all calves, with an onset of symptoms at day 5 after inoculation (supplemental fig. 7A). The animals treated with dornase alfa had a trend towards lower LRTD scores (P = 0.07). Interestingly, 4 out of the 6 animals in the control group had to be sacrificed prematurely due to reaching their humane end-point based on acute deterioration (to a maximal clinical disease score of 4): 3 calves on day 7 and 1 calf on day 8, as opposed to 2 out of 6 calves in the dornase alfa group based on persistent severe disease (with four subsequent clinical scores of 3, both on day 8). However, this difference between the groups did not reach statistical significance in survival analysis (supplemental fig. 7B). The remaining animals (2 in the control groups versus 4 in the dornase alfa group) were slowly recovering from their LRTD on day 9 at the study ending. The average weight gain per day was 150 gram/day higher in the dornase alfa treated group as compared to the control group (supplemental fig. 7C, 508 ± 114 gram versus 363 ± 146 gram respectively, P = 0.08).

In addition to our histopathological observation that dornase alfa treatment causes substantial decrease in NETs-induced airway obstruction, the extent of hypercapnia was significantly reduced in dornase alfa treated calves at peak disease (day 7), as compared to saline treated animals of which three calves suffered from acute ventilatory failure (51.5 ± 2.8 mmHg versus 61.7 ± 4.7 mmHg, supplemental fig. 7D, P = 0.04). Blood oxygenation parameters were however not different between groups (supplemental fig. 7E-F), which could be explained from the widespread alveolar histopathological changes regardless of treatment.



Supplemental fig. 7: Clinical disease severity and ventilation and oxygenation parameters

(A) Mean (± SD) clinical scores of lower respiratory tract disease (LRTD) severity (supplemental table 1) in dornase alfa (white circles) and control (black circles) treated calves infected with bovine respiratory syncytial virus (bRSV). (N = 6 per group, *P* = 0.07). **(B)** Kaplan Meier curve of survival of control calves (black circles) and dornase alfa treated calves (white circles) during the study (*P* = 0.16). **(C)** Average (± SD) weight gain in kg per day during the study (study day -9 until the day of sacrifice) in dornase alfa treated animals (white circles) and control calves (black circles, *P* = 0.08). **(D)** Arterial blood gas analysis for the levels of pCO_2, pO_2 **(E)** and Haemoglobin-saturation **(F)** during severe bovine respiratory syncytial virus (RSV)-induced lower respiratory tract disease in control calves (black circles) and dornase alfa treated calves (white circles)**.** N = 6 calves per group. Data are represented as mean + individual data points. * *P* = 0.04.

References

1. Cortjens B, de Boer OJ, de Jong R, Antonis AF, Sabogal Pineros YS, Lutter R, et al. Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease. The Journal of pathology. 2016;238(3):401-11.
2. Jones JE, Causey CP, Knuckley B, Slack-Noyes JL, Thompson PR. Protein arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. Current opinion in drug discovery & development. 2009;12(5):616-27.
3. Viuff B, Tjornehoj K, Larsen LE, Rontved CM, Uttenthal A, Ronsholt L, et al. Replication and clearance of respiratory syncytial virus: apoptosis is an important pathway of virus clearance after experimental infection with bovine respiratory syncytial virus. The American journal of pathology. 2002;161(6):2195-207.