Pulmonary and systemic responses to aerosolized lysate of Staphylococcus aureus and Escherichia coli in calves

Laura L. Bassel1, Carmon Co1, Alaina Macdonald1, Laurel Sly2, Erin E. McCandless2, Joanne Hewson3, Raksha Tiwari2, Shayan Sharif1, Laura Siracusa1, Mary Ellen Clark1 and Jeff L. Caswell1*

Abstract

Background: Constitutive and inducible defenses protect the respiratory tract from bacterial infection. The objective of this study was to characterize the response to an aerosolized lysate of killed bacteria, as a basis for studying the regulation and in vivo effects of these inducible innate immune responses.

Results: Bacterial lysate consisting of heat-killed and sonicated Staphylococcus aureus and Escherichia coli was aerosolized to 6 calves and systemic and pulmonary innate immune and inflammatory responses were measured in the first 24 h relative to baseline. Evaluated parameters included clinical parameters (body temperature and heart and respiratory rates), blood acute phase proteins and leukocyte counts, and leukocytes and proteins in bronchoalveolar lavage fluid. Mild clinical signs with increased heart rates and rectal temperatures developed following administration of the lysate, with resolution by 24 h. Serum haptoglobin and plasma fibrinogen concentrations were elevated at 24 h relative to baseline. Bronchoalveolar lavage fluid (BALF) had increased cellularity and increased proportion of neutrophils, as well as higher concentrations of interleukin (IL)-8, IL-10 and total protein at 24 h relative to baseline. Mass spectrometry identified 965 unique proteins in BALF; 19 proteins were increased and 26 proteins were decreased relative to baseline. The upregulated proteins included those involved in innate immunity including activation of complement, neutrophils and platelets. At postmortem examination, calves receiving higher doses of lysate had areas of lobular consolidation and interlobular edema. Histologically, neutrophils were present within bronchioles and to a lesser extent within alveoli. Calves receiving highest doses of lysate had patchy areas of neutrophils, hemorrhage and hyaline membranes within alveoli.

Conclusions: Aerosolization of bacterial lysate stimulated an innate immune response in lungs and airways, with alveolar damage observed at higher doses. Such a stimulus could be of value for investigating the effects of inducible innate immune responses on occurrence of disease, or for evaluating how stress, drugs or genetics affect these dynamic responses of the respiratory tract.

Keywords: Cattle, Inflammation, Innate immunity, Lung, Bacteria, Bronchoalveolar lavage fluid, Diffuse alveolar damage, Pathology, Proteomics

© The Author(s). 2020 Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Background
The respiratory tract has not only static defences but also dynamic cellular and humoral responses to inhaled substances. For the most part, these responses protect the lungs from infection and other threats. Pattern recognition receptors on epithelial cells and leukocytes (including alveolar macrophages), as well as humoral factors such as complement proteins, act as sentinels to detect various pathogen-associated molecular patterns (PAMPs) that are unique to microbes. Once these receptors bind their ligands, signaling cascades are activated that induce the production of various mediators of inflammation and host defense, leading to rapid recruitment of neutrophils and monocytes as well as increased production of host defense proteins such as defensins. This may help eliminate pathogens but excessive inflammation can damage tissue and impair pulmonary function [1]. Thus, inflammatory responses should be inducible under circumstances where they are needed, but tightly controlled and quickly downregulated when the threat has passed.

In mice, administering PAMPs by aerosol to the respiratory tract has been shown to stimulate innate immune responses that protect against various pathogen challenges. For example, aerosolization of a lysate of nontypeable Haemophilus influenzae (NTHi) bacteria induced pulmonary inflammation characterized by increased concentrations of IL-6 and tumor necrosis factor (TNF-α) as well as increased neutrophils in BALF [2]. In pulmonary tissue collected 2 h following administration of NTHi, numerous genes related to host defense were upregulated including chemokines, other cytokines, pattern recognition receptors, antimicrobial peptides and oxygen radicals [3]. Similarly, administration of aerosolized Toll-like receptor (TLR) ligands to mice increased IL-6, TNF, and CXCL2 concentrations in BALF with no observed signs of illness or behavioural changes [4]. In these mice, neutrophils in BALF increased by 4 h, peaked at 48 h and returned to baseline by 7 days [4]. Aerosolization of NTHi prior to challenge with influenza A virus, Streptococcus pneumoniae, Bacillus anthracis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Yersinia pestis, Franciscella tularensis, or Aspergillus fumigatus protected against mortality [2, 3, 5]. Thus, upregulation of respiratory innate immune responses prior to pathogen challenge can be protective against bacterial and viral pathogens in mice.

Induction of innate immune defenses has also been demonstrated in cattle in response to various innate immune agonists and inflammatory cytokines. In primary cultures of bovine tracheal epithelial cells, administration of lipopolysaccharide (LPS, a TLR4 agonist), Pam3CSK4 (a TLR1/2 agonist), flagellin (a TLR5 agonist), IL-1β, TNF-α or IL-17 resulted in the induction of innate defenses such as tracheal antimicrobial peptide (TAP), lingual antimicrobial peptide (LAP), and lactoferrin [6–11]. Similarly, adenosine-5′-triphosphate, which has been shown to have antimicrobial effects [12], was released from cultured pulmonary epithelial cells in response to LPS, heat-killed bacteria and IL-1β [13]. Lipopolysaccharide induced expression of inflammatory cytokines (IL-1α, IL-1β), TNF-α, and IL-8 and the release of histamine and leukotriene B4 in cultured pulmonary epithelial cells [14, 15]. Similarly, isolated bovine alveolar macrophages increased expression of TNF-α, IL-1β and IL-8 in response to LPS [16, 17]. Following instillation of LPS into the lungs of cattle, neutrophils and macrophages were identified histologically within alveoli [18], and cell counts and the proportion of neutrophils in BALF increased, although TAP expression in bronchial mucosal biopsies did not change [8].

Thus, our hypothesis was that delivery of an aerosolized bacterial lysate to cattle would trigger innate immune responses without significant adverse effects. This was initially investigated using cell culture experiments to determine whether a lysate of killed Escherichia coli and Staphylococcus aureus upregulated TAP and LAP expression in cultured bovine tracheal epithelial cells. In vivo studies evaluated the clinical, hematologic and BALF changes and postmortem findings in calves following aerosol delivery of the bacterial lysate. The purpose was to establish a dose of bacterial lysate that induced an innate immune response without causing adverse effects, for use in further experiments. Thus, relatively low numbers of calves were used in the study, and comparisons were made between pre-treatment and post-treatment data in the same calf. We identified an inflammatory stimulus that could be tested as a novel method to prevent respiratory disease at times when immune defenses are compromised, such as in stressed beef calves at the time of arrival to feedlots. Furthermore, the use of a standardized stimulus could be useful to measure how stress or pharmacologic interventions affect the inducible responses of the respiratory tract, and to investigate the contribution of genetics or prior life events to these dynamic respiratory innate immune responses.

Results
Induction of TAP and LAP expression in tracheal epithelial cells
Treatment of primary cultures of bovine tracheal epithelial cells with a lysate of heat-killed E. coli and S. aureus significantly increased relative TAP gene expression compared to that of cells treated with medium alone (P = 0.002; Fig. 1a). Similarly, relative LAP gene expression was upregulated
in tracheal epithelial cells following administration of bacterial lysate. Treatment with bacterial lysate did not affect expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) based on analysis of the crossing points ($P > 0.05$).

**Clinical and clinical pathologic findings**

Six 3-month-old Holstein bull calves received aerosolized lysate of heat-killed *E. coli* and *S. aureus* (calves 1–6). All calves had clinical scores of zero prior to the administration of aerosol. Between 1 and 12 h following lysate administration, all 6 calves that received bacterial lysate developed subtle clinical signs consisting of mildly increased respiratory effort and mild depression that resolved by 18 h (Fig. 2; Additional file 1). All 6 calves coughed at one or more time points following administration of the lysate. Two calves (5 and 6) were reluctant to rise at 2 and 4 h after administration of the lysate, respectively. One calf (1) had a reduced appetite at 2 h following lysate administration. There was no association between the severity or duration of clinical signs and the dose of bacterial lysate. Two control calves (calves C1 and C2) received only aerosolized phosphate-buffered saline (PBS) to ensure that the aerosolization procedure did not affect the measured parameters; these 2 calves did not cough or show any clinical signs following aerosolization of PBS.

Rectal temperature was increased relative to baseline in calves that received aerosolized bacterial lysate ($P = 0.02$), with significant elevations at 4 and 8 h (Fig. 3a; Additional file 1). Heart rate increased following administration of bacterial lysate (one-way analysis of variance [ANOVA], $P < 0.05$); however, upon adjustment for multiple comparisons (Dunnett’s post hoc test), there were no significant differences in heart rate between baseline and any single post-aerosolization time point (Fig. 3b). Respiratory rate was not affected by the administration of aerosolized lysate ($P = 0.18$; Fig. 3c). In calves that received PBS, rectal temperature, heart rate, and respiratory rate did not change.

In calves treated with bacterial lysate, serum haptoglobin concentration significantly increased between baseline (mean: 0.17 g/L; 95% CI: 0.15, 0.18) and 24 h following aerosolization of lysate (mean: 0.27 g/L; 95% CI: 0.19, 0.70; paired t-test; $P = 0.044$) (Fig. 4a). Plasma fibrinogen concentration increased numerically between baseline (mean: 3.28 g/L; 95% CI: 2.60, 3.97) and 24 h following aerosolization of bacterial lysate (mean: 3.64 g/L; 95% CI = 3.29, 3.99) but the difference was not significant ($P = 0.058$; paired t-test of log-transformed values) (Fig. 4b). Administration of aerosolized bacterial lysate did not change hematologic...
values from baseline including total blood leukocytes ($P = 0.92$), neutrophils ($P = 0.88$), monocytes ($P = 0.08$) and lymphocytes ($P = 0.19$) (Fig. 4d-f).

**Changes in bronchoalveolar lavage fluid**

The cellular composition of BALF changed significantly following administration of bacterial lysate (Figs. 5 and 6; Additional files 2 and 3). Administration of bacterial lysate significantly increased the number of nucleated cells ($P = 0.036$) and the proportion of neutrophils ($P < 0.0001$) relative to baseline, while the proportion of macrophages was reduced ($P < 0.0001$) and the proportion of lymphocytes was unchanged ($P = 0.95$). Microscopically, cytocentrifuge preparations of the fluid from calves administered bacterial lysate contained large numbers of non-degenerate neutrophils as well as large macrophages with foamy cytoplasm containing occasional phagocytosed neutrophils (Fig. 7). Macrophages were significantly larger in post-lysate samples compared to baseline, based on morphometric analysis of the area of these cells in the stained cytocentrifuge preparation ($P < 0.0001$; Additional file 4).

Protein concentrations in BALF (as determined by BCA protein assay) were higher at 24 h after delivery of aerosolized bacterial lysate (mean: $122 \mu g/mL; 95\% \text{ CI: } 58, 186$) compared to baseline (mean: $55 \mu g/mL; 95\% \text{ CI: } 33, 77$) ($P = 0.016$, Additional file 5). In this analysis, data from one calf was excluded because the post-aerosol protein concentration was an outlier ($870.8 \mu g/mL$) as determined by Grubb’s test.

Cytokine concentrations in BALF were measured before and 24 h after aerosolization of bacterial lysate (Fig. 8). Following aerosolization, there was increased concentration of IL-8 ($P = 0.005$) and IL-10 ($P = 0.048$) compared to baseline. Following log transformation, the post-lysate concentration of IL-8 was positively correlated with the dose of bacterial lysate ($R^2 = 0.68$, $P = 0.012$). Interleukin 17 concentration increased numerically following aerosolization of bacterial lysate, but this change was not statistically significant ($P = 0.25$). The following cytokines were generally below the lower limit of the assay and were not statistically analyzed: interferon (IFN) $\alpha$ (< 9.8 pg/mL), IL-1$\beta$ (< 9.8 pg/mL), IL-2 (< 39.1 pg/mL), IL-6 (< 156.3 pg/mL), TNF-$\alpha$ (< 39.1 pg/mL), and IFN-$\gamma$ (< 2.4 pg/mL).

BALF samples collected before and 24 h after administration of aerosolized bacterial lysate were analyzed by tandem mass tag (TMT)-labelled quantitative mass spectrometry. There were 965 different proteins identified in the BALF (Additional file 6). Individual paired t-tests identified 19 unique proteins that had increased abundance (Table 1) and 26 proteins that had reduced abundance (Table 2) following aerosolization of bacterial lysate. The 19 proteins with increased abundance in post-lysate BALF included the following protein classes (as determined by PANTHER analysis):
transfer/carrier proteins (PC00219; n = 3), signaling molecules (PC00207; n = 2), receptors (PC00197; n = 2), defense/immunity proteins (PC00090; n = 2), hydrolases (PC00121; n = 4), and 1 of each of enzyme modulator (PC00095), oxidoreductase (PC00176), and extracellular matrix protein (PC00102) (Additional file 7). The 26 proteins that were decreased in post-lysate BALF included the following protein classes: membrane traffic proteins (PC00150; n = 3), hydrolase (PC00121; n = 3), enzyme modulator (PC00095; n = 3), nucleic acid binding (PC00171; n = 6), and cytoskeletal proteins (PC00085; n = 2). The remaining protein classes had only a single protein in each category (Additional file 7). Reactome analysis recognized 13 of the 19 significantly upregulated proteins and identified overrepresentation of pathways related to complement cascade, platelet activation and degranulation, neutrophil degranulation, innate immune system, and noncanonical NF-κB signaling (Additional file 8).

Fig. 4 Hematologic changes in calves after aerosol administration of bacterial lysate. Blood samples were collected at baseline and 24 h after aerosolization of Staphylococcus aureus and Escherichia coli lysate (n = 6) or saline (n = 2). Horizontal bars indicate the mean. a Serum haptoglobin, b plasma fibrinogen, c total blood leukocytes, d blood neutrophils, e blood monocytes, and f blood lymphocytes. * P < 0.05
Pathologic findings
At postmortem examination, the calculated lung to heart weight ratio was positively associated with the dose of aerosolized bacterial lysate ($R^2 = 0.96$, $P = 0.003$; Fig. 9). Grossly, lesions were predominately in the cranioventral areas of lung and included red mottling of the lung tissue, interlobular edema, and scattered discrete angular red-purple foci of lobular consolidation (Additional file 9). In the calves that received $10^{12}$ colony forming unit (CFU)-equivalents of bacterial lysate, lungs were mottled red with mild interlobular edema. The calves that received $10^{10}$ and $10^{11}$ CFU-equivalents had small, multifocal areas of lobular consolidation in the cranioventral lung lobes, affecting less than 5% of the total lung parenchyma. The calf that received $10^8$ CFU-equivalents had no gross lesions. The calf that received $10^9$ CFU-equivalents had an area of consolidation of a cranial lung lobe that was determined by histopathology to have been present for greater than 3 days prior to euthanasia (that is, preceding aerosolization of bacterial lysate).

Histologically, bronchioles and to a lesser extent alveoli contained neutrophils in airspaces and occasionally in the bronchiolar lamina propria and alveolar septa. Small numbers of neutrophils were visible in bronchiolar lumens and rarely in alveoli of the calves that received the lower doses of bacterial lysate ($10^8$ and $10^9$ CFU-equivalents). In the calves that received the higher doses ($10^{10}$ to $10^{12}$ CFU-equivalents), neutrophils were more numerous in bronchiolar lumens and alveoli (Fig. 10; Additional file 10). In the calves that received the highest doses ($10^{10}$ to $10^{12}$ CFU-equivalents), there were areas of acute alveolar damage evidenced by hyaline membranes and intra-alveolar neutrophils, macrophages and brightly eosinophilic (protein-rich) edema fluid (Fig. 11). The histologic lung lesions were ranked, based on severity of inflammation by 2 independent observers, without knowledge of the treatment status. The severity of inflammatory lesions in the lung corresponded to increasing doses of bacterial lysate.

Discussion
The pulmonary innate immune system is critical for detecting and responding to inhaled pathogens, yet there has been relatively little investigation into ways to stimulate or increase the effectiveness of these responses. In situations where a pathogen reaches the lung, the course of disease depends on many factors including host immunity and pathogen virulence. There is considerable variation among individuals in both the magnitude of inducible innate immune defenses [19, 20] and the severity of disease following bacterial pathogen challenge [21]. Thus, administration of aerosolized bacterial lysate in the current study could provide a standardized stimulus.
to investigate the genetic or acquired basis for this variability.

Evaluating the response to bacterial challenge is complicated by survival or host-mediated killing of the bacteria, and by the effects of bacterial virulence factors that may modulate the host innate immune response. By using killed bacterial lysate, we were able to characterize pulmonary and systemic responses to an inflammatory stimulus delivered to the lung, independent of pathogen survival. Gaining an understanding of these responses to bacterial lysate can allow us to compare innate immune responsiveness between individuals, examine the effects of various external influences, and investigate the impacts of immunomodulatory drugs on these responses.

Initial in vitro experiments confirmed that bacterial lysate upregulated the host defense peptides LAP and TAP in cultured tracheal epithelial cells. This is consistent with previous studies that demonstrated upregulation of TAP and LAP in response to the purified TLR agonists LPS and Pam3CSK4 [8, 10, 11]. Similarly, administration of non-typeable *Haemophilus influenzae* lysate to cultured murine epithelial cells resulted in upregulation of 18 of the 30 antimicrobial peptides examined and enhanced bacterial killing [3]. As the focus of this investigation was on the in vivo effects of aerosolization of bacterial lysate, we did not investigate additional genes in cultured epithelial cells or the in vitro responses of other cell types.

We used a bacterial lysate rather than purified agonists to stimulate innate immune responses. It has been shown that combinations of agonists can induce synergistic or complementary innate immune responses [22, 23]. In the bovine lung, it is likely that the simultaneous stimulation of multiple pattern recognition receptors by lysed bacteria, similar to that in natural pathogen exposure, could similarly induce a more robust response than individual purified agonists, although this was not specifically investigated in these experiments.

The aerosol treatments were well-tolerated by the calves. Although mild clinical signs including elevated respiratory effort, cough, and nasal discharge developed shortly after aerosolization of bacterial lysate, these

Fig. 6 Bronchoalveolar lavage fluid cells (mean ± SEM) before and after aerosol administration of bacterial lysate to 6 Holstein bull calves. a Total nucleated cell count. b Percentage of neutrophils. c Percentage of macrophages. d Percentage of lymphocytes (y-axis scale is different than b and c). * P < 0.05, ** P < 0.01; (paired t-test)
Fig. 7 Cytocentrifuge preparation of bronchoalveolar lavage fluid from a calf that received $10^{11}$ CFU-equivalents of *Escherichia coli* and *Staphylococcus aureus* lysate. There are large numbers of macrophages and neutrophils. Macrophages are large, with foamy cytoplasm and frequent binucleation (arrowheads). Occasional phagocytosed neutrophils are present within the cytoplasm of macrophages (arrows). Wright stain. Bar = 25 μm.

Fig. 8 Cytokine concentrations in bronchoalveolar lavage fluid prior to and 24 h following aerosolization of *Staphylococcus aureus* and *Escherichia coli* lysate ($n=6$) or saline ($n=2$). Horizontal bars indicate the mean. **a** Interleukin-8. **b** Interleukin-10. **c** Interleukin-17. *P* < 0.05, **P** < 0.01; (paired t-test).
resolved by 24 h. Similarly, in mice that received a sublethal dose of *Pseudomonas aeruginosa*, lung function and rectal temperature were most significantly decreased and behavioural signs of illness were highest between 6 and 12 h post-inoculation [24].

Neutrophils were markedly increased in BALF following aerosolization of bacterial lysate. BALF was sampled from caudodorsal areas of lungs to minimize any effects of pre-existing lung inflammation. As gross and histologic lesions were more numerous in the cranioventral than caudodorsal lung lobes, the analysis of BALF from the caudodorsal region may have underestimated the magnitude of the response to the aerosolized bacterial lysate. Experiments in mice demonstrated that neutrophils appeared in lung lavage fluid at 4 h after treatment with the TLR agonists oligodeoxynucleotide and Pam2CSK4 [4]. In that study, the number of neutrophils in BALF peaked at 48 h and returned to baseline by 7 days. In the current study, there was no apparent relationship between the dose of lysate and the magnitude of the increase in neutrophils in BALF. This suggests that each of the tested lysate doses were above the threshold for a maximal response. In support of this, neutrophil recruitment in response to oligodeoxynucleotide and Pam2CSK4 in mice is reported to follow a sigmoidal dose-response curve, with a plateau at higher doses [4]. In cattle, aerosolization of *Mannheimia (Pasteurella) haemolytica* significantly increased the neutrophil:macrophage ratio in BALF, beginning at 30 min and increasing up to 4 h after challenge [25, 26]. Conversely, although inhalation of endotoxin from *Salmonella typhimurium* induced increases in the percentage of neutrophils within the BALF, inhalation of *Staphylococcus epidermidis* did not alter the cell populations [25]. This is consistent with a pilot experiment we conducted (data not shown) where aerosolization of heat-killed *S. aureus* did not induce clinical effects or significantly increased neutrophil numbers in BALF. Thus, the inclusion of *E. coli* lysate augmented the pulmonary inflammatory response compared to *S. aureus* alone. Similarly, killed Gram-negative bacteria were found to contribute to robust induction of inflammatory responses [25]. In the present study, at 24 h after administering bacterial lysate, macrophages in BALF demonstrated evidence of activation with a larger cell size than at baseline, vacuolated cytoplasm, and frequent binucleation. The phagocytosis of apoptotic neutrophils (efferocytosis) observed in these calves is thought to be important in the resolution of inflammation [27].

The most consistent histologic observations were neutrophils within bronchiolar walls and lumens, whereas

### Table 1 Bronchoalveolar lavage fluid proteins that were significantly elevated following aerosolization of bacterial lysate relative to baseline

| Accession | Description                              | Ratio of post-lysate to baseline | P-value |
|-----------|------------------------------------------|----------------------------------|---------|
| G3MYZ3    | Afamin                                   | 2.06                             | 0.04    |
| Q7SIH1    | Alpha-2-macroglobulin                     | 3.05                             | 0.04    |
| P01888    | Beta-2-microglobulin                      | 2.25                             | 0.00    |
| Q0V8R6    | Beta-hexosaminidase subunit alpha         | 1.67                             | 0.01    |
| F1NO76    | Ceruloplasmin                             | 2.92                             | 0.04    |
| P81187    | Complement factor B                       | 2.22                             | 0.00    |
| Q28085    | Complement factor H                       | 3.39                             | 0.04    |
| ASPJT7    | ECM1 protein                              | 2.78                             | 0.02    |
| Q29437    | Primary amine oxidase, liver isozyme      | 3.23                             | 0.01    |
| P82943    | Regakine-1                                | 2.50                             | 0.04    |
| Q29443    | Serotransferrin                           | 1.92                             | 0.01    |
| G3N1U4    | Serpin A3–3                               | 2.46                             | 0.05    |
| Q2K1S7    | Tetrancetin                               | 3.02                             | 0.04    |
| F1MMR5    | Tetraticopeptide repeat domain 38         | 2.30                             | 0.03    |
| Q3ZB57    | Vitronectin                               | 2.24                             | 0.02    |
| E1BH06    | Uncharacterized protein*                  | 2.46                             | 0.05    |
| F1MCF8    | Uncharacterized protein                   | 1.40                             | 0.02    |
| F1MJK3    | Uncharacterized protein                   | 4.34                             | 0.04    |
| F1MVK1    | Uncharacterized protein                   | 4.19                             | 0.05    |

The table shows bronchoalveolar lavage fluid proteins that were significantly elevated (n = 19) following aerosolization of bacterial lysate relative to baseline, as determined by a paired t-test. Tandem mass-tag mass spectrometry was performed on bronchoalveolar lavage fluid from 4 calves obtained at baseline and 24 h after aerosol administration of bacterial lysate, with data analysis and protein normalization in Proteome Discoverer 2.2 against Uniprot Bovine Database.

*Four uncharacterized proteins were increased following treatment.*
Neutrophils were more variably observed within alveoli. Neutrophils were more numerous in calves that received higher doses of bacterial lysate. The dearth of neutrophils in baseline BALF and their abundance after stimulation confirmed that neutrophil infiltration occurred after and in response to the bacterial lysate. Neutrophils, lymphocytes and plasma cells were also present within bronchial and tracheal mucosa but these can be present in conventionally reared calves [28].

At higher doses, the aerosolized bacterial lysate induced histologically evident lung injury, including intra-alveolar neutrophils, hyaline membranes, and hypercellularity of alveolar septa. In addition, the gross lung:heart weight ratios increased with increasing dose, presumably due to edema as a result of increased vascular permeability. However, the presence of edema did not affect respiratory rates at the time of euthanasia. In mice, lung weights were also increased following aerosolization of oligodeoxynucleotide and Pam2CSK4 [4]. Aerosolization of LPS has been used to model septic

**Table 2** Bronchoalveolar lavage fluid proteins that were significantly reduced following aerosolization of bacterial lysate relative to baseline

| Accession | Description | Ratio of post-lysate to baseline | P-value |
|-----------|-------------|---------------------------------|---------|
| A6QLJ0    | Mammalian ependymin-related protein 1 | 0.30 | 0.03 |
| P31404    | V-type proton ATPase catalytic subunit A | 0.33 | 0.02 |
| Q5E9J1    | Heterogeneous nuclear ribonucleoprotein F | 0.35 | 0.04 |
| Q3T0D0    | Heterogeneous nuclear ribonucleoprotein K | 0.35 | 0.03 |
| Q5E9E2    | Myosin regulatory light polypeptide 9 | 0.37 | 0.03 |
| G3MYX8    | Tyrosine-protein kinase receptor | 0.41 | < 0.001 |
| P04272    | Annexin A2 | 0.41 | 0.01 |
| Q3T0F7    | Myotrophin | 0.42 | 0.04 |
| Q3T0M0    | Vacuolar protein sorting-associated protein | 0.44 | 0.03 |
| Q3SZA6    | Syndecan binding protein (Syntenin) | 0.52 | 0.02 |
| Q2HJH1    | Aspartyl aminopeptidase | 0.54 | 0.05 |
| F1MC48    | IQ motif containing GTPase activating protein 1 | 0.56 | < 0.001 |
| Q3T0S6    | 60S ribosomal protein L8 | 0.56 | 0.02 |
| A0A140T843| Beta-2-glycoprotein 1 | 0.57 | 0.05 |
| Q2YDE4    | Proteasome subunit alpha type-6 | 0.57 | 0.05 |
| F1MG05    | Elongation factor 1-gamma | 0.58 | 0.02 |
| F1MSE7    | Sushi domain containing 2 | 0.60 | 0.05 |
| P11116    | Galectin-1 | 0.62 | 0.04 |
| Q32PH8    | Elongation factor 1-alpha 2 | 0.65 | 0.05 |
| F1MM32    | Sulphydryl oxidase | 0.68 | 0.01 |
| G3MXB5    | Uncharacterized protein | 0.69 | 0.00 |
| A6QLG5    | 40S ribosomal protein S9 | 0.69 | 0.01 |
| Q0IIM3    | Heat shock protein 105 kDa | 0.71 | 0.05 |
| Q3ZC44    | Heterogeneous nuclear ribonucleoprotein A/B | 0.72 | 0.03 |
| Q18MR9    | Protein kinase C and casein kinase substrate in neurons 2 | 0.72 | 0.03 |
| A5PKK0    | FAM151B protein | 0.81 | 0.05 |

**Fig. 9** Relationship of challenge dose of *Staphylococcus aureus* and *Escherichia coli* lysate with lung:heart weight ratios. The data show the ratio of lung and heart weights measured at the time of postmortem examination, 24 h after 4 calves were administered $10^8$, $10^9$, $10^{10}$ or $10^{11}$ CFU-equivalents of *Staphylococcus aureus* and *Escherichia coli* lysate by aerosol. The ratio of lung to heart weight was correlated with the dose of bacterial lysate ($R^2 = 0.96, P = 0.003$).
Fig. 10 Histologic sections of bronchioles from calves that received aerosolized bacterial lysate 24 h prior to euthanasia. At lower doses of bacterial lysate (a, calf 1, $10^8$ CFU-equivalents; and b, calf 2, $10^9$ CFU equivalents), the bronchioles have no or few neutrophils within their lumens. At higher doses of bacterial lysate (c, calf 3, $10^{10}$ CFU-equivalents; and d, calf 4, $10^{11}$ CFU-equivalents) there are neutrophils within bronchioles. Bar = 40 μm

Fig. 11 Histologic sections of lungs from calves receiving $10^{10}$ (a), $10^{11}$ (b), $10^{12}$ (c, d) and CFU-equivalents of *Escherichia coli* and *Staphylococcus aureus* lysate. There are neutrophils and macrophages in alveoli (a), fibrin (b) or eosinophilic fluid (c) in alveoli (asterisks), thickened hypercellular alveolar septa (c), and hyaline membranes (d, arrows) indicating diffuse alveolar damage.
lung injury, similar to what was seen in the calves receiving a high dose of lysate [29–31]. Based on these findings, doses of $10^8$ to $10^{10}$ CFU-equivalents of bacterial lysate were chosen for future studies.

Aerosolization of bacterial lysate in the present study significantly increased BALF protein concentration and inflammatory cytokines, consistent with other studies that delivered aerosolized lysates or innate immune agonists [30]. Interleukin 8 and IL-10 concentrations were elevated in BALF after stimulation. Interleukin 8 has been shown to recruit neutrophils to the bovine lung [32]. Interleukin 10 functions to downregulate inflammatory responses and inhibits release of pro-inflammatory cytokines from macrophages and other leukocytes; its induced expression in response to bacterial infection or inflammatory cytokines is thought to represent a feedback loop to protect against excessive inflammation and tissue damage [33]. Other cytokines were below the limit of quantification (TNF-α, IFN-α, IL-1β, IL-2, IL-6). This may reflect the fact that BALF was sampled from caudodorsal areas of lung whereas lesions were more severe in cranioventral areas. Further, this may be due to the timing of the sample collection, as IL-1β and TNF-α were shown to peak and return to baseline within 8 h of exposure to *M. haemolytica* [34]. Mouse studies found that the cytokines IL-6, CCL2 and TNF peaked within 8 h and returned almost to normal following aerosol administration of CpG oligodeoxynucleotide and Pam2CSK4 [4]. Similarly, following non-lethal administration of *Pseudomonas aeruginosa*, TNF-α levels peaked at 4 h and returned to baseline by 24 h [24]. It is therefore possible that changes in cytokine concentrations occurred at an earlier time point.

Mass spectrometry identified numerous proteins within bovine BALF, and there were significant increases in the levels of several proteins related to innate immunity and the acute phase response, including complement components, metal-scavenging proteins, protease inhibitors, and chemokines. Many of the upregulated proteins identified by our analysis were involved in innate immune pathways including complement activation and neutrophil degranulation. Activation of neutrophil-related pathways corresponds to the increased number of these cells in bronchoalveolar lavage fluid and in histological sections. The overrepresentation of pathways related to platelet activation and degranulation are consistent with their increasingly recognized role in pulmonary innate immunity. It should be noted that this analysis does not determine the cellular source of the detected proteins, nor whether the increased levels result from greater synthesis of the protein, secretion from infiltrating cells such as leukocytes, exudation of proteins from plasma, or reduced degradation or clearance.

These experiments investigated a range of concentrations of bacterial lysate from $10^8$ to $10^{12}$ CFU-equivalents. The lung: heart weight ratios, concentrations of IL-8 in BALF, and histologic lesions of inflammation and alveolar damage were greater in calves receiving the higher doses of bacterial lysate. We did not determine a minimum dose to elicit pulmonary neutrophil recruitment, and it is possible that all tested doses were above a response threshold.

**Conclusions**

In vitro studies demonstrated that the bacterial lysate induced gene expression of host defense peptides. In vivo aerosol administration of bacterial lysate stimulated a transient systemic inflammatory response with short-term elevations in rectal temperature and heart rate, mild increases in blood biomarkers of inflammation, but no differences in blood leukocytes. Between baseline and 24 h after administering lysate, the BALF had a significant increase in neutrophil numbers, enlargement of macrophages with foamy cytoplasm, and increased concentrations of total protein, IL-8 and IL-10. Mass spectrometric analysis of BALF proteins identified 965 proteins, with altered levels of several proteins related to complement activation, neutrophils and platelets after administration of the lysate. Understanding and characterizing the pulmonary inflammatory responses could provide a platform to investigate innate immune responses, measure the effect of host or environmental influences on these responses, and evaluate immunomodulatory therapeutics. Further studies will investigate the effects of this immune stimulant on development of respiratory disease.

**Methods**

**Preparation of bacterial lysate**

An isolate of *Staphylococcus aureus* from a case of bovine mastitis and an isolate of *Escherichia coli* from a case of bovine diarrhea were kindly provided by the Animal Health Laboratory (AHL), University of Guelph. The *E. coli* isolate was considered enterotoxigenic based on identification of F41, F5/K99 and STa genes but was not enteropathogenic or verotoxigenic based on absence of eaeA, hlyA, or Shiga toxins 1 or 2 genes. A stock suspension of each isolate was streaked onto a blood agar plate and cultured at 37 °C for 24 h. A single colony of *Staphylococcus aureus* and of *E. coli* were each inoculated into separate flasks containing 250 mL tryptose soy broth and incubated at 37 °C with shaking for 16 h (stationary phase). The concentration of viable bacteria (colony forming units (CFU)/mL) was determined from aliquots of the bacterial preparations, by plating serial dilutions onto Columbia agar with 5% sheep blood (Oxoid Canada, Nepean, Ontario, Canada), incubating overnight at 37 °C, and counting the number of bacterial colonies. Other aliquots of the bacterial preparations were killed by incubation in a 65 °C water bath for 90 min. Bacterial killing was confirmed by lack of growth on blood agar.
The suspension was centrifuged at 4 °C at 10000 x g for 15 min and washed twice with cold PBS. The bacterial pellets were re-suspended in PBS to create a final stock solution containing 1 x 10^{11} CFU-equivalents/mL. The heat-killed bacteria were sonicated using a dismembranator (Model 120, Fisher Scientific) at 90% amplitude alternating 20 s pulses with 30 s rest for a total of 5 min. Aliquots of bacterial lysates were stored at −20 °C. Concentrations of the bacterial lysate are reported as CFU-equivalents.

**Cell culture experiments**

Primary cultures of bovine tracheal epithelial cells were established as previously described [11, 20]. Once the cells reached 80% confluency, triplicate wells were treated with medium only (negative control) or a combination of 10^{7} CFU-equivalents each of *S. aureus* and *E. coli* lysate. Cells treated with 0.1 μg/mL LPS (Sigma Aldrich, MO, USA, L9143) were used as a calibrator. Following treatment, the cells were incubated for 16 h before being harvested for RNA extraction.

RNA extraction and cDNA synthesis were carried out as previously described [20]. Real-time reverse transcription quantitative PCR was used to evaluate the effect of bacterial lysate on TAP and LAP expression relative to expression of the reference gene GAPDH as previously described [10, 11, 20]. Stability of expression of GAPDH across treatments was assessed using ANOVA. Template-negative wells were included in each run, and data of technical triplicates was assessed using ANOVA. Template-negative wells were included in each run, and data of technical triplicate samples were averaged. The specificity of the reaction was confirmed by examining melting curves and identification of a single peak at approximately 82.8 °C (TAP), 86.9 °C (LAP) and 85.4 °C (GAPDH).

**Aerosolization of bacterial lysate to calves**

Use of animals in these studies was approved by the Animal Care Committee of the University of Guelph (AUP #3286) according to Canadian Council on Animal Care guidelines. Calves were obtained from the Elora Dairy Research Centre (Ontario Ministry of Agriculture, Food and Rural Affairs). Eight 3-month-old Holstein bull calves were housed in pairs within climate-controlled, biosecurity level 2 pens in an isolation facility. Shavings were used as bedding, and hay, water and calf starter pellets (Sharpe Farms Supplies Limited, Guelph, Ontario) were offered *ad libitum*.

An aerosol of *S. aureus* and *E. coli* lysate was administered to 6 calves. Calves were randomly assigned to receive either 10^{6} (calf 1), 10^{5} (calf 2), 10^{10} (calf 3), 10^{11} (calf 4) or 10^{12} (calf 5 and 6) CFU-equivalents suspended in a total volume of 10 mL PBS. The bacterial lysates or PBS were delivered by aerosol using a compressor (Precision Medical, Northampton, PA) that produced approximately 25 psi of air pressure and was attached to a Whisper Jet nebulizer (Marquest, Englewood, Colo) connected to a small Equine Aeromask (Trudell Medical International, London, ON) placed over the calf’s muzzle. A prior study showed that this system delivered aerosol to the upper respiratory tract as well as the bronchioles and alveoli (Bassel et al., 2019). Personnel wore powered air-purifying respirators (PAPRs) and N95 respirators were placed over the one-way exhalation valves on the calf masks to reduce room air contamination. Two control calves (calf 1 and C2) were randomly assigned to receive only PBS, to ensure that the aerosolization procedure did not affect the measured parameters.

**Clinical and hematologic responses**

Calves were assessed prior to and at 1, 2, 4, 6, 12, 18 and 24 h following administration of aerosolized bacterial lysate or PBS without knowledge of treatment group. At these times, rectal temperature, respiratory rate and effort, heart rate, and mentation were assessed. Additional clinical signs including lack of appetite, coughing or nasal discharge were noted when present. Clinical scores were assigned as described in Additional file 11. Blood was collected into sodium citrate anticoagulant for measuring plasma fibrinogen and without anticoagulant for evaluation of serum haptoglobin (AHL, University of Guelph) prior to and 24 h following aerosolization of bacterial lysate or PBS.

**Bronchoalveolar lavage fluid analysis**

Bronchoalveolar lavage fluid was collected from all calves prior to and 24 h following administration of the bacterial lysate. Calves were sedated with xylazine and BALF was collected from the right caudodorsal lung for the baseline sample collection and the left caudodorsal lung for the post-aerosol sample collection, by infusing 120 mL sterile saline (120 mL, 0.9%) through an endoscope and retrieving the lavage fluid by manual suction through a 60 mL syringe. The sample was placed on ice until further processing (within 2 h). Samples were filtered through gauze prior to determination of total nucleated cell counts using electrical impedance (Z2 Coulter counter, Beckman Coulter, Mississauga, ON) and cytocentrifuge preparations were prepared using 200 μL of fluid. Differential cell counts were performed on 200 cells on the Wright-stained slide preparations. Olympus CellSens software was used to trace the outline of individual cells and measure the area of 15 macrophages in a single central 400x field.

**Mass spectrometry analysis**

For mass spectrometry, the remaining BALF was centrifuged (500 x g, 5 min, 4 °C), then desalted and concentrated by centrifugal filtration (Amicon Ultra-15, Millipore, Bedford, MA, USA). Protein concentrations were measured
using a Bradford assay [35]. Tandem mass tag (TMT) mass spectrometry (SPARC BioCentre, The Hospital for Sick Children, Toronto, Canada) was conducted on four pre-and post-aerosolization samples (calves 1–4). The normalized protein concentrations were reported. Samples were reduced, alkylated, digested, and TMT labelled according to manufacturer’s directions (Thermo Fisher TMT 10 Plex, Product 90,110). Labelled peptides from all samples were combined and lyophilized. Peptides were cleaned up using a C18 ZipTip (Millipore) and then lyophilized.

Samples were analyzed on a Thermo Scientific Orbitrap Fusion-Lumos Tribid Mass Spectrometer (ThermoFisher, San Jose, CA) outfitted with a nanospray source and EASY-nLC 1200 nano-LC system (ThermoFisher, San Jose, CA) and equipped with ETD mode as described in Additional file 12. Data analysis was performed using Proteome Discoverer 2.2 against a Uniprot Bovine Database (6002 sequences). TMT modifications of lysine and the peptide N-terminals as well as carbamidomethyl of cysteine were considered fixed modifications while oxidation of methionine and protein N-terminal acetylation were considered variable. Parent mass tolerance was set to 10 ppm, fragment mass tolerance was set to 0.6 Da. Reporter ion quantification for the 8 TMT channels was done on the MS3, and lot-specific correction factors were used. Proprotein quantification for the 8 TMT channels was done on 10,000 pg/mL in 1X DPBS. The panel 2 calibrators were diluted to a concentration of 4,000 pg/mL in 1X Dulbecco’s PBS without calcium or magnesium, followed by 4-fold serial dilutions into 1X DPBS. The panel 2 calibrators were diluted to a concentration of 40,000 pg/mL in 1X DPBS followed by 4-fold serial dilutions.

The detection antibodies were sulfo-tagged following the MSD quick guide conjugation protocol using a challenge ratio of 1:20. Panel 1 detection antibodies were used at the following concentrations: IL-1β at 1 μg/mL (Biorad), IL-2 at 0.5 μg/mL (R&D Systems), IL-6 at 2 μg/mL (R&D Systems), IFN-α at 1 μg/mL (Kingfisher Biotech) and TNF-α at 0.5 μg/mL (R&D systems). Panel 2 detection antibodies were used at the following concentrations: IL-8 at 0.5 μg/mL (Mabtech), IL-10 at 1 μg/mL (Biorad), IL-17A at 0.2 μg/mL (Kingfisher Biotech) and IFN-γ at 1 μg/mL (Mabtech).

Cytokines were quantified using the U-plex assay platform (MSD, Rockville, MD) assembled according to manufacturer’s instructions using a chemiluminescent readout (Additional file 12). For soluble protein levels, a BCA protein kit (Thermofisher, Rockford, IL) was used. The cytokine concentrations were normalized to the total protein levels for each sample.

Post-mortem examination
All but one of the lysate-treated calves were euthanized at 24 h following administration of aerosols, by intravenous injection of pentobarbital. Gross postmortem examination was performed within 2h of death including visual inspection and palpation of respiratory tissues. The lungs (trachea removed) and heart were weighed and the lung: heart weight ratio was calculated for each calf. Samples of nasal mucosa, trachea and cranioventral, caudodorsal and caudoventral regions of lung were placed in 10% formalin, and histologic sections were routinely prepared and stained with hematoxylin and eosin.

Statistical analysis
Data were compared between samples obtained before and after aerosolization of bacterial lysate within the same animal. Descriptive statistics, t-tests and ANOVA were performed (Graphpad Prism v8.0, San Diego, CA, USA) and 2-sided tests were considered significant when \( P < 0.05 \). Outcome variables were evaluated for normality using a D’Agostino-Pearson omnibus K2 test, Shapiro-Wilk test and Kolmogorov-Smirnov test and transformed as indicated. Grubb’s test, with alpha set at 1%, was used to detect outliers, which were subsequently removed from further analysis.
ANOVA compared the effects of bacterial lysate on normalized ratios of TAP: GAPDH and LAP: GAPDH expression in cultured tracheal epithelial cells. Repeated measures of clinical parameters including rectal temperatures, respiratory rate, and heart rate were evaluated using repeated measures one-way ANOVA with a Geisser-Greenhouse correction to adjust for unequal variability of differences. Multiple comparisons of post-aerosolization values against baseline (time 0) values were evaluated using a Dunnett’s test. Residuals were evaluated to determine whether ANOVA assumptions were met. For clinical scores, a repeated measure non-parametric Friedman test was conducted, with post hoc Dunn’s tests to compare post-aerosolization clinical scores with baseline.

Paired t-tests were used to compare baseline and post-stimulation concentrations of serum haptoglobin, plasma fibrinogen, hematology values and BALF parameters (cell counts and cytokines). For the cytokine assays, analytes that were below the limit of detection were assigned a value that was equal to the lower limit of detection divided by the square root of 2 [36]. Outcome variables that were not normally distributed and were not normalized following transformation were assessed non-parametrically using a Wilcoxon matched-pairs sign rank test.

Two-sided tests with \( \alpha < 0.05 \) were considered significant. Data from the study is provided as Additional file 13.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12917-020-02383-7.

Additional file 10. Histologic lesions in respiratory tissues from 4 calves treated with aerosolized Staphylococcus aureus and Escherichia coli lysate.

Additional file 11. Clinical scoring system.

Additional file 12. Additional methods for cytokine analysis and mass spectrometry.

Additional file 13. Data.

Abbreviations

AHL: Animal Health Laboratory; ANOVA: One-way analysis of variance; BALF: Bronchoalveolar lavage fluid; BCA: Bicinchoninic acid; CFU: Colony forming units; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IFN: Interferon; IL: Interleukin; LAP: Lingular antimicrobial peptide; LPS: Lipopolysaccharide; NTHi: Nontypeable Haemophilus influenzae; PAMPs: Pathogen-associated molecular patterns; PBS: Phosphate-buffered saline; TAP: Tracheal antimicrobial peptide; TLR: Toll-like receptor; TMT: Tandem mass tag; TNF: Tumor necrosis factor

Acknowledgements

The authors wish to thank Jonathan Krieger of SPARC BioCentre Molecular Analysis, The Hospital for Sick Children, Toronto, Canada for assistance with mass spectrometry; and Laura Wright, the staff of the Flora Dairy Research Centre; Mary Fowler, Tony Cenija, Linda Groocock and Veronique Carson for their exceptional care of the research calves.

Authors’ contributions

LLB conceived and designed the study, performed the laboratory work, analyzed the data and drafted the manuscript. CC, AM, LS2 and MEC contributed to study design, performed the laboratory work, and analyzed data. LS1, EM, RT contributed to study design and performed laboratory work. JH and SS contributed to study design, and critically appraised the manuscript. JLC conceived and designed the study, provided laboratory supervision and contributed to writing the manuscript. All authors approved the final version of the manuscript.

Funding

This work was supported by Zoetis, the Natural Sciences and Engineering Research Council of Canada (NSERC, CRDPJ 476331), Beef Farmers of Ontario (13-05), and the Ontario Ministry of Agriculture, Food and Rural Affairs (UofG2013–1488 and 27337). Dr. Bassel was supported by the OVC Scholarship program. Zoetis scientists (LS1, EM, RT) contributed to study design, performed laboratory work (cytokine analysis), and wrote the methods for the manuscript. Calves were provided by the Ontario Ministry of Agriculture, Food and Rural Affairs. The funding bodies did not otherwise contribute to the design of the study and collection, analysis and interpretation of data, or writing the manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Use of animals in these studies was approved by the Animal Care Committee of the University of Guelph (AUP #3286) according to Canadian Council on Animal Care guidelines. Calves were provided by the funding agency (Ontario Ministry of Agriculture, Food and Rural Affairs; UofG2013–1488) from the Elora Dairy Research Centre for the purpose of this research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1, Canada. 2Global Therapeutics Research, Veterinary Medicine Research and Development, Zoetis Inc, Kalamazoo, MI, USA. 3Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada.
Received: 10 January 2020 Accepted: 17 May 2020
Published online: 29 May 2020

References

1. Pechous RD. With friends like these: the complex role of neutrophils in the progression of severe pneumonia. Front Cell Infect Microbiol. 2017;11:60.

2. Tuvim E, Clement D, Gilbert T. Augmented Lung Inflammation Protects against Influenza A Pneumonia. PLoS ONE. 2009;4(1):e17176.

3. Evans S, Clement L, Kontoyiannis L, et al. Stimulated Innate Resistance of Lung Epithelium Protects Mice Broadly Against Bacteria and Fungi. Am J Respir Cell Mol Biol. 2010;42:240–50.

4. Alfaro G, Valverde M, Quinton W, et al. Safety, tolerability, and biomarkers of the treatment of mice with aerosolized Toll-like receptor ligands. Front Pharmacol. 2014;5.

5. Clement CG, Evans SE, Evans CM, Hawke D, Kobayashi R, Reynolds PR, et al. Stimulation of lung innate immunity protects against lethal pneumococcal pneumonia in mice. Am J Respir Crit Care Med. 2008;177:1322–30.

6. Russell JP, Diamond G, Tarver AP, Scanlin TF, Bevins CL. Coordinate tracheal epithelial cells. Vet Immunol Immunopathol. 2018;203:40–4.

7. Bourque LA, Raverty S, Co C, Lillie BN, Daoust P-Y, Clark ME, et al. Benzoxa pyrene suppresses tracheal antimicrobial peptide gene expression in bovine tracheal epithelial cells. Vet Immunol Immunopathol. 2018;203:40–6.

8. Taha-Abdelaziz K, Wyer L, Berghuis L, Bassel LL, Clark ME, Caswell JL. Comparison of innate immune agonists for induction of tracheal antimicrobial peptide gene expression in tracheal epithelial cells of cattle. Vet Res. 2014;45:105.

9. Lin Y, Qiu D, Huang L, Zhang S, Song C, Wang B, et al. A novel chalcone derivative, 1,2H17, ameliorates lipopolysaccharide-induced acute lung injury in rats: comparative assessment of intratracheal instillation and aerosol inhalation. Toxicology. 2013;304:158–66.

10. Roos AB, Berg T, Ahlgren KM, Gronewald J, Nord M. A method for generating pulmonary Neutrophilia using aerosolized lipopolysaccharide. J Vis Exp. 2014. https://doi.org/10.3791/51470.

11. Liu F, Liu W, Pauluhn J, Trabel H, Wang C. Lipopolysaccharide-induced acute lung injury in rats: comparative assessment of intratracheal instillation and aerosol inhalation. Toxicology. 2013;304:158–66.

12. Lin Y, Qiu D, Huang L, Zhang S, Song C, Wang B, et al. A novel chalcone derivative, 1,2H17, ameliorates lipopolysaccharide-induced acute lung injury via upregulating HO-1 activity. Int Immunopharmacol. 2019;71:100–8.

13. Mitchell GB, Albright BN, Caswell JL. Effect of interleukin-1 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. Infect Immun. 2003;71:1643–9.

14. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol. 2011;29:71–109.

15. Malazdrewich C, Ames TR, Abrahamsen MS, Maheswaran SK. Pulmonary expression of tumor necrosis factor alpha, Interleukin-1 Beta, and Interleukin-8 in the acute phase of bovine pneumonic Pasteurellosis. Vet Pathol. 2001;38:297–310.

16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.

17. Hornung RW, Reed LD. Estimation of average concentration in the presence of nondetectable values. Appl Occup Environ Hyg. 1990;5:46–51.

18. Fabregat A, Jupé S, Matthews L, Sidiroopoulos K, Gillespie M, Garapati P, et al. The Reactome pathway knowledgebase. Nucleic Acids Res. 2018;46:D649–55.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.