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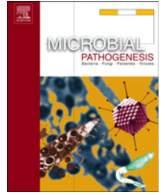

Histophilus somni causes extracellular trap formation by bovine neutrophils and macrophages

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A B S T R A C T

Histophilus somni (formerly *Haemophilus somnus*) is a Gram-negative pleomorphic coccobacillus that causes respiratory, reproductive, cardiac and neuronal diseases in cattle. *H. somni* is a member of the bovine respiratory disease complex that causes severe bronchopneumonia in cattle. Previously, it has been reported that bovine neutrophils and macrophages have limited ability to phagocytose and kill *H. somni*. Recently, it was discovered that bovine neutrophils and macrophages produce extracellular traps in response to *Mannheimia haemolytica*, another member of the bovine respiratory disease complex. In this study, we demonstrate that *H. somni* also causes extracellular trap production by bovine neutrophils in a dose- and time-dependent manner, which did not coincide with the release of lactate dehydrogenase, a marker for necrosis. Neutrophil extracellular traps were produced in response to outer membrane vesicles, but not lipooligosacchride alone. Using scanning electron microscopy and confocal microscopy, we observed *H. somni* cells trapped within a web-like structure. Further analyses demonstrated that bovine neutrophils trapped and killed *H. somni* in a DNA-dependent manner. Treatment of DNA extracellular traps with DNase I freed *H. somni* cells and diminished bacterial death. Treatment of bovine monocyte-derived macrophages with *H. somni* cells also caused macrophage extracellular trap formation. These findings suggest that extracellular traps may play a role in the host response to *H. somni* infection in cattle.

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1. Introduction

Bovine respiratory disease (BRD) is a leading cause of economic loss to the dairy and beef industries. An estimated 25 percent of calves experience respiratory illness in the first year of life [1,2]. BRD is a multi-factorial disease caused by a combination of viral and bacterial pathogens [3]. Several viruses, including bovine herpes virus-1, bovine respiratory syncytial virus, parainfluenza-3, bovine viral diarrhea virus, and bovine coronavirus have been implicated in the initiation of BRD. These in turn predispose cattle to severe secondary infection by bacterial pathogens such as *Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*, *Mycoplasma bovis*, and *Arcanobacterium pyogenes* [3].

H. somni is a Gram-negative, pleomorphic coccobacillus that resides in the upper respiratory and reproductive tracts of diary and beef cattle. *H. somni* is an opportunistic pathogen that causes a wide variety of clinical syndromes such as pneumonia, reproductive disease, polyarthritis, septicemia, myocarditis and an acute neurological disease known as thrombotic meningoencephalitis [4,5]. Despite being a normal resident of the microflora, *H. somni* has a variety of virulence factors including phosphocholine modification, phase variation of its lipooligosacchride (LOS), exopolysacchride production and secreted immunoglobulin Fc binding proteins such as IbpA [6]. *H. somni* also inhibits oxygen radical formation by bovine leukocytes [5]. Co-incubation of *H. somni* with bovine alveolar macrophages, blood monocytes and neutrophils reduces the ability of these cells to phagocytose, produce oxygen radicals and kill the bacteria. Those *H. somni* cells that are phagocytosed appear to survive within the leukocytes despite the production of nitric oxide [5]. Viable *H. somni* cells, but not killed cells nor culture supernatant, impair leukocyte function [5].

Neutrophil extracellular trap (NET) formation is an active form of cell death in which activated neutrophils release nuclear deoxyribonucleic acid (DNA) that is studded with antimicrobial proteins

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such as elastase, histones, and myeloperoxidase [7–9]. NETs are produced by human, murine, bovine, fish, feline and chicken leukocytes [7,10–17] in response to prokaryotic and eukaryotic pathogens [18]. Upon formation, NETs can trap and kill a wide variety of Gram-positive and Gram-negative bacteria, fungi, protozoa [18] and viruses [19]. NET formation results in little or no lactate dehydrogenase (LDH) release, indicating that necrosis is not the chief cause of DNA release [7]. Similarly, NET formation occurs in the presence of inhibitors of apoptosis [20,21].

In addition to neutrophils, macrophages, mast cells, and eosinophils also produce extracellular traps (ETs) [20,22–26]. Macrophages have recently been discovered to produce macrophage ETs (METs) composed of nuclear DNA and histones, which are released in response to Gram-positive and Gram-negative bacteria and nano-particles [20,22,23]. Mast cells also produce ETs containing nuclear DNA, histones, tryptase, and cathelicidin LL-37 in response to *Streptococcus pyogenes* [25]. Interestingly, eosinophils produce ET composed of mitochondrial DNA rather than nuclear [24]; although, some have demonstrated mitochondrial DNA is also found in NETs produced by neutrophils that do not die in the process of NET formation [27,28]. Despite some differences between leukocyte traps, several authors have confirmed the ability of these ETs to trap and kill pathogens [20,23–26].

Recent research from our laboratory has demonstrated that *M. haemolytica*, an important member of the BRD complex, causes the formation of ETs by bovine neutrophils and macrophages [20,21]. ET formation is mediated at least in part by the *M. haemolytica* leukotoxin, a member of the RTX (repeats-in-toxin) family of exotoxins, produced by *M. haemolytica* [20,21]. Here, we demonstrate that another BRD pathogen, *H. somni,* which does not produce an RTX exotoxin, also causes neutrophil and macrophage ET formation. We present data that that outer membrane vesicles (OMVs), and not LOS of *H. somni*, is required for NET formation.

2. Materials and methods

2.1. Neutrophil and macrophage isolation

Whole blood was collected by venipuncture from healthy Holstein cows housed at the University of Wisconsin–Madison Dairy Cattle Center using 0.38% (v/v) sodium citrate as anticoagulant. Blood was centrifuged at 2100× *g* for 12 min and the buffy coat removed. The buffy coat, containing mononuclear cells, was suspended in Hank's balanced salt solution (HBSS; Cellgro, Manassas, VA) with 4 mM ethylenediaminetetraacetic (EDTA) (without calcium or magnesium), layered onto Histopaque-1083 (Sigma–Aldrich, Saint Louis, MO) and centrifuged at 2100× *g* for 25 min at room temperature. Mononuclear cells were removed and contaminating red blood cells (RBCs) lysed in a 1:10 dilution of lysis buffer (150 mM ammonium chloride, 10 mM Tris (pH 7.5)) while rotating at 8 rpm for 10 min. Cells were pelleted at 1000× *g* and washed 3× in HBSS with 4 mM EDTA. Mononuclear cells were resuspended in RPMI-1649 (Cellgrow) with 1% FBS and incubated for 2 h on 100 mm carboxyl coated dishes (Becton, Dickinson and Company, Franklin Lakes, NJ). Nonadherent cells were removed by repeated washing and the medium replaced with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The adherent monocytes were cultured for 7 days at 37 °C, during which time the media was changed twice. Monocytes with greater than 99% viability, as determined by trypan blue staining and light microscopy, were deemed acceptable for further use.

Bovine neutrophils (bPMNs) were isolated by diluting the red blood cell (RBC) pellet in a 1:3 dilution of lysis buffer (150 mM ammonium chloride, 10 mM tris (pH 7.5)) while rotating at 8 rpm for 10 min. bPMNs were pelleted at 1000× *g* and washed 4× with Hank's balanced salt solution (HBSS; Cellgro, Mannasa, VA) to remove contaminating fibrinogen and platelets. Cells were resuspended in serum- and phenol red-free RPMI-1640 medium (Cellgro) and examined by light microscopy. A purity of >98% bPMNs was determined by microscopic inspection and >99% viability as determined by trypan blue exclusion.

2.2. Bacteria

H. somni (formally *Haemophilus somnus)* strain 649, generously provided by Dr. Lynette Corbeil, was initially isolated from a clinical case of bovine abortion. Bacteria were grown as previously described [29–31]. Briefly, a frozen aliquot of stationary-phase *H. somni* cells was thawed and inoculated into brain-heart infusion (BHI) broth supplemented with 0.5% yeast extract and 0.01% thiamine monophosphate (TMP; Sigma–Aldrich). The bacteria were cultured without shaking for 16 h at 37 °C with 5% CO_2. Bacteria were washed 3× and resuspended in RPMI-1640 without phenol red or serum. Bacteria were enumerated by growth on tryptic soy agar supplemented with 5% sheep red blood cells (RBCs; Becton Dickson).

2.3. Quantification of extracellular DNA

Neutrophil extracellular DNA was quantified as previously described [21]. Briefly, neutrophils or macrophages were incubated for the indicated times with various stimuli and then pelleted at 250× *g* for 3 min. The supernatant was removed, micrococcal nuclease buffer with 0.1 U/μL micrococcal nuclease was added (New England Biolabs, Ipswich, MA), and incubated for 15 min at 37 °C (as described by the manufacturer). A 1:400 dilution of PicoGreen (Invitrogen, Calsbad, CA) in 10 mM Tris base buffered with 1 mM EDTA was added to an equal volume of the nuclease-treated leukocyte mixture. Fluorescence was determined at an excitation wavelength of 484 nm and an emission wavelength of 520 nm using an automated plate reader (DTX 800 Multimode detector, Beckman Coulter, Brea, CA). NET production was quantified as fold increase in DNA release compared to untreated control neutrophils.

2.4. Reagents

DNase I (source: bovine pancreas), PMA, *Escherichia coli* lipopolysaccharide (LPS) and cytochalasin D were purchased from Sigma–Aldrich. *H. somni* LOS was kindly provided by Dr. Tom Inzana (Blacksburg, VA) [32]. MPER was purchased from Pierce (Rockford, IL) and used to make bPMN lysates. LDH release was determined using CytoTox 96 Non-radioactive Cytotoxicity Assay as described by the manufacturer (Promega, Madison, WI).

2.5. Immunofluorescence and electron microscopy

To perform immunofluorescence microscopy, bPMNs (3×10^6) were incubated on poly-L-lysine (Electron Microscopy Sciences, Hatfield, PA) coated glass slides (Fisher Scientific, Hanover Park, IL) for 30 min. Slides were then incubated at 37 °C with 10^8 fluorescein-labeled *H. somni* cells, *H. somni* cells and 360 U DNase, 100 nM PMA, 250 nM *H. somni* LOS, or 100 μg of *H. somni* OMVs for 1 h. Slides were washed 3× with PBS and fixed for 10 min with 4% paraformaldehyde (PFA). The slides were then washed, blocked with 1% bovine serum albumin (BSA; Pierce) in PBS for 20 min at room temperature and then incubated for 1 h with 1 μM TOPRO. Following that slides were washed and examined by confocal microscopy (Nikon Eclipse TE2000-U, Nikon Corporation, Tokyo, Japan).

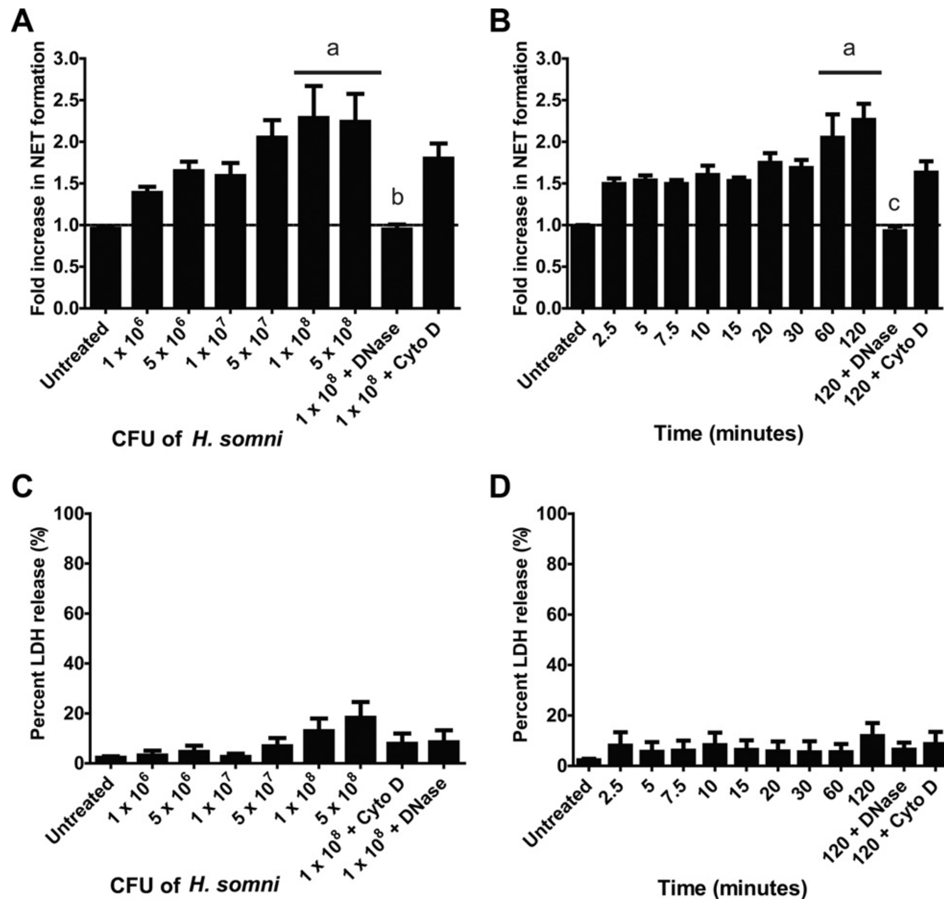

Fig. 1. Bovine neutrophils produce NETs in a dose- and time-dependent manner in response to *H. somni* cells. 10^6 bPMN were incubated with various concentrations of *H. somni* cells for 120 min (A and C) or for various times with 10^8 *H. somni* cells (B and D). bPMNs were incubated with 180 U DNase I or 10 μg/mL cytochalasin D (Cyto D) as controls. Extracellular DNA was quantified by the addition of PicoGreen (1:400 dilution) and fluorescence quantified by an automated plate reader at excitation wavelength of 484 nm and an emission wavelength of 520 nm. LDH release was quantified using the CytoTox 96 Non-radioactive Cytotoxicity Assay as described by the manufacturer. Data represent the mean ± standard error of the mean (S.E.M.) of 5 independent experiments. a) indicates $p < 0.05$ as compared to untreated bPMNs; b) indicates $p < 0.05$ as compared to bPMNs treated with 10^8 *H. somni* cells; and c) indicates $p < 0.05$ as compared to bPMNs treated with 10^8 *H. somni* cells for 120 min.

For scanning electron microscopy, bPMNs (3×10^6) were incubated on poly-L-lysine coated glass slides for 30 min, washed twice with PBS and then incubated with 10^8 *H. somni* cells for 1 h. Slides were washed, fixed with 4% paraformaldehyde in PBS, post fixed with 2.5% glutaraldehyde, and prepared as previously described [21]. Images were taken with a Hitachi S900 at the Biological and Biophysical Preparation, Imaging and Characterization Laboratory at the University of Wisconsin – Madison (Hitachi, Japan).

2.6. Bacterial trapping and killing

H. somni cells were grown to log phase as described earlier, washed 3× in PBS, and resuspended for 15 min on ice in 0.5 mg/mL fluorescein isothiocyanate (FITC; Sigma–Aldrich) in 50 mM sodium carbonate buffer. The *H. somni* cells were then washed 3× with PBS and resuspended at a concentration of 5×10^9 bacteria/mL in serum-free RPMI-1640. Cell viability after FITC labeling was assessed by dilution plating both FITC-labeled and unlabeled *H. somni* cells. Dilutions were plated on blood agar and CFU estimated.

To inhibit phagocytosis, 10 μg/mL cytochalasin D was incubated at 37 °C with 5% CO_2 for 30 min with bPMNs (10^6), the cells were then washed and resuspended in RPMI-1640 without phenol red or serum. As a control, cytochalasin D-treated and untreated bPMNs (10^6) were incubated for the length of the experiment with 180 U DNase I. Treated (Cyto D, DNase, Cyto D + DNase) and untreated bPMNs were washed and then incubated with FITC-labeled or unlabeled *H. somni* cells (10^7) in RPMI-1640 for 60, 120 or 180 min at 37 °C.

To quantify the bacterial cells trapped within NETs, samples were washed 3× with PBS and fluorescence determined using an automated plate reader. To determine bacterial killing by NETs, bPMN were incubated with *H. somni* suspensions for various times at 37 °C. Following this, samples were removed, were serially diluted in PBS and plated on TSA with 5% sheep RBC. As a control, *H. somni* was incubated in RPMI without bPMNs and samples removed and plated on blood agar to quantify total CFU. The percent bactericidal activity attributable to NETs was determined as described previously [12,21].

2.7. *H. somni* outer membrane vesicles (OMVs)

H. somni OMVs were prepared and purified as previously described [33]. Briefly, *H. somni* was grown to log phase in BHI broth supplement with 0.5% yeast extract and 0.01% TMP at 37 °C with 5% CO_2. The culture was centrifuged at 10,000× *g* for 30 min, the supernatant collected and centrifuged again at 20,000× *g* for 30 min. These cell-free supernatants were ultracentrifuged at 105,000× *g* for 30 min at 4 °C to collect the OMVs. *H. somni* OMNVs were dissolved in RPMI without phenol red and stored at −80 °C until used in an experiment. Protein concentrations were

determined using bicinchoninic acid (BCA) as described by the manufacture (Pierce).

2.8. Statistical analysis

Group means were compared by ANOVA, followed by the Tukey–Kramer pairwise comparison test, as performed by the Instat statistical package (GraphPad, San Diego, CA). The level of significance was set at $p < 0.05$.

3. Results

3.1. H. somni causes NET formation

We observed NET formation in response to *H. somni* cells in both a dose- and time-dependent manner (Fig. 1). To confirm that PicoGreen bound extracellular DNA, we incubated *H. somni*-treated bPMNs with DNase I, which cleaves extracellular DNA. As expected, DNase I reduced the PicoGreen fluorescence (Fig. 1). We used cytochalasin D, an inhibitor of actin polymerization, to confirm NET formation results from the release of extracellular DNA. NET formation occurred in response to *H. somni* despite cytochalasin D treatment (Fig. 1).

NET formation has been classified as independent of necrosis and apoptosis [7,9]. During necrosis, significant lactate dehydrogenase (LDH) is released [7,9]. We quantified LDH release and found little to no significant LDH release when bPMNs were incubated with *H. somni* cells (Fig. 1 C,D). Likewise, NET formation was not inhibited when bPMNs were treated with Z-VAD-FMK, a pan-caspase inhibitor of apoptosis (data not shown).

We and others have observed that 10–33% of human and bovine neutrophils undergo NET formation when exposed to a stimulus [8,9]. We report previously that repeated exposure of bPMNs to *M. haemolytica* led to a significant increase in NET formation [21]. Here, we found a similar effect when bPMNs were repeatedly exposed to *H. somni* cells (Fig. 2A). Furthermore, no significant LDH release was seen (Fig. 2B).

3.2. H. somni outer membrane vesicles (OMVs) cause NET formation

H. somni has several virulence factors that contribute to its pathogenesis including lipooligosacchride (LOS) and outer membrane proteins that are found in OMVs [5].We purified *H. somni* OMVs and incubated these with bPMNs at various concentrations and for various times. We observed a dose- and time-dependent increase in NET formation in response to purified *H. somni* vesicles (Fig. 3). Various molecules are found in OMVs including LOS, immunoglobulin-binding protein (IbpA) and others [6]. The ability of lipopolysacchride (LPS) to cause NET formation has been controversial, with some authors demonstrating a need for a second stimulus [28,34]. Recently, purified LOS from non-typable *Haemophilus influenzae* was reported to cause NET formation by human neutrophils [35]. We did not observe NET formation when bPMNs were incubated with various concentrations of LOS (kind gift from Dr. Inzana) for 2 h at 37 °C (data not shown). Non-typable *H. influenzae* LOS required 5 h of incubation to trigger NET formation [35]. We tried various concentrations (10 pg–70 μg) of *H. somni* LOS for up to 8 h and did not observe NET formation at any concentration or time point (data not shown). These data indicate that OMVs cause NET formation, but LOS alone is not the causative agent.

3.3. NETs trap and kill H. somni cells

Several authors have found that bPMNs trap and kill pathogens [12,14,21]. Here, we found that *H. somni* cells were trapped and killed by bovine NETs in a time-dependent manner (Fig. 4 A,B). As expected, bPMNs treated with DNase I, to degrade extracellular DNA, had a reduced ability to trap and kill *H. somni* cells (Fig. 4 C,D). bPMNs treated with cytochalasin D were able to trap and kill *H. somni*, although at a reduced level compared to untreated bPMNs (Fig. 4 C,D). Treatment of cytochalasin D-treated bPMNs with DNase I did reduce NET-mediated trapping and killing (Fig. 4 C,D).

We used scanning electron microscopy (SEM) to examine the ultrastructure of the *H. somni*-induced NETs. *H. somni*-treated bPMNs produced NETs that ensnared *H. somni* cells (see arrows) in a web-like structure where some fibrils are longer than 20 μm (Fig. 5A). In a higher magnification picture, *H. somni* cells are seen trapped in fibrils of DNA that appear to be extruded from a bPMN (Fig. 5B). Untreated bPMNs did not produce NETs (Fig. 5C). Similarly, using confocal microscopy we found *H. somni* caused NET formation by approximately 25% of bPMNs (Fig. 6). We also found *H. somni* cells (green) trapped within a matrix of DNA (red). Addition of DNase I freed the bacterial cells from the matrix (Fig. 6A). NETs were also observed when bPMNs were incubated with *H. somni* OMVs and PMA, but not in response to *H. somni* LOS (Fig. 6).

3.4. H. somni causes macrophage extracellular traps (METs)

Recently, we and other researchers have demonstrated that human [22], murine [23], and bovine macrophages [20] produce

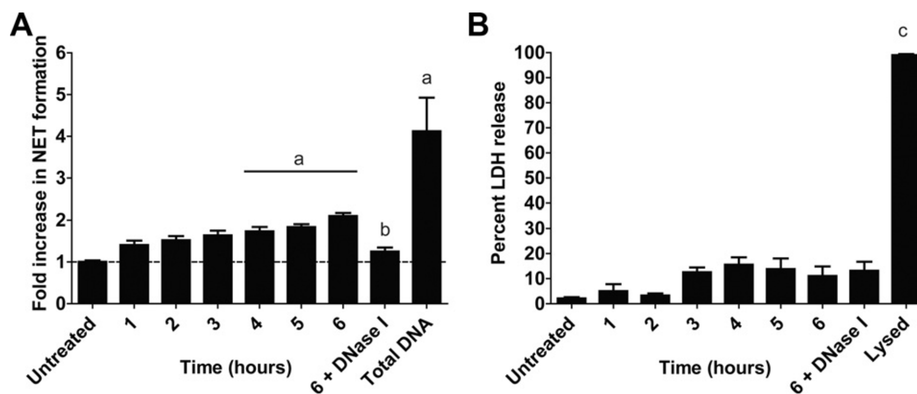

Fig. 2. Repeated exposure to *H. somni* cells causes an increase in NET formation. 10^8 *H. somni* cells were added to 10^6 bPMNs every hour for one to 6 h at 37 °C and (A) NET formation and (B) LDH release were measured. As a control, 180 U of DNase I was also added to one group of cells treated hourly for 6 h. Total DNA was determined by lysing 10^6 bPMNs and DNA quantified as described previously. Data represent the mean ± S.E.M. of 5 independent experiments. a) indicates $p < 0.05$ as compared to bPMNs treated for 1 h; b) indicate $p < 0.05$ as compared to bPMNs treated for 6 h; and c) indicates $p < 0.05$ as compared to untreated bPMNs.

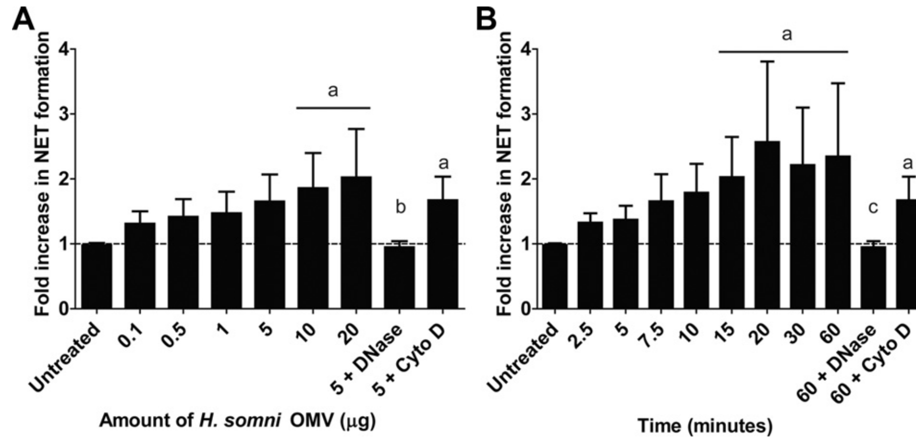

Fig. 3. *H. somni* OMVs cause a dose- and time-dependent increase in NET formation by bovine neutrophils. 10^6 bovine neutrophils were incubated with various concentrations of purified *H. somni* OMVs for 120 min (A) or for various times with 5 μg *H. somni* OMVs at 37 °C (B). In some experiments, 180 U of DNase I or 10 μg/mL cytochalasin D (Cyto D) were also added. Extracellular DNA was quantified as previously described. Data represent the mean ± S.E.M. of 5 independent experiments. a) indicates $p < 0.05$ as compared to untreated bPMNs; b) indicate $p < 0.05$ as compared to bPMNs treated with 5 μg *H. somni* OMVs; and c) indicates $p < 0.05$ as compared to bPMNs treated for 60 min.

macrophage extracellular traps (METs) in response to various stimuli. We found that *H. somni* cells caused the formation of METs by bovine monocyte-derived macrophages in a dose-dependent manner (Fig. 7). MET formation was reduced when bovine macrophages were incubated with DNase I, which cleaves extracellular DNA, but was not affected when bovine macrophages were incubated with cytochalasin D (Fig. 7).

4. Discussion

The results of this study add *H. somni* to the list of pathogens that cause NET formation [12,14]. In particular, *M. haemolytica*, a primary bacterial pathogen that causes BRD, has been shown to cause NET and MET formation [20,21]. NET formation is believed to be an active form of cell death that is independent of necrosis and

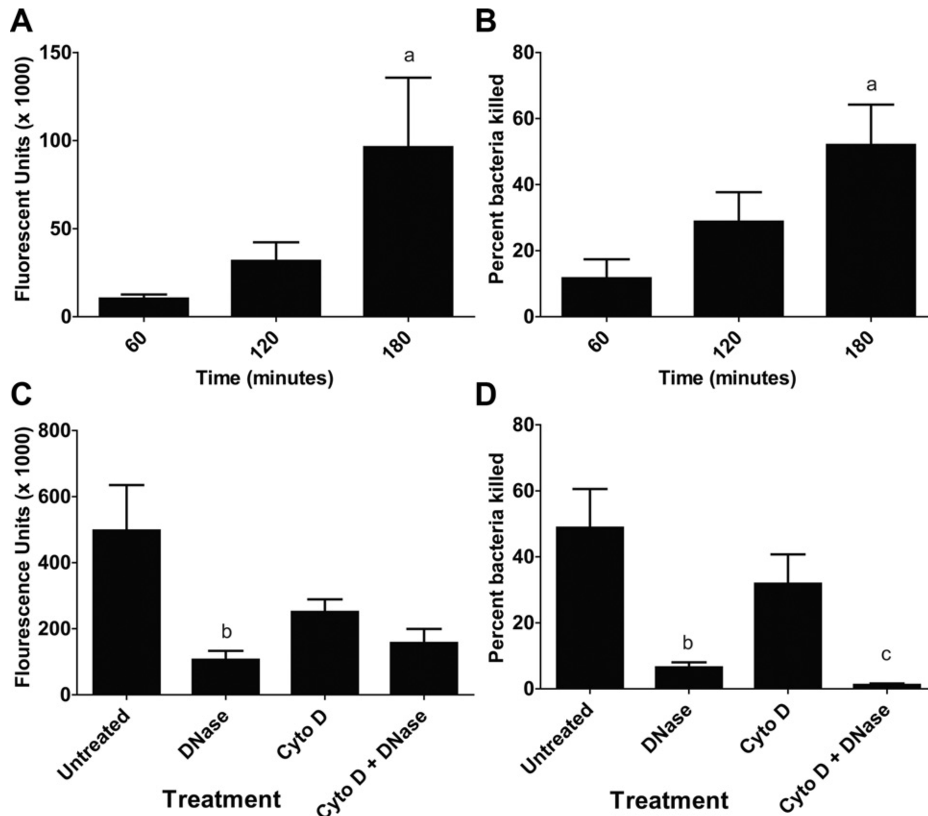

Fig. 4. NETs trap and kill *H. somni* cells. 10^6 untreated bPMNs were incubated with fluorescein- labeled 10^8 *H. somni* cells (A) or unlabeled *H. somni* cells (B) for 60, 120 or 180 min at 37 °C. In another experiment, 10^6 treated and untreated bPMNs were incubated with fluorescein-labeled 10^8 *H. somni* cells (C) or unlableled *H. somni* cells (D) for 120 min at 37 °C. Additional, treatment groups include: 180 U DNase I, 10 μg/mL cytochalasin D (Cyto D), or 180 U DNase I and 10 μg/mL cytochalasin D (Cyto D + DNase). Fluorescence (A) was measured using an automated plate reader. Bacterial survival (B) was estimated by plating serial dilutions of lysates on TSA + 5% sheep RBC plates. Data represent the mean ± S.E.M. of 5 independent experiments a) indicates $p < 0.05$ as compared to bPMNs treated for 60 min; b) indicates $p < 0.05$ as compared to untreated bPMNs; and c) indicates $p < 0.05$ as compared to cytochalasin D treated bPMNs.

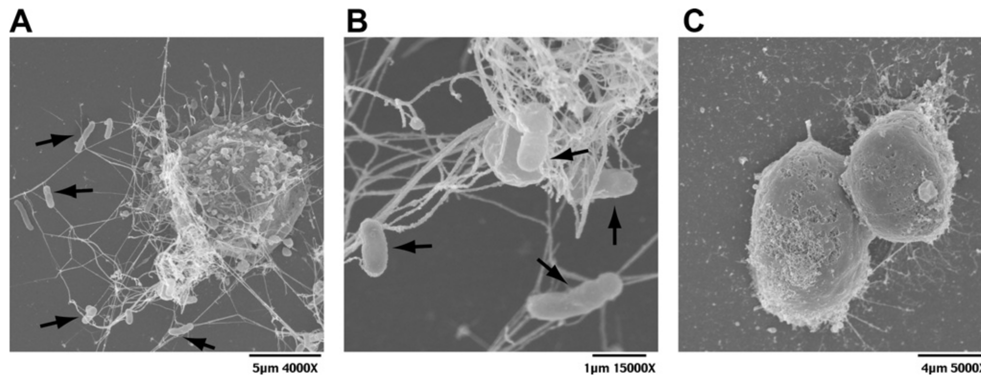

Fig. 5. Scanning electron photomicrograph of NETs formed by bovine neutrophils in response to *H. somni* cells. 10^8 *H. somni* cells were incubated with 3×10^6 bPMNs for 60 min at 37 °C. Cells were washed, fixed, and processed for SEM as described in the Materials and Methods. (A), Micrograph demonstrating a large web-like structure with several *H. somni* cells (arrows) trapped within a network of fibrils great than 20 μM in length (4,000×). (B), Enlargement of (A), to illustrate individual fibrils with *H. somni* cells attached (15,000×). (C), Controls cells incubated in RPMI do not exhibit extracellular fibrils (5,000x). Photomicrographs are of representative cells from 3 independent experiments.

apoptosis. Extracellular DNA release by bPMNs in response to *H. somni* cells occurred without significant release of LDH, implicating NET formation rather than necrosis as the cause for extracellular DNA (Fig. 1). Similarly, cytochalasin D did not significantly diminish NET formation in response to *H. somni* cells (Fig. 1), which others have argued is a hallmark for NETosis [7,9]. Nor was NET formation significantly diminished when the pan-caspase inhibitor, Z-FAD-FMK, was added to inhibit apoptosis (data not shown) [9]. The amount of extracellular DNA was reduced when bovine DNase I was incubated with NETs produced in response to *H. somni* cells (Fig. 1). Repeated exposure to *H. somni* cells (hourly for up to 6 h) further increased NET formation without a significant increase in LDH release (Fig. 2). *M. haemolytica*, another BRD pathogen, elicits a similar response when bPMNs are repeatedly exposed to *M. haemolytica* cells [21].

An important virulence factor for *H. somni* is its truncated lipopolysaccharide, LOS, which has been shown to activate caspase-3 and -8 and induce apoptosis in bovine endothelial cells [36–38]. LOS has also been demonstrated to induce platelet aggregation and activation leading to endothelial cell death and a proinflammatory response [39–42]. Interestingly, NET formation was not observed in response to purified *H. somni* LOS at various concentrations (10 pg–70 μg) nor for various lengths of time (10 min–8 h) (data not shown). Our findings differ from a report that found LOS purified from *H. influenzae* causes NET formation in a model of otitis media [35], and alters the ability of NETs to kill *H. influenzae* cells. Mutants of *H. influenzae*, with LOS core and lipid A assembly defects, were more susceptible to NET killing than *H. influenzae* with native LOS. These date indicate that LOS may cause NET formation, but the NETs formed are limited in their ability to kill *H. influenzae* cells [43]. *H. influenzae* [44–46] and *H. somni* [47–49] both undergo random phase and antigenic variation of the LOS *in vitro* or in response to the specific host immune response leading to changes in the composition or structure of the LOS [50–52]. *H. somni* LOS can also be modified by the addition of phosphorylcholine, which has been demonstrated to be important for colonization of the bacteria to bovine upper respiratory tract [32] and platelet aggregation [39]. Here, we did not observe NET formation in response to LOS purified from *H. somni* 649, which produces LOS with phosphorylcholine modifications.

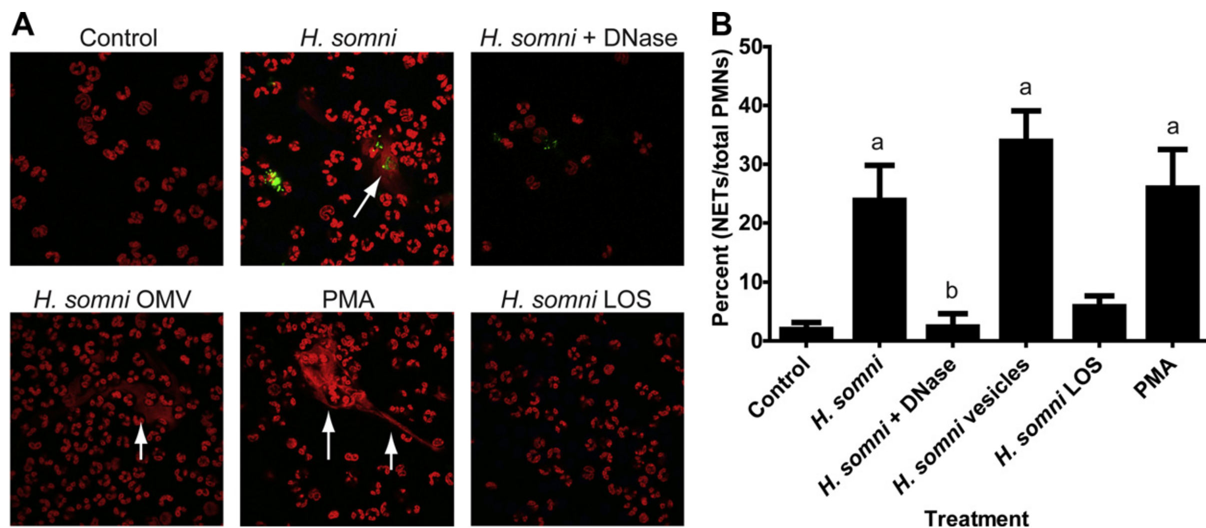

Fig. 6. Confocal photomicrographs demonstrate extracellular DNA released from bovine neutrophils in response to *H. somni* cells, OMVs or PMA. In panel (A), 3×10^6 bPMNs were allowed to attach to poly-L-lysine-treated glass slides and then incubated with RPMI (control) or with various stimulants for 60 min at 37 °C. Stimulants include: 1) 10^8 fluorescein-labeled *H. somni* cells, 2) 10^8 fluorescein-labeled *H. somni* cells with 360 U DNase I, 3) 100 nM PMA, 4) 250 nM *H. somni* LOS, or 5) 100 μg of *H. somni* OMVs. Cells were fixed, stained for DNA using TORPO and examined by confocal microscopy. Arrows indicate characteristic NETs. Panel (B) illustrates the percentage of bPMNs that formed NETs based on scoring 500 bPMNs in multiple micrographs. a) indicates $p < 0.05$ as compared to untreated bPMNs and b) indicates $p < 0.05$ as compared bPMNs incubated with *H. somni* cells. Photomicrographs are of representative cells from 3 independent experiments.

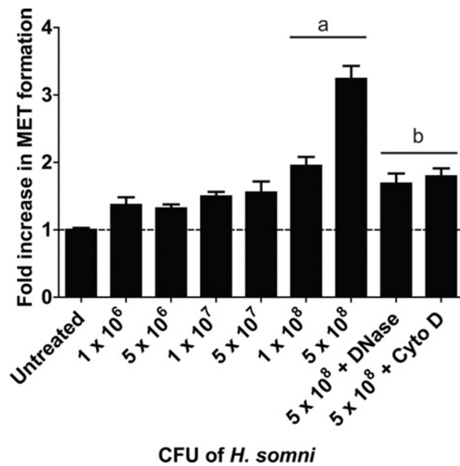

Fig. 7. *H. somni* cells cause MET formation by bovine monocyte-derived macrophages. 10^5 adherent, bovine monocyte-derived macrophages were incubated with various concentrations of *H. somni* cells for 120 min. In some experiments, 180 U DNase I or 10 μg/mL cytochalasin D (Cyto D) were added as controls. Extracellular DNA was quantified as previously described. Data represent the mean ± S.E.M. of 5 independent experiments. a) indicates $p < 0.05$ as compared to untreated macrophages and b) indicates $p < 0.05$ as compared with macrophages incubated with 5×10^8 *H. somni* cells.

OMVs have recently been studied as vaccine candidates for various Gram-negative pathogens such as *Neisseria meningitidis*, *Shigella boydii*, and *Vibrio cholerae* [53–59]. We examined whether *H. somni* OMVs, containing outer membrane proteins and cytosolic components, could cause NET formation. We found that *H. somni* OMVs stimulated NET formation in a dose- and time-dependent response, although the response varied among experiments (Fig. 3). Corbeil and colleagues [60–62] have demonstrated that recombinant immunoglobulin-binding protein (IbpA) subunits protect calves and mice from severe *H. somni* disease. Vaccination of calves with three subunits of IbpA (A3, A5, and DR2) led to a lower percentage of gross lesions and histological abnormalities than control calves and *H. somni* was not recovered from these pneumonic lesions [62]. Because IbpA is secreted in OMVs, future investigations could address the role of IbpA and other OMV proteins in NET formation.

NETs have been reported to trap and kill a variety of bacteria, parasites, and fungi (for a review see [18]). Here, we demonstrate that bPMNs trap and kill *H. somni* cells in a time-dependent manner (Fig. 4). Incubation of NET with DNase I reduced DNA-mediated trapping and killing, indicating that most bacterial cells (>40%) were killed in NETs rather than by phagocytosis. Pre-incubation of bPMNs with cytochalasin D, an inhibitor of actin polymerization, did not significantly reduce the trapping and killing of *H. somni* cells. Cytochalasin D inhibits phagocytosis, but not NET formation, providing further evidence that most bacterial cells are trapped and killed via NET formation (Fig. 4). *H. somni* has been known to be rather resistant to killing via phagocytosis [5]. Perhaps NET formation is a more prominent form of controlling *H. somni in vivo*.

It has been long noted that BRD pathogens cause clusters of “streaming leukocytes” within the alveoli of infected cattle [63,64]. These elongated leukocytes with streaming nuclei are also referred to as “oat cells” [63,64] in which the origins of the streaming leukocytes were mostly produced from neutrophils, with some arising from macrophages [65]. Histological section of a BRD lung also showed extensive neutrophil infiltration and vast amounts of extracellular DNA that have been theorized to be produced by “streaming leukocytes” [21]. Here, we provide photomicrographs that demonstrate elongated bPMNs having a similar morphological structure *in vitro* as previously reported as ‘streaming leukocytes’ [63–66]. Extracellular DNA (i.e. “streaming nuclei”) are seen that have trapped *H. somni* cells (Figs. 5 and 6 (see arrows)). Using confocal microscopy, we confirm that approximately 25–30 percent of bPMNs form NETs when incubated with *H. somni* cells, *H. somni* OMVs, or PMA, but not when incubated with *H. somni* LOS (Fig. 6). These data are similar to previous reports in which 10 to 33 percent of neutrophils underwent NET formation [8,9,21]. We infer that ‘streaming leukocytes’ and free DNA found in the alveolar spaces of BRD afflicted cattle [21] reflect in part NET formation elicited by BRD pathogens such as *H. somni* or *M. haemolytica*.

Recently, macrophages have been demonstrated to produce ETs in response to various stimuli [20,22,23], including *M. haemolytica* [20]. Here, we demonstrate that another BRD pathogen, *H. somni*, also causes MET formation by bovine monocyte-derived bovine macrophages (Fig. 7). We also observed MET formation by bovine alveolar macrophages in response to *H. somni* (data not shown). Macrophages are sentinel cell in the lungs and other tissue sites that detect invaders and release chemoattractant proteins for other leukocytes including neutrophils. The role METs play in the recruitment of inflammatory leukocytes has not been investigated.

The benefits of NETs have been demonstrated primarily by their ability trap and kill various pathogens *in vitro* [18,19]. However, it has been suggested that NETs may play a role in vasculitis, deep vein thrombosis (DVT), systemic lupus erythematosous, pre-eclampsia, gout and arthritis [67–75]. These observations may be relevant to the pathogenesis of *H. somni* infection, which produces a wide array of clinical syndromes including arthritis, vascular inflammation, multiple thrombotic lesions and an acute form of vasculitis called thrombotic meningoencephalitis (TME). The latter is characterized by fibrinopurulent meningitis with abscess formation, hemorrhaging, and thrombotic vasculitis throughout the central nervous system that can result in death within 12–24 h after the onset of symptoms [4]. It has been demonstrated that deposition of NETs in inflamed kidneys, and circulating myeloperoxidase-DNA complexes, can trigger vasculitis and promote an autoimmune response against neutrophil components [69]. Similarly, others have demonstrated that histones and chromatin, which are present in NETs, cause up regulation of thrombin leading to venous thrombi formation in mice that is similar to DVT seen in humans [76]. Interestingly, intravenous administration of histones exacerbated DVT formation, but administration of DNase I, which degrades NETs, protected mice from venous thrombosis [76]. Neutrophils from human cancer patients treated with granulocyte colony-stimulating factor (G-CSF) are sensitized to form NETs upon exposure to a second stimulus, leading to a prothrombotic state in these patients [71]. Platelet-induced NET formation has also been implicated in thrombus formation in a mouse model of transfusion-related acute lung injury, where the administration of an anti-histone antibody and DNase I protected mice for acute lung injury [77]. The connection between *H. somni*-induced vasculitis or TME and NET formation has not been addressed; however, further research should focus on how NETs may play a role in the vascular syndromes seen in *H. somni* infected animals. Perhaps, DNase I should be examined to determine if DNase I could alleviate vascular symptoms.

5. Conclusion

The results of this study provide a new perspective to older observations regarding the interactions of bovine neutrophils and macrophages with *H. somni*. Several reports indicated that bovine neutrophil function was inhibited by various components of *H. somni* (then called *H. somnus*) [78–82]. Despite being

a somewhat fastidious organism in the laboratory, *H. somni* was resistant to killing by bovine neutrophils and mononuclear phagocytes. This resistance was attributed to a weak oxidative burst by neutrophils in the presence of metabolically active *H. somni*, or high and low molecular weight fractions released from the organism [83]. Bovine macrophages also were ineffective at killing *H. somni* and could support limited intracellular multiplication of the organism [84]. Despite these *in vitro* observations, it is clear that host innate defense mechanisms are able to restrict growth of *H. somni* in the respiratory tract and at other sites, as the infections tend to be subacute rather than acute in onset (except for TME) [4,5,50]. The observations in the present study may illustrate one way in which neutrophils and macrophages can battle effectively with *H. somni*. If the organism triggers extracellular trap formation *in vivo*, similar to what was observed *in vitro*, this could provide one means by which the host restrains multiplication and slows the progression of *H. somni* infection.

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References

[1] Genicot B, Mouligneau F, Rollin F, Lindsey JK, Close R, Lekeux P. Economic, clinical and functional consequences of a treatment using metrenperone during an outbreak of shipping fever in cattle. Vet Rec 1993;132(10):245–7.
[2] Yates WD. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can J Comp Med 1982;46(3):225–63.
[3] Collier JR, Brown Jr WW, Chow TL. Microbiologic investigations of natural epizootics of shipping fever of cattle. J Am Vet Med Assoc 1962;140:807–10.
[4] Harris FW, Janzen ED. The *Haemophilus somnus* disease complex (Hemophilosis): a review. Can Vet J 1989;30(10):816–22.
[5] Siddaramppa S, Inzana TJ. *Haemophilus somnus* virulence factors and resistance to host immunity. Anim Health Res Rev 2004;5(1):79–93.
[6] Zekarias B, Mattoo S, Worby C, Lehmann J, Rosenbusch RF, Corbeil LB. *Histophilus somni* IbpA DR2/Fic in virulence and immunoprotection at the natural host alveolar epithelial barrier. Infect Immun 2010;78(5):1850–8.
[7] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303(5663):1532–5.
[8] Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. Nat Rev 2007;5(8):577–82.
[9] Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biology 2007; 176(2):231–41.
[10] Chuammitri P, Ostojic J, Andreasen CB, Redmond SB, Lamont SJ, Palic D. Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. Vet Immunol Immunopathol 2009;129(1–2):126–31.
[11] Ermert D, Urban CF, Laube B, Goosmann C, Zychlinsky A, Brinkmann V. Mouse neutrophil extracellular traps in microbial infections. J Innate Immun 2009; 1(3):181–93.
[12] Grinberg N, Elazar S, Rosenshine I, Shpigel NY. Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic *Escherichia coli*. Infect Immun 2008; 76(6):2802–7.
[13] Katzenback BA, Belosevic M. Isolation and functional characterization of neutrophil-like cells, from goldfish (*Carassius auratus.*) kidney. Dev Comp Immunol 2009;33(4):601–11.
[14] Lippolis JD, Reinhardt TA, Goff JP, Horst RL. Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. Vet Immunol Immunopathol 2006;113(1–2):248–55.
[15] Palic D, Andreasen CB, Ostojic J, Tell RM, Roth JA. Zebrafish (*Danio rerio*) whole kidney assays to measure neutrophil extracellular trap release and degranulation of primary granules. J Immunol Methods 2007;319(1–2):87–97.
[16] Palic D, Ostojic J, Andreasen CB, Roth JA. Fish cast NETs: neutrophil extracellular traps are released from fish neutrophils. Dev Comp Immunol 2007; 31(8):805–16.
[17] Wardini AB, Guimaraes-Costa AB, Nascimento MT, Nadaes NR, Danelli MG, Mazur C, et al. Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. J Gen Virol 2010:259–64.
[18] Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. Cell Death Differ 2012;18(4):581–8.
[19] Saitoh T, Komano J, Saitoh Y, Misawa T, Takahama M, Kozaki T, et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe 2012;12(1):109–16.
[20] Aulik NA, Hellenbrand KM, Czuprynski CJ. *Mannheimia haemolytica* and its leukotoxin cause macrophage extracellular trap formation by bovine macrophages. Infect Immun 2012;80(5):1923–33.
[21] Aulik NA, Hellenbrand KM, Klos H, Czuprynski CJ. *Mannheimia haemolytica* and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. Infect Immun 2010;78(11):4454–66.
[22] Bartneck M, Keul HA, Zwadlo-Klarwasser G, Groll J. Phagocytosis independent extracellular nanoparticle clearance by human immune cells. Nano Lett 2010; 10(1):59–63.
[23] Chow OA, von Kockritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL, et al. Statins enhance formation of phagocyte extracellular traps. Cell Host Microbe 2010;8(5):445–54.
[24] Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E, et al. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. Nat Med 2008;14(9):949–53.
[25] von Kockritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, et al. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. Blood 2008;111(6): 3070–80.
[26] Lin AM, Rubin CJ, Khandpur R, Wang JY, Riblett M, Yalavarthi S, et al. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. J Immunol 2011;187(1):490–500.
[27] Keshari RS, Jyoti A, Kumar S, Dubey M, Verma A, Srinag BS, et al. Neutrophil extracellular traps contain mitochondrial as well as nuclear DNA and exhibit inflammatory potential. Cytometry A 2012;81(3):238–47.
[28] Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ 2009;16(11):1438–44.
[29] Behling-Kelly E, Kim KS, Czuprynski CJ. *Haemophilus somnus* activation of brain endothelial cells: potential role for local cytokine production and thrombosis in central nervous system (CNS) infection. TJ Thromb Haemost 2007;98(4):823–30.
[30] Behling-Kelly E, McClenahan D, Kim KS, Czuprynski CJ. Viable H*aemophilus somnus* induces myosin light-chain kinase-dependent decrease in brain endothelial cell monolayer resistance. Infect Immun 2007;75(9): 4572–81.
[31] Behling-Kelly E, Vonderheid H, Kim KS, Corbeil LB, Czuprynski CJ. Roles of cellular activation and sulfated glycans in *Haemophilus somnus* adherence to bovine brain microvascular endothelial cells. Infect Immun 2006;74(9): 5311–8.
[32] Elswaifi SF, St Michael F, Sreenivas A, Cox A, Carman GM, Inzana TJ. Molecular characterization of phosphorylcholine expression on the lipooligosaccharide of *Histophilus somni*. Microb Pathog 2009;47(4):223–30.
[33] Tagawa Y, Bastida-Corcuera F, Corbeil LB. Immunological characterization of the major outer membrane protein of *Haemophilus somnus*. Vet Microbiol 2000;71(3–4):245–54.
[34] Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. Nat Med 2007;13(4):463–9.
[35] Juneau RA, Pang B, Weimer KE, Armbruster CE, Swords WE. Nontypeable *Haemophilus influenzae* initiates formation of neutrophil extracellular traps. Infect Immun 2011;79(1):431–8.
[36] Sylte MJ, Corbeil LB, Inzana TJ, Czuprynski CJ. *Haemophilus somnus* induces apoptosis in bovine endothelial cells in vitro. Infect Immun 2001;69(3): 1650–60.
[37] Sylte MJ, Kuckleburg CJ, Inzana TJ, Bertics PJ, Czuprynski CJ. Stimulation of P2X receptors enhances lipooligosaccharide-mediated apoptosis of endothelial cells. J Leukoc Biol 2005;77(6):958–65.
[38] Sylte MJ, Leite FP, Kuckleburg CJ, Inzana TJ, Czuprynski CJ. Caspase activation during *Haemophilus somnus* lipooligosaccharide-mediated apoptosis of bovine endothelial cells. Microb Pathog 2003;35(6):285–91.
[39] Kuckleburg CJ, Elswaifi SF, Inzana TJ, Czuprynski CJ. Expression of phosphorylcholine by *Histophilus somni* induces bovine platelet aggregation. Infect Immun 2007;75(2):1045–9.

[40] Kuckleburg CJ, McClenahan DJ, Czuprynski CJ. Platelet activation by *Histophilus somni* and its lipooligosaccharide induces endothelial cell proinflammatory responses and platelet internalization. Shock 2008;29(2):189–96.
[41] Kuckleburg CJ, Sylte MJ, Inzana TJ, Corbeil LB, Darien BJ, Czuprynski CJ. Bovine platelets activated by *Haemophilus somnus* and its LOS induce apoptosis in bovine endothelial cells. Microb Pathog 2005;38(1):23–32.
[42] Kuckleburg CJ, Tiwari R, Czuprynski CJ. Endothelial cell apoptosis induced by bacteria-activated platelets requires caspase-8 and -9 and generation of reactive oxygen species. J Thromb Haemost 2008;99(2):363–72.
[43] Hong W, Juneau RA, Pang B, Swords WE. Survival of bacterial biofilms within neutrophil extracellular traps promotes nontypeable *Haemophilus influenzae* persistence in the chinchilla model for otitis media. J Innate Immun 2009; 1(3):215–24.
[44] Fujita K, Hirano T, Kodama S, Suzuki M. Prognostic impact of phosphorylcholine expression in nontypeable *Haemophilus influenzae* in otitis media with effusion. Acta Otolaryngol 2009;129(8):832–8.
[45] Tong HH, Blue LE, James MA, Chen YP, DeMaria TF. Evaluation of phase variation of nontypeable *Haemophilus influenzae* lipooligosaccharide during nasopharyngeal colonization and development of otitis media in the chinchilla model. Infect Immun 2000;68(8):4593–7.
[46] Weiser JN, Pan N, McGowan KL, Musher D, Martin A, Richards J. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. J Exp Med 1998;187(4):631–40.
[47] Howard MD, Cox AD, Weiser JN, Schurig GG, Inzana TJ. Antigenic diversity of *Haemophilus somnus* lipooligosaccharide: phase-variable accessibility of the phosphorylcholine epitope. J Clin Microbiol 2000;38(12):4412–9.
[48] Howard MD, Willis L, Wakarchuk W, St Michael F, Cox A, Horne WT, et al. Genetics and molecular specificity of sialylation of *Histophilus somni* lipooligosaccharide (LOS) and the effect of LOS sialylation on Toll-like receptor-4 signaling. Vet Microbiol 2011;153(1–2):163–72.
[49] St Michael F, Inzana TJ, Cox AD. Structural analysis of the lipooligosaccharide-derived oligosaccharide of *Histophilus somni* (*Haemophilus somnus*) strain 8025. Carbohydr Res 2006;341(2):281–4.
[50] Inzana TJ, Gogolewski RP, Corbeil LB. Phenotypic phase variation in *Haemophilus somnus* lipooligosaccharide during bovine pneumonia and after in vitro passage. Infect Immun 1992;60(7):2943–51.
[51] Inzana TJ, Hensley J, McQuiston J, Lesse AJ, Campagnari AA, Boyle SM, et al. Phase variation and conservation of lipooligosaccharide epitopes in *Haemophilus somnus*. Infect Immun 1997;65(11):4675–81.
[52] Inzana TJ, Iritani B, Gogolewski RP, Kania SA, Corbeil LB. Purification and characterization of lipooligosaccharides from four strains of *Haemophilus somnus*. Infect Immun 1988;56(11):2830–7.
[53] Mirlashari MR, Hagberg IA, Lyberg T. Platelet-platelet and platelet-leukocyte interactions induced by outer membrane vesicles from *N. meningitidis*. Platelets 2002;13(2):91–9.
[54] Mitra S, Barman S, Nag D, Sinha R, Saha DR, Koley H. Outer membrane vesicles of *Shigella boydii* type 4 induce passive immunity in neonatal mice. FEMS Immunol Med Microbiol 2012.
[55] van de Waterbeemd B, Streefland M, van Keulen L, van den Ijssel J, de Haan A, Eppink MH, et al. Identification and optimization of critical process parameters for the production of NOMV vaccine against *Neisseria meningitidis*. Vaccine 2012;30(24):3683–90.
[56] Avila-Calderon ED, Lopez-Merino A, Jain N, Peralta H, Lopez-Villegas EO, Sriranganathan N, et al. Characterization of outer membrane vesicles from *Brucella melitensis* and protection induced in mice. Clin Dev Immunol 2011; 2012:352493.
[57] Bishop AL, Tarique AA, Patimalla B, Calderwood SB, Qadri F, Camilli A. Immunization of mice with *Vibrio cholerae* outer-membrane vesicles protects against hyperinfectious challenge and blocks transmission. J Infect Dis 2011; 205(3):412–21.
[58] Nakao R, Hasegawa H, Ochiai K, Takashiba S, Ainai A, Ohnishi M, et al. Outer membrane vesicles of *Porphyromonas gingivalis* elicit a mucosal immune response. PloS One 2011;6(10):e26163.
[59] Collins BS. Gram-negative outer membrane vesicles in vaccine development. Discov Med 2011;12(62):7–15.
[60] Geertsema RS, Worby C, Kruger RP, Tagawa Y, Russo R, Herdman DS, et al. Protection of mice against *H. somni* septicemia by vaccination with recombinant immunoglobulin binding protein subunits. Vaccine 2008;26(35): 4506–12.
[61] Lo KL, Kimball RA, Lehmann J, Gershwin LJ, Worby C, Corbeil LB. Antibody responses of calves to *Histophilus somni* recombinant IbpA subunits. Comp Immunol Microbiol Infect Dis 2012;35(5):453–9.
[62] Geertsema RS, Zekarias B, La Franco Scheuch L, Worby C, Russo R, Gershwin LJ, et al. IbpA DR2 subunit immunization protects calves against *Histophilus somni* pneumonia. Vaccine 2011;29(29–30):4805–12.
[63] Brogden KA, DeBey B, Audibert F, Lehmkuhl H, Chedid L. Protection of ruminants by *Pasteurella haemolytica* A1 capsular polysaccharide vaccines containing muramyl dipeptide analogs. Vaccine 1995;13(17):1677–84.
[64] Caswell JL, Middleton DM, Sorden SD, Gordon JR. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. Vet Pathol 1998;35(2):124–31.
[65] Ackermann MR, DeBey BM, Stabel TJ, Gold JH, Register KB, Meehan JT. Distribution of anti-CD68 (EBM11) immunoreactivity in formalin-fixed, paraffin-embedded bovine tissues. Vet Pathol 1994;31(3):340–8.
[66] Yener Z, Ilhan F, Ilhan Z, Saglam YS. Immunohistochemical detection of *Mannheimia (Pasteurella) haemolytica* antigens in goats with natural pneumonia. Vet Res Commun 2009 Apr;33(4):305–13.
[67] Gupta A, Hasler P, Gebhardt S, Holzgreve W, Hahn S. Occurrence of neutrophil extracellular DNA traps (NETs) in pre-eclampsia: a link with elevated levels of cell-free DNA? Ann N Y Acad Sci 2006;1075:118–22.
[68] Hakkim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A 2010;107(21):9813–8.
[69] Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009;15(6):623–5.
[70] Logters T, Paunel-Gorgulu A, Zilkens C, Altrichter J, Scholz M, Thelen S, et al. Diagnostic accuracy of neutrophil-derived circulating free DNA (cf-DNA/NETs) for septic arthritis. J Orthop Res 2009;27(11):1401–7.
[71] Demers M, Krause DS, Schatzberg D, Martinod K, Voorhees JR, Fuchs TA, et al. Cancers predispose neutrophils to release extracellular DNA traps that contribute to cancer-associated thrombosis. Proc Natl Acad Sci U S A 2012.
[72] Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler Thromb Vasc Biol 2012;32(8):1777–83.
[73] Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers Jr DD, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A 2012;107(36):15880–5.
[74] Knight JS, Kaplan MJ. Lupus neutrophils: 'NET' gain in understanding lupus pathogenesis. Curr Opin Rheumatol 2012.
[75] Mitroulis I, Kambas K, Chrysanthopoulou A, Skendros P, Apostolidou E, Kourtzelis I, et al. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PloS One 2011;6(12): e29318.
[76] Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. J Thromb Haemost 2011;10(1):136–44.
[77] Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. J Clin Invest 2012;122(7):2661–71.
[78] Gomis SM, Godson DL, Beskorwayne T, Wobeser GA, Potter AA. Modulation of phagocytic function of bovine mononuclear phagocytes by *Haemophilus somnus*. Microb Pathog 1997;22(1):13–21.
[79] Howard MD, Boone JH, Buechner-Maxwell V, Schurig GG, Inzana TJ. Inhibition of bovine macrophage and polymorphonuclear leukocyte superoxide anion production by *Haemophilus somnus*. Microb Pathog 2004;37(5):263–71.
[80] Pfeifer CG, Campos M, Beskorwayne T, Babiuk LA, Potter AA. Effect of *Haemophilus somnus* on phagocytosis and hydrogen peroxide production by bovine polymorphonuclear leukocytes. Microb Pathog 1992;13(3):191–202.
[81] Sample AK, Czuprynski CJ. Elimination of hydrogen peroxide by *Haemophilus somnus*, a catalase-negative pathogen of cattle. Infect Immun 1991;59(7): 2239–44.
[82] Chiang YW, Kaeberle ML, Roth JA. Identification of suppressive components in *Haemophilus somnus* fractions which inhibit bovine polymorphonuclear leukocyte function. Infect Immun 1986;52(3):792–7.
[83] Czuprynski CJ, Hamilton HL. Bovine neutrophils ingest but do not kill *Haemophilus somnus* in vitro. Infect Immun 1985;50(2):431–6.
[84] Lederer JA, Brown JF, Czuprynski CJ. *Haemophilus somnus*, a facultative intracellular pathogen of bovine mononuclear phagocytes. Infect Immun 1987;55(2):381–7.