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Histophilus somni causes extracellular trap formation by bovine neutrophils and macrophages

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ABSTRACT

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 lipooligosaccharide 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 lipooligosaccharide (LOS), exopolysaccharide 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.
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 pathogenic [18]. Mast cells also produce ETs containing nano-particles [20,22,23]. Mast cells also produce extracellular traps (ETs) [20,22–26]. 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 leukotxin, 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 2100g 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 2100g 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 1000g and washed 3× in HBSS with 4 mM EDTA. Mononuclear cells were resuspended in RPMI-1649 (Cellgro) with 1% FBS and incubated for 2 h on 100 mm barbital 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, Manassas, VA) to remove contaminating fibrinogen and platelets. Cells were suspended 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% CO2. 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, Callsbad, CA) in 10 mM Tris base buffered 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 × 106) 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 105 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).
For scanning electron microscopy, bPMNs \((3 \times 10^6)\) 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 \(3 \times \) 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 \(3 \times \) with PBS and resuspended at a concentration of \(5 \times 10^7\) 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 \(\mu\)g/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 \(3 \times \) with PBS and fluorescence determined using an automated plate reader. To determine bacterial killing by NETs, bPMNs 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 \(\times \) g for 30 min, the supernatant collected and centrifuged again at 20,000 \(\times \) g for 30 min. These cell-free supernatants were ultracentrifuged at 105,000 \(\times \) g for 30 min at 4 °C to collect the OMVs. *H. somni* OMVs 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 pancaspase 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 [8,9]. We report previously that repeated exposure of bPMNs to neutrophils undergo NET formation when exposed to a stimulus.

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

H. somni has several virulence factors that contribute to its pathogenesis including lipooligosaccharide (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 lipopolysaccharide (LPS) to cause NET formation has been controversial, with some authors demonstrating a need for a second stimulus [28,34]. Recently, purified LOS from nontypable 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). Nontypable 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. 5 A). In a higher magnification picture, H. somni cells are seen trapped in fibrils of DNA that appear to be extruded from a bPMN (Fig. 5 B). Untreated bPMNs did not produce NETs (Fig. 5 C). 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. 6 A). 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. 10⁶ H. somni cells were added to 10⁶ 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 10⁶ 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 untreated bPMNs; and c) indicates $p < 0.05$ as compared to untreated bPMNs.
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...
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 data 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. 5. Scanning electron photomicrograph of NETs formed by bovine neutrophils in response to H. somni cells. 10⁵ H. somni cells were incubated with 3 × 10⁵ 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 greater than 20 μm in length (4,000x). (B), Enlargement of (A), to illustrate individual fibrils with H. somni cells attached (15,000x). (C), Controls cells incubated in RPMI do not exhibit extracellular fibrils (5,000x). Photomicrographs are of representative cells from 3 independent experiments.

Fig. 6. Confocal photomicrographs demonstrate extracellular DNA released from bovine neutrophils in response to H. somni cells, OMVs or PMA. In panel (A), 3 × 10⁶ 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) 10⁶ fluorescein-labeled H. somni cells, 2) 10⁶ 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.
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 pneumatic 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 erythematosus, preeclampsia, 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 meningocenchephalitis (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 chro-matin, 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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