ABSTRACT

Title of Thesis: **BOVINE NEUTROPHILS RELEASE EXTRACELLULAR TRAPS UPON STIMULATION WITH *OSTERTAGIA OSTERTAGI***

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*Ostertagi*a *ostertagi* is a widespread parasite that causes significant production losses in the cattle industry. Recently discovered neutrophil extracellular traps (NETs) have been demonstrated as important effector mechanisms of neutrophils against various pathogens including parasitic worms. Exposure of neutrophils to *O. ostertagi* extract resulted in a significant release of extracellular DNA and co-localization of NET associated proteins histone and neutrophil elastase confirmed these structures of DNA as NETs. In response to both live and heat-killed *O. ostertagi* larvae, there was a similarly strong release of NETs. *O. ostertagi* induced NETs were significantly diminished by inhibition of enzymes NADPH oxidase, neutrophil elastase, and myeloperoxidase. Interestingly, NETs were also released in response to non-pathogenic nematode *C. elegans* indicating a potential conserved response to

nematodes. Mouse neutrophils demonstrated a similar NET response to *O. ostertagi* however there was no response to *C. elegans.* Surprisingly, these NET responses did not appear to be dependent on production of reactive oxygen species (ROS) as has been previously reported. This is the first report indicating *O. ostertagi*-induced NET formation and indicates a potential role for NETs in the response against *O. ostertagi* infection.

BOVINE NEUTROPHILS RELEASE EXTRACELLULAR TRAPS UPON STIMULATION WITH *OSTERTAGIA OSTERTAGI.*

by

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List of Abbreviations

ABAH	4-Aminobenzoic acid hydrazide
AFU	Arbitrary fluorescence units
Ag	Antigen
APC	Antigen presenting cell
B6	C57BL/6 mouse
BM	Bone marrow
BSA	Bovine serum albumin
CGD	Chronic granulomatous disease
CMK	Suc-Ala-Ala-Pro-Val chloromethyl ketone
DNase	deoxyribonuclease
DPI	diphenylene iodonium
FBS	Fetal bovine serum
H2O2	Hydrogen Peroxide
IL	interleukin
IFN	interferon
LPS	Lipopolysaccharide
M	molar
MNase	micrococcal nuclease
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate

NET	neutrophil extracellular trap
OO	*Ostertagia ostertagi*
PBL	Peripheral blood
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PKC	Protein kinase C
PFA	Paraformaldehyde
PMA	phorbol 12-myristate 13-acetate
RFU	Relative fluorescence units
RLU	Relative light units
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WT	Wild type

Chapter 1: Introduction

Significance

Despite continued efforts to develop effective vaccines against the parasite *O. ostertagi,* there currently exists no commercially available vaccine against this parasite (1). Infection with *O. ostertagi* causes significant production losses in the beef industry and is of particular concern for the organic beef market where anti-helminthic use is prohibited (2, 3). Cattle infected with the parasite O. ostertagi are not able to efficiently clear the parasite and develop an impaired protective immunity, resulting in repeated or chronic infections, even though there is evidence of immune cell infiltration into the local environment (4). This suggests the parasite may be influencing the local inflammatory environment to prevent or avoid clearance. Neutrophils, one of the immune systems first-responders, circulate throughout the body in large numbers and possess a surprisingly complex immunological repertoire (5). Interactions between *O. ostertagi* and immune cells, particularly innate immune cells such as neutrophils, is not well known. While neutrophils possess many different functions, the recently discovered mechanism for the release of neutrophil extracellular traps (NETs) has been demonstrated to be an important effector mechanism of neutrophils against a wide array of pathogens including parasites and worms (6).

With each new discovery, the mechanisms of NET formation and their role in inflammation is becoming increasingly complex (7). Multiple mechanisms for NET

formation have been proposed, however the mechanism of NET formation in response to extracellular pathogens, particularly worms, is largely unknown. Additionally, once released, NETs can contribute to not only immunological defense, but can play roles in resolution of inflammation as well (6, 8). If *O. ostertagi* does induce NET release, this would not only contribute another parasite to the growing list of infections NETs are involved in, but also advance our understanding of the role neutrophils play in *O. ostertagi* infection, potentially highlighting targets or strategies for the development of vaccines or possible treatments against this disease. While NETs have demonstrated the capacity to successfully capture other extracellular parasites, no report currently exists on the ability of *O. ostertagia* to induce NETs (9). Therefore, the objective of my research is to determine whether *O. ostertagia* is capable of inducing NETs and the potential mechanisms involved in this response.

O. ostertagia

Ostertagia ostertagi (*O. ostertagi*), also known as the brown stomach worm is a gastrointestinal nematode parasite in cattle and is considered one of the most economically significant in the industry (10). Once ingested by the bovine host, Ostertagia larvae invade the glands of the abomasum, causing cellular hyperplasia and infiltration by local immune cells (11). Despite the infiltration and accumulation of immune cells, there is no immunological control of Ostertagia infection, causing significant production loss. This is exacerbated by the lack of protective immunological memory formation, meaning cattle can be repeatedly infected, increasing pathology

and conversely production losses. Parasitic infection by *O. ostertagi* is of importance in grass-fed cattle. Exposure to the parasite is much more difficult to control in a grass-fed setting, where they can readily encounter the parasite, as opposed to cattle which are fed by feedlot. Grass-fed beef, which can include both organic and non-organic products, is a market undergoing massive growth, as consumers seek healthier or more "natural" meat products. Grass-fed beef will comprise 30 percent to 40 percent of the total beef market sector within the next 10 years (12), with demand for grass-fed beef growing at an annual rate of 25-30 percent for the past decade (13). As the market grows, *O. ostertagi* will only continue to cost producers more, thus a better understanding of the mechanisms of infection and the bovine immune response is crucial. The life cycle of *O. ostertagi* has been well-defined, beginning with the ingestion of the infective third stage larvae (L3s). These larvae then exsheath in the rumen, travel to the abomasum, and invade abomasal gastric glands. A prepatent period of ~3 weeks (14) involves multiple molting's until it reaches its adult stage and leaves the abomasal glands. This is followed by mating and the laying of eggs by the adult worms, which are shed by its host into the environment. In ~2 weeks, the worms hatch and develop to the infective L3 stage, which can survive for extended periods of time waiting for it to be ingested by a new host (10). During infection, progressive weight loss, anorexia, diarrhea, hypoproteinemia, and edema under the lower jaw are possible pathologies (15). Hyperplasia and thickening of the mucosa in the affected tissues leads to lesions, which are slightly raised, pale-colored, circular nodules of 2-3 mm in diameter. In chronic infections, the entire abomasal mucosa can become hyperplasic

and bleeding. Treatment for parasites such as Ostertagia are limited to anthelmintics. However there is a concern for anthelmintic drug resistance in cattle parasites, which is amplified by the overuse and misuse of drugs in the cattle industry (15). Additionally, the use of such products is prohibited in organic farming, limiting the options available to farmers and producers.

While there have been attempts at developing a vaccine, their efficacy has been limited and none has reached the market (16). Clearly, the interactions between parasite and host immunology need to be further understood for better treatments and more effective vaccines to be developed, as the burden of GI parasites such as *O. ostertagi* will only grow larger. Parasites have evolved to evade or influence host immune responses to their benefit, and most of these mechanisms are not well understood (17). In Ostertagia specifically, immunosuppression has been observed when worm extract was co-cultured with antigen-specific lymphocytes (18). However, there has been little attention paid to the role that innate immune cells, such as neutrophils, a crucial first-responder, play in Ostertagia infection. Neutrophils produce and release a variety of chemokines and cytokines which can influence the local immune response, and as they are present in such large numbers early in infection, they may play a large role in the establishment of infection (10).

Neutrophil Biology

Neutrophils, also known as polymorphonuclear leukocytes, are one of the most abundant white blood cells found in mammals and provide a first line of defense against

pathogens and injury. They are produced in significant quantities. In humans in particular, an estimated 10^{11} neutrophils are produced and released into the circulation daily (19). Neutrophils have been highlighted to have roles in various inflammatory conditions such as atherosclerosis, cancer, autoimmunity, and many others (10, 11, 14–16, 20). Neutrophils are normally considered to be short-lived effector cells with circulating lifespans of about 8 hours in humans (13, 17) and 1.5 hours to 8 hours in mice (18, 21). Neutrophils complete their maturation process in the bone marrow (22) with a large reservoir of mature neutrophils remaining in the bone marrow to replace peripheral neutrophils or supplement them at times of increased demand such as during infection or inflammation (23). Once released into the peripheral blood, neutrophils are transcriptionally silent. No major change in gene expression is associated with the release of neutrophils from the bone marrow to the circulation. However, major transcriptional changes occur once neutrophils migrate into tissues (24). Gene expression profiling of neutrophils has been conducted in multiple studies comparing disease states ex-vivo (25–28), as well as in-vitro following stimulation with agents such as bacteria, LPS, and by phagocytosis of IgG- and complement-coated latex beads (26, 28–33). It has been recognized that there are differences among different stimuli in vitro (28, 31), as well as differences between in-vitro and in-vivo stimulation (26). This suggests that neutrophils generate distinct transcriptional responses depending on the type of stimuli, likely with multiple, and not individual, signaling pathways contributing to the transcriptional response of neutrophils. Neutrophils can produce and release a variety of chemokines and cytokines, influencing the local inflammatory

environment. The earliest neutrophil cytokine signal released is to recruit more neutrophils, with large amounts of IL-8 being released (34). Neutrophils can also produce IL-1β and TNF-α which induce other cells to produce neutrophil attracting chemokines (35, 36).

Neutrophil Killing

Neutrophils main immunological function is to kill or impede microbial threats. One of the main mechanisms by which neutrophils kill is by producing a wide array of proteins that can directly kill or impair pathogens, and the majority of these are stored in granules. Neutrophil granules can be classified into three distinct subsets based on the presence of certain granule proteins. Primary or azurophilic granules (myeloperoxidase), secondary granules (lactoferrin), and tertiary granules (gelatinase) (24, 37). Azurophilic granules contain MPO (myeloperoxidase), but can also carry defensins, lysozyme, bactericidal/permeability-increasing protein (BPI), and a number of serine proteases: neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G (CG) (37–39). The second class of granules, the secondary granules, are smaller, do not contain MPO, and are characterized by the presence of the glycoprotein lactoferrin. They also contain a wide range of antimicrobial compounds including NGAL, hCAP-18, and lysozyme (39). The third class, the tertiary granules, also MPO-negative, are smaller than secondary granules, and contain few antimicrobials, but they serve as a storage location for several metalloproteases, such as gelatinase and leukolysin. There are three categories of antimicrobials found within neutrophils: proteins that bind to

microbial membranes (cationic proteins), proteins that prevent access to essential nutrients, and enzymes (5). These proteins are normally stored in the aforementioned granules so far hundreds of antimicrobial peptides have been described (40). However, before a neutrophil can release its various anti-microbial weapons, it must reach its target and be activated. Once the neutrophil has left circulation and crossed the endothelium, various chemoattractant and inflammatory stimulants, both host and pathogen-derived, will predominantly dictate their behavior (41). These chemoattractant will bind their respective receptors including pattern recognition receptors such as Toll-like receptors (TLRs), continuing the neutrophils activation. In neutrophils, all but TLR3 are expressed, and their stimulation contributes to further activation (42, 43). However, the signals and inputs neutrophils receive during activation are complex and the exact effects of these signals are incompletely understood.

Neutrophil Heterogeneity

The concept of neutrophil heterogeneity has existed for some time, but has recently become the subject of much interest, with growing evidence of neutrophil populations with distinct functions under both homeostatic and pathological conditions. Multiple strategies have been used to identify distinct neutrophil populations, including distinct cell surface markers, cell maturity status, immunological functions, and tissue residency. Subsets of circulating human neutrophils have been linked with autoimmunity based on expression of CD177 and OLFM4 (44, 45). Aged neutrophils

and neutrophils that have undergone reverse transmigration also display changes in their cell surface markers (46, 47). Novel neutrophil populations have recently been identified during various conditions such as infection (48–51), autoimmunity (52), cancer (53, 54), cardiovascular disease (55, 56), and pregnancy (57). However, it is still not understood whether these examples are distinct neutrophil subsets that are derived from separate lineages or are simply different activation or polarization states of a common neutrophil. This has been particularly relevant in the cancer field. Contradictory roles for neutrophils in this disease setting have been established, with a large number of studies identifying pro-tumoral functions (42, 43, 58–60) while others demonstrate anti-tumor properties (61–65). These populations have been termed N1 and N2 to describe anti-tumor and pro-tumor neutrophil populations, respectively. A similar dichotomy has been seen in the blood of tumor-bearing mice and cancer patients based on density gradient separation (69, 70). Neutrophil populations were found in the low-density fraction, collectively termed low-density neutrophils (LDNs), and the high-density layer, termed high-density neutrophils (HDNs). The HDN and LDN populations displayed anti-tumor and pro-tumor functions, respectively (69). The similarities between the N1/N2 polarized neutrophils and circulating mature HDNs/LDNs has prompted a new nomenclature of NC1and NC2 (69). These polarization schemes have also been adopted in other models. Clearly, the functional variation and possible plasticity of what has long been considered a relatively straightforward cell is only beginning to be unraveled, and it appears that much is still to be learned regarding the abilities and functions of neutrophils. While the

predominant investigation on the abilities of neutrophils is reserved for humans and mice, work in other species has revealed many similarities but also key differences between neutrophils from different species.

Bovine Neutrophils

Bovine neutrophils, while similar to human and mouse neutrophils, do possess distinct features. For example, the composition of bovine neutrophil granules is not the same. Lysozyme, a bactericidal enzyme of azurophilic granules is abundant in other species, yet is present at very low levels in bovine granules(71). Bovine neutrophils also possess an additional, unique granule, that isn't present in humans or mice (72) and lack receptors for N-formylated peptides (73). While in humans, neutrophils make up a large portion of the circulating leukocytes (50-70%), neutrophils in cattle make up about 25% (74). In cattle, neutrophils also have half-lives of around 9 hours (75). In contrast to human neutrophils, bovine neutrophils do not express FcγRI and FcγRII receptors, and have a special receptor for IgG2, the Fcγ2 receptor, which is distinct from all other Fcγ receptors(76).

Bovine neutrophils, as in other species, interact with other immune cells. Research into neutrophil degranulation products has suggested that these neutrophil products (secretory vesicles, primary, secondary, and tertiary granules) can influence the activation, adhesion and the migration of three bovine monocyte subsets, as well as affecting monocyte-macrophage differentiation(38), something similar to what is seen in humans(77).

Bovine neutrophils can respond to toll-like receptor ligands. So far, 10 TLRs has been identified in cattle (78). The TLRs in cattle have been associated with the recognition of Mycobacterium tuberculosis and *M. bovis* by macrophages (79), bovine respiratory disease (80), and *Escherichia coli* mediated mastitis(81). In bovine neutrophils the detection of mRNA for TLR1, TLR2, TLR4, TLR6, TLR7 and TLR10, but not TLR3, TLR5, TLR8, TLR9 has been reported previously(82).

Stimulation of bovine neutrophils with LPS has been shown to induce changes in mRNA expression within 2 hours, increasing expression of IL-8, SOD2, TNF-a and BPI (83). Exposure to LPS also can influence the production of inflammatory mediators including IL-1β, IL-12, IFN-γ, IL-8, and TNF-a(84, 85). Suggesting that cattle neutrophils are capable of rapidly changing and regulating RNA expression, a potentially overlooked area of neutrophil function, as neutrophils are predominantly believed to be short-lived and contain relatively little RNA.

Neutrophil Extracellular Traps

A current area of active study and research comes from the discovery of neutrophil extracellular traps (NETS). NETosis, or the process of NET formation, is thought to be a novel form of extracellular bacterial killing, whereby neutrophils release a mesh of chromatin fibers which contains anti-microbial peptides and enzymes such as NE and MPO attached (86). NET formation has still not been well defined, however. It's is widely considered that the involvement of the reactive oxygens species (ROS) pathway is necessary (87), although the actual mechanisms by which is involved are

currently unknown (88). Other key molecules believed to be necessary for NETosis are MPO NADPH oxidase, and neutrophil elastase (89–91). Involvement of the Raf-MEK-ERK pathway, which is upstream from the NADPH oxidase pathway, is also involved in NET formation (92).

Autophagy has been associated with NET formation, and NET formation is beginning to be considered a variation of the autophagy process (93). In addition it appears that the ROS pathways is involved in the autophagy process, which may establish a connection between ROS and NETs However, the use of non-specific autophagy inhibitors has made it difficult to make a definitive connection (93). A reason for the difficulty in researching NETS is the lack of or more specifically inability to develop knockout models for NETS. Additionally, as NETs are predominantly DNA, it is difficult to conclude the source of any extracellular DNA, or whether the release of the DNA from a neutrophil is indeed a NET structure. Neutrophils, like most cells, would be unlikely to survive and develop without DNA or histones and basic function of neutrophils would be greatly impacted without the other factors essential for NET formation such as NADPH oxidase, MPO, and NE. This is the main reason as to why most NET research is performed *ex-vivo* or *in vitro*, and why the evidence for NET function *in-vivo* is predominantly indirect, although there has been an increasing trend towards novel intravital imaging techniques to directly observe NET formation and activity (94, 95), which may greatly enhance our understanding of the NET formation process.

NETs have been implicated to have roles in various infectious models including fungal (96), bacterial (86), viral (97), and protozoan (98) infections, not to mention their potentially significant role in sterile inflammatory conditions including autoimmune disorders such as systemic lupus erythematous (SLE) (99). NETs have been characterized in several mammals including humans (91), mice (100), goats (101), sheep (102), cattle (103), and dolphins (104). Similar structures have been observed in the neutrophil-like cells of other vertebrates such as chickens (105) and fish (106), and even non vertebrates such as gastropods (107). Release of extracellular structures has been observed in other immune cells such as macrophages (108), eosinophils (109), mast cells (110) and even the root-tip cells from plants (111) suggesting that extracellular trap formation may be an evolutionarily conserved if not primitive defense mechanism. The capacity of these NETs to kill so far has been model-dependent, as the evidence has so far been contradictory (98, 112).

Whether or not neutrophils survive post-NET release is also debatable. While NETs seem to remain anchored to the neutrophil that release them (113), neutrophils have been shown to continue to perform basic immune functions post-NET release *in-vivo* (113) while *in-vitro* findings suggest that NETosis leads to lysis of the cell (91). Currently, there are three distinct forms of NET release theorized, although this is still greatly debated (7, 114). There are the typical or "classical" NETs, of which there is significant evidence and involve the death of the cell (115). Instances of neutrophils surviving post-NET release is limited but growing. They are considered to be the result

of either mitochondrial release of DNA (116, 117), or from the release of relatively small amounts of DNA trafficked by vesicles (114, 118)

The determinants that lead to NET formation are not yet clear, as neutrophils have other effector functions such as phagocytosis, degranulation, ROS, etc. Imaging has shown that only about 30-50% of neutrophils undergo NET formation even under artificial stimulation using the potent NET inducer phorbol 12-myristate 13-acetate (PMA), although the variability is quite donor-dependent (119). Additionally, there is evidence that the release of NETs can be context dependent, where PMA may activate a certain pathway, another may activate a completely different pathway (120, 121). The possibility of species specific differences between host neutrophils, has also not been adequately addressed. One current theory regarding the neutrophil "decision" to under NET formation, posits that the formation of NETs is due to the inability of neutrophils to properly phagocytize a pathogen, which would explain NETs being released against large fungal hyphae and the viruses which are very small (122). While an attractive theory, there is currently no accepted model for why some neutrophils release NETs and others do not under the same conditions.

Although neutrophils have proven capable of releasing NETs in response to a small number of nematode parasites (107, 123), including one recent report in cattle (9), it is currently unknown whether stimulation with *O. ostertagi* would induce the release of NETs, and what the mechanisms underlying this response would be. Based on the prevalence of the NET responses to various pathogens including relatively large,

extracellular parasites and fungi (122, 124), I hypothesize that *O. ostertagi* is capable of inducing the release of NETs from bovine neutrophils in a similar manner.

Chapter 2: Materials and Methods

Cattle

The Wye Angus herd is a closed herd maintained by the Wye Research and Education Center, University of Maryland Experimental Station (Queenstown, MD) (125) . The steers were maintained on the pasture of orchard grass, alfalfa, or clover, and fed with alfalfa, and bailage in winter (126). Helminth-free Holstein steers were maintained on the campus of Beltsville Agricultural Research Center (BARC), Beltsville, MD. Jugular venous blood was obtained for neutrophil and serum isolation. Animal Care and Use Protocols were approved by both the BARC and UMD Institutional Animal Care and Use Committees**.**

Parasite propagation and parasitic antigen preparation

O. ostertagi adult worms and stage four larvae (L4) were propagated in helminth-free calves as described previously (127). Briefly, 4-6 months old, helminth-free Holstein steers were inoculated with a bolus dose of O. ostertagi L3 on Day 0 and euthanized on Day 9 for L4 larvae or Day 21 for adult worm, and the abomasum and abomasal content were collected for parasite isolation. Parasites were collected from the abomasal tissue using the Baermann technique (L4) or from the abomasal content using the gel migration method (adult). Live L4 larvae were washed 3 times in cell culture medium containing antibiotics prior to use in co-cultures. Adult parasites were washed 2 times with cold PBS and homogenized. The homogenate was centrifuged at

20,000g for 30 min at 4°C and soluble extract (hereafter referred to as "OO extract") was stored at -20°C prior to experimental use.

For the C. elegans experiments, a mixed population (early stage larvae to adult) of soil nematode Caenorhabditis elegans was grown at 20°C in axenic liquid mCeHR-2 medium supplemented with 20 µM hemin (128). Both live and heat-killed (60°C, 60 min) worms were spun down at 800g and washed twice with PBS before being resuspended in RPMI media (129).

Tissue collection and histochemistry

Tissue samples were collected, fixed and paraffin embedded from infected and non-infected steers at the time of euthanasia as described previously (4). Tissue were sectioned and 5 µm sections were rehydrated through xylene and ethanol (100–50%) to water. Sections were then incubated in Hoechst 33342 (Invitrogen, 2 µg/mL) to visualize DNA and cell nuclear shape for 5 min and mounted in anti-fade reagent (Fisher Scientific). Images were taken using a laser scanning confocal microscope (Zeiss LSM 510 system, Thornwood, NY, USA).

Bovine Neutrophil Isolation

Jugular vein blood was collected cattle using vacutainers containing EDTA or no additive (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Neutrophils were isolated as previously described (130) with minor modifications; briefly, blood was transferred to 15 mL conical tubes (Fisher) and centrifuged for 20 min at 1,000*g* at 4°C. Following centrifugation, the plasma, buffy coat, and one-third of the red blood

cell pellet were discarded. The remaining cells were resuspended in 5 mL ACK lysis buffer to remove red blood cells. The cell suspension was gently mixed and incubated for 5 min at room temperature. The solution was then centrifuged for 10 min (200*g* at 4°C) and the supernatant decanted. The pellet was washed with 15 mL of calcium- and magnesium-free PBS (CMF-PBS) and centrifuged for 5 min (850*g* at 4°C). For complete red blood cell lysis, ACK treatment was repeated. Cells were then washed twice with 15 mL of CMF-PBS and centrifuged for 5 min (850*g* at 4°C). After the final wash, the pellet was resuspended in 1 mL of RPMI 1640 lacking phenol red (Gibco), and neutrophil concentrations were measured using the trypan blue exclusion method on a hemocytometer.

Mouse Neutrophil Isolation

Neutrophils were isolated from bone marrow of tibias and femurs of adult mice by density gradient centrifugation as described previously (131–133) and resuspended in phenol red-free RPMI 1640 medium containing 2% FBS.

NET Quantification

Neutrophils were re-suspended in RPMI 1640 medium containing 2% FBS and lacking phenol red. Cells were deposited in triplicate into 96-well flat-bottom plates (Nunc, Fisher Scientific) and incubated for 30 min at 37°C and 5% CO_2 prior to stimulation. Cells were then stimulated for up to 3 hours with OO extract (3 µg/mL) or Toll-like receptor 4 (TLR4) ligand LPS (100 ng/mL) in a final volume of 200 µL per well. Dose dependency of OO extract was evaluated using further dilutions (1:10 and

1:100) in RPMI medium. For live worm experiments, neutrophils were cultured with either *C. elegans* (20 or 40 worms/well) or *O. ostertagi* L4 larvae (40 worms/well) in triplicate.

Following stimulation, micrococcal nuclease was added (5 U/well, New England Biolabs, Ipswich, MA, USA) and incubated for 15 min. Samples were centrifuged (800*g*, 5 min) and the supernatants (100 µL/well) were transferred to a black 96-well flat-bottom plate (Nunc). The samples were stained with the fluorescent DNA dye Sytox Green (5 µM final concentration, Invitrogen, Carlsbad CA, USA) and incubated at RT in the dark for 10 min (134). NET formation was quantified in arbitrary fluorescent units (AFU) by spectrofluorometric analysis with an excitation wavelength of 485 nm and an emission wavelength of 525 nm using an automated plate reader (Biotek, Winooski, VT, USA). For negative controls, unstimulated neutrophils in regular RPMI medium lacking phenol red were used. Neutrophil stimulation with phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA 100 nM final concentration) served as a positive control (103).

Inhibition assays

Specific inhibitors were used for blockage of NET formation (9, 135) as described previously. The following inhibitors were used: the NE inhibitor Suc-Ala-Ala-Pro-Val chloromethyl ketone (CMK; 1mM final concentration, Sigma), NADPH oxidase (NOX) inhibitor diphenylene iodonium (DPI; 10 µM final concentration, Sigma), and the MPO inhibitor 4-Aminobenzoic acid hydrazide (ABAH; 100 µM final

concentration, Sigma). TLR4 signaling inhibitor CLI-095 (1μg/mL final concentration; Invivogen, San Diego, CA). Cells were pre-incubated with inhibitors for 30 min at 37°C prior to stimulation as described above (9, 135).

Visualization of NETs and Detection of NET-associated proteins

Isolated neutrophils (3×10^5) were seeded on 13 mm round glass coverslips pre-treated with poly-L-lysine (Sigma) in 24-well plates and were allowed to adhere for 30 min at 37°C (136). Cells were then stimulated as described previously for up to 3 hours. Following treatment, coverslips were washed with PBS and fixed in 2% paraformaldehyde for 15 min at RT. Coverslips were then washed 3 times with PBS and blocked with 2% BSA (Sigma) for 30 min to prevent non-specific binding. To detect histone or NE, coverslips were incubated with anti-histone (H3) antibody (Fisher Scientific) at 1:1000 or anti-NE antibody (Abcam, Cambridge, MA, USA) at 1:200 for 1 h at room temperature (RT). Following first antibody incubation, coverslips were washed twice with PBS and incubated 30 min at RT with anti-mouse IgG-PE (Biolegend, San Diego, CA, USA) diluted at 1:500 in blocking buffer. Coverslips were subsequently stained with Sytox Green (1:1000, 15 min), washed twice with PBS, and mounted on glass slides using anti-fade mounting buffer (Fisher Scientific). Images were taken using a laser scanning confocal microscope (Zeiss LSM 510 system, Thornwood, NY, USA).

For imaging of unfixed cells, cells were seeded on 24-well plates without coverslips under similar conditions. Hoechst 33342 (20μM): is a cell permeable DNA

dye and was present in media throughout stimulation. Sytox Green (5µM) was added 15 minutes prior to imaging.

Measurement of DNA in sera from OO-challenged and uninfected control cattle

Cattle were infected with OO L3 and blood samples were collected at days 0, 15 and 29 post infection. Serum sample (10µL) was added to 90 µL of PBS, followed by addition of 100 uL of Sytox Green (1:200) per well in a black 96-well flat bottom plate (Nunc) and incubated in the dark for 15 minutes at RT. Fluorescence was quantified as described above in "NET Quantification".

Chemiluminescent measurement of ROS production

ROS production was measured by chemiluminescence as described previously (91, 137). Neutrophils (1 x 10^5) were resuspended in RPMI 1640 containing 11 mM HEPES, 55 mM Luminol (Fisher), and 1.2 U/mL horse radish peroxidase (Sigma). Ninety µL of the cells was then plated in a white 96-well flat bottom microplate (Nunc) and subsequently stimulated with 10 µL of stimuli as described in "NET quantification". Chemiluminescence was recorded for 1 s per well every 2 min for 30 min prior to addition of stimuli, and for an additional 2 h using an automated plate reader set to 37°C.

Statistical Analysis

Data were analyzed using two-tailed Student's *t* test or one-way analyses of variance (ANOVA) with repeated measurements to compare stimulation conditions

using the Tukey-Kramer multiple comparisons test (GraphPad InStat Software, Inc., La Jolla, CA). P values of <0.05 were considered significantly different.

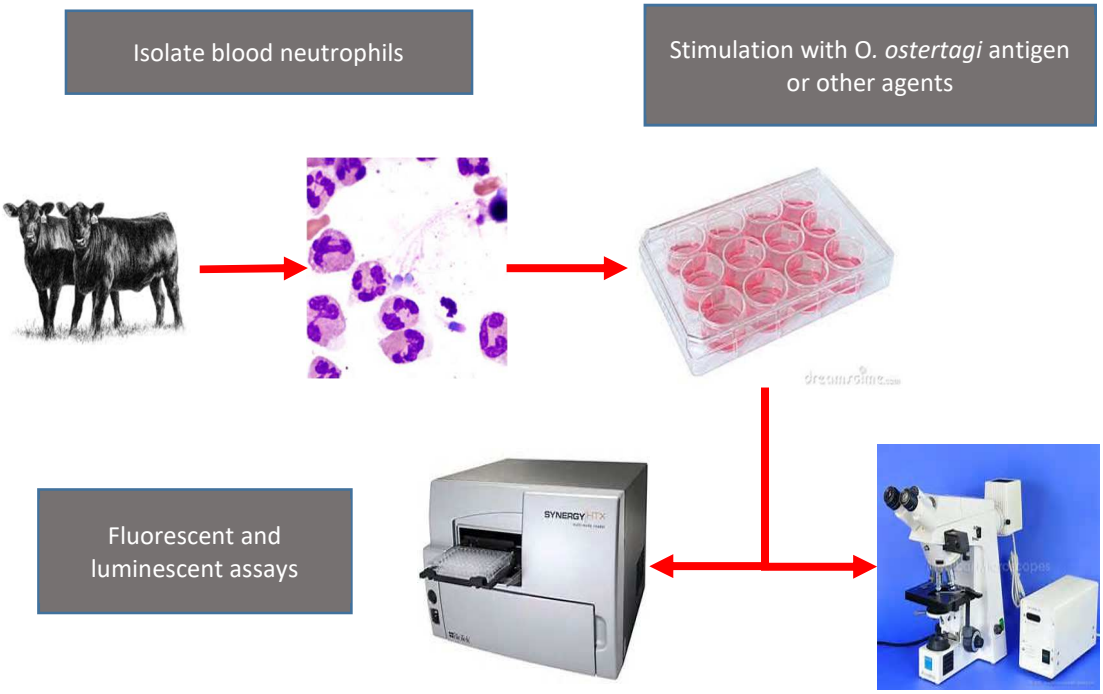


Figure 1. Overall scheme of experimental design. Neutrophils were isolated from Holstein steers and stimulated *in vitro* with *O. ostertagi* extract or other experimental stimuli in the presence or absence of inhibitors. Following a 3-hour stimulation period, release of NETs was measured by fluorescent quantification and reactive oxygen species (ROS) measured by luminol enhanced chemiluminescence.

Chapter 3: Results

Ostertagia ostertagi induces NET Release

NETs consist predominantly of DNA and a small list of proteins (138), including neutrophil elastase and histone. Histones are normally strongly associated with DNA, and get released within NETs, possibly contributing anti-microbial properties to the NET structure (92, 139, 140). Neutrophil elastase (NE) is a serine proteinase secreted by neutrophils during inflammation, and is normally involved in the destruction of microorganisms and tissues (141). It is also one of the predominant proteinases present in NETs, as being bound to DNA prolongs and preserves its proteolytic activity (94), although the exact reason for its association with the NET structure is still unclear (86). The co-localization of NE, DNA and histone has been observed in multiple models of NETs including cattle (142), and is routinely used as a marker for NETs when present alongside DNA (138). Studies on other ruminant parasites have demonstrated their ability to induce NETs in bovine neutrophils (9, 135, 142), suggesting the strong possibility that *O. ostertagi* could induce NET formation as well. Following stimulation with OO extract, NETs were observed and displayed typical structures (Fig. 2). Sytox Green staining revealed that stimulation of bovine neutrophils with OO extract led to the release of a dense network of DNA fibers spreading outwards from the cell (Fig. 2A-b and 2B-b). Localization of DNA appeared to be outside of the cell as determined by staining with permeable (Hoechst) and impermeable (Sytox green) dyes on unfixed cells (Supplemental Figure 1). These DNA

structures co-localized with histone (Fig. 2A-h) and NE (Fig. 2B-h), affirming their identity as NETs (9, 91, 142). Unstimulated neutrophils demonstrated normal multilobed nuclei and lacked extracellular DNA structures (Fig. 2A-c and 2B-c). Stimulation with the well-established neutrophil activator and NET inducer PMA (143) led to the formation of similar extracellular DNA structures (Fig. 2A-a and 2B-a) which also co-localized with histone (Fig. 2A-g) and NE (Fig. 2B-g), consistent with previous reports (9, 135, 142).

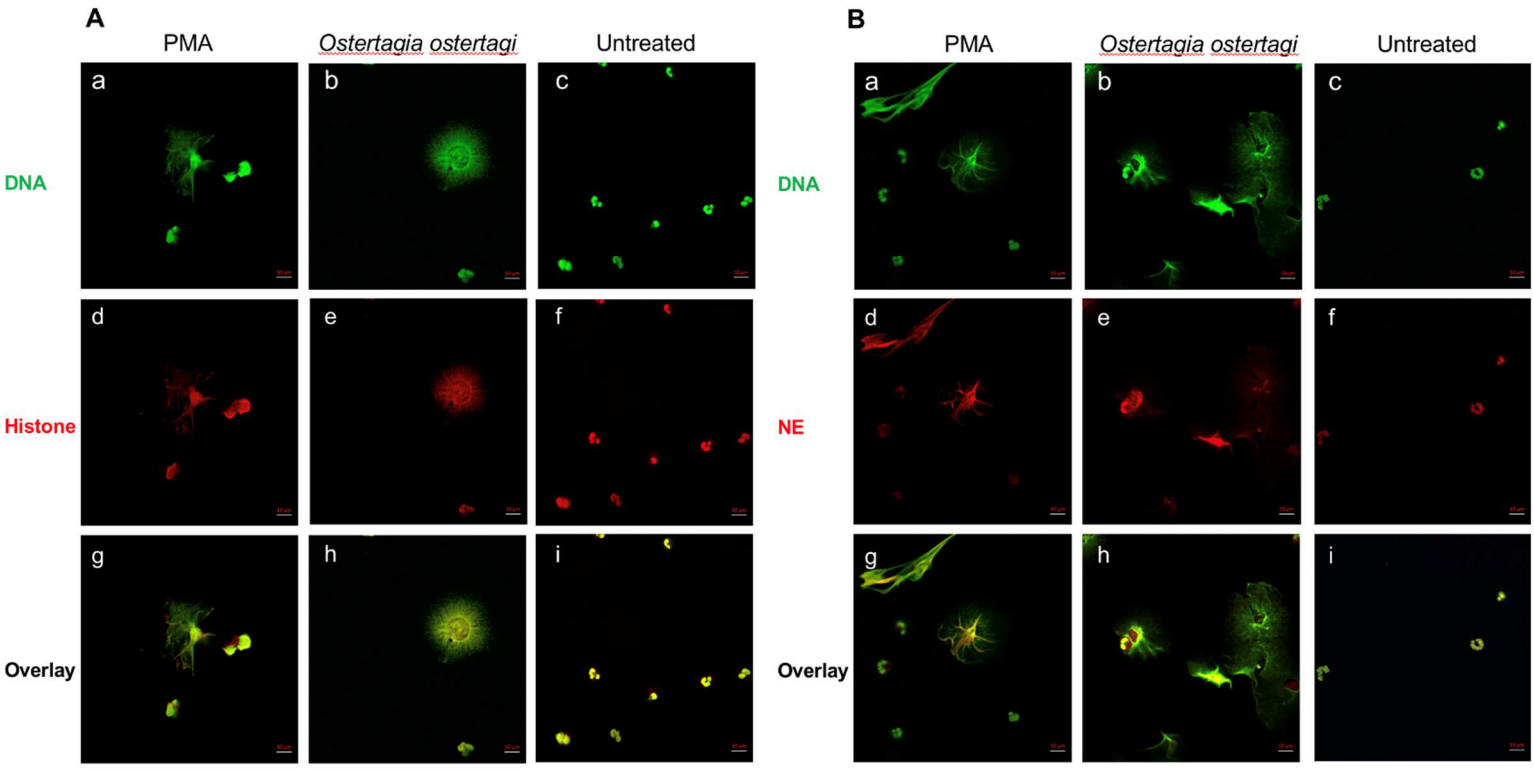


Figure 2 Co-localization of DNA with histone (H3) and neutrophil elastase (NE). Histone (H3) (A) and neutrophil elastase (NE) (B), in PMA stimulated (a, d, g), Ostertagia ostertagi (OO) extract stimulated (b, e, h) and unstimulated (c, f, i) bovine neutrophils and associated neutrophil extracellular trap structures. a-c: DNA stained with Sytox Green (green). d-f: Histone (H3) staining in A, and NE staining in B within NET structures (red). g-i: Overlay of NET-DNA with histone (A) or NE (B). The neutrophils were incubated with each stimulus for 3 hours before staining.

O. ostertagi induced NET release is dose and time dependent

The release of NETs by neutrophils has been demonstrated to occur relatively rapidly in various models including in humans, mice, and cattle (9, 121), although the kinetics seem to be stimuli and possibly species dependent (121). To further characterize the role of OO extract in NET release, the time- and dose-dependent responses were examined *in vitro*. The release of extracellular DNA was detectable 30 min following incubation with PMA, LPS and OO extract (Fig. 3A), and was significantly higher in cells treated with PMA, LPS or OO extract than unstimulated cells ($p < 0.01$). The amount of NETs released was significantly greater up to 90 min post stimulation in cells treated with PMA when compared to the amounts induced by LPS or OO extract, and in general, PMA appeared to induce higher average NETs than those stimulated by other treatments (Fig. 3A). This may reflect the artificial nature of PMA-induced NET release, as PMA is able to directly activate protein kinase C (PKC), whereas LPS and possibly OO extract may have to work through different pathways which require multi-stepped signal transductions (121). NET release in response to OO extract illustrated a clear time-dependent pattern like that of LPS (Fig. 3A). Maximal NET release was observed at 180 min for all treatments, similar to previous reports (9, 86, 91), and thus all subsequent experiments were conducted for this amount of time. To investigate the dose response of neutrophils to OO extract, purified bovine neutrophils were incubated with different concentrations of extract for 180 min. The neutrophils displayed a clear dose-dependent NET response, with maximal production

demonstrated at 3 µg/mL of OO extract (Fig. 3B). All subsequent experiments were conducted using OO extract at this concentration (3 µg/mL). Treatment with DNase I lowered the detection of NETs to the level of the controls (Fig. 2B), suggesting that the increase in detected DNA was predominantly of extracellular origin and not attributable to the presence of apoptotic or necrotic neutrophils. These results were consistent with the detection of extracellular DNA in NETs induced by PMA, LPS, and OO in Fig.2. Interestingly, the unstimulated negative control (Fig. 3B) seemed to have a small but consistent release of extracellular DNA, as treatment with DNase I led to a statistically significant decrease in the measured fluorescence. While the exact source of this DNA is unclear, it is possibly due to low levels of background activation during the isolation process, given neutrophils sensitive predisposition for activation (144).

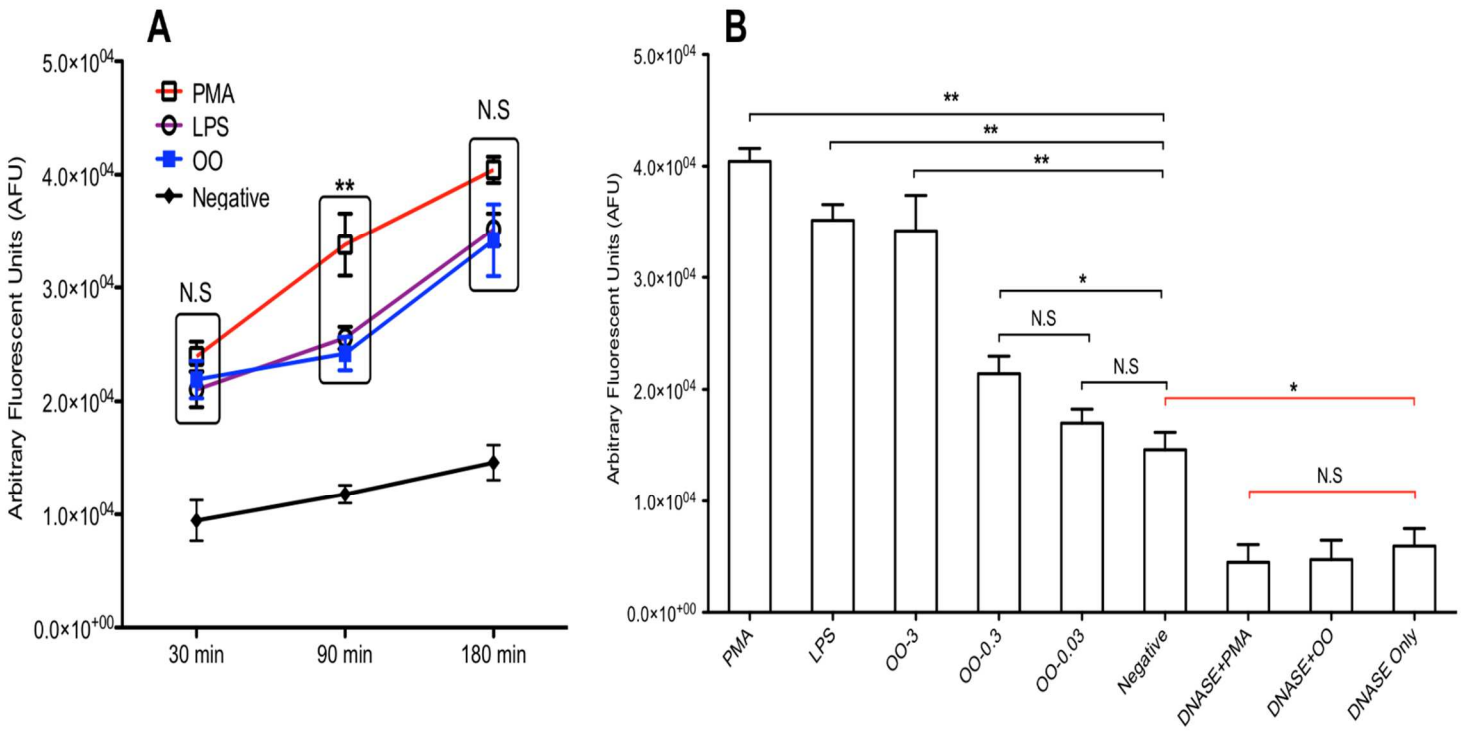


Figure 3. DNA release following *Ostertagia ostertagi* (OO) soluble extract treatment of bovine neutrophils A. Kinetics of OO extract-induced DNA release over a period of 3 hours. Asterisks indicate statistical significance on all the data in each square using one-way ANOVA. B. Dose dependent effect of Ostertagia soluble extract on DNA release by neutrophils in incubation for 3 hours. Experiments were performed using cells from five cattle. Data are expressed as means +/- SEM. Asterisks indicate statistical significance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Black lines: t-test. Red lines: One-way ANOVA. Data were representative of at least three experiments with similar results.

OO -induced NET release is MPO-, NE-, and NADPH oxidase-dependent

While the exact mechanism of NET formation and release are still not completely understood (94), certain key proteins have proven to be essential in nearly all models. NETs have been widely demonstrated to be dependent on the enzymes NE, myeloperoxidase (MPO), and NADPH oxidase, and bovine NETs in particular have been reported to be dependent on these proteins (8, 9, 135, 142). NADPH oxidase is a crucial enzyme for neutrophil activity. Its activation initiates and mediates the production of ROS during the respiratory burst and is widely associated with NET induction (55). MPO is a potent peroxidase which also is crucial to the respiratory burst pathway, and alongside NE, are both abundant in the primary granules of neutrophils and are commonly found within NET structures (6, 138, 145, 146). The serine protease NE and peroxidase MPO, both have demonstrated potential roles in the chromatin decondensation crucial for NET release (147), and are both involved in various NET models (9, 148, 149). To further investigate the characteristics of OO extract-induced NET release, inhibition of these molecules was performed using specific inhibitors for NE (CMK), MPO (ABAH), and NADPH oxidase (DPI) (59). Inhibition of each of these molecules individually demonstrated significant reductions in NET release following OO extract stimulation ($p \leq 0.01$, Fig. 4), indicating key roles for each of these enzymes in OO extract-induced NET release. Interestingly, addition of inhibitors to the unstimulated control did not affect the low levels of DNA released by neutrophils (Fig. 4), suggesting an independent mechanism for the release of this DNA.

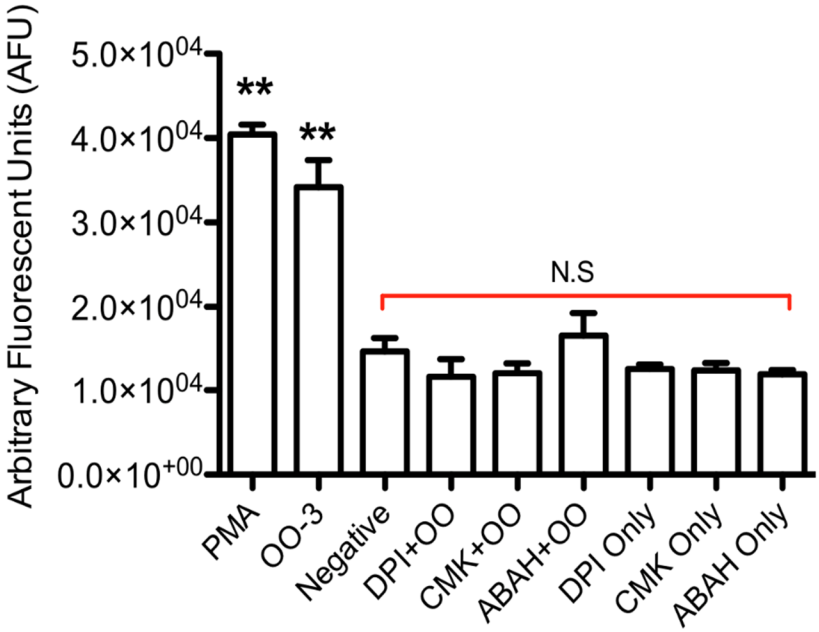


Figure 4. Blockage of OO-triggered NETosis using inhibitors. Inhibition of NADPH Oxidase with DPI, NE with CMK, and MPO with ABAH, respectively. Data were expressed as means +/- SEM from five cattle. Data were expressed as means +/- SEM from triplicate using cells from one cow. Red lines: One-way ANOVA. Data were representative of at least three experiments with similar results.

OO induced NET release is TLR4 independent

While the OO extract-induced NET release was dependent on NADPH oxidase, MPO and NE, none of these molecules are directly involved in the initial recognition of the target pathogen or signal. The OO extract used in these experiments contains whole parasite content, which may also may include some bacteria from the parasites own flora (150). Endosymbionts have recently been reported to be the source of ligands inducing NET formation human helminth infection with *Onchocerca volvulus* (151). The bacteria that may be present within *Ostertagia ostertagi* contribute many immune stimulating components, such as LPS from gram-negative bacterial walls, which binds to the toll-like receptor-4 (TLR4) present on the neutrophil surface (81). To investigate this possibility, neutrophils were treated with TLR4 inhibitor CLI-095, before stimulation with OO extract (152). Inhibition of TLR4, however, had no effect on the NET release induced by OO extract (Fig. 5), indicating that OO extract-stimulated NET release is not dependent on TLR4.

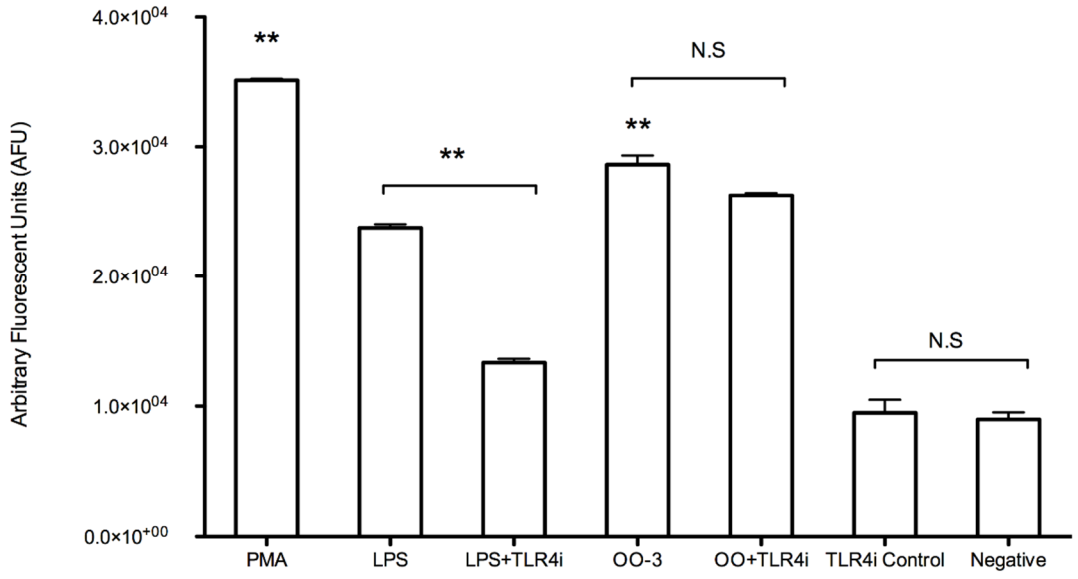


Figure 5. Inhibition of TLR4 with its inhibitor CLI-095 (TLR4i). Data were expressed as means +/- SEM from triplicate using cells from two cows. Red lines: One-way ANOVA. Data were representative of at least three experiments with similar results.

DNA in serum does not correlate with infection

NETs are released into the extracellular environment, and have demonstrated the ability to enter the systemic circulation at detectable levels (138). Multiple reports have investigated the potential for NETs, or their individual components, as viable diagnostic markers (138, 153, 154). While there has been some limited success in the use of cell free DNA as a NET specific marker in sterile inflammatory conditions (145, 154), its relevance in infectious models is unknown. To determine if the levels of cell-free DNA in blood were associated with OO infection, which could be related to entry of OO-induced NETs into circulation, we evaluated the cell-free DNA concentration in the sera of uninfected controls and cattle infected with *O. ostertagi* for 15 or 29 days, with day 0 samples collected immediately prior to infection. Serum DNA concentration did not appear to correlate to infection status and did not significantly differ between control and infected animals at days 15 and 29 post-challenge (Fig. 6). While extracellular DNA was detectable in the sera, levels were predictably lower (2 log-fold) than in the supernatant of *in vitro* stimulated neutrophils (Fig 2). The serum DNA was extracellular and sensitive to DNase I treatment, similar to those present *in vitro* (Fig. 2B). Therefore, cell-free DNA levels in serum do not appear to be affected by *O. ostertagi* infection, raising the possibility that NETs may be sequestered at the sites of infection, although DNA staining of tissue sections of infected tissue showed no obvious indications of a significant NET presence (Supplemental Figure 2). Alternatively, NETs are released but rapidly cleared, possibly by endogenous mechanisms including DNases (145).

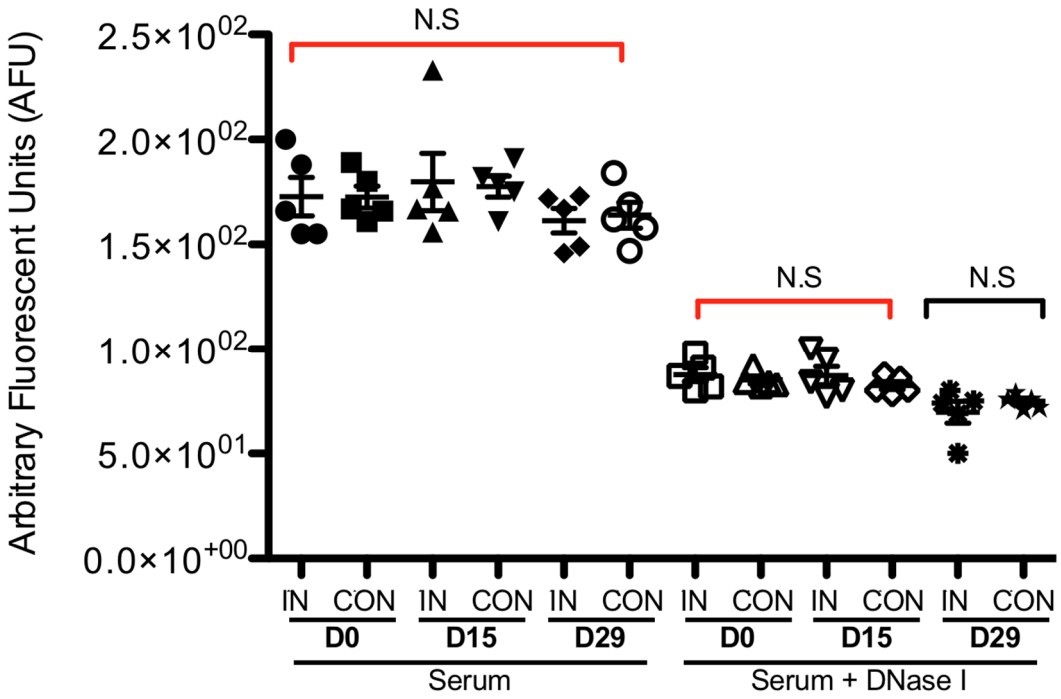


Figure 6. DNA concentrations in sera of cattle experimentally infected with *Ostertagia ostertagi*. Infected and uninfected controls had serum isolated on Days 0, 15, or 29 post infection. Black lines: t-test. Red lines: One-way ANOVA. IN, infected; CON, control**.**

O. ostertagi infection possibly suppressed NET response

It has long been known that neutrophils can be transitioned into a state of enhanced responsiveness, a process termed "priming" Exposure to a priming agent such as pro-inflammatory mediator TNFα (155) or microbial products such as LPS (156) enhances various neutrophil functions including respiratory burst, phagocytic ability, granule release, and chemotaxis (157). Recent reports suggest enhanced NET formation may also be an effect of neutrophil priming (157, 158). To investigate whether *O. ostertagi* infection alters NET release either through priming or some other mechanism, neutrophils were isolated from animals undergoing *O. ostertagi* infection (11 days post-challenge) and stimulated with OO extract. The neutrophils from both infected cattle released significant amounts of NETs in response to PMA or LPS stimulation. However, OO extract induced a relatively lower amount of NETs compared to unstimulated controls ($p \leq 0.01$, Fig. 7A). To compare the level of the NET response between healthy and acutely infected cattle, the NET release by neutrophils from the two infected cattle was calculated as a percentage of each corresponding PMA positive control. Interestingly, both animals appeared to have a diminished response to LPS or OO extract than those from healthy cattle (Fig. 7B). These data suggest that the ability of neutrophils to form NETs is not enhanced, but may instead be inhibited by *O. ostertagi* infection, possibly contributing to the parasites survival *in vivo*.

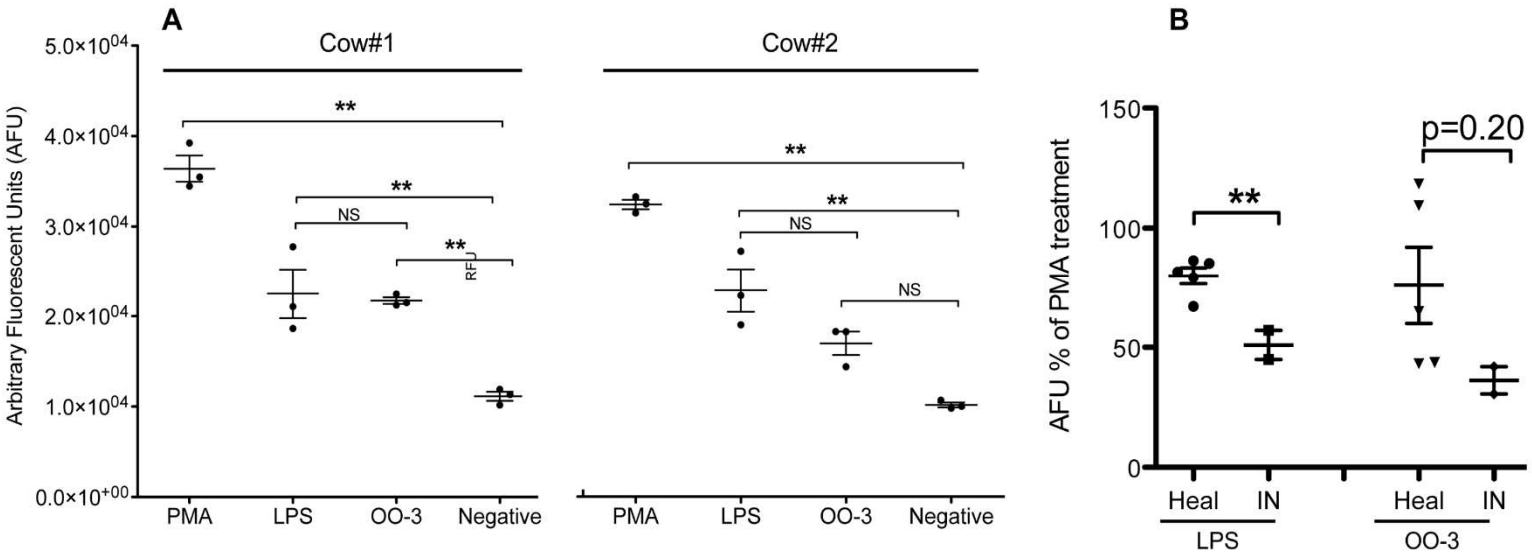


Figure 7. Suppression of NET release in infected cattle neutrophils. A. The neutrophils were purified from the blood samples of O. ostertagi infected cattle 11 days post infection and assayed for NET release in triplicate. Data are expressed as mean +/- SEM. OO-3: 3 ug/mL of OO-soluble extract. Data were analyzed by Student t-test. B. Comparison of neutrophils from healthy (Heal) or infected (IN, the same as in A) in response to LPS or OO extract. Background AFU (Negative control) was subtracted from all the data to obtain exclusive (responsive) AFU. The AFU % of PMA treatment: each value was divided by the exclusive AFU of PMA treatment of the neutrophils from the same cow.

Bovine neutrophils lack detectable ROS following OO exposure

The role of NADPH oxidase and ROS has been widely reported in various models of NET release including in cattle (9, 114, 135), however the exact mechanisms of this involvement, and roles for specific ROS remains unclear.

The NADPH oxidase complex mediates neutrophil production of ROS (159). Inhibition of NET release following incubation with the NADPH oxidase inhibitor DPI indicated the importance of this enzyme in OO extract-induced NET formation. The importance of NADPH oxidase in the release of NETs made it likely that the main NADPH oxidase output, ROS, would be detected in significant quantities following stimulation with PMA, LPS or OO extract, based on Figs. 3 and 4. Confirming the role of the NADPH oxidase complex in the formation of ROS, neutrophils treated with the inhibitor DPI showed significant reductions in ROS production following PMA stimulation ($p \leq 0.01$, Fig. 8A-B). Surprisingly however, OO extract did not appear to induce any significant production of ROS by neutrophils at any point over the 2-hour stimulation period, compared to the strong ROS response detected in PMA-stimulated neutrophils (Fig. 8A). Similarly, when measured as total ROS released over time (the area under each curve), OO extract-treated neutrophils showed a complete lack of ROS production ($p \geq 0.05$, Fig. 8B). Taken together, these results suggest a ROS-independent pathway for NET release by this parasite and indicate the possibility that the ROS products of the NADPH oxidase complex pathway may be dispensable in OO extract-induced NET formation.

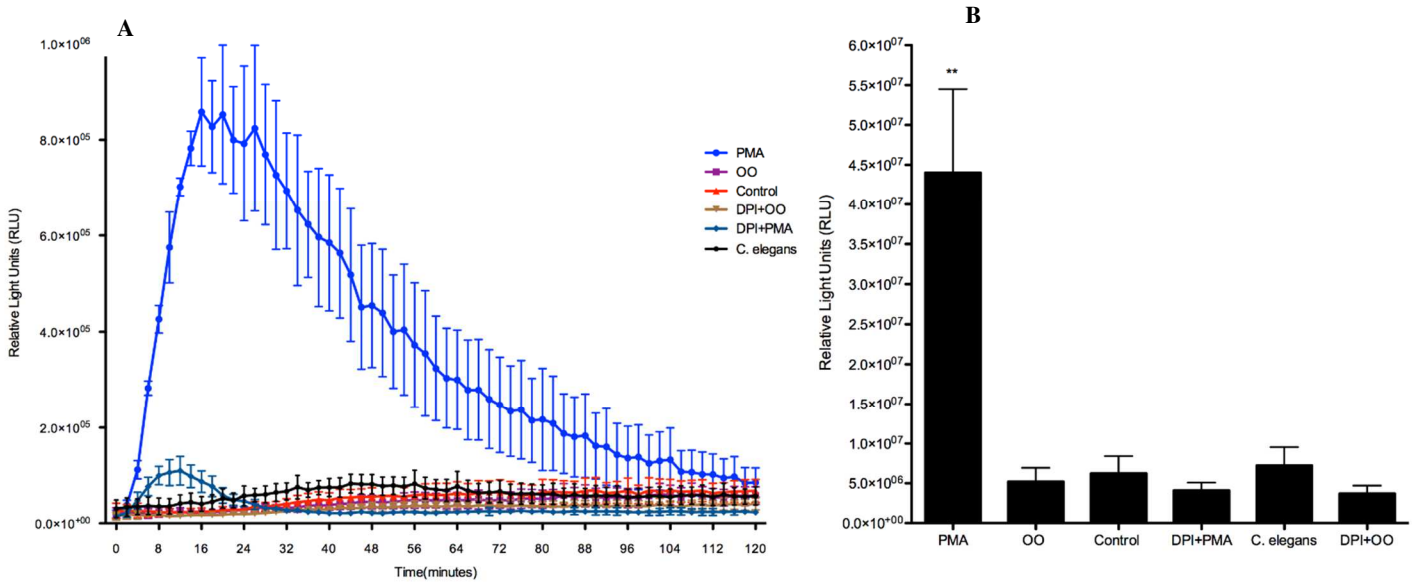


Figure 8 Reactive oxygen species (ROS) production by OO-stimulated neutrophils. ROS was measured by luminol-enhanced chemiluminescence assay. A. Kinetics of ROS release by neutrophils under treatments over a period of 2 hours. B. Total ROS production measured by area under each curve for the whole period of 2 hours. Experiments were performed in triplicate using cells from three cattle. Data are expressed as means +/- SEM. Red lines: One-way ANOVA. Data were representative of two experiments with similar results.

Exogenous ROS induces NET release

As there was a noticeable lack of detectable ROS following stimulation with OO extract, it raised the question as to what the role of ROS products may be in bovine NET formation. Specific ROS products such as superoxide (160), hypochlorous acid (161, 162), or hydrogen peroxide (91, 162) have been demonstrated to be able to directly stimulate NET formation in the absence of any other stimuli. To test this possibility in bovine neutrophils, exogenous hydrogen peroxide (H2O2) was used as a source of ROS. Neutrophils exposed to varying concentrations of H2O2 displayed a significant, dose-dependent NET release ($p \leq 0.01$, Fig.9), equivalent to that of PMA or OO extract-treated neutrophils. Even with DPI inhibition eliminating the endogenous ROS response, H2O2 was able induced NET release ($p \leq 0.01$, Fig. 9). Even though there seems to be a significant lack of detectable ROS produced by neutrophils in response to OO extract, H2O2, a predominant ROS product, demonstrated the ability to directly stimulate NET formation in bovine neutrophils.

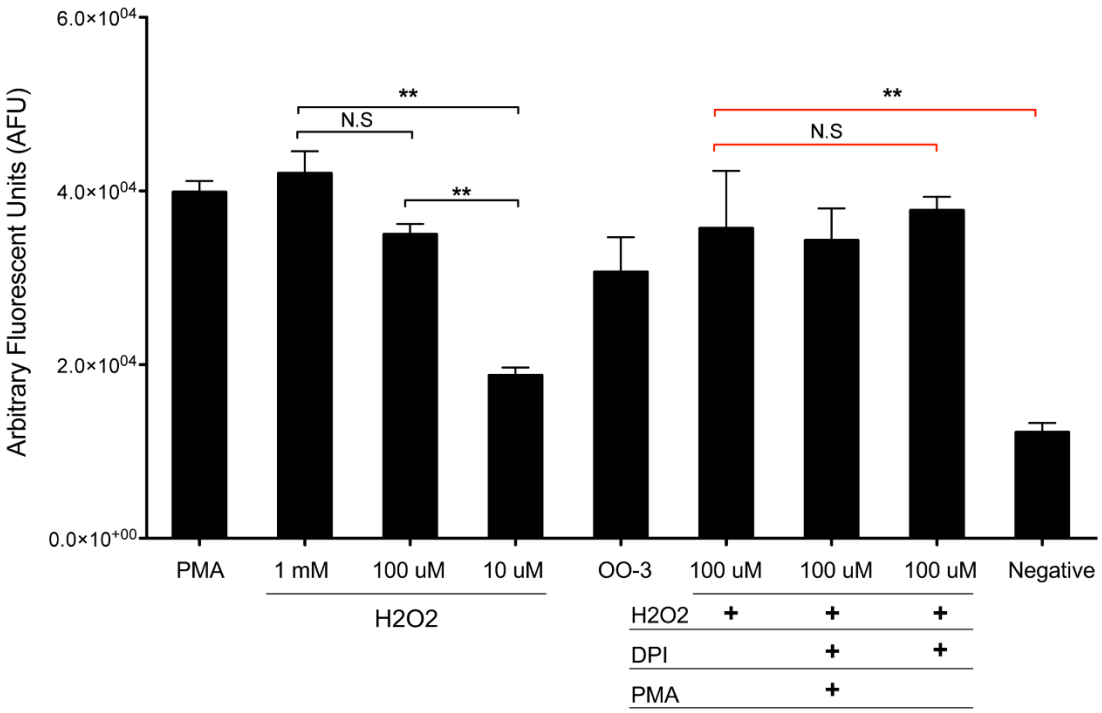


Figure 9. Effect of exogenous ROS (H2O2) on NET release. Dose-dependent effect of an exogenous ROS product, hydrogen peroxide (H2O2), on DNA release by neutrophils for 3 hours. Experiments were performed in triplicate using cells from three cattle. Data are expressed as means +/- SEM. Black lines: t-test. Red lines: One-way ANOVA. Data were representative of two experiments with similar results.

Bovine neutrophils release NETs in response to live nematodes

While the OO extract demonstrated the ability to induce significant NET release from bovine neutrophils, the ability of live, whole parasite to induce NETs warranted investigation. To determine the ability of live parasite to induce NETs, live L4 stage *O. ostertagi* were used to stimulate bovine neutrophils *in vitro*. Additionally, to determine the possibility of a conserved response to nematodes, *C. elegans,* a lab strain of a non-pathogenic soil nematode, was also examined for induction of NET release. Reinforcing the OO extract experiments, neutrophils stimulated with either 20 or 40 live L4 *Ostertagia* larvae released significantly higher amounts of NETs compared to unstimulated controls in both cattle samples ($p \leq 0.05$, Fig. 10A). Unexpectedly, bovine neutrophils exposed to *C. elegans* showed significant NET release ($p \leq 0.01$, Fig. 10B), albeit not as large as in PMA-stimulated neutrophils. *C. elegans*-induced NET release was independent of viability of the worms, as heat-killed worms (60°C, 60 min) induced equivalent amounts of NET release (Fig. 10B). Inhibition experiments confirmed the dependence of the *C. elegans*-induced NET response upon NE, MPO, and NADPH oxidase ($p \leq 0.01$, Fig. 10B), indicating their similarity to the OO extract-induced NETs. These data suggest a bovine NET response to different nematodes, unrelated to their pathogenicity, raising the possibility that NET release may be a conserved defensive mechanism against a broad range of nematodes or similar parasites.

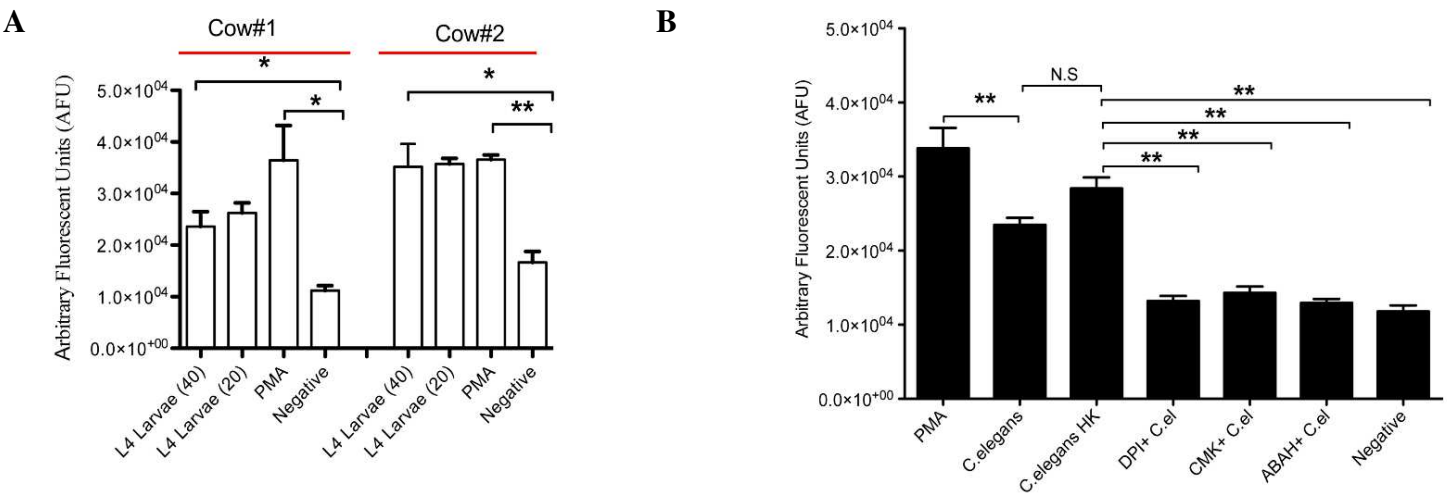


Figure 10. NET release following stimulation with live worms. The induction of NET release by neutrophils is a conserved response against nematodes. NET release by bovine neutrophils from two cattle following stimulation with live L4 Ostertagia larvae (A), or adult *C. elegans* (B). Data are expressed as means +/- SEM. The data in D represent two independent experiments in triplicate. C.el: *C.elegans*. HK: heat-killed. Black lines: t-test. Red lines: One-way ANOVA.

OO-induced NET formation is not bovine-specific

O. ostertagi is a nematode parasite of ruminants, most predominantly in cattle and to a lesser degree sheep and goats. It is not known to establish infection or cause disease in rodents, the most probable reason for the lack of reports investigating the effects of *O. ostertagi* on mouse immune cells, although OO extract has once previously been reported to have the ability to influence mouse immune cell activity (163). To address the possibility of a conserved neutrophil response to *O. ostertagi,* particularly with a species not normally associated with this parasite, the ability of *O. ostertagi* to elicit NET formation in mouse neutrophils was investigated. Mouse bone marrow neutrophils from C57BL/6 mice were stimulated with OO extract and their NETs release was examined. Interestingly, there was a significant increase in NET release by OO extract-treated mouse neutrophils ($p \leq 0.001$, Fig.11A), which was inhibited by the NADPH oxidase inhibitor DPI ($p \leq 0.001$, Fig. 11A*),* although not as completely inhibited as in bovine neutrophils (Fig 4). Additionally, *C. elegans*, was not able to induce murine neutrophils to form NETs (Fig. 11A), in contrast to bovine neutrophils (Fig 10A). Consistent with bovine neutrophils, neutrophils from mice did not produce ROS following OO extract stimulation (Fig. 11B), further indicating that *O. ostertagi* mediates NET release independent of ROS production. These data suggest that NET release by neutrophils in response to OO extract may be a conserved response, potentially mediated by broad or conserved host-pathogen pattern recognition mechanisms (164, 165).

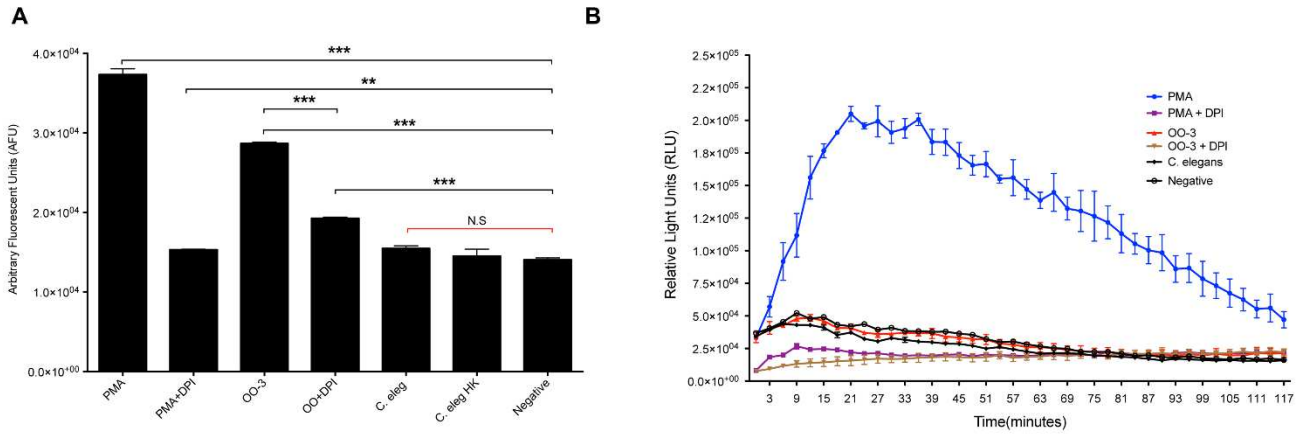


Figure 11. OO-induced NET release in murine neutrophils. The induction of NET release by neutrophils is a conserved function of the nematodes in different host species. Bone marrow-derived mouse neutrophils stimulated with O. ostertagi soluble extract (OO) were assayed for NET release 3 h post-treatment (A) or ROS production over a period of 2 h using luminol-enhanced chemiluminescence assay. Data are expressed as means +/- SEM. The data D represent two independent experiments in triplicate. C.el: *C. elegans*. HK: heat-killed. Black lines: t-test. Red lines: One-way ANOVA.

Discussion

The role and mechanisms of NET release initially focused on its role in bacterial infection (86, 91). As different disease models, including both infectious and sterile inflammation, have been investigated, the list of diseases in which NETs are thought to play a role in has constantly grown (5). However, the role of NETs has largely been limited to murine and human models of disease, and even in those models, relatively few reports exist on the role of NETs on nematode infections (123, 151, 166). Bovine neutrophils have been shown to release NETs, upon stimulation from various parasites (102, 135, 167, 168), and have recently demonstrated the ability to release NETs in response to a similar nematode parasite *H. contortus* (9). In this report, we provide the first evidence of a NET response elicited by *O. ostertagi*, one of the most detrimental GI nematode parasites to the cattle industry (82, 83). OO extract was able to induce bovine neutrophils to form NETs, a reaction which was MPO, NE and NADPH oxidase dependent, enzymes often reported to be involved in a majority of NET models inclding multiple parasite-dependent NET models in cattle (83, 108, 135). Supporting the relevance of the OO extract-induced NET release, live OO L4 larvae were similarly able to induce significant NET release in bovine neutrophils. Surprisingly, the nematode *C. elegan*s, a free-living soil nematode which is demonstrably non-hazardous, non-infectious, non-pathogenic, and non-parasitic (169), was also able to cause NET release in bovine neutrophils. OO extract also demonstrated the ability to

induce NETs in both murine and bovine neutrophils, suggesting the presence of shared mechanisms in NET formation between species in response to nematodes.

During infection, while currently unknown, the concentration of parasite content or antigen released by *O. ostertagi* can be logically assumed to be relatively low compared to what is normally used *in vitro*. Additionally, there is a good chance of direct interaction of parasites with immune cells, especially during the acute phase of *O. ostertagi* infection into the abomasal mucosal. To simulate this *in vivo* situation, the response of bovine neutrophils to low concentrations of adult stage *O. ostertagi* soluble extract was examined. The capacity of these bovine neutrophils to release significant levels of NETs in response to even low concentrations of parasite extract increase the likelihood of the viability of this response *in vivo*, a situation where the actual abundance of parasite antigen is likely much lower than in typical *in vitro* settings. To validate the ability of bovine neutrophils to release NETs against intact parasites and not just parasite extract, live L4 stage *O. ostertagi* were used to stimulate neutrophils. A similar NET response was observed between the live parasite and to the soluble extract alone. These results suggest that neutrophils can release NETs in response against live intact parasites and parasite soluble extract. However, whether this response can occur in an *in vivo* setting remains to be confirmed.

While increases in extracellular DNA were not detected in the sera of infected animals, this does not necessarily diminish the possibility of NET production *in vivo*. NETs are extracellular structures and thereby subjected to controls by powerful homeostatic machinery in the host, such as degradation by DNase I (94) or

macrophages (170). Released NETs may also potentially not be able to enter the systemic circulation, becoming sequestered in the infected tissue. The lack of visible NETs in infected tissues does not necessarily contradict our findings *in vitro.* Infection of a parasite such *O. ostertagi* is a dynamic process and it is difficult to know at what stage of infection parasites may be at a given time. It may be that if NETs are released *in vivo*, they may only be released during the very early stages as Ostertagia invades the tissue.

While NETs, including bovine NETs, have been shown to be released in response to infectious parasites of various origins, there is a lack of literature on their release against commensal or strictly non-infectious parasites. To explore the possibility that there might be a bovine NET response against other nematodes, not specific to *O. osteragi*, the bovine neutrophil NET response to the non-parasitic nematode *C. elegans* was investigated. The release of NETs against *C. elegans* was a rather surprising result. This nematode causes neither pathology nor is it associated with any known immune reaction from the mammalian immune system. Interestingly, the NET response was observed in both live and heat-killed *C. elegans,* diminishing the possibility of a mechanical activation of the NET response. These results however, must be interpreted with caution. The possibility of the release of certain damage or danger signals from the cultured *C. elegans* was not addressed in these experiments. The response of *C. elegans* to various temperatures has been extensively studied, and this free-living soil nematode is known to be unable to survive prolonged exposure to high temperatures, including the homeostatic temperature of mammals and the

common culture condition for cells 37C, which is considered a lethal dose within 180 minutes (171). It is possible the bovine neutrophils are responding to the potential release of these signals. However, the increasing number of reports detailing NET formation in multiple host species in response to various different microbes reinforces the idea of NETs being an ancient and conserved aspect of the innate immune system shared among vertebrates and plants (172, 173), and it may be a common mechanism for neutrophils to release NETs against potential parasites (104).

While similar, there are distinct differences in the immune systems between species that are still being unraveled. With a significantly smaller body of literature, the immune system of cattle is not as well understood as the murine system. The ability of bovine neutrophils to respond to *C. elegans,* whereas murine neutrophils do not, suggests a greater responsiveness of bovine neutrophils and potentially a greater role for bovine neutrophils in immune defense. An additional possibility for the discrepancy in host neutrophil response may be related to the strain of mouse. It is widely established that each strain of mouse has quite a large variation in its immune response to parasites (174). While the C57/B6 is of a wildtype background, the lab environment in which the strain has been bred may have led to the development of a diminished immune response against certain mediators and organisms.

It has been demonstrated previously that OO soluble extract contains certain immunoregulators that can regulate T cell (175, 176) and macrophage (127) responses. Given the heterogeneity of the OO soluble extract used, it is likely that multiple mediators present in the extract could be independently or synergistically initiating the

NET response, each potentially using different pathways or receptors, such as the various pattern recognition receptors present on the surface or in the endosomes of neutrophils (e.g. TLR's) (124). In regards to the intact L4 larvae and *C. elegans,* in addition to the potential neutrophil response to microbe size (122), there may also be recognizable molecules present on the surface of these worms that neutrophils are binding. Additionally, the presence of any such surface molecules is likely to be similar in the soluble extract as well. For example, various *Candida albicans* cell surface components are capable of inducing NETs via various receptors including TLRs, C-lectin family (Dectin-1), and complement receptors (CD11b/CD18; Mac-1) (177). In addition, OO soluble extract is known to contain lectin (178). Certain lectin receptors, such as C-type lectin receptor, Mincle, have been shown to be involved in the NET response in mouse neutrophils (87). Therefore, NET release in response to OO soluble extract may be triggered by the components such as lectin, which is conserved across many species (179). It is likely that multiple signals are simultaneously mediating the *O. ostertagi* response, leading to possibly redundant mechanisms for NET formation, a common strategy in many immunological functions, and further research is needed to determine which components are mediating this response (180, 181).

The importance of NE, MPO, and NADPH oxidase in the NET pathway has been demonstrated in many reports (5, 9, 114). Inhibition of these molecules demonstrated their critical role in the *O. ostertagi*-mediated NET response. NADPH oxidase specifically has long been known to be crucial for NET release as patients suffering from chronic granulomatous disease (CGD), a deficiency in the NADPH

oxidase enzyme, are unable to release NETs (91, 182). Chemical inhibition of the NADPH oxidase enzyme with DPI completely abrogated the formation of NETs following *O. ostertagi* stimulation, confirming the importance of this enzyme, whose main physiological function is the initiation of the respiratory burst and release of ROS (183). However, upon investigation of the release of ROS following *O. ostertagi* stimulation, there was no discernable release of ROS throughout the assay. While the predominant mechanism for NET formation and release has involved NADPH oxidase and ROS, there has been increasing evidence for NADPH oxidase derived ROS independent pathways, of which there are currently believed to be two, termed "vital NETosis," due to the survival of the neutrophil post NET release (7, 184). Multiple reports exist of a NADPH oxidase-independent NET pathway which involves the dependence exclusively on mitochondrial ROS and results in the release of mitochondrial DNA (116), the other, which is less understood, is truly independent of any ROS, and is the result of small packets of DNA being released extracellularly (7, 184). However, while it is difficult to exclude the possibility of a NADPH oxidase-independent pathway in *O. ostertagi*-mediated NET formation, the complete lack of any ROS detected during stimulation diminishes the likelihood of a mitochondrial-ROS pathway. Interestingly, LPS is often considered an inducer of NETs (120), yet it is well established that LPS alone is a poor stimulator of ROS (120) in unprimed neutrophils, which was also observed.

Both bovine and murine neutrophils demonstrated no measurable ROS response following *O. ostertagi* or *C. elegans* stimulation. This suggests a potential

difference between the non-physiological PMA-induced NET pathway and the *O. ostertagi* induced NET pathway, a concern that has been raised previously regarding the use of PMA (121). In addition, while DPI is the most widely used inhibitor of NADPH oxidase, DPI can affect various other cellular processes as well, particularly in the mitochondria (185). Data resulting from use of DPI may require more cautious interpretation than has been previously considered.

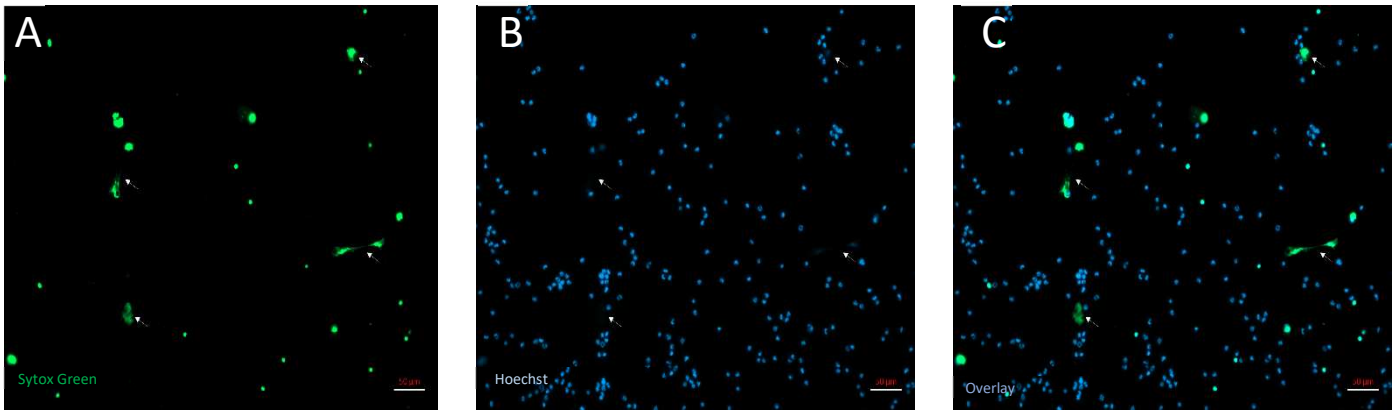
ROS are a heterogeneous mixture of oxygen-containing molecules with high chemical reactivity. The presence of unpaired electrons in some of these species render them extremely unstable and reactive. ROS include peroxides, hypochlorous acid, hydroxyl radicals, singlet oxygen, and the superoxide anion, as well as a few other compounds. The importance of ROS in NET formation was realized quickly following its initial discovery (91), however, the exact ways in which ROS is involved in NET formation are not known. NETosis has usually been considered a novel form of cell death, but recent results suggest it may be directly linked to or be a variant of the autophagy process (93, 115). It has recently been determined that the regulation of autophagy, is closely tied to ROS (88, 186), although the exact mechanisms by which ROS is involved in the signaling network behind autophagy are still incompletely understood, particularly their contribution to cell remodeling.

Thus, ability of ROS itself, independent of NADPH oxidase, to induce NETs was investigated. Previous reports in humans (91) and chickens (105) have indicated that ROS on its own can influence NET production. In the current work, NET release was assayed in bovine neutrophils following addition of exogenous ROS in the form

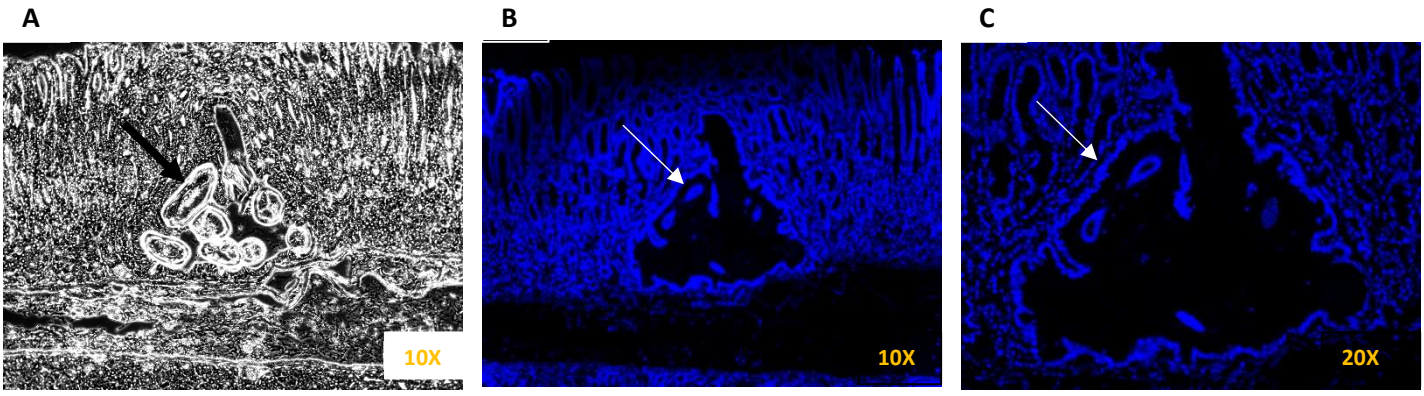
of H2O2, which induced NETs, even in the presence of NADPH oxidase inhibitor, confirming that bovine neutrophils are also capable of forming NETs in the presence of ROS alone without NADPH oxidase activity. Interestingly, it has been shown that certain organisms such as *Candida albicans* possess the ability to detoxify or degrade ROS utilizing superoxide dismutase (SOD) and catalases (187, 188) as a strategy against neutrophils. It is plausible that similar enzymes may be present within worms such as *O. ostertagi.* Paradoxically, it has also been reported that *C. albicans* is able to produce its own ROS (189), enough to rescue NET formation in CGD-patient isolated neutrophils. However, these explanations do not address the clearly demonstrated lack of detectable ROS production. Although the possibility of non-neutrophil sources of ROS or rapid degradation of ROS cannot be completely dismissed, these results suggest that the formation of NETs may be dependent upon multiple factors, of which ROS production may be a dispensable one. Much is not understood regarding the role of ROS in NET formation, particularly its role in the main mechanisms of NETosis, such as chromatin decondensation, histone citrullination, binding of enzymes to DNA, and membrane rupture. Recent work has indicated that the involvement of NADPH oxidase-derived ROS in NET formation vary on the specific molecular stimuli (190). These authors found that NET formations require NADPH oxidase-derived ROS when induced with PMA or by certain bacterial stimulation, but not if the induction occurs via the bacterial calcium ionophore ionomycin. Investigation of NET formation in the neutrophil-like granulocytes of carp suggests that a stimulus-dependent requirement for ROS may be an evolutionarily conserved pattern within vertebrates (173).

These data clearly demonstrate the capability of *O. ostertagi* to induce NET formation, suggesting a potential role for NET formation in the innate immune response of cattle to the parasite. Furthermore, the response of NET formation is not specific to live, fully intact parasite but can also be induced with parasite antigen alone. Surprisingly, the *O. ostertagi*-mediated NET response appears to be independent of ROS but requires NADPH oxidase activity. While this result is difficult to interpret, a plausible hypothesis is that multiple pathways are involved in the NET response to *O. ostertagi*, which is clearly distinct from the PMA-induced NETs extensively studied. The theory of context or stimuli specific mechanisms for NET release has been gathering evidence, and it's likely that as more of the specific mechanisms underlying NET release are discovered, there will be a much more complex picture of the process of NET formation than is currently understood. In addition, it is certain that the *in vivo* context presents additional complexity compared to the simplified, single cell type models used *in vitro*. Also, the contribution of cytokines, chemokines, and interactions with other cells in the local milieu can alter the response as well as outcome. It is also possible that these NETs themselves are interacting with and affecting the local immune response or activity of other immune cells, which warrants further investigation. While the actual ability of NETs to entrap or potentially kill *O. ostertagi* and its relevance *in vivo* remain unclear, *O. ostertagi* appears capable of inducing NETs *in vitro* and suggest that NETs may be a potentially overlooked and important immune defense mechanism against infection with nematode parasites such as *O. ostertagi*.

Supplemental Figures



Supplemental Figure 1. Intracellular vs extracellular localization of DNA following Ostertagia *ostertagi* stimulation for three hours on live, unfixed cells using permeable and impermeable DNA dyes A: DNA stained with cell-impermeable Sytox Green (green). B: DNA stained with cell-permeable Hoechst 33342 (blue). C: Overlay of live cells and cells releasing NETs. White arrows indicate potential NETs.



Supplemental Figure 2. DNA staining of histological section from the fundic region of the abomasum using Hoechst 33342. A. Bright field with clear distinction of region where *O. ostertagi* parasite(s) were located at time of sectioning. B. DNA staining of same region at 10x magnification. C. Same region at 20x magnification. Arrows indicate location of parasite.

Bibliography

1. Rinaldi, M., and P. Geldhof. 2012. Immunologically based control strategies for ostertagiosis in cattle: where do we stand? *Parasite Immunol.* 34: 254–264.

2. Cabaret, J., M. Bouilhol, and C. Mage. 2002. Managing helminths of ruminants in organic farming. *Vet. Res.* 33: 625–640.

3. Gasbarre, L. C., L. L. Smith, E. Hoberg, and P. A. Pilitt. 2009. Further characterization of a cattle nematode population with demonstrated resistance to current anthelmintics. *Vet. Parasitol.* 166: 275–80.

4. Tuo, W., L. Li, Y. Lv, J. Carrillo, D. Brown, W. C. Davis, J. Song, D. Zarlenga, and Z. Xiao. 2016. Abomasal mucosal immune responses of cattle with limited or continuous exposure to pasture-borne gastrointestinal nematode parasite infection. *Vet. Parasitol.* 229: 118–125.

5. Amulic, B., C. Cazalet, G. L. Hayes, K. D. Metzler, and A. Zychlinsky. 2012. Neutrophil Function: From Mechanisms to Disease. *Annu. Rev. Immunol.* 30: 459–489.

6. Kruger, P., M. Saffarzadeh, A. N. R. Weber, N. Rieber, M. Radsak, H. von Bernuth, C. Benarafa, D. Roos, J. Skokowa, and D. Hartl. 2015. Neutrophils: Between host defence, immune modulation, and tissue injury. *PLoS Pathog.* 11: e1004651.

7. Yipp, B. G., and P. Kubes. 2013. NETosis: how vital is it? *Blood* 122: 2784–2794.

8. Guimarães-Costa, A. B., N. C. Rochael, F. Oliveira, J. Echevarria-Lima, and E. M. Saraiva. 2017. Neutrophil Extracellular Traps Reprogram IL-4/GM-CSF-Induced Monocyte Differentiation to Anti-inflammatory Macrophages. *Front. Immunol.* 8: 523.

9. Muñoz-Caro, T., M. C. Rubio R, L. M. R. Silva, G. Magdowski, U. Gärtner, T. N. McNeilly, A. Taubert, and C. Hermosilla. 2015. Leucocyte-derived extracellular trap formation significantly contributes to Haemonchus contortus larval entrapment. *Parasit. Vectors* 8: 607.

10. Nathan, C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.* 6: 173–182.

11. Camps, M., T. Rückle, H. Ji, V. Ardissone, F. Rintelen, J. Shaw, C. Ferrandi, C. Chabert, C. Gillieron, B. Françon, T. Martin, D. Gretener, D. Perrin, D. Leroy, P.-A. Vitte, E. Hirsch, M. P. Wymann, R. Cirillo, M. K. Schwarz, and C. Rommel. 2005. Blockade of PI3Kγ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat. Med.* 11: 936–43.

12. Conejeros, I., E. Jara, M. D. Carretta, P. Alarcón, M. . Hidalgo, and R. A. Burgos. 2012. 2-Aminoethoxydiphenyl borate (2-APB) reduces respiratory burst, MMP-9 release and CD11b expression, and increases l-selectin shedding in bovine neutrophils. *Res. Vet. Sci.* 92: 103–110.

13. Dancey, J. T., K. A. Deubelbeiss, L. A. Harker, and C. A. Finch. 1976. Neutrophil kinetics in man. *J. Clin. Invest.* 58: 705–715.

14. Kolaczkowska, E., and P. Kubes. 2013. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13: 159–175.

15. Lämmermann, T., P. V. Afonso, B. R. Angermann, J. M. Wang, W. Kastenmüller, C. A. Parent, and R. N. Germain. 2013. Neutrophil swarms require LTB4 and integrins at sites of cell death in vivo. *Nature* 498: 371–375.

16. McDonald, B., K. Pittman, G. B. Menezes, S. A. Hirota, I. Slaba, C. C. M. Waterhouse, P. L. Beck, D. A. Muruve, and P. Kubes. 2010. Intravascular Danger Signals Guide Neutrophils to Sites of Sterile Inflammation. *Science (80-.).* 330: 362–366.

17. McMillan, R., and J. L. Scott. 1968. Leukocyte labeling with 51-Chromium. I. Technic and results in normal subjects. *Blood* 32: 738–54.

18. Suratt, B. T., S. K. Young, J. Lieber, J. A. Nick, P. M. Henson, and G. S. Worthen. 2001. Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* 281.

19. Furze, R. C., and S. M. Rankin. 2008. The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. *FASEB J.* 22: 3111–9.

20. Coffelt, S. B., M. D. Wellenstein, and K. E. de Visser. 2016. Neutrophils in cancer:

neutral no more. *Nat. Rev. Cancer* 16: 431–446.

21. Basu, S., G. Hodgson, M. Katz, and A. R. Dunn. 2002. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood* 100: 854–61.

22. Theilgaard-Monch, K., L. C. Jacobsen, R. Borup, T. Rasmussen, M. D. Bjerregaard, F. C. Nielsen, J. B. Cowland, and N. Borregaard. 2005. The transcriptional program of terminal granulocytic differentiation. *Blood* 105: 1785–1796.

23. Boxio, R., C. Bossenmeyer-Pourié, N. Steinckwich, C. Dournon, O. Nü ße, and O. Nüsse. 2004. Mouse bone marrow contains large numbers of functionally competent neutrophils. *J. Leukoc. Biol.* 75: 604–611.

24. Borregaard, N., O. E. Sørensen, and K. Theilgaard-Mönch. 2007. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* 28: 340–345.

25. Jarvis, J. N., K. Jiang, M. B. Frank, N. Knowlton, A. Aggarwal, C. A. Wallace, R. McKee, B. Chaser, C. Tung, L. B. Smith, J. L. McGhee, Y. Chen, J. Osban, K. M. O'Neil, and M. Centola. 2009. Gene expression profiling in neutrophils from children with polyarticular juvenile idiopathic arthritis. *Arthritis Rheum.* 60: 1488–1495.

26. Theilgaard-Mönch, K., S. Knudsen, P. Follin, and N. Borregaard. 2004. The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J. Immunol.* 172: 7684–93.

27. Radom-Aizik, S., F. Zaldivar, S.-Y. Leu, P. Galassetti, and D. M. Cooper. 2007. Effects of 30 min of aerobic exercise on gene expression in human neutrophils. *J. Appl. Physiol.* 104: 236–243.

28. Kotz, K. T., W. Xiao, C. Miller-Graziano, W.-J. Qian, A. Russom, E. A. Warner, L. L. Moldawer, A. De, P. E. Bankey, B. O. Petritis, D. G. Camp, A. E. Rosenbach, J. Goverman, S. P. Fagan, B. H. Brownstein, D. Irimia, W. Xu, J. Wilhelmy, M. N. Mindrinos, R. D. Smith, R. W. Davis, R. G. Tompkins, M. Toner, H. V Baker, U. G. J. Balis, T. R. Billiar, S. E. Calvano, J. P. Cobb, J. Cuschieri, C. C. Finnerty, R. L. Gamelli, N. S. Gibran, B. G. Harbrecht, D. L. Hayden, L. Hennessy, D. N. Herndon, M. G. Jeschke, J. L. Johnson, M. B. Klein, S. F. Lowry, R. V Maier, P. H. Mason, G. P. McDonald-Smith, J. P. Minei, E. E. Moore, A. B. Nathens, G. E. O′Keefe, L. G. Rahme, D. G. Remick, D. A. Schoenfeld, M. B. Shapiro, J. Sperry, J. D. Storey, R. Tibshirani, H. S. Warren, M. A. West, B. Wispelwey, and W. H. Wong. 2010. Clinical microfluidics for neutrophil genomics and proteomics. *Nat. Med.* 16: 1042–1047.

29. Fessler, M. B., K. C. Malcolm, M. W. Duncan, and G. S. Worthen. 2002. A Genomic and Proteomic Analysis of Activation of the Human Neutrophil by Lipopolysaccharide and Its Mediation by p38 Mitogen-activated Protein Kinase. *J. Biol. Chem.* 277: 31291–31302.

30. Malcolm, K. C., P. G. Arndt, E. J. Manos, D. A. Jones, and G. S. Worthen. 2003. Microarray analysis of lipopolysaccharide-treated human neutrophils. *Am. J. Physiol.*

- *Lung Cell. Mol. Physiol.* 284: L663–L670.

31. Zhang, X., Y. Kluger, Y. Nakayama, R. Poddar, C. Whitney, A. Detora, S. M. Weissman, and P. E. Newburger. 2003. Gene expression in mature neutrophils: early responses to inflammatory stimuli. *J. Leukoc. Biol.* 75: 358–372.

32. Kobayashi, S. D., J. M. Voyich, A. R. Whitney, and F. R. DeLeo. 2005. Spontaneous neutrophil apoptosis and regulation of cell survival by granulocyte macrophage-colony stimulating factor. *J. Leukoc. Biol.* 78: 1408–1418.

33. Subrahmanyam, Y. V. B. K., S. Yamaga, Y. Prashar, H. H. Lee, N. P. Hoe, Y. Kluger, M. Gerstein, J. D. Goguen, P. E. Newburger, and S. M. Weissman. 2001. RNA expression patterns change dramatically in human neutrophils exposed to bacteria. *Blood* 97: 2457–68.

34. Scapini, P., J. A. Lapinet-Vera, S. Gasperini, F. Calzetti, F. Bazzoni, and M. A. Cassatella. 2000. The neutrophil as a cellular source of chemokines. *Immunol. Rev.* 177: 195–203.

35. Sica, A., K. Matsushima, J. Van Damme, J. M. Wang, N. Polentarutti, E. Dejana, F. Colotta, and A. Mantovani. 1990. IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. *Immunology* 69: 548–53.

36. Kasama, T., Y. Miwa, T. Isozaki, T. Odai, M. Adachi, and S. Kunkel. 2005. Neutrophil-Derived Cytokines: Potential Therapeutic Targets in Inflammation. *Curr.*

Drug Target -Inflammation Allergy 4: 273–279.

37. Faurschou, M., and N. Borregaard. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* 5: 1317–27.

38. Hussen, J., M. Koy, W. Petzl, and H.-J. Schuberth. 2016. Neutrophil degranulation differentially modulates phenotype and function of bovine monocyte subsets. *Innate Immun.* 22: 124–137.

39. Nüsse, O., and M. Lindau. 1988. The dynamics of exocytosis in human neutrophils. *J. Cell Biol.* 107: 2117–23.

40. Brogden, K. A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* 3: 238–250.

41. Borregaard, N. 2010. Neutrophils, from Marrow to Microbes. *Immunity* 33: 657–670.

42. Sabroe, I., S. K. Dower, and M. K. B. Whyte. 2005. The Role of Toll-Like Receptors in the Regulation of Neutrophil Migration, Activation, and Apoptosis. *Clin. Infect. Dis.* 41: S421–S426.

43. Parker, L. C., M. K. B. Whyte, S. K. Dower, and I. Sabroe. 2005. The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J. Leukoc. Biol.* 77: 886–92.

44. Watanabe, J., G. K. Marathe, P. O. Neilsen, A. S. Weyrich, K. A. Harrison, R. C. Murphy, G. A. Zimmerman, and T. M. McIntyre. 2003. Endotoxins stimulate neutrophil adhesion followed by synthesis and release of platelet-activating factor in microparticles. *J. Biol. Chem.* 278: 33161–8.

45. Pliyev, B. K., M. V. Kalintseva, S. V. Abdulaeva, K. N. Yarygin, and V. G. Savchenko. 2014. Neutrophil microparticles modulate cytokine production by natural killer cells. *Cytokine* 65: 126–129.

46. Swain, S. D., T. T. Rohn, and M. T. Quinn. 2002. Neutrophil Priming in Host Defense: Role of Oxidants as Priming Agents. *Antioxid. Redox Signal.* 4: 69–83.

47. Doerfler, M. E., R. L. Danner, J. H. Shelhamer, and J. E. Parrillo. 1989. Bacterial lipopolysaccharides prime human neutrophils for enhanced production of leukotriene B4. *J. Clin. Invest.* 83: 970–7.

48. Guthrie, L. A., L. C. McPhail, P. M. Henson, and R. B. Johnston. 1984. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme. *J. Exp. Med.* 160: 1656–71.

49. Worthen, G. S., C. Haslett, A. J. Rees, R. S. Gumbay, J. E. Henson, and P. M. Henson. 1987. Neutrophil-mediated Pulmonary Vascular Injury: Synergistic Effect of Trace Amounts of Lipopolysaccharide and Neutrophil Stimuli on Vascular Permeability and Neutrophil Sequestration in the Lung. *Am. Rev. Respir. Dis.* 136: 19–

28.

50. Kitchen, E., A. G. Rossi, A. M. Condliffe, C. Haslett, and E. R. Chilvers. 1996. Demonstration of reversible priming of human neutrophils using platelet-activating factor. *Blood* 88: 4330–7.

51. de Oliveira, S., E. E. Rosowski, and A. Huttenlocher. 2016. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat. Rev. Immunol.* 16: 378–391.

52. Kansas, G. 1996. Selectins and their ligands: current concepts and controversies. *Blood* 88.

53. McEver, R. P., and R. D. Cummings. 1997. Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J. Clin. Invest.* 100: 485–91.

54. Yago, T., B. Shao, J. J. Miner, L. Yao, A. G. Klopocki, K. Maeda, K. M. Coggeshall, and R. P. McEver. 2010. E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin L 2-mediated slow leukocyte rolling. *Blood* 116: 485–494.

55. Mueller, H., A. Stadtmann, H. Van Aken, E. Hirsch, D. Wang, K. Ley, and A. Zarbock. Tyrosine kinase Btk regulates E-selectin–mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) □2 and PI3K□

pathways. .

56. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7: 678–689.

57. Constantin, G., M. Majeed, C. Giagulli, L. Piccio, J. Y. Kim, E. C. Butcher, and C. Laudanna. 2000. Chemokines Trigger Immediate β2 Integrin Affinity and Mobility Changes. *Immunity* 13: 759–769.

58. Liu, S., W. B. Kiosses, D. M. Rose, M. Slepak, R. Salgia, J. D. Griffin, C. E. Turner, M. A. Schwartz, and M. H. Ginsberg. 2002. A Fragment of Paxillin Binds the 4Integrin Cytoplasmic Domain (Tail) and Selectively Inhibits 4-Mediated Cell Migration. *J. Biol. Chem.* 277: 20887–20894.

59. Prince, L. R., M. K. Whyte, I. Sabroe, and L. C. Parker. 2011. The role of TLRs in neutrophil activation. *Curr. Opin. Pharmacol.* 11: 397–403.

60. El-Benna, J., P. M.-C. Dang, and M.-A. Gougerot-Pocidalo. 2008. Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin. Immunopathol.* 30: 279–289.

61. Aida, Y., and M. J. Pabst. 1990. Priming of neutrophils by lipopolysaccharide for enhanced release of superoxide. Requirement for plasma but not for tumor necrosis factor-alpha. *J. Immunol.* 145: 3017–25.

62. Follin, P., and C. Dahlgren. 1992. Phagocytosis by lipopolysaccharide-primed human neutrophils is associated with increased extracellular release of reactive oxygen metabolites. *Inflammation* 16: 83–91.

63. Forehand, J. R., J. S. Bomalski, and R. B. Johnston. 1991. Mechanisms of Lipopolysaccharide Priming for Enhanced Respiratory Burst Activity in Human Neutrophils. In Springer US. 65–73.

64. Fittschen, C., R. A. Sandhaus, G. S. Worthen, and P. M. Henson. 1988. Bacterial lipopolysaccharide enhances chemoattractant-induced elastase secretion by human neutrophils. *J. Leukoc. Biol.* 43: 547–56.

65. Condliffe, A., E. Kitchen, and E. Chilvers. 1998. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin. Sci.* 94: 461–71.

66. Inaba, M., T. Takahashi, Y. Kumeda, T. Kato, F. Hato, Y. Yutani, H. Goto, Y. Nishizawa, and S. Kitagawa. Increased basal phosphorylation of mitogen-activated protein kinases and reduced responsiveness to inflammatory cytokines in neutrophils from patients with rheumatoid arthritis. *Clin. Exp. Rheumatol.* 26: 52–60.

67. Sela, S., R. Shurtz-Swirski, M. Cohen-Mazor, R. Mazor, J. Chezar, G. Shapiro, K. Hassan, G. Shkolnik, R. Geron, and B. Kristal. 2005. Primed Peripheral Polymorphonuclear Leukocyte: A Culprit Underlying Chronic Low-Grade Inflammation and Systemic Oxidative Stress in Chronic Kidney Disease. *J. Am. Soc. Nephrol.* 16: 2431–2438.

68. Suematsu, M., M. Suzuki, T. Kitahora, S. Miura, K. Suzuki, T. Hibi, M. Watanabe, H. Nagata, H. Asakura, and M. Tsuchiya. 1987. Increased respiratory burst of leukocytes in inflammatory bowel diseases--the analysis of free radical generation by using chemiluminescence probe. *J. Clin. Lab. Immunol.* 24: 125–8.

69. DeLeo, F. R., J. Renee, S. McCormick, M. Nakamura, M. Apicella, J. P. Weiss, and W. M. Nauseef. 1998. Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J. Clin. Invest.* 101: 455–463.

70. Blouin, E., L. Halbwachs-Mecarelli, and P. Rieu. 1999. Redox regulation of β2-integrin CD11b / CD18 activation. *Eur. J. Immunol.* 29: 3419–3431.

71. Rausch, P. G., and T. G. Moore. 1975. Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. *Blood* 46: 913–9.

72. Gennaro, R., B. Dewald, U. Horisberger, H. U. Gubler, and M. Baggiolini. 1983. A novel type of cytoplasmic granule in bovine neutrophils. *J. Cell Biol.* 96: 1651–61.

73. Brown, G. B., and J. A. Roth. 1991. Comparison of the response of bovine and human neutrophils to various stimuli. *Vet. Immunol. Immunopathol.* 28: 201–218.

74. Paape, M. J., D. D. Bannerman, X. Zhao, and J.-W. Lee. 2003. The bovine neutrophil: Structure and function in blood and milk. *597 Vet. Res* 34: 597–627.

75. Carlson, G. P., and J. J. Kaneko. 1975. Intravascular granulocyte kinetics in developing calves. *Am. J. Vet. Res.* 36: 421–5.

76. Zhang, G., J. R. Young, C. A. Tregaskes, P. Sopp, and C. J. Howard. 1995. Identification of a novel class of mammalian Fc gamma receptor. *J. Immunol.* 155.

77. van der Does, A. M., H. Beekhuizen, B. Ravensbergen, T. Vos, T. H. M. Ottenhoff, J. T. van Dissel, J. W. Drijfhout, P. S. Hiemstra, and P. H. Nibbering. 2010. LL-37 Directs Macrophage Differentiation toward Macrophages with a Proinflammatory Signature. *J. Immunol.* 185: 1442–1449.

78. McGuire, K., M. Jones, D. Werling, J. L. Williams, E. J. Glass, and O. Jann. 2006. Radiation hybrid mapping of all 10 characterized bovine Toll-like receptors. *Anim. Genet.* 37: 47–50.

79. Werling, D., J. Piercy, and T. J. Coffey. 2006. Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells—Potential role in pathogen discrimination? *Vet. Immunol. Immunopathol.* 112: 2–11.

80. Hodgson, P. D., P. Aich, A. Manuja, K. Hokamp, F. M. Roche, F. S. L. Brinkman, A. Potter, L. A. Babiuk, and P. J. Griebel. 2005. Effect of stress on viral-bacterial synergy in bovine respiratory disease: novel mechanisms to regulate inflammation. *Comp. Funct. Genomics* 6: 244–50.

81. De Schepper, S., A. De Ketelaere, D. D. Bannerman, M. J. Paape, L. Peelman, and C. Burvenich. 2008. The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of Escherichia coli mastitis in dairy cattle. *Vet. Res* 39.

82. Conejeros, I., R. Patterson, R. A. Burgos, C. Hermosilla, and D. Werling. 2011. *Induction of reactive oxygen species in bovine neutrophils is CD11b, but not dectin-1-dependent*,.

83. Revelo, X. S., and M. R. Waldron. 2012. In vitro effects of Escherichia coli lipopolysaccharide on the function and gene expression of neutrophils isolated from the blood of dairy cows. *J. Dairy Sci.* 95: 2422–2441.

84. Sohn, E. J., M. J. Paape, E. E. Connor, D. D. Bannerman, R. H. Fetterer, and R. R. Peters. 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-α, IL-1β, IL-12 and IFN-γ. *Vet. Res.* 38: 809–818.

85. Worku, M., and A. Morris. 2009. Binding of different forms of lipopolysaccharide and gene expression in bovine blood neutrophils. *J. Dairy Sci.* 92: 3185–3193.

86. Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, and A. Zychlinsky. 2004. Neutrophil Extracellular Traps Kill Bacteria. *Science (80-.).* 303.

87. Rochael, N. C., A. B. Guimarães-Costa, M. T. C. Nascimento, T. S. DeSouza-Vieira, M. P. Oliveira, L. F. Garcia e Souza, M. F. Oliveira, and E. M. Saraiva. 2015. Classical ROS-dependent and early/rapid ROS-independent release of Neutrophil Extracellular Traps triggered by Leishmania parasites. *Sci. Rep.* 5: 18302.

88. Scherz-Shouval, R., and Z. Elazar. 2011. Regulation of autophagy by ROS:

physiology and pathology. *Trends Biochem. Sci.* 36: 30–38.

89. Papayannopoulos, V., K. D. Metzler, A. Hakkim, and A. Zychlinsky. 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191: 677–691.

90. Patel, S., S. Kumar, A. Jyoti, B. S. Srinag, R. S. Keshari, R. Saluja, A. Verma, K. Mitra, M. K. Barthwal, H. Krishnamurthy, V. K. Bajpai, and M. Dikshit. 2010. Nitric oxide donors release extracellular traps from human neutrophils by augmenting free radical generation. *Nitric Oxide* 22: 226–234.

91. Fuchs, T. A., U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann, and A. Zychlinsky. 2007. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* 176: 231–241.

92. Li, P., M. Li, M. R. Lindberg, M. J. Kennett, N. Xiong, and Y. Wang. 2010. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J. Exp. Med.* 207: 1853–62.

93. Remijsen, Q., T. Vanden Berghe, E. Wirawan, B. Asselbergh, E. Parthoens, R. De Rycke, S. Noppen, M. Delforge, J. Willems, and P. Vandenabeele. 2011. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* 21: 290–304.

94. Kolaczkowska, E., C. N. Jenne, B. G. J. Surewaard, A. Thanabalasuriar, W.-Y. Lee,

M.-J. Sanz, K. Mowen, G. Opdenakker, and P. Kubes. 2015. Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nat. Commun.* 6: 6673.

95. Tanaka, K., Y. Koike, T. Shimura, M. Okigami, S. Ide, Y. Toiyama, Y. Okugawa, Y. Inoue, T. Araki, K. Uchida, Y. Mohri, A. Mizoguchi, and M. Kusunoki. 2014. In Vivo Characterization of Neutrophil Extracellular Traps in Various Organs of a Murine Sepsis Model. *PLoS One* 9: e111888.

96. Urban, C. F., U. Reichard, V. Brinkmann, and A. Zychlinsky. 2006. Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell. Microbiol.* 8: 668–676.

97. Wardini, A. B., A. B. Guimaraes-Costa, M. T. C. Nascimento, N. R. Nadaes, M. G. M. Danelli, C. Mazur, C. F. Benjamim, E. M. Saraiva, and L. H. Pinto-da-Silva. 2010. Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus. *J. Gen. Virol.* 91: 259–264.

98. Guimarães-Costa, A. B., M. T. C. Nascimento, G. S. Froment, R. P. P. Soares, F. N. Morgado, F. Conceição-Silva, and E. M. Saraiva. 2009. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. *Proc. Natl. Acad. Sci. U. S. A.* 106: 6748–53.

99. Khandpur, R., C. Carmona-Rivera, A. Vivekanandan-Giri, A. Gizinski, S. Yalavarthi, J. S. Knight, S. Friday, S. Li, R. M. Patel, V. Subramanian, P. Thompson,

P. Chen, D. A. Fox, S. Pennathur, and M. J. Kaplan. 2013. NETs Are a Source of Citrullinated Autoantigens and Stimulate Inflammatory Responses in Rheumatoid Arthritis. *Sci. Transl. Med.* 5: 178ra40-178ra40.

100. Ermert, D., C. F. Urban, B. Laube, C. Goosmann, A. Zychlinsky, and V. Brinkmann. 2009. Mouse Neutrophil Extracellular Traps in Microbial Infections. *J. Innate Immun.* 1: 181–193.

101. Silva, L. M. R., T. Muñoz Caro, R. Gerstberger, M. J. M. Vila-Viçosa, H. C. E. Cortes, C. Hermosilla, and A. Taubert. 2014. The apicomplexan parasite Eimeria arloingi induces caprine neutrophil extracellular traps. *Parasitol. Res.* 113: 2797–2807.

102. Yildiz, K., S. Gokpinar, A. N. Gazyagci, C. Babur, N. Sursal, and A. K. Azkur. 2017. Role of NETs in the difference in host susceptibility to Toxoplasma gondii between sheep and cattle. *Vet. Immunol. Immunopathol.* 189: 1–10.

103. Lippolis, J. D., T. A. Reinhardt, J. P. Goff, and R. L. Horst. 2006. Neutrophil extracellular trap formation by bovine neutrophils is not inhibited by milk. *Vet. Immunol. Immunopathol.* 113: 248–255.

104. Villagra-Blanco, R., L. M. R. Silva, A. Aguilella-Segura, I. Arcenillas-Hernández, C. Martínez-Carrasco, A. Seipp, U. Gärtner, R. Ruiz de Ybañez, A. Taubert, and C. Hermosilla. 2017. Bottlenose dolphins (Tursiops truncatus) do also cast neutrophil extracellular traps against the apicomplexan parasite Neospora caninum. *Int. J. Parasitol. Parasites Wildl.* 6: 287–294.

105. Chuammitri, P., J. Ostojić, C. B. Andreasen, S. B. Redmond, S. J. Lamont, and D. Palić. 2009. Chicken heterophil extracellular traps (HETs): Novel defense mechanism of chicken heterophils. *Vet. Immunol. Immunopathol.* 129: 126–131.

106. Palić, D., J. Ostojić, C. B. Andreasen, and J. A. Roth. 2007. Fish cast NETs: Neutrophil extracellular traps are released from fish neutrophils. *Dev. Comp. Immunol.* 31: 805–816.

107. Lange, M. K., F. Penagos-Tabares, T. Muñoz-Caro, U. Gärtner, H. Mejer, R. Schaper, C. Hermosilla, and A. Taubert. 2017. Gastropod-derived haemocyte extracellular traps entrap metastrongyloid larval stages of Angiostrongylus vasorum, Aelurostrongylus abstrusus and Troglostrongylus brevior. *Parasit. Vectors* 10: 50.

108. Aulik, N. A., K. M. Hellenbrand, and C. J. Czuprynski. 2012. Mannheimia haemolytica and its leukotoxin cause macrophage extracellular trap formation by bovine macrophages. *Infect. Immun.* 80: 1923–33.

109. Yousefi, S., J. A. Gold, N. Andina, J. J. Lee, A. M. Kelly, E. Kozlowski, I. Schmid, A. Straumann, J. Reichenbach, G. J. Gleich, and H.-U. Simon. 2008. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat. Med.* 14: 949–53.

110. von Köckritz-Blickwede, M., O. Goldmann, P. Thulin, K. Heinemann, A. Norrby-Teglund, M. Rohde, and E. Medina. 2008. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* 111: 3070–80.

111. Hawes, M. C., G. Curlango-Rivera, F. Wen, G. J. White, H. D. VanEtten, and Z. Xiong. 2011. Extracellular DNA: The tip of root defenses? *Plant Sci.* 180: 741–745.

112. Menegazzi, R., E. Decleva, and P. Dri. 2012. Killing by neutrophil extracellular traps: fact or folklore? *Blood* 119.

113. Yipp, B. G., B. Petri, D. Salina, C. N. Jenne, B. N. V Scott, L. D. Zbytnuik, K. Pittman, M. Asaduzzaman, K. Wu, H. C. Meijndert, S. E. Malawista, A. de Boisfleury Chevance, K. Zhang, J. Conly, and P. Kubes. 2012. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat. Med.* 18: 1386–1393.

114. Stoiber, W., A. Obermayer, P. Steinbacher, and W.-D. Krautgartner. 2015. The Role of Reactive Oxygen Species (ROS) in the Formation of Extracellular Traps (ETs) in Humans. *Biomolecules* 5: 702–23.

115. Mitroulis, I., K. Kambas, A. Chrysanthopoulou, P. Skendros, E. Apostolidou, I. Kourtzelis, G. I. Drosos, D. T. Boumpas, and K. Ritis. 2011. Neutrophil Extracellular Trap Formation Is Associated with IL-1β and Autophagy-Related Signaling in Gout. *PLoS One* 6: e29318.

116. Douda, D. N., M. A. Khan, H. Grasemann, and N. Palaniyar. 2015. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc. Natl. Acad. Sci. U. S. A.* 112: 2817–22.

117. Lood, C., L. P. Blanco, M. M. Purmalek, C. Carmona-Rivera, S. S. De Ravin, C.

K. Smith, H. L. Malech, J. A. Ledbetter, K. B. Elkon, and M. J. Kaplan. 2016. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat. Med.* 22: 146–153.

118. Zhang, K., P. Kubes, M. Sugai, M. G. Bowden, M. Hussain, S. M. Robbins, F. H. Y. Green, M. G. Surette, C. Fahey, B. G. Yipp, C. D. Sibley, F. H. Pilsczek, D. Salina, and K. K. H. Poon. 2010. Staphylococcus aureus Response to Neutrophil Extracellular Trap Formation in A Novel Mechanism of Rapid Nuclear A Novel Mechanism of Rapid Nuclear Neutrophil Extracellular Trap Formation in Response to Staphylococcus aureus. *J. Immunol. aureus. J. Immunol.* 185: 7413–7425.

119. Brinkmann, V., C. Goosmann, L. I. Kühn, and A. Zychlinsky. 2012. Automatic quantification of in vitro NET formation. *Front. Immunol.* 3: 413.

120. Pieterse, E., N. Rother, C. Yanginlar, L. B. Hilbrands, and J. van der Vlag. 2016. Neutrophils Discriminate between Lipopolysaccharides of Different Bacterial Sources and Selectively Release Neutrophil Extracellular Traps. *Front. Immunol.* 7: 484.

121. van der Linden, M., G. H. A. Westerlaken, M. van der Vlist, J. van Montfrans, and L. Meyaard. 2017. Differential Signalling and Kinetics of Neutrophil Extracellular Trap Release Revealed by Quantitative Live Imaging. *Sci. Rep.* 7: 6529.

122. Branzk, N., A. Lubojemska, S. E. Hardison, Q. Wang, M. G. Gutierrez, G. D. Brown, and V. Papayannopoulos. 2014. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat. Immunol.* 15:

1017–25.

123. Bonne-Année, S., L. A. Kerepesi, J. A. Hess, J. Wesolowski, F. Paumet, J. B. Lok, T. J. Nolan, and D. Abraham. 2014. Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval Strongyloides stercoralis. *Microbes Infect.* 16: 502–11.

124. Schönrich, G., and M. J. Raftery. 2016. Neutrophil Extracellular Traps Go Viral. *Front. Immunol.* 7: 366.

125. Zhao, C., F. Tian, Y. Yu, J. Luo, Q. Hu, B. J. Bequette, R. L. Baldwin VI, G. Liu, L. Zan, M. Scott Updike, and J. Song. 2012. Muscle transcriptomic analyses in Angus cattle with divergent tenderness. *Mol. Biol. Rep.* 39: 4185–4193.

126. Carrillo, J. A., Y. He, Y. Li, J. Liu, R. A. Erdman, T. S. Sonstegard, and J. Song. 2016. Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare. *Sci. Rep.* 6: 25948.

127. Qu, G., R. Fetterer, L. Leng, X. Du, D. Zarlenga, Z. Shen, W. Han, R. Bucala, and W. Tuo. 2014. Ostertagia ostertagi macrophage migration inhibitory factor is present in all developmental stages and may cross-regulate host functions through interaction with the host receptor. *Int. J. Parasitol.* 44: 355–367.

128. Samuel, T. K., J. W. Sinclair, K. L. Pinter, and I. Hamza. 2014. Culturing

<em>Caenorhabditis elegans in Axenic Liquid Media and Creation of Transgenic Worms by Microparticle Bombardment. *J. Vis. Exp.* e51796.

129. Sinclair, J., K. Pinter, T. Samuel, S. Beardsley, X. Yuan, J. Zhang, K. Meng, S. Yun, M. Krause, and I. Hamza. 2017. Inter-organ signalling by HRG-7 promotes systemic haem homeostasis. *Nat. Cell Biol.* 19: 799–807.

130. Garcia, M., T. H. Elsasser, D. Biswas, and K. M. Moyes. 2015. The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro. *J. Dairy Sci.* 98: 918–26.

131. Feng, Y., L. Zou, R. Si, Y. Nagasaka, and W. Chao. Bone marrow MyD88 signaling modulates neutrophil function and ischemic myocardial injury. .

132. Swamydas, M., and M. S. Lionakis. 2013. Isolation, purification and labeling of mouse bone marrow neutrophils for functional studies and adoptive transfer experiments. *J. Vis. Exp.* e50586.

133. Bruhn, K. W., K. Dekitani, T. B. Nielsen, P. Pantapalangkoor, and B. Spellberg. 2016. Ly6G-mediated depletion of neutrophils is dependent on macrophages. *Results Immunol.* 6: 5–7.

134. Martinelli, S., M. Urosevic, A. Daryadel, P. A. Oberholzer, C. Baumann, M. F. Fey, R. Dummer, H.-U. Simon, and S. Yousefi. 2004. Induction of Genes Mediating Interferon-dependent Extracellular Trap Formation during Neutrophil Differentiation.

J. Biol. Chem. 279: 44123–44132.

135. Villagra-Blanco, R., L. M. R. Silva, T. Muñoz-Caro, Z. Yang, J. Li, U. Gärtner, A. Taubert, X. Zhang, and C. Hermosilla. 2017. Bovine Polymorphonuclear Neutrophils Cast Neutrophil Extracellular Traps against the Abortive Parasite Neospora caninum. *Front. Immunol.* 8: 606.

136. Gabriel, C., W. R. McMaster, D. Girard, and A. Descoteaux. 2010. Leishmania donovani Promastigotes Evade the Antimicrobial Activity of Neutrophil Extracellular Traps. *J. Immunol.* 185: 4319–4327.

137. Liu, L., C. Dahlgren, H. Elwing, and H. Lundqvist. 1996. A simple chemiluminescence assay for the determination of reactive oxygen species produced by human neutrophils. *J. Immunol. Methods* 192: 173–8.

138. Masuda, S., D. Nakazawa, H. Shida, A. Miyoshi, Y. Kusunoki, U. Tomaru, and A. Ishizu. 2016. NETosis markers: Quest for specific, objective, and quantitative markers. *Clin. Chim. Acta* 459: 89–93.

139. HIRSCH, J. G. 1958. Bactericidal action of histone. *J. Exp. Med.* 108: 925–44.

140. Saffarzadeh, M., C. Juenemann, M. A. Queisser, G. Lochnit, G. Barreto, S. P. Galuska, J. Lohmeyer, and K. T. Preissner. 2012. Neutrophil Extracellular Traps Directly Induce Epithelial and Endothelial Cell Death: A Predominant Role of Histones. *PLoS One* 7: e32366.

141. Belaaouaj, A., K. S. Kim, and S. D. Shapiro. 2000. Degradation of outer membrane protein A in Escherichia coli killing by neutrophil elastase. *Science* 289: 1185–8.

142. Muñoz-Caro, T., S. J. Mena Huertas, I. Conejeros, P. Alarcón, M. A. Hidalgo, R. A. Burgos, C. Hermosilla, and A. Taubert. 2015. Eimeria bovis-triggered neutrophil extracellular trap formation is CD11b-, ERK 1/2-, p38 MAP kinase- and SOCE-dependent. *Vet. Res.* 46: 23.

143. Desai, J., S. V. Kumar, S. R. Mulay, L. Konrad, S. Romoli, C. Schauer, M. Herrmann, R. Bilyy, S. Müller, B. Popper, D. Nakazawa, M. Weidenbusch, D. Thomasova, S. Krautwald, A. Linkermann, and H.-J. Anders. 2016. PMA and crystal-induced neutrophil extracellular trap formation involves RIPK1-RIPK3-MLKL signaling. *Eur. J. Immunol.* 46: 223–229.

144. Hromadkova, L., L. Zarybnicka, J. Vavrova, Z. Bilkova, and Z. Sinkorova. 2014. The impact of pre-analytical treatment and sorting on human neutrophil function. *Vet. Med. (Praha).* 59: 543–552.

145. Park, J.-H., J.-E. Kim, J.-Y. Gu, H. J. Yoo, S. H. Park, Y. I. Kim, I. S. Nam-Goong, E. S. Kim, and H. K. Kim. 2016. Evaluation of Circulating Markers of Neutrophil Extracellular Trap (NET) Formation as Risk Factors for Diabetic Retinopathy in a Case-Control Association Study. *Exp. Clin. Endocrinol. Diabetes* 124: 557–561.

146. Swain, D. K., M. S. Kushwah, M. Kaur, T. K. Patbandha, A. K. Mohanty, and A.

K. Dang. 2014. Formation of NET, phagocytic activity, surface architecture, apoptosis and expression of toll like receptors 2 and 4 (TLR2 and TLR4) in neutrophils of mastitic cows. *Vet. Res. Commun.* 38: 209–219.

147. Parker, H., and C. C. Winterbourn. 2012. Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps. *Front. Immunol.* 3: 424.

148. Reichel, M., T. Muñoz-Caro, G. Sanchez Contreras, A. Rubio García, G. Magdowski, U. Gärtner, A. Taubert, and C. Hermosilla. 2015. Harbour seal (Phoca vitulina) PMN and monocytes release extracellular traps to capture the apicomplexan parasite Toxoplasma gondii. *Dev. Comp. Immunol.* 50: 106–115.

149. Metzler, K. D., T. A. Fuchs, W. M. Nauseef, D. Reumaux, J. Roesler, I. Schulze, V. Wahn, V. Papayannopoulos, and A. Zychlinsky. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 117: 953–9.

150. Dirksen, P., S. A. Marsh, I. Braker, N. Heitland, S. Wagner, R. Nakad, S. Mader, C. Petersen, V. Kowallik, P. Rosenstiel, M.-A. Félix, and H. Schulenburg. 2016. The native microbiome of the nematode Caenorhabditis elegans: gateway to a new host-microbiome model. *BMC Biol.* 14: 38.

151. Tamarozzi, F., J. D. Turner, N. Pionnier, A. Midgley, A. F. Guimaraes, K. L. Johnston, S. W. Edwards, and M. J. Taylor. 2016. Wolbachia endosymbionts induce neutrophil extracellular trap formation in human onchocerciasis. *Sci. Rep.* 6: 35559.

152. Wang, H., L. Mo, X. Xiao, S. An, X. Liu, J. Ba, W. Wu, P. Ran, P. Yang, and Z. Liu. 2017. Pplase of Dermatophagoides farinae promotes ovalbumin-induced airway allergy by modulating the functions of dendritic cells in a mouse model. *Sci. Rep.* 7: 43322.

153. Altrichter, J., S. Zedler, R. Kraft, E. Faist, S. R. Mitzner, M. Sauer, J. Windolf, M. Scholz, and T. Lögters. 2010. Neutrophil-derived circulating free DNA (cf-DNA/NETs), a potential prognostic marker for mortality in patients with severe burn injury. *Eur. J. Trauma Emerg. Surg.* 36: 551–557.

154. Vallés, J., A. Lago, M. T. Santos, A. M. Latorre, J. I. Tembl, J. B. Salom, C. Nieves, and A. Moscardó. 2017. Neutrophil extracellular traps are increased in patients with acute ischemic stroke: prognostic significance. *Thromb. Haemost.* 117: 1919–1929.

155. Dewas, C., P. M.-C. Dang, M.-A. Gougerot-Pocidalo, and J. El-Benna. 2003. TNF-α Induces Phosphorylation of p47phox in Human Neutrophils: Partial Phosphorylation of p47phox Is a Common Event of Priming of Human Neutrophils by TNF-α and Granulocyte-Macrophage Colony-Stimulating Factor. *J. Immunol.* 171.

156. El-Benna, J., M. Hurtado-Nedelec, V. Marzaioli, J.-C. Marie, M.-A. Gougerot-Pocidalo, and P. M.-C. Dang. 2016. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Immunol. Rev.* 273: 180–193.

157. Miralda, I., S. M. Uriarte, and K. R. McLeish. 2017. Multiple Phenotypic Changes

Define Neutrophil Priming. *Front. Cell. Infect. Microbiol.* 7: 217.

158. Hazeldine, J., P. Harris, I. L. Chapple, M. Grant, H. Greenwood, A. Livesey, E. Sapey, and J. M. Lord. 2014. Impaired neutrophil extracellular trap formation: a novel defect in the innate immune system of aged individuals. *Aging Cell* 13: 690–8.

159. Mayadas, T. N., X. Cullere, and C. A. Lowell. 2014. The Multifaceted Functions of Neutrophils. *Annu. Rev. Pathol. Mech. Dis.* 9: 181–218.

160. Al-Khafaji, A. B., S. Tohme, H. O. Yazdani, D. Miller, H. Huang, and A. Tsung. 2016. Superoxide induces Neutrophil Extracellular Trap Formation in a TLR-4 and NOX-dependent mechanism. *Mol. Med.* 22: 621.

161. Palmer, L. J., P. R. Cooper, M. R. Ling, H. J. Wright, A. Huissoon, and I. L. C. Chapple. 2012. Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clin. Exp. Immunol.* 167: 261–8.

162. Akong-Moore, K., O. A. Chow, M. von Köckritz-Blickwede, and V. Nizet. 2012. Influences of chloride and hypochlorite on neutrophil extracellular trap formation. *PLoS One* 7: e42984.

163. Cross, D. A., and P. H. Klesius. 1989. Soluble extracts from larval Ostertagia ostertagi modulating immune function. *Int. J. Parasitol.* 19: 57–61.

164. Thomas, C. J., and K. Schroder. 2013. Pattern recognition receptor function in neutrophils. *Trends Immunol.* 34: 317–328.

165. Klesius, P. H. 1988. Immunity to Ostertagia ostertagi. *Vet. Parasitol.* 27: 159–67.

166. Chuah, C., M. K. Jones, M. L. Burke, H. C. Owen, B. J. Anthony, D. P. McManus, G. A. Ramm, and G. N. Gobert. 2013. Spatial and temporal transcriptomics of Schistosoma japonicum-induced hepatic granuloma formation reveals novel roles for neutrophils. *J. Leukoc. Biol.* 94: 353–365.

167. Aulik, N. A., K. M. Hellenbrand, H. Klos, and C. J. Czuprynski. 2010. Mannheimia haemolytica and its leukotoxin cause neutrophil extracellular trap formation by bovine neutrophils. *Infect. Immun.* 78: 4454–66.

168. Wilson-Welder, J. H., A. T. Frank, R. L. Hornsby, S. C. Olsen, and D. P. Alt. 2016. Interaction of Bovine Peripheral Blood Polymorphonuclear Cells and Leptospira Species; Innate Responses in the Natural Bovine Reservoir Host. *Front. Microbiol.* 7: 1110.

169. Bürglin, T. R., E. Lobos, and M. L. Blaxter. 1998. Caenorhabditis elegans as a model for parasitic nematodes. *Int. J. Parasitol.* 28: 395–411.

170. Farrera, C., and B. Fadeel. 2013. Macrophage clearance of neutrophil extracellular traps is a silent process. *J. Immunol.* 191: 2647–56.

171. Zevian, S. C., and J. L. Yanowitz. 2014. Methodological considerations for heat shock of the nematode Caenorhabditis elegans. *Methods* 68: 450–7.

172. Hawes, M., C. Allen, B. G. Turgeon, G. Curlango-Rivera, T. Minh Tran, D. A.

Huskey, and Z. Xiong. 2016. Root Border Cells and Their Role in Plant Defense. *Annu. Rev. Phytopathol.* 54: 143–161.

173. Pijanowski, L., L. Golbach, E. Kolaczkowska, M. Scheer, B. M. L. Verburg-van Kemenade, and M. Chadzinska. 2013. Carp neutrophilic granulocytes form extracellular traps via ROS-dependent and independent pathways. *Fish Shellfish Immunol.* 34: 1244–1252.

174. Sacks, D., and N. Noben-Trauth. 2002. The immunology of susceptibility and resistance to Leishmania major in mice. *Nat. Rev. Immunol.* 2: 845–858.

175. Gómez-Muñoz, M. ., A. Canals-Caballero, S. Almeria, P. Pasquali, D. . Zarlenga, and L. . Gasbarre. 2004. Inhibition of bovine T lymphocyte responses by extracts of the stomach worm Ostertagia ostertagi. *Vet. Parasitol.* 120: 199–214.

176. Almería, S., A. Canals, D. S. Zarlenga, and L. C. Gasbarre. 1997. Isolation and phenotypic characterization of abomasal mucosal lymphocytes in the course of a primary Ostertagia ostertagi infection in calves. *Vet. Immunol. Immunopathol.* 57: 87–98.

177. Zawrotniak, M., O. Bochenska, J. Karkowska-Kuleta, K. Seweryn-Ozog, W. Aoki, M. Ueda, A. Kozik, and M. Rapala-Kozik. 2017. Aspartic Proteases and Major Cell Wall Components in Candida albicans Trigger the Release of Neutrophil Extracellular Traps. *Front. Cell. Infect. Microbiol.* 7: 414.

178. Klesius, P. H. 1993. Regulation of immunity to Ostertagia ostertagi. *Vet. Parasitol.* 46: 63–79.

179. Sattler, S., H. Ghadially, and E. Hofer. 2012. Evolution of the C-Type Lectin-Like Receptor Genes of the DECTIN-1 Cluster in the NK Gene Complex. *Sci. World J.* 2012: 1–11.

180. Rosen, H., and B. R. Michel. 1997. Redundant contribution of myeloperoxidase-dependent systems to neutrophil-mediated killing of Escherichia coli. *Infect. Immun.* 65: 4173–8.

181. Soehnlein, O. 2009. An elegant defense: how neutrophils shape the immune response. *Trends Immunol.* 30: 511–2.

182. Heyworth, P. G., A. R. Cross, and J. T. Curnutte. 2003. Chronic granulomatous disease. *Curr. Opin. Immunol.* 15: 578–84.

183. Bedard, K., and K.-H. Krause. 2007. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. *Physiol. Rev.* 87: 245–313.

184. Delgado-Rizo, V., M. A. Martínez-Guzmán, L. Iñiguez-Gutierrez, A. García-Orozco, A. Alvarado-Navarro, and M. Fafutis-Morris. 2017. Neutrophil Extracellular Traps and Its Implications in Inflammation: An Overview. *Front. Immunol.* 8: 81.

185. Holland, P. C., and H. S. Sherratt. 1972. Biochemical effects of the hypoglycaemic compound diphenyleneiodonnium. Catalysis of anion-hydroxyl ion exchange across

the inner membrane of rat liver mitochondria and effects on oxygen uptake. *Biochem. J.* 129: 39–54.

186. Mitroulis, I., I. Kourtzelis, K. Kambas, S. Rafail, A. Chrysanthopoulou, M. Speletas, and K. Ritis. 2010. Regulation of the autophagic machinery in human neutrophils. *Eur. J. Immunol.* 40: 1461–1472.

187. Frohner, I. E., C. Bourgeois, K. Yatsyk, O. Majer, and K. Kuchler. 2009. Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol. Microbiol.* 71: 240–52.

188. Hosseinzadeh, A., and C. F. Urban. 2013. Novel Insight into Neutrophil Immune Responses by Dry Mass Determination of Candida albicans Morphotypes. *PLoS One* 8: e77993.

189. Kenny, E. F., A. Herzig, R. Krüger, A. Muth, S. Mondal, P. R. Thompson, V. Brinkmann, H. von Bernuth, and A. Zychlinsky. 2017. Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife* 6.

190. Parker, H., M. Dragunow, M. B. Hampton, A. J. Kettle, and C. C. Winterbourn. 2012. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J. Leukoc. Biol.* 92: 841–849.