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#### Authors

Li, Ronald HL

Ng, Geena

Tablin, Fern

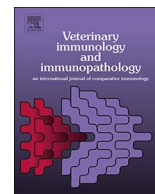
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## Research paper

# Lipopolysaccharide-induced neutrophil extracellular trap formation in canine neutrophils is dependent on histone H3 citrullination by peptidylarginine deiminase

Ronald H.L. Li\*, Geena Ng, Fern Tablin

Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California Davis, Davis, CA, United States



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## ABSTRACT

Neutrophils release neutrophil extracellular traps (NETs), which are extracellular chromatin decorated with histones and antimicrobial proteins. Although known for antimicrobial properties, overzealous production of NETs (NETosis) may lead to cytotoxicity and multiple organ failure in sepsis. Pathogen-induced NETosis has been extensively studied in mice but its importance in dogs remains largely unknown. This study sought to characterize *in vitro* NETosis induced by *E. coli* LPS, including assessing the role of peptidylarginine deiminase (PAD) in canine NETosis. Neutrophils ( $1 \times 10^6$  cells/ml) from healthy dogs were isolated and treated with 100  $\mu\text{g/ml}$  LPS, 100 nM phorbol 12-myristate 13-acetate (PMA), or buffer for either 90 or 180 min. NETs were assessed using fluorescence microscopy of living neutrophils and immunofluorescent microscopy. Supernatant and cellular debris were purified and cell-free DNA was quantified by spectrophotometry. The role of PAD was assessed by treating LPS- and PMA-activated neutrophils with 50, 100 or 200  $\mu\text{M}$  of the PAD inhibitor, Cl-amidine. *In vitro* NETosis was characterized by co-localization of cell-free DNA, citrullinated histone H3, and myeloperoxidase. LPS stimulation resulted in intracellular citrullination of histone H3. Compared to PMA chemically-induced NETosis, LPS resulted in smaller NETs with less extracellular citrullinated histone H3. Cl-amidine decreased citrullination of histones and NET production in either LPS- or PMA-stimulated neutrophils demonstrating that neutrophil PAD is essential for these cellular processes.

## 1. Introduction

Neutrophils are the primary effector cells of innate immunity during bacterial infection. In addition to phagocytosis and degranulation, neutrophils release NETs composed of extracellular DNA decorated with citrullinated histones and antimicrobial proteins (Brinkmann and Zychlinsky, 2007). The exact mechanisms of how NETs contribute to innate immunity is complex and not entirely known. Using fluorescently labelled *E. coli*, an *in vivo* study showed bacterial trapping by NETs within the liver sinusoids of septic mice (Clark et al., 2007). Entrapped bacteria were killed by high local concentrations of NET antimicrobial components such as neutrophil elastase, myeloperoxidase (MPO), histones, and bactericidal permeability-increasing protein (Medina, 2009).

In addition to bacteria, exposure to viruses, fungi and pathogen associated molecular patterns such as LPS induce NET formation or

NETosis in mice and people (Branzk and Papayannopoulos, 2013; Narasaruju et al., 2011; Pieterse et al., 2016; Rebordao et al., 2014). Although one study, using the chemical phorbol 12-myristate 13-acetate (PMA), demonstrated that canine neutrophils could produce NETs *in vitro*, the cellular mechanisms regulating NETosis are poorly understood in dogs (Jeffery et al., 2015). Studies in murine and human neutrophils demonstrated that NETosis and the release of histones and DNA require post-translational modification of histones. Citrullination or deimination of histones converts arginine and mono-methyl arginine to citrulline, resulting in a loss of positive charge in the N terminus. This reaction, catalyzed by the enzyme, peptidylarginine deiminase 4 (PAD4), leads to chromatin decondensation during NETosis (Leshner et al., 2012; Wang et al., 2009). Accordingly, PAD4 knockout mice were unable to produce NETs in the presence of either LPS or bacteria (Li et al., 2010).

High circulating concentrations of plasma cell-free DNA (cfDNA),

Abbreviations: cfDNA, cell-free DNA; cDNA, cellular DNA; citH3, citrullinated histones H3; DPBS, Dulbecco's phosphate-buffered saline; DAMPs, Danger-associated molecular patterns; MPO, myeloperoxidase; PAD, peptidylarginine deiminase; PMA, phorbol 12-myristate 13-acetate; RT, room temperature

\* Corresponding author. Present address: Department of Veterinary Surgical & Radiological Sciences, School of Veterinary Medicine, University of California Davis, Davis, One Shield Avenue, University of California, Davis, CA, 95616, United States.

E-mail address: [rhli@ucdavis.edu](mailto:rhli@ucdavis.edu) (R.H.L. Li).

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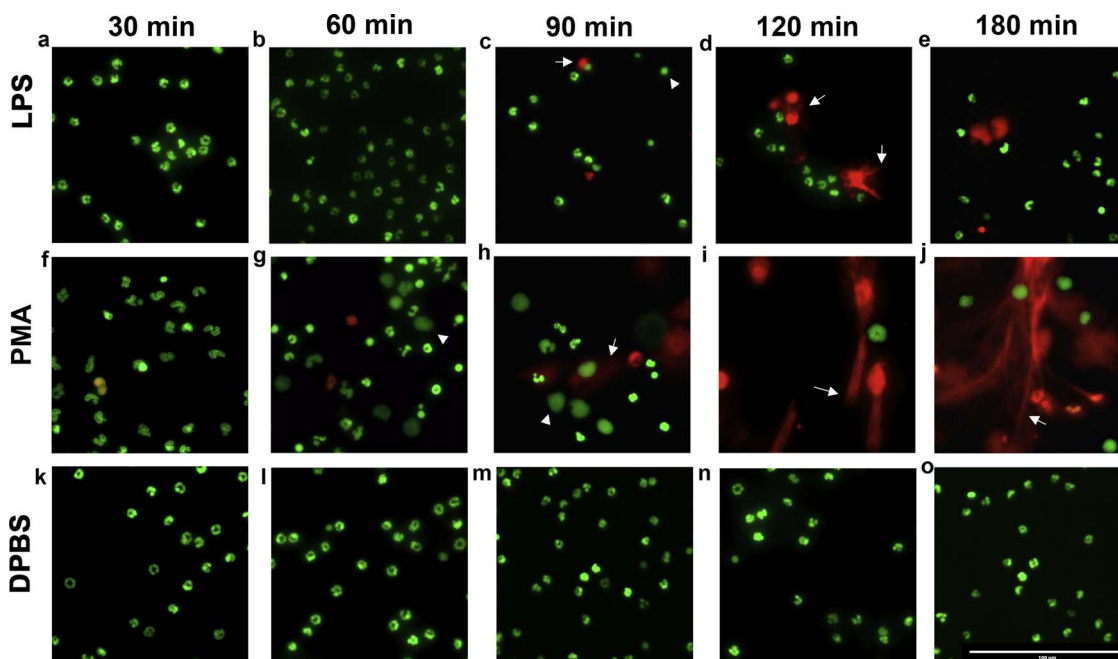


Fig. 1. Representative immunofluorescent images of live canine neutrophils.

Neutrophils were incubated for 30, 60, 90, 120 or 180 min with 100  $\mu\text{g}/\text{ml}$  LPS (a–e), 100 nM phorbol-myristate acetate (PMA) (positive control) (f–j), or DPBS (k–o). Nucleic acid in live cells was stained using a cell-permeant nucleic acid dye, Syto Green, whereas cell-free (cf) DNA and neutrophils with compromised plasma membranes were stained red with a cell-impermeant dye, Sytox Orange. Unstimulated neutrophils did not produce cfDNA at any time point (k–o). Compared to DPBS- and LPS-treated neutrophils (b, l), most nuclei of PMA-stimulated neutrophils had lost their lobulated appearance by 60 min (g, arrowhead). By 90 min, cfDNA could be seen surrounding decondensed chromatin (arrows) in both LPS-(c) and PMA-(h) treated neutrophils. LPS stimulation resulted in chromatin decondensation in some neutrophils (arrowhead). At 120 and 180 min, LPS- (d,e) and PMA-stimulated neutrophils (i,j) had non-lobulated nuclei and permeable plasma membranes surrounded by cfDNA (arrows). The experiment was replicated twice using neutrophils from 4 independent donors and yielded similar results. Scale bar = 100  $\mu\text{m}$ . Original 40 $\times$  magnification.

considered by some researchers as a biomarker of NETosis, is associated with a poor prognosis in human septic patients, suggesting that overzealous production of NETs is detrimental to the host (Dwivedi et al., 2012; Gould et al., 2015). High circulating cfDNA may, instead, be a marker of disease severity and not NETosis as other processes such as necrosis and apoptosis also can result in the release of cfDNA. Likewise, serum citrullinated histone H3 (citH3), a hallmark of NETs, can be found in critically ill human beings with bacterial infection and is associated with septic shock and mortality in a rodent model of sepsis (Hirose et al., 2014; Li et al., 2014). Extracellular histones released via NETosis act as damage-associated molecular patterns (DAMPs) to initiate apoptosis, cytotoxicity, vascular necrosis and systemic inflammation (Chen et al., 2014; Li et al., 2016; Narasaraaju et al., 2011). Recently, cfDNA also have been measured in healthy dogs and dogs with sepsis and immune-mediated haemolytic anemia, respectively (Smith et al., 2017; Jeffery et al., 2015; Letendre and Goggs, 2017). Because disruption of NETosis may be a potential treatment strategy that can modulate the morbidity and mortality associated with sepsis in dogs, a better understanding of the signalling mechanisms leading to NETosis, relevant to infections, is needed. In this study, we sought to characterize *in vitro* LPS-induced NETosis in canine neutrophils, to determine the role of PAD in histone citrullination and NETosis in canine neutrophils.

## 2. Materials and methods

### 2.1. Study population

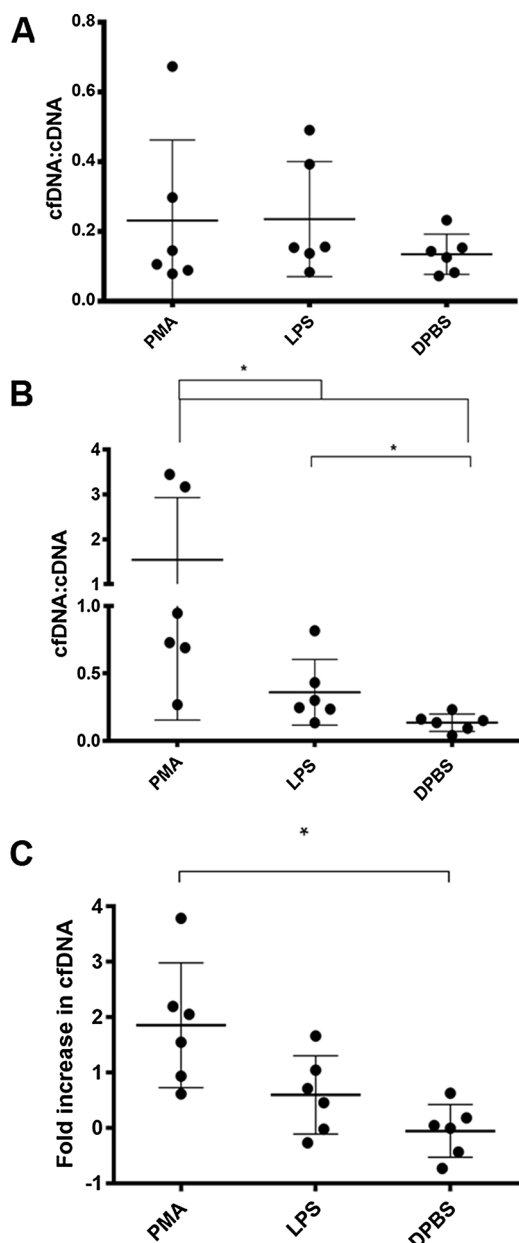
The study protocol was approved by the Institutional Animal Care and Use Committee at the University of California, Davis (protocol number:18338). Clinically healthy dogs owned by students, clients or staff members weighing more than 5 kg were eligible for enrolment.

### 2.2. Neutrophil isolation

Blood was drawn from either the cephalic or jugular vein (8–16 ml) and collected into sodium heparin tubes. Neutrophil isolation was carried out under sterile conditions using a modified protocol (Oh et al., 2008). In brief, blood was first diluted (1:1) with Dulbecco's phosphate-buffered saline (DPBS) and transferred to 3% dextran (30 min at room temperature (RT)). Leukocyte-rich plasma was layered onto Ficoll-Paque separation media and centrifuged at 400  $\times$  g (30 min, RT, no brake). The polymorphonuclear cell layer was retrieved and residual erythrocytes were lysed in ultrapure water for 30–60 s before adding an equal volume of cold 1.8% NaCl solution followed by centrifugation at 112  $\times$  g (10 min, 4  $^{\circ}\text{C}$ , no brake). Neutrophils were resuspended in DPBS (pH 7.3, 1.9 mM  $\text{CaCl}_2$ , 5 mM dextrose, 0.9 mM  $\text{MgCl}_2$ , 1% BSA) and diluted to a final concentration of  $1 \times 10^6$  cells/ml. Neutrophil count was determined by an automatic cell analyser (Coulter ACT diff, Beckman-Coulter Inc, Miami, FL) and verified by hemocytometer. Neutrophil viability was determined by trypan blue exclusion test and ranged from 98 to 100%. (Strober, 2015)

### 2.3. Fluorescent microscopy of live neutrophils

Isolated neutrophils ( $1 \times 10^6/\text{ml}$ ) were incubated with DPBS alone, 100 nM PMA (positive control), or 100  $\mu\text{g}/\text{ml}$  LPS (*E. coli* O55:B5, InvivoGen, San Diego, CA) for 30, 60, 90, 120, or 180 min at 37  $^{\circ}\text{C}$  in poly-L-lysine coated culture wells. SYTOX Orange dye (Thermo Fisher Scientific, Waltham, MA) (1  $\mu\text{M}$ , 10 min, RT) was used to label cfDNA and neutrophils with compromised plasma membranes. For detection of intracellular nucleic acids in live cells, cells were stained with 1  $\mu\text{M}$  SYTO Green Fluorescent Nucleic Acid Stain (SYTO 16, Thermo Fisher Scientific, Grand Island, NY). Fluorescent images were acquired using EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA).



**Fig. 2.** Isolated canine neutrophils release cfDNA in response to LPS and PMA. Neutrophils from 6 dogs were incubated with 100 nM PMA, 100  $\mu\text{g}/\text{ml}$  LPS or DPBS for either 90 (A) or 180 min (B). (A) At 90 min, PMA and LPS-stimulated neutrophils did not release significantly more cfDNA compared to DPBS-treated neutrophils. (B) At 180 min, PMA-treated neutrophils released significantly more cfDNA compared to LPS- and DPBS-treated neutrophils. LPS-treated neutrophils released more cfDNA than DPBS-treated neutrophils. (C) PMA-induced neutrophils had a significantly higher fold increase in cfDNA from 90 to 180 min than DPBS-treated neutrophils. (\*  $p < 0.05$ ).

#### 2.4. Neutrophil cell-free DNA quantification

Cell-free components and intact cells ( $1 \times 10^6/\text{ml}$ ) were separated by centrifugation (1500  $\times$  g, 10 min) and purified using a commercially available kit (Puregene Genra Systems, Minneapolis, MN), according to the manufacturer's protocol. cfDNA and cellular DNA (cDNA) concentrations ( $\text{ng}/\mu\text{l}$ ) were quantified by spectrophotometry (Nanodrop 2000, Thermo Scientific, Waltham, MA) at 260 nm as previously described. (Desjardins and Conklin, 2010) DNA purity was confirmed by the ratio of absorbance at 260 and 280 nm. Relative DNA release was assessed as the ratio of cfDNA concentration to cellular DNA concentration (cfDNA:cDNA). The fold increase in cfDNA released over time was calculated by the formula:

$$\text{Fold increase in cfDNA} = \log_{10}(\text{cfDNA:cDNA})_{180\text{minutes}} - \log_{10}(\text{cfDNA:cDNA})_{90\text{minutes}}$$

#### 2.5. In vitro neutrophil extracellular trap formation and PAD inhibition

Isolated neutrophils ( $1 \times 10^6$  cells/ml) were incubated in DPBS and activated with either 100  $\mu\text{g}/\text{ml}$  LPS or 100 nM PMA, seeded onto poly-D-lysine coated coverslips, and incubated at 37  $^{\circ}\text{C}$  for 180 min. To inhibit PAD, cells were pre-treated with 50, 100 or 200  $\mu\text{M}$  Cl-amidine (EMD Millipore, Billerica, MA) or DMSO (vehicle control), for 15 min at 37  $^{\circ}\text{C}$  before incubation with 100 nM PMA or 100  $\mu\text{g}/\text{ml}$  LPS (37  $^{\circ}\text{C}$ , 180 min).

#### 2.6. Immunofluorescence

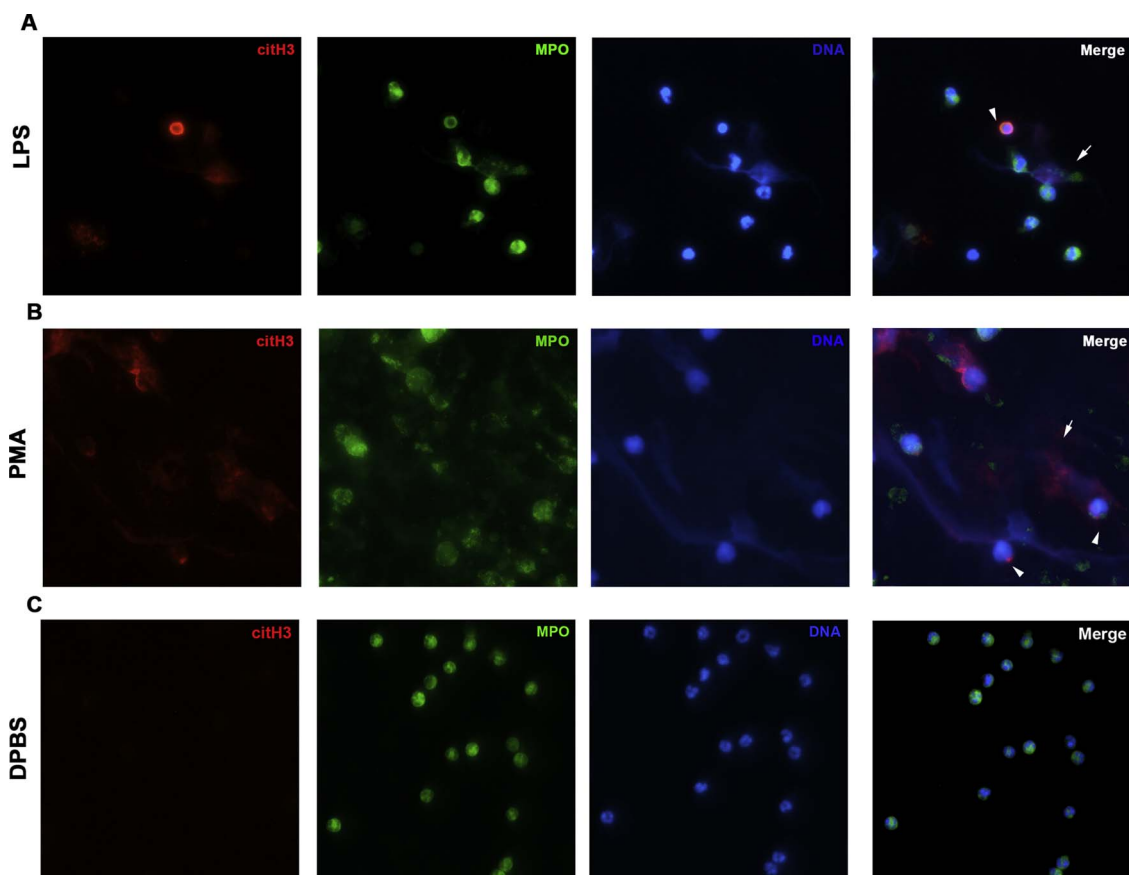
Neutrophils were fixed in 4% paraformaldehyde, permeabilized with 1% NP-40 (Surfact-Amps, NP-40, Pierce, Rockford, IL) and washed 3  $\times$  with DPBS. Because primary ab used to detect citrullinated histones H3 (citH3) and MPO were from the same species (rabbit), we utilized a modified double immunolabeling protocol (Negoescu et al., 1994). After blocking with 5% goat serum (1 h, 37  $^{\circ}\text{C}$ ), cells were incubated with 1:400 rabbit polyclonal anti-citrullinated histone H3 (citH3) ab (ab5103, Abcam, Cambridge, MA) (1 h, 37  $^{\circ}\text{C}$ ). After washing 3 times with DPBS, cells were incubated with 1:200 polyclonal goat anti-rabbit ab conjugated with Alexa Fluor 568 (A-11011, Thermo Fisher Scientific, Waltham, MA) (1 h, 37  $^{\circ}\text{C}$ ). To prevent MPO primary ab from binding to the first secondary ab (Type I interference), cells were blocked in 10% rabbit serum (4  $^{\circ}\text{C}$ , overnight) and incubated with 50  $\mu\text{g}/\text{ml}$  unconjugated goat anti-rabbit Fab fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at RT. Cells were then washed 3  $\times$  with DPBS and incubated with 1:200 polyclonal rabbit anti-human MPO ab<sup>13</sup> (1 h, 37  $^{\circ}\text{C}$ ) followed by a secondary ab (A-11008, Thermo Fisher Scientific, Waltham, MA) (1:200 Alexa Fluor 488 conjugated polyclonal goat anti-rabbit IgG). Cells were stained with 300 nM 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Interference controls were prepared by excluding incubation with either primary abs in the second immuno-labelling step (Type II inference).

#### 2.7. Assessment of nuclear morphology, intracellular citH3 expression and NET quantification

Fluorescent images were acquired using an EVOS FL Cell Imaging System. Ten random fields were captured at 40  $\times$  magnification for each experimental condition. Each sample was randomly assigned to a number and the images were acquired and analyzed in a blinded manner using available software (ImageJ, v1.50 g, NIH). NETs were identified based on co-localization of cfDNA, extracellular MPO, and citH3 (Narasaraju et al., 2011). The quantity of NETs released was expressed as a ratio (number of NETs: total number of neutrophils) in 10 random fields for each experimental condition. To quantify citH3 within a NET, corrected total fluorescence (CTF) was measured as previously described. (Gavet and Pines, 2010) In brief, an outline was drawn around each NET and area, mean fluorescence and integrated density were measured under the respective channel. The mean background fluorescence was acquired by measuring 3 adjacent mean gray values. The CTF of citH3 (CTF<sub>citH3</sub>) within each NET were calculated using the formula:

$$\text{CTF}_{\text{citH3}} = \text{integrated density} - (\text{area of selected NET} \times \text{mean fluorescence of background}).$$

The mean CTF was calculated based on the number of NETs released. Intracellular citH3 expression was quantified as the ratio of the number of neutrophils with intracellular citH3 to the number of neutrophils without intracellular citH3 ( $\text{Cells}_{\text{CitH3}} : \text{Cells}_{\text{noCitH3}}$ ) for each



**Fig. 3.** Representative immunofluorescent images of *in vitro* NETosis in canine neutrophils. Cells were fixed, permeabilized and stained for citrullinated histone H3 (citH3) (red) and myeloperoxidase (MPO) (green) after treatment with (A) LPS, (B) PMA or (C) DPBS for 180 min. DNA was stained with DAPI (blue). (A, merge) Note the well-defined NET (arrow) with extracellular citH3 and MPO granules produced by a LPS-stimulated neutrophil. Intracellular citH3 in a cell with a non-lobulated nucleus (arrow head). (B) Neutrophils stimulated by PMA were entangled in a vast network of NETs (arrows) decorated with extracellular citH3 and MPO granules. Neutrophils expressing intracellular citH3 (arrow heads) were surrounded by NETs. (C) No NETs were generated from unstimulated neutrophils. Scale bar = 50  $\mu$ m. Original magnification 40x.

experimental condition in 10 random fields.

To assess the changes in nuclear morphology due to NETosis and PAD inhibition, the ratio of neutrophils with non-lobulated nuclei to lobulated nuclei were quantified in living neutrophils.

### 3. Statistical analysis

Based on preliminary data, a sample size of 6 dogs was estimated to detect a 25% increase in NETs formation with a power > 80% and alpha-priori of 0.05. Normality of data was tested by examining normal quartile plots and Shapiro-Wilk test. Data measured as ratios were logarithmically transformed and analysed using either *t*-tests or Wilcoxon rank-sum test for paired data. One-way repeated measures ANOVA was used for detection of overall differences between the means of dose-response studies. Data that violated the assumption of sphericity were analyzed using Greenhouse-Geisser correction. An alpha-priori of < 0.05 was considered statistically significant. Normally distributed data are presented as mean  $\pm$  SDs. Non-normally distributed data are presented as medians and interquartile ranges. Data were analyzed using commercially available software (Prism 7.0, GraphPad Software Inc., La Jolla, CA).

## 4. Results

### 4.1. Fluorescence microscopy of live neutrophils

Nuclear morphology, plasma membrane integrity and the presence

of extracellular DNA in living neutrophils isolated from 5 dogs were characterized by fluorescence microscopy shown in Fig. 1. At 30 min, neutrophils treated with LPS, PMA or DPBS maintained their lobulated nuclei and intact plasma membranes (Fig. 1 a, f, k). By 60 min, the nuclei of most LPS-stimulated neutrophils remained lobulated ( $0.068 \pm 0.064$ ) compared to the non-lobulated round nuclei (arrow head) noted in most PMA-stimulated neutrophils ( $3.31 \pm 3.18$ ) ( $p = 0.0047$ ) (Fig. 1 b,g). By 90 min, extracellular DNA (Fig. 1c, h, arrows) could be seen surrounding LPS- and PMA-treated neutrophils that had undergone chromatin decondensation but maintained an intact plasma membrane. After 120 min of LPS treatment, a significantly higher number of neutrophil nuclei had lost their lobulated appearance compared to unstimulated neutrophils ( $0.34 \pm 0.17$  vs.  $0.094 \pm 0.038$ ,  $p = 0.030$ ). LPS-stimulated neutrophils also had lost their plasma membrane integrity ( $0.075 \pm 0.050$ ) compared to unstimulated neutrophils ( $0.0096 \pm 0.014$ ) ( $p = 0.004$ ) and a portion of those neutrophils also were surrounded by cfDNA (Fig. 1d, n, arrows). At 120 min, PMA-stimulated neutrophils had significantly higher numbers of non-lobulated nuclei ( $11.48 \pm 4.44$ ) and loss of plasma membrane integrity ( $2.42 \pm 1.89$ ) compared to LPS-stimulated neutrophils ( $p = 0.0002$ ,  $p = 0.0007$ , respectively). PMA-stimulated neutrophils were also surrounded by vast strands of cfDNA. (Fig. 1 i,j) Unstimulated neutrophils maintained intact plasma membranes and lobulated nuclei (Fig. 1k – o) (arrows).



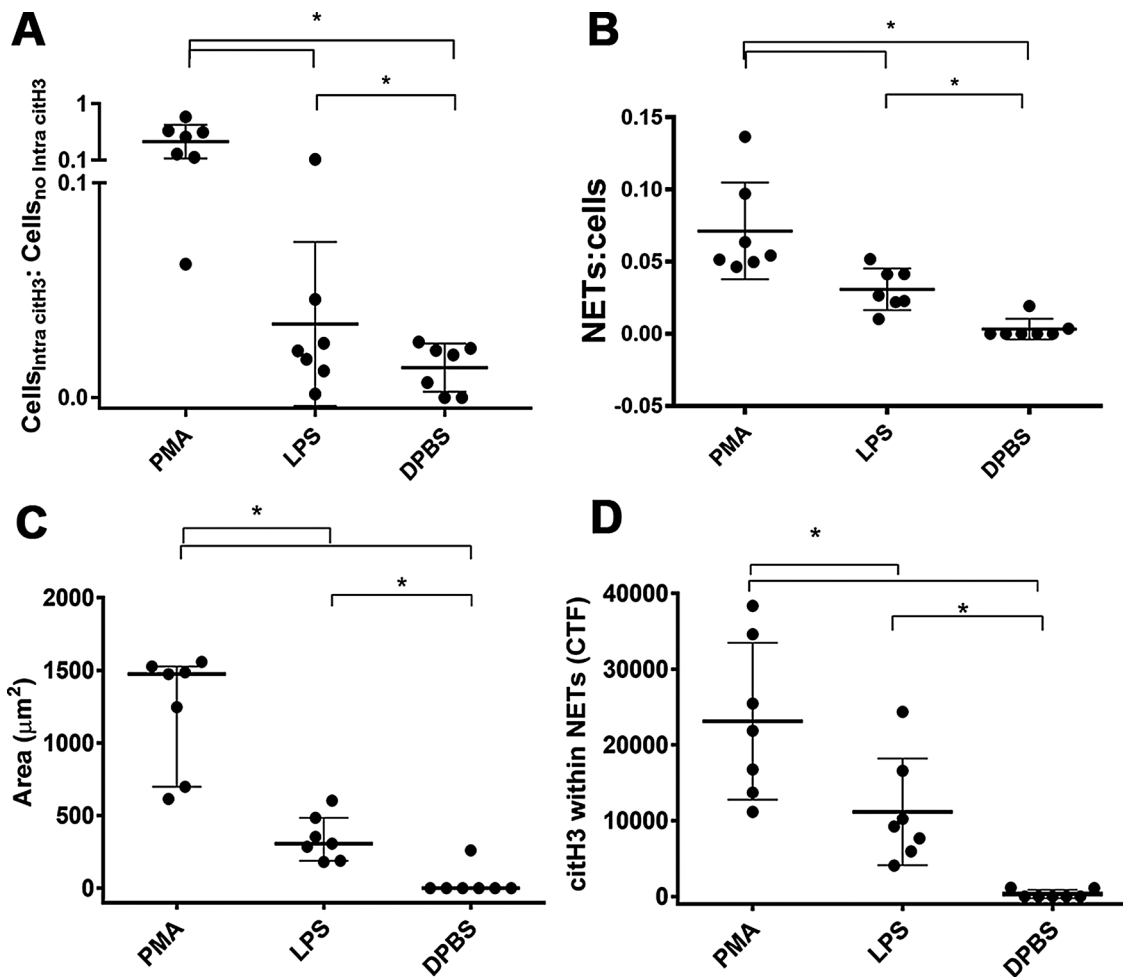


Fig. 4. Quantification and comparison of *in vitro* LPS- and PMA- induced NETosis.

Neutrophils isolated from 7 dogs were incubated with 100  $\mu\text{g}/\text{ml}$  LPS, 100 nM PMA, or DPBS for 180 min. (A) PMA-stimulated neutrophils had the highest number of cells expressing intracellular citH3 compared to LPS and DPBS-treated neutrophils. (B) LPS stimulation resulted in NETosis and unstimulated neutrophils did not produce any NETs. PMA stimulation resulted in significantly greater NET production than LPS stimulation. (C) PMA-stimulated neutrophils also released NETs that were significantly larger in area ( $\mu\text{m}^2$ ) compared to LPS-stimulated neutrophils. (D) Extracellular citH3 within NETs were measured as corrected total fluorescence (CTF). Only PMA- and LPS-treated neutrophils produced extracellular citH3 within NETs. (\*  $p < 0.05$ ).

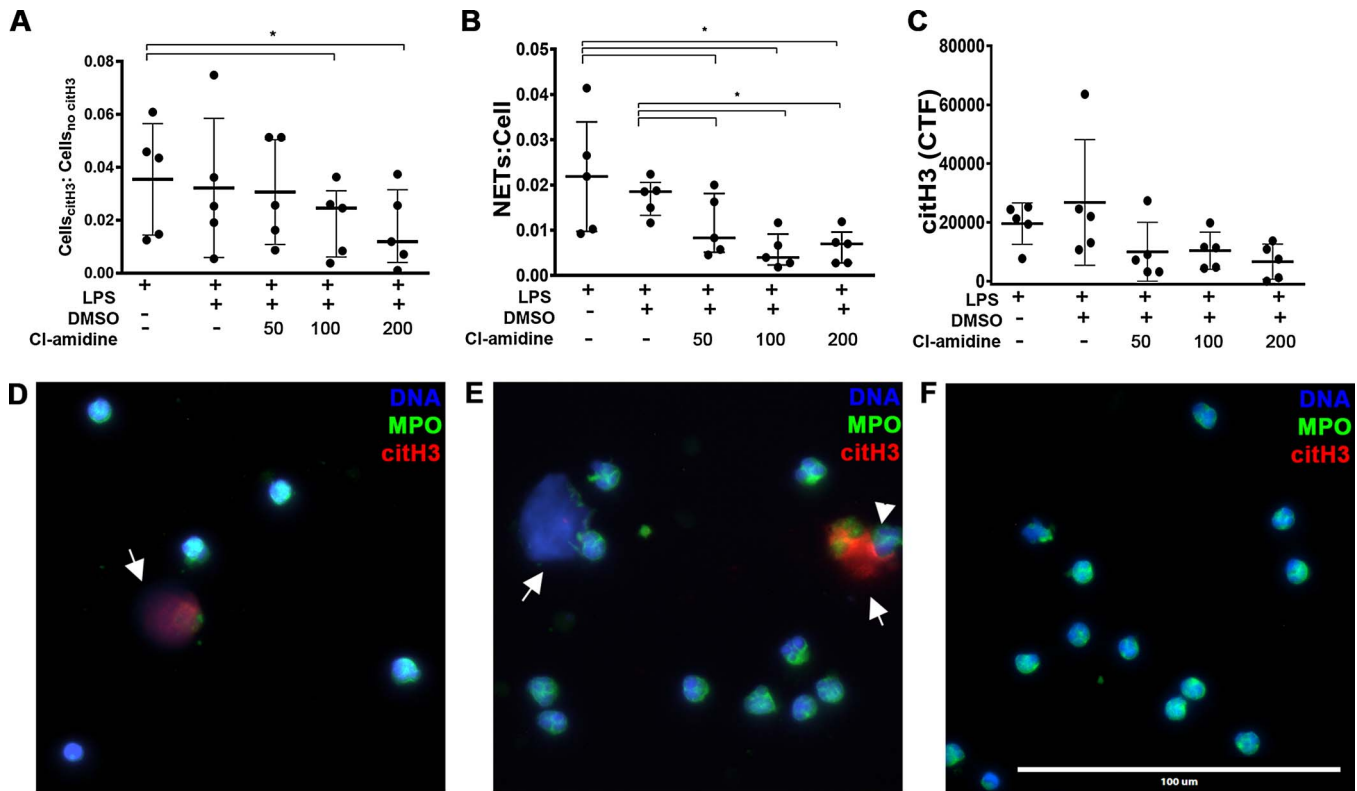
#### 4.2. LPS induced cfDNA release from canine neutrophils

cfDNA:cDNA was quantified by spectrophotometry 90 or 180 min after stimulation (Fig. 2). At 90 min, LPS-treated neutrophils released more cfDNA ( $0.24 \pm 0.16$ ) than DPBS-treated neutrophils ( $0.12 \pm 0.073$ ) but the difference did not reach statistical significance ( $p = 0.14$ ). Similarly, cfDNA released from PMA-treated neutrophils ( $0.23 \pm 0.23$ ) was not significantly different from LPS-treated neutrophils ( $p = 0.54$ ) and no differences were found between DPBS- and PMA-treated neutrophils ( $p = 0.37$ ) (Fig. 2A). At 180 min, neutrophils treated with PMA or LPS released significantly higher concentrations of cfDNA ( $1.35 \pm 1.37$ ,  $0.36 \pm 0.24$ , respectively) than unstimulated neutrophils ( $0.13 \pm 0.064$ ,  $p = 0.0040$  and  $0.0023$ , respectively). PMA-treated neutrophils also released significantly more cfDNA than LPS-treated neutrophils ( $p = 0.042$ ) (Fig. 2B). The mean fold increase in cfDNA over 90–180 min of LPS stimulation was  $0.60 \pm 0.71$ , which did not differ significantly from either PMA-stimulated ( $1.86 \pm 1.13$ ) ( $p = 0.078$ ) or unstimulated neutrophils ( $0.054 \pm 0.48$ ) ( $p = 0.12$ ) (Fig. 2C). The mean fold increase in cfDNA in PMA-treated neutrophils was significantly higher than DPBS-treated neutrophils ( $p = 0.0041$ ).

#### 4.3. LPS induced intracellular citH3 expression and NETs decorated with citH3 and MPO

Expression of intracellular citH3 was noted in PMA- and LPS-stimulated neutrophils (Fig. 3A, B Merge, arrow heads, 180 min). The ratio of neutrophils expressing intracellular citH3 was significantly higher in LPS-stimulated neutrophils ( $0.034 \pm 0.041$ ) compared to neutrophils incubated with DPBS alone ( $0.014 \pm 0.011$ ) ( $p = 0.047$ ). As expected, PMA stimulation resulted in the highest number of neutrophils with intracellular citH3 ( $0.40 \pm 0.27$ ) compared to LPS- and DPBS-treated neutrophils ( $p = 0.0004$ ,  $p = 0.014$ , respectively) (Fig. 4A).

NET components including cfDNA, extracellular citH3, and MPO were identified using immunofluorescence microscopy as shown in Fig. 3. Neutrophils activated by LPS released NETs, which were discrete web-like scaffolds of cfDNA decorated with extracellular citH3 and MPO granules, often surrounding nearby cells (Fig. 3A Merge, arrow). LPS-treated neutrophils produced significantly more NETs than unstimulated neutrophils ( $0.031 \pm 0.014$  vs  $0.0036 \pm 0.014$ ,  $p = 0.016$ ). Compared to neutrophils stimulated by PMA ( $0.07 \pm 0.034$ ), LPS-treated neutrophils produced significantly fewer NETs ( $p = 0.0006$ ) (Fig. 4B). PMA-stimulated neutrophils produced clusters and strands of cfDNA decorated with citH3 and MPO (Fig. 3B, arrows).



**Fig. 5.** Peptidylarginine deiminase inhibition by Cl-amidine suppresses LPS-induced histone citrullination and NETosis.

Neutrophils from 5 dogs were treated with 50, 100, or 200  $\mu\text{M}$  Cl-amidine or DMSO (vehicle control) before stimulation with 100  $\mu\text{g}/\text{ml}$  LPS for 180 min. (A) Pre-treatment with 100 or 200  $\mu\text{M}$  Cl-amidine led to significantly fewer cells expressing intracellular citH3 compared to LPS. (B) Pre-treatment with all concentrations of Cl-amidine significantly decreased NET formation compared to LPS and vehicle control. (C) Extracellular citrullinated histone H3 (citH3) within NETs did not differ between with Cl-amidine treatment. (D–F) Representative immunofluorescent images of neutrophils treated with LPS (D), LPS with DMSO (E) and LPS with 200  $\mu\text{M}$  Cl-amidine (F). In the absence of Cl-amidine, LPS induced NETosis (arrows; D,E) and expression of intracellular citH3 (arrow head; E). Neutrophils pre-treated with Cl-amidine (F) maintained lobulated nuclei and only a small number of cells expressed intracellular citH3. Scale bar = 100  $\mu\text{m}$ . Original magnification 40x. (\*  $p < 0.05$ ).

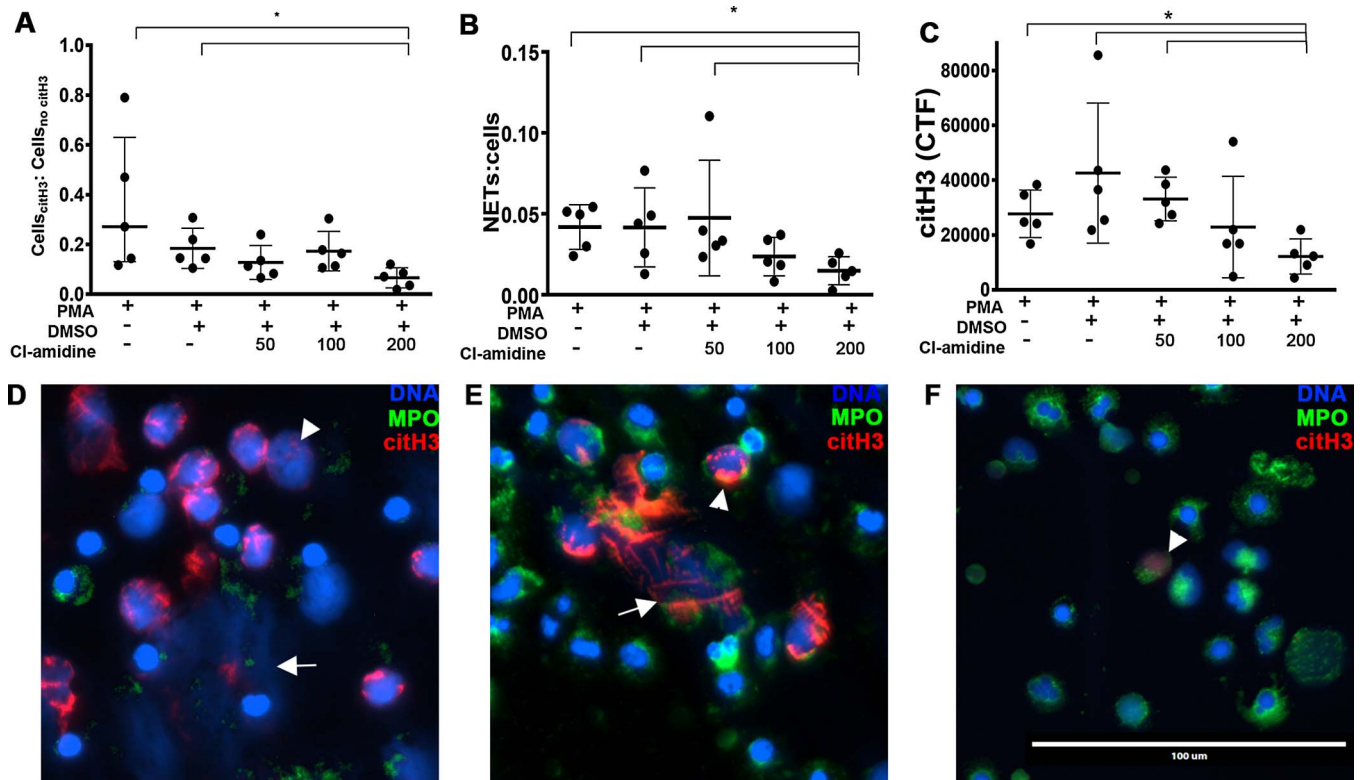
When comparing the NET structures produced by activated neutrophils, LPS stimulation resulted in significantly smaller NETs than PMA (343.2  $\mu\text{m}^2$  (188.3–484.8) vs. 1475  $\mu\text{m}^2$  (697.9–1527),  $p = 0.016$ ) (Fig. 4C). Compared to unstimulated neutrophils (0 CTF; 0–1087), LPS-treated neutrophils produced significantly higher levels of extracellular citH3 within NETs (9260 CTF; 5962–16595) ( $p = 0.016$ ) (Fig. 4D). Compared to PMA-treated neutrophils (21878 CTF; 13713–34628), LPS-treated neutrophils produced NETs that were decorated with significantly less extracellular citH3 ( $p = 0.016$ ) (Fig. 4D).

#### 4.4. Histone H3 citrullination and NETosis are dependent on PAD in canine neutrophils

To determine if PAD is required for hypercitrullination of histones and NETosis in canine neutrophils, LPS- or PMA-stimulated neutrophils were incubated with varying concentrations of Cl-amidine. Stimulated neutrophils were treated with equal volumes of DMSO as vehicle control. The number of LPS-activated neutrophils expressing intracellular citH3 differed with varying concentrations of Cl-amidine ( $p = 0.042$ ). Pre-treatment of LPS-activated neutrophils with 100 or 200  $\mu\text{M}$  Cl-amidine resulted in significantly fewer cells (Cells<sub>IntraCitH3</sub>: Cells<sub>noIntraCitH3</sub>) with intracellular citH3 compared with cells without Cl-amidine (0.019  $\pm$  0.013 vs. 0.053  $\pm$  0.032,  $p = 0.0044$ ; 0.023  $\pm$  0.019 vs. 0.053  $\pm$  0.032,  $p = 0.028$ , respectively) (Fig. 5A). Cl-amidine also decreased NET formation in LPS-treated neutrophils ( $p = 0.0074$ ). LPS-activated neutrophils pre-treated with 50, 100, or 200  $\mu\text{M}$  of Cl-amidine produced significantly less NETs (0.010  $\pm$  0.0057, 0.0052  $\pm$  0.0039, 0.0064  $\pm$  0.0038, respectively) than ones without Cl-amidine (0.022  $\pm$  0.013) ( $p = 0.0108$ ,

$p = 0.013$ ,  $p = 0.040$ , respectively). Similarly, LPS-activated neutrophils treated with DMSO (0.016  $\pm$  0.0052;  $p = 0.010$ ) produced significantly more NETs than neutrophils treated with 50, 100, or 200  $\mu\text{M}$  Cl-amidine ( $p = 0.021$ ,  $p = 0.025$ ,  $p = 0.032$ , respectively) (Fig. 5B). However, levels of citH3 within NETs did not differ significantly with Cl-amidine treatment ( $p = 0.23$ ) (Fig. 5C). Representative images of LPS-activated neutrophils (Fig. 5D), with DMSO (Fig. 5E) or with 200  $\mu\text{M}$  Cl-amidine (Fig. 5F) demonstrate the effects of PAD inhibition.

PAD inhibition by Cl-amidine also resulted in significant decreases in the number of PMA-stimulated cells expressing intracellular citH3 ( $p = 0.031$ ). 200  $\mu\text{M}$  Cl-amidine led to a significantly lower number of neutrophils expressing intracellular citH3 than neutrophils treated with PMA alone (0.067  $\pm$  0.040 vs. 0.36  $\pm$  0.28,  $p = 0.0079$ ) or with DMSO (0.067  $\pm$  0.040 vs. 0.18  $\pm$  0.080,  $p = 0.012$ ). (Fig. 6A) As expected, PMA-induced NETosis decreased as a result of Cl-amidine treatment ( $p = 0.0074$ ). Post hoc tests revealed that PMA-activated neutrophils pre-treated with 200  $\mu\text{M}$  Cl-amidine produced significantly fewer NETs (0.015  $\pm$  0.0087) compared to those with PMA alone (0.042  $\pm$  0.014) ( $p = 0.014$ ) or with DMSO (0.042  $\pm$  0.024) ( $p = 0.029$ ) (Fig. 6B). Treatment with 200  $\mu\text{M}$  Cl-amidine also resulted in significantly less NET production than that in neutrophils treated with 50  $\mu\text{M}$  Cl-amidine (0.048  $\pm$  0.024) ( $p = 0.030$ ) (Fig. 6B). Compared to neutrophils treated with PMA alone, NETs released by neutrophils pre-treated with 200  $\mu\text{M}$  Cl-amidine had significantly lower CTF<sub>citH3</sub> (27727  $\pm$  8698 vs. 12152  $\pm$  6445,  $p = 0.0038$ ). Treatment with 200  $\mu\text{M}$  Cl-amidine resulted in significant reduction in extracellular citH3 within NETs compared to PMA and DMSO (42594  $\pm$  25580,  $p = 0.035$ ) and 50  $\mu\text{M}$  Cl-amidine (33152  $\pm$  7952,  $p = 0.0036$ ) (Fig. 6C). Representative images of PMA-activated



**Fig. 6.** Peptidylarginine deiminase inhibition by Cl-amidine suppresses *in vitro* PMA-induced histone citrullination and NETosis in dogs.

Neutrophils from 5 dogs were treated with 50, 100, or 200  $\mu\text{M}$  Cl-amidine or DMSO (vehicle control) before stimulation with 100 nM PMA for 180 min. (A) Pre-treatment with 200  $\mu\text{M}$  Cl-amidine led to significantly fewer cells expressing intracellular citH3 compared to PMA and vehicle control. (B) 200  $\mu\text{M}$  Cl-amidine significantly decreased NET formation compared to PMA, DMSO and 50  $\mu\text{M}$  Cl-amidine. (C) Neutrophils treated with 200  $\mu\text{M}$  Cl-amidine released significantly less citH3 within NETs than neutrophils treated with PMA alone, DMSO or 50  $\mu\text{M}$  Cl-amidine. (C). (D-F) Representative immunofluorescent images of neutrophils treated with PMA (D), PMA with DMSO (E) and PMA with 200  $\mu\text{M}$  Cl-amidine (F). Note the extent of NETosis (arrows) and expression of intracellular citH3 (arrow heads) in the absence of Cl-amidine (D, E). Nuclei were round with decondensed chromatin. Neutrophils pre-treated with Cl-amidine before PMA stimulation (F) maintained lobulated nuclei and only a small number of cells expressed intracellular citH3. Scale bar = 100  $\mu\text{m}$ . Original magnification 40x. (\* $p < 0.05$ ).

neutrophils (Fig. 6D), with DMSO (Fig. 6E) or with 200  $\mu\text{M}$  Cl-amidine (Fig. 6F) demonstrate the effects of PAD inhibition.

## 5. Discussion

This is the first study to show that, in the presence of *E. coli* LPS, canine neutrophils undergo histone hypercitrullination and NETosis *in vitro*, which are dependent on PAD.

*E. coli*, one of the most common bacteria identified in septic dogs, is often associated with infections such as septic peritonitis, urinary tract infections and bacterial pneumonia (Dickinson et al., 2015; Proulx et al., 2014; Wong et al., 2015). Found on the outer membrane of *E. coli* and other Gram-negative bacteria, LPS is recognized by Toll-like receptor 4 and acts as pathogen-associated molecular pattern to initiate inflammation and innate immunity during bacterial infections. As the primary effector cells of innate immunity, neutrophils are among the first blood cells to encounter bacteria and LPS. In dogs, low-dose LPS induces neutrophilia, neutrophil integrin activation, and transendothelial migration to effected tissues and organs (Yu et al., 2012). Neutrophil accumulation within vital organs may lead to organ dysfunction, which is a major cause of morbidity and mortality in sepsis (Brown et al., 2006; Kenney et al., 2010). There is increasing evidence to suggest that excessive NETosis in sepsis can also lead to organ injury (Czaikoski et al., 2016; Hidalgo et al., 2002; Matthijsen et al., 2007). In our study, we found that LPS-mediated NETosis results in the release of MPO from canine neutrophils. MPO, which is stored in the primary granules of neutrophils, catalyzes the formation of hypochlorite from hydrogen peroxide (Stelmazynska and Zgliczynski, 1974). Hypochlorite has potent bactericidal properties but also induces oxidative

damage and cytotoxicity (Matthijsen et al., 2007; Hidalgo et al., 2002). In addition to the role of histones in chromatin organization, recent evidence confirms that histones can either translocate to the cell surface or extracellular space where they function as DAMPs. (Allam et al., 2013; Kawai et al., 2016). The NET-mediated release of extracellular histones and MPO in our study suggest that NETosis during Gram-negative infection and sepsis maybe a cause of organ injury in dogs. In mice, NET inhibition results in attenuation of organ dysfunction and reduced mortality following LPS challenge. The exact mechanisms of NET-associated organ injury and whether NETs could be a therapeutic target in septic dogs require further investigations.

We showed that when living neutrophils are exposed to LPS, their chromatin undergoes decondensation, a process not unique to NETosis. Using a specific biomarker of histone citrullination, we were able to clearly demonstrate LPS-induced NETosis (Leshner et al., 2012; Yoon et al., 2014). Similar to human neutrophils, our findings indicate that LPS is capable of inducing histone H3 citrullination in canine neutrophils (Neeli et al., 2008). Histone citrullination, facilitated by PAD4, results in a net loss of positive charge of histones and, in turn, obliterates electrostatic interactions between DNA and histones causing chromatin decondensation and release of cfDNA during NETosis (Wang et al., 2009). By using the pan-PAD inhibitor, Cl-amidine, we showed that PAD is essential for LPS-induced NETosis in dogs. Since PAD inhibition diminishes the expression of intracellular citH3, our data indicate that histone H3 citrullination by PAD is an important cellular event during NETosis. Among the 5 PAD enzymes expressed in people and mice, PAD4 is highly expressed in granulocytes (Lewis et al., 2015). Interestingly, LPS stimulation of human neutrophils results in a rise in intracellular calcium; however, the signalling pathway leading to PAD4



activation is unknown (Yee and Christou, 1993).

PMA, a potent protein kinase C activator, was used as a positive control in our experiments to induce histone hypercitrullination and NETosis by significant generation of reactive oxygen species. Compared to PMA-induced NETosis, considerably lower concentrations of Cl-amidine were required to inhibit LPS-mediated intracellular histone H3 citrullination and NETosis. This highlights that LPS-mediated PAD activation likely reflects a more subtle physiological process compared to PMA-chemically mediated stimulation. The degree of histone citrullination and NETosis may also vary with different LPS serotypes. Using 7 different serotypes of LPS, Pieterse et al. (2016) found that only LPS derived from *Pseudomonas aeruginosa* and *E.coli O128:B12* elicited NETosis in a manner similar to PMA in human neutrophils. The selectivity of NETosis in response to LPS serotypes in dogs requires further investigation.

The present study has several limitations. It remains controversial whether NETosis induced by high-dose LPS in an isolated system free of plasma proteins and other blood cells is truly reflective of NETosis *in vivo* (Pieterse et al., 2016; Liu et al., 2016). In our case, canine neutrophils may be relatively more resistant to LPS; thus a considerably higher dose of LPS is required to elicit NETosis in a purified serum- and platelet-free system. The lack of fold increase in cfDNA release over time suggests that LPS-induced canine NETosis may require additional stimulation by cytokines such as interleukin-8, and molecular patterns like N-Formylmethionine-leucyl-phenylalanine (Itakura and McCarty, 2013). *In vitro* studies have shown that activation of platelets by LPS augments NETosis in human neutrophils (Pieterse et al., 2016). Platelets have also been shown to upregulate NETosis in *in vivo* murine septic models (Clark et al., 2007). Our laboratory is currently investigating the effects of LPS-mediated platelet activation on NETosis in dogs. Given studies in other model systems, identification of NETs in tissues from septic animals may provide more insight in this matter. Finally, since Cl-amidine inhibits all PAD family members irreversibly, the precise role of PAD4, in canine neutrophils remains to be understood.

## 6. Conclusion

Our study shows that canine neutrophils respond to *E. coli* LPS by PAD-dependent histone H3 hypercitrullination and NET production. Further studies of canine NETosis both *in vitro* and *in vivo* will increase our understanding of the function of NETs in sepsis.

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