Veterinary Immunology and Immunopathology 193–194 (2017) 29–37

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

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

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ARTICLE INFO

Keywords:
Sepsis
Citrullinated histone
Peptidylarginine deiminase
Cell-free DNA

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 (1×10^6 cells/ml) from healthy dogs were isolated and treated with 100 μg/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 μM 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; Narasaraju 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 moleculat patterns; MPO, myeloperoxidase; PAD, peptidylarginine deiminase; PMA, phorbal 12-myristate 13-acetate; RT, room temperature

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http://dx.doi.org/10.1016/j.vetimm.2017.10.002
Received 28 April 2017; Received in revised form 11 October 2017; Accepted 20 October 2017

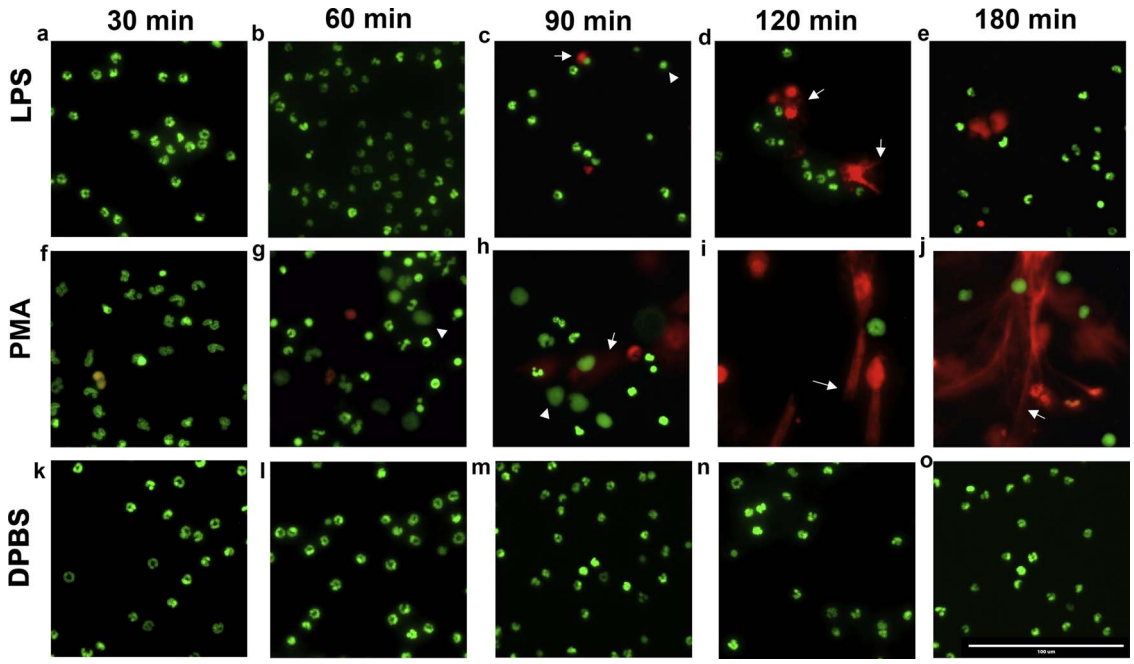

Fig. 1. Representative immunofluorescent images of live canine neutrophils.
Neutrophils were incubated for 30, 60, 90, 120 or 180 min with 100 μg/ml LPS (a-e), 100 nM phorbol-myristate acetate (PMA) (positive control) (f-j), or DPBS (k-o). Nucleic acid in live cells was stained using a cell-permeant 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 μm. Original 40 x magnification.

considered by some researchers as a biomarker of NETosis, is associated with a poor prognosis in human septic patients, suggesting that over-zealous 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; Narasaraju 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 x 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 x g (10 min, 4 °C, no brake). Neutrophils were resuspended in DPBS (pH 7.3, 1.9 mM $CaCl_2$, 5 mM dextrose, 0.9 mM $MgCl_2$, 1% BSA) and diluted to a final concentration of 1×10^6 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 (1×10^6/ml) were incubated with DPBS alone, 100 nM PMA (positive control), or 100 μg/ml LPS (*E. coli* O55:B5, InvivoGen, San Diego, CA) for 30, 60, 90, 120, or 180 min at 37 °C in poly-L-lysine coated culture wells. SYTOX Orange dye (Thermo Fisher Scientific, Waltham, MA) (1 μM, 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 μM 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).