Protoporphyrin IX derived from dual-species anaerobic biofilms of Fusobacterium necrophorum and Porphyromonas levii attenuates bovine neutrophil function

Joey S. Lockhart a,*, Thibault Allain a, Simon A. Hirota b,c, Andre G. Buret a, Douglas W. Morck a,d,e

a Department of Biological Sciences, University of Calgary, Canada
b Department of Physiology & Pharmacology, University of Calgary, Canada
c Department of Microbiology, Immunology & Infectious Disease, University of Calgary, Canada
d Animal Health Unit, University of Calgary, Canada
e Department of Comparative Biology & Experimental Medicine, University of Calgary, Canada

ARTICLE INFO

Keywords:
Anaerobe
Biofilm
Neutrophil
Bovine
Protoporphyrin ix
ROS

ABSTRACT

Host immune cells and clinical interventions often fail to eradicate biofilm-mediated infections, resulting in chronic inflammation. The role of the biofilm three-dimensional structure in this tolerant phenotype has been studied extensively; however, the impact of small molecules released from biofilm-bacteria in modulating host immune function is less well understood. A model of mixed-species biofilms composed of Fusobacterium necrophorum and Porphyromonas levii was developed to evaluate bovine neutrophil responses to bioactive molecules released from either biofilm or planktonic bacteria. We hypothesized that different soluble extracellular factors (ECFs) would be released from planktonic and biofilm bacteria, resulting in altered neutrophil function. Neutrophils exposed to ECFs from planktonic bacteria showed significantly elevated levels of reactive oxygen species (ROS). In contrast, biofilm components from these same species of bacteria failed to induce such a response. Size-exclusion filtration of ECFs revealed that the bioactive molecule causing neutrophil ROS responses was below 3 kDa. Intensive heat, nuclease, lipase, or protease treatments of the <3 kDa fractions did not alter neutrophil functional responses. Protoporphyrin IX (PPIX) is an important heme precursor and growth requirement for many anaerobes. Porphyromonas species can accumulate environmental PPIX at the cell surface as a strategy to protect the bacteria from oxidative stress and we investigated the direct interaction of bovine neutrophils with PPIX. In the present study, evidence suggests that the accumulation of protoporphyrin in these dual-species biofilm ECFs attenuates neutrophil ROS production and chemotaxis. The diminished neutrophil response to biofilm ECFs via the action of PPIX may represent a biofilm immune-evasion strategy that could assist in explaining the ineffectual host clearance of biofilm-mediated infections involving these bacteria.

1. Introduction

Biofilms are complex communities of surface-adhered microorganisms encased in a matrix composed mainly of extracellular polysaccharides, proteins, water, lipids, DNA, and other small molecules [1–3]. Within the confines of a biofilm, bacteria produce various communication molecules and metabolites that are different from molecules released from bacteria growing as a planktonic culture [4]. This study explores how these unique biofilm-derived products may impact host innate immune function.

When immune cells encounter bacterial biofilms in vivo, whether they are attached to an artificial substrate or are formed on a biotic material, the bacteria may persist resulting in chronic infections [5]. Immune cell responses are often thwarted by biofilms, which leads to local tissue damage in a perpetual cycle of inflammation. In this fashion, biofilms serve as a protected nidus of infection and may enable periodic bacteremia or septicemia. Neutrophils are critical first responder cells recruited to sites of infection [6]. Human neutrophils carry out phagocytosis, degranulate, and release eDNA as Neutrophil Extracellular Traps (NETs) in response to biofilms, as has been demonstrated in an infection...
model with *Staphylococcus aureus* [7] as well as other experimental systems. While neutrophils can adhere to biofilms, penetrate these structures, and release pro-inflammatory cytokines in response to biofilms, biofilm persistence remains the cause of many recurrent bacterial infections [8–10]. There is clear evidence for a complex interplay between the host immune system and anaerobic bacterial biofilms, particularly in the context of periodontal pathogens (reviewed by Ref. [11]). The diffusion barrier of the extracellular matrix, along with decreased metabolic activity of microbes and increased incidence of horizontal gene transfer within the biofilm are features that may confer resistance to common antibiotics used in veterinary and human medicine [12–16]. Collectively, the ability of biofilm-bacteria to avoid host immune cells, and the increased natural resistance to antimicrobials, present a significant challenge for successful clinical intervention of biofilm-mediated infections [17]. The impact of extracellular factors (ECFs) released from bacterial biofilms on neutrophil function remains largely unknown, particularly in the context of bovine neutrophils and their interactions with anaerobic opportunistic veterinary pathogens.

*Fusobacterium necrophorum* and *Porphyromonas levi* are two species of opportunistic anaerobic pathogens of cattle that are known to cause necrotic infections that often involve biofilm formation [18–20]. When *Fusobacterium* and *Porphyromonas* grow as a dual-species culture, certain virulence mechanisms are upregulated to benefit both species. Examples of this include an increased ability to invade epithelial cells and an augmentation in biofilm-biomass production when in co-culture compared to single species cultures [21–25]. This synergy is observed in other species of anaerobes, such as *F. nucleatum* and *Parvimonas micra* [26], and *F. nucleatum* and *Prevotella intermedia* [27]. Because dual-species bacteria are frequently located together in a wide range of necrotic infections, and since *Porphyromonas* spp. can positively impact *Fusobacterium* biofilm formation [22], we employed a dual-species model of biofilm-growth for the experiments in the current study. The role of ECFs released from biofilm-anaerobes and their impact on innate immunity is poorly understood. Many anaerobes (including *Porphyromonas*) are auxotrophic for protoporphyrin IX (PPIX), a small (562 g/mol), thermostable metabolite that is the final intermediate in heme synthesis [28,29]. *Porphyromonas* spp. can break down heme, sequester PPIX and accumulate the molecule at the cell surface to maintain a local concentration of PPIX in biofilm ECFs inhibiting bovine neutrophil ROS and chemotaxis.

### 2. Materials and methods

#### 2.1. Bacteria, planktonic cultures, and biofilm generation

*Fusobacterium necrophorum* ssp necrophorum (ATCC 27852) and *Porphyromonas levi* (ATCC 29147) were streaked at –80° C in Brain Heart Infusion (BHI) medium, supplemented with hemin (5 µg/mL), vitamin K₁ (0.2 µg/mL) and 10% glycerol. Cultures were grown and maintained within a Bactron™ Anaerobic chamber under strict anaerobic conditions (5% hydrogen, 5% carbon dioxide and 90% nitrogen) at 37 °C. Primary cultures were grown on Fastidious Anaerobic Agar (FAA) plates supplemented with hemin (5 µg/mL) and vitamin K₁ (0.2 µg/mL) and 5% (v/v) sterile defibrinated sheep blood (Dalynn Biologicals HS30-400). Secondary streak plates were prepared on FAA and used to generate 30 mL of mixed-species planktonic growth with slight modifications to the direct colony suspension method [32]. Briefly, 3–5 colonies of *F. necrophorum* or *P. levi* were swabbed off secondary plates and added to 2 mL of BHI medium to match a 1.0 MacFarland standard. Five hundred microlitres of each standardized suspension was diluted in 14.5 mL BHI to generate two equal 1.0 × 10⁷ CFU/mL starting cultures. Mixed-species planktonic culture of *F. necrophorum* and *P. levi* (1:1 ratio) was grown for 17 h. Mixed-species biofilms were initiated in the same fashion; once starting cultures were mixed in a 1:1 ratio, 1.5 mL was added to the wells of a 12-well cell culture plate. A Transwell™ insert (0.4 µm pore size) was added on top of the culture and 500 µL sterile BHI placed on top of the Transwell™. Biofilms were incubated on a shaker at 150 rpm for 5 days at 37 °C and 50% of the medium was replenished every 48 h to ensure consistent nutrients for biofilm development. Membranes were assessed for biofilm formation using a modified crystal violet (CV) biomass assay [34]. Membranes were washed twice in sterile 0.9% NaCl and stained for 10 min in 0.1% CV. Excess CV was washed from membranes with 0.9% NaCl and allowed to dry overnight. CV was solubilized in 30% acetic acid and absorbance at 550 nm measured in a plate spectrophotometer to determine relative biofilm biomass present. Culture purity was ensured with gram staining and viability was assessed with differential viable CFU counts. The dual-species biofilms were visualized with Scanning Electron Microscopy (SEM) to confirm the presence of the two species of interest on the membranes. Transwell membranes with dual species biofilm were washed in 2 mL of 0.9% NaCl to remove loosely-adherent planktonic bacteria and then fixed for 3 h in 2 mL 0.1 M cacodylate buffer pH 7.2 (Sigma CO125) containing 2.5% glutaraldehyde (Electron Microscopy Sciences 16365). The membranes were air-dried for 5 days at room temperature in sealed sterile 12-well culture dishes and then mounted on stubs and sputter-coated with gold-palladium. The biofilms were observed with a Phillips XL30ESEM microscope at an accelerating voltage of 20 kV, and images were taken at magnifications of 100×, 1,000x and 10,000x. Dispersed planktonic cultures were generated from 5-day old biofilms. Membranes containing biofilms were washed in 0.9% NaCl twice to remove any planktonic organisms. Biofilm bacteria were dispersed from membranes via sonication for 5 min into BHI medium. This medium was used as a starting culture for a new 17 h planktonic growth composed of reverted-biofilm bacteria.

#### 2.2. Ultrafiltration and preparation of bacterial extracellular fractions (ECFs) from planktonic and biofilm bacteria

Medium from 5-day biofilm growth was pooled and volume matched with a 17h planktonic culture. Cultures were serially diluted and plated for viable CFU counts and to verify that both planktonic and biofilm growth consisted of a relative 1:1 ratio of *F. necrophorum* and *P. levi* (Supplementary Fig. 1). Samples were centrifuged at 1200 g for 30 min at 4 °C and the supernatants were passed through 0.2 µm syringe filters to remove any large cellular debris and bacteria. Aerobic and anaerobic post-filter controls were plated to ensure there were no contaminants. Each filtrate was then diluted 1/3 with Hank’s Balanced Salt Solution
(HBSS) and 10 mL were placed on an Amicon™<3 kDa (Millipore) ultrafiltration apparatus. The tubes were centrifuged at 1200 g for 45 min at 4 °C, and the filtrates containing solely molecules <3 kDa (planktonic and biofilm ECFs) were aliquoted and stored at −80 °C. Dispersed planktonic ECFs were generated following the same protocol.

2.3. Protease, lipase, and nuclease treatments of bacterial ECFs

Frozen bacterial ECFs were thawed on ice immediately prior to treatment with one of the following: trypsin, proteinase K, Flavourzyme™, DNase or lipase. ECFs were incubated with 50 μg/mL Trypsin (Sigma T1426) for 1 h at 37 °C. Proteinase K (Invitrogen AM2546) treatment was performed at 37 °C for 1 h with a final concentration of 200 μg/mL per sample. ECFs were incubated with 0.1% (v/v) Flavourzyme™ (Sigma P6110) for 1 h at 37 °C to hydrolyze peptides within the samples. Crude lipase (Sigma L5126) (50 mg/mL) was added to each ECF sample and incubated at 45 min at 45 °C to digest small lipids in the preparations. ECFs were treated with 500 μg/mL DNase (Roche 11284932001) for 45 min at 45 °C. Samples containing proteases, lipase or DNase were all inactivated following treatment by boiling for 15 min prior to neutrophil exposure and assessment of neutrophil ROS.

2.4. Animals, blood collection and neutrophil isolation

All animal procedures were conducted under the Guidelines of the Canadian Council on Animal Care. Procedures were approved by the University of Calgary Veterinary Sciences Animal Care Committee under protocols AC15-0104 and AC19-0058. Whole blood was aseptically collected from healthy mixed-breed heifers via jugular venipuncture into Vacutainers™ (BD 364606) with 1.5 mL acid citrate dextrose. Approximately 100 mL was collected per experiment. Blood was pooled into Vacutainers™ with 1.5 mL acid citrate dextrose. Solutions were maintained on ice and all centrifugation steps performed at 4 °C to prevent inadvertent activation of neutrophils. Neutrophil viability was assessed with trypan blue exclusion and purity was assessed with a cytopsin preparation followed by DiffQuik™ staining. Neutrophil suspensions used in all experiments were at least 95% viable and 90% pure.

2.5. Assessment of neutrophil activity

A previously established method of measuring ROS using the intracellular fluorescent ROS indicator 2′7′-dichlorodihydrofluorescein diacetate (DCF-DA) was employed to detect neutrophil-derived ROS following exposure to the bacterial ECFs (Lockhart et al., 2017). Neutrophils (1.2×10⁶ cells/mL) were isolated as described above and resuspended in HBSS with 10 μM DCF-DA (Sigma D6868). Cells were incubated at 37 °C + 5% CO₂ for 25 min to allow DCF-DA to enter neutrophils. Bacterial ECF stimulant or appropriate control (150 μL/well) were added to duplicate wells of a blank 96-well plate, followed by 50 μL of cells or 50 μL HBSS/DCF-DA for cell-free controls. Fluorescence was measured in a plate fluorometer at an excitation wavelength of 485 nm and emission wavelength of 530 nm every 20 min for 120 min. All ROS results are reported at the 120 min time-point. Background fluorescence from the neutrophil-free controls were subtracted from the experimental wells to determine overall neutrophil ROS. Phorbol 12-myristate 13-acetate (PMA) (Sigma P8139) served as the positive control in all neutrophil experiments and neutrophils exposed to BHI medium or HBSS were the vehicle (unstimulated) controls. A commercially available bovine multiplex assay was employed to assess the release of cytokines from stimulated neutrophils. Five hundred microlitres of freshly isolated peripheral neutrophils (1.2×10⁶ cells/mL) were exposed to 500 μL of bacterial ECFs, PMA (5000 nm), PMA (100 nm) + LPS (50 μg/mL) (Sigma L3024), or BHI medium for 2 h at 37 °C + 5% CO₂. Following incubation, cells were pelleted by centrifugation at 1200g and the supernatants were carefully removed to fresh microtubes and stored at −80 °C until analysis. Frozen samples were submitted for a bead-based multiplex cytokine array (Eve Technologies, Calgary Alberta, Canada).

2.6. Neutrophil chemotaxis assay

Chemotaxis was measured using slight modifications to a previously described Transwell™ transmigration assay (16,36). Neutrophil suspensions (100 μL/well) in HBSS were gently pipetted on a Transwell™ (8 μm pore size) insert and incubated at 37 °C + 5% CO₂ for 10 min. Appropriate stimulant (600 μL/well) was added underneath the Transwell™ membranes and the apparatus incubated for 2 h at 37 °C + 5% CO₂ to allow for migration. Membranes were carefully removed with sterile forceps and the quantity of transmigrated neutrophils in each well was assessed via trypan blue staining and viable cell counts.

2.7. Quantification of protoporphyrin IX

PPIX in the planktonic and biofilm ECFs was detected using plate fluorometry and the auto-fluorescent properties of PPIX (37–39). Briefly, PPIX (Sigma P8293) was solubilized (10 mg/mL) in DMSO and a standard curve of known PPIX concentrations from 0 μM to 300 μM in HBSS was generated and assessed for fluorescence at an excitation wavelength of 410 nm and emission wavelength of 612 nm in a plate fluorometer. The standard curve was used to interpolate the concentrations of PPIX in the <3 kDa bacterial ECFs and <3 kDa BHI medium.

2.8. Statistical analyses

All reported values represent means ± SEM. Unless otherwise stated, all data were assessed for normal distribution with a Shapiro-Wilk normality test. Comparisons between groups with normal distribution were performed using a parametric one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. In cases where the data did not exhibit a normal distribution, the non-parametric Kruskal Wallis (KW) analysis was employed. An unpaired, two-tailed Student’s t-test was employed when comparing two independent variables. In all experiments, statistical significance was established at p < 0.05. All experiments consisted of multiple replicates from different animals and a minimum of three independent experiments. Statistical analyses were performed using GraphPad Prism 9 software (La Jolla, CA, USA).

3. Results

3.1. Bovine neutrophils produce significantly less ROS when exposed to ECFs from biofilm bacteria compared to planktonic bacterial ECFs

Anaerobic bacterial biofilms were generated on polycarbonate Transwell™ membranes using previously established methods (Fig. 1A) (40). A crystal violet biomass assay confirmed the presence of robust mixed-species biofilms on the membranes and within the wells of the culture plate (Fig. 1B). SEM images confirmed the presence of both P. levii and F. necrophorum attached to the polycarbonate membrane (Fig. 1C). ECFs from five-day biofilms and planktonic overnight cultures were collected and processed to investigate the impact of extracellular products on neutrophil function. Neutrophils that were directly exposed to biofilm ECFs produced significantly less ROS than neutrophils treated with ECFs from planktonic cultures of the same bacteria (p < 0.0001) (Fig. 2). The stimulatory or inhibitory potential of a small molecule...
Effector released into the ECFs was further characterized via size-exclusion ultra-filtration and subsequent neutrophil exposure to these refined products. There was a similar trend among neutrophils exposed to ECFs <3 kDa, where neutrophils did not respond with the same intensity to refined biofilm ECFs compared to ECFs from planktonic cultures (Fig. 2). To reinforce these findings, additional parameters of neutrophil activation such as chemotaxis and cytokine release in response to the bacterial ECFs were also assessed (Supplementary Fig. 2). Biofilm dispersed F. necrophorum and P. levii were isolated and re-grown as overnight planktonic cultures. The attenuated neutrophil ROS response to biofilm ECFs was reversed when biofilm-dispersed planktonic organisms were evaluated under the same experimental conditions (Fig. 3).

3.2. Extreme heat, trypsin, proteinase K or Flavourzyme™ treatments do not attenuate the ECFs <3 kDa-induced neutrophil ROS response

High-heat treatment by boiling ECFs for 15 min at 100 °C did not alter observed differential neutrophil ROS response between groups, suggesting that the bioactive molecule in the <3 kDa ECFs is thermostable (Fig. 4A). Autoclaving the bacterial ECFs also had no effect on subsequent neutrophil responses and there were no significant differences in pH levels of the <3 kDa ECFs (data not shown). Trypsin (Fig. 4B) and proteinase K (Fig. 4C) treatments did not significantly alter neutrophil ROS responses to the <3 kDa ECFs. Similarly, no differences in neutrophil ROS responses were observed when planktonic and biofilm supernatants were pre-treated with Flavourzyme™, a specialized peptidase mixture known to cleave peptides to the individual amino-acid level (Fig. 4D) [41,42]. Together these data suggested that the molecule responsible for the decreased neutrophil response to biofilm ECFs was not a peptide.

3.3. Crude lipase or DNase treatments of planktonic and biofilm ECFs does not attenuate the observed differential neutrophil ROS responses

The potential role of bioactive lipids on neutrophil activation was assessed by treating the bacterial fractions with lipase. Crude lipase treatment did not alter the relative neutrophil responses in planktonic and biofilm ECFs (Fig. 5A). Similarly, treatment with DNase had no effect on ROS production (Fig. 5B).

3.4. PPIX released from Fusobacterium and Porphyromonas dual-species biofilms attenuates neutrophil ROS and chemotaxis

Bacteria that are incapable of heme synthesis possess mechanisms to acquire iron from environmental sources, and one heat-stable molecule of interest that P. levii can stockpile from host sources is PPIX. The accumulation of PPIX, an essential heme precursor, was quantified in the bacterial ECFs by measuring the fluorescent properties of PPIX. Biofilm <3 kDa ECFs contained significantly more PPIX (approximately 8 μM) than planktonic <3 kDa ECFs or BHI medium (p < 0.05) (Fig. 6A). Co-culture of cells stimulated with PMA and containing increasing concentrations of PPIX (1.5, 15, 25, 50 μM) significantly impaired neutrophil oxidative responses as the concentration of PPIX increased (p < 0.0001) (Fig. 6B). The attenuation of neutrophil ROS by PPIX revealed maximal inhibition at 50 μM PPIX, which was the highest concentration of exogenous PPIX tested. Additionally, exogenous PPIX at 25 and 50 μM inhibited neutrophil chemotaxis towards the positive stimulus (p <
Clinical reports from a variety of chronic infections in which immunity appears to be suppressed indicate that host-defense mechanisms are deficient when triggered by biofilms [7,8]. An example of this deficiency is the inability of neutrophils to properly migrate towards positive stimuli in the presence of *P. aeruginosa* biofilm extracellular polysaccharides [16]. We focused on the interaction of neutrophils with soluble ECFs released from planktonic bacteria and biofilms composed of the same species. The Costerton group demonstrated that neutrophils could mount a limited oxidative response towards bacterial biofilms of *P. aeruginosa* grown in vitro [43,44]. The authors provided convincing evidence that neutrophils respond to biofilm bacteria with a significantly reduced oxidative burst than neutrophils exposed to a similar number of planktonic organisms. These findings were corroborated in our neutrophil activity assay, where ROS production was significantly elevated in neutrophils exposed to planktonic ECFs compared to biofilm ECFs (Fig. 2). This stimulation profile was also observed when neutrophils were exposed to ECFs that had been passed through 3 kDa cutoff filters, suggesting the differential release of bioactive small molecules in planktonic cultures compared with biofilms (Fig. 2). In all neutrophil experiments, PMA served as the positive control, as it is known to trigger ROS production, cytokine release, and chemotaxis [45-47]. While cytotoxic effects may play a role in the significant ROS generation in response to planktonic ECFs, this does not account for the observed differences in ROS for the <3 kDa filtered samples (Supplementary Fig. 3). Neutrophil activation and cell death was expected due to the presence of large stimulatory molecules in the whole ECFs such as lipopolysaccharide (LPS) and *F. necrophorum* leukotoxin (~330 kDa) [48-50]. LPS causes neutrophil activation via TLR4-signaling pathways and *F. necrophorum* leukotoxin stimulates granululization, ROS production and apoptosis of bovine neutrophils at low concentrations [51]. At high concentrations, leukotoxin causes necrosis, which would release substantial amounts of pro-inflammatory ROS [51]. Lockhart and colleagues demonstrated that LPS does play a significant role in the activation of neutrophils, however removal of LPS from ECF preparations did not alter ROS production from neutrophils in those studies [40]. Treatment of the <3 kDa ECFs with polymyxin B had no effect on neutrophil ROS production, demonstrating that fragments of LPS (i.e., Lipid A, ~1.8 kDa) are not the cause of the differential neutrophil response to biofilm ECFs compared to planktonic ECFs. Furthermore, there was no significant difference in cytotoxic effects on neutrophils following exposure to planktonic <3 kDa compared with biofilm <3 kDa ECFs (Supplementary Fig. 3). Together, these data suggest that additional compounds of interest participate in the stimulation or inhibition of neutrophil activity independent of cytotoxic effects in our experimental system.

The biofilm phenotype is known to confer significant alterations to gene expression compared with planktonic organisms [52-54]. This switch is mediated by various second messengers, but bis-(3′–5′)-cyclic dimeric GMP (c-di-GMP) has been shown to play a crucial role in *Pseudomonas aeruginosa* [55], reviewed by Refs. [56,57]. To investigate whether the phenotypic changes were reversible in our experimental system, biofilm-bacteria were harvested and immediately re-grown as planktonic cultures. Upon neutrophil stimulation with ECFs from these reverted cultures, high levels of ROS production were again observed (Fig. 3). Consistent with our hypothesis, the biofilm phenotype failed to elevate ROS. More research is warranted to determine whether reduced c-di-GMP levels in the planktonic form of growth of these anaerobes may contribute to this phenomenon. Indeed, recent findings suggest that c-di-GMP promotes biofilm formation in other anaerobic bacteria such as *Clostridium difficile* [58].

Short peptide sequences that activate human and murine neutrophils have been described, however less is understood regarding bovine neutrophils [59,60]. A protein BLAST search revealed that bovine neutrophils do not possess short peptide receptors that are analogous to the...
human formylated peptide receptors (FPR1/2) (UniProt Accession no. P21462). This is corroborated by experimental evidence demonstrating a lack of neutrophil response to formylated peptides in many ruminant species [46, 61]. Treatment of the bacterial ECFs with heat, trypsin, proteinase K, or Flavourzyme did not attenuate the differential neutrophil ROS responses (Fig. 4A–D). This provides evidence for a bioactive, heat-stable, non-peptide molecule that is differentially expressed between the two modes of bacterial growth.

Lipids are known to influence neutrophil activity by, at least in part, stimulating the release of neutrophil extracellular traps [62]. To investigate the potential role of bacterial-derived bioactive lipids in our experimental system, ECFs were treated with lipase prior to neutrophil exposure (Fig. 5A). Crude lipase treatment did not alter subsequent neutrophil ROS responses, indicating that differential expression of lipids in the two growth modes was not relevant in this setting. Human neutrophils can recognize and respond to bacterial DNA via the TLR9 receptor and subsequent signaling cascades [63]. However, bovine neutrophils lack mRNA expression for TLR9 and are unable to respond to foreign DNA in a similar fashion [64]. In keeping with these observations, DNase treatment did not alter bovine neutrophil responses to planktonic and biofilm ECFs (Fig. 5B). These results suggest that bovine neutrophils are responding to something other than extracellular DNA within these bacterial ECFs.

PPIX is a heme precursor that

---

**Fig. 4.** Neutrophil oxidative responses to bacterial ECFs following high-heat or various protease treatments, assessed via DCF fluorescence at 485 nm and 530 nm. PMA (7500 nM) served as the positive control; BHI and HBSS indicate levels of ROS in unstimulated neutrophils. (A) Neutrophil ROS following exposure to heat treated ECFs for 15 min at 100°C; n = 5, 11. (B) Neutrophil ROS following exposure to trypsin treated ECFs; n = 4. (C) Neutrophil ROS following exposure to proteinase K treated ECFs; n = 3. (D) Neutrophil ROS following exposure to 0.1% Flavourzyme™ treated ECFs; n = 4. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

**Fig. 5.** Neutrophil oxidative responses to bacterial ECFs following lipase or DNase treatments, assessed via DCF fluorescence at 485 nm and 530 nm. PMA (7500 nM) served as the positive control; BHI and HBSS indicate levels of ROS in unstimulated neutrophils. (A) Neutrophil ROS following exposure to lipase treated bacterial ECFs; n = 3, 4. (B) Neutrophil ROS following exposure to DNase treated bacterial ECFs; n = 5. ***p < 0.001.
More research is necessary to determine the structure and properties of <3 kDa ECFs responsible for increased ROS secretion remains unknown. Increased PPIX accumulation, the stimulatory compound in planktonic neutrophil function by biofilm ultimately leading to altered innate immune responses. Neutrophils that is differentially expressed in the two modes of bacterial growth, activation upon exposure. In this study, we describe a small (<3 kDa), heat-stable, non-proteinaceous, non-lipidic and non-nucleic acid compound that is differentially expressed in the two modes of bacterial growth, ultimately leading to altered innate immune responses. Neutrophils produce high levels of ROS upon exposure to bioactive molecules present in the planktonic ECFs. The findings reported here describe a newly discovered mechanism whereby PPIX is elevated in biofilm bacteria versus planktonic organisms, and that PPIX accumulation in biofilms inhibits neutrophil function (ROS and chemotaxis). These observations offer new insights into strategies that biofilm bacteria employ to evade host immune responses.

**Funding**

All funding for this project was provided by University of Calgary Project Number 10002461 granted to DW Morck.

**CRediT authorship contribution statement**

**Joey S. Lockhart:** Conceptualization, Methodology, Investigation, Writing – original draft. **Thibault Allain:** Conceptualization, Writing – original draft. **Simon A. Hirota:** Conceptualization, Writing – original draft. **Andre G. Buret:** Conceptualization, Writing – original draft. **Douglas W. Morck:** Conceptualization, Supervision, Funding acquisition, Writing – original draft.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Data availability**

Data will be made available on request.

**Acknowledgments**

The authors would like to acknowledge Peter Spackman for help with blood collection from research animals and Wayne Jansen for procuring reagents and supplies for the study. Select figures from the PhD dissertation by Joey Scott Lockhart are included in this manuscript.
Bae GH, Lee HY, Jung YS, Shim JW, Kim SD, Baek SH, et al. Identification of novel Styrt B. Species variation in neutrophil biochemistry and function. J Leukoc Biol 2008;19:135-8. https://doi.org/10.1002/jlb.18.6.135.

Narayanan SK, Nagaraja TG, Chengappa MM, Stewart GC. Cloning, sequencing, and expression of the leukotxin gene from Fusobacterium nucleatum. Infect Immun 2001;69:5447. https://doi.org/10.1128/IAI.69.9.5447-5455.2001.

Degroote RL, Weigand M, Hauck SM, Deeg CA. IL8 and PMA trigger the regulation of biofilm exopolysaccharide production by Neisseria meningitidis. Front Microbiol 2020;11:1835. https://doi.org/10.3389/fmicb.2020.01835.

Trevani AS, Chorny A, Salamone G, Vermeulen M, Gamberale R, Schettini J, et al. Bacterial DNA activates human neutrophils by a CpG-independent pathway. Eur J Immunol 2003;33:3164-74. https://doi.org/10.1002/eji.200324334.

Dawson LF, Peltier J, Hall CL, Harrison MA, Derakhshan M, Shaw HA, et al. Neutrophil activation by heme: implications for inflammatory processes. Blood 2002;99:4160-5. https://doi.org/10.1182/blood.V99.11.4160.

Chua SL, Liu Y, Yam JKH, Chen Y, Vejborg RM, Tan BGC, et al. Dispersed cells from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 2006;61:1196-210. https://doi.org/10.1111/j.1365-2958.2006.05310.x.

Porphyromonas gingivalis Herrera D, et al. Comparative gene expression analysis of planktonic and biofilm cells. BMC Microbiol 2019;19:1-11. https://doi.org/10.1186/s12866-019-1423-9.

Degroote RL, Weigand M, Hauck SM, Deeg CA. IL8 and PMA trigger the regulation of biofilm exopolysaccharide production by Neisseria meningitidis. Front Microbiol 2020;11:1835. https://doi.org/10.3389/fmicb.2020.01835.

Vallelian F, Schaer CA, Deuel JW, Ingoglia G, Humar R, Buehler PW, et al. Regulation of biofilm exopolysaccharide production by Neisseria meningitidis. Front Microbiol 2011;2:12. https://doi.org/10.3389/fmicb.2011.00012.

Chua SL, Liu Y, Yam JKH, Chen Y, Vejborg RM, Tan BGC, et al. Dispersed cells from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 2006;61:1196-210. https://doi.org/10.1111/j.1365-2958.2006.05310.x.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Chua SL, Liu Y, Yam JKH, Chen Y, Vejborg RM, Tan BGC, et al. Dispersed cells from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 2006;61:1196-210. https://doi.org/10.1111/j.1365-2958.2006.05310.x.

Luo YP, Jiang L, Kang K, Fei DS, Meng XL, Nan CC, et al. Hemin inhibits NLRP3 inflammasome activation in sepsis-induced acute lung injury, involving heme oxygenase-1. Int Immunopharm 2014;12:3818-27. https://doi.org/10.1016/J.BIIOX.2013.10.029.

Miglior JJ, Torres JS, Ferreira WA, Fabris FCZ, Leonardo FC, Costa FF, et al. Heme induces significant neutrophil adhesion in vitro via an afsb-dependent pathway. Blood 2018;132. https://doi.org/10.1182/BLOOD-2018-11-897483.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Vallelian F, Schaer CA, Deuel JW, Ingoglia G, Humar R, Buehler PW, et al. Revisiting the putative role of heme as a trigger of inflammation. Pharmacology Research & Perspectives 2018;6:000392. https://doi.org/10.1002/prp2.392.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.

Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Res Commun 2011;417:14-23. https://doi.org/10.1016/J.BBRC.2011.07.099.