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The role of extracellular DNA in neutrophil extracellular traps

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The role of extracellular DNA in neutrophil extracellular traps

by

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Abstract

Pseudomonas aeruginosa is an opportunistic pathogen and the primary pathogen in lung infections in patients with Cystic Fibrosis (CF). Neutrophils are recruited to the site of infection to clear the invading bacteria by phagocytosis and neutrophil extracellular trap (NET) formation. NETs are composed of an extracellular lattice of genomic DNA enmeshed with various antimicrobial components normally found within the neutrophil. NETs trap and kill bacteria; however, the killing attributes of NETs are poorly understood. Here we show that the DNA is an important antimicrobial component of NETs. *P. aeruginosa* senses and responds to DNA in NETs by inducing genes required to survive NET killing. *P. aeruginosa* uses various surface modifications for NET resistance and secreted enzymes to degrade and neutralize NETs. These defensive strategies promote survival during neutrophil encounters and illustrate a dynamic example of innate immune evasion by *P. aeruginosa*.

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Dedication

To my parents, Mary and Ralph Halverson and my sisters, Erika and Dayna Halverson. Thank-you for being a strong supportive force during this entire process. As well as to my aunt and uncle, Janice and Don Halverson and to my cousins Kourtney and Brody Halverson, thank-you for helping me adjust and get settled into Calgary.

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List of Symbols, Abbreviations and Nomenclature

-	Dash
#	Hash
%	Percent
@	At
~	Approximately
˚C	Degree Celsius
<	Less than
=	Equals
°	Circle
µF	Microfarad
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometer
3’	Three Prime
3D	Three Dimensional
5’	Five Prime
A	Adenine
α	Alpha
ACD	Anticoagulant Citrate Dextrose Solution
Amp	Ampicillin
BHI	Brain Heart Infusion
BM2	Basal media 2
BP	Band Pass
BSC	Biological Safety Cabinet
C	Cytosine
C5a	Complement Component 5a
Ca^{2+}	Calcium
$CaCl_2$	Calcium Chloride
cAMP	Cyclic adenosine monophosphate
CAPs	Cationic Antimicrobial Peptides
CCM	Cell Culture Media
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFU	Colony Forming Units
CIAP	Calf Intestinal Alkaline Phosphatase
CO_2	Carbon Dioxide
CPS	Counts Per Second
Cy-5	Cyanine-5
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4’,6-diamidino-2-phenylindole
ddH_2O	Double Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphates
DPI	Diphenylene iodonium
eDNA	Extracellular DNA
Em	Emission
EPS	Exopolysaccharides
Ex	Excitation
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
Fe^{2+}	Iron
$FMNH_2$	Reduced riboflavin mononucleotide
FOV	Field of View
FSC	Forward Scatter
g	Grams
G	Guanine
GAS	Group A Streptococcus
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GI	Gastrointestinal
Gm	Gentamicin
Gs	Gravity
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine-5'-triphosphatase
H_2O_2	Hydrogen Peroxide
$HBSS^-$	Hank's Balance Salt Solution (No Cations)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Histopaque	Ficoll-Hypaque Density Media
HIV	Human Immunodeficiency Virus
HNP-1	Human Neutrophil Peptide
HOCl	Hypochlorous Acid
Hr	Hour
HSA	Human Serum Albumin
IFN	Interferon
IL	Interleukin
IM	Inner Membrane
Kan	Kanamycin
Kb	Kilobases
KCl	Potassium Chloride
kV	Kilovolt
L	Litre
L-Ara4N	Aminoarabinose (4-amino-4-deoxy-L-arabinose)
LB	Luria broth
LDH	Lactate Dehydrogenase
LOS	Lipooligosaccharides
LP	Long Pass
LPS	Lipopolysaccharide

LTA	Lipoteichoic Acid
M	Molar
MDR	Multiple drug resistance
mg	Milligram
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
MIC	Minimum Inhibitory Concentration
min	Minute
mL	Millilitre
mM	Millimolar
Mn^{2+}	Manganese
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaP	Sodium Phosphate
NETs	Neutrophil Extracellular Traps
NK cells	Natural Killer cells
nm	Nanometer
nM	Nanomolar
O_2	Oxygen
O_2^-	Superoxide
OD	Optical Density
OE	Over Expressing
OM	Outer Membrane
OMV	Outer Membrane Vesicles
P1	Population 1
P2	Population 2
P3	Population 3
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFT	Pore Forming Toxins
PI	Propidium Iodide
PMA	Phorbol 12-Myristate 13-Acetate
PMN	Polymorphonuclear leukocytes
PMT	Photomultiplier tube
PTase	Phosphatase
PVL	Paton-Valentine leukocidin
Q	Quadrant
r	Resistant
RBCs	Red Blood Cells
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
RND	Resistance-nodulation-cell division
ROS	Reactive Oxygen Species

rpm	Rotations per Minute
RPMI 1640	Roswell Park Memorial Institute medium
rRNA	Ribosomal Ribonucleic Acid
s	Second
SCIN	Staphylococcal Complement Inhibitor
SCV	Small Colony Variants
SIC	Streptococcal Inhibitor of Complement
SOD	Superoxide Dismutase
SP	Short Pass
SSC	Size Scatter
ssDNA	Salmon sperm DNA
T	Thymine
T0	Time Zero
T1	Time 1 hour
T3SS	Type 3 Secretion System
T4	Time 4 hour
TCS	Two component system
Tet	Tetracycline
TLR	Toll-Like Receptors
TNFR	Tumour Necrosis Factor Receptor
TNF-α	Tumour Necrosis Factor-alpha
TOTO-1	Quinolinium (TOTO-1 Iodide)
U	Unit
w/v	Weight per volume
X	Times
Zn^{2+}	Zinc
Δ	Delta
σ	Sigma
Ω	Ohm

Epigraph

"Success is the sum of small efforts, repeated day in and day out" – Robert Collier

Chapter One: Introduction

1.1 The opportunistic pathogen- *Pseudomonas aeruginosa*

Bacteria belonging to the genus *Pseudomonas* are Gram-negative, rod-shaped organisms with often one or more polar flagella for motility[1]. These bacteria often show a great metabolic diversity and flexibility, in terms of their nutritional requirements[1; 2]. It is this flexibility that allows these organisms to be found in various environments and niches, such as the soil, water, and even medical devices[3; 4; 5]. Many Pseudomonads are involved in bioremediation, since these bacteria are well adapted to metabolize chemical pollutants in the environment[6]. However, there are some species that can be pathogenic to humans, animals, insects and even plants[3; 7; 8]. One of the most medically important species of this genus is the opportunistic pathogen, *Pseudomonas aeruginosa*.

P. aeruginosa is very well adapted to survive in a variety of different locations, making it a great colonizer of different environments[9]. *P. aeruginosa* is able to use a wide range of organic substrates for food and possesses a large arsenal of virulence factors, which enable this bacterium to infect damaged tissue or individuals with reduced immunity, by either colonizing the skin or the mucosal surfaces found in humans and animals[3; 10]. Upon establishing an infection, *P. aeruginosa* can have a very devastating effect on the host.

P. aeruginosa can cause various infections that can range from mild, localized diseases, such as dermatitis and folliculitis, to more serious infections that include burn or wound infections, otitis media, urinary tract infections, and include serious systemic infections of the respiratory tract, endocarditis, bacteremia, and septicemia[11; 12; 13; 14; 15]. *P. aeruginosa* is considered a critical pathogen for those who have a compromised immune system, such as burn

victims, individuals with cancer or HIV, or people who suffer from the genetic disease cystic fibrosis (CF).

Due to its high levels of intrinsic antibiotic resistance, *P. aeruginosa* is among the top three hospital-acquired infections[3]. *P. aeruginosa* causes a variety of acute and chronic diseases, where the latter are thought to be due to biofilm growth[16]. The ability to grow and form a biofilm can be considered a long-term survival strategy for withstanding the environment or the host immune system. Chronic or biofilm-related infections include those associated with colonized indwelling catheters and foreign-body implants[17], chronic sinusitis and wound infections[3], as well as the lifelong lung infection experienced by individuals with cystic fibrosis.

1.2 Cystic Fibrosis

Cystic fibrosis is an autosomal recessive genetic disorder that is caused by mutations in the cAMP-regulated chloride channel gene for the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is required to regulate the salt/ion components of sweat, digestive juices and mucus[18; 19]. The most common CFTR mutation is the ΔF508, which is a deletion of the amino acid phenylalanine at position 508 in the protein. This mutation prevents the CFTR protein from properly localizing to the cell membrane, by not being able to escape the endoplasmic reticulum for further processing[20]. The defect in chloride ion transport due to a *cftr* mutation in the apical membranes of lung epithelial cells results in altered ion transport and mucus production. The thick mucus is the result of reduced chloride secretion and increased sodium reabsorption in the airway epithelium, leading to reduced water content of the mucous along with a decreased fluid surrounding the surface epithelial cells in the airway[18; 19; 21; 22]. As a result of this viscous mucus production, mucocilliary clearance in the airway is blocked, which

prevents the normal clearance of inhaled bacteria and ultimately leads to chronic colonization and polymicrobial infections.

Infants with CF are colonized and tend to develop bacterial infections early in life[21]. During childhood, the initial colonizing bacteria include *Staphylococcus aureus* and *Haemophilus influenz*a, which are ultimately replaced by new pathogens, such as *Pseudomonas aeruginosa*[23; 24; 25]. It is now appreciated that CF infections are very diverse and complex microbial communities[26] that have been identified using culture-dependent and culture-independent methods such as pyrosequencing and phylogenetic analysis based on the hypervariable sequences of the 16S rRNA gene[26; 27; 28]. The use of these methods has even identified that the *Streptococcus milleri* group can establish chronic pulmonary infections in CF patients[29]. Despite the complex microbiology and potential for other pathogens to play a role, *P. aeruginosa* is considered the predominant CF pathogen and is found in 50-80% of adult CF patients. Patients colonized with *P. aeruginosa* tend to experience persistent and recurring lung infection and increased mortality[23]. The CF lung is perhaps the best-studied example of an infection site where *P. aeruginosa* survives by growing in a biofilm[11; 30; 31; 32; 33; 34]. *P. aeruginosa* has been observed in large aggregates encased in exopolysaccharides which results in protection from recruited neutrophils[35; 36]. During this chronic infection, *P. aeruginosa* experiences selection pressures and undergoes various mutations in this environment, which results in isolates that are adapted for long-term survival. A classic example is the development of the mucoid phenotype due to the hyper-production of alginate, which provides several capsule functions that contribute to resilience to the innate immune system and antibiotics. The quorum sensing profiles of isolates in the CF lungs are similar to that of biofilms and not planktonic cultures, providing indirect evidence for biofilm formation in the CF lung[34].

1.3 *Pseudomonas aeruginosa* and the biofilm lifestyle

Many bacteria can live as part of a multicellular community known as a biofilm. These biofilms are often attached to a surface, either abiotic or biotic, and are encased in an extracellular matrix composed of a variety of polymers that include primarily exopolysaccharides and DNA[37; 38; 39; 40]. The extracellular DNA found within a biofilm may be released from dead bacteria, lysed host cells or from small outer membrane vesicles (OMV) released from living cells[38; 40; 41]. The CF lung is rich in DNA, which may also come from the extracellular traps produced by neutrophils in their attempt to eliminate this biofilm-type infection[30; 42; 43]. DNA has been shown to be a universal structural component during the initial phase of biofilm formation in all biofilm-forming organisms[38; 39; 44]. Biofilm development can be disrupted when a DNase is added during the initial stages of construction[38]. To allow for adequate flow of nutrients to support the biofilm, the overall structure consists of large microcolonies separated by water channels[45]. Biofilms can be found in a variety of environments in nature, in hospitals and within infected hosts.

The formation of a biofilm involves a series of steps that include the initial surface attachment, formation and maturation of three-dimensional microcolonies, and the dispersion of the mature biofilm[46]. Biofilms can develop on inert surfaces and materials, as well as on dead tissue and living surfaces, such as the mucosal surface[16]. Biofilms display a variety of phenotypic differences from their planktonic counterparts, that include reduced motility[47; 48], increased exopolysaccharide (EPS) production[49], as well as increased resistance to antibiotics and the immune system[50; 51]. These adaptations allow for long-term survival and make eradication of biofilms very difficult. Current research in our lab is focussed on the antibiotic resistance and immune evasion mechanisms of cells in biofilms. Biofilms were once viewed as cells in a matrix

that are passively protected from antibiotics and components of the immune system. While this is true, we now appreciate that cells in biofilms express unique genes and phenotypes when compared to planktonic cells, some of which contribute to antibiotic resistance. In addition, there are many regulators involved in controlling *P. aeruginosa* biofilm formation[52; 53; 54; 55], which is further evidence that the biofilm lifestyle is dynamic and highly regulated.

1.3.1 Biofilms as the cause of chronic infections

Before the development of improved sanitation and hygiene practices, bacterial infections were one of the leading causes of death in human society[56; 57]. The next greatest stride in the fight against infection was the discovery of vaccines and antibiotics as a means of prevention and treatment, respectively[57]. Most antibiotics are developed against planktonic cultures, however bacteria that exist as a biofilm are more resistant to all classes of antibiotics and other antimicrobial agents[50; 51; 58]. This resistance to antibiotics allow the bacterial biofilms to persist, ultimately leading to chronic and recurring infections. In particular, *P. aeruginosa* biofilms have been linked to various chronic infections, such as burns, sinusitis and the hallmark lung infections in patients with cystic fibrosis[34; 59; 60]. In the developed world, the majority of bacterial infections are thought to involve biofilm formation (up to 80%) and thus make biofilms clinically important[57]. Some of the most common biofilm associated diseases include urinary tract infections, otitis media, endocarditis, and cystic fibrosis pneumonia[57]. The number of diseases associated with bacterial biofilms is increasing and it is thought that some undiagnosed chronic diseases might in fact have a biofilm origin[61]. One of the trademark features of a biofilm is its resistance to both antimicrobial and antibiotic compounds, which contributes to bacterial persistence in a human host.

1.3.2 Antibiotic resistance of biofilms

Since the discovery of penicillin, antibiotics are often viewed as miracle drugs to help cure many different bacterial infections. However, with constant and improper usage in hospitals and agriculture, antibiotic resistant strains of bacteria are rapidly and continually emerging. Due to their innate multidrug tolerance and increased resistance to the host's immune response, biofilms are of significant concern and challenge in the medical world[62; 63]. Depending on the antimicrobial agent, *P. aeruginosa* biofilm cells can be 10 to 1000-fold more resistant than their planktonic counterparts[12; 50; 61]. Various factors have been described that contribute to the antimicrobial tolerance exhibited by biofilms[64; 65].

1.3.3 Mechanisms of antibiotic resistance in biofilms

Biofilm cells are encased in an extracellular matrix that acts as barrier to diffusion and can reduce the exposure of cells to certain antimicrobial agents[61]. For example, DNA in CF sputum has long been known to bind aminoglycosides and antimicrobial peptides[65; 66]. It has recently been shown that extracellular DNA in the *P. aeruginosa* biofilm matrix shields the biofilm cells from aminoglycoside killing[66]. Alginate is another anionic polymer and has been shown to bind antimicrobial peptides with electrostatic and hydrophobic interactions, contributing to the barrier of mucoid isolates[67]. By hindering the diffusion of antibiotics through the biofilm matrix, it may render the compound ineffective and unable to reach the target cells[68; 69]. This effect is likely short-term, as antibiotics will accumulate more slowly, and does not contribute to limit the diffusion of all antibiotics. The antibiotic poperacillin is unable to diffuse into *P. aeruginosa* biofilms while the antibiotics rifampicin and vancomycin are able to cross into *Staphylococcus epidermidis* biofilms[69].

Biofilms are aggregates of heterogeneous cells in different growth states. There is often a subpopulation of cells that have reduced metabolic rates, typically cells found deep within the biofilm[61]. Nutrient or oxygen limitation in these deeper layers plays a role in 'antibiotic indifference', as some antimicrobial agents are active against rapidly dividing cells or in aerobic conditions[68], thus, these slow growing cells are protected from these compounds. Antibiotics such as ciprofloxacin, which interferes with bacterial replication, was found to kill metabolically active cells found on the top layer of the biofilm, while the population of cells with a reduced metabolic activity were protected[64]. The opposite effect can be witnessed with the use of antimicrobial agents that target the cell membrane, such as colistin. This compound has been shown to kill cells deep within the biofilm structure, while metabolically active cells in the top layer are able to survive[70].

Persister cells are a unique subpopulation of metabolically dormant cells that are very tolerant to antibiotic treatment[71]. Persister cells are present at up to 1% of cells in stationary phase planktonic cultures, but can also be present in biofilms[71]. It was proposed that antibiotic treatment and the immune system can clear most planktonic and biofilm cells during an infection, but persisters in the biofilm may be afforded extra protection. Upon removal of antibiotic, persisters regain their normal growth and antibiotic susceptibility, but also repopulate the biofilm and thus may be involved in recurring infections[71]. The persister phenotype is not present in all cells and is transient, which makes it difficult to study, however the mechanism of persister cell formation is being examined[68]. Small colony variants are another subpopulation within biofilms and have also been shown to be very difficult to eliminate with antibiotics.

Small colony variants (SCV) are mutant isolates that arise in the CF lung or during *in vitro* biofilm formation[72]. In addition, SCVs have slow growth rates, increased EPS production

and aggregation, which make them very difficult to treat with antibiotics[73]. Small colony variants are able to persist in mammalian cells and are less susceptible to antibiotics compared to their wild-type counterparts, which can lead to latent or recurring infections[73]. There are two major groups of SCVs found in clinical isolates, SCVs that are defective in electron transport and SCVs that are deficient in thymidine biosynthesis[73]. *P. aeruginosa* SCVs have been found in patients with cystic fibrosis and can play a role in the progression of the reoccurring lung infection experienced by these patients. Patients with *P. aeruginosa* SCVs experienced more compromised lung function and require daily inhalation of tobramycin or colistin[74]. As well, *P. aeruginosa* SCVs have been shown to be more resistant to aminoglycosides and more damaging to the host cells, ultimately leading to increases persistence in the CF lung[73].

It is also possible the conventional or novel mechanisms of antibiotic resistance may be uniquely expressed in biofilms[61; 68]. For example, *P. aeruginosa* contains a putative efflux pump, encoded by *PA1874-PA1877* that is highly expressed in biofilm cells that provides resistance to antibiotics such as tobramycin, gentamicin and ciprofloxacin[75]. The presence of the *ndvB* gene in *P. aeruginosa* is important for biofilm-specific resistance, whereby periplasmic cyclic glucans interact with tobramycin and prevent it from accessing its cellular target[76].

1.4 The protective role of the exopolysaccharide matrix in biofilms

The biofilm extracellular matrix is composed primarily of extracellular DNA (eDNA) and EPS[37; 38]. Exopolysaccharides have both structural functions in maintaining biofilm formation, but also have capsule functions required for immune evasion. For example, it is very difficult for antibodies and phagocytic cells to penetrate deep within the biofilm matrix, however antibodies do form immune complexes at the biofilm surface[62]. Biofilms can also reduce elements of the immune response such as the oxidative burst of neutrophils in response to *P.*

aeruginosa biofilms, when compared to planktonic cells[77]. During the course of a chronic infection, *P. aeruginosa* is able to evolve from a non-mucoid strain to a rough, mucoid strain[32]. Isolates of *P. aeruginosa* from CF patients frequently undergo conversion to a mucoid, alginate overproducing strain, as a result of mutation and the selection pressure in the lung. Alginate is a major component of the biofilm matrix of mucoid strains and also has many capsule functions[37]. Alginate is involved in inhibiting neutrophil chemotaxis, limiting phagocytosis by neutrophils and macrophages, and even inhibits complement activation[62; 78]. Mucoid strains have also been shown to be less susceptible to opsonic antibodies[62]. The dense extracellular matrix and outer layer of cells can protect the interior of the biofilm community and contributes to the multifactorial immune evasion in biofilms.

The early *P. aeruginosa* colonizers of the CF lung are non-mucoid isolates, which express the Pel and Psl exopolysaccharides. Pel is a glucose-rich, cellulose-like polymer essential for biofilm and pellicle formation at the air-liquid interface, and has been shown to be involved with cell-to-cell interactions in *P. aeruginosa* PA14 biofilms[79; 80]. Pel has been shown to specifically contribute to increased antibiotic resistance[81] and *in vivo* biofilm formation in the *Drosophila* GI tract[82]. Psl is rich in mannose and galactose and is involved in the initial attachment and biofilm maturation[83]. For mature biofilms, Psl is associated with the mushroom caps and in forming a peripheral meshwork covering the cap region[83; 84]. Psl is also required for intracellular survival in neutrophils and macrophages[79]. Isolates with a 'small colony' phenotype are frequently isolated from CF patients' lungs, which have the general properties of increased EPS production, aggregation, antibiotic resistance and immune system evasion. The small colony phenotype has been shown to arise from mutations leading to increased production of Pel and Psl

EPS in non-mucoid isolates[85]. The EPS hyper-producing isolates (mucoid, small colony) are thought to provide an increased long-term survival advantage in the CF lung.

1.4.1 Extracellular DNA in biofilms

Whereas the importance of bacterial exopolysaccharides is well studied, the accumulation and roles for extracellular DNA have only recently been discovered. Extracellular DNA was first shown to function as a matrix component in *P. aeruginosa* biofilms, where it contributed to biofilm architecture and maintaining the microcolony structure[38]. The addition of DNA degrading enzymes prevented the early stages of biofilm formation. Extracellular DNA is a universal polymer in the biofilm matrix, helps to maintain the 3D structure of biofilms in both Gram-positive and Gram-negative bacteria[39; 63] and is required in the cell-cell interconnections[38]. In *P. aeruginosa*, matrix DNA is localized on the surface of young biofilms and concentrated along the stalk structure of mature mushroom–shaped microcolonies[39]. The source of eDNA in biofilms cultivated *in vitro* is likely from dead bacteria but during infection is probably also derived from immune cells. The eDNA found in biofilms is controlled by quorum sensing regulated release of eDNA, suggesting a specific mechanism to secrete DNA[39], or through the general release of DNA found in outer membrane vesicles that bleb from the outer membrane of living cells [40; 86]. Our lab has reported that *P. aeruginosa* can utilize DNA as a sole nutrient source of carbon, nitrogen and phosphate. DNA utilization as a nutrient may permit a growth advantage in infection sites where DNA accumulates[87]. Given the ubiquitous nature of eDNA in biofilms, our lab is interested in identifying novel functions for this biofilm matrix polymer.

1.5 DNA-induced antibiotic resistance in biofilms

Our lab hypothesized that the accumulation of DNA in biofilms, and possibly other matrix polymers, could induce the expression of unique genes that would help to account for the

novel phenotypes of cells in biofilms. To test this hypothesis, planktonic cultures were supplemented with exogenous DNA in an attempt to identify genes that were induced or repressed by extracellular DNA. This experimental system identified a number of genes that are influenced by the presence of extracellular DNA. The ability to induce bacterial gene expression was related to the cation chelating activity of DNA, which our lab showed is a very efficient chelator of divalent metal cations including Mg^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+} [12]. At high concentrations (>5-10 mg/mL), DNA has a fast-killing antimicrobial activity that results from stripping Mg^{2+} cations from the surface and disrupting inner and outer membrane integrity of bacterial cells[12]. The implications of the antimicrobial activity will be discussed later.

When present at sub-lethal concentrations of DNA, the cation chelating property of DNA functions to protect *P. aeruginosa* by imposing a localized cation-limited environment and inducing genes controlled by Mg^{2+} sensing PhoPQ and PmrAB two component systems. The PhoPQ/PmrAB systems both respond to limiting Mg^{2+} conditions and activate the expression of many Mg^{2+}-regulated genes. The early studies used Mg^{2+} limitation as a convenient expression condition in the lab, but this was not recognized as an environmental condition that *P. aeruginosa* would encounter, as Mg^{2+} is not limiting in the soil or during infection in humans.

Some of PhoPQ/PmrAB-controlled genes are involved in modifying the charge and structure of LPS and thus contribute to resistance to cationic antimicrobial peptides (CAPs)[88; 89]. CAPs are short, amphipathic peptides that bind to and disrupt the outer and cytoplasmic membranes resulting in cell death. The presence of resistance mechanisms to CAPs may enable *P. aeruginosa* to overcome an essential component of the innate immune system. The observation that DNA induces the expression of antibiotic resistance genes suggested that DNA is more than just a structural component of biofilms.

1.6 DNA-induced surface modifications in *Pseudomonas aeruginosa*

1.6.1 Aminoarabinose-modified LPS

The presence of eDNA in biofilms leads to increased expression of *PA3552-PA3559* (*arn*) genes and increased resistance to cationic antimicrobial peptides[12]. These genes encode for an LPS modification allowing for the covalent addition of aminoarabinose to the phosphates of the core lipid A[13]. Aminoarabinose masks the negatively charged phosphates in the lipid A core, blocking the antimicrobial peptide binding sites and protecting the membrane from peptide damage (Figure 1.1). CF isolates of *P. aeruginosa* were described that stably express these LPS phenotypes, which are not normally expressed in the most commonly studied strains under standard growth conditions[90]. DNA-mediated induction of this operon can be blocked by adding excess Mg^{2+}, indicating that cation chelation and sequestering is required for expression[12]. Mutants lacking the aminoarabinose-modified LPS do not have increased resistance in DNA-enriched planktonic and biofilm cultures to both antimicrobial peptides and aminoglycosides[12]. Although aminoglycosides act on the bacterial ribosome, they are cationic and surface modification to mask negative charges will limit their entry into the cell. This modification is an example of a DNA-induced mechanism that is biofilm-specific allowing protection of *P. aeruginosa* against antibiotic exposures or host antimicrobial peptides in DNA-rich environments like biofilms formed in the CF lung[12].

1.6.2 Surface spermidine production

The presence of extracellular DNA also induces the expression of a novel cell surface modification and antimicrobial peptide resistance mechanism. This mechanism involves the addition of polyamines to the bacterial membrane. Polyamines are small organic hydrocarbons containing positively charged amino groups at physiological pH. The primary bacterial

polyamines include putrescine, cadavarine, and spermidine and they are generally known for their cytoplasmic functions and are involved in numerous and diverse cellular processes[91]. Polyamines bind nucleic acids and proteins and therefore modulate DNA transcription and protein translation[92]. They protect DNA from oxidative damage and thus have an antioxidant function. Recently, polyamines have been implicated in virulence, biofilm formation and antibiotic resistance[93].

Our lab has shown that *P. aeruginosa* produces putrescine and spermidine on the outer cell surface[94]. Spermidine production on the surface protects the outer membrane from antimicrobial peptide treatment, likely by electrostatic interactions that mask additional negative surface charges (Figure 1.1). Surface polyamines also protect the membrane lipids from oxidative damage by reactive oxygen species (ROS). ROS are another major antimicrobial component produced by cells of the innate immune system to kill bacterial pathogens. Exogenous DNA strongly induces the expression of spermidine synthesis genes *PA4773-PA4775*, which results in surface spermidine production and protection of the membrane from antimicrobial peptide and ROS damage[94].

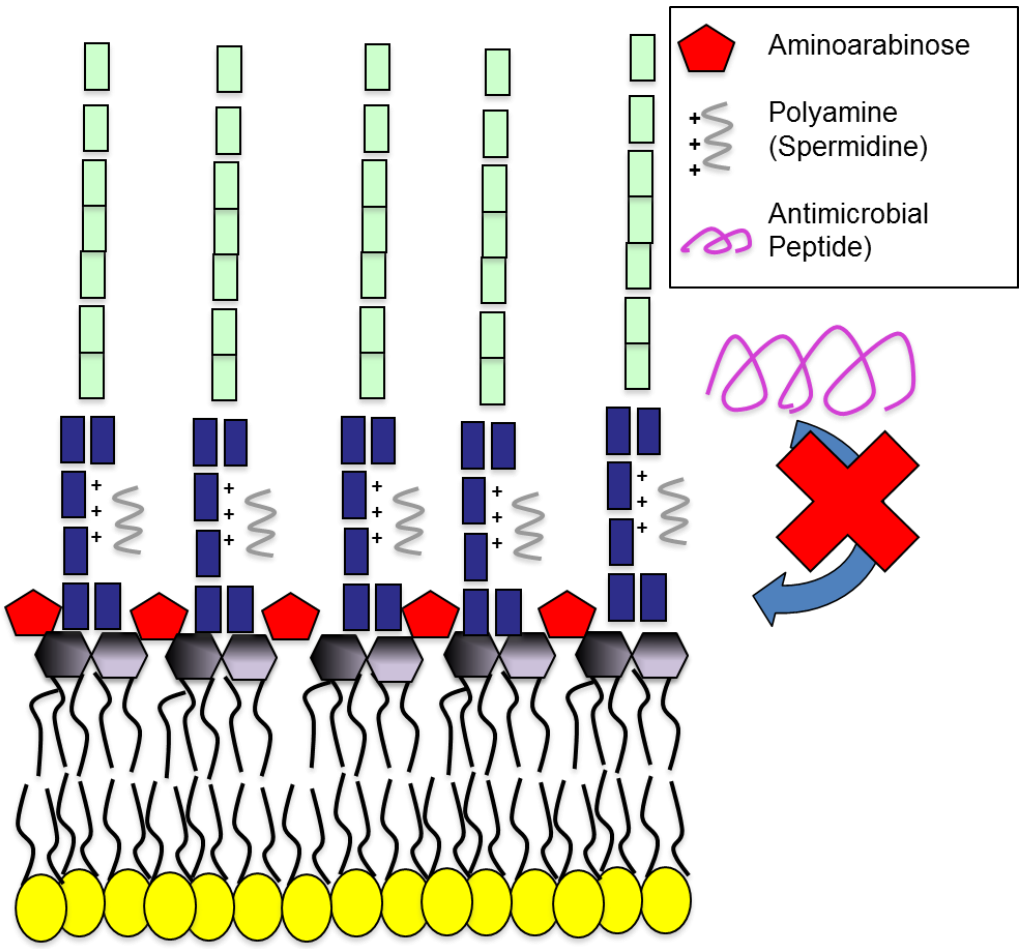


Figure 1.1. Cell surface modifications with spermidine and aminoarabinose protection.

Outer surface modification to phosphates found in LPS. Magnesium (Mg^{2+}) cations normally bind to phosphates in the lipid A and inner core of LPS. Cationic antimicrobial peptides compete with Mg^{2+} for binding sites. The displacement of cations causes a partition in the membrane and pore formation, compromising the membrane permeability. Polyamines likely bind to core phosphates and aminoarabinose binds to lipid A phosphates, helping protect the membrane from cationic antimicrobial peptides. Figure adapted from Lewenza (2013).

1.6.3 Exopolysaccharide production

In addition to mutation events that influence EPS production in CF isolates, there are environmental conditions that influence *psl/pel* expression and therefore EPS production. The *pel/psl* biosynthesis genes are controlled by a complex regulatory pathway that involves the GacS, RetS and LadS two component sensor proteins[5; 53; 54; 96; 97; 98]. The RetS sensor represses *pel/psl* expression and biofilm formation, and is required for expression of the type III secretion system (T3SS), which is generally considered a delivery system of effector molecules involved in acute virulence[99]. Thus, *P. aeruginosa* can express either the determinants for biofilm formation, or the acute virulence factor encoded by the T3SS, which suggests that this organism can respond to environmental conditions and adjust its pathogenic lifestyle[54; 96].

Given the influence of eDNA on the antibiotic resistance phenotype in planktonic cells, we also examined the possible influence of DNA and limiting Mg^{2+} on the biofilm phenotype. Surprisingly, *P. aeruginosa* forms large aggregates in limiting Mg^{2+} growth media and forms pronounced biofilms, which is due to increased expression of the *pel/psl* EPS biosynthesis genes[52]. This was ultimately due to the PhoPQ-mediated repression of the *ret*S biofilm repressor under limiting Mg^{2+} conditions[52]. Similarly, the addition of DNA imposes a Mg^{2+} limitation on *P. aeruginosa*, which also promotes biofilm formation, which can be blocked if excess Mg^{2+} is also added with exogenous DNA[52]. In the CF lung infections, DNA is very concentrated (up to 20 mg/ml) and originates from dead bacteria and immune cells. Taken together, it is likely that eDNA can chelate cations in the lung and induce expression of the defining phenotypes of biofilms: increased aggregation, antibiotic resistance and resistance to the immune system.

1.7 Neutrophils - First Line of Defense

Neutrophils are a major component of the innate immune system. Neutrophils have distinct physiological characteristics, which include a multi-lobed nucleus, and the presence of various granules. They are one of the first lines of defense against invading pathogens, circulating the bloodstream ready to combat any foreign intruders[100; 101]. Neutrophils have a short lifespan; after maturation, neutrophils are released into the bloodstream and circulate and/or marginate for 10-24 hours before migrating into tissues where they may function for up to 1-2 days before undergoing apoptosis, and are finally cleared by macrophages or dendritic cells[102; 103; 104]. The neutrophil lifespan is highly regulated to ensure there is proper removal of spent/effete neutrophils in order to prevent accidental release of cytotoxic molecules that could potentially damage host tissue[102; 105]. Neutrophils contain a variety of antimicrobial strategies that are employed when trying to eliminate pathogens from the body.

1.7.1 Neutrophil killing mechanisms

Neutrophils are primarily phagocytes, thus they combat invading microbes by ingesting them. Neutrophils contain multiple granules that are used during the phagocytic process, but they can also be released through the process of degranulation to kill extracellular bacteria and recruit additional leukocytes to infected or inflamed area. This process involves the expulsion of granular contents into the extracellular space. Neutrophils contain different type of granules that contain various antimicrobial agents. One set of granules are the azurophilic granules, which are comprised of neutrophil elastase, cathepsin G, defensins, and myeloperoxidase (MPO). There are also the specific granules, that contain alkaline phosphatase, lysozyme, NADPH oxidase, collagenase, lactoferrin and cathelicidin, as well as the tertiary granules containing gelatinase and cathepsin[106; 107; 108]. Neutrophils also contain secretory vesicles that contain plasma proteins such

as albumin and can also act as internal reservoirs of membrane/cytokine receptors[109]. Neutrophils have also been shown to produce extracellular traps (NETs). These NETs are composed of genomic DNA from the neutrophil combined with cytosolic and granular proteins[107].

1.7.2 Neutrophils and Cystic Fibrosis

Neutrophil migration into the lung is an early event in the increased inflammatory response observed in the CF lung[110; 111; 112; 113]. The chronic biofilm infection in the CF lung leads to a persistent neutrophil accumulation and necrosis, resulting in viscous sputum with high concentrations of DNA, actin and granule proteins[114]. A study by Morris *et al.*, has shown that neutrophils from CF patients had a lower phagocytosis capacity, which has been linked to a reduced intraphagolysosomal hypochlorous acid (HOCl) production due to impaired CFTR function[30; 115]. CF neutrophils were also reported to have an increase in oxidative burst, releasing more oxidants[114] and elastase[116]. This can cause severe damage to the CF airway since the elastase can degrade nearly all the structural proteins of the lung[114]. In the CF lung, the increased bacterial load and biofilm formation reduce the efficiency of phagocytosis. The neutrophils that settle in the biofilms may be unable to migrate away[117; 118]. Neutrophils that accumulate in the biofilm may result in self-injury, causing a release of oxidants, which in turn can compromise host defense mechanisms. DNA and actin from necrotic neutrophils can also serve as a biological matrix and are incorporated into *P. aeruginosa* biofilms[114; 118].

1.7.3 Neutrophils and **Pseudomonas aeruginosa** *biofilms.*

A study by Jesaitis *et al*. examined the interaction of neutrophils *to P. aeruginosa* biofilms[119]. They showed a unique interaction of the neutrophils to the biofilm, in that the neutrophils will settle onto the biofilm but are not capable of migrating away from the point of contact[119]. The neutrophils are still able to "attack" the biofilm by either mounting a respiratory

burst or phagocytosis of the biofilm cells. Since the neutrophils appear to be immobilized onto the biofilm surface, the bacteria on the surface may act as a protective wall that serves as a sink for various reactive oxidant species[119]. This ROS sink may also have a detrimental effect on the neutrophils that pile up on this wall, leading to self-injury and compromised host defense activity[119; 120]. Some uninjured bacteria may be able to escape and colonize other areas as well[119]. *P. aeruginosa* contains other mechanisms to protect itself from neutrophils and host defenses.

When examining the morphology of the neutrophils in contact with the biofilm, they appear to remain rounded and inactivated. It may be possible that exotoxins produced by *P. aeruginosa* may play a role in the neutrophils maintaining their rounded morphology[119]. The exotoxins ExoS, ExoY and ExoT can be directly injected into eukaryotic cells via a type III secretion system[121]. Our lab has shown that mucoid biofilms also have increased expression and production of type III secreted effectors, which accumulate in the biofilm matrix[122].

P. aeruginosa uses a single, unipolar flagellum for swimming and initial attachment during biofilm formation[32]. Flagellin is a potent activator of the host immune response, via Toll-like receptor 5, which can induce various genes involved in the pro-inflammatory response, such as IL-8[32], however, in the presence of neutrophil elastase, there is a down regulation of transcription and expression of flagellin that may help *P. aeruginosa* evade the immune system[123]. *P. aeruginosa* also produces rhamnolipids that have been shown to increase its tolerance towards immune cells[51; 124]. In the presence of polymorphonuclear leukocytes (PMNs), such as neutrophils, *P. aeruginosa* biofilms respond by up regulating the production of toxic compounds, such as rhamnolipids[124]. These rhamnolipids serve as a protective shield surrounding the biofilm cells and are capable of destroying approaching PMNs via necrosis[51; 124]. Lastly, *P. aeruginosa* produces two extracellular proteases, alkaline protease and elastase, that

inhibit phagocytosis, and the activity of NK and T-cells, as well as inactivate various cytokines and cleave immunoglobulin and inactivate complement[62].

1.8 Neutrophil extracellular traps (NETs)

1.8.1 Induction of NETs - NETosis

It has been shown that activated neutrophils are able to generate neutrophil extracellular traps[107]. The induction of NETs is still currently under investigation; however, certain compounds have been identified to promote NET formation. Certain bacteria (either whole cells, LPS, or pili) and inflammatory signals (IL-8, IFN I+II, C5a)[102; 108; 125] as well as fungi[126], protozoa[127], platelet-activating factor[128] and M1 protein (a matrix protein of the influenza virus that forms a coat inside the viral envelope)[129] have been described as factors that activate neutrophils to release NETs.

The activation pathway for NETs can involve different receptors, such as Toll-like receptors (TLRs). With the receptors stimulated, it leads to the activation of protein kinase C causing a signal transduction cascade that induces the assembly and activation of the NADPH oxidase complex[130]. This complex will oxidise NADPH, releasing two electrons that are transferred to oxygen (O_2), resulting in the formation of superoxide (O_2^-)[130]. With the use of superoxide dismutase (SOD), the O_2^- dismutates into hydrogen peroxide (H_2O_2) which is then converted to hypochlorite (HOCl) by myeloperoxidase (MPO)[130]. These ROS are required for NET formation, since the addition of the oxidase inhibitor diphenylene iodonium (DPI) can block the NET induction[130]. The H_2O_2 produced is a potent inducer of NETs at physiological concentrations[100] and with the introduction of exogenous catalase that can degrade H_2O_2, NET formation is blocked[130]. Conversely, NET formation can be increased with the catalase inhibitor

3-amino-1,2,4-triaxole. These ROS may act as second messengers and promote the downstream signalling events that culminate in NET formation[131].

NET formation is an active process that begins with the rearrangement of the nuclear granular components within the neutrophil[130]. Under activating conditions *in vitro*, neutrophils will flatten and become motile and phagocytic with the respiratory burst peaking within the first hour[100]. It is only after one hour that major morphological changes begin. The nucleus starts to lose its lobular appearance and the chromatin segregates into euchromatin and heterochromatin regions[100; 130]. The nuclear membrane begins to separate and after 2 hours, the membrane continuity is lost and a chain of individual vesicles have formed[132]. At this point the chromatin comes into direct contact with the cytoplasmic components while at the same time, the granules dissolve and the NET components can freely mix within the cell[100; 130]. Net release into the extracellular space occurs 15-60 minutes after nuclear membrane dissolution. Originally, it was believed that neutrophils are alive until the release of the NETs, where the cell membrane ruptures and the cell dies[100; 107].

Recent *in vivo* studies have shown that the activation and release of NETs can happen in a much shorter time frame and that after NET release, the neutrophils may remain viable. When exposed to *S. aureus*, neutrophils appear to release extracellular traps in a distinct mechanism that is more rapid than previously described[128]. When incubated with the bacteria for at least 10 minutes, there was substantial NET release with minimal neutrophil lysis, death or apoptosis suggesting that neutrophils have a coordinated mechanism of chromatin release to defend against invading bacteria[128]. This rapid NET release begins with the nucleus of the neutrophil losing its lobed appearance and condensing to sphere-like structures while a separation of the inner and outer nuclear envelope develops forming vesicles[128]. The separated membranes and the vesicles

are filled with nuclear DNA, which is then released through the plasma membrane into the extracellular space through budding; DNA-containing vesicles can be seen in the extracellular space suggesting that intact vesicles are released[128]. This type of NET release has been witnessed *in vivo* showing intact neutrophils following this release[133]. Only a subgroup of neutrophils that form extracellular traps become anuclear, a larger population of PMNs undergoing NETosis still contain diffuse intracellular DNA[133]. Other studies have shown that anuclear PMNs are able to crawl, transmigrate, phagocytose and kill bacteria[134; 135; 136]. It appears that neutrophils have different mechanisms and methods to release extracellular traps that are involved in defending the body from diverse microbial invaders.

1.8.2 Killing components of NETs

NETs are thought to provide a high local concentration of antimicrobial compounds to bind, disarm and kill microbes extracellularly[30; 101; 106; 107; 128; 137; 138]. NETs are complex, extracellular structures that are composed of neutrophil chromatin and specific proteins from the granules and the cytoplasm within the neutrophil[107; 108; 139]. The chromatin acts as the backbone of the NET structure and treatment with DNases, but not proteases, can degrade these structures[106]. NETs contain proteins from azurophilic granules, which comprise of neutrophil elastase, cathepsin G and myeloperoxidase (MPO). Proteins from specific granules, such as lactoferrin, and tertiary granules containing gelatinase have also been found in NETs[102; 107; 108]. However, various cytoplasmic proteins, such as actin and tubulin are not incorporated into the NET structure[107]. A study by Parker *et al,* found that MPO present in NETs is still active and can contribute to bacteria killing[140]. When the NETs are supplied with H_2O_2, effective killing of *S. aureus* was observed but was not due to the H_2O_2 itself. Killing was blocked in the presence of

an MPO inhibitor and HOCl scavenger, indicating that killing is MPO-dependent and is most likely the result of the generation of HOCl in the presence of H_2O_2[140].

Histones have been shown to act as an effective antimicrobial agent and promote cell lysis by interfering with the bacterial membrane permeability. The membrane activity is attributed to low molecular weight peptides that are liberated from intact histones[141; 142; 143]. The antimicrobial activity of histones, specifically histone H2A, might be the result of the presence of a buforin I sequence. Buforin I has strong amino acid homology to the N-terminus of histone H2A[144]. Buforin I is present in the stomach of *Bufo bufo gargarizans* (Asiatic toad) and has been shown to have a strong antimicrobial activity to various kinds of microorganisms[144]. Blocking the histones with anti-histone antibodies has been shown to reduce the killing efficiency of NETs[107; 132]. Another component of NETs that has been shown to have antimicrobial activity is calprotectin, a cytosolic protein that is vital in eliminating fungal infections[145]. The incorporation of calprotectin in the NET structure is essential for antifungal activity in mice since calprotectin-deficient mice were less effective at clearing *Candida albicans* infections[145].

1.8.3 Microbial susceptibility to NETs

Different Gram-negative and Gram-positive bacteria have been exposed to neutrophils that were activated to make NETs. Some bacteria appear sensitive to NETs while other bacteria are able to survive. Typically bacteria that are susceptible to NETs show a reduction in cell number when incubated with neutrophils that have released NETs. When Brinkmann *et al* (2004) first showed that neutrophils are able to expel these extracellular traps; it was found that both *Salmonella typhimurium* and *Shigella flexneri* are sensitive to NET killing[107]. Other bacteria, such as *E. coli*[146], *S. aureus*[130; 147], *Listeria monocytogenes, Mycobacterium canetti,* and *Mycobacterium tuberculosis*[148] have also shown vulnerability to NETs. Not all bacteria appear to

be killed by NETs, since Group A streptococcus[149], *Streptococcus pneumonia*[137], and *P. aeruginosa*[30; 126; 150] are able to survive exposure to these structures. Thus, it appears that the effectiveness of the killing due to NETs varies between organisms. Previous studies have shown that the azurophilic granule components have varied capacity to kill different bacterial species[151; 152; 153; 154]. As well, certain bacterial species are able to avoid killing by NETs through different processes and strategies.

Bacteria are not the only microbes that can be trapped and killed in NETs. Various fungi, protozoa and even viruses have been shown to have varying degrees of susceptibility to NETs. Studies have shown that fungi such as *Aspergillus fumigatus*[155], *Candida albicans*[156] and *Candida glabrata*[157] show inhibited growth when exposed to NETs while *Cryptococcus gatti*[157] is able to survive NET killing. Other organisms, such as the protozoa *Plasmodium falciparium* and *Leishmania donovani* are able to be trapped in and survive these extracellular traps, respectively[158; 159] while other protozoa like *Toxoplasma gondii* show a reduction in parasite number when incubated with NETs[160].

Studies examining viruses and the role of NETs have found that some viruses can modulate the NET formation, such as influenza A H1N1[161] while other viruses have been shown to have reduced infectivity when in contact with NETS, such as the human immunodeficiency virus (HIV)-1[162]. Thus there appears to even be differences in susceptibility to NETs, not only in bacteria, but in other microorganisms.

1.8.4 NETs and Cystic Fibrosis

It has been reported that NETs form in airway fluids from patients with cystic fibrosis and mouse cystic fibrosis lung disease and has been correlated with impaired obstructive lung function[30; 42; 43; 163]. A study by Young *et al*. showed that decreased susceptibly to NET-mediated

killing develops over time in the CF airway[30]. As well, the development of a mucoid phenotype allows for a decreased interaction with NETs (from the increased alginate production) thus interfering with the killing by NET-associated granule proteins. *Streptococcus pneumonia* can counteract the killing of NETs through the expression of polysaccharide capsule and lipoteichoic acid (LTA) modifications, as well as through the production of an extracellular DNase[137; 164]. These bacterial DNases are able to degrade NETs and defend against NET killing. Our lab has shown that *P. aeruginosa* contains a two gene operon that encodes for a putative phosphatase (*PA3910*) and a secreted enzyme with DNase activity (*PA3909*)[87]. The presence of these two enzymes might allow for the degradation of NETs, allowing for the survival of *P. aeruginosa.* As an added bonus, it appears that *P. aeruginosa* is able to degrade DNA and use the degradation products (carbon, nitrogen, phosphate). This allows *P. aeruginosa* to increase its pool of available nutrients[87] which may contribute to its long-term survival.

1.9 Hypothesis

We have shown that eDNA induces the expression of genes required for antimicrobial peptide resistance and for aggregation. These observations suggest that eDNA found in biofilms plays a greater role than acting merely as a structural component. The various surface modifications that include aminoarabinose modification of LPS and surface spermidine production have been shown to be required for antimicrobial peptide resistance under *in vitro* conditions. I aimed to test if these DNA-induced surface modifications are important for surviving interactions with innate immune cells that produce antimicrobial peptides. I was particularly interested in neutrophils because 1) they are the most heavily recruited cell to the biofilm infections in the CF lung, 2) they utilize antimicrobial peptides to kill bacteria, and 3) they form DNA-rich structures (NETs), which contribute to DNA accumulation at the site of

infection[101; 106; 107; 165; 166]. The central hypothesis of this study is that the DNA-induced responses by *P. aeruginosa* are used to defend against NET killing and can actually be activated by the DNA backbone of neutrophil extracellular traps. I also propose that the known antimicrobial activity of DNA will provide a broad spectrum of bacterial killing. In this way, DNA may contribute to both the structure and killing by NETs. If growth in the presence of eDNA promotes immune evasion, this may help to explain the long-term survival of *P. aeruginosa* in DNA rich infection sites like the CF lung.

1.9.1 Objectives

Objective #1: Examine if *P. aeruginosa* is able to sense and respond to neutrophil extracellular traps.

Objective #2: Determine if the DNA in NETs plays a role in bacterial killing.

Objective #3: Examine the resistance mechanisms that aid in the survival of *P. aeruginosa* to NETs.

Chapter Two: Materials and Methods

2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2.1. *Pseudomonas aeruginosa* PA01 and *lux*-tagged PA01::p16*Slux*[167] were used as the standard wild-type strains. *Staphylococcus aureus* ATCC 25923[128] was used as a representative Gram-positive species while *Escherichia coli* DH5α (Invitrogen Cat#:18265-017) was used as Gram-negative control. Bacterial cultures were routinely grown at 37°C in LB or BM2 defined minimal media with 0.5 mM $MgSO_4$, unless otherwise stated. BM2 growth medium includes the following components: 0.1 M HEPES pH 7.0, 7 mM ammonium sulfate, 20 mM sodium succinate pH 6.7, 10 µM iron sulfate, 1600 µM phosphate buffer pH 7.2, 1.62 µM manganese sulfate, 2.45 µM calcium chloride, 13.91 µM zinc chloride, 4.69 µM boric acid, 0.67 µM cobalt chloride[87]. *S. aureus* strains where grown overnight in BHI media. The *lux*-reporter strains, *PA4774::lux*, *PA4774::lux, PA4773/4::lux,* and *PA3553::lux* were recovered from our mutant library strain collection[88]. The *PA4774c.c* mutant strain was chromosomally complemented by the integration of the *PA4773-4775* genes into the chromosome, as previously described[168]. Mutants in genes encoding for a secreted DNase (*eddB*) or phosphatase (*eddA*) were recovered from the mini-Tn*5-lux* library in our lab[88] and from a mini-Tn*5-lacZ/phoA* mutant library (University of Washington)[169]. When necessary, the following antibiotics were used: 50 µg/mL tetracycline for *P. aeruginosa* mini-Tn*5-lux* mutants and mini-Tn*5-lacZ/phoA* mutants, and 50 µg/mL kanamycin for *E. coli* DH5α/pσ70-*lux*.

2.1.1 Confirmation of DNase and PTase mutants

Polymerize chain reaction (PCR) amplification was performed to confirm wild type gene sizes of *eddA* (PTase) and *eddB* (DNase) in various mutants obtained from Dr. Bob Hancock, to

confirm the correct site of transposon insertion. The EddB-F and EddB-R primers were used to amply the *eddB* gene while the *eddA* gene was amplified using the EddA-F and EddA-R primers (Table 2.1). Since the obtained mutants have a transposon insertion, the respective gene in each mutant should not be amplified due to the transposon insertion making the gene of interest too large to amplify. The *P. aeruginosa* PA01 wild-type was added as a positive control for each condition. Genomic DNA (gDNA) was isolated from each strain using the Qiagen DNA isolation kit. The PCR tubes were set up to contain a total volume of 20.0 µL made up of: 2.0 µL of 10X PCR Buffer, 0.6 µL of $MgCl_2$, 0.2 µL of dNTP, 1.0 µL of the Forward Primer, 1.0 µL of the Reverse Primer, 0.16 µL of *Taq* Polymerase (Invitrogen-10342-020), 1.0 µL DMSO, 12.84 µL of ddH_2O and 1.0 µL gDNA. PCR reactions were run in the thermocycler (MyCycler) using the following conditions: 94°C for 2 min then denaturing at 94°C for 30s, annealing at 52°C for 30s, then extension at 74°C for 3 min and holding at 4°C. Following the PCR, samples are run on a 1% agarose gel using the Invitrogen 1 Kb+ Ladder (Cat# 10787-018) as a molecular marker. Negative controls were run using ddH_2O instead of gDNA.

Table 2.1. List of bacterial strains, plasmids, and primer sequences used in the study

Strain	Description	Reference
PA01	Wild type *P. aeruginosa*	RE Hancock
PA01::p*16Slux*	Wild type *P. aeruginosa* transcriptional fusion	Riedel *et al* (2007)
PA01 Tn*7::gfp*	Wild type *P. aeruginosa* containing a stable GFP protein	Koch *et al.* (2007)
S. aureus	Wild type *S. aureus* ATCC 25923	Pilsczek *et al.* (2010)
E. coli	Wild type *E. coli* DH5α	Invitrogen Cat#: 18265-017
E. coli DH5α/pσ70-*lux*	DH5α expressing a σ70 promoter fused to *luxCDABE* in the pSC101 vector	This Study
PA4773/4::lux	Intergenic *PA4773/4774::lux* transposon mutant and transcriptional fusion, 11_A9	McPhee *et al.* (2003)
PA4773-lux	*PA4773::lux* transposon mutant and transcriptional fusion, 50_F5	Lewenza *et al.* (2005)
PA4774::lux	*PA4774::lux* transposon mutant and transcriptional fusion, 38_F9	Lewenza *et al.* (2005)
PA4774c.c	*PA4774::lux* chromosomally complemented with *PA4773-PA4775* integrated into *att*Tn*7* site	Johnson *et al.* (2012)
PA3553::lux	*PA3553::lux* transposon mutant and transcriptional fusion, 53_D10	Lewenza *et al.* (2005)
eddB::lux	*PA3909::lux* transposon mutant and transcriptional fusion, 41_A5	Mulcahy *et al.* (2010)
eddB::lacZ	*PA3909 lacZwpo894H8* mutant in PA01 background with ISlacZ/hah transposon, Tet^r	U Washington
*eddB::lacZ/*pEddB	*eddB::lacZ* complemented with *eddB* cloned into pUCP22, Amp^r	This Study
eddA::lacZ	*PA3910 lacZwpo1qtDo5* mutant in PA01 background with ISlacZ/hah transposon, Tet^r	U Washington
eddA::phoA	*PA3910 phoAwpo6q2Fo7* mutant in PA01 background with ISlacZ/hah transposon, Tet^r	U Washington

Strain	**Description**	**Reference**
eddA::phoA/pEddB	*PA3910 phoA* complemented with *eddB* cloned into pUCP22, Amp^r	This study
Δ*psl*	WFPA800 *psl* promoter deletion mutant	Ma *et al.* (2006)
Psl-OE	WFPA801 arabinose inducible *psl* promoter to over express Psl	Ma *et al.* (2007)
pelB::lux	*pelB::lux* transposon mutant and transcriptional fusion, 66_B7	Lewenza *et al.* (2005)
pelD::lux	*pelD::lux* transposon mutant and transcriptional fusion, 53_C4	Lewenza *et al.* (2005)
Plasmids	**Description**	**Reference**
pUCP22	Amp^r, multicopy broad -host-range expression vector	West *et al.* (1994)
pσ70-*lux*	Kan^r , low copy expression vector with σ70 promoter-*luxCDABE* fusion in pSC101	Davidson *et al* (2010)
Primers	**Application**	**5'-3' Sequence**
EddB-F	PCR amplification, pUCP22 cloning	AGAACAGTTCCCCCTTCG
EddB-R	PCR amplification pUCP22 cloning	ATGCAGAAAGGCCACCGAG
EddA-F	PCR amplification	TTTCCCCTGACCAAACGG
EddA-R	PCR amplification	ATACTCCTTTATCGGAATGG

[a] Abbreviations; Amp^r, ampicillin resistant; Kan^r, kanamycin resistance, Tet^r, tetracycline resistant

2.2 Construction and complementation of bacterial strains

2.2.1 Generation of lux-tagged reporter strain E. coli DH5α with pσ70-lux plasmid

We introduced a plasmid-encoded *lux* fusion that contained the sigma 70 promoter upstream of the *luxCDABE* genes into *E. coli* DH5α. This plasmid construct was provided by Dr. Michael Surette[174]. The plasmid (pSC101) with sigma (σ)70-*luxCDABE* fusion[174] was purified and introduced into competent *E. coli* DH5α using heat shock transformation. For each transformation, 50 µL of cells were mixed with 5 µL of plasmid and incubated on ice for 30 min flowed by a heat shock at 42˚C for 30s, without shaking. The tubes were then placed on ice for 2 min followed by the addition of 250 µL of pre-warmed LB media then placed in a 37˚C incubator for 1 hour and then plated on LB agar plates containing 50 µg/mL of kanamycin. Positive luminescent colonies were frozen for long-term storage in 7% DMSO (Sigma-Aldrich D8418).

2.2.2 Cloning of DNase and complementation of DNase mutant

The medium copy pUCP22 plasmid vector was used to clone the *eddB* gene for complementation analysis. The *eddB* gene was amplified using the EddB-F and EddB-R primers (Table 2.1). The PCR conditions were set as: 94°C for 2 min then denaturing at 94°C for 30s, annealing at 52°C for 30s, then extension at 74°C for 3 min and holding at 4°C. The PCR product was cloned into the TOPO vector (Invitrogen) in *E. coli* and then digested with *Eco*R1 and subcloned into the pUCP22 plasmid vector. The pUCP22 derived construct pEddB was electroporated into both the *eddB::lacZ* and *eddA::phoA* mutants. Electroporation conditions were set at Ec2 (25 µF, 200 Ω and 2.5 kV) on the MicroPulser (BioRad) and pulsed once. One millilitre of LB was added to the cuvettes to help recover transformed cells during 1 hour incubation at 37°C with shaking. After the incubation, 100 µL of cells were plated onto an LB

agar plate containing 100 µg/mL of ampicillin. A DNase supernatant assay was used to confirm the presence of active DNase (*eddB*), as described below. DNase positive transformants were frozen for long-term storage in 7% DMSO.

2.3 DNase supernatant assay

The DNase supernatant assay was performed as described by Mulcahy *et al.* (2010)[87]. *P. aeruginosa* strains were grown in DNase inducing condition; BM2 media with low phosphate (400 µM) supplemented with 10 mM $MgSO_4$ and 10 mM $CaCl_2$. Overnight cultures of the designated strains were normalized to an OD of 1.0 (measured at 600 nm). The supernatants were collected by centrifugation at 800 rpm for 10 min. Fifteen microliters of supernatant was incubated with 2 µg of chromosomal salmon sperm DNA (ssDNA) (BioLynx Inc. UB14405S2) for 3 hours at 37°C. DNA degradation was visualized on ethidium bromide stained 1% agarose gels.

2.4 Neutrophil isolation

Neutrophils were isolated from healthy donors (Ethics approval ID#23187) using a protocol previously described[128] (Table 2.2). All media and solutions were kept at 4˚C for the isolation. Blood from the donors was collected in a 30 mL syringe that contains 4 mL of ACD. The ACD is prepared by adding 7.36 g Citric Acid, 14.71 g Sodium Citrate and 9.91 g Dextrose (Aldrich 158708500G) into 250 mL ddH_2O. Typically 20 mL of blood was collected from each blood draw. The ACD-Blood mixture was then gently emptied into a 50 mL conical tube containing 12 mL of a 6% Dextran/0.9% NaCl solution (Dextran- Fluka: 09184-506-F). The mixture was then allowed to settle for 45-60 min, or until separation of RBCs and plasma was complete. After this time, the supernatant (plasma) was pipetted into a fresh 50 mL conical tube and centrifuged (Eppendorf Centrifuge 5810R) for 12 min at 1150 rpm at 4˚C with a low brake

applied for stopping. The supernatant was discarded and the pellet resuspended in 12 mL ddH_2O. After 20s, 4.0 mL of KCl solution was added and the solution is then diluted to 50 mL with PBS and inverted several times. The tube was then spun at 1300 rpm for 6 min at 4˚C using a high brake. These steps were repeated until the pellet appears white, with no RBCs remaining. Once completed, the supernatant was discarded and the pellet resuspended in 2.5 mL of PBS. The mixture was then layered on top of 3 mL of histopaque (Ficol-Hypaque density media) (Sigma 1077) then spun at 1500 rpm for 30 min at 4˚C using a low brake. After this spin the supernatant was removed using a transfer pipet and the pellet resuspended in 2 mL of $HBSS^-$ (no cations) (Invitrogen 14175-095) Finally, the cell concentration was determined using a haemocytometer where by 10 µL of a 1 in 10 dilution was mixed with 10 µL of Trypan blue (used to stain the cells) (Invitrogen 15250).

Table 2.2. Donor list of volunteers for neutrophil isolation

Donor	Gender	Age Range	Smoker (Y/N)	Time in Study
D001	M	20-25	N	03/ 2011– Present
D002	F	30+	N	03/2011 – 06/ 2012
D003	F	20-25	N	05/ 2011 – Present
D004	F	25-30	N	05/ 2011 – 08/2011
D005	F	25-30	N	05/ 2011 – Present
D006	F	30+	N	05/ 2011 – 06/ 2012
D007	M	20-25	N	05/ 2011 – 08/ 2012
D008	M	20-25	N	05/2011 – Present
D009	F	20-25	N	06/ 2011 – Present
D010	M	25-30	N	11/ 2011 – Present
D011	M	30+	N	11/ 2011 – 08/ 2012
D012	M	20-25	N	11/ 2011 – Present
D013	F	20-25	N	02/ 2012 – 08/ 2012
D014	F	18-20	N	06/ 2012 – 08/ 2012
D015	M	18-20	N	06/ 2012 – 08/ 2012
D016	M	20-25	N	09/ 2012 – Present
D017	F	30+	N	09/ 2012 – 01/ 2013
D018	M	30+	N	11/ 2012 – Present

2.5 NET quantification assay

The quantification of NETs was performed using a protocol previously described[128]. Cell culture media (CCM) was made by adding 48.5 mL of RPMI 1640 (Invitrogen 11875), 0.5 mL of 1.0 M HEPES and 1.0 mL of human serum albumin (HSA) (Innovative Research 11144) (2%) to a 50 mL conical tube. A total of 100 mL of CCM was needed for the assay. Two 15 mL conical tubes were filled with CCM. The first tube contained 240 µL of cytochalasin D (from a stock of 1 mg/mL) (Sigma-Aldrich 331562) added to 5.76 mL of CCM to make a final concentration of 40 µg/mL. The second tube contained 2.4 mL of DNase I (stock solution 25 800 kunitzU/mL) (VWR 31149) to 3.6 mL of CCM to have a final concentration of 10 000 kunitzU/mL. Isolated neutrophils were diluted into CCM to make a final concentration of $2.0x10^6$ cells/mL. A 96-well black, clear-bottom plate (Thermo Scientific 265301) was coated with HSA and allowed to air dry in the Biological safety cabinet (BSC). Once dried, 100 µL of the isolated neutrophils were added to the 96-well plate. The cells were allowed to adhere to the plate for 30 minutes, incubated at 37˚C in 5% CO_2 incubator. From the prepared tube of cytochalasin D, 50 µL of solution was added to every well and the plate was incubated for 15 min at 37˚C. The addition of cytochalasin D was meant to inhibit phagocytosis as previously described[30; 149; 155; 175]. Mid-log bacterial cultures were used for all experiments (OD_{600} = 0.2-0.4). Cultures were diluted in CCM to obtain $2.0x10^7$ CFU/mL and 100 µL was added to the wells to get a multiplicity of infection (MOI) of 10:1 (bacteria to neutrophils). For conditions of bacteria alone, 100 µL of bacteria was added to 100 µL of fresh CCM. For DNase treated samples, the designated wells has 50 µL of DNase added. The plate was then centrifuged (Eppendorf Centrifuge 5810R) at 800 x gravity (Gs) for 10 min. Next, 10 µL of Sytox green (5 mM stock) (Invitrogen 57020) was added to each well at a concentration of 2.5 µM and

fluorescence (Green: Ex 490/8; Em 535/25) was measured with the plate reader (Perkin Elmer 1420 Multilabel Counter Victor^3 or Perkin Elmer 2030 Multilabel reader Victor 3X) for 1 to 4 hours.

2.5.1 NET microscopy in 96-well microplates

Following the NET-quantification assay described above, the plate was removed from the Victor^3 plate reader after a 2-3 hour incubation, once a strong signal of Sytox green had been detected. The clear bottom plate was viewed using the inverted Leica DMI 4000B microscope equipped with an ORCA R2 digital camera and Metamorph software (Quorum Angstrum Optigrid) for image acquisition using the 63X objective. To measure green fluorescence, the excitation Ex 490/20 and emission Em 525/36 filters were used.

2.6 NET microscopy on glass cover slips

Glass cover slips were placed in each well of a 6-well tissue culture plate (BD Falcon 353046) and coated with HSA then allowed to air dried in the BSC. Isolated neutrophils were then added to each designated well at $2.0x10^6$ cells/mL (volume = 1.0 mL). The neutrophils were incubated in the 5% CO_2 incubator at 37˚C for 30 min to allow the neutrophils to adhere to the cover slip. Cytochalasin D (stock 1 mg/mL) was added (250 µL) to each well to give a final concentration of 10 µg/well. The plate was allowed to incubate for 15 min in the 5% CO_2 incubator at 37˚C. PMA was then added (100 µL) to the wells at 250 nM/mL (25 nM/well). PMA is added to activate the neutrophils as previously described[107]. The plates were then incubated in the 5% CO_2 incubator at 37˚C for 15 min. Mid-log bacteria were diluted in $HBSS^-$ to make $1.0x10^8$ CFU/mL sample. One millilitre of bacterial sample was added to the designated wells to give a final concentration of $5.0x10^7$ CFU/mL (MOI of 50:1). For wells that do not contain bacteria or of just bacteria alone, 1.0 mL of $HBSS^-$ was added. The plate was then

centrifuged at 800 x gravity (Gs) for 10 min, then incubated for 1-4 hours in the 5% CO_2 incubator at 37˚C. Following the incubation the slides were fixed with 4% paraformaldehyde (VWR BDH0502-1LP) diluted in $HBSS^-$ and incubated in the 5% CO_2 incubator at 37˚C for 30min. The cover slips were washed with 250 µL of 10% FBS (Invitrogen A12617DJ) in PBS then treated with either DNA stains and/or various primary and secondary antibodies.

2.6.1 Extracellular DNA staining of NETs

After the slides had been fixed with 4% paraformaldehyde, various combinations of DNA stains were used to visualize the NETs (Table 2.3). After the DNA dyes were added to the samples, the plate was covered in aluminum foil and incubated for 30 min at room temperature. The cover slips were washed three times with sterile ddH_2O or $HBSS^-$ and allowed to air dry for 20 min. Once dry the cover slips were mounted onto slides with polyvinyl alcohol mounting media with DABCO, anti-fading (Fluka 100931400). The slides were then viewed under the microscope.

2.6.2 Anti-myeloperoxidase antibody visualization

For NET visualization with antibodies, the anti-human MPO antibody (3.2 g/L) was diluted into 10% FBS in PBS (1/500) and 30 µL was added to the cover slips and incubated for 30 min at 37˚C then washed twice with sterile PBS. Next, 40 µL of secondary antibody (anti rabbit Cy 5) diluted in 10% FBS in PBS was added to each well along with 100 µL of the DNA stain DAPI (stock 5 mg/mL), which gave a final concentration of 5 µg/mL. The plate was covered with aluminum foil and allowed to incubate for 15 min at room temperature. Following the incubation the cover slips were washed twice with PBS and then mounted on slides with mounting media and viewed under the microscope.

2.6.3 NET visualization with anti-DNA and anti-histone antibodies

Anti-DNA and anti-histone antibodies where obtained from Dr. M. Fritzler (Table 2.3). The antibodies were identified and validated from auto-immune patient sera. The anti-histone antibodies were identified and validated according to Fritzler *et al.* (1982)[176]. Antibodies to double stranded DNA were detected by the *Crithidia lucilliae* indirect immunofluorescence test and other nuclear antibodies by immunodiffusion, and then validated by an addressable laser bear immunoassay[176; 177; 178]. After the cover slips were fixed and washed, the primary antibodies were then added, either the anti-DNA or the anti-histone. From the stocks, the anti-DNA and anti-histone antibodies were diluted in 10% FBS in PBS (1 in 10 and 1 in 500, respectively). From the diluted solutions, 30 µL of primary antibody was added to the respective wells and incubated for 30 min at 37°C then washed twice with PBS. Next, 20 µL of anti-human secondary antibodies were added which contain the Alexa Flour 647 fluorophore and diluted in 10% FBS in PBS (1/500). The cover slips were allowed to incubate for 15 min at room temperature, covered with aluminum foil then washed twice with sterile PBS and mounted onto slides with mounting media and viewed under the microscope.

2.6.4 Analysis of NET structures under various treatments

Cover slips are prepared as described above and isolated neutrophils were coated onto the cover slips and activated with PMA and cytochalasin D. No bacteria were added; instead the NETs were treated with DNase, phosphatase (PTase) or Mg^{2+}. For wells designated for Mg^{2+} treatment, 100 µL of 10 mM $MgSO_4$ was added to the wells. For the addition of DNase, 100 µL of 2580 kunitzU/mL DNase I was added to the wells and for the PTase condition, 100 µL of 500 U/mL calf intestinal alkaline phosphatase (CIAP) (Invitrogen 18009-027) was added to the designated wells. Untreated wells had 100 µL of $HBSS^-$ added. The plates were incubated for 1-4

hours in the 5% CO_2 incubator at 37˚C then fixed with 4% paraformaldehyde in $HBSS^-$ and incubated again in the 5% CO_2 incubator at 37˚C for 30 min. The cover slips were then washed with 250 µL of 10% FBS in PBS. Using the staining techniques previously described the samples were treated with the anti-MPO antibodies and the anti-rabbit Cy5 secondary antibody were used to visualize the NETs. DAPI was also used to stain extracellular DNA and help visualize the neutrophils. Once prepared the cover slips were mounted onto slides and viewed under the microscope.

2.6.5 Microscope imaging analysis

All slides were viewed using the Leica DMI 4000B inverted microscope equipped with ORCA R2 digital camera and Metamorph Quorum Angstrum Optigrid software for image acquisition using the 63X or 100X objectives. The following excitation and emission filters were used for blue fluorescence (Ex 390/40; Em 455/50) red fluorescence (Ex 555/25; Em 605/52), far red fluorescence (Ex 645/30; Em 705/72) and green fluorescence (Ex 490/20; Em 525/36). These images are then formatted and analyzed using the Imaris 7.0.0 imaging software.

Table 2.3. List of DNA stains and antibodies used for NET microscopy

Stain	Stock	Dilution*	Volume Added	Product Information
Sytox Red	5 µM	1 in 10	100 µL	Invitrogen- 534859
Syto 9	5 mM	1 in 1000	100 µL	Sigma- 34854
PI	1 mM	1 in 200	100 µL	Sigma- P4170
TOTO-1	1 mM	1 in 200	100 µL	Invitrogen- T3600
DAPI	500 mg/mL	1 in 1000	200 µL	Invitrogen- D1306
Primary Antibody				
Anti-MPO	3.2 g/L	1 in 500	50 µL	DakoCytomation- A0398
Anti-DNA	-	1 in 10	30 µL	Fritzler *et al.* (1982)
Anti-Histone	-	1 in 500	30 µL	Tan *et al.* (1982)
Secondary Antibody				
Anti-rabbit Cy 5	1.5 mg/mL	1 in 500	40 µL	R DeVinney Jackson ImmunoResearch 60354
Anti-rabbit Alexa Fluor 350	2.0 mg/mL	1 in 500	40 µL	R DeVinney Invitrogen A21068
Anti-human Alexa flour 647	2.0 mg/mL	1 in 500	40 µL	R DeVinney Invitrogen A21445
*Dilutions are done in 10% FBS in PBS				

2.7 NET killing assay - Plate counts

Neutrophil extracellular trap killing has often been examined using plate counting methods where a reduction in cell number (CFU/mL) indicated bacterial killing[30; 107]. Isolated human neutrophils were prepared and treated in a black, clear-bottom 96-well plate as described above. Wells that will not contain any neutrophils are filled with 100 µL of fresh CCM. Mid-log bacteria were diluted in CCM to give a concentration of $2.0x10^6$ CFU/mL (an MOI of 1:1). One hundred microliters of bacteria were added to 100 µL of neutrophils. Bacteria alone conditions were just added to 100 µL of fresh CCM. A solution of 2580 kunitzU/mL DNase I was then added to the appropriate wells at 50 µL, while the remaining wells have fresh CCM added to them. From the wells, 15 µL was removed and added to 135 µL of $HBSS^-$ solution and a series of 1/10 dilutions was done in a fresh clear 96-well plate (BD Falcon 353072). Following the serial dilutions, 5 µL of sample was stamped onto LB agar plates to obtain plate count data for time zero (T_0). The 96-well plate was then placed in the centrifuge and spun for 10 min at 800 x gravity at room temperature then incubated for 1 hour. Following the incubation, 50 µL of DNase I solution was added to every well, mixed, and allowed to incubate for 30 min at 37°C. This DNase was added to release any bacteria trapped in the NETs. Another serial dilution was performed as previously described to generate a 1 hour time point (T_1) plate count. The plate count data from T_1 was normalized with the T_0 time point. The conditions with neutrophils were compared to the bacteria alone conditions, as well as the bacteria with neutrophils and DNase conditions. The percent survival was calculated by subtracting the T_0 plate counts from the T_1 plate counts and dividing the bacteria and neutrophil conditions (with or without DNase) by the bacteria alone conditions and multiplying by one hundred.

2.7.1 Modifications to NET killing assay - Protection to NETs

Repeated plate killing assays are modified to use $HBSS^-$ instead of the CCM to prevent the addition of excess cations, which may have inhibited DNA killing, The modified plate killing assay was also used to examine the effects on NET killing when samples were treated with DNase, PTase and Mg^{2+}. The plates were prepared as described above, up until the incubation. Once the bacteria had been added to the appropriate wells, solutions of the various treatments were added to specific wells. For DNase treated samples, 50 µL of a 2580 kunitzU/mL DNase I solution was added to the appropriate wells. For PTase treated wells, 50 µL of a 100 U/mL was added and for the Mg^{2+} treated cells, 50 µL of a 30 mM $MgSO_4$ (in HBSS-) was added to the wells, for a final volume 5 mM Mg^{2+}. The incubation time was increased to 4 hours to allow for greater interaction with NETs. Following the incubation the samples were treated with a DNase to free any trapped bacteria from the NETs. Plate counts were done at T_0 and T_4 to measure the protective effects of each treatment on *P. aeruginosa* PA01. The CFU counts were used to calculate the percent survival of the bacteria as described above.

2.8 Viability and gene expression assay with extracellular DNA

Overnight strains of various *lux*-reporter strains were subcultured into 10 mL of BM2 media with 0.5 mM Mg^{2+} and allowed to grow for 3-4 hours (to reach an OD_{600} of 0.2-0.4). Sodium phosphate (NaP) buffer was added to each well of a black clear bottom 96-well plate (100 µL to each well). A 4% ssDNA solution (w/v) was made by adding 4.0 mg to 10 mL NaP buffer. Once made, 100 µL of solution was added to the row 1 and 7 of a black, clear-bottom 96-well plate to create a 2% DNA solution. Next, 1 in 2 serial dilutions were made by transferring 100 µL of solution form row 1 to row 2 and continued until row 6. This was repeated for rows 7 to 10. Fifty microliters of 2580 kunitzU/mL DNase, 100 U/mL PTase, or 30 mM $MgSO_4$ was

added to the appropriate wells. The plate was then allowed to incubate at 37°C for 1 hour to permit the treatments to act on DNA. Following the incubation, mid-log cultures were washed twice with PBS then 100 µL of bacteria were added to the wells at a concentration of $2.0x10^7$ CFU/mL. The plate was then placed in the Wallac Victor^3 reader and the gene expression or viability (luminescence, CPS) was measure for 2 hours, with reading every 10 minutes. After the reading, the plate was removed and samples were stamped (5 µL) on LB agar plates and placed in the 37°C incubator overnight.

2.8.1 Viability and gene induction assay with NETS

A black, clear-bottom plate was prepared with adhered human neutrophils, which were activated as described above. Mid-log bacteria were diluted in $HBSS^-$ to give a concentration of $2.0x10^7$ CFU/mL, which gave an MOI of 10:1 (bacteria to neutrophils). A solution of 2580 kunitzU/mL DNase I was added to the appropriate wells at 50 µL. For PTase treated wells, 50 µL of a 100 U/mL CIAP was added to those designated wells. For the Mg^{2+} treated cells, 50 µL of a 30 mM $MgSO_4$ was added to the wells with a final concentration of 5 mM Mg^{2+}. Untreated wells were had 50 µL of $HBSS^-$ added to ensure the volumes of all wells are equal. The plate was placed in the centrifuge and spun at 800 x gravity for 10 min at room temperature. The plate was then placed in the Victor^3 plate reader and the optical density (growth, OD_{600}) and gene expression (luminescence, CPS) was read every 20 minutes for 3-4 hours. Depending on the strain used, the CPS can be used to determine gene expression or viability. Plate counting methods, described above, were also done in combination.

2.9 Bacterial viability using flow cytometry

The samples for flow cytometry were prepared in a black, clear-bottom 96 well microplates in the exact same way as described in the luminescence gene induction and viability

assays. Before the plate was placed in the centrifuge for the final spin, 15 µL was removed from the wells and placed in 135 µL of $HBSS^-$ solution for a dilution series (done in a clear 96 well plate). The dilutions were stamped on LB agar plates (5 µL) to generate T_0 plate counts. The sample plate was then placed in the $Victor^3$ plate reader at 37°C to read the wells that contain reporter strains (as described above). The plate was incubated for 3-4 hours then T_4 plate counting was done in the same fashion as the T_0 point. The remainder of the sample (~200 µL) was placed in 5 mL polystyrene round-bottom sample tubes, stained with the live/dead reagent kit (Molecular Probes) to measure bacterial viability. The stains used were Syto 9 (3.34 mM stock) and PI (20 mM) stock; 2 µL of each dye was added to the tubes giving a final concentration of 0.02 mM and 0.2 mM, respectively. One tube of just mid-log bacteria was left unstained to determine the quadrant gating parameters for the unstained events. After the dyes were added the tubes were quickly spun at 300 x gravity using a bench-top centrifuge and allowed to incubate at room temperature for 15-20 min.

The tubes were then analyzed using the BD LSRII flow cytometer (BD Bioscience, San Jose, USA) equipped with a blue laser (488nm) and a green laser (532nm). Unstained, mid-log cells were used to gate the forward scatter (FSC) and size scatter (SSC) parameters, while stained sampled were analyzed with the FSC and SSC signals along with green and red signals. For fluorescence signalling, the appropriate PMT (photomultiplier tube) voltages were used; for the PE-TR (PI- 532 nm with the Bandpass filter 610/20 and Longpass filter 600) and FITC (Syto9-488 nm with Bandpass filter 525/50 and Longpass filter 505) parameter. All detectors were set to the logarithmic amplification with the following voltages, 500, 240, 596, and 489 and threshold was set at 200 for both FSC and SSC. For each sample, 50 000 events were acquired using the BD FACSDiva software 6.1.3. The Hierarchical gating strategy was used to determine double

positive population of cells (stained with both Syto 9 and PI). Gate P1 is the total population of FSC and SSC gated events, as determined from bacteria alone control and then applied to all other samples. P2 is the population of events stained by Syto 9 and P3 is the population stained with both Syto 9 and PI. For graphical representation, the files were analyzed using the FlowJo software and the FACSDiva software was utilized to determine percentage of cells stained with both dyes. Graphical representation of the samples was done with PI vs. Syto 9 gradient gating. The bi-exponential scale was used for the samples.

2.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism v4.0 software. One-way ANOVA with Bonferroni posts tests and Student T-Tests were used to calculate significant differences between the various conditions for the plate count, CPS and flow cytometry, live dead staining. Significant differences refer to P-values < 0.05.

Chapter Three: *Pseudomonas aeruginosa* is a weak NET inducer and is tolerant to NET killing

3.1 Establishing the methodology to monitor bacterial killing

Since the discovery of NETs a variety of methods have been established to isolate, visualize and quantitate the release of these structures. When working with human neutrophils, the first step is to properly isolate the cells with minimal activation. Using a protocol previously described, neutrophils were isolated from whole blood separating them from red blood cells using a combination of gradient separation and hypotonic lysis[128]. To separate the neutrophils from the resulting cell suspension, gradient centrifugation was used[128]. Proper care was needed to prevent early activation of the cells. Once isolated, the neutrophils were treated with various compounds that allow for the generation of the extracellular traps. Both PMA and cytochalasin D have been shown to help activate the neutrophils to produce NETs and inhibit phagocytosis, respectively[30; 107; 155; 175]. Both compounds together allow for optimal NET production. To access and validate the NET production, previously described microscopy and extracellular DNA quantification methods were used[128; 179]. Both methods involve staining extracellular DNA released from the neutrophils as an indicator for NET production. Using these methods, we moved forward to monitor the ability of *P. aeruginosa* to survive exposure to these traps. Previous studies have shown that *P. aeruginosa* is tolerant to NETs compared to other bacteria, such as *S. aureus*[30; 126; 147; 150].

3.2 Visualization of NET structures

3.2.1 DNA-staining to image NETs

The NET structure is made up of a lattice of neutrophil DNA laced with the various granular contents found within the neutrophil. By using DNA-stains, we visualized the NET structure by targeting the DNA backbone of the extracellular traps. Isolated neutrophils were

allowed to adhere to cover-slips and treated with cytochalasin D. After an hour of incubation, the cells were fixed and stained to visualize the NETs. We used a combination of stains, which included Sytox red, Syto 9, Propidium iodide (PI) and TOTO-1. Sytox red is a dead eukaryotic cell stain that has a high-affinity to nucleic acids. The stain can easily penetrate cells with compromised plasma membranes but will not cross the membrane of metabolically active cells. Syto 9 is a green fluorescent nucleic acid stain that shows a large fluorescence enhancement upon binding nucleic acids, such as RNA and DNA in both live and dead eukaryotic cells, as well as in Gram-positive and Gram-negative bacteria. Propidium iodide is a nucleic acid dye that can stain both DNA and RNA and is membrane impermeant and generally excluded from viable bacterial cells. PI is commonly used for identifying dead bacteria in a population and as a counterstain in multicolor fluorescent techniques. TOTO-1 dye is a cell-impermeant, high-affinity nucleic acid stain based upon a symmetric cyanine dye dimer that is almost nonfluoresent in the absence of extracellular nucleic acids.

Using two combinations of DNA dyes, Sytox red and Styo 9 (Figure 3.1A), and TOTO-1 and PI (Figure 3.1B), the DNA in the neutrophil extracellular traps from neutrophils activated with PMA was observed. With the Sytox red stain, a unique staining pattern was seen where the dye appeared to stain granular like structures within inactivated neutrophils, but also extracellular DNA in NETs (Figure 3.1A). We were able to confirm that PMA induced NET formation, which were readily stained with Syto 9, TOTO-1 and Sytox red.

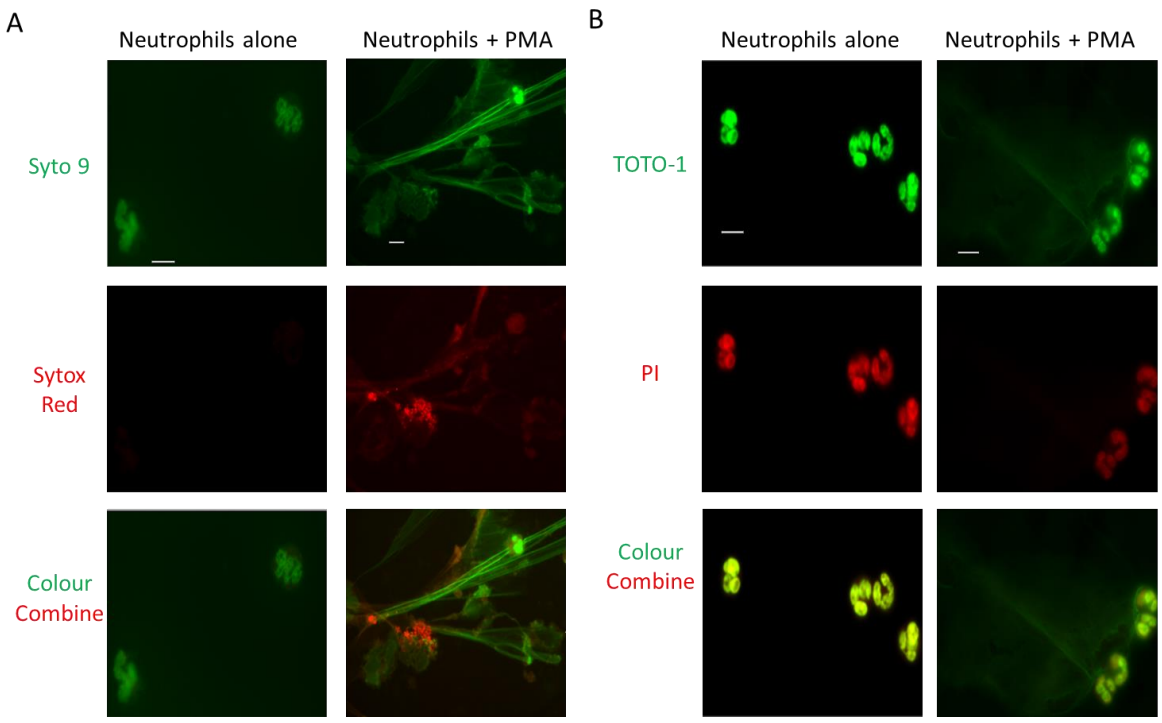


Figure 3.1. Staining of PMA-induced NETs with DNA stains.

NETs were stained with two combinations of dyes (A) Syto 9 and Sytox Red and (B) TOTO-1 and PI. The top panel of images shows green fluorescence of neutrophils unstimulated or stimulated with PMA. The middle panels show red fluorescence of unstimulated and stimulated neutrophils. The bottom panel shows the merge images of the above panels. All images were taken after 1 hour of PMA treatment using the 63X objective. All images were captured with similar acquisition settings. The scale bar represents 10 μm.

3.2.2 NET visualization using Sytox green

After visualization of NETs, we then quantitated NET formation using Sytox green, another extracellular DNA stain as previously described[128]. Sytox green fluorescence was measured to estimate and compare the amount of DNA released from neutrophils when exposed to PMA, *P. aeruginosa* PA01, or *S. aureus* ATCC 25923[128]. Sytox green is a high affinity nucleic acid stain that penetrates cells with compromised plasma membranes. However, it will not cross the membranes of live cells and is a useful stain for extracellular DNA in NETs.

Bacterial-neutrophil coincubations in 96-well microplates (clear bottom) were monitored for Sytox green and NET formation throughout a 3 hour window. After 3 hours, the microplate was imaged using an inverted microscope to visualize the NETs. In Figure 3.2, it is shown that under non-activating conditions, there was a low, basal level of eDNA released from the neutrophils. When incubated with PMA, *P. aeruginosa* PA01, or *S. aureus* ATCC 25923, there was a qualitative increase in Sytox green staining, indicating that there was more DNA and NETs released from these neutrophils (Figure 3.2). These images confirm that only extracellular DNA in NETs, or neutrophils that may have a compromised membrane, were stained with Sytox green. This supports previous publications showing that DNA can be detected from neutrophils that have released the DNA as a NET or are currently undergoing the NETosis process. Quantitative analysis of NET formation is discussed later. For further image analysis and to confirm the presence of true NET structures, immunostaining for proteins and antimicrobial factors known to be present in NETs was performed.

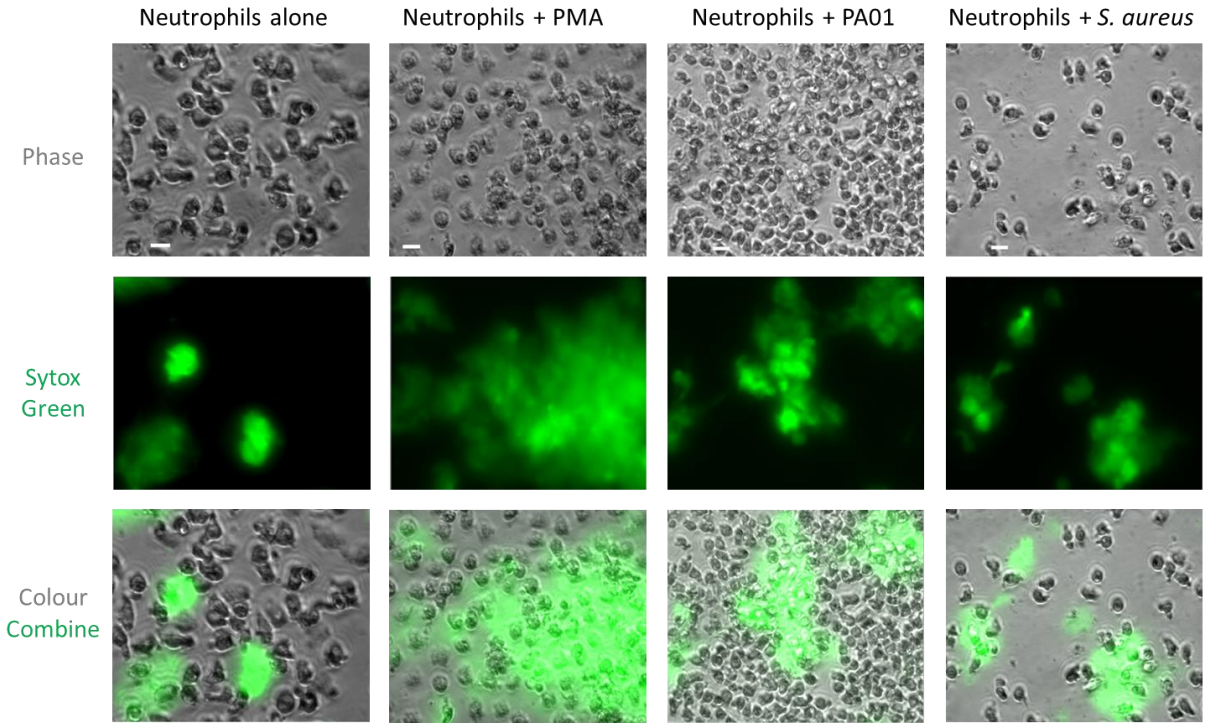


Figure 3.2. Sytox green staining of extracellular DNA released from neutrophils.

The top panel of images shows phase contrast of untreated neutrophils, or stimulated neutrophils with PMA, *P. aeruginosa* PA01 or *S. aureus*. The middle panels show Sytox green fluorescence and eDNA release. The bottom panel shows the merge of the above panels. All images were taken after 3 hours of neutrophil and bacterial co-incubation using the 63X objective. The scale bars represent 10 μm.

3.2.3 Using anti-MPO antibodies to image NETs

Another method to visualize NET structures is to target specific components of the NETs, and to show colocalization with DNA. Myeloperoxidase (MPO) has been previously shown to be incorporated in NETs though proteomic analysis and immunofluorescence assays[102; 107; 140; 145]. MPO is a peroxidase enzyme that is found in neutrophil granulocytes[140]. It is a lysosomal protein that is stored in azurophilic granules of the neutrophil. MPO plays an important role in oxidant metabolism in neutrophils and is a key contributor to phagosomal killing[180; 181]. The enzyme reacts with H_2O_2 and chloride ions to generate HOCl, which is a powerful antimicrobial oxidant. In previous studies, MPO has been shown to be a constituent of NETs, and the use of anti-MPO antibodies allowed for specific visualization of MPO in DNA-containing NET structures[182].

Following NET induction, neutrophils were adhered to cover slips and activated with PMA, *P. aeruginosa* or *S. aureus.* The cells were incubated with the primary polyclonal rabbit anti-human MPO antibody to bind to the MPO, and then to a secondary goat anti-rabbit-Cy 5 antibody in order to visualize the MPO in NETs. The fluorophore Cy-5 (Cyanine-5) has fluorescence emission in the red or far-red spectrum. In combination with the anti-MPO antibody, the nucleic acid stain DAPI was used to bind to DNA, inside or outside the neutrophil. DAPI is a nucleic acid stain that binds strongly to the A-T rich regions in DNA. DAPI can pass through intact cell membranes and can be used to stain both live and fixed cells. In Figure 3.3, the MPO is shown in red (Cy 5) and the DNA is shown in blue (DAPI). The nuclei of the neutrophils tend to stain stronger than extracellular DNA using DAPI. There was staining of extracellular DNA that colocalized with anti-MPO staining, but this pattern was faint compared to that of the nucleus-staining pattern. Both *P. aeruginosa* and *S. aureus* were able to induce NETs decorated with MPO, which has been previously reported[130; 140].

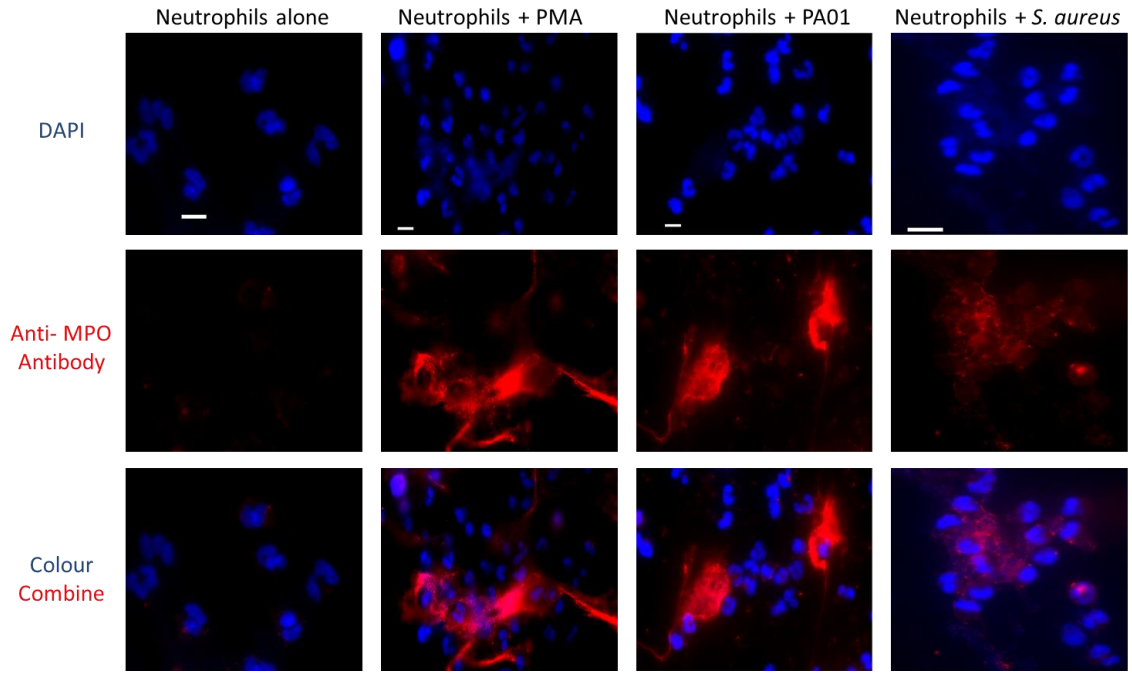


Figure 3.3. Neutrophil extracellular trap imaging using anti-MPO antibodies and DAPI.

The top panel of images shows DAPI stained neutrophils unstimulated, or stimulated with PMA, *P. aeruginosa* PAO1 and *S. aureus*. The middle panels shows anti-MPO antibody binding in NETs. The bottom panel shows the merged image of the above panels. All images were taken after 3 hours of co-incubation using the 63X objective. The scale bars represent 10 μm.

3.2.4 Using anti-DNA and anti-histone antibodies to image NETs

Along with MPO, histones are also incorporated into the NET structure[107]. Histone-derived peptides are able to function as effective antimicrobial agents and promote bacterial lysis[141; 143]. Both histones and MPO are commonly used as markers of NETs, when shown to colocalize with DNA. The anti-DNA and anti-histone antibodies were identified, validated from auto-immune patient sera and provided by Dr. Marvin Fritzler[176; 177; 178]. Neutrophils were seeded onto cover slips and activated with PMA and cytochalasin D and incubated for up to 30 min. Mid-log bacteria, Gfp-tagged *P. aeruginosa*[170] were then added to the coverslips and incubated for up to 4 hours, followed by fixing and washing. The primary antibodies were added, either the anti-DNA or the anti-histone, and the secondary antibody, anti-human Alexa Flour 647 was used next. The fluorophore, Alexa Flour 647 is a fluorescent dye with an absorbance at 650 nm and emission at 665 nm showing up in the red spectrum. Some slides were prepared using the anti-human MPO antibodies with the secondary antibody, anti-rabbit Cy 5.

Distinct NET structures were visualized with all three antibodies (Figure 3.4). The images confirm that DNA, histones, and MPO are incorporated in the NETs. The merged images show bacteria (PA01 Tn*7::gfp*) trapped in the NETs. There is a subtle difference in the localization of bacteria and antibody signal for the different antibodies. For both the anti-DNA and anti-histone antibodies, there was a strong overlap with antibody and bacteria. With respect to the anti-MPO antibodies there was a more distinct spacing, in that the bacteria appeared to "fill in" the areas where there was minimal anti-MPO signal. Following the imaging of NETs, we moved to quantitative assays to measure the amount of NETs released when exposed to either bacterial or chemical stimuli.

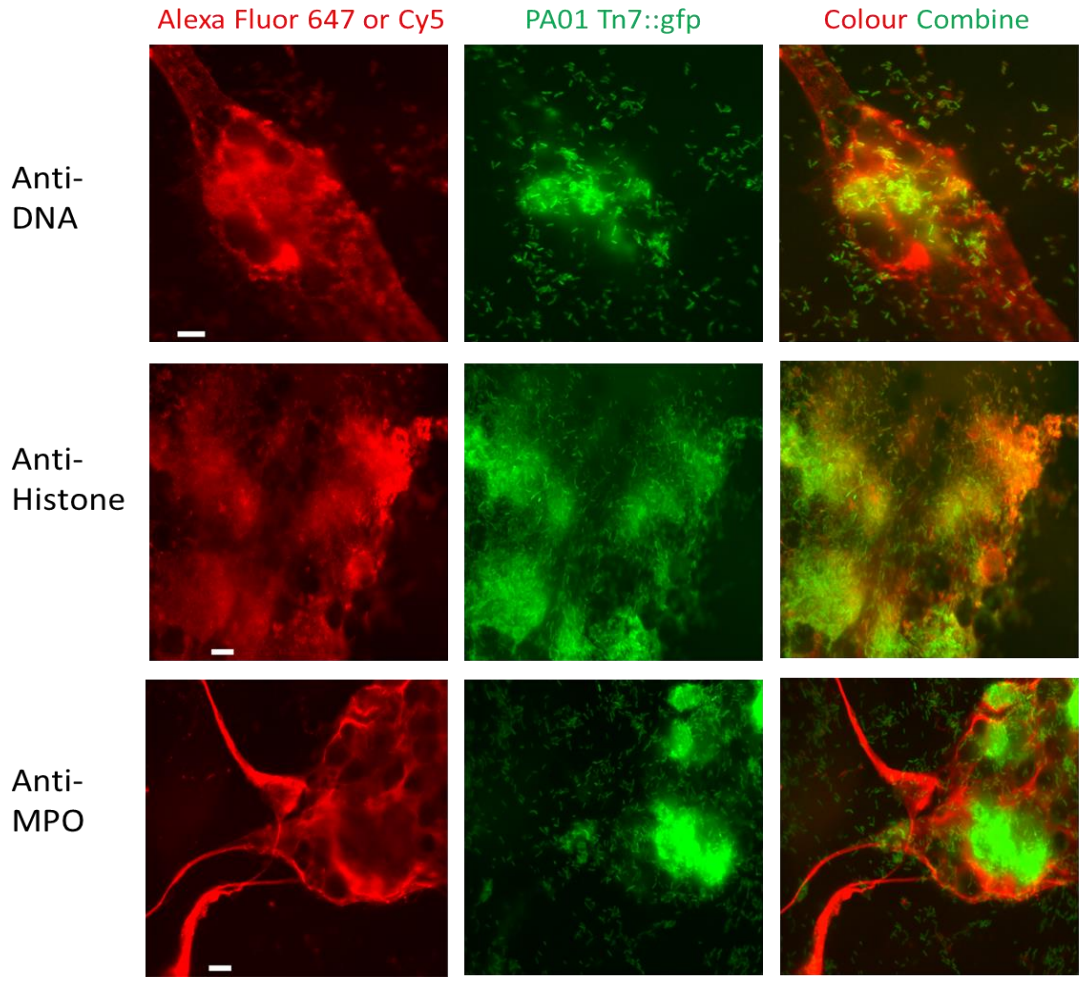


Figure 3.4. Bacterial aggregates in NETS visualized anti-DNA and anti-histone antibodies

The left column of images shows red fluorescence of antibody (anti-DNA, anti-histone, or anti-MPO) labelled NETs. The middle column shows the green fluorescence of *P. aeruginosa* Tn*7::gfp* expressing stable GFP. The right column is shows the merge of the previous panels. All images were taken after 3 hours of co-incubation using the 63X objective. The scale bars represent 10 μm.

3.3 Induction of neutrophil extracellular traps

Various compounds have been identified to induce NETs and NETosis, such as bacteria or inflammatory signals[102; 108; 125]. Even though NET formation is stimulated by pathogens, it is not clear if the process of NETosis during host-pathogen interactions is a programmed mechanism, a high-jacking of host pathways by pathogen factors, or an incidental component of neutrophil lysis[102]. When first discovered, it was demonstrated that the formation of NETs is the last step in a process of active neutrophil death[128]. This response occurred when exposed to either PMA, as well as to *S. aureus* and that over the period of approximately three hours[100; 128]. A study by Pilsczek *et al.* showed that *S. aureus* could induce NETs in a more rapid fashion than previously described[128]. It was also shown that this rapid nuclear neutrophil extracellular trap formation is induced by Gram-positive bacteria *in vivo*[133]. Since different bacteria are able to induce NETs, we compared bacterial induction of NETs and quantified the release of DNA.

3.4 NET quantification using Sytox green

Having established an assay to isolate neutrophils and induce NETs with PMA or bacterial stimuli, we wanted to quantify the NETs formed by these various stimuli. The neutrophils were incubated with PMA or the bacteria of interest for 1 to 3 hours. The amount of signal detected was measured on 1420 Mulitlabel Counter Victor 3V fluorescence plate reader. It has been shown that *S. aureus* is able to induce NETs to a much greater magnitude and at an earlier time point than other Gram-positive and even Gram-negative bacteria, including *P. aeruginosa*[128]. After the first hour of NET induction (Figure 3.5A), both *S. aureus* and *E. coli* induced more NET formation than *P. aeruginosa*. At the early time point of 1 hour, *P. aeruginosa* did not increase the amount of extracellular DNA released from the neutrophils above the background levels from unstimulated neutrophils (Figure 3.5A). To confirm that Sytox

green was detecting extracellular DNA, exogenous DNase I was added to the samples. DNase I addition reduced the Sytox green signal and thus reduced the amount of eDNA staining both in stimulated and unstimulated neutrophils. After 3 hours of co-incubation, the amount of eDNA released from neutrophils increased significantly when stimulated with PMA, *S. aureus, E. coli*, or *P. aeruginosa* (Figure 3.5B). The presence of exogenous DNase I after 3 hours of incubation still caused a reduction in Sytox green staining, and thus NET formation in all samples. These data suggest that *P. aeruginosa* is a weak inducer of NETs at early time points when compared to *S. aureus* and *E. coli*, consistent with data from previous publications[128]. Using Sytox green staining, along with the NET quantification assay, showed that *P. aeruginosa* was able to induce NETs, however this appears to be delayed compared to that of *S. aureus* and *E. coli* and even that of the known activator PMA. Since it appears that *P. aeruginosa* is a weak inducer of NETs, we then examined its ability to survive exposure to NETs.

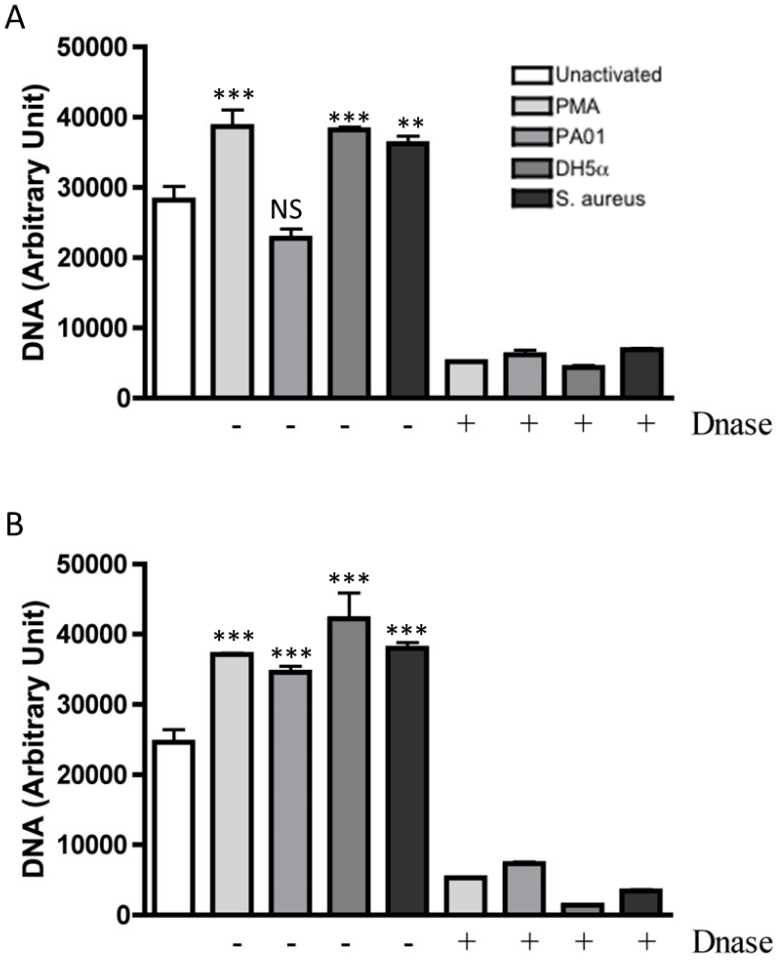


Figure 3.5. *Pseudomonas aeruginosa* shows a delayed response in NET induction

Neutrophils were co-incubated with PMA, *P. aeruginosa*, *S. aureus*, or *E. coli* and NET formation was measured after (A) 1 hour and (B) 3 hours. Extracellular DNA was stained with Sytox green and quantitated as an indicator of NET formation. Asterisks indicate a significant difference in extracellular DNA release between activated and inactivated neutrophils (white bar) (**P<0.01, ***P<0.001). A plus sign (+) indicates the addition of DNase during the co-incubation of NETs with PMA and each bacterial species. Each value shown is an average from 6 replicates with error bars representing the standard error of the mean (SEM). The experiment was performed at least three times and a representative experiment is shown.

3.5 Monitoring bacterial killing by neutrophil extracellular traps

During an infection, neutrophils are one of the first lines of defense against bacteria[100; 101]. These cells will migrate to the site of infection in an attempt to rid the area of the invading pathogens. They will try to engulf and kill the organism by exposing them to a variety of antimicrobial peptides, proteins and reactive oxygen species[102; 103; 108]. Neutrophils will also combat pathogens through the use of NETs[107]. NETs appear to function to help limit both microbial spread and the collateral damage from granular contents. Microbes can become trapped and killed by the NETs based on the high local concentration of antimicrobial components enmeshed in the NETs[107].

We have demonstrated the production of NETs in human neutrophils using DNA staining as well as anti-MPO, anti-DNA and anti-histone antibodies that colocalize with eDNA staining of NET-like structures. The next step was to assess the killing ability of NETs on *P. aeruginosa*. For these experiments, we wanted to optimize NET formation, and thus maximize bacterial killing by NETs. In previous reports it was observed that NETs can reduce bacterial numbers by 10 to 100-fold, but they generally do not provide sterilizing immunity during a co-incubation of neutrophils mixed at up to 10:1 ratios, and up to 1.0×10^7 CFU of bacteria per experiment[30; 107]. Therefore, we chose to maximize NET formation using a combination of PMA induction, and an additional treatment of neutrophils with cytochalasin D to prevent phagocytosis[30; 149; 155; 175]. These NET-activated neutrophils can only kill by releasing antimicrobial NET structures, and not through the conventional phagocytosis pathway. In this experimental system, we can monitor the efficiency of NET killing of *P. aeruginosa* and other bacterial species.

There is a debate in the literature regarding the killing function of NETs. The trapping function of NETs and their ability to ensnare bacterial, fungal, viral and protozoan pathogens has

been well established. One recent study confirmed that the viability of bacteria trapped in NETs can be assessed by plate counting of bacteria that were released from NETs with exogenous addition of DNase[175]. When the bacteria were plated from these wells it was found that the killing due to NETs was considerably reduced, suggesting that live bacteria were released from the DNase treatment[175]. However, various other studies have shown that different microbes can in fact be killed by NETs. Despite the apparent controversy regarding NET killing[175], a variety of studies have shown NET-mediated killing of different microbes using the final addition of DNase to release and quantitate bacterial viability. *P. aeruginosa* has been shown to survive exposure to NETs compared to other bacteria[30; 126; 150].

3.6 *Pseudomonas aeruginosa* is tolerant to NET killing.

Neutrophils were obtained from healthy donors and activated with PMA-cytochalasin D to produce NETs and co-incubated with *P. aeruginosa*. NET killing was determined by measuring the bacterial numbers at time zero, at 1 and 4 hours, and by adjusting for changes in bacterial cell number between inactivated or activated neutrophils. For the initial experiment, only *P. aeruginosa* and *S. aureus* were used (Figure 3.6). A DNase treatment was also used as a control to confirm that the killing witnessed was attributed to the NET structures. Bacterial survival was presented as percent survival, which was calculated using CFU counts from the time zero (T_0) and 1 (or 4) hour time points (T_1 or T_4) that were normalized to the bacteria alone condition and using the formula:

$$\% \text{ survival} = [(T_{1\ (\text{bacteria + NETs})} - T_{0\ (\text{bacteria + NETs})}) / (T_{1(\text{bacteria alone})} - T_{0(\text{bacteria alone})}) \times 100].$$

The bacteria alone control was necessary to correct for any bacterial growth that may have occurred during the incubation period and to demonstrate that the NETs were actually killing bacteria, rather than simply inhibiting growth. If the T_1 or T_4 counts are less than the T_0 counts,

this generates a negative % survival value that indicates 100-fold increase in bacterial killing or greater. Figure 3.5 indicated that *P. aeruginosa* was generally more tolerant to NET killing than the Gram-positive organism *S. aureus. P. aeruginosa* was reproducibly tolerant to NET killing, showing % survival values that range between 30-80 % (data not shown). To confirm killing due to NETs, exogenous DNase was added with the NET sensitive *S. aureus*, and showed that the presence of DNase at the beginning of the co-incubation with *S. aureus* disabled NET killing, leading to increased survival of *S. aureus* (Figure 3.6). This was not done with *P. aeruginosa* since it has previously been shown to be tolerant to NET-killing[30; 126; 150].

This basic NET killing experiment was repeated with another Gram-negative species *E. coli* DH5α. For these experiments, the co-incubation period was increased to 4 hours, as reports in the literature reported that longer incubation times allow for greater NET formation and killing (Figure 3.6). From these NET killing experiments where bacterial viability was monitored by plate counting, we made the following general conclusions: (1) *P. aeruginosa* PA01 is more tolerant to NET killing than *S. aureus* and *E. coli*, (2) NET killing of *S. aureus* and *E. coli* improved with 4 hour incubation times and (3) DNase addition during co-incubation effectively blocked killing of *S. aureus* and *E. coli* (Figure 3.7). This last observation is important because it suggests that NET killing is mediated by contact of bacteria with DNA-containing NET structures, presumably by antimicrobial compounds found in neutrophil granules but that are loaded into NET structures. In later chapters, we will revisit the mechanisms of bacterial killing and discuss the components of NETs that are implicated in bacterial killing.

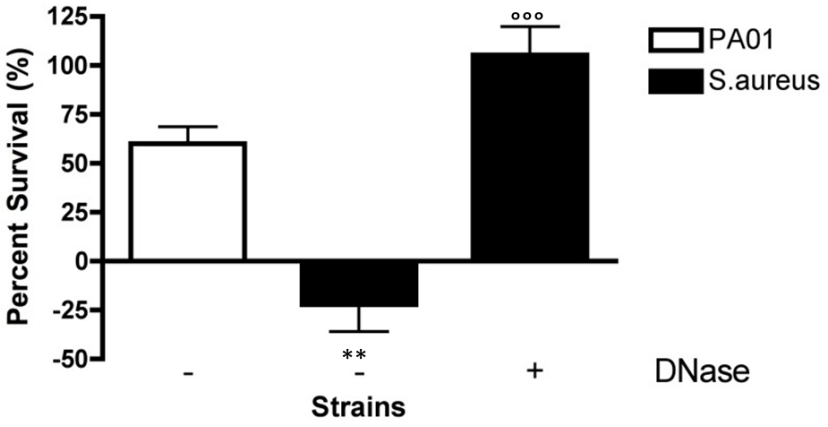


Figure 3.6. *Pseudomonas aeruginosa* is more tolerant than *S. aureus* to NET killing.

Activated neutrophils were incubated with either *P. aeruginosa* or *S. aureus* for 1 hour. The percent survival was calculated from the CFU counts before and after incubation with neutrophils and was normalized to bacterial counts in the absence of neutrophils. Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 (**P<0.01). A plus sign (+) indicates the addition of DNase during the co-incubation of NETs with bacteria. Circles indicate a significant difference in bacterial survival when treated with DNase compared to the non-DNase treated sample (°°°P<0.001). Each value shown is an average of three experiments with error bars representing the SEM.

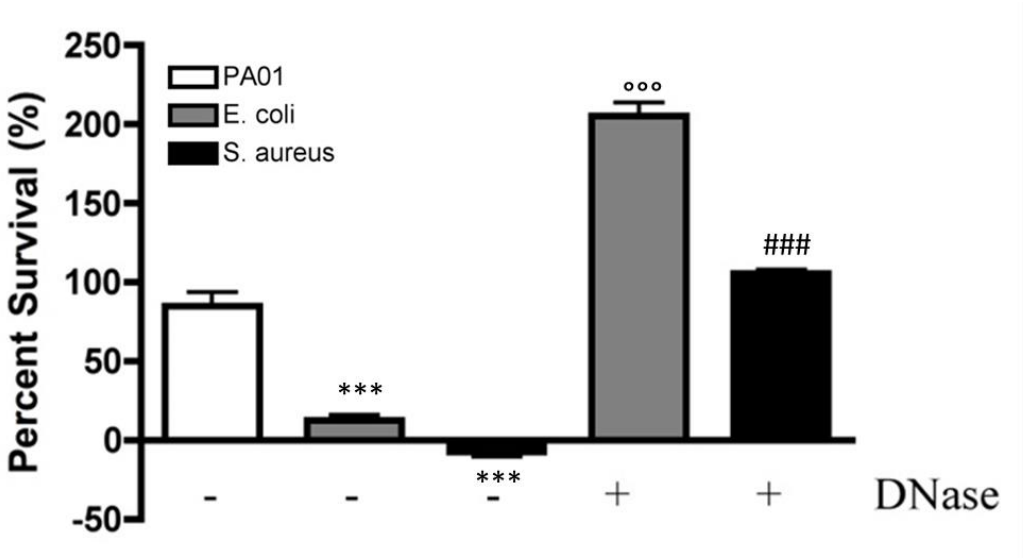


Figure 3.7. Bacterial susceptibility to NET-killing.

Activated neutrophils were incubated with *P. aeruginosa*, *E. coli*, or *S. aureus* for 4 hours. The percent survival was calculated from the CFU counts before and after incubation with neutrophils was normalized to bacterial counts in the absence of neutrophils. Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 (***P<0.001). A plus sign (+) indicates the addition of DNase during the co-incubation of NETs with bacteria. Circles and hash signs indicate a significant difference in bacterial survival when treated with DNase compared to the non-DNase treated sample (°°°P<0.001, ###P<0.001). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed three times with a representative experiment shown.

3.7 Use *of luxCDABE* reporter system to measure gene expression

The production and emission of light from a living organism is known as bioluminescence, a naturally occurring form of luminescence that occurs when energy is released from a chemical reaction in the form of light. Some organisms, eukaryotic and prokaryotic, contain the enzyme luciferase that emits light in the presence of oxygen and the substrate luciferin[183]. Luciferin in bacterial species is a reduced riboflavin mononucleotide ($FMNH_2$) that is oxidized in association with a long-chain aldehyde and an oxygen molecule[183]. Bioluminescence has been utilized as tool to measure bacterial viability and as s gene reporter, where bacteria express a gene or promoter of interest that is linked to the reporter. The activity of the reporter protein is measured and the resulting product is taken as a representation for the cellular response of the target gene[184].

For this study, the reporter system used is the *luxCDABE* cassette, derived from *Photorhabdus luminescens* (formally *Xenorhabdus luminescens*)[183]. The *luxCDE* genes encode a fatty acid reductase complex that results in the synthesis of a fatty aldehyde luciferin substrate, which is catalyzed by the luciferase subunits, encoded by *luxAB,* to produce luminescence[185]. The *lux* reporter is an ideal gene expression reporter due to its sensitivity, dynamic range, high-throughput potential, and the ability to monitor light production in real-time without the need for the addition of enzyme substrates[88].

3.8 Using the *lux*-reporter system to monitor bacterial survival when exposed to NETs

To date, the only assay used to measure bacterial viability during NET exposure has been direct plate counting. The limit to this assay is that bacterial viability is only reduced by 10 to 100-fold, compared to antibiotic treatments, which can reduce survival many more orders of magnitude. Therefore, in an attempt to develop further evidence of bacterial killing, and to

generate an assay with a greater throughput, we developed a *lux*-based viability assay to measure survival during NET exposure. We previously used the *lux*-reporter strain PA01::p*16Slux* to monitor survival during exposure to antimicrobial peptides and in response to lethal concentration of cation chelators DNA or EDTA[12]. In this assay, the *lux*-reporter strain is resuspended in buffer to limit growth and then treated with an antimicrobial. A decrease in luminescence (CPS) was previously shown to be the result of bacterial death and a decrease in viability[12]. For this purpose, a second *lux*-tagged, *E. coli* strain (DH5α/pσ70-*lux*) was constructed to measure bacterial viability in another Gram-negative organism. *E. coli* DH5α was transformed with a plasmid-encoded transcriptional *lux*-reporter plasmid pσ70-*lux*, which is a very low copy number (1-2 per cell) plasmid[174]. The plasmid contains the *lux* genes fused with a highly expressed copy of the σ70 promoter (personal communication, Dr. Michael Surette).

Both strains were grown in the presence of NETs for up to 4 hours in the Wallac Victor[3] plate reader and viability/CPS was read every 20 min. Both strains showed a basal level of expression/viability during this time. When exposed to NETs, a slow decrease in light production and therefore an increased amount of killing was observed for PA01 (Figure 3.8A). In contrast, there was a more rapid reduction in light production and thus viability for *E. coli* (Figure 3.8C). In addition to plotting the raw CPS values, we also normalized the CPS values relative to the T_0 CPS value and the bacteria alone condition, and presented the data as a percentage of CPS/viability. Consistent with plate counting data in Figure 3.7, PA01 was killed more slowly and was ultimately more tolerant to NET killing than *E. coli*. Using *lux*-reporter strains provides a more sensitive method to examine bacterial killing due to NETs.

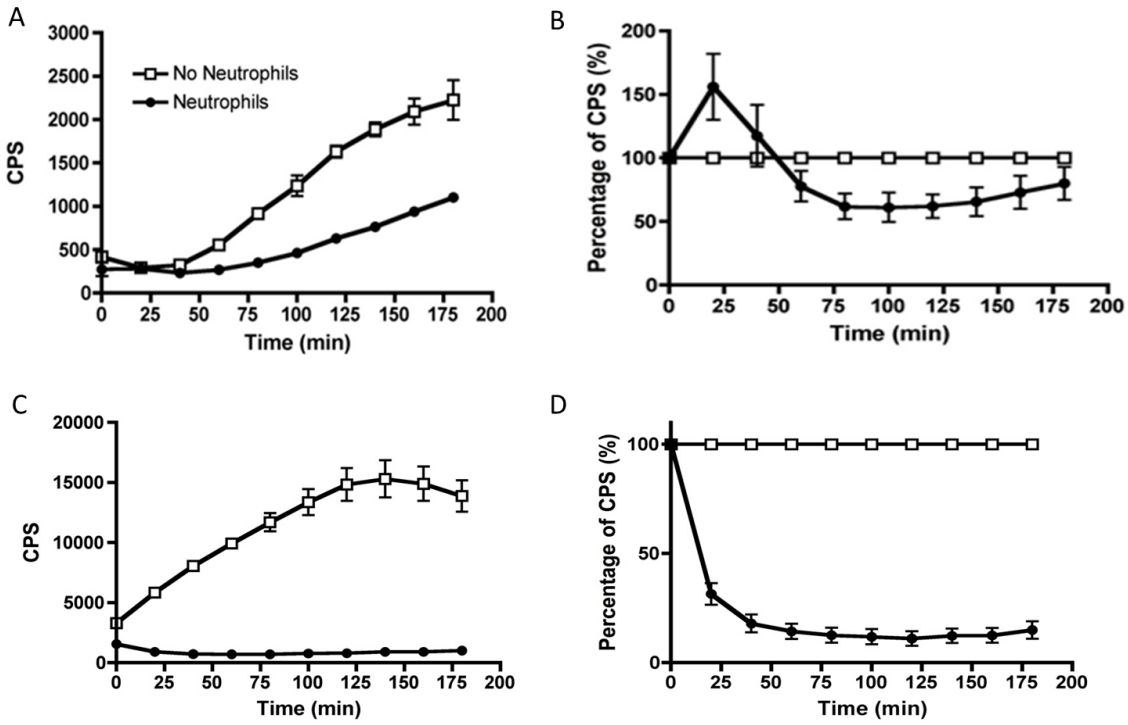


Figure 3.8. *Lux*-based measurements as an indicator of bacterial killing by NETs

P. aeruginosa PA01::p*16Slux* and *E. coli* DH5α/pσ70-*lux* were incubated with NETs for up to 4 hours with luminescence/viability (CPS) measured every 20 min. The raw CPS values of PA01::p*16Slux* (A) and *E. coli* DH5α/pσ70-*lux* (C) are shown. The CPS values were then normalized to the bacteria alone condition (representing 100%) PA01::p*16Slux* (B) and *E. coli* DH5α/pσ70-*lux* (D).

3.9 Using flow cytometry to measure bacterial viability

Flow cytometry allows for a sensitive and quantitative fluorescence method to count and assess bacterial viability of a large population of cells. Flow cytometry uses a flow cytometer that measures the scattering of light by cells as they pass by a laser[15]. The instrument can count the total population of cells, or depending on the technique, specific populations of cells can be counted. Often staining of cells with dyes or antibodies are needed for this procedure[15].

Typically, a laser of a single wavelength is directed onto a focused stream of liquid[186]. Various detectors are set up and pointed at where the stream passes through the light beam. One detector is in line with the light beam, this detects the forward scatter (FSC) while several others are perpendicular to it to detect the side scatter (SSC) (Figure 3.9A)[186]. The fluorescence detectors measure the light emitted from specific stains or fluorescent tags from antibodies. The combination of scattered and fluorescent light is analyzed and specific information can be determined about the physical and chemical structure of each individual particle passing through the light[186]. From the FSC, the cell volume is determined while the SSC can determine the inner complexity of the particle, such as the presence of cytoplasmic granules[186]. Flow cytometry is commonly used to examine larger cells, specifically in blood samples, such as leukocytes. The cytometer can detect particles from 0.2-150 μm[186], thus bacterial cells can be detected, but are on the lower end of the range.

3.9.1 Live/Dead bacterial staining with flow cytometry

To determine bacterial viability when incubated with NETs, live-dead staining was used to measure the amount of dead bacteria. The FSC and SSC light and fluorescence are split into defined wavelengths and channeled by a set of mirrors within the flow cytometer. The fluorescence is filtered so that each sensor will detect only a specific wavelength, these sensors

are known as photomultiplying tubes (PMTs) which will convert the energy of a photon into an electronic signal, or voltage (Figure 3.9B)[186]. Within the flow cytometer, for each PMT there are specific filters that are specific for the wavelengths they detect. There are the band pass (BP) filters that allow transmission of photons that have wavelengths within a specific range. The short pass (SP) filters only allow transmission of photons below a specific wavelength. The long pass (LP) filters allow transmission of photons above a specific wavelength[186]. These filters are set for each specific PMT based on the excitation wavelength they are to detect. As a fluorescing cell passes through the laser, a peak/pulse is created over time in the number of photons. These are detected by the PMTs and converted to a voltage pulse, which is known as an event[186]. Measuring the voltage pulse area, it will correlate to the intensity of fluorescence for that event. For the live-dead staining, events are determined based on cells stained with Syto 9 and/or PI. The staining pattern of the events can be graphed in a dot-plot with a gating system set up to define the parameters. For the graphical representation, a bi-exponential scale is used as opposed to the 4-5 decade log scale typically used. The log scale can cause a large number of cells to appear compressed against the axis. The bi-exponential (Logicle) scaling incorporates linear scaling, with space for values below zero, for low values together with a log scaling for high values[187]. The bi-exponential scaling gets rid of the "compressing" of events and allows for proper visualization of events with negative or dim fluorescence and ensures that the "visual" population center better correlates with the statistical center (median)[187].

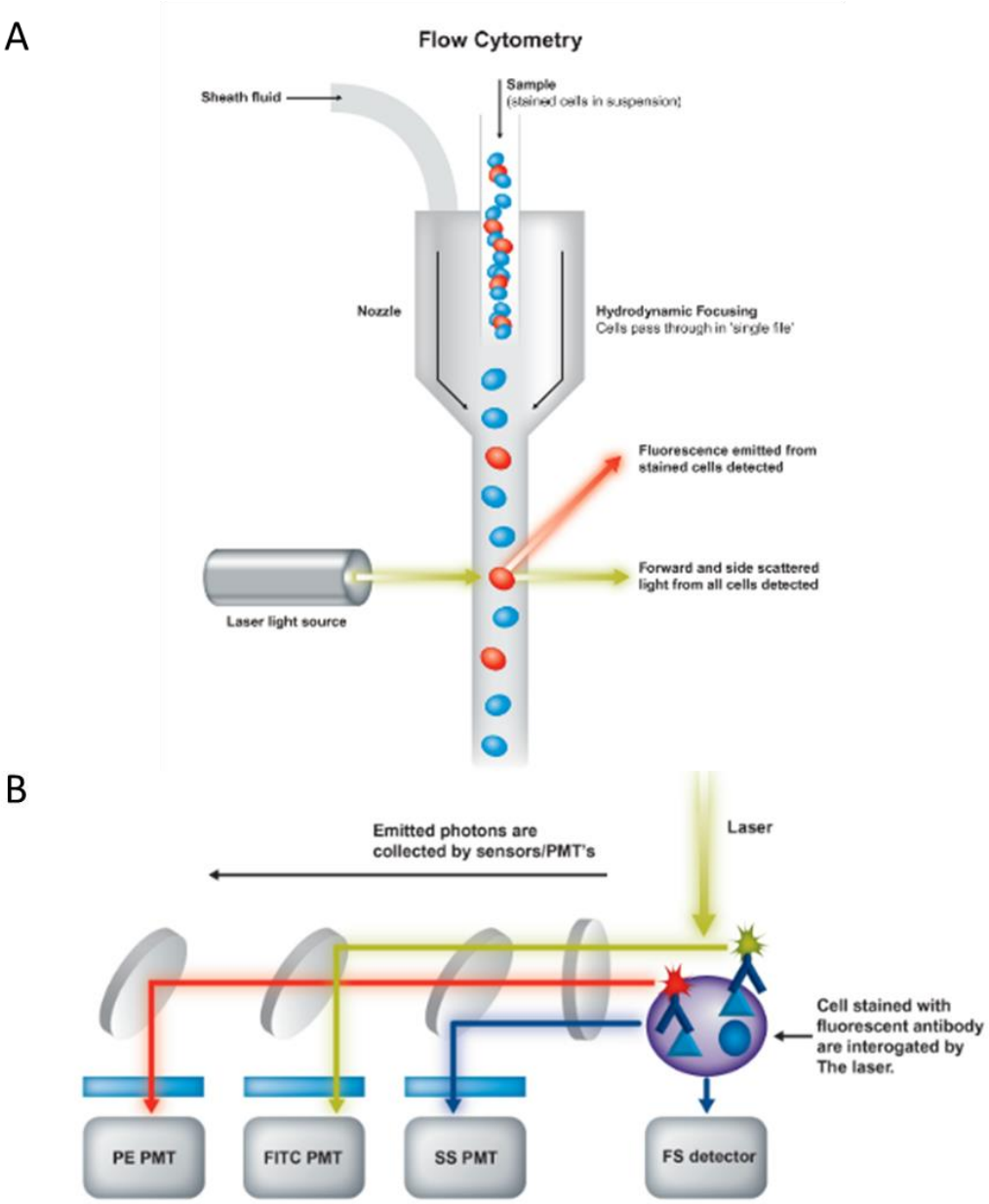


Figure 3.9. Schematic for the representation of flow cytometry and detection of scatter and fluorescence light.

General schematic of flow cytometry detection (A). Representation of how scatter and fluorescent light it measured in flow cytometry (B). Images were obtained from: http://docs.abcam.com/pdf/protocols/Introduction_to_flow_cytometry_May_10.pdf

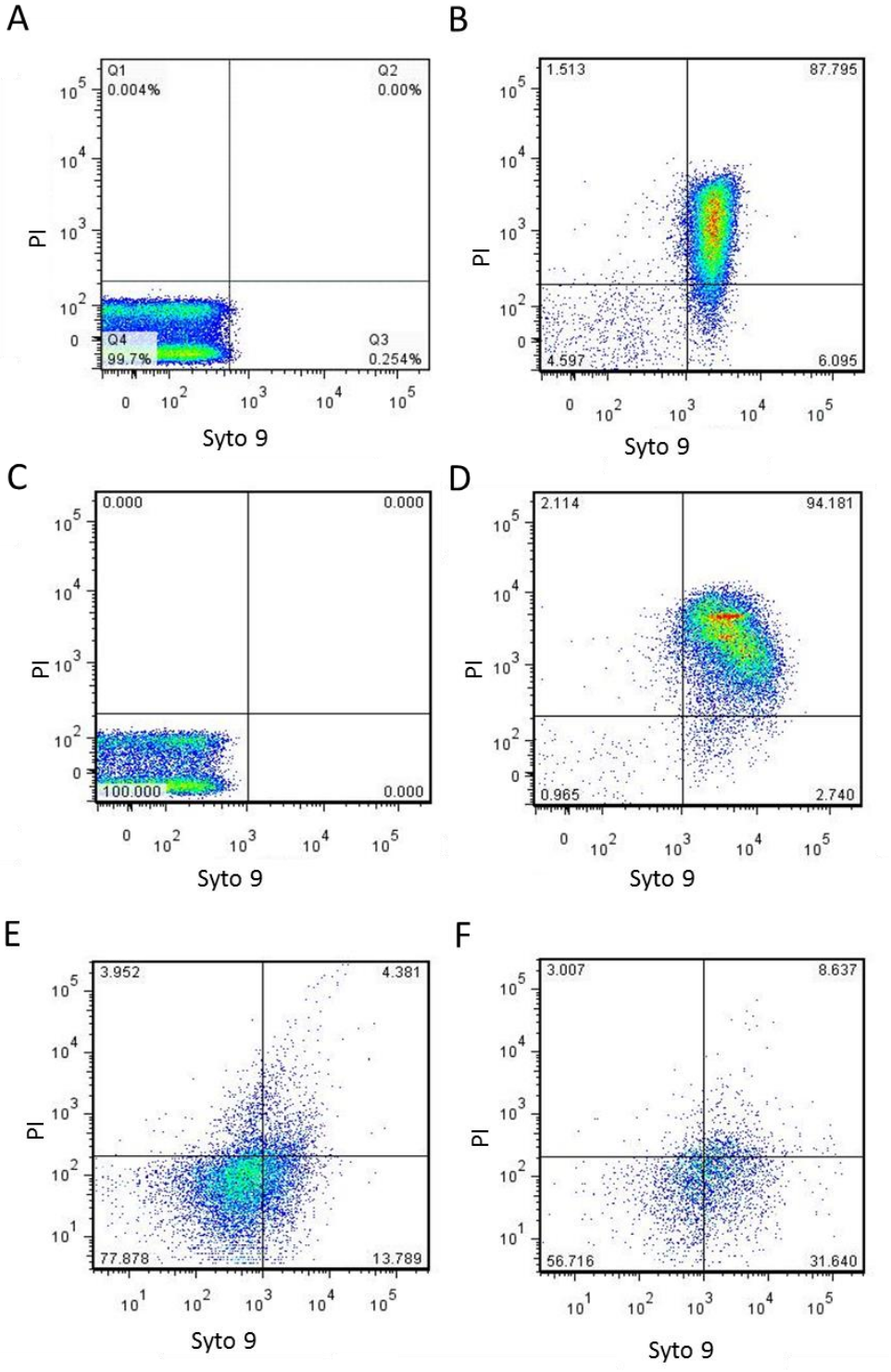
3.10 Live-dead staining of bacteria trapped in NETs using flow cytometry

3.10.1 Determining FSC, SSC and fluorescence parameters for live and dead bacteria

Initial experiments were performed to determine Population 1 used for the hierarchal gating strategy. Samples of *P. aeruginosa* and *E. coli* bacteria cells grown to the mid-log and stained with PI and Styo 9 and analyzed. Population 1 was determined form the stained events and applied to the various other sample tubes. Using defined gating parameters for live mid-log phase bacteria, extensive controls were performed to measure the fluorescence profiles of live (syto 9-stained) and dead (syto 9/PI dual-stained) bacterial cells (Figure 3.10). For each experiment, 50,000 cells (or events) were counted, and each experiment was performed at least three times. When using the gating parameter for bacterial events, 50 000 events were not obtained for neutrophil alone samples, confirming the absence of any bacteria-like objects in neutrophil samples. Unstained samples of both *P. aeruginosa* and *E. coli* were double negative for Syto 9 and PI staining (Figure 3.10A, C). A mid-log phase culture of *P. aeruginosa* was boiled for 15 minutes to kill the cells and served as a positive "dead" control. For both *P. aeruginosa* and *E. coli* cells, the boiled, dead bacteria stained double positive for both Syto 9 and PI (Figure 3.10B, D). Two other control samples were analyzed that consisted of PMA-cytochalasin D-activated neutrophils or inactivated neutrophils (Figure 3.10E, F). These controls were performed to determine if there was any contribution from neutrophils that may have been mistaken as dead bacterial cells. The neutrophil controls showed minimal events that were double positive for Syto 9 and PI, and therefore did not resemble dead cells.

Figure 3.10. Flow cytometry to detect live and dead stained bacterial.

(A) Unstained cells from mid-log phase *P. aeruginosa* cultures. (B) Boiled, dead cells from mid-log *P. aeruginosa* cultures. (C) Unstained cells from mid-log phase *E. coli* cultures. (D) Boiled, dead cells from mid-log *E. coli* cultures. (E) A sample of PMA and cytochalasin D -activated neutrophils and (F) unactivated neutrophils were used to determine if the neutrophils generated any background signal with similar gating and fluorescence properties of bacteria. The numbers in each quadrant represent the percentage of events (cells) that were double negative (bottom left), single positive (bottom right, top left) or double positive (top right) for live/dead staining.


A
Q1
0.004%
Q2
0.00%
Q4
99.7%
Q3
0.254%
PI
Syto 9
B
1.513
87.795
4.597
6.095
PI
Syto 9
C
0.000
0.000
100.000
0.000
PI
Syto 9
D
2.114
94.181
0.965
2.740
PI
Syto 9
E
3.952
4.381
77.878
13.789
PI
Syto 9
F
3.007
8.637
56.716
31.640
PI
Syto 9

3.10.2 Flow cytometry detects bacterial sensitivity to NETs

Bacteria were incubated with PMA and cytochalasin D-activated neutrophils for up to 4 hours in a 96-well plate. Both *P. aeruginosa* PA01 and *E. coli* DH5α were used to examine their sensitivity to NETs. Following the incubation, samples were mixed to resuspend neutrophil-bacterial coincubations in the 96-well plate. Samples were prepared for the flow cytometry by placing ~200 µL into polystyrene round-bottom sample tubes for subsequent live/dead staining using PI and Syto 9. The bacteria alone controls for *P. aeruginosa* PA01 and *E. coli* DH5α contained low levels of dead cells, 3.9% and 8.9% of 50,000 cells, respectively, (Figure 3.11A, C). When the bacteria were incubated with NETs, there was an increase in the dead bacterial population for both *P. aeruginosa* PA01 and *E. coli* DH5α (Figure 3.11B, D).

To quantitate NET killing, the amount of dead cell staining was represented as a percent of events stained with both PI and Syto 9, compared to all events stained with Syto 9 (Figure 3.11E). When incubated with NETs, *E. coli* DH5α showed more PI (dead) staining compared to *P. aeruginosa* PA01 incubated with NETs. This supports previous data indicating that *P. aeruginosa* is more tolerant to NETs. However, the *E. coli* DH5α alone condition showed a higher level of PI staining without incubation with NETs. This indicates that the starting population of cells already contained dead or dying (membrane compromised) cells before the addition of neutrophils. One explanation for this difference between *P. aeruginosa* and *E. coli* may have been the culture conditions. *P. aeruginosa* is routinely grown in BM2 defined growth medium and *E. coli* was grown in LB medium, and they may have grown to different stages of growth at the time of sampling. Future experiments were performed in BM2 medium, to correct for possible differences in growth medium and rate. Importantly, we were able to detect and quantitate an increase in ‘dead’ or PI-stained bacterial cells after incubation with NETs. We

observed the occurrence of “doublets” or even “triplets” that appear as horizontal linear patterns in the flow cytometry dot-plots (Figure 3.11). This may be due to the aggregation of cells in groups of 2 or 3 being detected. The aggregation may be the result of cells tethered together by the NET structures.

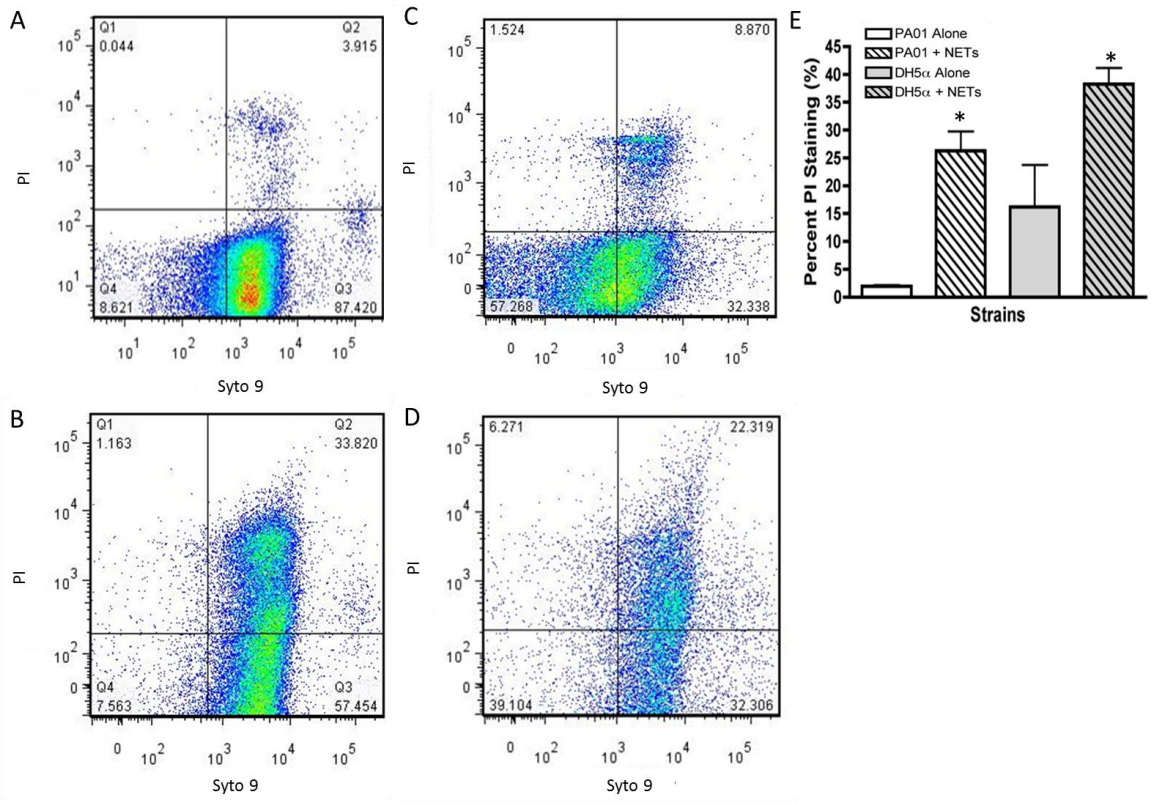


Figure 3.11. NET exposure results in PI staining for *P. aeruginosa* and *E. coli.*

P. aeruginosa PA01 and *E. coli* DH5α were incubated with NETs for up to 4 hours and stained with PI and Syto 9 to measure live and dead cells. Mid-log phase PA01 (A) and *E. coli* DH5α (C) were used as baseline controls to compare to bacteria incubated with NETs. Live/dead staining in PA01 coincubated with NETs (B) and *E. coli* DH5α with NETs (D). The percentage of PI staining was calculated as percentage of total cells (E). Asterisks indicate a significant difference in PI staining between bacteria incubated with NETs compared to bacteria alone (*$P<0.05$). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed at least three times with a representative experiment shown.

3.11 Summary of findings

Different DNA stains were used to visualize NETs induced by PMA. To confirm that DNA containing structures also contained additional NET components, we used a panel of antibodies to detect MPO and histones. The antibody staining for neutrophil granule proteins used in combination with extracellular DNA dyes confirmed the presence of NETs when induced by bacteria or with PMA.

Using a NET quantification assay, we showed that *P. aeruginosa* has a delayed induction of NETs compared to both *S. aureus* and *E. coli.* Both *S. aureus* and *E. coli* were able to induce NETs within the first hour when incubated with neutrophils, and to comparable levels as seen with PMA-activated neutrophils (Figure 3.5A). It is only after 3 hours that *P. aeruginosa* was able to stimulate NETs to a level similar to that of neutrophils incubated with PMA or *S. aureus* or *E. coli* (Figure 3.5B).

Using two novel assays, the *lux*-based viability reporter and flow cytometry of live/dead stained bacteria, we were able to confirm the plate counting results to conclusively show that NETs kill bacteria. These assays are quantitative and sensitive approaches to measure bacterial viability when exposed to NETs. In the *lux*-based assay, both *P. aeruginosa* PA01 *p16S::lux* and *E. coli* DH5α/pσ70-*lux* showed a decrease in luminescence when incubated in the presence of NETs, suggesting bacterial killing (Figure 3.8) Using flow cytometry, live/dead staining was used to indicate bacterial death, but may also be an indicator of membrane damaged cells. These novel methods were used for additional experiments to explore the bacterial response to NETs and the NET resistance strategies used by *P. aeruginosa*.

Chapter Four: NETs can induce DNA-responsive antimicrobial peptide resistance genes in *P. aeruginosa*

4.1 Antimicrobial peptide killing and bacterial resistance strategies

One of the ways the host protects itself from bacterial pathogens is through the use of cationic antimicrobial peptides (CAPs). CAPs are short peptides containing a large number of cationic and hydrophobic amino acid residues, which impart an amphipathic peptide nature. Bacterial membranes contain negatively charged membrane phospholipids and lipopolysaccharide in the outer leaflet of the outer membrane. The negatively charged phosphates and carbohydrate groups on the bacterial surface are the initial sites of CAP binding[188]. The peptides then partition into the hydrophobic layer of the membrane through hydrophobic interactions between peptides and lipids, which ultimately disrupts membrane integrity and leads to bacterial killing[188]. CAPs act preferentially on the bacterial cell membrane, and less on mammalian cell membranes, which contain more zwitterionic or neutral phospholipid species[189]. The CAPs can either attach to and insert into membrane bilayers to form pores by 'barrel-stave', 'carpet' or 'toroidal-pore' mechanisms, or alternately, they may penetrate into the cell to bind intracellular molecules which are crucial to cell living[190].

4.2 Bacterial resistance to cationic antimicrobial peptides

4.2.1 Trapping and proteolytic degradation

Certain pathogens are able to produce compounds that are able to bind and trap CAPs, preventing their antimicrobial action. *S. aureus* secretes staphylokinase that can bind and form complexes with human neutrophil peptide-1 and 2 (HNP-1 and 2) and results in greater than 80% reduction in activity of these CAPs against *S. aureus*[191]. Certain strains of Group A streptococcus (GAS) secrete a Streptococcal inhibitor of complement (SIC) that can protect GAS by directly

binding to and inactivating CAPs[192]. On the surface of epithelial cells are negatively charged proteoglycan molecules. These provide another mechanism bacteria have developed to protect against CAPs. Extracellular proteases secreted by GAS, *Enterococcus faecalis,* and *P. aeruginosa* degrade these cell-surface proteoglycans causing a release of dermatan sulphate, which can bind and inactivate human α-defensins[193]. *P. aeruginosa* is able to enhance the shedding of the heparin sulfate proteoglycan, syndecan-1, from various host cells using the virulence factor, LasA, that can serve to neutralize CAPs[194]. Other pathogens can secrete or express on their cell surface, proteases that can recognize and cleave CAPs before they reach the bacterial membrane. Some examples include aureolysin from *S. aureus*, elastase from *P. aeruginosa* and streptopain SpeB from *Streptococcus pyogenes* that each degrade and inactivate cathelicidin LL-37[195; 196].

4.2.2 Use of efflux pumps

Bacteria often contain membrane-associated active transporters that are involved in effluxing harmful compounds from the cells, such as antibiotics. These pumps are primarily used to export hydrophobic drugs; they have relatively low specificity and may accept a subset of antimicrobial peptides as substrates[197]. In both *Neisseria gonorrhoeae* and *Neisseria meningitidis,* the resistance-nodulation-cell division (RND)-type efflux pump (MtrCDE) has been show to allow resistance to specific CAPs, protegrin-1 and cathelicidin LL-37[198]. It was recently shown that the multiple drug resistance (MDR) RND-type efflux pumps from *E. coli* and *P. aeruginosa* were unable to confer resistance to specific human CAPs, indicating that most MDR exporters do not mediate broad range CAP resistance and are specific to certain types of CAPs[199].

4.2.3 Cell surface modifications

In Gram-positive bacteria there is the presence of a thick cell wall composed of cross-linked polymers of teichoic or lipoteichoic acids attached to peptidoglycan[188]. In Gram-negative bacteria, there is a multilayer membrane structure including a peptidoglycan matrix in the periplasmic space[188]. CAPs must transverse these envelope structures to reach their target site. The toxic effect of CAPs toward bacteria is primarily due to binding and disruption of the surface membrane or cell wall[200; 201]. Both Gram-negative and Gram-positive bacteria have developed strategies to evade CAP activity. This typically involves the modification of anionic cell surface components with cationic molecules that will reduce the anionic charge and cause a repulsion of the positively charged CAPs[188]. Besides changing the net charge of the bacterial cell surface, direct surface structural changes are often used to protect against CAPs. Some of these structural changes include acylation of lipid A, the modification of lipopolysaccharides (LPS) and lipooligosaccharides (LOS) and the formation of capsules[188]. Both Gram-positive and Gram-negative bacteria employ a variety of different methods to alter their cell surface. One of the most studied mechanisms is the lipid A modification in *P. aeruginosa* and other Gram-negative bacteria.

4.2.4 Modification of lipid A with aminoarabinose

In Gram-negative bacteria, the outer layer of the outer membrane serves as the site of attachment for the lipid A. Lipid A is an anionic dimer containing glucosamine linked to fatty acid chains and flanked by polar phosphate groups[188]. The lipid A moiety is the first site of interaction with CAPs. Covalent modifications of the phosphate group of lipid A with aminoarabinose (L-Ara4N), an amino sugar derived from arabinose, reduces the overall negative charge of the LPS and reduces the affinity for CAPs[188]. Elimination of the L-Ara4n reduced the

polymyxin B minimum inhibitory concentration (MIC) by 94-fold in *Salmonella enterica* suggesting that LPS charge contributes significantly to the interactions between CAPs and LPS and that the structure and/or location of the L-Ara4N modification to lipid A have an inhibitory effect on CAP binding[202]. The addition of L-Ara4N to lipid A is mediated by the PhoPQ two component system (TCS) through the post-translational activation of PmrAB regulatory system[171]. In *S. enterica*, the *pmrHFIJKLM* genes are regulated directly by the PmrAB TCS and are necessary for the L-Ara4N addition to the lipid A and polymyxin B resistance[203]. The function of the *pmr* genes and their regulation by PhoPQ/PmrAB has been characterized in other human pathogens such as *P. aeruginosa* and *Klebsiella pneumonia*, where the addition of L-Ara4N increased resistance to CAPs[204; 205].

4.3 Regulation of the *pmr* genes in *P. aeruginosa*

The two-component response regulators constitute one of the larger families of regulatory proteins in *P. aeruginosa* [206]. Within the system, one protein that acts as a sensor protein that detects specific stimuli, leading to autophosphorylation of the sensor and subsequent phosphotransfer to a cognate response regulator protein [207]. This phosphorylated response regulator protein then recognizes and binds to a specific DNA sequence, leading to modulation of transcription from that promoter[207]. In *P. aeruginosa* there are two separate two-component systems, PmrAB and PhoPQ that respond to magnesium limitation and separately regulate different operons[89]. The PhoPQ system autoregulates the *oprH-phoP-phoQ* operon under Mg^{2+}-limiting conditions and is also involved in resistance to CAPs and polymyxin B[89; 208]. The PmrAB system is also involved in regulating resistance to CAPs and polymyxin B in response to Mg^{2+}-limiting conditions[89]. This regulatory redundancy occurs because both PmrAB and PhoPQ regulate the expression of the *pmr/arn* genes (*PA3552-3559*), while PmrAB autoregulates the

PA4773-4775 operon in response to Mg^{2+} concentrations[89; 171]. Magnesium-limiting environments are encountered in areas rich with extracellular DNA, such as in bacterial biofilms[52]. DNA binds and sequesters divalent metal cations, and creates a localized cation-limited environment, activating the PhoPQ and PmrAB two-component systems and upregulating many Mg^{2+} regulated genes. Some of the genes are involved in the modification of the LPS structure that contributes resistance to CAPs and ROS[88; 89; 94].

4.4 DNA-induced surface modifications

Extracellular DNA has been shown to increase the expression of the *PA3553-3559* (*arn*) genes allowing for the addition of aminoarabinose to the phosphates of the core lipid A blocking the antimicrobial peptide binding sites and protecting the membrane [13] (Figure 1.1). Polyamine synthesis is also induced by eDNA to help protect the bacterial membrane from antimicrobial damage. In particular, *P. aeruginosa* produces putrescine and spermidine on the outer cell surface [94]. Spermidine is a polyamine compound found in the ribosomes and living tissues that have various metabolic functions. It is encoded by the *PA4773-4775* operon, regulated by the PmrAB two-component system. Spermidine protects the outer membrane from antimicrobial peptide damage, likely by the electrostatic interactions that mask additional negative surface charges (Figure 1.1). Surface polyamines also protect the membrane lipids from damage by ROS. The induction of spermidine synthesis genes by eDNA, suggests that *P. aeruginosa* is generating its own organic cations to substitute for the absence of divalent metal cations. Since the backbone structure of NETs is DNA, we hypothesized that *P. aeruginosa* may be able to sense and respond to the DNA and modify its cell surface to protect itself from the various antimicrobial peptides in NETs.

4.5 *Pseudomonas aeruginosa* is able to sense and respond to NETs

Our lab has shown that eDNA can function as a cation chelator and create an environment of magnesium limitation. It is this magnesium limitation that induces the expression of *PA3553-3559* and *PA4773-4775,* allowing *P. aeruginosa* to modify its cell surface protecting it from CAPs. DNA was added exogenously to planktonic cultures at concentrations that are relevant and present in the DNA-rich CF lung (1-20 mg/ml). Although flow chamber biofilms did not appear to accumulate high DNA concentrations, mutants that hyper-produce eDNA did form biofilms with increased expression of the DNA-induced operons[209]. In this work we wanted to examine a new and "real-life" context for DNA-induced gene expression; the interaction of *P. aeruginosa* with NETs. Since the backbone of NETs is made up of DNA, we wanted to examine if there is enough DNA present in these structures to be able to induce the surface modification genes. As histone-derived peptides are an antimicrobial component of NETs, this would provide an interesting example of a pathogen being able to sense, respond and defend against the immune system.

Before examining bacterial-NET interactions, we developed strategies to specifically block and neutralize the antimicrobial, cation chelating activity of DNA. To target and block DNA killing, we used three distinct treatments; deoxyribonuclease (DNase), excess magnesium ions (Mg^{2+}) and phosphatase (PTase) addition. DNase is an obvious treatment since it degrades DNA and NETs, while proteases are unable to do so[106; 137; 149]. Since DNA acts as a cation chelator[12], excess Mg^{2+} was added to neutralize the cation binding and gene induction activities of DNA. Finally, PTase was used to remove the phosphate groups present on the DNA, since we propose that the negative charge of the phosphates allow DNA to bind cations and disrupt membranes, inducing the various genes[12].

4.6 Examining NET integrity

The first step to this series of experiments was to determine the effects of these three treatments on NET integrity. Neutrophils were seeded onto cover-slips and activated to produce NETs with PMA and cytochalasin D. Designated wells were treated with DNase, excess Mg^{2+} or PTase and incubated for 4 hours. The slides were fixed and stained using DAPI to stain the nuclei of the cells and rabbit-anti-human MPO antibodies with the secondary anti-body, the anti-rabbit Cy5 to detect the MPO that is incorporated into the NETs. Neutrophil extracellular traps were seen from activated neutrophils (Figure 4.1). When treated with DNase, the NET structures were completely lost (Figure 4.1B), while incubated with excess magnesium ions (Figure 4.1C), or PTase (Figure 4.1D), did not disrupt the NET structures. The dual staining with DAPI and the anti-MPO antibody overlap; showing both extracellular DNA and MPO present in the NETs. These data confirm that only DNase treatment completely dissolves NET structure.

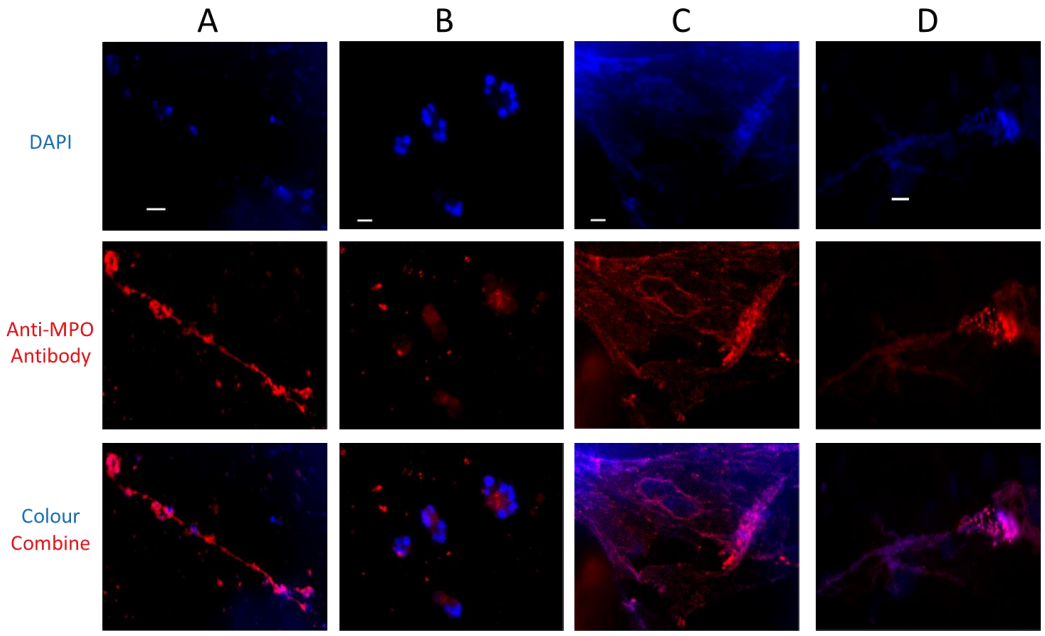


Figure 4.1. NET structure integrity under various conditions

Neutrophils were activated with PMA and cytochalasin D, and then (A) left untreated, (B) treated with DNase, (C) excess 5 mM magnesium, or (D) PTase for 4 hours. The cells were fixed and stained with DAPI (Blue) and the anti-MPO antibody, using the anti-rabbit Cy 5 (Red) as a secondary antibody. The top panel shows DAPI stained cells and eDNA. The middle panel shows anti-MPO antibody binding to NETs. The bottom panel is a merge of the two above panels. The images are obtained using an Olympus Microscope at 63X objective with the scale bar representing 10 μm.

4.6.1 Surface modification genes are induced in the presence of NETs

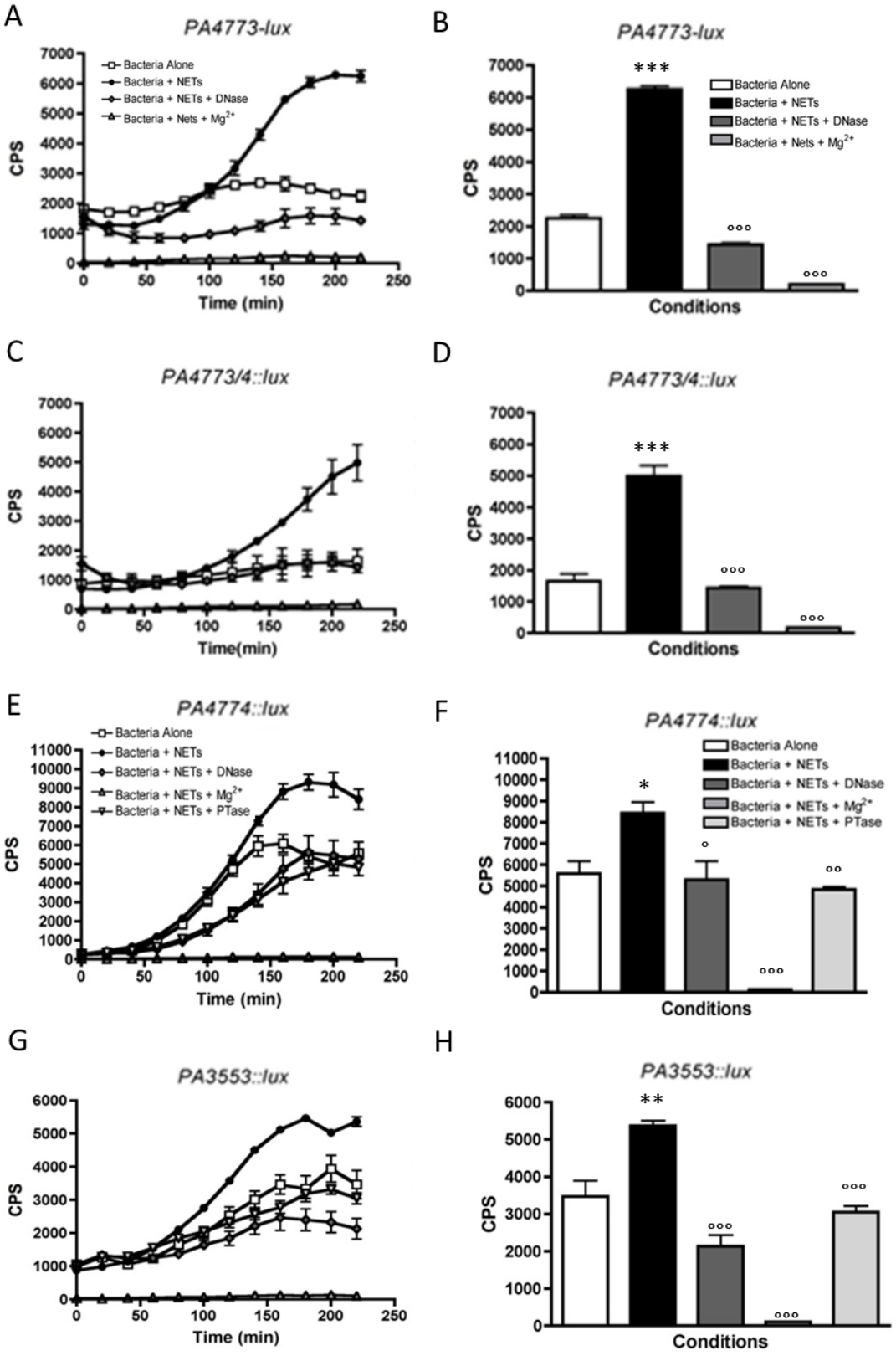
To monitor the transcriptional response of *P. aeruginosa* during interactions with NET-activated neutrophils, strains expressing *lux*-reporter fusions to various genes were used. The *PA3553::lux* strain was used as a transcriptional reporter of the *arn/pmr* operon, while *PA3773/4::lux* and *PA4774::lux* were used as transcriptional reporters for spermidine synthesis genes. These strains are dual purpose strains, as the transposon insertion inactivates the genes of interest, but also introduces a *lux* reporter fusion[88]. To test the response of wild type *P. aeruginosa*, we used a *PA4773-lux* reporter, in which the *PA4773* promoter drives the *lux* reporter expression from a construct that was introduced into the chromosome at the neutral *att* site (constructed by Lori Johnson)[94] . For this assay, overnight cultures were subcultured in BM2 with 0.5 mM Mg^{2+} to repress expression of all these Mg^{2+}-regulated genes. Typically, 0.02 mM Mg^{2+} is limiting and leads to very high expression levels, and increasing to 0.5 mM Mg^{2+} leads to much lower basal levels of expression before the introduction of neutrophils.

Strains were incubated with NETs up to 4 hours in the Wallac Victor^3 plate reader and gene expression/CPS was read every 20 min. To test if the reporter strains were responding specifically to the DNA in NETs, there was the addition of DNase, excess Mg^{2+} or PTase to designated wells. From Figure 4.2 all the strains show a significant 2 to 3-fold gene induction in the presence of NETs as compared to the basal levels of the bacteria alone. With the addition of DNase, Mg^{2+}, or PTase the gene expression is significantly reduced, below the basal levels of control conditions. Since DNase, Mg^{2+} and PTase all target DNA, these data strongly suggest that it is the DNA responsible for the bacterial gene expression response. DNase should degrade the NET, Mg^{2+} saturates and neutralizes DNA, and PTase cleaves the cation chelating phosphate moiety, all of which prevent NET-mediated gene induction. These experiments are performed in

Hank's Buffered Salt Solution minus divalent cations ($HBSS^-$). For NET induction, we propose that the DNA scaffold is binding and sequestering trace Mg^{2+} from the media or cell surface, and is able to create a magnesium-limited environment that activates both the PmrAB and PhoPQ TCS. These experiments provide strong evidence that sufficient DNA is released from $1x10^6$ neutrophils that were PMA activated, and suggest that *P. aeruginosa* can sense and respond to the DNA under these conditions.

Figure 4.2. Surface modification genes are induced in the presence of NETs.

Bacterial reporter strains were coincubated with activated neutrophils to produce NETs and treated with DNase, excess Mg^{2+}, or PTase. The kinetics of gene expression (total CPS) for (A) wild-type *PA4773-lux*, (C) the intergenic transposon insertion mutant *PA4773/4::lux*, (E) *PA4774:::lux* and (G) *PA3553::lux* was determined throughout 4 hours. The final time points for CPS readings (4 hrs.) were also graphed for *PA4773-lux* (B), *PA4773/4::lux* (D) *PA4774:::lux* (F) and *PA3553::lux* (H), to compare the gene expression under the various treatments. Asterisks indicate a significant difference in gene induction when compared to bacteria alone (*P<0.05, **P<0.01, ***P<0.001). Circles indicate a significant reduction in gene expression of treated samples compared to untreated bacteria incubated in the presence of NETs (°P<0.05, °°P<0.01, °°°P<0.001). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed at least three times and a representative experiment is shown.


A
PA4773-lux
Bacteria Alone
Bacteria + NETs
Bacteria + NETs + DNase
Bacteria + Nets + Mg2+
CPS
Time (min)
B
PA4773-lux
***
Bacteria Alone
Bacteria + NETs
Bacteria + NETs + DNase
Bacteria + Nets + Mg2+
CPS
Conditions
C
PA4773/4::lux
CPS
Time(min)
D
PA4773/4::lux
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E
PA4774::lux
Bacteria Alone
Bacteria + NETs
Bacteria + NETs + DNase
Bacteria + NETs + Mg2+
Bacteria + NETs + PTase
CPS
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PA4774::lux
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CPS
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4.7 Summary of Findings

By developing multiple strategies to specifically target the DNA found in NETs, I provided evidence that *P. aeruginosa* was able to sense and respond to DNA present in the NET structures. NETs treated with DNase, Mg^{2+} or PTase all prevented NET-mediated gene expression responses, but only DNase treatment effectively degraded the NETs. The experiment with PTase provided the first data to address and support the hypothesis that phosphates are the cation chelating moiety of DNA. It is important to note that during these bacterial-neutrophil interactions, we showed in Chapter 3 that the wild-type strain was being killed by NETs. Therefore, we are only measuring the gene expression from those cells that are not being killed. We have not normalized gene expression per cell in these experiments, and given the cell numbers are decreasing over time, the fold changes may be underrepresented, if we were able to normalize to viable cells. As well, with the presence of neutrophils in the wells, we could note normalize to OD, since it would take into consideration both population of cells and not just the light-producing bacteria.

Using the *lux* gene reporter system, various transcriptional fusions to genes required for the production of aminoarabinose-modified LPS and spermidine synthesis showed a significant increase in gene expression in the presence of NETs. The implication is that these genes/surface modifications are also required to defend against NETs, which will be explored in Chapter 6.

Chapter Five: DNA contributes to the antimicrobial activity of neutrophil extracellular traps.

5.1 Antimicrobial activity of DNA

Deoxyribonucleic acid (DNA) is made up of multiple linked nucleotides, making it a polynucleotide. The phosphates present in the DNA backbone make the DNA a polyanion and give it a very strong negative charge (Figure 5.1). Due to this negative charge, it can bind strongly to divalent metal cations including Mg^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+} [12]. At high concentrations (>10 mg/mL), DNA can act as a fast-killing antimicrobial agent to planktonic cultures. Bacterial surfaces are highly negatively charged and consequently have high levels of Mg^{2+} and Ca^{2+} bound to the surface[210]. It was proposed that DNA can strip Mg^{2+} cations from the surface of bacterial membranes, since it was shown to disrupt both the inner and outer membrane integrity of bacterial cells[12]. This ultimately leads to lysis and bacterial death. Since we already showed that the DNA in NETs can upregulate certain surface modification genes, we suspect that the cation chelating of DNA found in these structures may actually contribute to the killing of microbes that become captured. This might help to explain the broad spectrum killing activity of NETs.

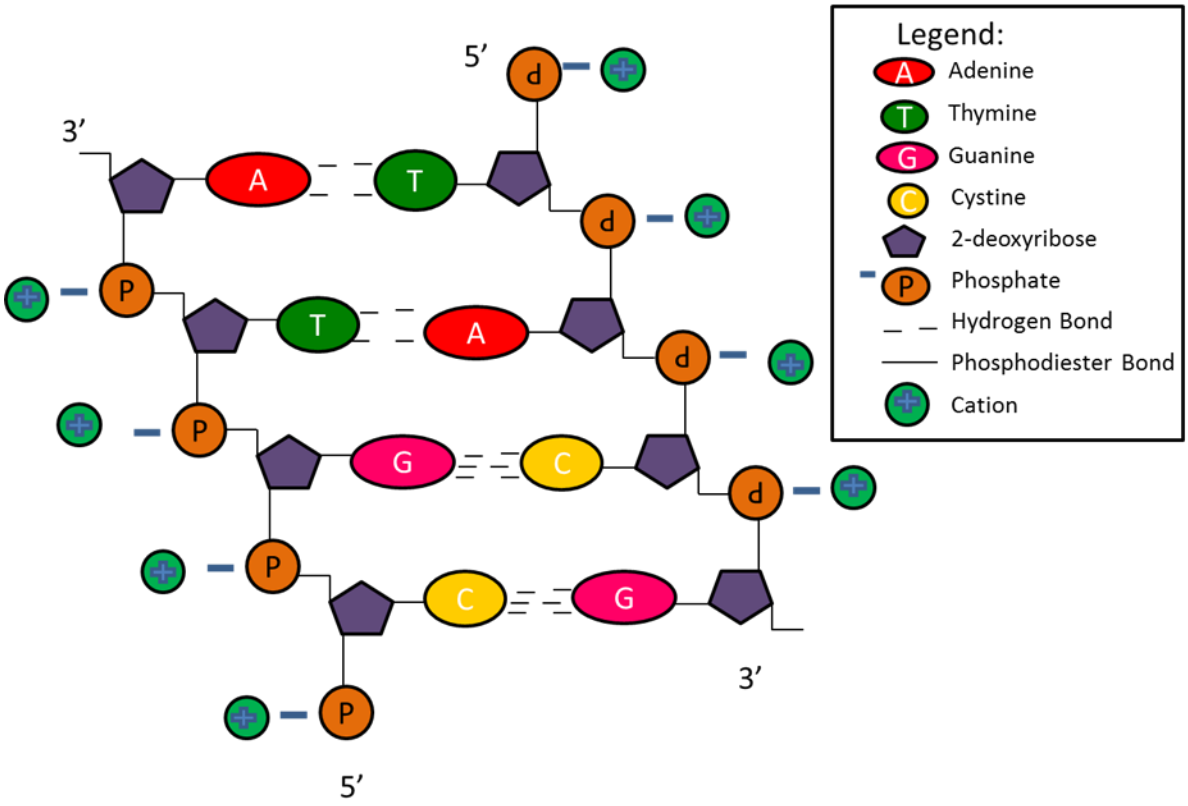


Figure 5.1. The phosphates of DNA allows for cation chelation.

There are 4 bases that make up DNA; adenine (A), cytosine (C), guanine (G) and thymine (T). These four bases are attached to a sugar-phosphate group to make up a specific nucleotide. The nucleotides are bonded to the sugar (2-deoxyribose) and a phosphate group. The sugar-phosphate groups make up the backbone of DNA. DNA has a highly anionic nature due to the negative charges of the phosphate groups allowing it to attract positively charged ions (cations). DNA can disrupt the bacterial membrane by chelating these cations, leading to cell death.

5.2 *In vitro* killing by purified DNA can be blocked by DNase, Mg^{2+}, and PTase.

We previously showed DNase, Mg^{2+}, and PTase can block the ability of DNA to induce specific genes in *P. aeruginosa*, which is ultimately a measure of the cation chelating activity of DNA. Here, we used the same approach to test if NET killing can be blocked by targeting DNA. Previous studies have shown that DNase degrades NETs and restores bacterial survival[30; 107; 175] and DNase can also decrease the ROS level[211]. The inhibition of ROS production may be caused by extracellular trap disassembly rather than direct MPO inhibition[211]. Excess magnesium ions can inhibit the antimicrobial activity of DNA, due to its ability to chelate cations[12]. To test if PTase can protect against NET killing, we first examined its ability to protect *P. aeruginosa* from the antimicrobial effects of purified extracellular DNA. Overnight cultures of the *P. aeruginosa* PA01::p*16Slux* strain were incubated with 0.5% eDNA (w/v) in a 96-well plate with some of the wells treated with DNase, PTase or 5 mM Mg^{2+}. For treated samples, the various conditions were allowed to incubate with the DNA for up to an hour before addition of the bacteria. The plate was placed in the Wallac Victor^3 plate reader and viability (luminescence, CPS) and growth (OD_{600}) was measured every 10 min for the 3 hours. Following the incubation, samples were stamped (~5 µL) on LB agar plates, counted and matched to the CPS readings.

At the end of the 3 hour incubation, there was a significant reduction in CPS from the bacteria incubated with a lethal concentration of 0.5% DNA (Figure 5.2A). In the presence of PTase, DNase, and DNase with Mg^{2+}, there was an increase in luminescence, indicating survival. The DNase with Mg^{2+} show better protection than just the DNase alone, because DNase, like other enzymes, often need cations for optimal enzyme activity[87]. There was a slight increase in light production for the bacteria incubated with just Mg^{2+} (Figure 5.2A). When samples were plated onto LB agar plates and allowed to grow overnight, there was no recovery of the bacteria

treated with 0.5% eDNA (Figure 5.2B). The conditions where the DNA was pretreated with DNase, Mg^{2+} or PTase showed bacterial growth. This suggests that the magnesium treatment did allow for bacterial recovery and confirmed that DNase and PTase can protect bacteria from extracellular DNA killing.

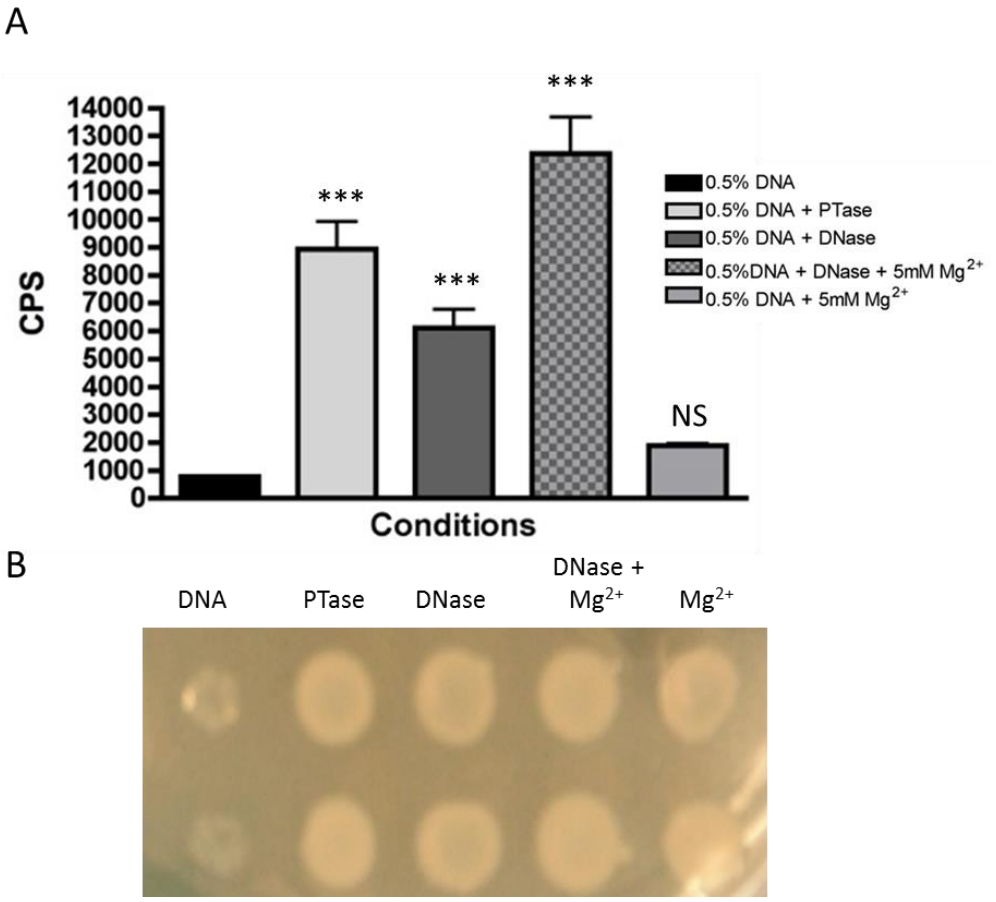


Figure 5.2. Phosphatase pre-treatment protects against extracellular DNA killing.

P. aeruginosa PA01::p*16Slux* was incubated with 0.5% DNA (w/v) that had been either treated with PTase, DNase, DNase and Mg^{2+}, or Mg^{2+} or left untreated. Final CPS readings after 3 hour incubation are shown (A). Each value shown is an average of at least three replicates with error bars representing the SEM. Asterisks indicate a significant difference in luminescence between treated and untreated samples (***P<0.001, NS= not significant). Following the incubation, 5 µL of sample was plated onto LB agar (B). Colonies were observed for each pre-treatment condition and no growth was observed in DNA alone. The experiment was performed at least three times and a representative experiment is shown.

5.3 Blocking NET DNA prevents bacterial killing

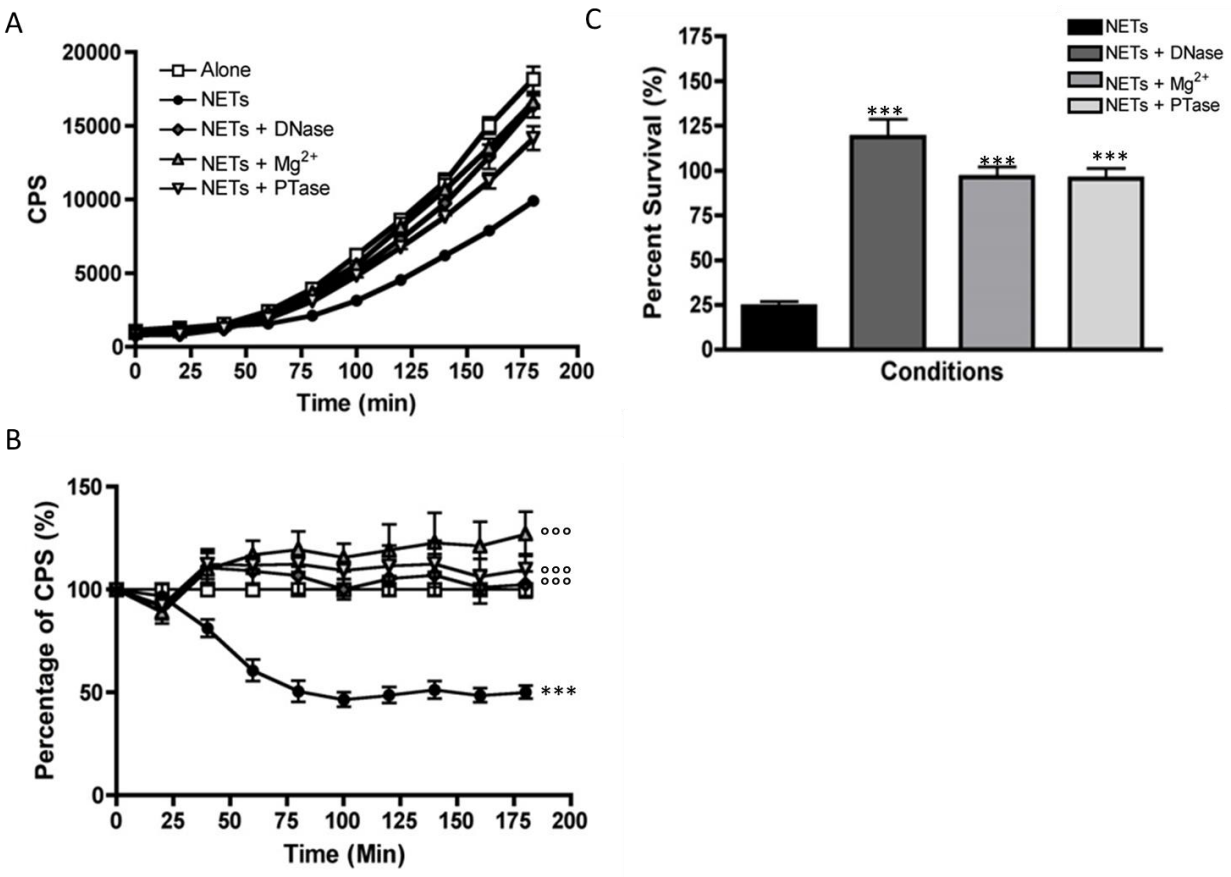
A combination of plate counting, *lux*-reporter viability assays (CPS) and flow cytometry were used to examine bacterial susceptibility to NETs when treated with DNase, excess Mg^{2+} and PTase. In a single experiment, we were able to obtain viability data from the three methods. The *P. aeruginosa* PA01::p*16Slux* strain was incubated with NET-activated neutrophils in a 96-well plate. Certain wells were left untreated or treated with DNase, PTase, or excess magnesium. The plate was placed in the Wallac Victor^3 plate reader and viability/CPS was read every 20 min for up to 4 hours. After the 4 hour time point, ~200 µL samples (in triplicate) were brought to the flow cytometer to measure the amount of dead bacteria with live/dead staining. Plate counting was also done at the initial (T_0) and final (T_4) time points.

5.3.1 Using the lux-reporter system to monitor bacterial survival

When the PA01::p*16Slux* was incubated with NET-activated neutrophils, there was a decrease in luminescence (Figure 5.3A), indicating the bacteria were being killed by the NETs. When DNase, Mg^{2+}, or PTase was added during the incubation, there was a return in the light production (Figure 5.3A). The raw CPS data was normalized to the initial CPS value at the first time point, and normalized to the bacteria alone condition (Figure 5.3B). This allowed for a better representation of the effects of NETs on the viability of the bacteria. To support the CPS data, plate counts were done as previously described. The plate counting data (Figure 5.3C) showed that when incubated with DNase, Mg^{2+} or PTase, there was an increase in bacterial survival to NETs compared to the untreated condition. In our experiments with exogenous DNase, we frequently observed bacterial survival and growth beyond the levels seen by the bacteria alone (Figure 5.4).

Figure 5.3. DNase, PTase and excess Mg^{2+} protect against NET killing.

Activated neutrophils producing NETs were incubated with PA01::p*16Slux* for up to 4 hours. Viability was measured based on the total light production (CPS) detected (measured every 20min) (A). The normalized CPS data is shown (B). Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 alone (***P<0.01). Circles indicate a significant difference in bacterial survival of treated samples compared to PA01::p*16Slux* incubated in the presence of NETs (°°°P<0.001). The percent survival was calculated from the CFU counts before and after incubation with neutrophils and was normalized to bacterial counts in the absence of neutrophils (C). Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 incubated in the presence of NETs (***P<0.001). Each value shown is an average of at least three replicates with error bars representing SEM. The experiment was performed at least three times and a representative experiment shown.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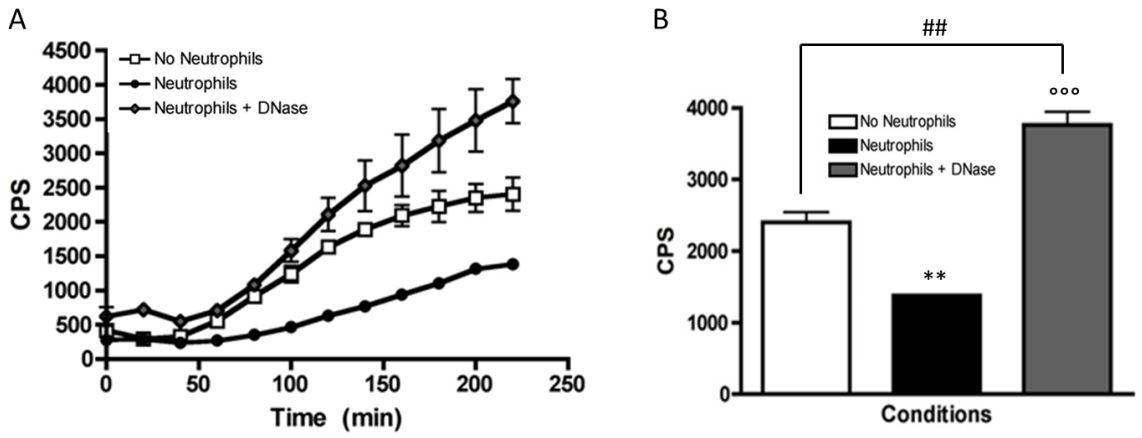


Figure 5.4. DNase degraded NETs contribute to bacterial growth

Activated neutrophils producing NETs were incubated with PA01::p*16Slux* for up to 4 hours with or without the addition of exogenous DNase. Viability was measured based on the total light production (CPS) detected (measured every 20min) (A). The CPS of the final time point (4 hours) is shown (B). Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01alone (**P<0.01). Circles indicate a significant difference in bacterial survival of DNase treated samples compared to PA01::p*16Slux* incubated in the presence of NETs (°°°P<0.001). Hash symbols indicate significant difference in bacteria survival incubated with neutrophils and DNase compared to PA01 alone (##P<0.01). Each value shown is an average of at least three replicates with error bars representing SEM. The experiment was performed at least three times and a representative experiment shown.

5.3.2 Live-dead staining of **P. aeruginosa** *incubated with NETs using flow cytometry*

Using the same samples from the luminescence (CPS) assay, following the 3-4 hour incubation, the 96-well plate was removed from the Wallac Victor^3 plate reader. Samples were prepared for the flow cytometer by placing ~200 µL into polystyrene round-bottom sample tubes and brought to the flow cytometer to measure live/dead staining using PI and Syto 9. Compared to the bacteria alone condition (Figure 5.5A) there was an increase in the population stained for both Syto 9 and PI, indicating "dead" bacteria when incubated with NETs (Figure 5.5B). This population drastically decreased when incubated with DNase (Figure 5.4C), and slightly decreased in the presence of 5 mM Mg^{2+} (Figure 5.5D). However, there appeared to be no change in the population when PTase was added (Figure 5.5E). The amount of PI staining was quantified as a percent of total live cells (Figure 5.5F). From the flow cytometry data it suggests that PTase had no protective effect, but when samples were plated onto LB agar there was a return in bacteria survival (Figure 5.3C). When *P. aeruginosa* was incubated with NETs, two dual Syto 9 and PI stained subpopulations were found, with one subpopulation showing higher PI fluorescence (Figure 5.6A- Black Arrow) compared to the second subpopulation (Figure 5.6A- White Arrow). Three out of seven sample sets showed these two highly PI stained populations. We propose that this highly PI stained subpopulation represents cells that are more permeable to PI due to increased membrane damage and cell death (Figure 5.6A- Black arrow), representing a true "dead" population. The other PI stained population was considered as a "dying" population of cells with membrane damage (Figure 5.6A- White Arrow). We were able to specifically measure this highly PI stained population and represent it as a percentage of the total PI and Syto 9 stained cells in the upper right quadrant, under the various treatments (Figure 5.6B). All three treatments reduced population size of true "dead" cells, with the DNase

treatment resulting in a statistically significant increase in survival. Some samples were also treated with a post-DNase treatment, with DNase added at the end of the incubation and allowed to incubate for 30 min. This late DNase treatment also restored bacterial viability, despite a 4 hour coincubation with NETS, suggesting that the late DNase treatment disabled NETs and restored bacterial recovery (Figure 5.7). We then examined if blocking the DNA in NETs can protect other species of bacteria, such as *E. coli.*

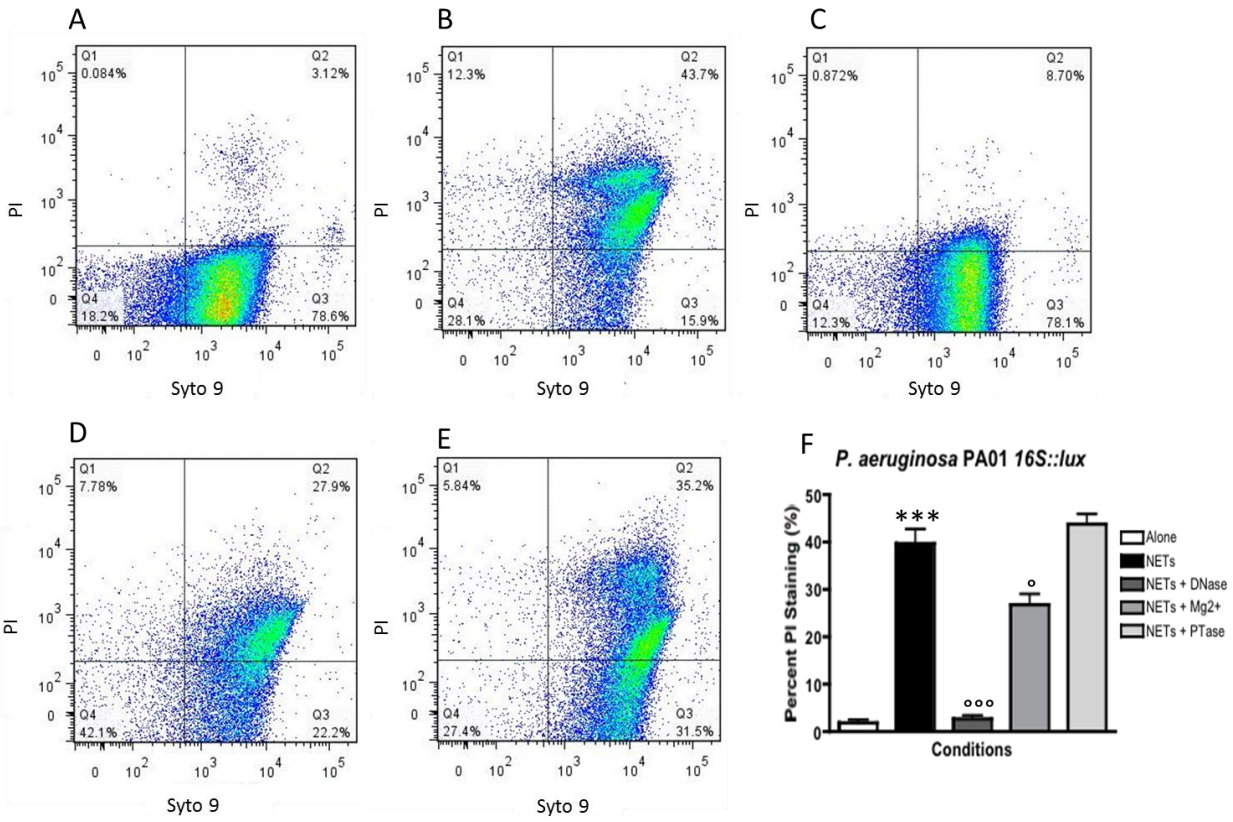


Figure 5.5. Bacterial susceptibility to NETs can be measured with flow cytometry

Samples of *P. aeruginosa* that were incubated with NETs for up to 4 hours were stained with PI and Syto 9 to measure live and dead cells. Mid-log phase PA01 was used as a base line control (A) to compare to the cells incubated with NETs (B). In some samples, DNase (C), 5 mM Mg^{2+} (D), or PTase (E) was added at the beginning of the incubation. The percentage of PI staining was calculated as percentage of total cells (F). Asterisks indicate a significant difference in PI staining between bacteria incubated with NETs compared to bacteria alone (***P<0.001). Circles indicate a significant difference in bacterial survival under treated conditions compared to bacteria incubated with NETs (°P<0.05, °°°P<0.001). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed at least three times and a representative one is shown.

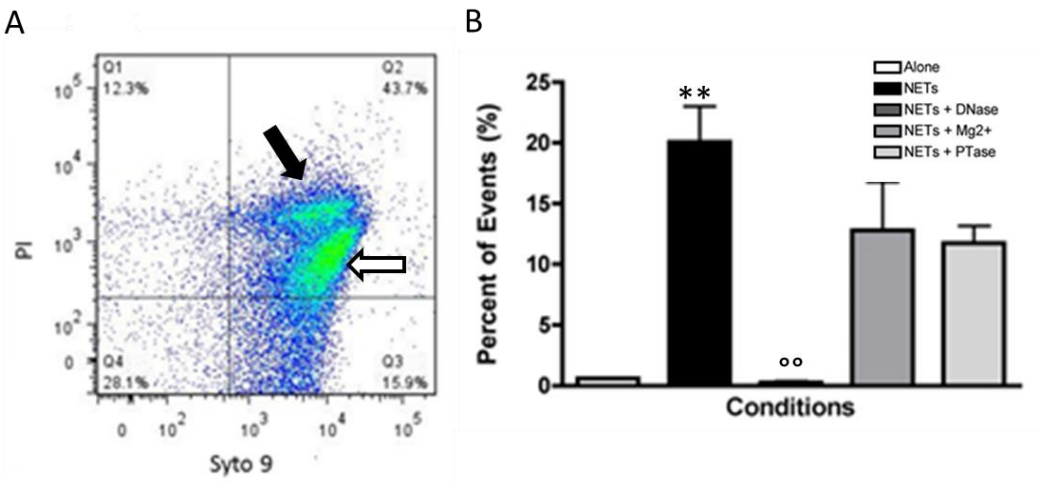


Figure 5.6. Dead and dying subpopulations of PI-stained bacterial cells.

Three out of seven flow cytometry samples of *P. aeruginosa* incubated with NETs showed two subpopulations of cells stained with both Syto 9 and PI. A) When incubated with NETs one population showed greater PI staining (Black arrow) compared to a second PI stained population (White Arrow). B) The amount of cells in the subpopulation of greater PI staining was represented as a percentage of total Syto 9 and PI-staining events under the various conditions. Asterisks indicate a significant increase bacterial killing when incubated with NETs compared to bacteria alone (**P<0.01). Circles indicate a significant increase in bacterial survival after DNase treatment compared to bacteria incubated with NETs (°°P<0.01). Each value shown is an average of at least three replicates with error bars representing the SEM.

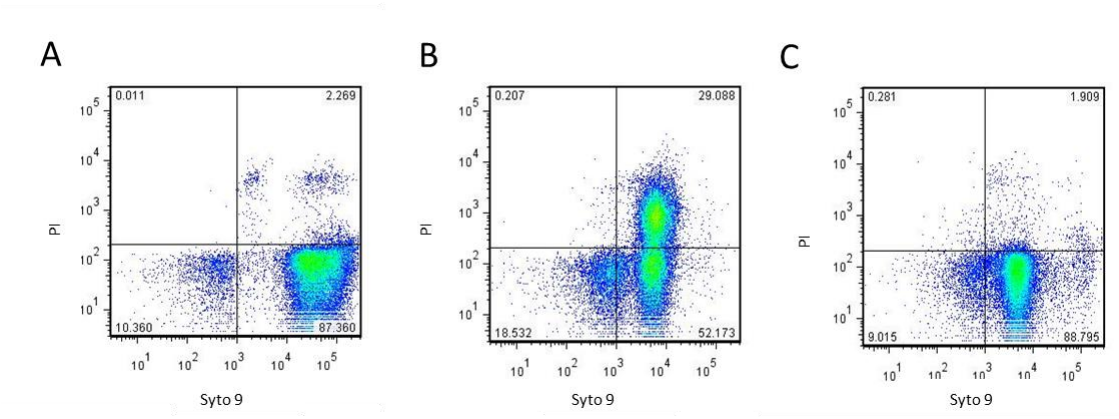


Figure 5.7. A post-DNase treatment protects against NETs.

Samples of *P. aeruginosa* that were incubated with NETs for up to 4 hours are stained with PI and Syto 9 to measure live and dead cells. Mid-log phase PA01 was used as a base line control (A) to compare to the cells incubated with NETs (B). A 30 min post-DNase treatment was performed following the incubation (C).

5.4 Blocking NET DNA can protect *E. coli* from NET killing

The viability assays described for *P. aeruginosa* were used to examine if blocking NET DNA restores survival and protects *E. coli*. Plate counting data showed that in the presence of DNase, Mg^{2+}, or PTase there was a return in bacterial survival (Figure 5.8). Flow cytometry data also showed a reduction in PI staining when NETs were treated with DNase and Mg^{2+}; however no reduction was witnessed for PTase treated samples (Figure 5.9). These results were consistent with PA01 samples tested for viability using flow cytometry. Detection of viability using the *lux*-reporter strain, *E. coli* DH5α/pσ70-*lux* was also attempted. It was shown previously that there was a dramatic decrease in luminescence when the bacteria were exposed to NETs. When samples were treated with DNase, Mg^{2+}, or PTase no restoration in survival was witnessed using the luminescence assay (data not shown).

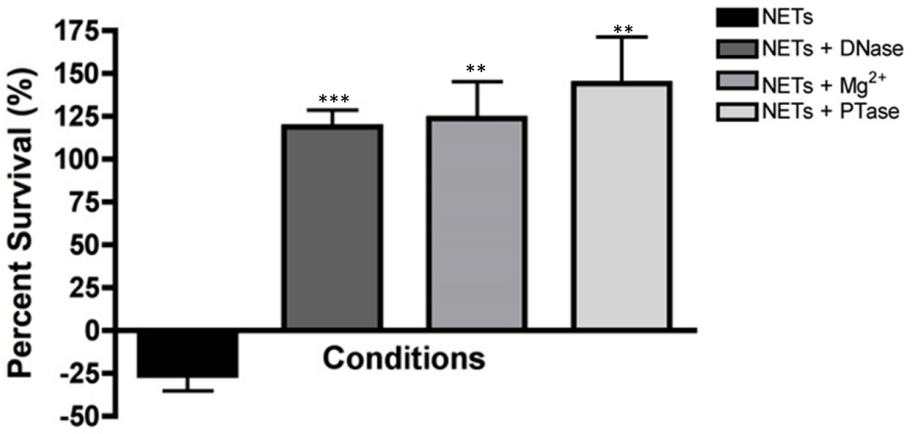


Figure 5.8. Blocking DNA protects *E. coli* DH5α from NET killing.

Activated neutrophils were incubated with *E. coli* DH5α/pσ70-*lux* for 4 hours. The percent survival was calculated from the CFU counts before and after incubation with neutrophils and was normalized to bacterial counts in the absence of neutrophils. Asterisks indicate a significant difference in bacterial survival when compared to *E. coli* DH5α/pσ70-*lux* incubated with NETs (**P<0.01, ***P<0.001). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed three times with a representative experiment shown.

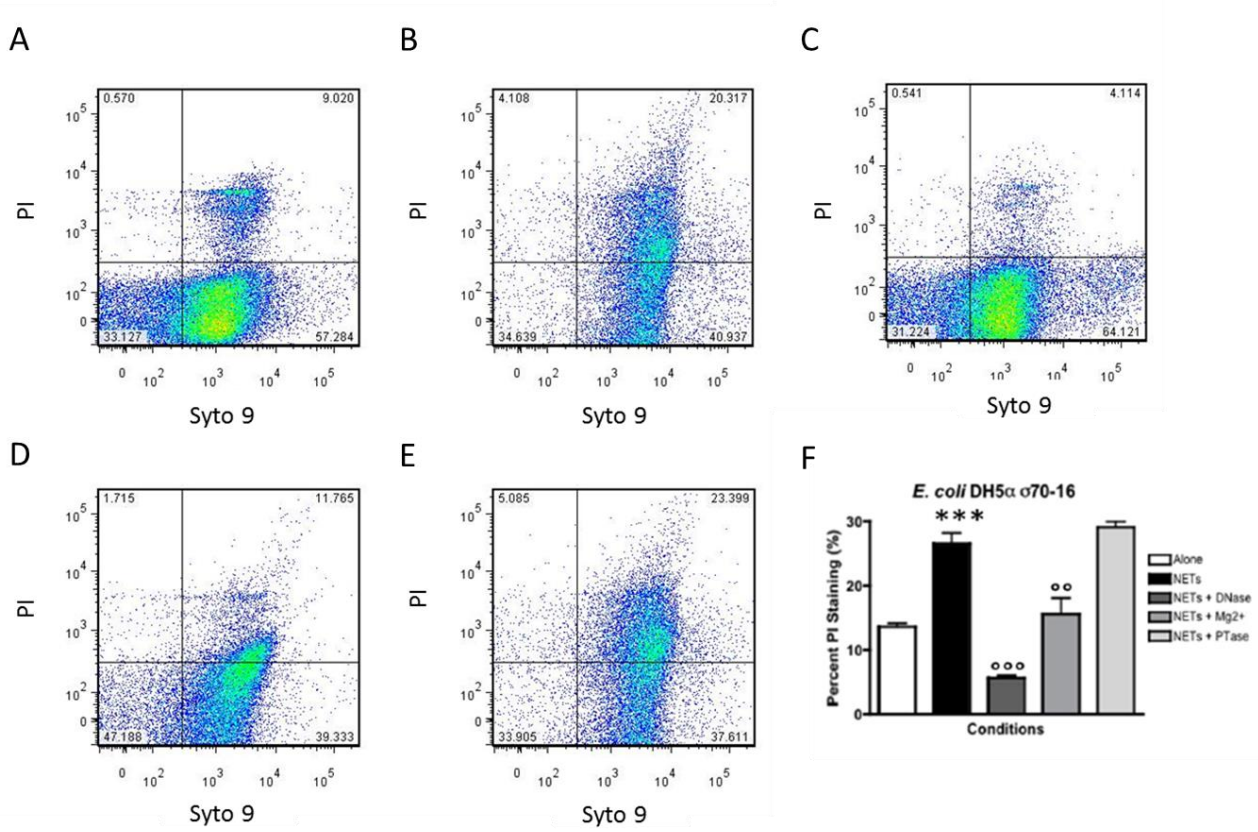


Figure 5.9. Using flow cytometry to measure NET-killing on *E. coli* DH5α

Samples *E. coli* DH5α/pσ70-*lux* that were incubated with NETs for up to 4 hours were stained with PI and Syto 9 to measure live and dead cells. Mid-log phase DH5α was used as a base line control (A) to compare to bacteria incubated with NETs (B). In some samples, DNase (C), 5 mM Mg^{2+} (D), or PTase (E) was added at the beginning of the incubation. The percentage of PI staining was calculated as percentage of total cells (F). Asterisks indicate a significant difference in PI staining between bacteria incubated with NETs compared to bacteria alone (***P<0.001). Circles indicate a significant difference in bacterial survival under treated conditions compared to bacteria incubated with NETs (°°P<0.01, °°°P<0.001). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed at least three times and a representative experiment shown.

5.5 Summary of findings

NETs are composed of the DNA and proteins found within the neutrophil. The negative charge of the phosphate backbone is what allows DNA to act as a cation chelator and antimicrobial agent. Multiple methods of monitoring bacterial viability indicated that targeting the DNA in NETs, with DNase, Mg^{2+}, or PTase treatments restored bacterial survival. This indicates that the DNA in NETs contributes to antimicrobial killing. Firstly, direct plate counts using both *P. aeruginosa* and *E. coli* show a return in bacterial survival when treated with DNase, Mg^{2+} and PTase (Figures 5.3, 5.8). Flow cytometry analysis after live/dead staining, both strains showed a large population of cells that appeared "dead" or "membrane damaged" in the presence of NETs. Within some sample sets of *P. aeruginosa*, we observed two subpopulations of events stained with both PI and Syto 9. One subpopulation showed more PI staining which we speculate to be "dead" cells, and the second subpopulation is likely a "dying" population. Out of the total dead cell population stained with PI and Syto 9, there was increased survival in the presence of excess magnesium and DNase, but not after the addition of PTase. However, upon closer examination of the highly PI stained subpopulation, DNase, excess Mg^{2+} and PTase treatments all restored survival, with the DNase treatment producing a statistically significant effect. A post-DNase treatment showed the loss of this "dead" population indicating that the NET-killing may be a slow process and this post-DNase treatment allows for cell recovery.

The use of the *lux*-reporter system in *P. aeruginosa* PA01 also adds a third method to examine bacterial viability when exposed to NETs. When incubated with DNase, Mg^{2+}, or PTase there was a return in luminescence, indicating a return in bacterial viability. With the incubation of DNase there was often an increase in luminescence, greater than the bacteria alone condition

suggestion that the DNase degraded NETs can be used by *P. aeruginosa* as a nutrient source and increase bacterial growth.

Chapter Six: Adaptations used by *Pseudomonas aeruginosa* to resist neutrophil extracellular trap killing

6.1 The neutrophil arsenal

The interaction between the host immune system and invading pathogens can be described as an arms race. Through years of evolution the immune system has developed strategies to combat or protect itself from pathogenic bacteria. However, these mechanisms may become less effective as the bacteria develop ways to counter these strikes. The immune system is constantly bombarded with different microbes. It is through maintaining a balance between the interactions of the immune system and bacteria that the host is able to remain healthy. If this balance is shifted to either side, there can be detrimental consequences. If a bacterial species over-runs the immune system, it can lead to septicemia and even sepsis causing death in the host. On the other hand, if the immune system becomes too active, it can lead to autoimmune diseases and cause a great deal of damage to the host.

When neutrophils come into contact with microbes, they will try and engulf the organism into a phagocytic vacuole termed a phagosome. The phagosome is then "filled" with various antimicrobial compounds by fusing with intracellular granules, forming a phagolysosome[212]. The microbes in the phagolysosome are killed though exposure to non-oxidative and oxidative mechanisms[213]. Neutrophils can also release the contents of their granules into the extracellular space through a process known as degranulation. Neutrophils contain four types of granules. The secretory vesicles are most likely to release their contents through degranulation, followed by gelatinase granules, specific granules and azurophilic granules[214]. A third type of microbe killing is through the production of NETs. Neutrophils release granule proteins and chromatin as fibres that form lattice structures to trap and kill pathogens[102; 107; 156; 212]. These NETs may kill microbes

through contact exposure to concentrated antimicrobial compounds within the NET or prevent the spread of microbes from the initial site of infection[212]. A final method to rid the host of pathogens is through apoptosis. In some cases, once a neutrophil has phagocytosed a number of microbes, it may undergo apoptosis or be cleared by macrophages, to help resolve the infection[215]. Despite these various methods of killing, microbes have developed ways to avoid or protect themselves to survive in the host.

6.2 Mechanism used by bacteria to evade neutrophil killing

6.2.1 Bacterial response to neutrophils

One of the hallmark features of neutrophil killing is the exposure to reactive oxygen species. Various microbes are able to respond to the presence of these ROS, especially when engulfed in a phagolysosome. Organisms such as *S. aureus*, *Streptococcus pyogenes*, *Candida albicans*, and *P. aeruginosa* show an upregulation of genes crucial to resist oxidative stress when phagocytosed[13; 216; 217]. Some organisms, such as *C. albicans* and *S. aureus* can increase the expression of oxidative stress genes, such as superoxide dismutase, catalase, and glutathione peroxidase[156; 218], which neutralize ROS and protect bacteria from the oxidative arsenal of neutrophils.

6.2.2 Avoiding neutrophil contact and preventing phagocytosis

Pathogens can avoid exposure to neutrophils by either moving to regions that the neutrophils cannot get access to or by preventing recruitment of the immune cells to the site of infection. *Listeria monocytogenes* is able to induce its own uptake into epithelial cells to avoid contact with neutrophils[219]. On the other hand, *S. aureus* is able to produce and secrete a chemotaxis inhibitory protein that binds to the formyl peptide receptor and the C5a receptor on neutrophils preventing the migration of the neutrophil to the site of infection[220]. A

polysaccharide capsular component on the fungus, *Crytococcus neoformans* can induce the loss of tumour necrosis factor receptor (TNFR). The loss of this receptor desensitizes the neutrophils to TNF-α, preventing the cells from being able to migrate to the infection site[221]. Thus, by preventing the interaction with neutrophils, certain organisms are able to survive in the host. Other microbes have established methods to survive phagocytosis by the leukocytes.

There are various ways microbes are able to prevent being engulfed by neutrophils. Some bacteria and fungi use physical barriers, such as the production of polysaccharide or polyglutamate capsules to prevent recognition of phagocytes[212]. The capsular antigens O75 and K5 found on uropathogenic *E. coli* can increase the bacterial resistance to phagocytosis[222]. Another method to survive phagocytosis is by preventing opsonisation. This method can be witnessed in *S. aureus,* which is able to secrete the complement inhibitor SCIN. SCIN inhibits the central complement convertases, ultimately reducing phagocytosis following opsonisation and efficiently block downstream effector functions[223]. A third mechanism to prevent phagocytosis is by inhibiting the actin cytoskeleton required for the formation of pseudopods needed for engulfment. *Yersinia* species use this approach and upon contact with the neutrophils, the bacteria use a type III secretion system to inject effector proteins into the cytoplasm of the host cell[224]. *In vivo,* these effector proteins are targeted to dendritic cells, macrophages and neutrophils. Four proteins, YopE, YopH, YopT and YopO inhibit the actin cytoskeleton and reduce neutrophil uptake[224].

6.2.3 Intracellular survival or neutrophil killing

Bacteria can also survive within the host cell or they will simply lyse and kill the neutrophil. Some of the intracellular strategies employed by certain organisms include inhibiting the fusion of the phagosome and lysosome, surviving inside the phagolysosome, and/or escaping

into the cytoplasm. The bacterium *S. pyogenes* is able to use all three of these strategies to withstand neutrophil killing. *S. pyogenes* is able to produce M and M-like proteins that can prevent degranulation and the fusion of the phagosome with azurophilic granules[225]. The bacterium is able to produce proteins H and M that allow *S. pyogenes* to survive within the phagolysosome, specifically the strong oxidative burst associated with being internalized[225]. By remaining in the phagolysosome, the bacteria are able to hide and avoid other immune cells, thus permitting long term survival. Once in the phagolysosome, *S. pyogenes* forms a large capsule that results in increased virulence and resistance to phagocytic killing[212]. This may allow it to escape the phagolysosome and survive within the cytoplasm of the neutrophils. The bacterium *Helicobacter pylori* is able to redirect the oxidative burst in neutrophils to the extracellular space, likely due to the redirected assembly of the NADPH-oxidase complex to the plasma membrane and to exclude the phagosomal membranes that surround the bacterium[226].

However, some microbes simply kill leukocytes by producing various toxins that target various cells of the innate immune system. *S. aureus* produces pore-forming toxins (PFTs), with one example being the α-toxin (α-haemolysin toxin). The α-toxin functions to bind to the plasma membrane of the target cell and oligomerizes into a ring structure, forming a transmembrane pore [227]. This will cause the cytoplasm to leak out of the leukocyte, leading to cell death. Other bacteria have been shown to be able to produce compounds that lead to the death of polymorphonuclear leukocytes (PMNs), such as neutrophils. *P. aeruginosa* produces rhamnolipids that have been shown to increase its tolerance towards immune cells[51; 124]. When exposed to PMNs, *P. aeruginosa* biofilms respond by upregulating the production of toxic compounds, such as rhamnolipids[124]. These rhamnolipids serve as a protective shield surrounding the biofilm cells and are capable of destroying approaching PMNs via necrosis[51; 124].

6.2.4 Bacterial survival to NET capture

One of the major components of NETs is the DNA backbone that has been shown to be a structural component of these traps. When treated with a deoxyribonuclease, the NET structure is degraded and NET killing is blocked. Various bacteria have been shown to produce extracellular nucleases. Specifically, the bacteria *S. pyogenes* and *Streptococcus pneumoniae* are able to express the extracellular DNases Sda1 and EndA respectively[137; 149]. Studies have shown that the streptococcal nucleases are able to degrade NETs and *S. pyogenes* strains lacking the *sda1* gene are more susceptible to NET killing[149]. This suggests that the production of an extracellular nuclease is a strong defense mechanism to NETs. The presence of a capsule has also been shown to aid in the protection against neutrophil extracellular traps. *Streptococcus pneumonia* can counteract the killing of NETs through the expression of polysaccharide capsule and lipoteichoic acid (LTA) modifications[164]. Pneumococci contain a *dlt* operon that mediates the incorporation of d-alanine residues into LTAs, thereby introducing positive charge onto the surface and protect the bacteria from antimicrobial peptides through electrochemical repulsion[164].

6.3 Lipopolysaccharide modifications aid in NET survival

Since the outer surface modifications of aminoarabinose-modified LPS and spermidine synthesis were upregulated in the presence of NETs, we hypothesized that these surface modifications would protect *P. aeruginosa* from NET killing, given their role in protecting from antimicrobial peptide damage and the known antimicrobial function of histone in NETs. Although histones are primarily a structural protein in the nucleosome and are important for chromatin structural organization, there are histone-derived peptides with known antimicrobial peptide activity[107; 144]. It has also been shown that the antimicrobial peptides in neutrophils,

defensins, are also incorporated into neutrophil extracellular traps[106]. Therefore, we examined if mutants in these pathways were more sensitive to NET killing.

Using the NET-killing assay, survival of the PA01 wild-type strain was compared to mutants defective in the production of aminoarabinose-modified LPS (*PA3553::lux*), and to two mutants in the spermidine synthesis genes (intergenic *PA4773/4::lux* and *PA4774::lux*). All three mutants were more susceptible to NET killing, as determined by plate counting for viable cells (Figure 6.1). We included in this panel of strains the chromosomally complemented *PA4774 c.c* strain, which expressed the *PA4773-PA4775* cluster at a neutral site in the chromosome of the *PA4774::lux* mutant strain[94]. The *PA4774c.c* complemented strain showed a return in survival compared to the *PA4774::lux* mutant. The *arn* operon (*PA3552-PA3559*) is required for the aminoarabinose modification of LPS. The transposon mutant in the second gene on this operon, *PA3553::lux*, has polar effects on the downstream genes, which is not possible to complement by the expression of the *PA3553* gene *in trans*. Due to the length of the *arn* operon and the polar mutations caused by transposon insertions, our lab has not yet attempted to genetically complement mutations in this cluster.

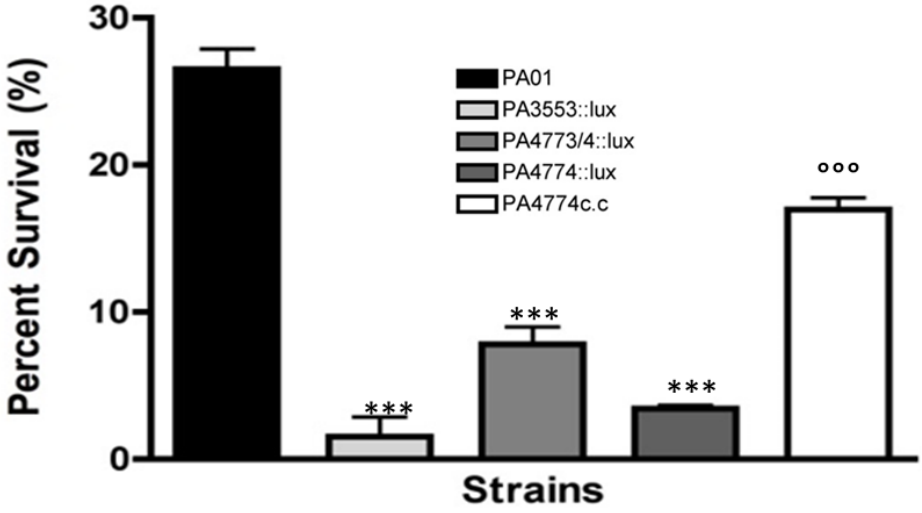


Figure 6.1. Outer surface modifications are required for NET survival

Activated neutrophils are incubated with either *P. aeruginosa* PA01 wild-type, *PA3553::lux, PA47743/4::lux, PA4774::lux* or *PA4774c.c* for 4 hours. The percent survival was calculated from the CFU counts before and after incubation with neutrophils and was normalized to bacterial counts in the absence of neutrophils. Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 (***P<0.001). Circles indicate a significant difference in bacterial survival when of *PA4774c.c* compared *to PA4774::lux* strain (°°°P<0.001). Each value shown is an average of at least three replicates with error bars representing the SEM. The experiment was performed three times with a representative experiment shown.

6.4 Exopolysaccharide/capsule production for biofilm formation and immune evasion

Exoploysaccharides are one of the major components of biofilm extracellular matrix. Non-mucoid *P. aeruginosa* isolates, the early colonizers of CF patients, produce two exopolysaccharides, Pel and Psl. Pel has been shown to specifically contribute to increased antibiotic resistance[81] while Psl is required for intracellular survival in neutrophils and macrophages[79]. Pel is also able to inhibit neutrophil phagocytosis and reduce the oxidative response by limiting complement-mediated opsonisation[228]. The Psl exopolysaccharide does have capsule-like functions, and we wanted to extend the study of possible capsule functions of Pel and Psl by testing their role in protecting *P. aeruginosa* from NET killing. The Pel and Psl exopolysaccharides are localized to the bacterial surface, and as such, may serve to shield the bacteria from direct contact with the various neutrophil components that are embedded in NETs.

6.4.1 Pel and Psl production protect P. aeruginosa *from NETs*

Using our co-incubation assay of NET-activated neutrophils with *P. aeruginosa*, we tested the requirement of the Psl and Pel capsules in protecting against the killing activity of NETs. In this assay, two independent *pel* transposon mutants, *pelB::lux and pelD::lux,* and the *Δpsl* strain were all significantly more sensitive to NETs than the PA01 control (Figure 6.2). We obtained a strain that overexpresses the Psl using an arabinose-controlled *psl* promoter[172] and predicted that this strain would be more tolerant than the wild type to NET killing. The Psl overexpressing (OE) strain was shown to restore survival during co-incubation with NET activated neutrophils (Figure 6.1). These data indicate that both the Psl and Pel exopolysaccharides have this novel capsule function of protecting from NETs.

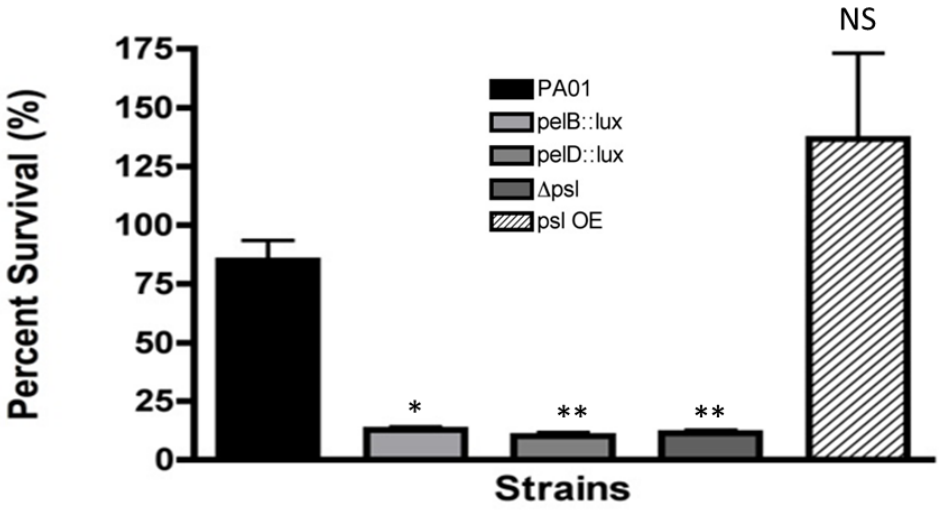


Figure 6.2. Exopolysaccharide production protects against NETs

Activated neutrophils were incubated with *P. aeruginosa* PA01, *pelB::lux, pelD::lux, Δpsl* and *psl* OE strains for 4 hours. The percent survival was calculated from the CFU counts before and after incubation with neutrophils and was normalized to bacterial counts in the absence of neutrophils. Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 wild type (*P<0.05, **P<0.01, NS= not significant). Each value shown is an average of at least three replicates with the error bars representing the SEM. The experiment was performed at least three times and a representative experiment shown.

6.5 Deoxyribonuclease production for DNA utilization as a nutrient

In the environment, there is often extracellular DNA (eDNA) present in various locations, such as in soil and water[229; 230]. Extracellular DNA is has been found in various infection sites, such as the CF lung where it can reach concentrations up to 20 mg/mL in sputum[166; 231; 232], and is even present circulating throughout the body in blood and other fluids[233]. In the biofilm matrix, eDNA serves as a structural component[38]. The eDNA found within the biofilm may come from dead cells, either bacterial or host, or even released through outer membrane vesicles[39; 40; 86]. In large microcolonies the centres may undergo dispersion triggered by a central hollowing that is accompanied by a virus or rhamnolipid-mediated lysis causing a release of DNA[41; 234]. This may serve as a means to provide DNA as a nutrient source to the neighbouring cells, especially to cells in the deepest part of the biofilm where the availability of nutrients is limited[87]. Given the abundance and stability of DNA in the environment, it is not surprising that *Shewenalla* spp. and *E. coli* are able to utilize DNA as a nutrient[87; 235]. Our lab has recently reported that *P. aeruginosa* can also utilize DNA as a sole source of phosphate, nitrogen and carbon, and the ability to recycle DNA may support growth in the environment, during infection, and in the nutrient limited regions of thick biofilms[236].

We identified a two-gene operon that encodes a secreted deoxyribonuclease (DNase) and phosphatase (PTase)[87]. These genes were highly induced in phosphate limiting conditions, and this suggested that they were involved in acquisition of phosphate[87]. It was also shown that the DNase-PTase genes were also highly induced in the presence of eDNA, supporting the hypothesis that they are involved in DNA utilization. A mutant in the DNase (*eddB::lux*) was shown to have a growth defect in the presence of eDNA, thereby confirming that this enzyme is required for DNA degradation and utilization[87]. The production of DNases has also been shown

to be important in bacterial virulence and one possible role of DNases is the degradation of neutrophil extracellular traps. Given the precedent for DNases contributing to degrading NETs, we hypothesized that *eddAB* (PTase-DNase) were also involved in degrading NETs and therefore contribute to NET resistance.

6.5.1 The eddB *DNase is required for DNA degradation*

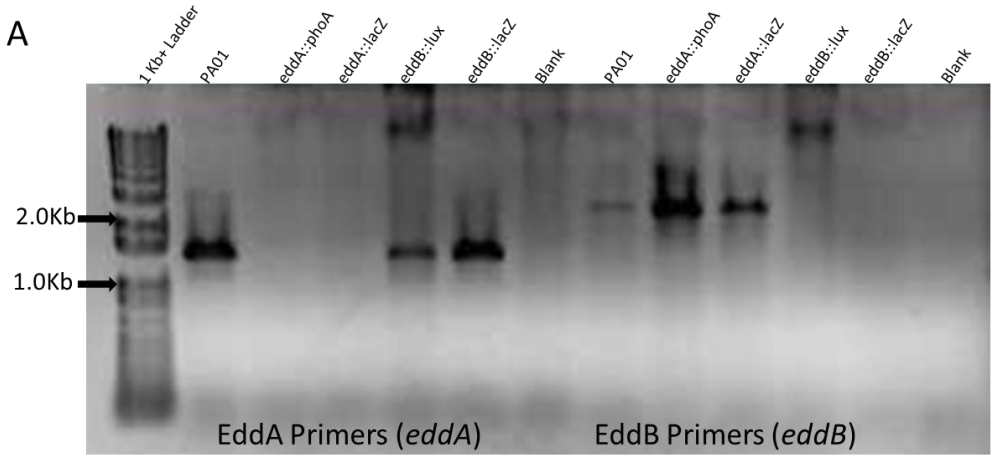
Our strain collection included a mini-Tn*5*-*lux* mutant in the DNase (*eddB::lux*). We acquired additional mini-Tn*5* mutants from other published mutant library collections, including a DNase mutant, *eddB::lacZ*, and PTase mutants, *eddA::lacZ* and *eddA::phoA*. To confirm the correct insertion of the transposon, PCR was performed to amplify the genes of interest (Figure 6.3A). Two sets of primers were used for the PCR, one set for the *eddA* gene (EddA-F and EddA-R) and the second set for the *eddB* gene (EddB-F and EddB-R). Each gene was amplified for all the mutant strains along with a PA01 wild-type control. Amplification of the *eddA* gene was not seen for both *eddA::lacZ* and *eddA::phoA;* however a band at ~2.3 Kb, the expected size of the *eddB* gene, was observed for these strains. In contrast, the *eddB* gene was unable to be amplified in the *eddB::lux* and *eddB::lacZ* while amplification of the *eddA* gene was witnessed with a band present at ~1.6 Kb (expected size) for these two strains. The PA01 wild-type strain showed amplification for both genes with bands at ~1.6 Kb and ~2.3 Kb for *eddA* and *eddB* respectively. Figure 6.3B shows a schematic of the transposon insertions for the mutant strains and successful or failed amplification of the genes of interest.

Complementation of two DNase mutants was then performed to restore DNase activity (done by Marina Tom). The *eddB* gene was first amplified using the EddB primers, cloned into the pUCP22 vector and directly transformed into the mutant strains (*eddB::lacZ* and *eddA::*phoA). The DNase construct could not be expressed in *E. coli*, likely because it is not

secreted and degrades genomic DNA. Complementation was performed for the *eddB::lacZ* strain to return DNase function and by complementing the *eddA::phoA* strain; we are able to observe that DNase activity in culture supernatants was also restored, despite the lack of PTase expression (Figure 6.3C). To monitor the DNase activity, a DNase supernatant assay was used. The DNase was previously shown to be secreted by the type II secretion system and was present and active in culture supernatants, as monitored using a DNA degradation assay. In this assay, 5 µg of DNA were incubated with 15 µl of culture supernatant of the *P. aeruginosa* strains grown under inducing conditions (limiting phosphate)[87]. From the DNase assay (Figure 6.3C), DNA degradation was not observed for the *eddB::lacZ* and *eddA::phoA* mutants, while degradation was seen in the PA01 wild-type and both complemented strains, *eddB::lacZ/*pEddB and *eddA::phoA*/pEddB.

Figure 6.3. Genetic organization of the DNase and PTase operon.

PCR amplification products of the *eddA* (PA3910) and *eddB* (PA3909) genes in PA01 wild-type, *eddA::lacZ, eddA::phoA, eddB::lux,* and *eddB::lacZ* were analyzed on a 1% agarose gel (A) A schematic of the transposon insertion for the mutant strains. Plus (+) indicates successful amplification and minus (-) indicates failed amplification. The black triangle (▼) indicates the positions of the transposon insertion (B). A DNase supernatant assay using supernatants from PA01 wild-type, *eddB::lacZ, eddB::lacZ/*pEddB, *eddA::phoA,* and *eddA::phoA/*pEddB cultures (C). For the PCR agarose gel and supernatant assay gel, the 1 Kb+ Ladder was used as a molecular marker.


A
1 Kb+ Ladder
PA01
eddA::phoA
eddA::lacZ
eddB::lux
eddB::lacZ
Blank
PA01
eddA::phoA
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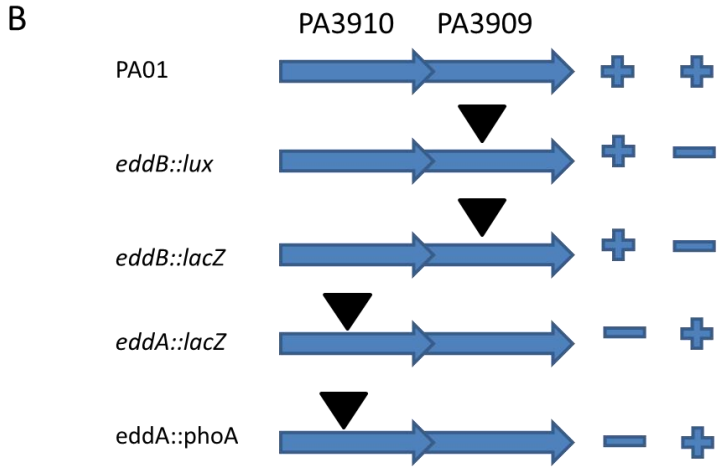
EddA Primers (eddA)
EddB Primers (eddB)


B
PA3910
PA3909
PA01
eddB::lux
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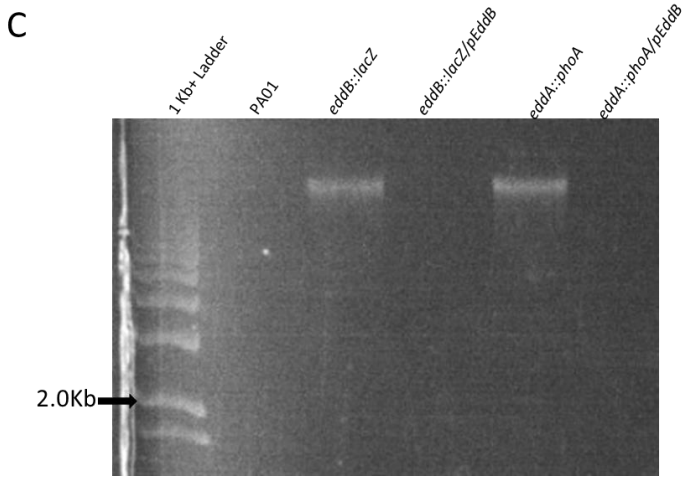
eddA::phoA


C
1 Kb+ Ladder
PA01
eddB::lacZ
eddB::lacZ/pEddB
eddA::phoA
eddA::phoA/pEddB
2.0Kb

6.5.2 The eddB DNase is required for surviving NET killing

Previous studies have shown that the production of a DNase aids in the protection against neutrophil extracellular traps. Using the NET-killing assay, with the various DNase and PTase mutants along with their respective complemented strains, were examined for susceptibility to NETs. When incubated with NET-activated neutrophils, *eddB::lacZ,* and *eddA::phoA* showed reduced survival when compared to the PA01 wild-type strain (Figure 6.4). This result suggested that both genes, DNase and PTase, were required to survive NET-killing; however, the PTase transposon insertion likely had a polar effect on expression of the DNase. Supernatant from the PTase mutant (*eddA::phoA*) did not have any DNA degradation ability (Figure 6.3C), possibly due to the polar effect on DNase expression. Both the *eddB::lacZ*/pEddB and *eddA::phoA*/pEddB strains showed better survival than the wild-type strain, producing statistically significant results showing survival greater than parental levels (Figure 6.4). This may be due to an over expression of the DNase allowing for better protection to NETs.

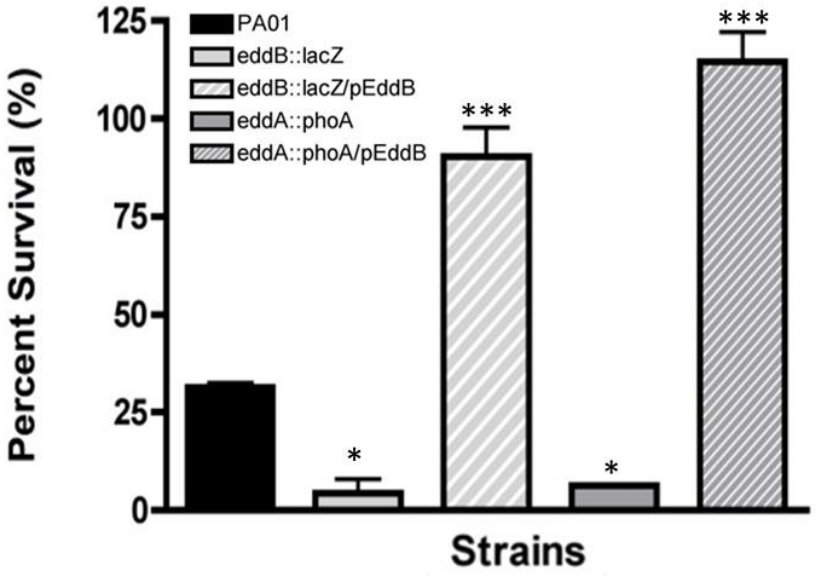


Figure 6.4. The *eddB* DNase gene is vital for NET survival

Neutrophils were co-incubated with *P. aeruginosa* PA01 wild-type, *eddB::lacZ,* *eddB::lacZ/*pEddB, *eddA::phoA,* and *eddA::phoA/*pEddB for 4 hours. The percent survival was calculated from the CFU counts before and after incubation with neutrophils and was normalized to bacterial counts in the absence of neutrophils. Asterisks indicate a significant difference in bacterial survival when compared to *P. aeruginosa* PA01 (*P<0.05, ***P<0.001). Each value shown is an average of at least three replicates with the error bars representing the SEM. The experiment was performed at least three times and a representative experiment is shown.

6.6 Summary of findings

Various bacterial species have developed strategies to evade neutrophil killing, which include the invasion of neutrophil extracellular traps. *P. aeruginosa* has been shown to be more tolerant to NETs than certain other bacteria. I previously showed that LPS modifications are induced in the presence of NETs. Here I show that the LPS modifications are important in bacterial survival to NETs and some preliminary data suggesting that EPS and DNase and PTase production are important for surviving NET-killing

The production of LPS modifications can aid in protecting the bacteria from antimicrobial compounds and ROS. Mutants deficient in the production of these LPS modifications are more sensitive to NET-killing. The Pel and Psl exopolysaccharides may serve as a capsule to protect the bacteria from NETs since deficient mutant strains are killed more easily by NETs. *P. aeruginosa* is also able to produce exogenous DNase and PTase. Mutants unable to produce the DNase showed greater sensitivity to NETs. Mutant stains complemented with the *eddB* gene showed a return in survival indicating that the *eddB* DNase is vital for surviving NETs. Mutants in the *eddA* gene (PTase) also showed sensitivity to NETs, indicating that PTase may be vital for NET survival. However, the PTase mutant expressing the DNase construct appeared to survive better exposed to NETs, even when lacking PTase production. This may indicate that PTase is not required for NET survival, or the over production of DNase in this mutant masked the lack of PTase production.

Chapter Seven: Discussion, Future Directions and Conclusions

7.1 Discussion

7.1.1 P. aeruginosa *is tolerant to NET-killing*

Neutrophil extracellular traps are a newly discovered mechanism of bacterial trapping and killing. The trapping function is well described, but the killing activity is poorly understood. Numerous labs are studying the signalling pathways and cell biology of how and when NETS are produced, both *in vitro* and *in vivo*, with only some studies attempting to identify the crucial antimicrobial components. NETs contain proteins from azurophilic, specific and tertiary granules, and even a cytosolic antifungal component[102; 107; 108], but their relative concentrations in a NET and their specific contributions have not been dissected. Most cytoplasmic proteins, such as actin and tubulin, are not incorporated into the NET structure[107].

The killing efficiency of NETs appears to vary between microbes, including bacteria. *S. aureus* is killed by NETs[106], however other bacteria, like *P. aeruginosa*, are more tolerant to NET exposure[30; 106]. From our study, we compared the relative survival of *P. aeruginosa* with other Gram-negative and Gram-positive bacteria, and confirmed that *P. aeruginosa* is quite tolerant to NET killing (Figures 3.6 and 3.7). Previous reports have also examined the kinetics of NET formation, and found *S. aureus* induces a rapid NET formation, possibly different from NETs induced by other stimuli or bacteria. I have also confirmed these observations, showing that the amount of early NET formation caused by *P. aeruginosa* appears less than that of *S. aureus* and *E. coli* (Figure 3.5). Recent studies have examined the specific bacterial products that are required for activating NET production in neutrophils. This is an important problem as it may shed light on the mechanism to explain when and why neutrophils produce NETs. Culture supernatant from *S. aureus* stimulates NET production[128]. One possible NET inducing molecule

is Panton-Valentine leukocidin (PVL), which is a secreted β-pore-forming toxin. It has been suggested that low concentrations of PVL may in fact induce NETs while higher concentrations will cause cell lysis[128]. *P. aeruginosa* is also able to induce NET formation with a secreted product called pyocyanin; a phenazine exotoxin[108]. Pyocyanin is a non-enzymatic NADPH oxidase and can execute the same chemical reaction as the members of the Nox NADPH oxidase family[107; 237]. In neutrophils, the Nox2-based NADPH oxidase is one of the most abundant and important oxidases involved in a variety of cell functions that include NET formation[102]. Pyocyanin has been shown to induce NETs at concentrations that would normally be found in CF sputum (>10 μM)[108]. Pyocyanin production is favoured in conditions of high cell populations and suboptimal nutrient availability, thus it was shown to be produced only after exponential phase of growth[238; 239]. Since mid-log cultures were incubated with neutrophils, there would have been a limited production of pyocyanin under our culture conditions, which would contribute to the delay in NET production by *P. aeruginosa*. Both *S. aureus* and *P. aeruginosa* appear to generate secreted, NET-inducing factors. Limiting early NET induction may be a strategy to avoid NET exposure, coupled with the ability to survive NET killing may compound the NET resistance of *P. aeruginosa*. Given the increased NET resistance of *P. aeruginosa,* it was interesting for us to examine the possible mechanisms that allow for its survival.

7.1.2 Cell surface modifications are required to resist NETs

The *pmr/arn* gene cluster, that facilitates aminoarabinose modification of lipid A, is well conserved in Gram-negative pathogens and is generally required for antimicrobial peptide resistance and virulence. The PhoPQ two-component system is also highly conserved and involved in controlling the *pmr/arn* cluster under Mg^{2+} limiting conditions. Although it was previously thought that *Salmonella* encounters Mg^{2+} limitation during intracellular macrophage

survival, it was shown that this environment was not Mg^{2+} limiting, but is acidified and contains antimicrobial peptides, both of which are also inducing signals for *pmr/arn* expression. This raises the question: when do bacteria encounter Mg^{2+} limiting environments? Mg^{2+} is present at millimolar amounts in the body and in the soil, unlike other metals like Fe^{2+} that are sequestered or insoluble[95]. Our lab proposes that the *pmr/arn* genes are expressed in DNA-rich environments like biofilms. My work has shown that the *pmr/arn*, as well as surface spermidine production, are both expressed in response to the DNA ejected during neutrophil NET formation (Figure 4.3).

The current of view of NET killing is that the antimicrobial factors are embedded in the DNA lattice. When bacteria are trapped in the NET, they encounter high, localized concentrations of antimicrobial factors, such as the histones, or histone-derived antimicrobial peptides. Consistent with this model, *P. aeruginosa* produces different surface modifications that shield the outer membrane from antimicrobial peptides/histones damage as a defense strategy. However, there are implications to our findings. Reactive oxygen species are produced early in the process of NET formation, and are generated from the MPO associated with the NETs[211]. We have shown that surface polyamines, consisting mostly of putrescine and lesser amounts of spermidine, have antioxidant activity and protect the membrane lipids from oxidative damage. Therefore, we propose that DNA-induced expression of the spermidine synthesis genes may also protect from NET-mediated oxidative damage, since mutants unable to modify the cell surface are most susceptible to NETs (Figure 6.1). Since these surface modification genes are induced by DNA, and our work has shown that DNA has *in vitro* antimicrobial activity and contributes to NET killing, it is also possible that *P. aeruginosa* is shielding its' surface against the membrane-disrupting action that results from stripping cations from the bacterial membrane. The covalent

addition of aminoarabinose to lipid A, or the production of polycations on the surface, may resist the killing activities of DNA. This hypothesis is currently being examined in the lab.

7.1.3 Capsules shield from NET killing

P. aeruginosa is able to express exopolysaccharide genes *pel* and *psl* under magnesium limiting conditions[52]. Pel has been shown to specifically contribute to increased antibiotic resistance[81] while Psl is required for intracellular survival in neutrophils and macrophages[79]. Psl has also been shown to inhibit neutrophil phagocytosis and reduce the oxidative response by limiting complement-mediated opsonisation[228]. Pel and Psl deficient strains showed less tolerance to NETs, and conversely, Psl overexpression resulted in increased tolerance to NETs (Figure 6.2). This preliminary observation suggests a new capsule function for Psl/Pel, to limit the antimicrobial effects of NETs. Consistent with this theme, *Streptococcus pneumoniae* can counteract the killing of NETs through the expression of a polysaccharide capsule and lipoteichoic acid (LTA) modifications[164].

7.1.4 DNases and PTases degrade and neutralize DNA in NETs.

Another way bacteria protect themselves from neutrophil extracellular traps is through the production of nucleases to degrade the structures[137; 149]. *P. aeruginosa* is also able to produce a deoxyribonuclease (*eddB*) and phosphatase (*eddA*) under phosphate starvation conditions or in the presence of exogenous DNA added as the sole phosphate source. *P. aeruginosa* uses the secreted DNase to break down extracellular DNA and use it as a nutrient source[87]. The role of DNase has also been shown to inhibit ROS production, limiting the formation of NETs, thus microorganism-derived DNase would contribute to their ability to evade killing[211].

We identified novel functions of the DNase and PTase, since mutants in these genes were killed more easily by NETs, compared to the wild-type strain (Figure 6.4). We cloned and

introduced the DNase into both mutants, which restores the survival phenotype in both strains (Figure 6.3). The production DNase and PTase allow *P. aeruginosa* to combat NET killing by degrading the NET. DNase and PTase production might also permit *P. aeruginosa* to utilize degraded NET DNA as a nutrient source. In our experiments with exogenous DNase, we frequently observed bacterial survival and growth beyond the levels seen by the bacteria alone (Figure 5.3 and 5.4), which is evidence that degraded NETs do support bacterial growth. Taken together, *P. aeruginosa* uses multiple strategies to defend against NET killing.

7.1.5 DNA in NETs can induce expression of bacterial defense genes

Prior to this study, we added exogenous DNA to planktonic cultures and measured the *P. aeruginosa* gene expression response. Using this approach, RNA-SEQ analysis was performed to identify the global bacterial response to extracellular DNA. This genome-wide method identified 255 genes that are significantly upregulated by exogenous DNA and illustrate that *P. aeruginosa* physiology and gene expression are greatly influenced by extracellular DNA (unpublished observation). For these experiments, the DNA concentrations used can be found in the CF lung, and therefore many of these genes might be expressed in the CF lung. DNA also accumulates in biofilms and may influence gene expression in biofilms, which extends our understanding of the possible functions of DNA in a biofilm. Although the *pmr* genes are not highly expressed in flow chamber biofilms in wild type strains, a mutant has been identified that accumulates large amounts of eDNA[95]. Interestingly, a microarray was performed on biofilms formed by the *bfmR* mutant, and both the *pmr* and *PA4773-4775* operons were upregulated compared to wild type biofilms[240]. Here, we extend these observations by examining the influence of NET DNA on *P. aeruginosa* gene expression.

In simple coincubation experiments with *lux*-tagged bacterial reporters and NET-activated neutrophils, we have shown that the DNA found in NETs can induce surface modification genes in *P. aeruginosa* (Figure 4. 3). It is very interesting that sufficient DNA can be released from $1x10^6$ neutrophils to induce a bacterial response. It should also be noted that only 10-30% of activated neutrophils make NETs[100; 102; 130; 211]. Here, *P. aeruginosa* is able to sense the NET structure, specifically the DNA, and respond to this antibacterial attack by expressing defense genes. This may explain why *P. aeruginosa* is persistent in causing lung infection in CF patients.

7.1.6 DNA is an antimicrobial component of neutrophil extracellular traps

This killing mechanism of NETs is generally attributed to granular proteins that are embedded in the DNA lattice. There is experimental evidence that blocking either histones or myeloperoxidase can limit or reduce the killing activity of NETs[107; 132; 140]. It is likely that these factors act in combination, and it is technically challenging to develop methods to inactivate multiple NET components and compare their contribution to NET killing. The discovery that DNA had antimicrobial activity was the initial motivation to investigate the hypothesis that DNA in NETs may also be an antimicrobial component. DNA is abundant in NETs, and DNA appears to have a broad spectrum of activity, since it is antimicrobial to several Gram-negative and Gram-positive organisms[12]. It is the negative charge from the phosphate backbone of DNA that likely permits the binding of cations, stripping of cations from bacterial membranes, and disrupting the inner and outer membrane integrity, resulting in lysis and death[12]. DNA is very efficient at binding various divalent cations, especially magnesium (Mg^{2+}), similar to the known chelator EDTA[12].

We provided several lines of evidence that DNA released in NETs has cation chelating and antimicrobial activity. As described above, NET formation led to induction of two gene clusters that were known to be DNA-induced. By neutralizing the DNA of the NET with excess cations, NETs no longer have the ability to induce expression of the bacterial genes (Figure 4.3), probably because DNA was saturated with Mg^{2+} and lacked sufficient capacity to maintain a Mg^{2+}-limited environment. The addition of DNase and subsequent NET degradation also blocked NET induction. We hypothesized that exogenous PTase would cleave phosphates from DNA, which are most likely the sites where the cations are binding. This hypothesis was confirmed, as PTase blocked expression of DNA-induced gene expression, although the NET structures remained intact (Figure 4.2). These data indicate that the cation chelating activity of DNA in NETs is responsible for inducing bacterial gene expression.

When examining the immunostaining pattern of anti-DNA and anti-histone antibodies, Gfp-tagged bacteria were colocalized with these structures, and had slightly different localization patterns to anti-MPO antibodies (Figure 3.4). Since DNA and histones are required for NET killing, this suggests killing is mediated by direct contact with specific NET components. MPO is also required but its action is mediated by diffusible ROS, and thus direct contact may not be needed. We next showed that Mg^{2+}, DNase and PTase also blocked *in vitro* bacterial killing from bactericidal concentrations of purified DNA (Figure 5.2). The final piece of evidence was the demonstration that targeting the DNA with excess cations or DNA-targeting enzymes also blocks bacterial killing by NETs (Figures 5.3, 5.5, 5.7, 5.8). This crucial experiment was performed with both *P. aeruginosa* and *E. coli*, whereby these treatments had a significant effect on survival in both organisms, as determined by plate counting.

The original NET description reported that exogenous DNase addition blocked NET killing and it was proposed that NET killing required bacterial contact and that the addition of DNase dissolved the lattice. As a result, the other embedded antimicrobial factors in NETs were released and effectively diluted[107]. We propose another interpretation of this early experiment. DNase addition degrades DNA, which is itself an important antimicrobial component of NETs. The other NET-bound factors are still present but released from the lattice, suggesting that they are not present at sufficient concentrations for bacterial killing.

7.1.7 Novel methods developed to monitor NET killing.

In order to better study the interactions of NETs and bacteria, more sensitive methods were needed, especially in the detection of bacterial viability. Typically, plate counting methods are used to assess NET killing, and really only show a 10 to100-fold decrease in bacterial numbers. For the plate counting assays, after the incubation with NETs, a DNase is added to release the dead bacteria. As reported in a recent study by Menegazzi *et al.* (2012), and in our experiments, DNase addition after coincubation with NETs and bacteria results in a greater recovery of bacteria[175]. The authors suggested that live bacteria are trapped in NETs, and that NETs do not actually kill[175]. However, we have clearly shown using multiple methods that NETs do kill, and that the ability of bacteria to utilize DNA as a nutrient supports growth of bacteria not trapped in NETs (figure 5.5).

Using *lux*-tagged bacteria allowed for a more precise means to measure bacterial killing by NETs. The kinetics of the killing could be monitored over time, which confirmed the slow kinetics of NET formation and killing that develops during a 4 hour experiment. Similarly, the gene induction kinetics using transcriptional reporters showed induction after 2 hours of induction, similar to the kinetics for NET production (Figure 4.3). Challenges arose when

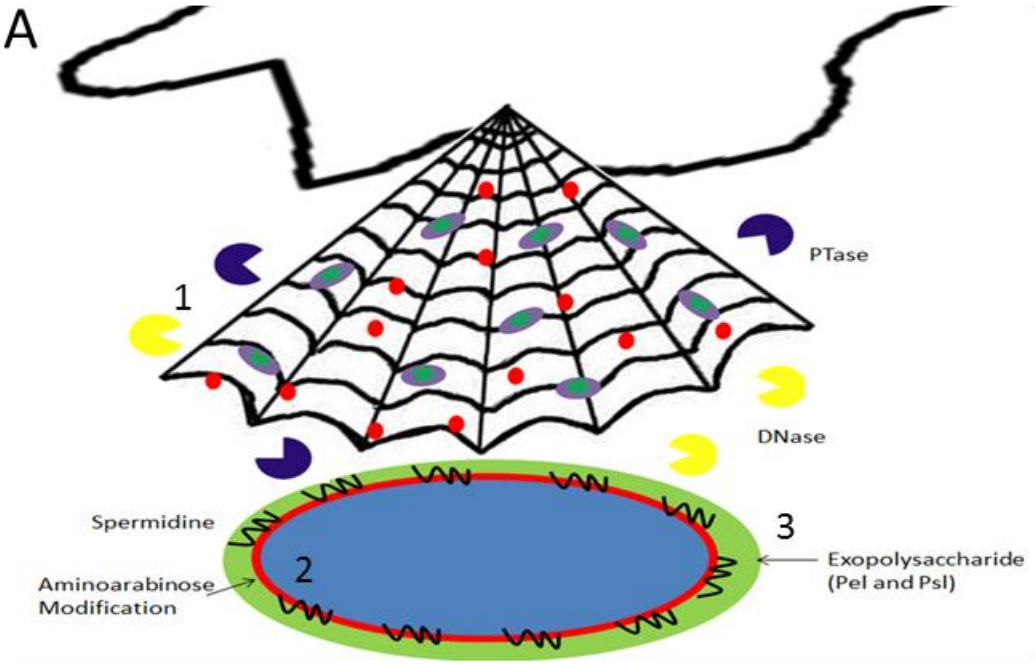
attempting to use the *E. coli* DH5α/pσ70-*lux* strain to measure viability. Plate counts indicated that DNase, PTase and Mg^{2+} increased *E. coli* survival (Figure 5.6), but this was not seen in viability experiments with *lux*-tagged bacteria (data not shown). Since *E. coli* was more sensitive to NETs, the protective treatments may not have had protective effects at the concentrations used, in the few hours following treatment. The pσ70-*lux* reporter will be expressed if the cells are in the exponential phase, but it is turned off when the cells enter stationary phase (Personal communication with Dr. Carla Davidson). The NET treated *E. coli* were likely being killed and entered into a non-growing, stationary phase, but were not able to be rescued and enter into a growing, exponential phase following the treatments. This may account for the inability to restore gene expression and luminescence of *E. coli* during the *lux*-based viability experiments.

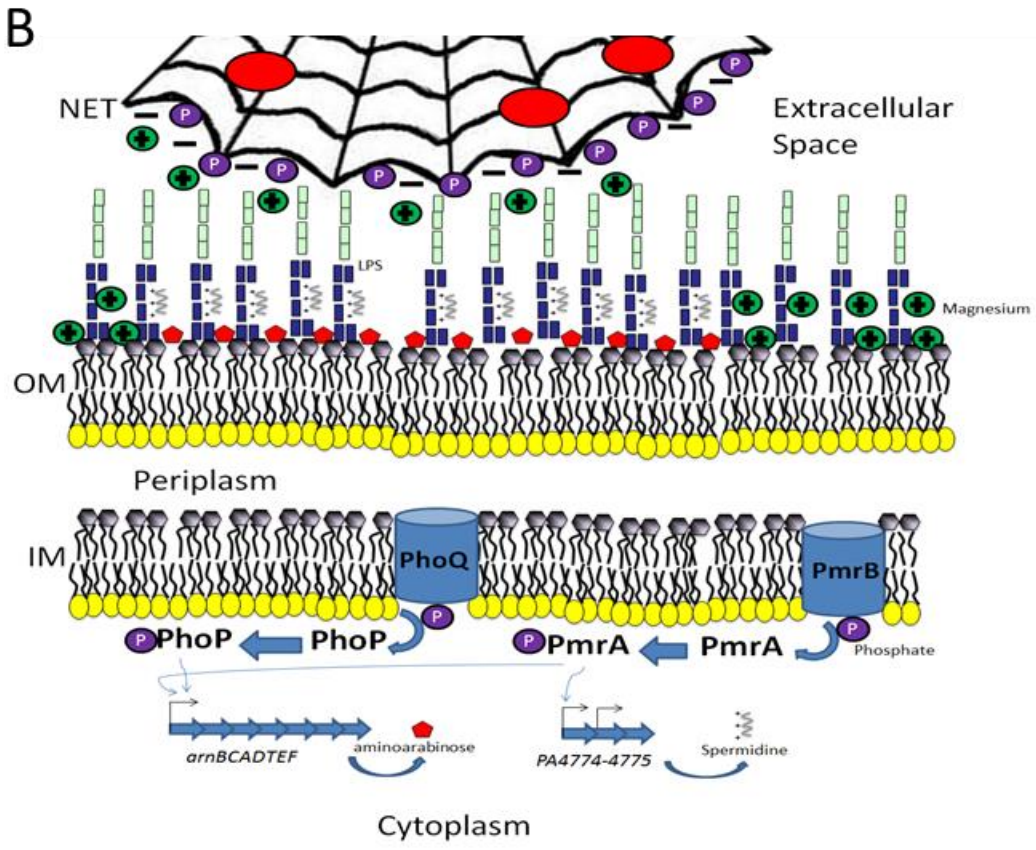
Flow cytometry provided a third and valuable approach to measure bacterial killing by NETs. Live/dead staining allowed for the detection of "dead" or "membrane damaged" bacteria when exposed to NETs (Figure 3.11), however there were some limits to the approach. For example, when attempting to block the antimicrobial activity of NETs with PTase, plate counts showed a return in survival for both *P. aeruginosa* and *E. coli*, while flow cytometry showed PTase treated cells still stained positive for propidium iodide (Figure 5.5 and 5.8). It may be possible that the PI staining was showing membrane-damaged cells, and when allowed to be grown on nutrient rich LB agar, the cells were able to recover. It is well established that propidium iodide is not an absolute indicator of dead cells, but also stains membrane compromised, or dying cells. Another possibility is that when the bacteria were in contact with the NETs, DNA from these structures ended up on the bacterial surface and it was the surface DNA that was being stained and detected. With the addition of the post-DNase, it removed the surface DNA and thus there was a loss of PI signal (Figure 5.5).

From these methods and techniques, we were able to show that the DNA in NETs can serve a variety of functions. The use of luminescence assays and flow cytometry allowed for more sensitive ways to measure bacterial killing and have generally supported plate count methods. The development of these methods have allowed for a deeper investigation into the mechanisms of NET and bacterial killing. Figure 7.1A summarizes the different mechanisms *P. aeruginosa* has at its disposal to protect against neutrophil extracellular traps, specifically the DNA. Figure 7.1B depicts a possible mechanism for the cation chelating effects of DNA, its effect on the bacterial membrane, and the sensing of surface Mg^{2+} levels by the PhoPQ and PmrAB two component systems.

Figure 7.1. Mechanisms used by *P. aeruginosa* to resist NET killing

Pseudomonas aeruginosa can use a variety of mechanisms to protect itself from neutrophil extracellular traps (A). Synthesis of DNase and PTase (1), modifying the LPS on the membrane (2) and production of Pel and Psl EPS (3). *P. aeruginosa* can also sense and respond to NETs by up-regulating antimicrobial defense genes (B) The DNA backbone of NETS creates a magnesium-limited environment that causes the sensor proteins PhoQ and PmrB along with their respective regulators, PhoP and PmrA to become activated. The phosphorylation of PhoP and PmrA activates the *arnBCADTEF* and *PA4773-4775* operons. Increased expression of the *arn* operon allows for the addition of aminoarabinose to the lipid A on the LPS and induction of the *PA4773-4775* operon results in the production of spermidine that can bind to the LPS on the membrane surface. Both aminoarabinose and spermidine addition protect the membrane from CAPs and ROS, which are used by the immune system to combat pathogens as part of neutrophil extracellular traps.


A
PTase
1
DNase
Spermidine
3
Exopolysaccharide
(Pel and Psl)
Aminoarabinose
Modification
2


B
NET
Extracellular
Space
LPS
Magnesium
OM
Periplasm
IM
PhoQ
PmrB
PhoP
PhoP
PmrA
PmrA
Phosphate
arnBCADTEF
aminoarabinose
PA4774-4775
Spermidine
Cytoplasm

7.2 Future Experiments

7.2.1 Examining NET-formation in vivo

The Kubes lab has developed elegant methods for observing NET formation in animals during an infection. In preliminary studies with Dr. Bjoern Petri, we are attempting to visualize NET formation in a skin infection model in mice as described in Yipp *et al.* (2012)[133]. Mice were anesthetized and a midline dorsal skin flap is separated and exposed to the spinning disk confocal intravital microscope. Before imaging, mice were injected with DNA stains and an antibody that recognizes neutrophils, and then injected with Gfp-tagged or Rfp-tagged *P. aeruginosa.* The direct interactions between bacteria, neutrophils and NETs were then examined.

As shown in Figure 7.2, we did observe DNA-staining NET structures, which were not present in uninfected mice, despite the recruitment of neutrophils to the surface tissues using MIP-2 treatment. During these experiments, we routinely observed phagocytosis of *P. aeruginosa*, and NET structures, but we never observed a NET in the process of NETosis. We also noticed that NET formation seemed to be less common than in control mice infected with *S. aureus*. The number of NETs and area of NET formation were quantified and confirmed that *S. aureus* is much stronger inducer of NETs *in vivo*, consistent with *in vitro* comparisons of these two bacteria (Figure 7.2). The long-term goal with this infection model is to try and develop methods for assessing bacterial viability of bacteria trapped in NETs, and to try and disable *in vivo* NETs with cations and enzymes that target the DNA, as a means to confirm our *in vitro* observations *in vivo.* Blocking NET-DNA has been shown to protect both *P. aeruginosa* and *E. coli.* Other bacteria that have been shown to be susceptible to NETs could also be tested. Another option may be to test a wide variety of microorganisms and examine if blocking NET-DNA can restore survival.

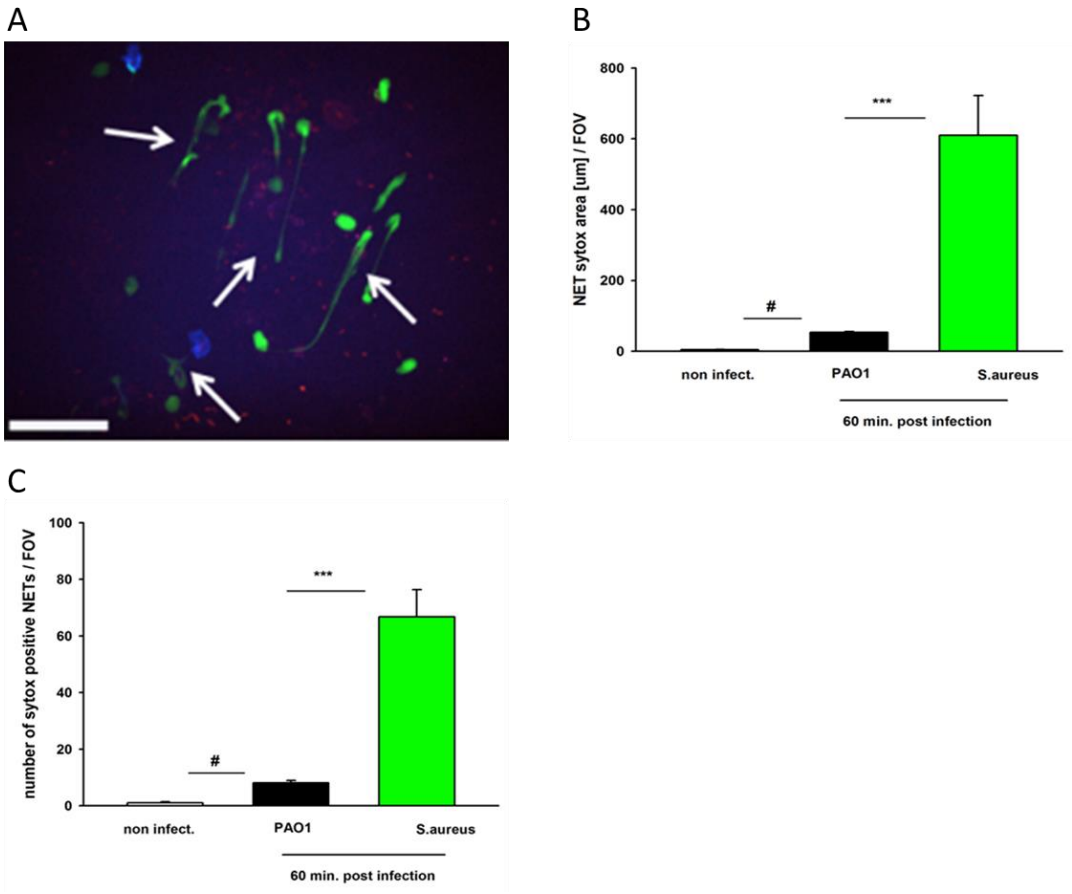


Figure 7.2. *Pseudomonas aeruginosa* is a poor inducer of NETs *in vivo*

Mice were injected with DNA stains and an antibody that recognizes neutrophils, and then injected Rfp-tagged *P. aeruginosa*. (A) DNA-staining NET structures of PA01 infected mice. Arrows pointing to released NET structures. (B) The total NET area was quantified along with (C) the total number of NETs of mice uninfected, infected with PAO1 or *S. aureus.* Hash symbol indicate a significant difference in NET area and number in PA01 infected mice compared to the uninfected control (#P<0.05). Asterisks indicate significant difference in NET area and number in mice infected with PA01 compared to *S. aureus* infected mice (***P<0.001). The values represent the average at least three fields of view (FOV) with error bars representing the standard deviation. The experiment was repeated three times with a representative experiment shown.

7.2.2 Compare NET resistance in biofilm cells vs. planktonic cells

The study to date has involved growing bacteria in liquid culture as planktonic cells grown in BM2 media with excess magnesium. Another approach would be to allow the bacterial cells to grow as a biofilm and observe the resistance to NETs. Flow chamber biofilms can be cultivated in flow chambers, and neutrophils can be injected into the biofilm to observe neutrophil-biofilm interactions. It was previously shown that when neutrophils encounter *P. aeruginosa* biofilms, the neutrophils settle on the biofilm and appear unable to migrate away[119]. The neutrophils do mount a respiratory burst and attempt phagocytosis on the biofilm cells, however they appear inactivated[119]. Staining techniques could be used to visualize either the neutrophils or the bacteria. It may be possible that the biofilms of certain NET-sensitive mutants may allow the neutrophils to penetrate deeper into the structure than that of the wild-type strain.

7.2.3 The role of exopolysaccharide capsules in protecting against NETs

The production of exopolysaccharides, such as Pel and Psl has been shown to protect *P. aeruginosa* from antimicrobial compounds, phagocytosis, the oxidative response in neutrophils, and prevent complement-mediated opsonisation[79; 228]. Another EPS, alginate, has been shown to convey protection of *P. aeruginosa* by scavenging ROS and to interfere with complement activation, chemotaxis, and neutrophil and macrophage phagocytosis[78; 241; 242; 243]. The role of alginate, which is a dominant adaptation in the CF lung, in protecting against neutrophil extracellular traps could be further studied. Since the over-production of alginate occurs when *P. aeruginosa* shifts from non-mucoid to mucoid morphology, mucoid strains could be tested for their susceptibility to NETs. Mucoid isolates from CF sputum is another approach to test bacterial resistance to NETs.

Pel and Psl production is induced under magnesium-limiting conditions by repressing *retS* expression and induces expression of the *pel/pel* EPS biosynthesis genes. The extracellular traps produced by neutrophils are able to create a magnesium-limited environment due to the cation chelating activity of extracellular DNA found within them. We could examine if *P. aeruginosa* exposed to NETs responds to these structures by upregulating the production of Pel and Psl. This could be explored using *lux*-reporter strains and monitoring gene expression. To study the genome-wide response to NETs, RNA-SEQ would be a suitable choice to examine the *P. aeruginosa* response to NET-activated neutrophils.

7.3 Conclusions

Neutrophil extracellular traps are a newly discovered mechanism used by the immune system to trap and kill pathogens. The killing by these structures has largely been attributed to histones, myeloperoxidase, and the various granular components released in the NET structure. The DNA present in the NETs has often been described as a structural component of these traps. However, due to the cation chelating activity of DNA, it may serve multiple roles in the traps. The DNA acts as an antimicrobial agent of NETs as well as an inducer of antimicrobial resistance genes in *P. aeruginosa*. The induction of these genes may help to protect the bacteria from NET killing and contribute to its persistence in chronic infections, such as the lung infection in cystic fibrosis patients. In this study, I have shown that by blocking the cation chelating activity of DNA, you can get a restoration in bacterial survival when exposed to NETs. The DNA in NETs generates a magnesium limited environment that can up-regulate the DNA-inducible surface modifications, which protect from NET killing. This study illustrates an interaction where a pathogen is able to respond and defend against the host innate immune response. Combined with the strategies to tolerate NET production, *P. aeruginosa* is a weak

inducer of NETs compared to other bacteria. These aspects combined may contribute to the ability of *P. aeruginosa* to cause persistent and reoccurring infections, especially in patients with cystic fibrosis.

References:

1. Vasil, M. L. (1986). *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. *J Pediatr* **108**, 800-5.
2. Madigan, M. T., Martinko, J. M. & Parker, J. (2003). *Brock biology of microorganisms*. 10th edit, Prentice Hall/Pearson Education, Upper Saddle River, N.J.
3. Cross, A. S. (1985). Evolving epidemiology of *Pseudomonas aeruginosa* infections. *Eur J Clin Microbiol* **4**, 156-9.
4. Peix, A., Ramirez-Bahena, M. H. & Velazquez, E. (2009). Historical evolution and current status of the taxonomy of genus Pseudomonas. *Infect Genet Evol* **9**, 1132-47.
5. Gooderham, W. J. & Hancock, R. E. (2009). Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. *FEMS Microbiol Rev* **33**, 279-94.
6. Marques, S. & Ramos, J. L. (1993). Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. *Mol Microbiol* **9**, 923-9.
7. Melotto, M., Underwood, W., Koczan, J., Nomura, K. & He, S. Y. (2006). Plant stomata function in innate immunity against bacterial invasion. *Cell* **126**, 969-80.
8. Seol, B., Naglic, T., Madic, J. & Bedekovic, M. (2002). *In vitro* antimicrobial susceptibility of 183 *Pseudomonas aeruginosa* strains isolated from dogs to selected antipseudomonal agents. *J Vet Med B Infect Dis Vet Public Health* **49**, 188-92.
9. Schaible, B., Taylor, C. T. & Schaffer, K. (2012). Hypoxia increases antibiotic resistance in *Pseudomonas aeruginosa* through altering the composition of multidrug efflux pumps. *Antimicrob Agents Chemother* **56**, 2114-8.
10. Kamei, A., Koh, A. Y., Gadjeva, M., Priebe, G. P., Lory, S. & Pier, G. B. (2010). Analysis of acquisition of *Pseudomonas aeruginosa* gastrointestinal mucosal colonization and horizontal transmission in a murine model. *J Infect Dis* **201**, 71-80.
11. Moreau-Marquis, S., Stanton, B. A. & O'Toole, G. A. (2008). *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. *Pulm Pharmacol Ther* **21**, 595-9.
12. Mulcahy, H., Charron-Mazenod, L. & Lewenza, S. (2008). Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *PLoS Pathog* **4**, e1000213.

13. Ernst, R. K., Moskowitz, S. M., Emerson, J. C., Kraig, G. M., Adams, K. N., Harvey, M. D., Ramsey, B., Speert, D. P., Burns, J. L. & Miller, S. I. (2007). Unique lipid a modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J Infect Dis* **196**, 1088-92.
14. Sibley, C. D., Duan, K., Fischer, C., Parkins, M. D., Storey, D. G., Rabin, H. R. & Surette, M. G. (2008). Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog* **4**, e1000184.
15. Nester, E. W. (2007). *Microbiology : a human perspective*. 5th edit, McGraw-Hill, Boston.
16. Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annu Rev Microbiol* **41**, 435-64.
17. Brouqui, P., Rousseau, M. C., Stein, A., Drancourt, M. & Raoult, D. (1995). Treatment of *Pseudomonas aeruginosa*-infected orthopedic prostheses with ceftazidime-ciprofloxacin antibiotic combination. *Antimicrob Agents Chemother* **39**, 2423-5.
18. Gadsby, D. C., Vergani, P. & Csanady, L. (2006). The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature* **440**, 477-83.
19. Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**, 194-222.
20. Araujo, F. G., Novaes, F. C., Santos, N. P., Martins, V. C., Souza, S. M., Santos, S. E. & Ribeiro-dos-Santos, A. K. (2005). Prevalence of deltaF508, G551D, G542X, and R553X mutations among cystic fibrosis patients in the North of Brazil. *Braz J Med Biol Res* **38**, 11-5.
21. Davis, P. B. (2006). Cystic fibrosis since 1938. *Am J Respir Crit Care Med* **173**, 475-82.
22. Silo-Suh, L., Suh, S. J., Phibbs, P. V. & Ohman, D. E. (2005). Adaptations of *Pseudomonas aeruginosa* to the cystic fibrosis lung environment can include deregulation of *zwf*, encoding glucose-6-phosphate dehydrogenase. *J Bacteriol* **187**, 7561-8.
23. Heijerman, H. (2005). Infection and inflammation in cystic fibrosis: a short review. *J Cyst Fibros* **4 Suppl 2**, 3-5.

24. FitzSimmons, S. C. (1993). The changing epidemiology of cystic fibrosis. *J Pediatr* **122**, 1-9.
25. Johansen, H. K., Kovesi, T. A., Koch, C., Corey, M., Hoiby, N. & Levison, H. (1998). *Pseudomonas aeruginosa* and *Burkholderia cepacia* infection in cystic fibrosis patients treated in Toronto and Copenhagen. *Pediatr Pulmonol* **26**, 89-96.
26. Sibley, C. D., Rabin, H. & Surette, M. G. (2006). Cystic fibrosis: a polymicrobial infectious disease. *Future Microbiol* **1**, 53-61.
27. Filkins, L. M., Hampton, T. H., Gifford, A. H., Gross, M. J., Hogan, D. A., Sogin, M. L., Morrison, H. G., Paster, B. J. & O'Toole, G. A. (2012). Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. *J Bacteriol* **194**, 4709-17.
28. Sibley, C. D., Grinwis, M. E., Field, T. R., Eshaghurshan, C. S., Faria, M. M., Dowd, S. E., Parkins, M. D., Rabin, H. R. & Surette, M. G. (2011). Culture enriched molecular profiling of the cystic fibrosis airway microbiome. *PLoS One* **6**, e22702.
29. Sibley, C. D., Parkins, M. D., Rabin, H. R., Duan, K., Norgaard, J. C. & Surette, M. G. (2008). A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc Natl Acad Sci U S A* **105**, 15070-5.
30. Young, R. L., Malcolm, K. C., Kret, J. E., Caceres, S. M., Poch, K. R., Nichols, D. P., Taylor-Cousar, J. L., Saavedra, M. T., Randell, S. H., Vasil, M. L., Burns, J. L., Moskowitz, S. M. & Nick, J. A. (2011). Neutrophil extracellular trap (NET)-mediated killing of *Pseudomonas aeruginosa*: evidence of acquired resistance within the CF airway, independent of CFTR. *PLoS One* **6**, e23637.
31. Mena, A., Smith, E. E., Burns, J. L., Speert, D. P., Moskowitz, S. M., Perez, J. L. & Oliver, A. (2008). Genetic adaptation of *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients is catalyzed by hypermutation. *J Bacteriol* **190**, 7910-7.
32. Murray, T. S., Egan, M. & Kazmierczak, B. I. (2007). *Pseudomonas aeruginosa* chronic colonization in cystic fibrosis patients. *Curr Opin Pediatr* **19**, 83-8.
33. Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**, 1251-4.

34. Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J. & Greenberg, E. P. (2000). Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**, 762-4.

35. Bjarnsholt, T., Jensen, P. O., Fiandaca, M. J., Pedersen, J., Hansen, C. R., Andersen, C. B., Pressler, T., Givskov, M. & Hoiby, N. (2009). *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol* **44**, 547-58.

36. Costerton, J. W., Lam, J., Lam, K. & Chan, R. (1983). The role of the microcolony mode of growth in the pathogenesis of *Pseudomonas aeruginosa* infections. *Rev Infect Dis* **5 Suppl 5**, S867-73.

37. Ryder, C., Byrd, M. & Wozniak, D. J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr Opin Microbiol* **10**, 644-8.

38. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. (2002). Extracellular DNA required for bacterial biofilm formation. *Science* **295**, 1487.

39. Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M. & Tolker-Nielsen, T. (2006). A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* **59**, 1114-28.

40. Schooling, S. R. & Beveridge, T. J. (2006). Membrane vesicles: an overlooked component of the matrices of biofilms. *J Bacteriol* **188**, 5945-57.

41. Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K. & Wozniak, D. J. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* **5**, e1000354.

42. Marcos, V., Zhou, Z., Yildirim, A. O., Bohla, A., Hector, A., Vitkov, L., Wiedenbauer, E. M., Krautgartner, W. D., Stoiber, W., Belohradsky, B. H., Rieber, N., Kormann, M., Koller, B., Roscher, A., Roos, D., Griese, M., Eickelberg, O., Doring, G., Mall, M. A. & Hartl, D. (2010). CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. *Nat Med* **16**, 1018-23.

43. Manzenreiter, R., Kienberger, F., Marcos, V., Schilcher, K., Krautgartner, W. D., Obermayer, A., Huml, M., Stoiber, W., Hector, A., Griese, M., Hannig, M., Studnicka, M., Vitkov, L. & Hartl, D. (2012). Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. *J Cyst Fibros* **11**, 84-92.

44. Yang, L., Barken, K. B., Skindersoe, M. E., Christensen, A. B., Givskov, M. & Tolker-Nielsen, T. (2007). Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. *Microbiology* **153**, 1318-28.
45. de Beer, D., Stoodley, P. & Lewandowski, Z. (1994). Liquid flow in heterogeneous biofilms. *Biotechnol Bioeng* **44**, 636-41.
46. Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S. & Tolker-Nielsen, T. (2003). Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* **48**, 1511-24.
47. O'Toole, G. A. & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**, 295-304.
48. Sauer, K. & Camper, A. K. (2001). Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J Bacteriol* **183**, 6579-89.
49. Davies, D. G. & Geesey, G. G. (1995). Regulation of the alginate biosynthesis gene algC in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl Environ Microbiol* **61**, 860-7.
50. Stewart, P. S. & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135-8.
51. Jensen, P. O., Bjarnsholt, T., Phipps, R., Rasmussen, T. B., Calum, H., Christoffersen, L., Moser, C., Williams, P., Pressler, T., Givskov, M. & Hoiby, N. (2007). Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* **153**, 1329-38.
52. Mulcahy, H. & Lewenza, S. (2011). Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. *PLoS One* **6**, e23307.
53. Parkins, M. D., Ceri, H. & Storey, D. G. (2001). *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol Microbiol* **40**, 1215-26.
54. Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. & Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Dev Cell* **7**, 745-54.
55. Petrova, O. E. & Sauer, K. (2009). A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. *PLoS Pathog* **5**, e1000668.

56. Cohen, M. L. (1998). Resurgent and emergent disease in a changing world. *Br Med Bull* **54**, 523-32.
57. Fux, C. A., Costerton, J. W., Stewart, P. S. & Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends Microbiol* **13**, 34-40.
58. Hall-Stoodley, L. & Stoodley, P. (2009). Evolving concepts in biofilm infections. *Cell Microbiol* **11**, 1034-43.
59. Kirketerp-Moller, K., Jensen, P. O., Fazli, M., Madsen, K. G., Pedersen, J., Moser, C., Tolker-Nielsen, T., Hoiby, N., Givskov, M. & Bjarnsholt, T. (2008). Distribution, organization, and ecology of bacteria in chronic wounds. *J Clin Microbiol* **46**, 2717-22.
60. Ramadan, H. H. (2006). Chronic rhinosinusitis and bacterial biofilms. *Curr Opin Otolaryngol Head Neck Surg* **14**, 183-6.
61. Davies, D. (2003). Understanding biofilm resistance to antibacterial agents. *Nat Rev Drug Discov* **2**, 114-22.
62. Kharazmi, A. (1991). Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. *Immunol Lett* **30**, 201-5.
63. Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S. & Qu, D. (2007). Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. *Microbiology* **153**, 2083-92.
64. Harmsen, M., Yang, L., Pamp, S. J. & Tolker-Nielsen, T. (2010). An update on *Pseudomonas aeruginosa* biofilm formation, tolerance, and dispersal. *FEMS Immunol Med Microbiol* **59**, 253-68.
65. Romling, U. & Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. *J Intern Med* **272**, 541-61.
66. Chiang, W. C., Nilsson, M., Jensen, P. O., Hoiby, N., Nielsen, T. E., Givskov, M. & Tolker-Nielsen, T. (2013). Extracellular DNA Shields against Aminoglycosides in *Pseudomonas aeruginosa* Biofilms. *Antimicrob Agents Chemother* **57**, 2352-61.
67. Kuo, H. H., Chan, C., Burrows, L. L. & Deber, C. M. (2007). Hydrophobic interactions in complexes of antimicrobial peptides with bacterial polysaccharides. *Chem Biol Drug Des* **69**, 405-12.

68. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-22.
69. Mah, T. F. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34-9.
70. Pamp, S. J., Gjermansen, M., Johansen, H. K. & Tolker-Nielsen, T. (2008). Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol Microbiol* **68**, 223-40.
71. Lewis, K. (2010). Persister cells. *Annu Rev Microbiol* **64**, 357-72.
72. Boles, B. R. & Singh, P. K. (2008). Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc Natl Acad Sci U S A* **105**, 12503-8.
73. Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M. & Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* **4**, 295-305.
74. Haussler, S., Tummler, B., Weissbrodt, H., Rohde, M. & Steinmetz, I. (1999). Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. *Clin Infect Dis* **29**, 621-5.
75. Zhang, L. & Mah, T. F. (2008). Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J Bacteriol* **190**, 4447-52.
76. Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S. & O'Toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* **426**, 306-10.
77. Jensen, E. T., Kharazmi, A., Lam, K., Costerton, J. W. & Hoiby, N. (1990). Human polymorphonuclear leukocyte response to *Pseudomonas aeruginosa* grown in biofilms. *Infect Immun* **58**, 2383-5.
78. Leid, J. G., Willson, C. J., Shirtliff, M. E., Hassett, D. J., Parsek, M. R. & Jeffers, A. K. (2005). The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. *J Immunol* **175**, 7512-8.
79. Colvin, K. M., Gordon, V. D., Murakami, K., Borlee, B. R., Wozniak, D. J., Wong, G. C. & Parsek, M. R. (2011). The *pel* polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. *PLoS Pathog* **7**, e1001264.

80. Friedman, L. & Kolter, R. (2004). Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Mol Microbiol* **51**, 675-90.
81. Chung, I. Y., Choi, K. B., Heo, Y. J. & Cho, Y. H. (2008). Effect of PEL exopolysaccharide on the *wspF* mutant phenotypes in *Pseudomonas aeruginosa* PA14. *J Microbiol Biotechnol* **18**, 1227-34.
82. Mulcahy, H., Sibley, C. D., Surette, M. G. & Lewenza, S. (2011). *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections in vivo. *PLoS Pathog* **7**, e1002299.
83. Lee, V. T., Matewish, J. M., Kessler, J. L., Hyodo, M., Hayakawa, Y. & Lory, S. (2007). A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. *Mol Microbiol* **65**, 1474-84.
84. Ma, L., Jackson, K. D., Landry, R. M., Parsek, M. R. & Wozniak, D. J. (2006). Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J Bacteriol* **188**, 8213-21.
85. Kirisits, M. J., Prost, L., Starkey, M. & Parsek, M. R. (2005). Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* **71**, 4809-21.
86. Kadurugamuwa, J. L. & Beveridge, T. J. (1995). Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol* **177**, 3998-4008.
87. Mulcahy, H., Charron-Mazenod, L. & Lewenza, S. (2010). *Pseudomonas aeruginosa* produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. *Environ Microbiol* **12**, 1621-9.
88. Lewenza, S., Falsafi, R. K., Winsor, G., Gooderham, W. J., McPhee, J. B., Brinkman, F. S. & Hancock, R. E. (2005). Construction of a mini-Tn5-*luxCDABE* mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes. *Genome Res* **15**, 583-9.

89. McPhee, J. B., Bains, M., Winsor, G., Lewenza, S., Kwasnicka, A., Brazas, M. D., Brinkman, F. S. & Hancock, R. E. (2006). Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg2+-induced gene regulation in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 3995-4006.
90. Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. L., Hackett, M. & Miller, S. I. (1999). Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science* **286**, 1561-5.
91. Kusano, T., Berberich, T., Tateda, C. & Takahashi, Y. (2008). Polyamines: essential factors for growth and survival. *Planta* **228**, 367-381.
92. McGinnis, M. W., Parker, Z. M., Walter, N. E., Rutkovsky, A. C., Cartaya-Marin, C. & Karatan, E. (2009). Spermidine regulates *Vibrio cholerae* biofilm formation via transport and signaling pathways. *FEMS Microbiol Lett* **299**, 166-74.
93. Shah, P. & Swiatlo, E. (2008). A multifaceted role for polyamines in bacterial pathogens. *Mol Microbiol* **68**, 4-16.
94. Johnson, L., Mulcahy, H., Kanevets, U., Shi, Y. & Lewenza, S. (2012). Surface-localized spermidine protects the *Pseudomonas aeruginosa* outer membrane from antibiotic treatment and oxidative stress. *J Bacteriol* **194**, 813-26.
95. Lewenza, S. (2013). Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*. *Front Microbiol* **4**, 21.
96. Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S. & Filloux, A. (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* **103**, 171-6.
97. Kuchma, S. L., Connolly, J. P. & O'Toole, G. A. (2005). A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 1441-54.
98. Ramsey, M. M. & Whiteley, M. (2004). *Pseudomonas aeruginosa* attachment and biofilm development in dynamic environments. *Mol Microbiol* **53**, 1075-87.
99. Ulrich, R. L. & DeShazer, D. (2004). Type III secretion: a virulence factor delivery system essential for the pathogenicity of *Burkholderia mallei*. *Infect Immun* **72**, 1150-4.

100. Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V. & Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231-41.
101. Papayannopoulos, V. & Zychlinsky, A. (2009). NETs: a new strategy for using old weapons. *Trends Immunol* **30**, 513-21.
102. Lu, T., Kobayashi, S. D., Quinn, M. T. & Deleo, F. R. (2012). A NET Outcome. *Front Immunol* **3**, 365.
103. Rigby, K. M. & DeLeo, F. R. (2012). Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol* **34**, 237-59.
104. Cartwright, G. E., Athens, J. W. & Wintrobe, M. M. (1964). The Kinetics of Granulopoiesis in Normal Man. *Blood* **24**, 780-803.
105. Bratton, D. L. & Henson, P. M. (2011). Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol* **32**, 350-7.
106. Segal, A. W. (2005). How neutrophils kill microbes. *Annu Rev Immunol* **23**, 197-223.
107. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. & Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-5.
108. Rada, B., Jendrysik, M. A., Pang, L., Hayes, C. P., Yoo, D. G., Park, J. J., Moskowitz, S. M., Malech, H. L. & Leto, T. L. (2013). Pyocyanin-enhanced neutrophil extracellular trap formation requires the NADPH oxidase. *PLoS One* **8**, e54205.
109. Borregaard, N., Sorensen, O. E. & Theilgaard-Monch, K. (2007). Neutrophil granules: a library of innate immunity proteins. *Trends Immunol* **28**, 340-5.
110. Konstan, M. W., Hilliard, K. A., Norvell, T. M. & Berger, M. (1994). Bronchoalveolar lavage findings in cystic fibrosis patients with stable, clinically mild lung disease suggest ongoing infection and inflammation. *Am J Respir Crit Care Med* **150**, 448-54.
111. De Rose, V., Oliva, A., Messore, B., Grosso, B., Mollar, C. & Pozzi, E. (1998). Circulating adhesion molecules in cystic fibrosis. *Am J Respir Crit Care Med* **157**, 1234-9.
112. Russell, K. J., McRedmond, J., Mukherji, N., Costello, C., Keatings, V., Linnane, S., Henry, M., Fitzgerald, M. X. & O'Connor, C. M. (1998). Neutrophil adhesion molecule

surface expression and responsiveness in cystic fibrosis. *Am J Respir Crit Care Med* **157**, 756-61.

113. Petit-Bertron, A. F., Tabary, O., Corvol, H., Jacquot, J., Clement, A., Cavaillon, J. M. & Adib-Conquy, M. (2008). Circulating and airway neutrophils in cystic fibrosis display different TLR expression and responsiveness to interleukin-10. *Cytokine* **41**, 54-60.
114. Walker, T. S., Tomlin, K. L., Worthen, G. S., Poch, K. R., Lieber, J. G., Saavedra, M. T., Fessler, M. B., Malcolm, K. C., Vasil, M. L. & Nick, J. A. (2005). Enhanced *Pseudomonas aeruginosa* biofilm development mediated by human neutrophils. *Infect Immun* **73**, 3693-701.
115. Morris, M. R., Doull, I. J., Dewitt, S. & Hallett, M. B. (2005). Reduced iC3b-mediated phagocytotic capacity of pulmonary neutrophils in cystic fibrosis. *Clin Exp Immunol* **142**, 68-75.
116. Taggart, C., Coakley, R. J., Greally, P., Canny, G., O'Neill, S. J. & McElvaney, N. G. (2000). Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-alpha and interleukin-8. *Am J Physiol Lung Cell Mol Physiol* **278**, L33-41.
117. Kobayashi, H., Kobayashi, O. & Kawai, S. (2009). Pathogenesis and clinical manifestations of chronic colonization by *Pseudomonas aeruginosa* and its biofilms in the airway tract. *J Infect Chemother* **15**, 125-42.
118. Downey, D. G., Bell, S. C. & Elborn, J. S. (2009). Neutrophils in cystic fibrosis. *Thorax* **64**, 81-8.
119. Jesaitis, A. J., Franklin, M. J., Berglund, D., Sasaki, M., Lord, C. I., Bleazard, J. B., Duffy, J. E., Beyenal, H. & Lewandowski, Z. (2003). Compromised host defense on *Pseudomonas aeruginosa* biofilms: characterization of neutrophil and biofilm interactions. *J Immunol* **171**, 4329-39.
120. Bass, D. A., DeChatelet, L. R., Burk, R. F., Shirley, P. & Szejda, P. (1977). Polymorphonuclear leukocyte bactericidal activity and oxidative metabolism during glutathione peroxidase deficiency. *Infect Immun* **18**, 78-84.
121. Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J., Sawa, T., Frank, D. W. & Wiener-Kronish, J. P. (2001). Type III protein secretion is

associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* **183**, 1767-74.

122. Horsman, S. R., Moore, R. A. & Lewenza, S. (2012). Calcium chelation by alginate activates the type III secretion system in mucoid *Pseudomonas aeruginosa* biofilms. *PLoS One* **7**, e46826.
123. Sonawane, A., Jyot, J., During, R. & Ramphal, R. (2006). Neutrophil elastase, an innate immunity effector molecule, represses flagellin transcription in *Pseudomonas aeruginosa*. *Infect Immun* **74**, 6682-9.
124. Alhede, M., Bjarnsholt, T., Jensen, P. O., Phipps, R. K., Moser, C., Christophersen, L., Christensen, L. D., van Gennip, M., Parsek, M., Hoiby, N., Rasmussen, T. B. & Givskov, M. (2009). *Pseudomonas aeruginosa* recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. *Microbiology* **155**, 3500-8.
125. Wartha, F., Beiter, K., Normark, S. & Henriques-Normark, B. (2007). Neutrophil extracellular traps: casting the NET over pathogenesis. *Curr Opin Microbiol* **10**, 52-6.
126. von Kockritz-Blickwede, M., Goldmann, O., Thulin, P., Heinemann, K., Norrby-Teglund, A., Rohde, M. & Medina, E. (2008). Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* **111**, 3070-80.
127. Guimaraes-Costa, A. B., Nascimento, M. T., Froment, G. S., Soares, R. P., Morgado, F. N., Conceicao-Silva, F. & Saraiva, E. M. (2009). *Leishmania amazonensis* promastigotes induce and are killed by neutrophil extracellular traps. *Proc Natl Acad Sci U S A* **106**, 6748-53.
128. Pilsczek, F. H., Salina, D., Poon, K. K., Fahey, C., Yipp, B. G., Sibley, C. D., Robbins, S. M., Green, F. H., Surette, M. G., Sugai, M., Bowden, M. G., Hussain, M., Zhang, K. & Kubes, P. (2010). A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* **185**, 7413-25.
129. Oehmcke, S., Morgelin, M. & Herwald, H. (2009). Activation of the human contact system on neutrophil extracellular traps. *J Innate Immun* **1**, 225-30.
130. Brinkmann, V. & Zychlinsky, A. (2007). Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol* **5**, 577-82.

131. Tonks, N. K. (2005). Redox redux: revisiting PTPs and the control of cell signaling. *Cell* **121**, 667-70.

132. Remijsen, Q., Kuijpers, T. W., Wirawan, E., Lippens, S., Vandenabeele, P. & Vanden Berghe, T. (2011). Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ* **18**, 581-8.

133. Yipp, B. G., Petri, B., Salina, D., Jenne, C. N., Scott, B. N., Zbytnuik, L. D., Pittman, K., Asaduzzaman, M., Wu, K., Meijndert, H. C., Malawista, S. E., de Boisfleury Chevance, A., Zhang, K., Conly, J. & Kubes, P. (2012). Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med* **18**, 1386-93.

134. Malawista, S. E., Montgomery, R. R. & van Blaricom, G. (1992). Evidence for reactive nitrogen intermediates in killing of staphylococci by human neutrophil cytoplasts. A new microbicidal pathway for polymorphonuclear leukocytes. *J Clin Invest* **90**, 631-6.

135. Malawista, S. E. & Van Blaricom, G. (1987). Cytoplasts made from human blood polymorphonuclear leukocytes with or without heat: preservation of both motile function and respiratory burst oxidase activity. *Proc Natl Acad Sci U S A* **84**, 454-8.

136. Malawista, S. E., Van Blaricom, G. & Breitenstein, M. G. (1989). Cryopreservable neutrophil surrogates. Stored cytoplasts from human polymorphonuclear leukocytes retain chemotactic, phagocytic, and microbicidal function. *J Clin Invest* **83**, 728-32.

137. Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A. & Henriques-Normark, B. (2006). An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Curr Biol* **16**, 401-7.

138. Clark, S. R., Ma, A. C., Tavener, S. A., McDonald, B., Goodarzi, Z., Kelly, M. M., Patel, K. D., Chakrabarti, S., McAvoy, E., Sinclair, G. D., Keys, E. M., Allen-Vercoe, E., Devinney, R., Doig, C. J., Green, F. H. & Kubes, P. (2007). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* **13**, 463-9.

139. Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I. & Simon, H. U. (2009). Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* **16**, 1438-44.

140. Parker, H., Albrett, A. M., Kettle, A. J. & Winterbourn, C. C. (2012). Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. *J Leukoc Biol* **91**, 369-76.
141. Hirsch, J. G. (1958). Bactericidal action of histone. *J Exp Med* **108**, 925-44.
142. Zhdan-Pushkina, S. M. & Dronova, N. V. (1976). [Differential sensitivity of bacteria to histones]. *Mikrobiologiia* **45**, 60-6.
143. Rose, F. R., Bailey, K., Keyte, J. W., Chan, W. C., Greenwood, D. & Mahida, Y. R. (1998). Potential role of epithelial cell-derived histone H1 proteins in innate antimicrobial defense in the human gastrointestinal tract. *Infect Immun* **66**, 3255-63.
144. Kim, H. S., Park, C. B., Kim, M. S. & Kim, S. C. (1996). cDNA cloning and characterization of buforin I, an antimicrobial peptide: a cleavage product of histone H2A. *Biochem Biophys Res Commun* **229**, 381-7.
145. Urban, C. F., Ermert, D., Schmid, M., Abu-Abed, U., Goosmann, C., Nacken, W., Brinkmann, V., Jungblut, P. R. & Zychlinsky, A. (2009). Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans. PLoS Pathog* **5**, e1000639.
146. Marin-Esteban, V., Turbica, I., Dufour, G., Semiramoth, N., Gleizes, A., Gorges, R., Beau, I., Servin, A. L., Lievin-Le Moal, V., Sandre, C. & Chollet-Martin, S. (2012). Afa/Dr diffusely adhering *Escherichia coli* strain C1845 induces neutrophil extracellular traps that kill bacteria and damage human enterocyte-like cells. *Infect Immun* **80**, 1891-9.
147. Doring, Y., Manthey, H. D., Drechsler, M., Lievens, D., Megens, R. T., Soehnlein, O., Busch, M., Manca, M., Koenen, R. R., Pelisek, J., Daemen, M. J., Lutgens, E., Zenke, M., Binder, C. J., Weber, C. & Zernecke, A. (2012). Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation* **125**, 1673-83.
148. Ramos-Kichik, V., Mondragon-Flores, R., Mondragon-Castelan, M., Gonzalez-Pozos, S., Muniz-Hernandez, S., Rojas-Espinosa, O., Chacon-Salinas, R., Estrada-Parra, S. & Estrada-Garcia, I. (2009). Neutrophil extracellular traps are induced by *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* **89**, 29-37.

149. Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Kotb, M., Feramisco, J. & Nizet, V. (2006). DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol* **16**, 396-400.

150. Khatua, B., Bhattacharya, K. & Mandal, C. (2012). Sialoglycoproteins adsorbed by *Pseudomonas aeruginosa* facilitate their survival by impeding neutrophil extracellular trap through siglec-9. *J Leukoc Biol* **91**, 641-55.

151. Bertram, T. A., Canning, P. C. & Roth, J. A. (1986). Preferential inhibition of primary granule release from bovine neutrophils by a *Brucella abortus* extract. *Infect Immun* **52**, 285-92.

152. Joiner, K. A., Ganz, T., Albert, J. & Rotrosen, D. (1989). The opsonizing ligand on *Salmonella typhimurium* influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. *J Cell Biol* **109**, 2771-82.

153. Palazzolo-Ballance, A. M., Reniere, M. L., Braughton, K. R., Sturdevant, D. E., Otto, M., Kreiswirth, B. N., Skaar, E. P. & DeLeo, F. R. (2008). Neutrophil microbicides induce a pathogen survival response in community-associated methicillin-resistant *Staphylococcus aureus. J Immunol* **180**, 500-9.

154. Nordenfelt, P., Bauer, S., Lonnbro, P. & Tapper, H. (2009). Phagocytosis of *Streptococcus pyogenes* by all-trans retinoic acid-differentiated HL-60 cells: roles of azurophilic granules and NADPH oxidase. *PLoS One* **4**, e7363.

155. McCormick, A., Heesemann, L., Wagener, J., Marcos, V., Hartl, D., Loeffler, J., Heesemann, J. & Ebel, F. (2010). NETs formed by human neutrophils inhibit growth of the pathogenic mold *Aspergillus fumigatus*. *Microbes Infect* **12**, 928-36.

156. Urban, C. F., Reichard, U., Brinkmann, V. & Zychlinsky, A. (2006). Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. *Cell Microbiol* **8**, 668-76.

157. Springer, D. J., Ren, P., Raina, R., Dong, Y., Behr, M. J., McEwen, B. F., Bowser, S. S., Samsonoff, W. A., Chaturvedi, S. & Chaturvedi, V. (2010). Extracellular fibrils of pathogenic yeast *Cryptococcus gattii* are important for ecological niche, murine virulence and human neutrophil interactions. *PLoS One* **5**, e10978.

158. Baker, V. S., Imade, G. E., Molta, N. B., Tawde, P., Pam, S. D., Obadofin, M. O., Sagay, S. A., Egah, D. Z., Iya, D., Afolabi, B. B., Baker, M., Ford, K., Ford, R., Roux, K. H. & Keller, T. C., 3rd. (2008). Cytokine-associated neutrophil extracellular traps and antinuclear antibodies in *Plasmodium falciparum* infected children under six years of age. *Malar J* **7**, 41.

159. Gabriel, C., McMaster, W. R., Girard, D. & Descoteaux, A. (2010). *Leishmania donovani* promastigotes evade the antimicrobial activity of neutrophil extracellular traps. *J Immunol* **185**, 4319-27.

160. Abi Abdallah, D. S., Lin, C., Ball, C. J., King, M. R., Duhamel, G. E. & Denkers, E. Y. (2012). *Toxoplasma gondii* triggers release of human and mouse neutrophil extracellular traps. *Infect Immun* **80**, 768-77.

161. Narasaraju, T., Yang, E., Samy, R. P., Ng, H. H., Poh, W. P., Liew, A. A., Phoon, M. C., van Rooijen, N. & Chow, V. T. (2011). Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol* **179**, 199-210.

162. Saitoh, T., Komano, J., Saitoh, Y., Misawa, T., Takahama, M., Kozaki, T., Uehata, T., Iwasaki, H., Omori, H., Yamaoka, S., Yamamoto, N. & Akira, S. (2012). Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* **12**, 109-16.

163. Papayannopoulos, V., Staab, D. & Zychlinsky, A. (2011). Neutrophil elastase enhances sputum solubilization in cystic fibrosis patients receiving DNase therapy. *PLoS One* **6**, e28526.

164. Wartha, F., Beiter, K., Albiger, B., Fernebro, J., Zychlinsky, A., Normark, S. & Henriques-Normark, B. (2007). Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell Microbiol* **9**, 1162-71.

165. Shak, S., Capon, D. J., Hellmiss, R., Marsters, S. A. & Baker, C. L. (1990). Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc Natl Acad Sci U S A* **87**, 9188-92.

166. Ranasinha, C., Assoufi, B., Shak, S., Christiansen, D., Fuchs, H., Empey, D., Geddes, D. & Hodson, M. (1993). Efficacy and safety of short-term administration of aerosolised recombinant human DNase I in adults with stable stage cystic fibrosis. *Lancet* **342**, 199-202.

167. Riedel, C. U., Casey, P. G., Mulcahy, H., O'Gara, F., Gahan, C. G. & Hill, C. (2007). Construction of *p16Slux*, a novel vector for improved bioluminescent labeling of gram-negative bacteria. *Appl Environ Microbiol* **73**, 7092-5.

168. Choi, K. H. & Schweizer, H. P. (2006). mini-Tn7 insertion in bacteria with single *att*Tn7 sites: example *Pseudomonas aeruginosa*. *Nat Protoc* **1**, 153-61.

169. Jacobs, M. A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S., Will, O., Kaul, R., Raymond, C., Levy, R., Chun-Rong, L., Guenthner, D., Bovee, D., Olson, M. V. & Manoil, C. (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **100**, 14339-44.

170. Koch, B., Jensen, L. E. & Nybroe, O. (2001). A panel of Tn7-based vectors for insertion of the *gfp* marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. *J Microbiol Methods* **45**, 187-95.

171. McPhee, J. B., Lewenza, S. & Hancock, R. E. (2003). Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**, 205-17.

172. Ma, L., Lu, H., Sprinkle, A., Parsek, M. R. & Wozniak, D. J. (2007). *Pseudomonas aeruginosa* Psl is a galactose- and mannose-rich exopolysaccharide. *J Bacteriol* **189**, 8353-6.

173. West, S. E., Schweizer, H. P., Dall, C., Sample, A. K. & Runyen-Janecky, L. J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from *pUC18/19* and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**, 81-6.

174. Davidson, C. J., Narang, A. & Surette, M. G. (2010). Integration of transcriptional inputs at promoters of the arabinose catabolic pathway. *BMC Syst Biol* **4**, 75.

175. Menegazzi, R., Decleva, E. & Dri, P. (2012). Killing by neutrophil extracellular traps: fact or folklore? *Blood* **119**, 1214-6.
176. Fritzler, M., Ryan, P. & Kinsella, T. D. (1982). Clinical features of systemic lupus erythematosus patients with antihistone antibodies. *J Rheumatol* **9**, 46-51.
177. Fritzler, M. J. (1985). Antinuclear antibodies in the investigation of rheumatic diseases. *Bull Rheum Dis* **35**, 1-10.
178. Tan, E. M., Fritzler, M. J., McDougal, J. S., McDuffie, F. C., Nakamura, R. M., Reichlin, M., Reimer, C. B., Sharp, G. C., Schur, P. H., Wilson, M. R. & Winchester, R. J. (1982). Reference sera for antinuclear antibodies. I. Antibodies to native DNA, Sm, nuclear RNP, and SS-B/La. *Arthritis Rheum* **25**, 1003-5.
179. Brinkmann, V., Laube, B., Abu Abed, U., Goosmann, C. & Zychlinsky, A. (2010). Neutrophil extracellular traps: how to generate and visualize them. *J Vis Exp*.
180. Hampton, M. B., Kettle, A. J. & Winterbourn, C. C. (1998). Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007-17.
181. Klebanoff, S. J. (2005). Myeloperoxidase: friend and foe. *J Leukoc Biol* **77**, 598-625.
182. Palic, D., Andreasen, C. B., Ostojic, J., Tell, R. M. & Roth, J. A. (2007). Zebrafish (*Danio rerio*) whole kidney assays to measure neutrophil extracellular trap release and degranulation of primary granules. *J Immunol Methods* **319**, 87-97.
183. Greer, L. F., 3rd & Szalay, A. A. (2002). Imaging of light emission from the expression of luciferases in living cells and organisms: a review. *Luminescence* **17**, 43-74.
184. van der Meer, J. R. & Belkin, S. (2010). Where microbiology meets microengineering: design and applications of reporter bacteria. *Nat Rev Microbiol* **8**, 511-22.
185. Hilpert, K. & Hancock, R. E. (2007). Use of luminescent bacteria for rapid screening and characterization of short cationic antimicrobial peptides synthesized on cellulose using peptide array technology. *Nat Protoc* **2**, 1652-60.
186. AbcamInc. (2013). A guide to flow cytometry. How does it work? What is it used for?, Vol. 2013. Abcam Inc, Abcam.com.
187. Herzenberg, L. A., Tung, J., Moore, W. A., Herzenberg, L. A. & Parks, D. R. (2006). Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* **7**, 681-5.

188. Anaya-Lopez, J. L., Lopez-Meza, J. E. & Ochoa-Zarzosa, A. (2013). Bacterial resistance to cationic antimicrobial peptides. *Crit Rev Microbiol* **39**, 180-95.

189. Matsuzaki, K. (2009). Control of cell selectivity of antimicrobial peptides. *Biochimica Et Biophysica Acta-Biomembranes* **1788**, 1687-1692.

190. Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* **3**, 238-250.

191. Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J. & Tarkowski, A. (2004). *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. *J Immunol* **172**, 1169-76.

192. Frick, I. M., Akesson, P., Rasmussen, M., Schmidtchen, A. & Bjorck, L. (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J Biol Chem* **278**, 16561-6.

193. Schmidtchen, A., Frick, I. M. & Bjorck, L. (2001). Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. *Mol Microbiol* **39**, 708-13.

194. Park, P. W., Pier, G. B., Preston, M. J., Goldberger, O., Fitzgerald, M. L. & Bernfield, M. (2000). Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of *Pseudomonas aeruginosa*. *J Biol Chem* **275**, 3057-64.

195. Sieprawska-Lupa, M., Mydel, P., Krawczyk, K., Wojcik, K., Puklo, M., Lupa, B., Suder, P., Silberring, J., Reed, M., Pohl, J., Shafer, W., McAleese, F., Foster, T., Travis, J. & Potempa, J. (2004). Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. *Antimicrob Agents Chemother* **48**, 4673-9.

196. Schmidtchen, A., Frick, I. M., Andersson, E., Tapper, H. & Bjorck, L. (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* **46**, 157-68.

197. Nizet, V. (2006). Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr Issues Mol Biol* **8**, 11-26.

198. Tzeng, Y. L., Ambrose, K. D., Zughaier, S., Zhou, X., Miller, Y. K., Shafer, W. M. & Stephens, D. S. (2005). Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. *J Bacteriol* **187**, 5387-96.

199. Rieg, S., Huth, A., Kalbacher, H. & Kern, W. V. (2009). Resistance against antimicrobial peptides is independent of *Escherichia coli* AcrAB, *Pseudomonas aeruginosa* MexAB and *Staphylococcus aureus* NorA efflux pumps. *Int J Antimicrob Agents* **33**, 174-6.

200. Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-95.

201. Yeaman, M. R. & Yount, N. Y. (2003). Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev* **55**, 27-55.

202. Tamayo, R., Choudhury, B., Septer, A., Merighi, M., Carlson, R. & Gunn, J. S. (2005). Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar typhimurium lipopolysaccharide core. *J Bacteriol* **187**, 3391-9.

203. Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K. & Miller, S. I. (2000). Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium. *Infect Immun* **68**, 6139-46.

204. Moskowitz, S. M., Ernst, R. K. & Miller, S. I. (2004). PmrAB, a two-component regulatory system of *Pseudomonas aeruginosa* that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *Journal of Bacteriology* **186**, 575-579.

205. Cheng, H. Y., Chen, Y. F. & Peng, H. L. (2010). Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in K*lebsiella pneumoniae* CG43. *J Biomed Sci* **17**, 60.

206. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-64.

207. Foussard, M., Cabantous, S., Pedelacq, J., Guillet, V., Tranier, S., Mourey, L., Birck, C. & Samama, J. (2001). The molecular puzzle of two-component signaling cascades. *Microbes Infect* **3**, 417-24.

208. Macfarlane, E. L., Kwasnicka, A., Ochs, M. M. & Hancock, R. E. (1999). PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol* **34**, 305-16.

209. Ueda, A. & Wood, T. K. (2010). Tyrosine Phosphatase TpbA of *Pseudomonas aeruginosa* Controls Extracellular DNA via Cyclic Diguanylic Acid Concentrations. *Environ Microbiol* **2**, 449-55.

210. Nicas, T. I. & Hancock, R. E. (1980). Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. *J Bacteriol* **143**, 872-8.

211. Munafo, D. B., Johnson, J. L., Brzezinska, A. A., Ellis, B. A., Wood, M. R. & Catz, S. D. (2009). DNase I inhibits a late phase of reactive oxygen species production in neutrophils. *J Innate Immun* **1**, 527-42.

212. Urban, C. F., Lourido, S. & Zychlinsky, A. (2006). How do microbes evade neutrophil killing? *Cell Microbiol* **8**, 1687-96.

213. Nathan, C. (2006). Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**, 173-82.

214. Faurschou, M. & Borregaard, N. (2003). Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* **5**, 1317-27.

215. Kobayashi, S. D., Braughton, K. R., Whitney, A. R., Voyich, J. M., Schwan, T. G., Musser, J. M. & DeLeo, F. R. (2003). Bacterial pathogens modulate an apoptosis differentiation program in human neutrophils. *Proc Natl Acad Sci U S A* **100**, 10948-53.

216. Foster, T. J. (2005). Immune evasion by staphylococci. *Nat Rev Microbiol* **3**, 948-58.

217. Voyich, J. M., Musser, J. M. & DeLeo, F. R. (2004). *Streptococcus pyogenes* and human neutrophils: a paradigm for evasion of innate host defense by bacterial pathogens. *Microbes Infect* **6**, 1117-23.

218. Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., Fierer, J. & Nizet, V. (2005). *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* **202**, 209-15.

219. Pizarro-Cerda, J. & Cossart, P. (2006). Subversion of cellular functions by *Listeria monocytogenes*. *J Pathol* **208**, 215-23.

220. de Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P. & van Strijp, J. A. (2004). Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent. *J Exp Med* **199**, 687-95.

221. Dong, Z. M. & Murphy, J. W. (1996). Cryptococcal polysaccharides induce L-selectin shedding and tumor necrosis factor receptor loss from the surface of human neutrophils. *J Clin Invest* **97**, 689-98.

222. Burns, S. M. & Hull, S. I. (1999). Loss of resistance to ingestion and phagocytic killing by O(-) and K(-) mutants of a uropathogenic *Escherichia coli* O75:K5 strain. *Infect Immun* **67**, 3757-62.

223. Rooijakkers, S. H., Ruyken, M., Roos, A., Daha, M. R., Presanis, J. S., Sim, R. B., van Wamel, W. J., van Kessel, K. P. & van Strijp, J. A. (2005). Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nat Immunol* **6**, 920-7.

224. Gruenheid, S. & Finlay, B. B. (2003). Microbial pathogenesis and cytoskeletal function. *Nature* **422**, 775-81.

225. Staali, L., Bauer, S., Morgelin, M., Bjorck, L. & Tapper, H. (2006). *Streptococcus pyogenes* bacteria modulate membrane traffic in human neutrophils and selectively inhibit azurophilic granule fusion with phagosomes. *Cell Microbiol* **8**, 690-703.

226. Allen, L. A., Beecher, B. R., Lynch, J. T., Rohner, O. V. & Wittine, L. M. (2005). *Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol* **174**, 3658-67.

227. Menestrina, G., Serra, M. D. & Prevost, G. (2001). Mode of action of beta-barrel pore-forming toxins of the staphylococcal alpha-hemolysin family. *Toxicon* **39**, 1661-72.

228. Mishra, M., Byrd, M. S., Sergeant, S., Azad, A. K., Parsek, M. R., McPhail, L., Schlesinger, L. S. & Wozniak, D. J. (2012). *Pseudomonas aeruginosa* Psl polysaccharide

reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell Microbiol* **14**, 95-106.

229. Nielsen, K. M., Johnsen, P. J., Bensasson, D. & Daffonchio, D. (2007). Release and persistence of extracellular DNA in the environment. *Environ Biosafety Res* **6**, 37-53.
230. Lorenz, M. G. & Wackernagel, W. (1994). Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol Rev* **58**, 563-602.
231. Ulmer, J. S., Herzka, A., Toy, K. J., Baker, D. L., Dodge, A. H., Sinicropi, D., Shak, S. & Lazarus, R. A. (1996). Engineering actin-resistant human DNase I for treatment of cystic fibrosis. *Proc Natl Acad Sci U S A* **93**, 8225-9.
232. Brandt, T., Breitenstein, S., von der Hardt, H. & Tummler, B. (1995). DNA concentration and length in sputum of patients with cystic fibrosis during inhalation with recombinant human DNase. *Thorax* **50**, 880-2.
233. Vlassov, V. V., Laktionov, P. P. & Rykova, E. Y. (2007). Extracellular nucleic acids. *Bioessays* **29**, 654-67.
234. Boles, B. R., Thoendel, M. & Singh, P. K. (2005). Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol Microbiol* **57**, 1210-23.
235. Pinchuk, G. E., Ammons, C., Culley, D. E., Li, S. M., McLean, J. S., Romine, M. F., Nealson, K. H., Fredrickson, J. K. & Beliaev, A. S. (2008). Utilization of DNA as a sole source of phosphorus, carbon, and energy by *Shewanella* spp.: ecological and physiological implications for dissimilatory metal reduction. *Appl Environ Microbiol* **74**, 1198-208.
236. Finkel, S. E. & Kolter, R. (2001). DNA as a nutrient: novel role for bacterial competence gene homologs. *J Bacteriol* **183**, 6288-93.
237. Rada, B. & Leto, T. L. (2008). Oxidative innate immune defenses by Nox/Duox family NADPH oxidases. *Contrib Microbiol* **15**, 164-87.
238. Huang, J., Sonnleitner, E., Ren, B., Xu, Y. & Haas, D. (2012). Catabolite repression control of pyocyanin biosynthesis at an intersection of primary and secondary metabolism in *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **78**, 5016-20.

239. Whooley, M. A. a. M., A.J. (1982). The regulation of pyocyanin production in *Pseudomonas aeruginosa*. *European Journal of Applied Microbiology and Biotechnology* **15**, 7.

240. Petrova, O. E., Schurr, J. R., Schurr, M. J. & Sauer, K. (2011). The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. *Mol Microbiol* **81**, 767-83.

241. Learn, D. B., Brestel, E. P. & Seetharama, S. (1987). Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. *Infect Immun* **55**, 1813-8.

242. Pedersen, S. S., Kharazmi, A., Espersen, F. & Hoiby, N. (1990). *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response. *Infect Immun* **58**, 3363-8.

243. Mai, G. T., Seow, W. K., Pier, G. B., McCormack, J. G. & Thong, Y. H. (1993). Suppression of lymphocyte and neutrophil functions by *Pseudomonas aeruginosa* mucoid exopolysaccharide (alginate): reversal by physicochemical, alginase, and specific monoclonal antibody treatments. *Infect Immun* **61**, 559-64.

Appendix:

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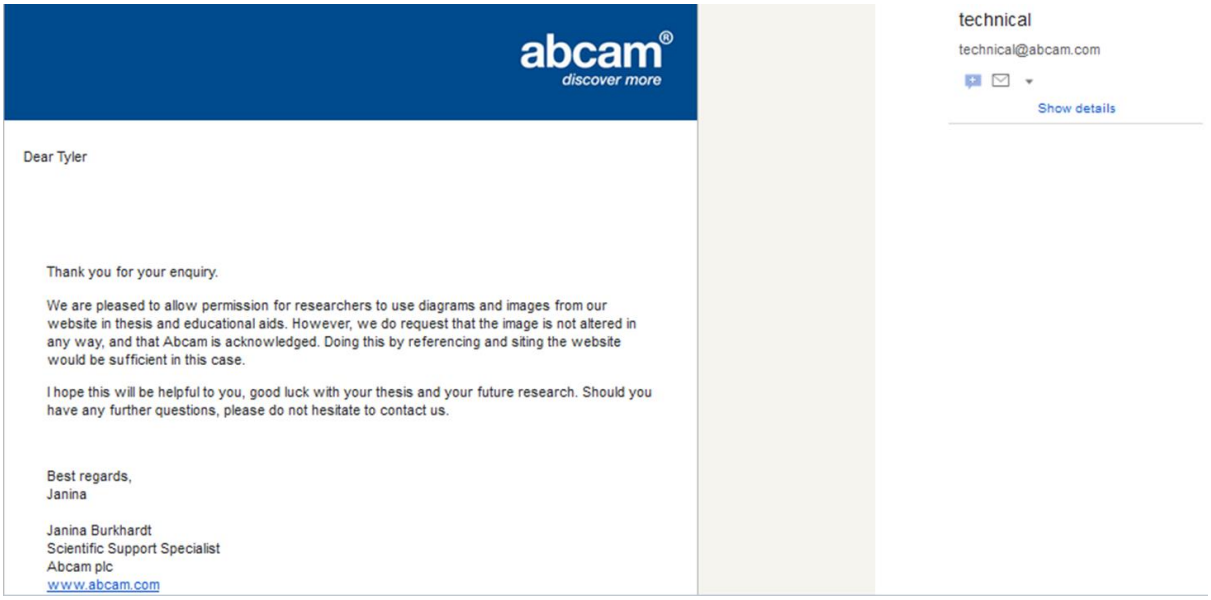

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Dear Tyler

Thank you for your enquiry.

We are pleased to allow permission for researchers to use diagrams and images from our website in thesis and educational aids. However, we do request that the image is not altered in any way, and that Abcam is acknowledged. Doing this by referencing and siting the website would be sufficient in this case.

I hope this will be helpful to you, good luck with your thesis and your future research. Should you have any further questions, please do not hesitate to contact us.

Best regards,
Janina

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