

**THE ROLE OF FLAGELLAR MOTILITY IN *PSEUDOMONAS AERUGINOSA*-
INDUCED FORMATION OF NEUTROPHIL EXTRACELLULAR TRAPS (NETs)**

by

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(Under the Direction of Balázs Rada)

ABSTRACT

Cystic fibrosis (CF) is a primarily pulmonary disease characterized by impaired mucociliary clearance and chronic bacterial infections. *Pseudomonas aeruginosa* is the main CF pathogen responsible for large-scale neutrophil recruitment in the airways. When neutrophils fail to clear bacteria, *P. aeruginosa*-induced neutrophil extracellular traps (NETs) likely form, during which neutrophil granulocytes release granule components with decondensed neutrophil DNA from the ruptured membrane. We aimed to determine the contribution of *P. aeruginosa*'s flagellum-powered motility to NET formation, and the role of MyD88 signaling in this pathway. Motile wild-type *P. aeruginosa* triggered significantly more NET formation than its motility- and flagellum-deficient counterparts. Additionally, MyD88 signaling was not involved in triggering NETosis. We propose bacterial motility is the primary virulence determinant responsible for triggering NETosis through a mechanism involving cooperation of multiple, MyD88-independent bacterial recognition receptors

acting synergistically. This work could lead to potential therapeutic targets for controlling NET formation in CF airways.

INDEX WORDS: Cystic Fibrosis, Neutrophil Extracellular DNA Traps, NETosis Mechanism, Bacterial Swimming Motility, *Pseudomonas aeruginosa*, Flagella, Motility Deficiency, Flagellum Deficiency, Myeloperoxidase, Human Neutrophil Elastase, Superoxide, MPO-DNA, Extracellular DNA, MyD88, TLR5, NLRC4.

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DEDICATION

This thesis is dedicated to family, friends, and pets that provided much needed support throughout these two years, as well as to those patients – past, present, and future – suffering from respiratory diseases around the globe. Together, we will make this world a better place.

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Lastly, I accept responsibility for ideas and interpretations constituting my thesis, unless otherwise cited.

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CHAPTER 1

INTRODUCTION

CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive disease occurring at a rate of 4×10^{-4} in primarily those of Caucasian descent (Rath *et al.*, 2014; Cant *et al.*, 2014). More than 70,000 CF patients are documented around the globe, with average life expectancies of greater than 35 years or, in some cases, greater than 50 years; early treatment intervention is key to extending life expectancy (Wang *et al.*, 2014; Ciofu *et al.*, 2014). CF affects all of the major mucosal organs, including, but not limited to, the airways, small intestine, and reproductive tract (Ciofu *et al.*, 2014). Specifically, patients suffering from CF lung disease experience gradually declining lung function typically attributed to infection (Rath *et al.*, 2014). Over time, multiple respiratory infections, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, become co-incident and eventually produce chronic bronchiectasis (Ciofu *et al.*, 2014). Failure to clear these bacterial infections is a consequence of defective CFTR function and serves as a chief contributor to elevated morbidity and mortality rates seen with CF patients (Ehre *et al.*, 2014; Rath *et al.*, 2014).

CF occurs as a result of a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) (Collins *et al.*, 1987). The CFTR manages

chloride (Cl^-) and bicarbonate (HCO_3^-) membrane transport responsible for preserving mucosal surface fluid homeostasis (Cant *et al.*, 2014; Le Gars, *et al.*, 2013). Thus, when the CFTR is defective, the result encompasses dehydration of accumulated mucus subsequently followed by bacterial infection – hallmarks of CF disease (Le Gars, *et al.*, 2013). In CF, six general mechanistic mutations, including the most common ΔF508 mutation, of the CFTR are well documented (Wang *et al.*, 2014). The ΔF508 mutation appears in approximately 70-85% of CF patients, while the other five types of mutations are only present at a frequency of 1-5% (Wang *et al.*, 2014; Le Gars, *et al.*, 2013). Four of the six mechanistic mutations relate to different aspects of the CFTR protein, including its production, synthesis, processing, and stability (Wang *et al.*, 2014). The other two mechanistic mutations relate to the CFTR's channel activity, including its conduction and regulation (Wang *et al.*, 2014). CFTRs exhibiting the ΔF508 phenotype are characteristically unstable (Wang *et al.*, 2014). KalydecoTM is the only drug currently approved for targeting CFTRs exhibiting the ΔG551 mutation, which affects a much smaller population than does the ΔF508 mutation (Wang *et al.*, 2014). Therefore, there is a large gap in the literature regarding possible targets and treatments of defective CFTRs in CF airways. Therapies are needed to combat the symptoms of dysfunctional CFTRs.

Effective treatments can only be developed with a better understanding of the link between CF disease progression and dysfunction of the CFTR. Improper chloride (Cl^-) and bicarbonate (HCO_3^-) secretion results from the faulty CFTR; this causes mucosal tissues to inappropriately regulate water flow and, thus,

produce unusually viscous and concentrated mucus, as well as maintain a more acidic epithelial surface pH known to hamper elimination of bacteria (Ehre *et al.*, 2014). This viscous, stationary mucus facilitates ideal bacterial growth conditions, inciting additional mucin secretion (known as mucus hypersecretion) and neutrophil transmigration (Ehre *et al.*, 2014; Rath *et al.*, 2014). Increased inflammation follows, leading to even greater neutrophil recruitment, which appears to further increase mucus thickness and diminish bacterial clearance, the latter of which is largely attributed to extracellular DNA release by recruited neutrophils (Lethem *et al.*, 1990; Ehre *et al.*, 2014). Additionally, studies have demonstrated that a steady presence of host-derived neutrophil elastase (NE) might contribute to CFTR dysfunction and/or intensify mutant CFTR deficiency (e.g., $\Delta F508$) through NE-driven degradation of functional CFTRs (Le Gars *et al.*, 2013). This process is thought to strengthen the inflammatory phenotype seen in chronically infected lungs, and, consequently, contribute to CF disease progression by disabling functional CFTRs – an effect that could continue in a therapeutic environment in which faulty CFTRs have been corrected (Le Gars *et al.*, 2013). Together, thick and dehydrated mucus, bacterial infection, damage inflicted by neutrophil-derived proteases (e.g., NE), and a sustained inflammatory response (triggered by increasingly stimulated neutrophil recruitment and pro-inflammatory cytokine release) create a dangerous and sometimes deadly environment in the lungs of CF patients (Ehre *et al.*, 2014; Le Gars *et al.*, 2013).

NEUTROPHIL INVOLVEMENT IN CYSTIC FIBROSIS AIRWAYS

Neutrophils are ephemeral granulocytes known to mature over several days in the bone marrow (Lu *et al.*, 2012). Following their maturation, neutrophils comprise approximately 60-70% of leukocytes flowing through the average human's bloodstream, where they remain for 10 to 24 hours before either migrating into infected tissue or being recycled – the latter of which is thought to be a combined function of the spleen, liver, and bone marrow but is not completely understood (Ng *et al.*, 2014; Lu *et al.*, 2012). These phagocytic cells primarily operate in controlling and eliminating infection, particularly that caused by extracellular bacteria, and can be sustained in the tissue for one to two days before initiating apoptosis and being cleared by macrophages and/or dendritic cells (Ng *et al.*, 2014; Lu *et al.*, 2012). Such tight regulation of a rather short lifespan is a necessity for neutrophils, since they are equipped with an arsenal of cytotoxic molecules capable of injuring not only invading pathogens but also host tissue if their functions are left unchecked (Lu *et al.*, 2012).

Neutrophils' most powerful bacterial killing mechanism engages production of microbicidal oxidants, mainly comprising superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) – the last of which is commonly known as household bleach (Ng *et al.*, 2014). Following neutrophil phagocytosis of a bacterium, cytoplasmic granules fuse with the phagosome, introducing NADPH-oxidase derived reactive oxygen species (ROS) and other cytotoxic molecules capable of destroying the bacterium (Rada and Leto, 2008; Rada *et al.*, 2008; Lu *et al.*, 2012). Bacterial killing is typically done within the

phagosome to avoid accidental release of cytotoxic molecules into the extracellular space, which could lead to host tissue damage (Lu *et al.*, 2012). Neutrophils then undergo programmed cell death, spurring clean up by local macrophages and/or dendritic cells; moreover, neutrophil lysis is typically bypassed in order to preclude release of dead bacteria and neutrophil-associated killing molecules into surrounding tissue (Lu *et al.*, 2012). In healthy hosts, these activities generally result in successful killing of phagocytosed bacteria, followed by down-regulation of inflammation and the immune response; however, a different outcome is observed in CF airways.

CF lung disease is clinically described, in part, by unresolved neutrophil-derived inflammation (Rath *et al.*, 2014). This unusually inflamed environment, generated by what are referred to as “uncontrolled neutrophil responses,” results in host tissue damage representative of CF lung pathology (Pazos *et al.*, 2015). Neutrophils are initially attracted to the infection site upon release of chemokines, leukotrienes, and prostaglandins from epithelial cells, macrophages, and mast cells present in infected tissue. Endothelial cells express E- or P-selectin capable of binding neutrophil surface mucins (Brinkmann and Zychlinsky, 2007). Next, neutrophil “rolling” begins along the endothelial surface, during which time chemokines – such as interleukin (IL)-8, complement component C5a, and bacterial *N*-formyl-peptides – stimulate neutrophils (Brinkmann and Zychlinsky, 2007). Neutrophils then express integrins that bind intercellular adhesion molecules (ICAMs) present on the endothelial cell surface, effectively halting the neutrophil “rolling” process (Brinkmann and Zychlinsky, 2007). Lastly, neutrophils

travel along a chemotactic gradient, transmigrating through gaps between endothelial cells and into infected tissue (Brinkmann and Zychlinsky, 2007). Beyond this traditional recruitment style, neutrophils can also stimulate their own migration via manufacture of cytokine mediators responsible for fostering microvascular leakage of neutrophils into tissues (Pazos *et al.*, 2015). Following entry into the tissue from the bloodstream, neutrophils can form aggregates at infection sites, a behavior referred to as “swarming,” which orchestrates localization of neutrophils within tissues (Pazos *et al.*, 2015). More research continues to be done on the mechanism of neutrophil entry into mucosal tissues (Pazos *et al.*, 2015).

Meanwhile, current literature is clear in its view that CF patients’ immune systems are still able to mount an efficacious inflammatory response, given such high neutrophil recruitment and infiltration of tissues (Ng *et al.*, 2014). However, despite this quantifiably large immune response, CF patients are still unable to effectively clear microbes and their corresponding infections. It is thus a lack of quality in the host’s immune response – something that cannot be rectified by boosted numbers of responding immune cells – that serves as the root of the immunological dilemma presented in CF airways (Cohen and Prince, 2012). Support of this claim is provided by studies of *Cftr*^{-/-} mice, which suffered significant impairment of their defense against *Pseudomonas aeruginosa* infection; this dysfunctional CFTR murine model is meant to mimic defective CFTR function in CF lungs (Ng *et al.*, 2014). These experiments demonstrated that *Cftr*^{-/-} mice still recruit similar numbers of neutrophils but fail to taper

neutrophil recruitment over time (Ng *et al.*, 2014). In contrast, control mice are able to auspiciously transition from early (indicated by neutrophils) to late (dominated by macrophages and lymphocytes) phase immunity, avoiding unremitting neutrophil-derived inflammation (Ng *et al.*, 2014). Therefore, CFTR dysfunction in CF airways is consistent with an inability to transition from early to late phase immunity, resulting in enduring neutrophil-derived inflammation and damage to host tissues (Ng *et al.*, 2014). This pernicious activity is further compounded by an alternative microbial killing mechanism – known as neutrophil extracellular trap (NET) formation – initiated by the high numbers of neutrophils recruited into CF patients' lung tissues.

NEUTROPHIL EXTRACELLULAR TRAPS (NETs)

Neutrophils are commonly known to fight invading pathogens via either phagocytosis or degranulation, but neutrophil extracellular traps (NETs) have proven to be a third possible mechanism in such defense (Brinkmann *et al.*, 2004). The process of NET formation by neutrophils, or NETosis, is associated with activation of the Raf-MEK-ERK pathway, after which simultaneous degradation of the nuclear envelope and peptidylarginine deiminase type 4 (PAD4)-mediated citrullination of histones present in the neutrophil's DNA occurs – the latter of which is known to promote chromatin decondensation (Lu *et al.*, 2012). This process represents the de-lobing of the typical 3 to 5 lobes of the neutrophil nuclei leading to eventual rupturing of the neutrophil plasma membrane, NET release, and neutrophil cell lysis (Brinkmann and Zychlinsky,

2007). Within approximately 45 to 240 minutes (depending upon when neutrophil cell death occurs) following stimulation, NETs are generally released (Brinkmann and Zychlinsky, 2007). This mechanism of neutrophil cell death is distinct from necrosis and apoptosis because neither caspases, DNA fragmentation, or complement components (from the host's perspective) nor disruptions of the cell membrane by bacterial toxins (from the bacterium's perspective) result in NET formation (Fuchs *et al.*, 2007; Brinkmann and Zychlinsky, 2007; Yoo, Floyd, *et al.*, 2014). Together, these findings point toward the possibility of a precise signaling cascade initiating NETosis, a pathway yet to be defined. Therefore, this represents a gap in knowledge that needs to be filled to most efficiently generate novel clinical treatments for NET-related symptoms in CF.

Current literature reports that host tissue damage in CF and sputum viscosity can, in part, be attributed to NET formation (Papayannopoulos *et al.*, 2011). This is because NETs are made up of a chromatin backbone decorated with neutrophilic granules, which non-specifically attack both pathogens and host tissue outside of the protective environment of the neutrophil granulocyte or phagosome (Fuchs *et al.*, 2007; Brinkmann and Zychlinsky, 2007). Moreover, the incredibly sticky nature of DNA bestows a glue-like phenotype upon NET-containing mucus, adding to the viscosity of mucus (Papayannopoulos *et al.*, 2011). In healthy airways, mucus acts as a physical barrier to bacterial infection through its continuous clearance from the lungs, effectively preventing bacterial attachment and colonization (Papayannopoulos *et al.*, 2011). In stark contrast, the thick, NET-containing sputum present in CF patients' airways is incredibly

challenging to clear, leading to persistence of mucus and, consequently, permitting bacterial attachment, colonization, and associated inflammation (Papayannopoulos *et al.*, 2011). Published studies emphasize that sputum viscosity can be lessened by deoxyribonuclease (DNase) treatment, since DNase degrades DNA in the presence of cations (Halverson *et al.*, 2015; Papayannopoulos *et al.*, 2011). Additionally, *in vitro* experiments with CF patient sputum demonstrate that neutrophil elastase (NE) degrades histones, thereby promoting exposure of DNA to nucleases and solubilization of sputum (Papayannopoulos *et al.*, 2011). However, DNA traps NE, thereby downregulating its proteolytic activity; this means that NE complexed with NETs can only be optimally freed to pursue bacterial killing under the condition of DNase treatment (Papayannopoulos *et al.*, 2011). Although, one must also take into consideration that free NE can damage both bacteria and host tissue indiscriminately (Lu *et al.*, 2012; Papayannopoulos *et al.*, 2011). On the other hand, in high concentration, DNA is thought to serve an antimicrobial purpose, since research shows that large concentrations of extracellular DNA cause massive disruptions in the bacterial cell envelope, eventually resulting in bacterial cell lysis and death (Mulcahy *et al.*, 2008). Thus, current literature is in conflict as to whether or not NETs are more beneficial or harmful to the host. This can be contemplated more thoroughly by examining the primary components associated with NET complexes.

COMPONENTS OF NETs: DNA, HISTONES, MPO, AND NE

CF patient sputum consistently contains sizeable quantities of free DNA, which has been inversely correlated with pulmonary function (Manzenreiter *et al.*, 2012). In contrast, free DNA is not found in healthy donor sputum (Manzenreiter *et al.*, 2012). The majority of CF sputum DNA originates from neutrophils (Lethem *et al.*, 1990). DNA found in CF airways shows characteristics of NET-DNA (Dwyer *et al.*, 2014). Large amounts of free, extracellular DNA in CF patients' airways have been successfully combatted with DNase treatments (Halverson *et al.*, 2015; Papayannopoulos *et al.*, 2011). Digestion of NETs by DNase releases active neutrophil elastase (NE) (Rahman and Gadjeva, 2014; Papayannopoulos *et al.*, 2011). This action may provide the transition between two steps of a proposed NET bactericidal mechanism in which full NET complexes initially capture and starve a bacterium before being partially degraded by DNase, after which an arsenal of antimicrobial peptides are released to finish inactivating the bacterium (Rahman and Gadjeva, 2014). Histones, neutrophil elastase (NE), and myeloperoxidase (MPO) are three of the chief components associated with DNA in NET complexes (Rahman and Gadjeva, 2014; Papayannopoulos *et al.*, 2011).

Histones are located within the neutrophil's DNA, and NE is responsible for degrading histones (Papayannopoulos *et al.*, 2011). MPO is specifically involved in the oxidative burst of neutrophils (Metzler *et al.*, 2011; Brinkmann and Zychlinsky, 2007). In this mechanism, the NADPH oxidase enzyme complex oxidizes NADPH, freeing one electron that transfers to oxygen (O₂) to generate

superoxide (O_2^-) (Brinkmann and Zychlinsky, 2007). Superoxide (O_2^-) then dismutates into hydrogen peroxide (H_2O_2), either spontaneously or through a reaction catalyzed by superoxide dismutase (SOD), an enzyme produced by some microbes resistant to the oxidative burst mechanism of neutrophils (Brinkmann and Zychlinsky, 2007). MPO then converts hydrogen peroxide (H_2O_2) into hypochlorous acid (HOCl), which attacks bacteria ensnared in NETs (Brinkmann and Zychlinsky, 2007). All three of these components cooperate during NET formation.

Upon activation of neutrophils, reactive oxygen species (ROS) are produced, including superoxide (O_2^-), which stimulates NE to exit the azurophilic granule and translocate across the nuclear membrane (Papayannopoulos *et al.*, 2010). Before NETs are released, NE must digest histones associated with the neutrophil's DNA, initiating decondensation of the chromatin; MPO is also thought to participate in this process (Papayannopoulos *et al.*, 2010). Peptidylarginine deiminase type 4 (PAD4)-mediated citrullination of histones also occurs at this time (Lu *et al.*, 2012). As such, citrullinated histones are present in CF airways (Manzenreiter *et al.*, 2012; Papayannopoulos *et al.*, 2011). Lastly, the neutrophil cell membrane ruptures, and NETs are released (Papayannopoulos *et al.*, 2010). Within the NET complex, histones act as adhesives, sticking to bacterial pathogens and holding them in place to be attacked and degraded by ROS, such as hypochlorous acid (HOCl), produced by MPO (Rahman and Gadjeva, 2014).

Clinical relevance of interactions between these four components – DNA, histones, MPO, and NE – has been established in immunofluorescence imaging of CF patient sputum in several studies illustrating overlapping DNA, citrullinated histone 3 (CitH3), MPO, and NE – an occurrence indicative of NET formation (Manzenreiter *et al.*, 2012; Yoo, Winn, *et al.*, 2014). Moreover, increased concentrations of NE and MPO in CF patient sputum are correlated with diminished lung function, quantified by forced expiratory volume occurring in one second (FEV₁) (Mayer-Hamblett *et al.*, 2007; Sagel *et al.*, 2012; Kim *et al.*, 2006). In experiments with neutrophils from patients entirely deficient in MPO (Δ MPO), NET formation failed to occur after stimulation with phorbol myristate acetate (PMA) (Metzler *et al.*, 2011). Additionally, experiments with a murine model compared effects on the CFTRs of wild-type and NE^{-/-} mice in the presence of *Pseudomonas aeruginosa* bacterial infection; in this study, researchers found that NE can exert destructive effects on the CFTR both *in vitro* and *in vivo* (Le Gars *et al.*, 2013). Thus, both MPO and NE have proven essential to NET formation and can be used in combination with measurements of other factors (e.g., histones and DNA) to establish whether or not NETosis has occurred.

Previous *in vitro* studies indicate that NET formation is not a completely comprehensive killer of all pathogens, but it can negotiate varying degrees of relief from the infectious burden imparted by a select number of microbes – among which *Pseudomonas aeruginosa* is included (Lu *et al.*, 2012). For example, some microbes evade NET-mediated killing by changing the charge of their cell surface, thereby diminishing their affinity for NETs, or by secreting

enzymes called DNases, which chop up DNA and thus reduce the chances of bacteria being ensnared by NETs (Lu *et al.*, 2012). In one study, infection with *Pseudomonas aeruginosa* led to cleavage of wild-type murine CFTRs within 20 hours, thereby establishing a possible interaction between *P. aeruginosa* and CFTRs (Le Gars *et al.*, 2013). This is in addition to *P. aeruginosa*'s already well known presence as the most prevalent pathogen in CF patients' airways.

PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa is a gram-negative, opportunistic bacterium that serves as the prevailing pathogen in CF lung disease, and many studies report on its relationship with decreased pulmonary function in CF patients (Foweraker, 2009; Lambiase *et al.*, 2006; West *et al.*, 2002). It typically survives as an aerobe, but previous research has shown that it can also grow anaerobically in CF lungs (Hassett *et al.*, 2009). One of the most distinguishing characteristics of *P. aeruginosa* infections in pulmonary disease is recruitment of considerable numbers of neutrophils (Williams *et al.*, 2010). Given that *P. aeruginosa* and neutrophils are both abundant in CF airways, they are believed to regularly make contact, sometimes via neutrophil phagocytosis of bacteria (Williams *et al.*, 2010). In other instances, *P. aeruginosa* and neutrophil interactions lead to large amounts of NET formation, which has been demonstrated both *in vitro* and *in vivo* (Young *et al.*, 2011; Rada *et al.*, 2013; Mayer-Hamblett *et al.*, 2007; Kim *et al.*, 2006). NET formation, however, has been shown to be more effective than phagocytosis in that NETs ensnare more bacteria than neutrophils and

macrophages are able to phagocytose, at least in a sepsis model (Clark *et al.*, 2007). Importantly, NET-mediated killing of bacteria is unaffected by the absence of functional CFTRs, while phagocytic killing is affected (Young *et al.*, 2011). However, *P. aeruginosa*'s adaptation to CF airways seems to include acquired resistance to NET-mediated killing (Yoo, Floyd, *et al.*, 2014; Young *et al.*, 2011). Regardless of the form, it is clear that interactions between *P. aeruginosa* and neutrophils are central to CF lung pathophysiology. Through continual exposure to neutrophils, *P. aeruginosa* faces hazards introduced by elevated levels of reactive oxygen species (ROS), which can damage *P. aeruginosa*'s DNA, thereby leading to more DNA mutations than normally expected (Hassett *et al.*, 2009). As such, these bacteria display fluctuating phenotypes of polysaccharide alginate (affects mucoidy), biofilm growth, antibiotic resistance, and flagellin, pilin, and other virulence factors (Hassett *et al.*, 2009; Garrett *et al.*, 1999). Variable expression of these virulence determinants is thought to contribute extensively to chronic lung infections (Young *et al.*, 2011).

Phenotypes of *P. aeruginosa* are thought to shift in distinct phases during lung infection. Initially, *P. aeruginosa* is present in planktonic form (Jain and Kazmierczak, 2014; Wolfgang *et al.*, 2004). After attachment to the mucosal surface, bacteria lose their motile phenotype, then form first microcolonies followed by biofilms, which protect them from harm inflicted by the host's immune system (Williams *et al.*, 2010; Klausen *et al.*, 2003). At this stage, the flagellum is lost, and bacterial growth occurs (Balloy *et al.*, 2014; Klausen *et al.*, 2003). Detachment from the biofilm then allows some bacteria, which have already

re-acquired their flagellum, to be released into the airways to colonize elsewhere (Balloy *et al.*, 2014; Williams *et al.*, 2010; Klausen *et al.*, 2003). Testing of clinical *P. aeruginosa* strains determined that early isolates prompt more robust NET formation than do their late isolate counterparts (when both early and late isolates were obtained from the same CF patient) (Yoo, Floyd, *et al.*, 2014). In a broad sense, these results suggest that *P. aeruginosa* may adapt to lower its NET-inducing qualities, allowing it to persist in a less detectable form as CF lung disease progresses (Yoo, Floyd, *et al.*, 2014). More specifically, these results concede a gap in knowledge regarding the contribution of motility by *P. aeruginosa* to recognition by neutrophils and subsequent induction of NET formation.

P. aeruginosa's movement largely relies on its flagellum, or swimming structure comprised of repeats of the flagellin protein (Skerrett *et al.*, 2006; Rossez *et al.*, 2015). Flagellin – a major virulence factor of both gram-positive and gram-negative bacteria – is recognized by Toll-like receptor (TLR) 5 present on neutrophils, alveolar macrophages, and respiratory epithelial cells (Balloy *et al.*, 2014). Other receptors reported to be involved in recognition of *P. aeruginosa* are listed in **Table 1**. Only TLR5 has been shown to be directly responsible for neutrophil recruitment upon flagella challenge, while others more so appear to play roles in responding to epithelial cell pro-inflammatory cytokine release (Adamo *et al.*, 2004). It has also been proposed that TLR4 and TLR5 cooperate in their recognition of flagellated bacteria, but no firm conclusions have been drawn as of yet, and their direct binding to flagellum is unlikely (Feuillet *et al.*,

2006). Other studies have suggested that TLR5 is possibly stored intracellularly within neutrophils and upregulated in its expression following flagella challenge through cooperative actions of TLR1 and TLR2 signaling; however, this has yet to be widely accepted throughout the literature (Koller *et al.*, 2008). Therefore, TLR5 and TLR4 remain the primary receptors recognized as being responsible for neutrophilic recognition of *P. aeruginosa*. NOD-like receptor caspase associated recruitment domain (CARD)-containing protein 4 (NLRC4) is also known to directly bind intracellular flagellum (Jain and Kazmierczak, 2014). Which neutrophil receptor(s) trigger NET formation, however, is still a mystery and, thus, represents a gap in knowledge in current literature.

TABLE 1. Pattern recognition receptors binding extracellular bacterial ligands reported to play a role in recognition of *Pseudomonas aeruginosa*. This is not a completely inclusive list but, rather, represents the receptors most commonly found in current literature. (*, possibly but not firmly established; #, in epithelial cells (not neutrophils); LPS, Lipopolysaccharide; LTA, Lipoteichoic Acid)

Receptor	Ligand(s)	Reference(s)
TLR5	Flagellin (protein)	Feuillet <i>et al.</i> , 2006; Skerrett <i>et al.</i> , 2006; Ramphal <i>et al.</i> , 2008; Rieber <i>et al.</i> , 2014; Koller <i>et al.</i> , 2008; Blohmke <i>et al.</i> , 2008; Williams <i>et al.</i> , 2010
TLR4	LPS; capsular alginate; exoenzyme S	Feuillet <i>et al.</i> , 2006; Skerrett <i>et al.</i> , 2006; Ramphal <i>et al.</i> , 2008; Clark <i>et al.</i> , 2007; Rieber <i>et al.</i> , 2014; Williams <i>et al.</i> , 2010
TLR2	Bacterial lipoproteins; LTA; peptidoglycan; Pam ₃ CSK4; pilin; capsular alginate; exoenzyme S; LPS*; flagellin [#]	Skerrett <i>et al.</i> , 2006; Ramphal <i>et al.</i> , 2008; Koller <i>et al.</i> , 2008; Adamo <i>et al.</i> , 2004 [#] ; Blohmke <i>et al.</i> , 2008; Williams <i>et al.</i> , 2010
TLR1	Pam ₃ CSK4	Koller <i>et al.</i> , 2008; Blohmke <i>et al.</i> , 2008

We aim to fill the aforementioned gap in knowledge based on preliminary data indicating that the flagellum is a major factor in *P. aeruginosa*-triggered NETosis (**Figure 3B**). This project proposes that motility of *P. aeruginosa* serves as the major virulence determinant contributing to NET formation by neutrophils (**Figure 1**). One reason this might be true is because motile *P. aeruginosa* are presumed to come into contact with neutrophils more frequently than non-motile *P. aeruginosa*. Swimming motility powered by the flagellar motor is likely responsible for this increased contact between neutrophils and *P. aeruginosa*. In our model, it is important for the flagellar motor to be functional, as a flagellum with a dysfunctional flagellar motor cannot move to initiate contact with additional neutrophils. Establishing the major virulence factor(s) responsible for triggering NET formation is important for the development of novel clinical therapies for CF patients, whose heavily infected airways suffer from an overabundance of NETosing neutrophils.

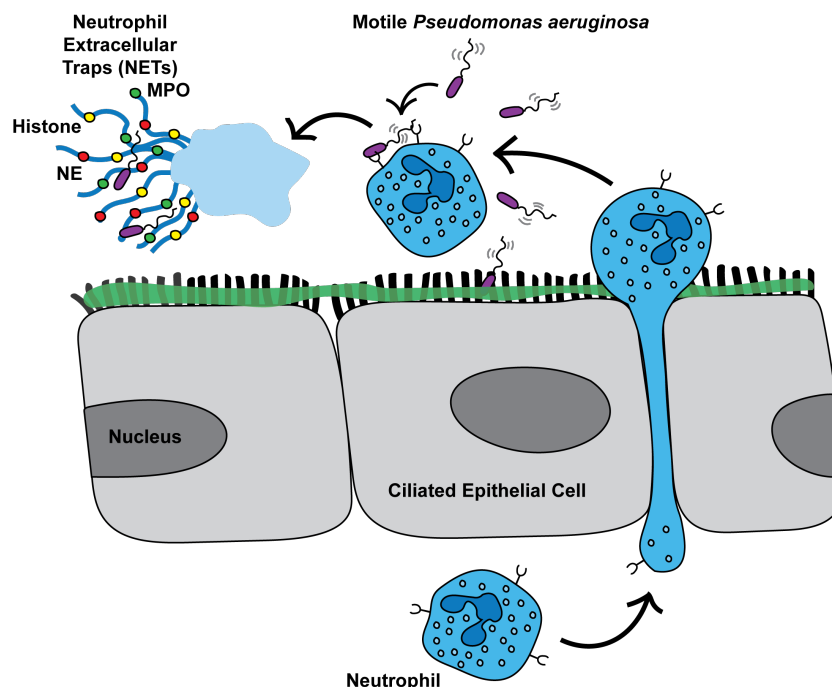


FIGURE 1. Proposed model in which motility of *Pseudomonas aeruginosa* is necessary for and more important in triggering maximal NET formation than is direct binding of flagellum in this process. Neutrophils transmigrate from the submucosa, across the lung mucosal epithelium, and into the lung mucosa to respond to motile *Pseudomonas aeruginosa*. Multiple receptors (specific for different bacterial ligands) on the neutrophil cell surface are proposed to bind ligands on motile *P. aeruginosa*. Bacterial motility is believed to promote contact between bacteria and neutrophils. This triggers NET formation in CF airways. Figure designed in Adobe Illustrator by Madison Floyd. (NETs, Neutrophil Extracellular Traps; MPO, Myeloperoxidase; NE, Neutrophil Elastase)

Five PAK background and two PAO1 background *P. aeruginosa* strains are used in the experiments included in this study. Their names and deficiencies are listed in **Table 2** (Doyle *et al.*, 2004). Experimentation for this project focused on determining the different effects of neutrophil stimulation with wild-type, motility-deficient ($Mot^{(-)}$), and flagellum-deficient ($Fla^{(-)}$) *P. aeruginosa* (Doyle *et al.*, 2004). Many of these deficient components can be seen in **Figure 2**.

TABLE 2. *Pseudomonas aeruginosa* strains used in this study. All PAK strains were provided by Dr. Linda McCarter of the University of Iowa. (WT, Wild-Type; $Mot^{(-)}$, Motility-Deficient; $Fla^{(-)}$, Flagellum-Deficient; Gen^r , Gentamicin-Resistant Vector; Tet^r , Tetracycline-Resistant Vector) (Doyle *et al.*, 2004)

Strain	Genetic Deficiency	Phenotype
PAK WT	None	Motile; flagellin expressed.
$Mot^{(-)}$ PAK LMP16	$\Delta motAB4::Gen^r motD2::Tet^r$	Non-motile; flagellin expressed.
$Mot^{(-)}$ PAK LMP50	$\Delta motB3::Gen^r motCD8::Tet^r$	Non-motile; flagellin expressed.
$Fla^{(-)}$ PAK LMP57	$\Delta flgC1::Tn5 (Tet^r)$	Non-motile; no flagellin.
PAO1 WT	None	Motile.
$Fla^{(-)}$ PAO1	$\Delta fliC$	Non-motile.

I hypothesized that motile wild-type *P. aeruginosa* triggers more robust NET formation than its motility- and flagellum-deficient counterparts. Through my studies, I aimed to establish motility as a major contributor to NET formation, as well as ascertain whether or not MyD88 signaling – used by TLRs 1, 2, 4, 5, 7 and 9 – is majorly involved in the NET signaling pathway (Koller *et al.*, 2008; Skerrett *et al.*, 2006). I show that motile wild-type *P. aeruginosa* triggers significantly more NET formation than its motility- and flagellum-deficient counterparts. Moreover, possession of a non-functional flagellum in motility-deficient *P. aeruginosa* fails to prompt significantly more NET formation than flagellum-deficient *P. aeruginosa*, despite the presence versus absence of TLR5 ligand flagellin in motility- and flagellum-deficient *P. aeruginosa*, respectively. I further demonstrate with murine neutrophils that MyD88 signaling – and, thus, binding of TLRs 1, 2, 4, 5, 6, and 9 – does not serve as a significant trigger of NET formation.

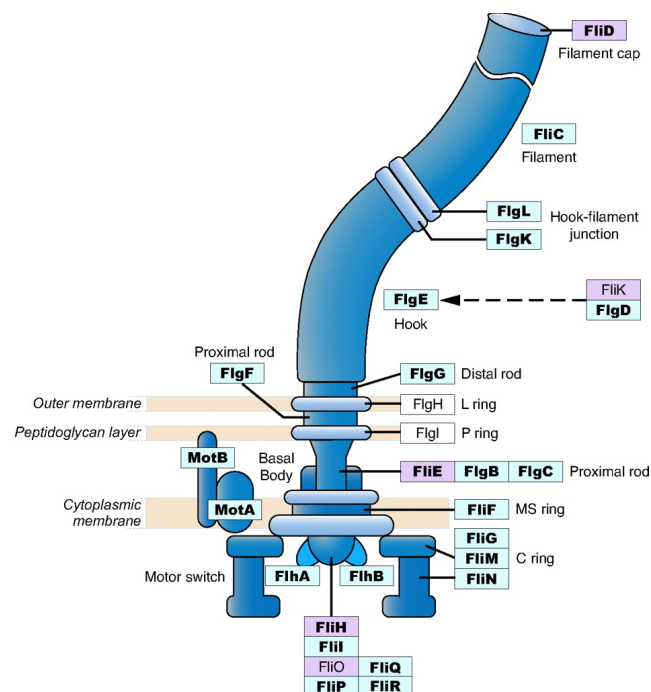


FIGURE 2. Primary components comprising the *Pseudomonas aeruginosa* flagellar motor and flagellum. Figure re-printed from Liu and Ochman, 2007, under open access terms and conditions as Creative Commons Attribution Noncommercial License.

CHAPTER 2

RESULTS: PART 1: FLAGELLAR MOTILITY OF *PSEUDOMONAS AERUGINOSA* CONTRIBUTES TO NET FORMATION

PROOF OF CONCEPT

Having already determined within our lab that *Pseudomonas aeruginosa* triggers NET formation (Yoo, Floyd, *et al.*, 2014; Yoo, Winn, *et al.*, 2014; Rada *et al.*, 2013), we sought to ascertain the importance of *P. aeruginosa*'s flagellar motility regarding induction of NETosis. Preliminary Western blotting established that flagellum-deficient ($\Delta flgC1$) bacteria exhibit a comprehensive absence of flagellin, the component protein of flagellum, while wild-type (WT) bacteria display a strong flagellin presence (not shown; Doyle *et al.*, 2004). Swimming motility requires functional flagellum, and testing confirms that WT *P. aeruginosa* generates significantly larger and more diffuse colonies, indicative of swimming motility, than does its $\Delta flgC1$ counterpart (**Figure 3A**). Growth and/or Brownian motion (spread of bacteria by dripping water resultant of condensation), but no true swimming motility, shapes the smaller, denser colonies of $\Delta flgC1$ *P. aeruginosa*.

Next, we demonstrate that motile WT *P. aeruginosa* PAK strain drives robust release of extracellular DNA, while flagellum-deficient $\Delta flgC1$ rouses only minimal NET release, consistent with that seen for untreated neutrophils (**Figure**

3B). This scenario provides our proof of concept by illustrating the contribution of bacterial flagellum to extracellular DNA release, a recognized marker correlated with CF lung disease severity (Kim *et al.*, 2006). Our data support other preliminary data (not shown) amassed by our lab, which demonstrated swimming motility and extracellular DNA release data comparable to that disclosed here.

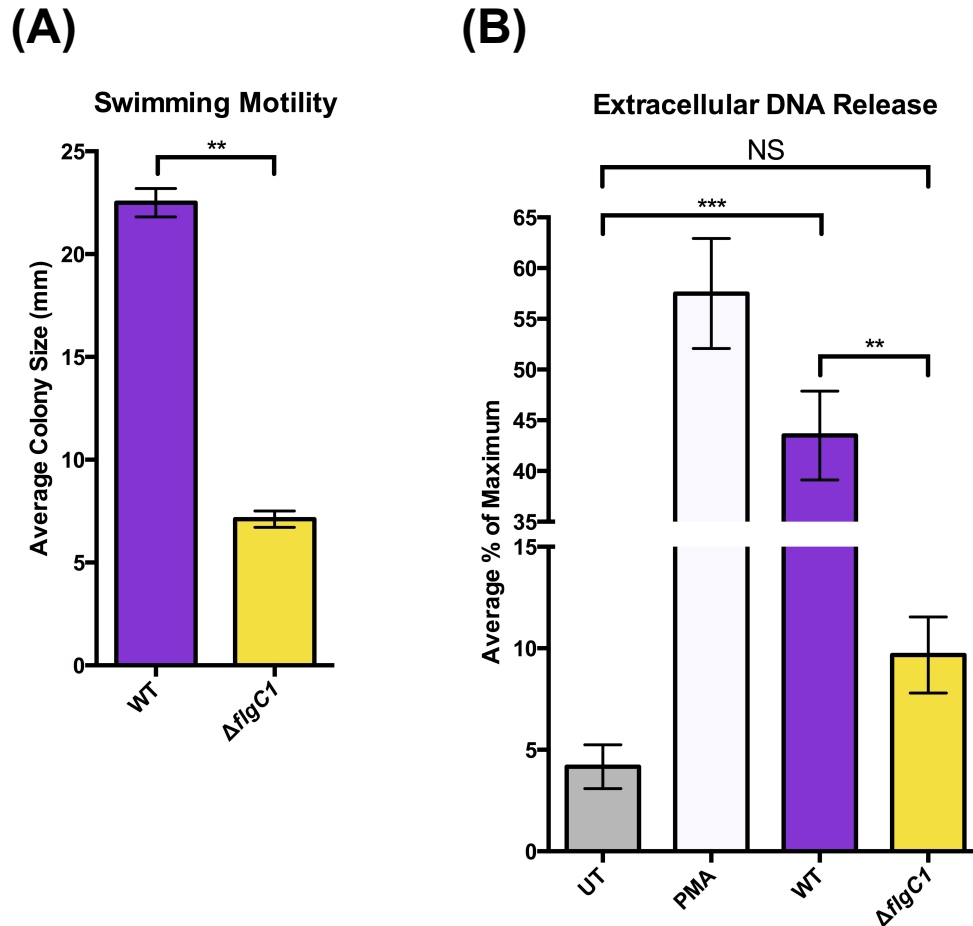


FIGURE 3. Flagellum deficiency impairs *Pseudomonas aeruginosa*-induced extracellular DNA release in human neutrophils. (A) PAK WT *P. aeruginosa* exhibits significantly larger colony size than does PAK $\Delta flgC1$, indicative of the presence and absence of swimming motility, respectively. Bacterial concentrations were set to 10^9 /ml, then diluted to 10^7 /ml and stabbed into swimming motility agar plates (0.3% agar) in duplicate. A negative control (NC)

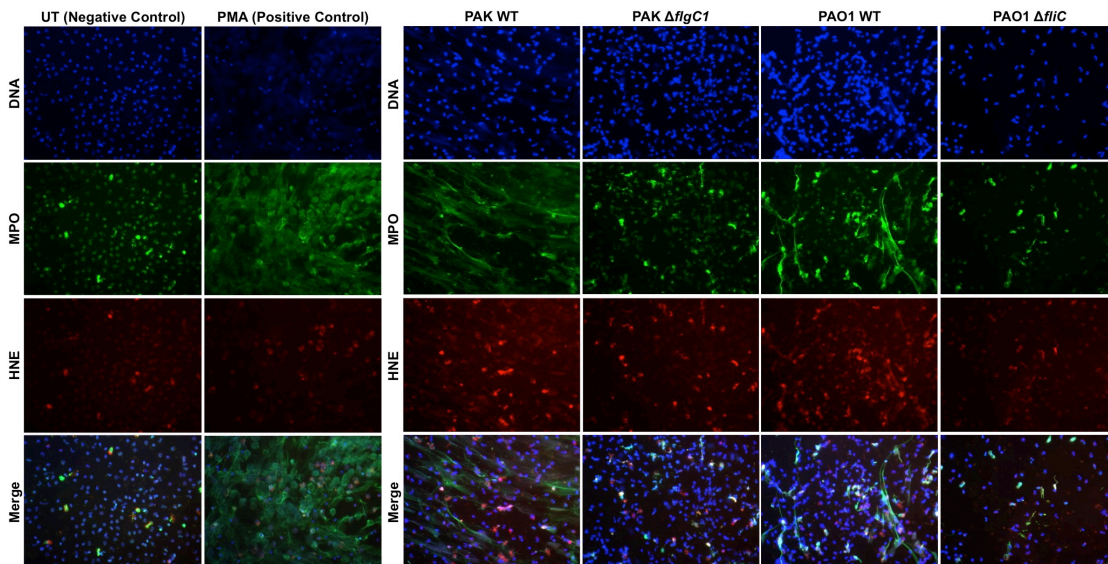
plate verified no agar-based contamination. Plates were incubated at room temperature for 48 hours. Diameter of colony size in millimeters is shown. ($n = 3$ experiments; mean $\pm$ SEM; student's t test; ** means $p < 0.01$) **(B)** Extracellular DNA release from healthy human neutrophils stimulated as described by the x-axis (10 MOI bacteria) was detected with Sytox Orange, a membrane impermeable dye. Healthy human neutrophils stimulated with PAK WT *P. aeruginosa* release significantly more extracellular DNA than those stimulated with flagellum-deficient PAK $\Delta flgC1$. Data shown represent signals generated over an average 6-hour time period. % of maximum refers to maximal signal induced by saponin, allowing experiments performed over several days to be compared. ($n = 6$ experiments; mean $\pm$ SEM; one-way ANOVA; *** means $p < 0.001$, ** means $p < 0.01$, and NS means no statistical significance) [UT, Untreated (HBSS); PMA, 100 nM Phorbol Myristate Acetate; WT, PAK Wild-Type; $\Delta flgC1$, Flagellum-deficient PAK Mutant; MOI, Multiplicity of Infection]

ASCERTAINING VISUAL CONFIRMATION OF THE CONTRIBUTION OF BACTERIAL FLAGELLUM TO *PSEUDOMONAS AERUGINOSA*-INDUCED NET FORMATION

Detecting co-localization of extracellular DNA with neutrophil granule components and histones in structures resembling NET morphology is required to define DNA release from neutrophils as NET formation (Rahman and Gadjeva, 2014; Papayannopoulos *et al.*, 2011). Previously, our group has shown that *Pseudomonas aeruginosa* PAO1 and PA14 triggers NET formation in human neutrophils by immunofluorescence detecting co-localization of DNA, MPO, and HNE (Yoo, Floyd, *et al.*, 2014; Yoo, Winn, *et al.*, 2014). Therefore, we pursued immunofluorescence staining of stimulated human neutrophils with two additional

NET-associated parameters: myeloperoxidase (MPO) and human neutrophil elastase (HNE). DNA, MPO, and HNE overlap in merged images of human neutrophils stimulated with wild-type (WT) and flagellum-deficient ($\Delta flgC1$ and $\Delta fliC$) PAK and PAO1 background *P. aeruginosa*, respectively (**Figure 4A**). WT *P. aeruginosa* strains induce NET formation more similar to that seen with PMA-stimulated human neutrophils. In contrast, flagellum-deficient *P. aeruginosa* strains induce very little NET formation, which is reminiscent of that seen with untreated human neutrophils. Insets of the merged images (**Figure 4B**) reveal intricate details of NETs shown in **Figure 4A**. These results will qualitatively validate quantitative data gathered going forward.

(A)



(B)

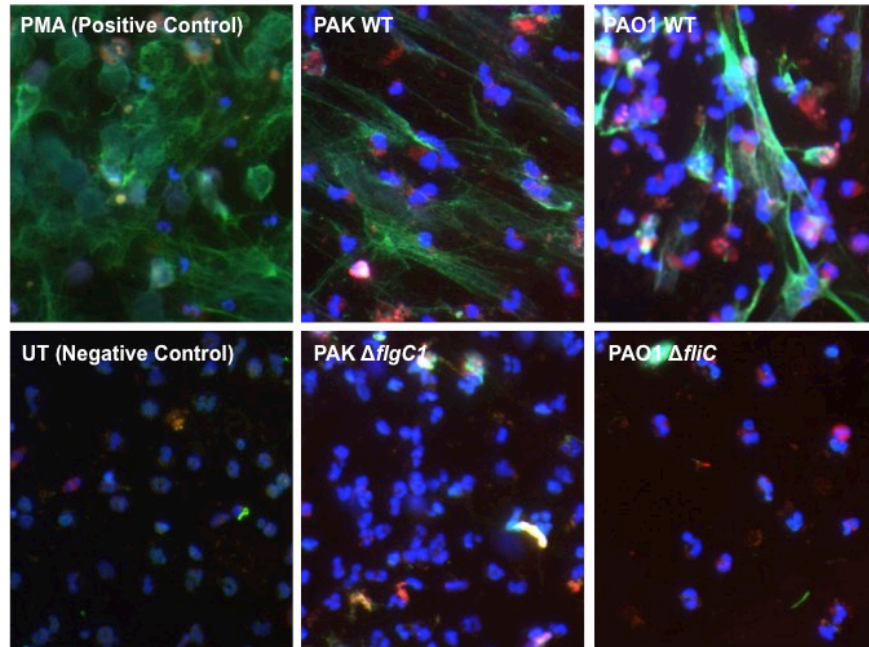
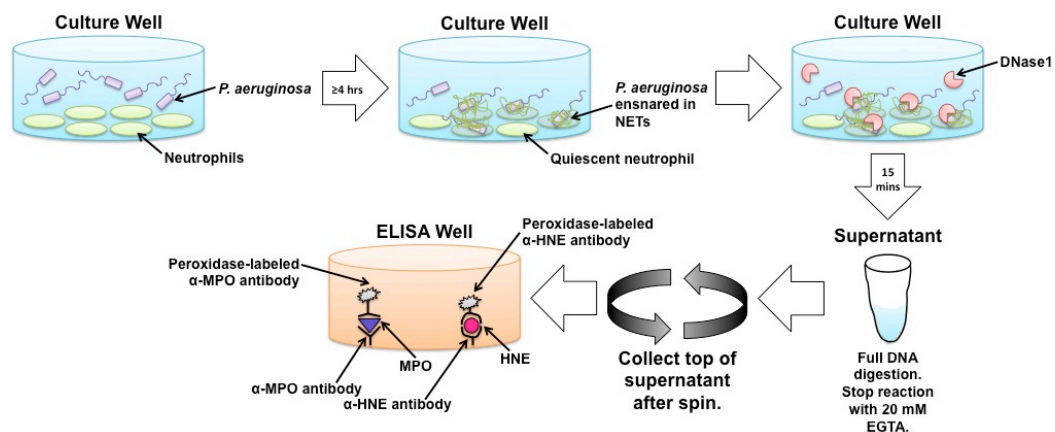


FIGURE 4. Flagellum deficiency abolishes *Pseudomonas aeruginosa*-induced NET formation, seen visually with DNA, MPO and HNE staining of human neutrophils. (A) Human neutrophils were stimulated as described in the figure (10 MOI bacteria) and incubated at 37°C for an average of 4 hours. Staining for DNA (**blue**), MPO (**green**), and HNE (**red**) is described in the Materials and Methods (Chapter 5). Images were taken at 20x magnification with a Zeiss AxioCam HRM fluorescent microscope and analyzed with Axioplan2 imaging software. (*n* = 3 experiments, one representative shown) **(B)** Zoomed-in insets of images presented in (A). [UT, Untreated (HBSS); PMA, 100 nM Phorbol Myristate Acetate; WT, PAK or PAO1 Wild-Type; $\Delta flgC1$, Flagellum-Deficient PAK Mutant; $\Delta fliC$, Flagellum-Deficient PAO1 Mutant; MPO, Myeloperoxidase; HNE, Human Neutrophil Elastase]

***PSUEDOMONAS AERUGINOSA* INDUCES RELEASE OF FREE MPO AND HNE DURING NET FORMATION**

MPO and HNE are well described in current literature, as well as by our own lab, as being associated with NET formation (Yoo, Winn, *et al.*, 2014). To quantitatively corroborate immunofluorescence imaging shown in **Figure 4**, we performed MPO and HNE ELISAs on supernatants of stimulated human neutrophils cultured as illustrated in **Figure 5A**. While no statistical significance is observed between WT and flagellum-deficient *P. aeruginosa* with either MPO (**Figure 5B**) or HNE (**Figure 5C**) release, a distinct pattern that is likely biologically significant nonetheless remains visible. One reason for finding no statistical significance may be that more “free” (i.e., not attached to extracellular DNA as a NET complex) MPO and HNE is released than is MPO or HNE released in complex with DNA. One potential mechanism for NET-independent MPO and HNE release could be degranulation (Papayannopoulos and Zychlinsky, 2009). Hence, we show that less MPO and HNE release is prompted by flagellum-deficient *P. aeruginosa* than is by WT *P. aeruginosa* (**Figures 5B and 5C**), despite being statistically insignificant by the $p < 0.05$ standard.

(A)



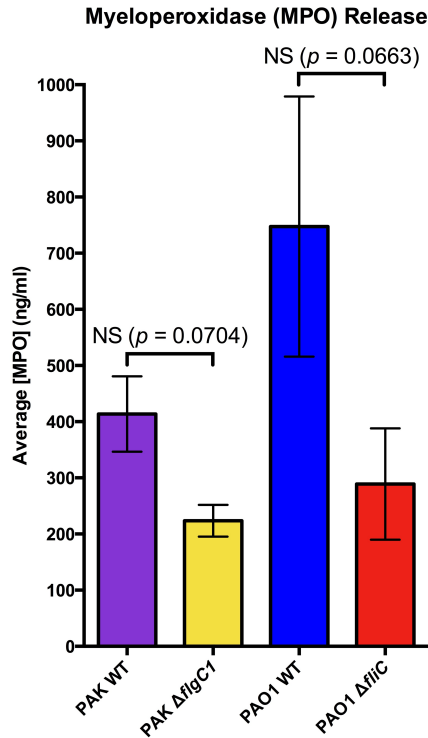
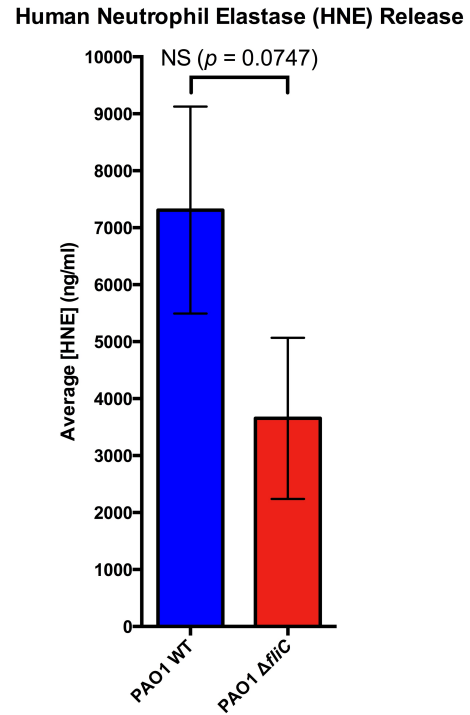
(B)**(C)**

FIGURE 5. MPO and HNE release during NET formation is greater following treatment with WT *Pseudomonas aeruginosa* than after stimulation with flagellum-deficient mutants. (A) Experimental scheme for MPO and HNE ELISAs portrayed in (B) and (C). 30 μ g/ml DNase1 was used to digest culture samples for both ELISAs. **(B)** PAK WT and PAO1 WT *P. aeruginosa*-stimulated human neutrophils release more free MPO during NET formation than flagellum-deficient PAK $\Delta flgC1$ - and PAO1 $\Delta fliC$ -stimulated human neutrophils. However, this difference is not statistically significant. Experiments were performed as illustrated in (A). ($n = 5$ experiments with PAK strains; $n = 4$ experiments with PAO1 strains; 10 MOI bacteria; mean $\pm$ SEM; student's t test; NS means no statistical significance) **(C)** PAO1 WT-stimulated human neutrophils release more HNE during NET formation than flagellum-deficient PAO1 $\Delta fliC$ -stimulated human neutrophils. However, this difference is not statistically significant. ($n = 4$ experiments; 10 MOI bacteria; mean $\pm$ SEM; student's t test; NS means no statistical significance) [WT, PAK or PAO1 Wild-Type; $\Delta flgC1$, Flagellum-Deficient PAK Mutant; $\Delta fliC$, Flagellum-Deficient PAO1 Mutant; MPO,

Myeloperoxidase; HNE, Human Neutrophil Elastase; MOI, Multiplicity of Infection]

MOTILITY AND FLAGELLUM DEFICIENCY EQUALLY ABOLISH SWIMMING ABILITY IN *PSEUDOMONAS AERUGINOSA*

Once we established the importance of the flagellum for swimming motility and NET formation, marked by extracellular DNA, MPO, and HNE release, we sought to ascertain the impact of a functional versus dysfunctional flagellar motor to swimming motility. We based this aim on unpublished data previously gathered in our lab in which Toll-like receptor (TLR) 5 neutralizing antibodies failed to inhibit *Pseudomonas aeruginosa*-induced NET formation (not shown). This directed us to study the involvement of bacterial motility, since flagellin – the component protein of flagellum recognized extracellularly by TLR5 – alone, in concentrations found to inhibit *P. aeruginosa*-stimulated superoxide production and known TLR5-mediated function in human neutrophils, clearly failed to majorly contribute to the NETosis mechanism (not shown). To test the contribution of motility to NET formation, we collaborated with Dr. Linda McCarter at the University of Iowa, who provided PAK background wild-type (WT) and motility- (Δmot LMP16 and Δmot LMP50) and flagellum-deficient ($\Delta flgC1$) mutant strains (Doyle *et al.*, 2004). Importantly, Δmot LMP16 and Δmot LMP50 exhibit $\Delta motAB4motD2$ and $\Delta motB3motCD8$ phenotypes, respectively, while $\Delta flgC1$ is deficient in a proximal rod protein of the flagellum (**Figure 2**) (Doyle *et al.*, 2004). It should be noted that while **Figure 2** does not denote flagellar motor domains C

and D, they are also present along with flagellar motor domains A and B (Doyle *et al.*, 2004).

We used these strains to conduct a swimming motility assay, which established that PAK WT moves significantly faster and more diffusely than two motility-deficient mutants (Δmot LMP16 and Δmot LMP50) and a flagellum-deficient mutant ($\Delta flgC1$) (**Figure 6**). Furthermore, motility-deficient mutants Δmot LMP16 and Δmot LMP50 displayed equally diminished swimming motility as compared to flagellum-deficient mutant $\Delta flgC1$ (**Figure 6**). These results help cement the necessity for a flagellum and a functional flagellar motor for swimming motility to occur in *P. aeruginosa*.

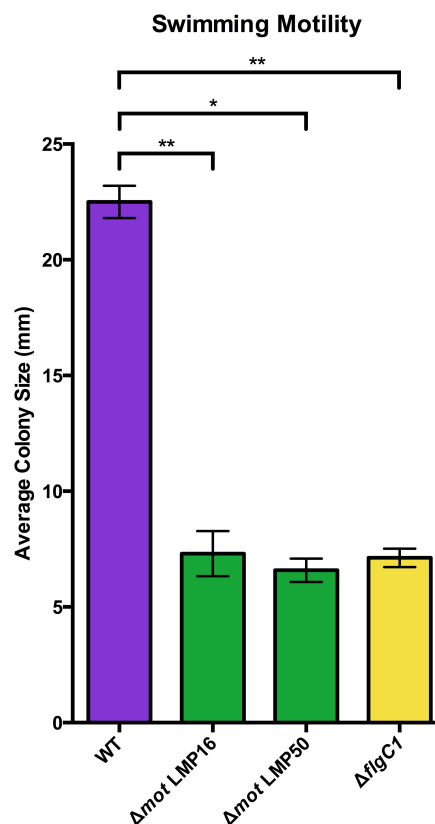


FIGURE 6. A flagellum and a functional flagellar motor are both required for swimming motility of *Pseudomonas aeruginosa*. PAK WT *P. aeruginosa*

exhibits significantly larger colony size than do PAK Δmot LMP16, Δmot LMP50, and $\Delta flgC1$, indicative of the presence and absence of swimming motility, respectively. Hence, motility and flagellum deficiency are detrimental to swimming motility ability of *P. aeruginosa*. Bacterial concentrations were set to 10^9 /ml, then diluted to 10^7 /ml and stabbed into swimming motility agar plates in duplicate. A negative control (NC) plate verified no agar-based contamination. Plates were incubated at room temperature for 48 hours. Diameter of colony size in millimeters is shown. ($n = 3$ experiments; mean $\pm$ SEM; one-way ANOVA; ** means $p < 0.01$ and * means $p < 0.05$) [WT, PAK Wild-Type; Δmot LMP16 and Δmot LMP50, Motility-Deficient PAK Mutants; $\Delta flgC1$, Flagellum-Deficient PAK Mutant]

FLAGELLAR MOTILITY IS CRUCIAL TO *PSEUDOMONAS AERUGINOSA*-TRIGGERED SUPEROXIDE PRODUCTION AND EXTRACELLULAR DNA RELEASE IN HUMAN NEUTROPHILS

The respiratory burst mediated by the NADPH oxidase in neutrophils has been shown to be required for NET formation stimulated by a wide variety of stimuli, including different bacteria (Almyroudis *et al.*, 2013; Lu *et al.*, 2012). Previous data from our group confirmed that the NADPH oxidase is required for *Pseudomonas aeruginosa*-induced NETs, since *P. aeruginosa* stimulates robust superoxide production in human neutrophils, and NADPH oxidase inhibitor diphenylene-iodonium (DPI) largely inhibits NET formation (Yoo, Floyd, *et al.*, 2014; Yoo, Winn, *et al.*, 2014). Here, we aimed to determine if PAK background *P. aeruginosa*-stimulated superoxide production is affected by mutations resulting in motility or flagellum deficiency. Our results show that motile PAK wild-

type (WT) *P. aeruginosa* induces significantly greater superoxide production (**Figure 7A**) and extracellular DNA release (**Figure 7B**) than its motility- (Δmot LMP16 and Δmot LMP50) and flagellum-deficient ($\Delta flgC1$) counterparts. Together, data presented in **Figure 7** show that having a flagellum and a functional flagellar motor for motility of *P. aeruginosa* is crucial to NET release. Additionally, motility- and flagellum-deficient mutants provoked superoxide and extracellular DNA values deemed not significantly greater than untreated (UT) samples (**Figure 7**), which also endorses the effect of bacterial motility on stimulation of NETosis.

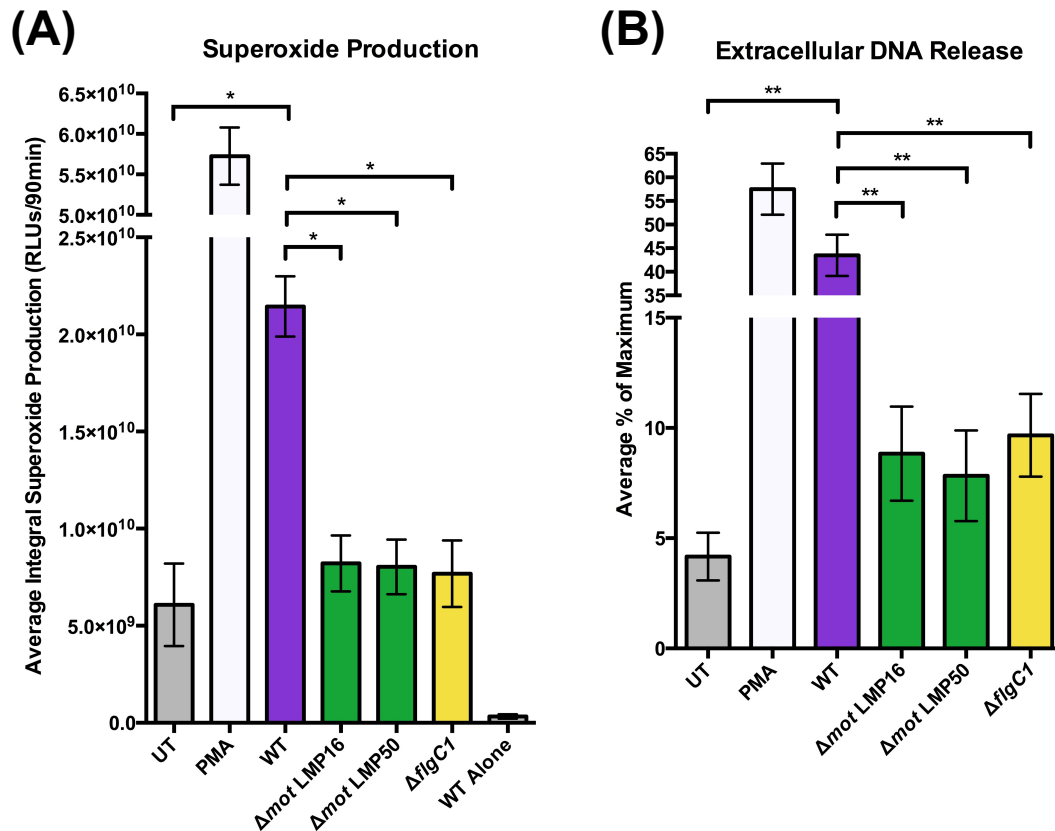


FIGURE 7. Motility is required for high levels of superoxide production and extracellular DNA release, two indicators of NET formation. (A) Superoxide production by human neutrophils is significantly greater in the presence of 10 MOI PAK WT *P. aeruginosa* than in the presence of 10 MOI motility- (Δmot

LMP16 and Δmot LMP50) or 10 MOI flagellum-deficient ($\Delta flgC1$) *P. aeruginosa*. Superoxide production by human neutrophils was measured every 30 seconds for 90 minutes in a Diogenes-based luminescence assay. ($n = 4$ experiments; mean $\pm$ SEM; one-way ANOVA; * means $p < 0.05$) **(B)** Extracellular DNA release from healthy human neutrophils stimulated as described by the x-axis (10 MOI bacteria) was detected with Sytox Orange, a membrane impermeable dye. Healthy human neutrophils stimulated with PAK WT *P. aeruginosa* release significantly more extracellular DNA than those stimulated with motility-deficient Δmot LMP16 or Δmot LMP50, as well as flagellum-deficient PAK $\Delta flgC1$. Data shown represent signals generated over an average 6-hour time period. % of maximum refers to maximal signal induced by saponin, allowing experiments performed over several days to be compared. ($n = 6$ experiments; mean $\pm$ SEM; one-way ANOVA; ** means $p < 0.01$) [UT, Untreated (HBSS); PMA, 100 nM Phorbol Myristate Acetate; WT, PAK Wild-Type; Δmot LMP16 and Δmot LMP50, Motility-Deficient PAK Mutants; $\Delta flgC1$, Flagellum-Deficient PAK Mutant; WT Alone, No Neutrophils; MOI, Multiplicity of Infection]

NEUTROPHIL MORPHOLOGY CHANGES IN RESPONSE TO STIMULATION BY *PSEUDOMONAS AERUGINOSA*

After obtaining quantitative data supporting NET formation in neutrophils stimulated with motile *P. aeruginosa*, we sought to visualize this process as confirmation of our numerical data. The most efficient way to do this was by photographing cells (**Figure 5A**); this was done after a 4-hour incubation at 37°C and prior to treatment with DNase1. Neutrophils undergoing NETosis are known to “de-lobe,” which can be seen as black spots in **Figure 8**. Additionally, neutrophils that have already ejected NETs appear as small halos in **Figure 8**;

however, these are more difficult to visualize. In contrast, neutrophils that have yet to enter the later stages of NETosis or remain quiescent appear as white spots in **Figure 8**. These data provide visual corroboration of quantitative data obtained thus far, as they show that NETosis is occurring to a greater extent in neutrophils stimulated with motile WT *P. aeruginosa* than in those stimulated with non-motile *P. aeruginosa* (Δmot LMP16, Δmot LMP50, and $\Delta flgC1$).

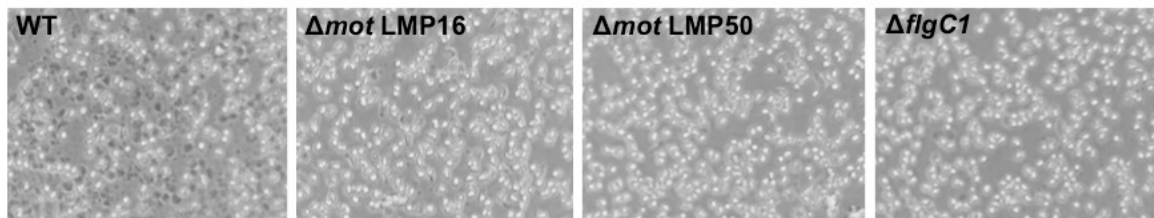


FIGURE 8. Large scale morphological changes are only seen in neutrophils treated with WT *Pseudomonas aeruginosa*, not with motility- and flagellum-deficient mutants. Healthy human neutrophils were cultured and stimulated as described in Figure 5A. All bacterial treatments were done at 10 MOI. Cells were photographed (with an iPhone 5S) at 20x with a light microscope after incubating for 4 hours at 37°C and prior to treatment with DNase1. Neutrophils undergoing the final stages of NETosis are seen as black, while quiescent neutrophils and those undergoing only the beginning stages of NETosis are seen as white. (one representative experiment of $n = 10$ total experiments) [WT, PAK Wild-Type; Δmot LMP16 and Δmot LMP50, Motility-Deficient PAK Mutants; $\Delta flgC1$, Flagellum-Deficient PAK Mutant; MOI, Multiplicity of Infection]

BACTERIAL MOTILITY MAY CONTRIBUTE TO INCREASED RELEASE OF FREE MPO DURING NET FORMATION

We then used supernatants cultured as those pictured in **Figure 8** to determine release of free MPO by WT, motility-deficient (Δmot LMP16 and Δmot

LMP50), and flagellum-deficient ($\Delta flgC1$) *P. aeruginosa* during NET formation. Unfortunately, we did not determine MPO release by healthy human neutrophils stimulated by WT *P. aeruginosa* to be significantly greater than MPO released by neutrophils treated with motility- or flagellum-deficient mutant strains (**Figure 9**). However, future experimentation to generate a larger experimental *n* number, or an investigation into other free MPO release pathways, could generate more fruitful results – possibly pointing to a contribution of *P. aeruginosa*'s motility to NET formation. This will be imperative to future understanding of the role of free MPO in NET formation, since a trend can still be seen in the data, despite that trend not being statistically significant at this time.

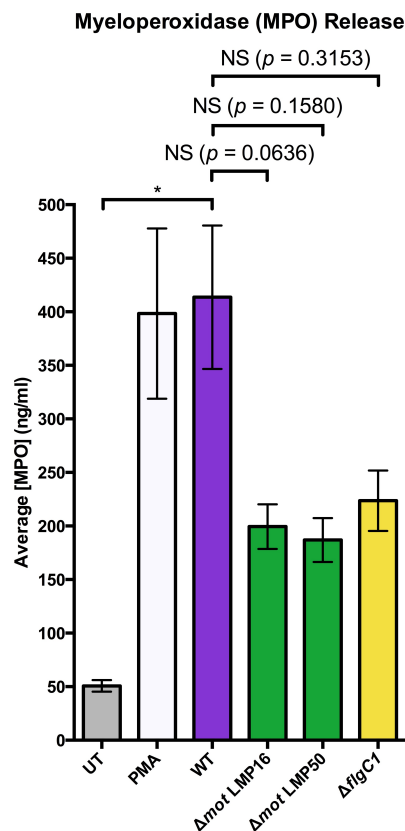


FIGURE 9. Motility of *Pseudomonas aeruginosa* does not serve as the only virulence determinant resulting in the release of free MPO. PAK WT

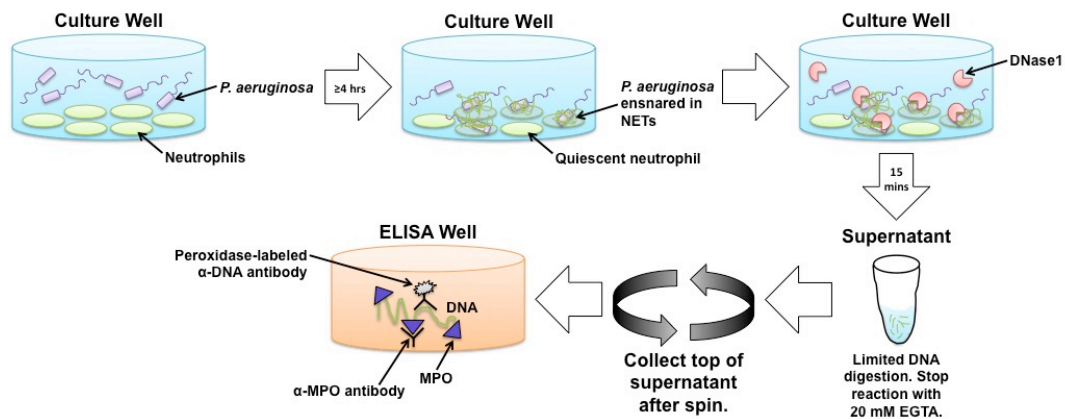
P. aeruginosa-treated healthy human neutrophils release more free MPO during NET formation than motility-deficient (Δmot LMP16 and Δmot LMP50) and flagellum-deficient $\Delta flgC1$ -stimulated human neutrophils. However, this difference is not statistically significant. Experiments were performed as illustrated in Figure 5A, with all bacterial treatments done at 10 MOI. 30 μ g/ml DNase1 was used to digest culture samples. ($n = 5$ experiments; mean $\pm$ SEM; one-way ANOVA; * means $p < 0.05$, and NS means no statistical significance) [UT, Untreated (HBSS); PMA, 100 nM Phorbol Myristate Acetate; WT, PAK Wild-Type; Δmot LMP16 and Δmot LMP50, Motility-Deficient PAK Mutants; $\Delta flgC1$, Flagellum-Deficient PAK Mutant; MPO, Myeloperoxidase; MOI, Multiplicity of Infection]

ESTABLISHING ASSAYS FOR DETECTING MPO COMPLEXED WITH EXTRACELLULAR DNA

We next aimed to quantitate NET release from *Pseudomonas aeruginosa*-stimulated human neutrophils. Current methods are not suitable to quantitate NETs in a reliable way. Quantitation of extracellular DNA is not NET-specific, and qualitative methods, such as immunofluorescence detection of MPO-DNA complexes, are difficult to objectively quantitate. Therefore, we established a NET-specific, quantitative ELISA assay to measure NETs (Yoo, Floyd, *et al.*, 2014). The NET complex we chose was MPO-DNA, schematically shown in **Figure 10A**. Notably, the MPO-DNA ELISA was previously described (Caudrillier *et al.*, 2012; Kessenbrock *et al.*, 2009) and modified for our purposes. In the *Immunology Letters* paper published by our lab, we provide data regarding optimization of this DNA complex ELISA assay (Yoo, Floyd, *et al.*, 2014). Part of

my contribution to the paper involved optimization of DNA digestion mediated by DNase1 in supernatants cultured and collected as shown in **Figure 10A**. Because phorbol myristate acetate (PMA) has already been shown to incite massive quantities of NET formation (**Figures 3B, 4, 7, and 9**), we used it as our NET-inducer for supernatants collected for our DNA gels. The representative DNA gel depicted in **Figure 10B** shows DNA bands for PMA-treated neutrophil supernatants digested with a range of DNase1 concentrations (0, 0.3, 1, 3, and 10 $\mu\text{g/ml}$), of which 1 $\mu\text{g/ml}$ proved to be the best concentration. This holds true in corresponding HNE-DNA ELISA data obtained from the same supernatants used in the DNA gel assay (Yoo, Floyd, *et al.*, 2014). Hence, we chose a 1 $\mu\text{g/ml}$ DNase1 concentration to use with all DNA complex ELISAs going forward. Also, untreated (UT) neutrophil supernatants produce no DNA bands (**Figure 10B**), supporting the idea that extracellular DNA is only released upon addition of a NET-inducing stimulant.

(A)



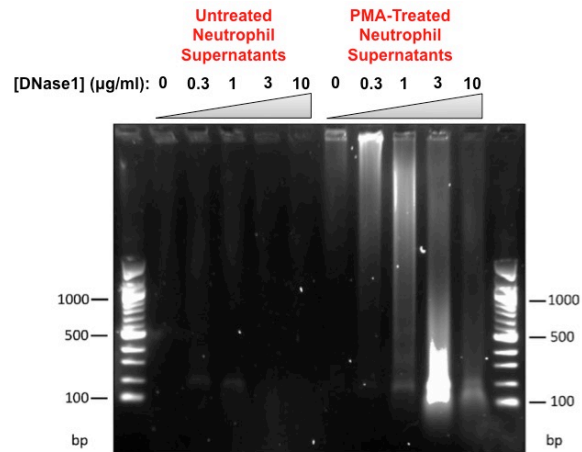
(B)

FIGURE 10. Optimization of DNA digestion by DNase1 for the MPO-DNA “NET” ELISA protocol. (A) Experimental scheme for MPO-DNA ELISAs portrayed in Figure 11. 1 µg/ml DNase1 was used to digest culture samples for both ELISAs. All bacterial stimulations were done at 10 MOI. **(B)** A dose-dependent effect of DNase1 of NET DNA is observed. DNase1 concentrations including 0, 0.1, 3, 1, and 10 µg/ml were used to digest NETs spewed by healthy human neutrophils either left untreated or stimulated with 100 nM PMA for 4 hours. The representative 0.8% agarose DNA gel shows 1 µg/ml as the optimal DNase1 concentration for limited digestion of NET DNA. ($n = 6$ experiments, with one representative shown) [MPO, Myeloperoxidase; HNE, Human Neutrophil Elastase; PMA, 100 nM Phorbol Myristate Acetate; MOI, Multiplicity of Infection]

BACTERIAL MOTILITY CONTRIBUTES TO INCREASED MPO-DNA NET COMPLEX RELEASE

Having optimized the MPO-DNA ELISA protocol based on preliminary data presented in **Figure 10**, we pursued MPO-DNA ELISAs with supernatants of healthy human neutrophils stimulated with WT, motility-deficient (Δmot LMP16 and Δmot LMP50), and flagellum-deficient ($\Delta flgC1$) *P. aeruginosa*. **Figure 11** shows that MPO-DNA release triggered by motility-deficient PAK strains is

significantly lower than that of the WT strain. Although the difference between the flagellum-deficient and WT strains did not demonstrate a statistically significant difference, the p value (0.0535) is very close to the $p < 0.05$ standard of significance. This shows that lacking a flagellum, similar to the effect of lacking a functional flagellar motor (Δmot LMP16 and Δmot LMP50) impairs the ability of *P. aeruginosa* to induce NET formation (**Figure 11**). These data are in line with those assessing extracellular DNA release (**Figure 3B**) and superoxide production (**Figure 7A**).

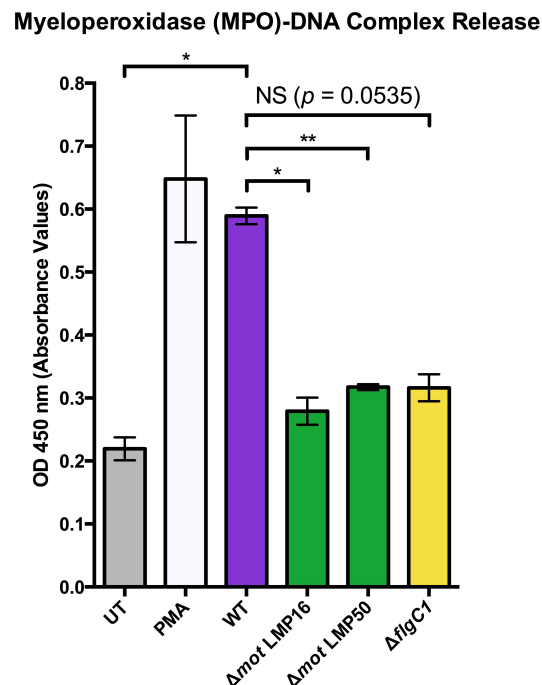


FIGURE 11. Motility of *Pseudomonas aeruginosa* significantly contributes to MPO-DNA NET complex release. PAK WT *P. aeruginosa*-treated healthy human neutrophils release larger numbers of MPO-DNA NET complexes than motility-deficient (Δmot LMP16 and Δmot LMP50) and flagellum-deficient $\Delta flgC1$ -stimulated human neutrophils. However, this difference is not statistically significant in either case. Experiments were performed as illustrated in Figure

10A. All bacterial stimulations were done at 10 MOI. 1 μ g/ml DNase1 was used to digest culture samples. ($n = 3$ experiments; mean $\pm$ SEM; one-way ANOVA; ** means $p < 0.01$, * means $p < 0.05$, and NS means no statistical significance) [MPO-DNA, Myeloperoxidase-DNA NET Complex; UT, Untreated; PMA, 100 nM Phorbol Myristate Acetate; WT, PAK Wild-Type; Δmot LMP16 and Δmot LMP50, Motility-Deficient PAK Mutants; $\Delta flgC1$, Flagellum-Deficient PAK Mutant; MOI, Multiplicity of Infection]

CHAPTER 3

RESULTS: PART 2: TOLL-LIKE RECEPTOR (TLR) SIGNALING THROUGH MYD88 DOES NOT MEDIATE *PSEUDOMONAS AERUGINOSA*-STIMULATED NETOSIS

VISUAL CONFIRMATION OF *PSEUDOMONAS AERUGINOSA*-INDUCED NET FORMATION IN THE MURINE SYSTEM

To test if the flagellum contributes to *Pseudomonas aeruginosa*-induced neutrophil extracellular trap (NET) formation via myeloid differentiation primary response 88 (MyD88), we aimed to compare NET formation in wild-type (WT) and MyD88-deficient (MyD88^{-/-}) murine neutrophils stimulated by *P. aeruginosa*. Once we confirmed NET formation in response to *P. aeruginosa* in the human system, we first sought similar confirmation in the murine system. Murine neutrophils were isolated from the bone marrow of the tibia and femur of each back leg. Other researchers have shown that murine neutrophils, like human neutrophils, are able to spew NETs in response to a variety of stimuli but have not yet been tested with *P. aeruginosa* (Ermert *et al.*, 2009; Kaplan and Radic, 2012). We show that stimulation of WT murine neutrophils with WT *P. aeruginosa* results in NET formation, demarcated by DNA and MPO staining (Figure 12).

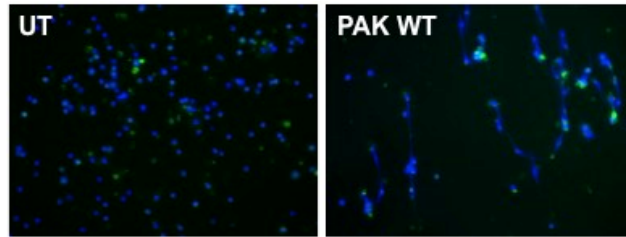
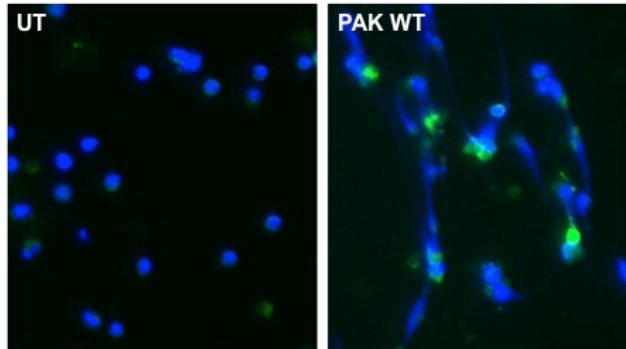
(A)**(B)**

FIGURE 12. *Pseudomonas aeruginosa*-induced NET formation confirmed visually with DNA and MPO staining of murine neutrophils. (A) Wild-type murine neutrophils were stimulated as described in the figure (10 MOI bacteria) and incubated at 37°C for an average of 5 hours. Staining for DNA (**blue**) and MPO (**green**) is described in the Materials and Methods (Chapter 5). Images were taken at 20x magnification with a Zeiss AxioCam HRM fluorescent microscope and analyzed with Axioplan2 imaging software. (*n* = 3 experiments, one representative shown) **(B)** Zoomed-in insets of images presented in (A). [UT, Untreated (HBSS); WT, PAK Wild-Type; MPO, Myeloperoxidase; MOI, Multiplicity of Infection]

QUANTITATIVE SUBSTANTIATION OF NET FORMATION TRIGGERED BY *PSEUDOMONAS AERUGINOSA* IN MURINE NEUTROPHILS

Once we visualized NET formation in the murine system, we sought to quantify it via an extracellular DNA release assay (**Figure 13**). Through an

experiment with solely wild-type murine neutrophils, we initially demonstrate that WT *P. aeruginosa* triggers more extracellular DNA release than its motility-deficient (Δmot LMP16 and Δmot LMP50) and flagellum-deficient ($\Delta flgC1$) counterparts (**Figure 13**). However, more repetitions need to be completed in the future before any distinguished conclusions can be made.

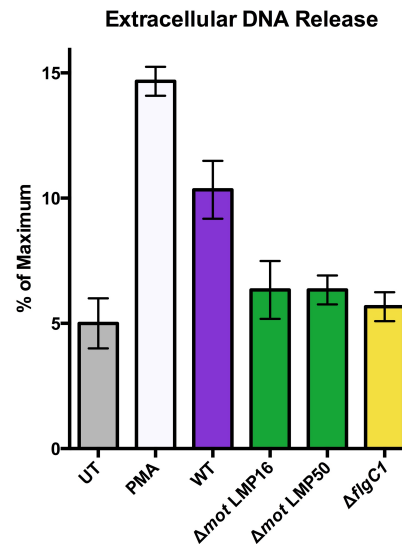


FIGURE 13. Motile *Pseudomonas aeruginosa* induces more extracellular DNA release than its non-motile counterparts in an assay with wild-type murine neutrophils. Extracellular DNA release from wild-type murine neutrophils stimulated as described by the x-axis (10 MOI bacteria) was detected with Sytox Orange, a membrane impermeable dye. Murine neutrophils stimulated with PAK WT *P. aeruginosa* release more extracellular DNA than those stimulated with motility-deficient Δmot LMP16 or Δmot LMP50, as well as flagellum-deficient PAK $\Delta flgC1$. Data shown represent signals generated over a 2-hour time period. % of maximum refers to maximal signal induced by saponin, allowing experiments performed over several days to be compared. ($n = 1$ experiment) [UT, Untreated (HBSS); PMA, 1 μ M Phorbol Myristate Acetate; WT, PAK Wild-Type; Δmot LMP16 and Δmot LMP50, Motility-Deficient PAK Mutants; $\Delta flgC1$, Flagellum-Deficient PAK Mutant]

***PSEUDOMONAS AERUGINOSA*-TRIGGERED NET FORMATION IS INDEPENDENT OF FLAGELLIN-BINDING RECEPTORS TLR5 AND NLRC4**

Once we established that *P. aeruginosa* triggers extracellular DNA release from wild-type murine neutrophils, we tested the contributions of known flagellum-binding receptors to *P. aeruginosa*-stimulated NET formation: Toll-like receptor (TLR) 5, an extracellular flagellin receptor, and NOD-like receptor caspase-associated recruitment domain (CARD)-containing protein 4 (NLRC4), known to interact with caspase-1 to induce apoptosis (Denes *et al.*, 2015). We investigated NLRC4 because it has been shown by our collaborators to contribute to bacterial recognition in *in vivo* murine models (Zhang *et al.*, 2014). We received TLR5/NLRC4^{-/-} mice from Dr. Andrew Gewirtz at Georgia State University. Our extracellular DNA release assay shows no significant difference in signals for WT and double knockout mice (**Figure 14**), indicative of TLR5 and NLRC4 not majorly contributing to NETosis signaling in murine neutrophils.

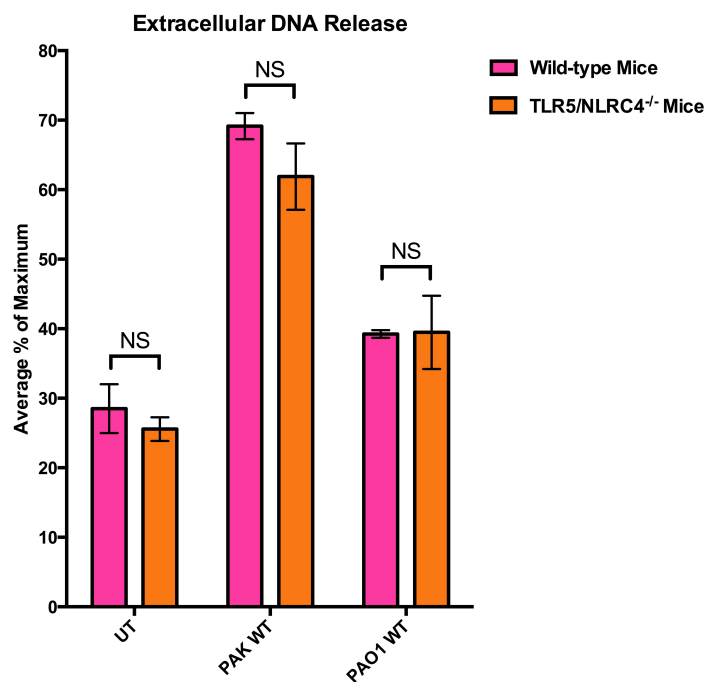


FIGURE 14. Major flagellin-binding receptors TLR5 and NLRC4 are not involved in the *Pseudomonas aeruginosa*-induced NETosis triggering mechanism. Extracellular DNA release from WT and TLR5/NLRC4 double knockout murine neutrophils stimulated as described by the x-axis was detected with Sytox Orange, a membrane impermeable dye. WT and TLR5/NLRC4^{-/-} murine neutrophils stimulated with 10 MOI PAK or PAO1 WT *P. aeruginosa* release similar amounts of extracellular DNA. Data shown represent signals generated over a 2-hour time period. % of maximum refers to maximal signal induced by saponin, allowing experiments performed over several days to be compared. (*n* = 4 experiments; mean±SEM; one-way ANOVA; NS means no statistical significance) [UT, Untreated (HBSS); WT, PAK or PAO1 Wild-Type; TLR5, Toll-Like Receptor 5; NLRC4, NOD-Like Receptor Caspase Associated Recruitment Domain (CARD)-Containing Protein 4]

TLRs 1, 2, 4, 5, 7, AND 9, WHICH SIGNAL THROUGH MYD88, ARE NOT PART OF THE *PSEUDOMONAS AERUGINOSA*-TRIGGERED NETOSIS SIGNALING PATHWAY

Because TLRs are widely reported as being involved with bacterial recognition, we employed a myeloid differentiation primary response gene 88 (MyD88)^{-/-} murine model obtained from collaborators. By knocking out MyD88 signaling, we effectively knocked out the signaling pathway for TLRs 1, 2, 4, 5, 7, and 9, allowing us to investigate the role of these receptors in the NETosis signaling pathway. Our results demonstrate that these TLRs are not the chief receptors involved in NETosis signaling, since WT and MyD88^{-/-} murine neutrophils stimulated with WT *P. aeruginosa* generated signals deemed not significantly different from one another (**Figure 15**).

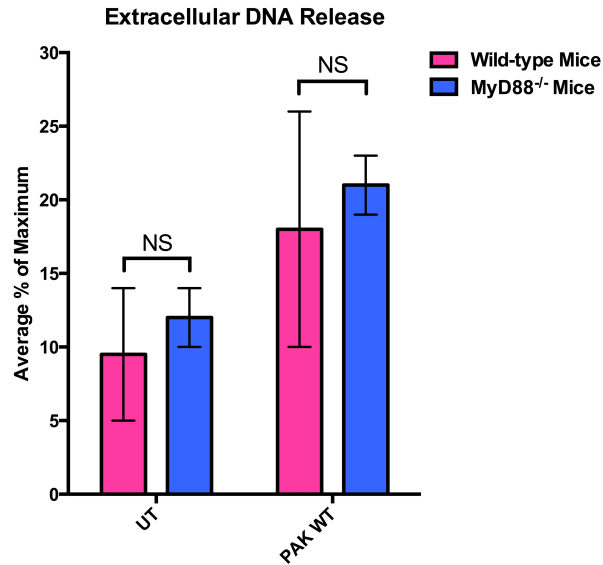


FIGURE 15. TLRs that signal through MyD88 are not involved in the *Pseudomonas aeruginosa*-induced NETosis signaling pathway. Extracellular DNA release from WT and MyD88 knockout murine neutrophils stimulated as described by the x-axis was detected with Sytox Orange, a membrane impermeable dye. WT and MyD88^{-/-} murine neutrophils stimulated with 10 MOI PAK WT *P. aeruginosa* release similar amounts of extracellular DNA. Data shown represent signals generated over a 2-hour time period. % of maximum refers to maximal signal induced by saponin, allowing experiments performed over several days to be compared. ($n = 2$ experiments; mean $\pm$ SEM; one-way ANOVA; NS means no statistical significance) [UT, Untreated (HBSS); WT, PAK Wild-Type; MyD88, Myeloid Differentiation Primary Response 88]

CHAPTER 4

DISCUSSION AND CONCLUSION

WHY IS *PSEUDOMONAS AERUGINOSA* MOVEMENT IMPORTANT?

Unpublished data from our lab involves extracellular DNA release assays in which Toll-like receptor (TLR) 5 neutralizing antibodies were pre-incubated with human neutrophils prior to stimulation with *Pseudomonas aeruginosa* (not shown). These results demonstrated that a major drop in extracellular DNA release (as compared to neutrophils left untreated by neutralizing antibodies) is, in fact, not seen following use of TLR5 neutralizing antibodies, as would be expected if TLR5 were the major receptor in the NETosis signaling pathway (not shown). Such surprising results prompted us to delve deeper into the contribution of bacterial movement, specifically that of *P. aeruginosa*, to the signaling mechanism of neutrophil extracellular trap (NET) formation, decidedly not largely occurring through the more predictable TLR5 route.

Bacterial movement is an important aspect of cystic fibrosis (CF) lung disease because variable flagellum expression is seen in clinical strains of *P. aeruginosa* (Mahenthiralingam *et al.*, 1994; Jain and Kazmierczak, 2014). Its expression is balanced differently depending on the stage of infection. For example, flagellar motility fosters dissemination, colonization, and biofilm formation – all of which are important factors during the first stages of infection

(Jain and Kazmierczak, 2014). On the other hand, bacteria want to avoid any unnecessary expression, since flagellin – the protein component of flagella – is a powerful antigen recognized by receptors such as TLR5 and NLRC4 (Jain and Kazmierczak, 2014). As such, bacteria are likely to downregulate flagella expression in later infection stages, except when promoting further spread and colonization. *P. aeruginosa* can exhibit three types of movement: swimming (flagella-dependent), twitching (dependent on the type IV pillus), and swarming (both flagella- and type IV pillus-dependent) (Jain and Kazmierczak, 2014). Flagella responsible for swimming motility are well recognized by the host immune system, and, as we established in our own data, also contribute to NET formation through an unknown mechanism related to their motility function (Jain and Kazmierczak, 2014). As such, flagella expression necessary for initial acute infection by *P. aeruginosa* is often downregulated to promote persistence by *P. aeruginosa* in a chronic infection setting (Jain and Kazmierczak, 2014; Mahenthiralingam *et al.*, 1994).

Together, previous research and our own work establish clinical relevance for the study of swimming motility, motility deficiency, and flagellum deficiency of *P. aeruginosa* by confirming that downregulated flagella expression is a way to evade both phagocytosis and NET formation in the host, which is critical to survival of *P. aeruginosa* during chronic infection, such as in the case of CF.

NET FORMATION OCCURS IN RESPONSE TO MOTILE *PSEUDOMONAS AERUGINOSA* IN BOTH HUMAN AND MURINE SYSTEMS

Swimming motility of *Pseudomonas aeruginosa* was previously correlated with bacterial virulence in both murine and human systems (Ledizet *et al.*, 2012; Feldman *et al.*, 1998; Montie *et al.*, 1982). We sought to add to this field by specifically focusing on motility as a factor distinct from flagellin protein presence, and future experimentation in our lab will continue this work. Our study demonstrated that the swimming motility of *P. aeruginosa* – characterized with a swimming motility assay (**Figure 6**) – had a significant impact, both qualitatively and quantitatively, on NET formation in an *in vitro* human system. Specifically, only *P. aeruginosa* strains with both a flagellum and a functional flagellar motor exhibited swimming motility (**Figure 6**). This extends beyond data previously collected by our lab, which demonstrated that wild-type (WT) *P. aeruginosa* is able to trigger NET formation (Yoo, Winn, *et al.*, 2014). Furthermore, we showed that motile *P. aeruginosa* evoked NET formation in several *in vitro* murine models (**Figures 14 and 15**), albeit to a lesser extent than seen in the human system (**Figure 7B**). This is in line with murine neutrophil work describing NET formation stimulated through other types of infections, such as bacterial pneumonia caused by *Streptococcus pneumoniae* (Moorthy *et al.*, 2013).

To validate our quantitative data, we visualized NET formation with immunofluorescence imaging of human (**Figure 4**) and murine (**Figure 12**) neutrophils, which illustrated that both cell types engaged NET formation upon treatment with motile wild-type (WT) *P. aeruginosa*. Moreover, human neutrophils

failed to initiate similar NET formation following treatment with flagellum-deficient *P. aeruginosa* (**Figure 4**), which is further supported with light microscopy images exemplifying morphological changes of neutrophils stimulated with *P. aeruginosa* (**Figure 8**). Thus, we were able to visually confirm the occurrence of NET formation in response to *P. aeruginosa* for both systems presented in this study, as well as the specific importance of bacterial motility in NETosis within the human system. Confidence in our presented quantitative data is supported by these qualitative results. We propose – based on results shown here, as well as preliminary data obtained by our lab and others – that the swimming motility of *P. aeruginosa* serves as its primary virulence determinant regarding triggering of NET formation (Ledizet *et al.*, 2012; Feldman *et al.*, 1998; Montie *et al.*, 1982).

NEUTROPHIL-MEDIATED ATTACK, SPECIFICALLY NET FORMATION, OF MOTILE *PSEUDOMONAS AERUGINOSA*

Before we could focus in on motility as a bacterial virulence factor, we first sought to establish neutrophil-mediated attack against *Pseudomonas aeruginosa* occurs *in vitro*, which we had already initiated in our lab's previous studies (Yoo, Winn, *et al.*, 2014). To do this, we looked at superoxide production, which is traditionally a major sign of neutrophil-mediated oxidative burst. Our results showed significantly more superoxide production by neutrophils exposed to motile wild-type (WT) *P. aeruginosa* than those stimulated by motility- or flagellum-deficient *P. aeruginosa* (**Figure 7A**). This suggested that swimming motility – not solely the presence of flagellin protein – was necessary to trigger

markedly higher levels of superoxide production by human neutrophils. We then went on to confirm that NETosis is the most probable attack mechanism with extracellular DNA release assays, in which neutrophils spewed significantly more NETs in response to motile WT *P. aeruginosa* than in reaction to motility- or flagellum-deficient *P. aeruginosa* (**Figure 7B**). Having established that motility plays a significant role in the extracellular DNA aspect of NET formation, we next sought to quantify known NET complex components myeloperoxidase (MPO) and human neutrophil elastase (HNE) in our human *in vitro* system.

MORE RESEARCH TO BE DONE WITH FREE MPO AND HNE, AS WELL AS MPO-DNA COMPLEX, RELEASE DURING NETOSIS TRIGGERED BY *PSEUDOMONAS AERUGINOSA*

Because myeloperoxidase (MPO) and human neutrophil elastase (HNE) are major components of NET formation, we studied their release – both in free form and complexed with DNA – in response to treatment with *Pseudomonas aeruginosa*. This work was based on previous studies by our lab in which both laboratory and clinical strains of *P. aeruginosa* were shown to induce significantly greater MPO, HNE, MPO-DNA, and HNE-DNA release compared to untreated samples (Yoo, Winn, *et al.*, 2014; Yoo, Floyd, *et al.*, 2014). In this study, we reported a trend toward more free MPO and HNE release following stimulation with motile wild-type (WT) *P. aeruginosa* than flagellum-deficient *P. aeruginosa* (**Figure 9**). While this difference was not deemed statistically significant, it is

likely biologically significant. This trend was again observed in assays testing for MPO-DNA NET complex release (**Figure 11**) in response to *P. aeruginosa*.

At this time, it is difficult to conclude the precise role of these components strictly regarding motility-based triggering of NETosis, leaving an opportune gap in knowledge for future studies to fill. In such studies, researchers should consider phagosomal killing, as well as the effects of MPO and HNE on the bacteria present in the assays, specifically regarding bacterial motility. Equally important in this endeavor will be research determining the precise signaling mechanism of NETosis, which should help shed light on the origin of MPO, HNE, and MPO-DNA measured in studies such as this one.

MYD88 SIGNALING IS NOT INVOLVED IN NET FORMATION IN RESPONSE TO *PSEUDOMONAS AERUGINOSA*

In pursuit of the NETosis signaling mechanism, we first targeted Toll-like receptor (TLR) 5, the major receptor known to recognize bacterial flagellin, as well as its less researched counterpart NOD-like receptor caspase associated recruitment domain (CARD)-containing protein 4 (NLRC4). We demonstrated that presence or absence of TLR5 and NLRC4 – generated through murine double knockout models – had no statistically significant effect on the ability of neutrophils to NETose in response to motile wild-type (WT) *Pseudomonas aeruginosa* (**Figure 14**). This was surprising, given the firm knowledge of TLR5's involvement in flagellin recognition (Rieber *et al.*, 2014; Williams *et al.*, 2010; Blohmke *et al.*, 2008; Koller *et al.*, 2008; Ramphal *et al.*, 2008; Skerrett *et al.*,

2006; Feuillet *et al.*, 2006); however, this stands to possibly support the idea that it is bacterial motility, not strictly the presence of flagellin, that promotes NET formation.

We then went on to test our hypothesis that TLRs signaling through MyD88 play a role in the *P. aeruginosa*-induced NETosis triggering mechanism. This was based on the rationale that TLRs are widely known for their recognition of virulence factors produced by many pathogens, as well as research demonstrating that MyD88^{-/-} mice inoculated with *P. aeruginosa* die swiftly due to an inability to mount an adequate host immune response (Skerrett *et al.*, 2006; Ramphal *et al.*, 2008). Our results, however, showed that presence or absence of MyD88 – generated through murine knockout models – had no statistically significant effect on NET formation by said neutrophils following stimulation with motile WT *P. aeruginosa* (**Figure 15**). This is in line with the idea that no single receptor is likely uniquely responsible for triggering NETosis. Specifically, current literature indicates that microbes elicit significantly greater NET production than simple agonists of single receptors, such as Toll-like receptors (TLRs), Fc receptors, and cytokine receptors; this suggests that simultaneous stimulation of multiple neutrophilic receptors is necessary to trigger NET formation (Brinkmann and Zychlinsky, 2007). In future studies, these receptors should be tested in a variety of combinations in order to determine which ones cooperate to generate synergistic effects resulting from bacterial recognition and killing through NET formation. The best approach for this will likely involve studying the interplay between the major receptor types, such as TLRs, NOD-like receptors (NLRs), Fc

receptors, complement receptors, and cytokine receptors. Only by analyzing interactions between many different types of receptors will we discover the true NETosis signaling mechanism, which could present potential targets for limiting, or, better yet, controlling NET formation believed to be responsible for lung tissue damage in cystic fibrosis (CF) patients.

CONCLUDING REMARKS

Our study investigated the contribution to NET formation of swimming motility powered by the flagellum of *Pseudomonas aeruginosa*, as well as the role of MyD88 signaling in the NETosis triggering mechanism. Based on our results, we propose that an unknown combination of receptors cooperate in their recognition of *P. aeruginosa* to synergistically trigger NET formation (**Figure 16**). Furthermore, it is our belief, based on research previously done in our lab where contact of *P. aeruginosa* with neutrophils was forced by centrifugation (not shown), that bacterial swimming motility increases contact of *P. aeruginosa* with neutrophils, thereby serving as an important factor in the triggering of NETosis.

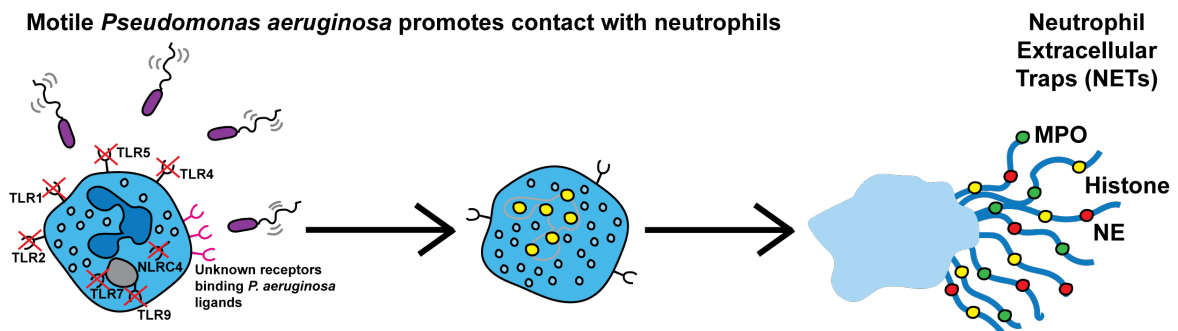


FIGURE 16. A graphical scheme of our results regarding motility-based triggering of NET formation by *Pseudomonas aeruginosa*. Our results collectively show which receptors are not singly involved in NETosis signaling.

However, other types of receptors – such as Fc, complement, NOD-like, and cytokine receptors – should also be tested in the future for their involvement in NETosis signaling. Additionally, comprehensive studies should look at multiple receptors – knocked out (in the murine system) or neutralized (in the human system) – to sufficiently determine synergistic effects of bacterial recognition. Figure designed in Adobe Illustrator by Madison Floyd. [TLR, Toll-Like Receptor; NLRC4, NOD-Like Receptor Caspase Associated Recruitment Domain (CARD)-Containing Protein 4; NE, Neutrophil Elastase; MPO, Myeloperoxidase; NETs, Neutrophil Extracellular Traps]

The research presented here represents the first stepping-stones toward novel therapeutic approaches for treating the negative NET-mediated effects seen in CF, such as lung tissue damage. Much remains to be discovered about the signaling pathway of NETosis, but receptors involved will likely make excellent therapeutic targets for controlling and/or limiting NET formation in CF airways. Most importantly, our data point toward the flagellum-powered swimming motility of *P. aeruginosa* as the main virulence determinant in NET formation. As such, the flagellar motor represents a potential novel therapeutic target for combatting excessive NET formation in CF airways. However, further research needs to be done regarding the detriment, if any, presented by limiting NET formation in the fight against chronic infection by *P. aeruginosa*. All in all, the potential ability to limit lung tissue damage in CF through targeting the motility of *P. aeruginosa* should absolutely be pursued, as it will likely greatly increase the quality of life of CF patients around the globe.

CHAPTER 5

MATERIALS AND METHODS

HUMAN SUBJECTS STATEMENT

Healthy human blood donors were recruited as described by an IRB-approved protocol (University of Georgia, IRB 2012-10769-9); each subject provided written consent. The majority of subjects ranged from ages 18 to 25, corresponding with part of the age range widely accepted for CF patients whose lungs tend to be primarily infected with *Pseudomonas aeruginosa*. The research performed in this thesis is in compliance with the ethical guidelines for medical research involving human subjects of the Declaration of Helsinki.

HUMAN NEUTROPHIL PURIFICATION AND SERUM PREPARATION

Four tubes of peripheral blood were acquired from each healthy human donor; anti-coagulant was added to three tubes, and the fourth was allowed to coagulate for serum collection. Blood was processed shortly thereafter. Following straining of blood to remove any debris, red blood cells were extracted over a twenty minute period via dextran sedimentation of anti-coagulant-treated blood. The leukocyte-rich plasma phase was obtained and washed with phosphate buffered saline (PBS), then centrifuged at 450 g for 5 minutes. Supernatant was discarded, and the leukocyte-rich pellet was re-suspended in PBS. A second spin

was completed at 400 *g* for 8 minutes, and the leukocyte-rich pellet was similarly re-suspended in PBS. This was then added to the top of a five-step Percoll gradient comprised of 0.66 g/ml, 0.71 g/ml, 0.76 g/ml, 0.81 g/ml, and 0.86 g/ml (Sigma-Aldrich). Cells were spun at 800 *g* for 30 minutes, this time with brakes. Neutrophils were collected from the 71-76-81 (w/v) % Percoll layers, then washed with PBS and centrifuged at 450 *g* for 10 minutes. Supernatant was discarded, and a second wash and spin was executed in PBS at 300 *g* for 5 minutes. After disposing of the supernatant, a 1:1 mixture of autologous serum (500 μ l) and RPMI-1640 medium (Life Technologies) plus HEPES (500 μ l) was used to re-suspend the neutrophil-containing pellet. Neutrophil cell count viability were determined with use of Trypan blue dye. Neutrophils were stored at room temperature until use within hours of being isolated.

Autologous (donor) serum was isolated by first collecting any non-clotted blood from the coagulated tube of healthy human peripheral blood. Tubes were spun at 10,000 *g* for 5 minutes. Supernatants were collected and spun again at 10,000 *g* for 5 minutes. Resulting supernatants were obtained and subjected to sterile filtration. Serum was stored at 4°C.

ANIMAL SUBJECTS STATEMENT

Collaborators at the University of Georgia (UGA) provided wild-type C57BL/6 (Jackson Laboratory) and FVB1 background mice. Dr. Wendy Watford, a collaborator at UGA, kindly provided myD88-deficient mice. Mice deficient in both TLR5 and NLRC4 receptors were kindly gifted by Dr. Andrew T. Gewirtz

(Georgia State University, Atlanta, GA). Same day isolation of bone marrow-derived neutrophils was performed as described in Dr. Balázs Rada's protocol entitled "Investigating bone marrow-derived neutrophil functions *ex vivo*," which was approved by the Institutional Animal Care and Use Committee (IACUC) of UGA.

MURINE NEUTROPHIL PURIFICATION AND SERUM PREPARATION

Blood for serum preparation was obtained via cardiac puncture within five minutes of mice being sacrificed. Mice were dissected, and the femur and tibia of each back leg was obtained. Bones were cleaned in PBS, then stored in RPMI-1640 medium (Life Technologies) plus 1% Pen-Strep. Both sides of each bone were trimmed, and bone marrow was flushed with RPMI-1640 medium (Life Technologies). Cells were strained, and then spun at 300 *g* for 10 minutes. The supernatant was discarded, and 1 ml of ACK buffer (Biowhittaker) was added to lyse any red blood cells (RBCs) over a 30-second time period. Phosphate buffered saline (PBS) solution was added immediately after 30 seconds had passed in order to stop the ACK buffer reaction. Cells were spun at 300 *g* for 10 minutes, after which the supernatant was discarded, the pellet was re-suspended in PBS, and another spin at 300 *g* for 10 minutes was completed. Pelleted cells were re-suspended in PBS. This was then added to the top of a two-step Percoll gradient comprised of 81% and 62% Percoll (Sigma-Aldrich). Cells were spun at 1,600 *g* for 30 minutes, this time with brakes. Neutrophils were collected from the 81%/62% Percoll interface, washed in PBS, and spun at 450 *g* for 10 minutes.

The supernatant was discarded, after which the pellet was re-suspended in PBS and spun again at 450 *g* for 10 minutes. After disposing of the supernatant, 500 μ l of MAC buffer (0.5% BSA in PBS, with 2 mM EDTA) was used to re-suspend the neutrophil-containing pellet. Neutrophil cell count and viability were determined with use of Trypan blue dye. Neutrophils were stored at room temperature until use within hours of being isolated. The purity of neutrophil preparations was assessed by surface staining of Gr-1 neutrophil marker by flow cytometry. Neutrophil preparations contained greater than 80% neutrophils, on average.

Murine serum was isolated by spinning coagulated blood at 10,000 *g* for 5 minutes. Supernatants were collected and spun again at 10,000 *g* for 5 minutes. Resulting supernatants were obtained. Serum was stored at 4°C.

***PSEUDOMONAS AERUGINOSA* STRAINS**

PAK background *Pseudomonas aeruginosa* strains were acquired through Dr. Linda McCarter at the University of Iowa. Wild-type (WT), motility-deficient $\Delta motAB4::Gen^r$ $motD2::Tet^r$ (Mot⁽⁻⁾ PAK LMP16), motility-deficient $\Delta motB3::Gen^r$ $motCD8::Tet^r$ (Mot⁽⁻⁾ PAK LMP50), and flagellum-deficient $\Delta flgC1::Tn5$ (Tet^r) (Fla⁽⁻⁾ PAK LMP57) strains were provided (Doyle *et al.*, 2004). Flagellin protein was found to be present in all but the flagellum-deficient mutant strain, as expected (data not shown; Doyle *et al.*, 2004). Deletions were made through use of gentamicin-resistant (Gen^r) and tetracycline-resistant (Tet^r) vectors, and strains were confirmed with growth on antibiotic-containing agar. Strains were

frozen in DMSO at -80°C. Immunofluorescence staining presented in Aim 1 and Aim 2, as well as extracellular DNA assays completed with TLR5/NLRC4 and MyD88 knock out murine neutrophils in Aim 2, was accomplished using PAK strains grown on *Pseudomonas aeruginosa*-specific agar plates. All other assays were done with PAK strains grown in Luria-Bertani (LB) broth. Strains were grown overnight for an average of 16-18 hours at 37°C.

PAO1 background *Pseudomonas aeruginosa* strains were stored at -80°C and included wild-type (WT) and flagellum-deficient $\Delta fliC$ (Fla⁽⁻⁾ PAO1). PAO1 strains for MPO and HNE ELISAs were cultured on *Pseudomonas aeruginosa*-specific agar plates overnight for an average of 16-18 hours at 37°C.

For all assays, bacteria were washed twice in HBSS (Mediatech) and spun at 10,000 *g* for 5 minutes. Bacterial concentration was then set at 10⁹/ml in 200 μ l (OD 0.6) by measuring absorbance at 600 nm with a BioTek Inc. Eon microplate spectrophotometer.

SWIMMING MOTILITY

Bacteria were cultured as previously described. 0.3% agar in Luria-Bertani (LB) broth was used to generate swimming motility agar plates. Plates were allowed to cool for 45 minutes before being stabbed with OD 0.6 bacterial samples diluted 1:100 in HBSS (Mediatech). Motility and growth occurred over a 48-hour time frame before colony sizes were measured in millimeters (mm). Duplicates were implemented to account for any variability in the assay.

EXTRACELLULAR DNA RELEASE

Human and murine neutrophils were washed and spun twice in HBSS (Mediatech) at 450 g. Cells were re-suspended in HBSS-based (Mediatech) concentrated media containing 20 mM HEPES, 10 mM glucose, 2% autologous (donor) serum, and 20 μ M Sytox Orange (a membrane-impermeable DNA-binding dye; Life Technologies). Human and murine neutrophils were seeded at 250,000 and 100,000 cells/well, respectively, in 50 μ l of media each in a poly D-lysine-coated 96-well black transparent-bottom microplates (Thermo Scientific). Cells were incubated at 37°C for 10-15 minutes, allowing time for neutrophils to adhere to the bottom of each well. Stimulants were added as 50 μ l each. Untreated samples consisted of adding HBSS. Phorbol myristate acetate (PMA) at 100 nM and 1 μ M concentrations for human and murine cells, respectively, was used as the positive control. 10 MOI *Pseudomonas aeruginosa* bacterial stimulations were employed. A maximal signal was generated with 10 mg/ml saponin (Sigma-Aldrich). Fluorescence (excited at 530 nm, emitted at 590 nm) was measured in a Varioskan Ascent (Thermo Scientific) fluorescence plate reader over 4-8 hours at 37°C. All signals were expressed as “% of maximum.” Quadruplicates or triplicates of sample treatments were done to account for any variability in the assay.

IMMUNOFLUORESCENCE STAINING AND MICROSCOPY

Bacteria were prepared as previously described, then pre-opsonized by treating 100 μ L of 10^9 /ml suspension with 10% autologous (donor) serum and

incubating at 37°C for 30 minutes. Human and murine neutrophils were washed and spun twice in HBSS (Mediatech) at 450 *g*. Cells were re-suspended in HBSS-based media comprised of 10 mM HEPES, 5 mM glucose, and 0.2% (for human cells) or 0.1% (for murine cells) autologous (donor) serum.

Coverslips were added to the bottoms of wells in a 24-well plate. Human neutrophils were added and incubated at 37°C for 15 minutes (allowing cells to adhere) before stimulants were added. Murine neutrophils and their corresponding stimulants were mixed in a microcentrifuge tube and periodically turned upside-down over the course of a 15-minute incubation at room temperature; this promoted contact between neutrophils and bacteria. These murine neutrophil and stimulant mixtures were then added to coverslips in a 24-well plate, as was similarly described for human neutrophils. 10 MOI bacterial stimulations were employed in all experiments. Phorbol myristate acetate (PMA) was used as a positive control in experiments with human neutrophils. HBSS (Mediatech) constituted untreated samples.

Cells and their corresponding stimulants were incubated on coverslips at 37°C for 3 hours or 5 hours, in the case of human and murine cells, respectively. Treatments were visually verified via light microscopy. Coverslips were removed from wells and added to parafilm wells generated on a test tube rack, where cells were fixed with 4% paraformaldehyde (PFA; Affymetrix) for 10 minutes at room temperature. Cells were washed with PBS for 5 minutes at room temperature. A 30-minute, room temperature blocking step followed, using a mixture of 5% bovine serum albumin (BSA), 5% normal donkey serum (NDS; Jackson

Immunoresearch Laboratories), and 0.05% Triton-X100. Cells were then treated overnight with primary antibodies mixed in media containing 1% BSA, 1% NDS (Jackson Immunoresearch Laboratories), and 0.05% Triton-X100 at 4°C. Human neutrophils were treated with MPO-7 monoclonal mouse anti-human MPO/FITC antibody (1:500, Dako) and polyclonal rabbit anti-histone H4 (citrulline 3, 1:1,000; Millipore). Murine neutrophils were treated with goat anti-mouse MPO antibody (1:1,000; R&D Systems) and anti-mouse IgG/FITC antibody (488 nm, 1:800; Life Technologies).

Next, cells were washed twice in PBS at room temperature for 5 minutes each. Human cells were then incubated with Alexa Fluor® 594-labeled donkey anti-rabbit secondary antibody (1:2,000 in PBS) against the histone primary antibody. Murine cells were treated with PBS alone. This treatment was done for 1 hour at room temperature, in the dark. Cells were then treated with DAPI (anti-DNA, 1:20,000; Molecular Probes) in PBS at room temperature for 2 minutes, followed by a single wash in PBS for 5 minutes at room temperature. Lastly, coverslips were somewhat dried, then mounted with ProLong Antifade (Molecular Probes) medium. Stained samples were analyzed by Zeiss AxioCam HRM fluorescence microscopy (Axioplan2 imaging software).

MPO, HNE AND MPO-DNA ELISAs

MPO supernatants were produced from 4.5-hour incubations of stimulated human neutrophils. Human neutrophils were washed and spun twice in HBSS (Mediatech) at 450 g. Cells were re-suspended in HBSS-based media comprised

of 10 mM HEPES, 5 mM glucose, and 0.2% autologous (donor) serum. All bacterial treatments were 10 MOI. 100 nm phorbol myristate acetate (PMA) was used as a positive control. Visual confirmation of NET formation was obtained via light microscopy. Wells were treated with 1 µg/ml or 30 µg/ml DNase1 (Roche) for MPO-DNA or MPO ELISAs, respectively; this treatment lasted 15 minutes. DNase1 activity was halted with 20 mM EGTA (Sigma-Aldrich) treatment. Supernatants were collected and spun down at 10,000 *g* for 10 minutes. The top 70 µl of every 120 µl (accounting for any pelleted debris) of supernatant was collected and stored at -80°C.

A commercial ELISA kit (R&D Systems) was used to measure MPO concentration in diluted (in PBS) human neutrophil supernatants. Preparation of a serially diluted standard was prepared from 125 ng/ml purified MPO; this standard was used to quantify MPO concentration in unknown samples. Graphs were generated with OD 450 nm absorbance values.

An ELISA was also used to measure HNE concentration. High-binding 96-well ELISA microplates (Greiner Bio-One) were coated with rabbit anti-HNE polyclonal (capture) antibody (1:2,000 in PBS; Calbiochem) and incubated overnight at 4°C. Plates were washed three times with 200 µl/well of 0.05% Tween20 in PBS, then blocked with 200 µl/well of 1% bovine serum albumin (BSA) at room temperature for 1 hour. Plates were then emptied but not washed. Supernatants were diluted in PBS and added to the plates. The standard was generated from 500 ng/ml purified HNE (1 mg/ml stock; CellSciences) and added to plates; this standard was used to quantify HNE concentration in unknown

samples. All samples were incubated overnight at 4°C. Plates were washed three times with 200 µl/well of 0.05% Tween20 in PBS, then coated with mouse anti-HNE (detection) antibody (1:1,000 in PBS; Calbiochem) and incubated at room temperature for 2 hours, in the dark. Plates were washed three times with 200 µl/well of 0.05% Tween20 in PBS, then coated with horseradish peroxidase (HRP)-linked donkey anti-mouse antibody (1:2,000; GE Healthcare) and incubated at room temperature for 30 minutes, in the dark. Plates were washed four times with 200 µl/well of 0.05% Tween20 in PBS. 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific) peroxidase substrate solution was employed to generate blue coloring from bound HRP antibody. The reaction was stopped with equal addition of 1 N hydrochloric acid (HCl; Sigma-Aldrich). Absorption values were read at 450 nm with a BioTeck Inc. Eon microplate spectrophotometer. Graphs were generated with OD 450 nm absorbance values.

MPO-DNA ELISA plates were coated with rabbit anti-MPO (1:2,000; Upstate) capture antibody; this was incubated overnight at 4°C. Plates were washed three times with 200 µl/well of 0.05% Tween20 in PBS, then blocked with 200 µl/well 5% BSA (containing 0.1% human albumin) in PBS at room temperature for a minimum of 2 hours. Next, plates were washed three more times with 200 µl/well of 0.05% Tween20 in PBS, then coated with HRP-conjugated mouse anti-DNA-POD secondary antibody (1:500; Roche) and incubated at room temperature for 30 minutes, in the dark. Plates were again washed three times with 200 µl/well of 0.05% Tween20 in PBS before adding TMB substrate (Thermo Scientific). Absorption values were read for 30 minutes

at 650 nm with a BioTeck Inc. Eon microplate spectrophotometer, after which an equal amount of 1 N HCl solution was added to stop the reaction. Absorption values were then read again, this time at 450 nm. Graphs were generated with OD 450 nm absorbance values.

SUPEROXIDE PRODUCTION

Human neutrophils were washed and spun twice in HBSS (Mediatech) at 450 g. Cells were re-suspended in HBSS-based media comprised of 10 mM HEPES, 5 mM glucose, and 1% autologous (donor) serum before being stimulated. All bacterial treatments were 10 MOI. 100 nM phorbol myristate acetate (PMA) was used as a positive control.

A Diogenes superoxide-specific chemiluminescence assay kit (National Diagnostics) was used to measure superoxide production. Chemiluminescence was measured every 30 seconds over 90-120 minutes in a 96-well white clear-bottom plate using a Varioskan microplate luminometer (Thermo Scientific). Integrated luminescence values were calculated and graphed as integrated relative luminescence units (RLUs) over time.

DNA GEL ELECTROPHORESIS

Human neutrophils were seeded at 250,000 cells/well in a poly D-lysine-coated 96-well plate, then stimulated with either HBSS (Mediatech) for the untreated control or phorbol myristate acetate (PMA). After a 4-hour incubation, DNase1 (Roche) treatments of 0, 0.3, 1, 3, and 10 µg/ml concentrations for each

condition (i.e., untreated or PMA) were completed for 15 minutes at room temperature. 20 mM EGTA (Sigma-Aldrich) in PBS was added to stop the DNase1 reaction, thereby ensuring limited DNA digestion. Supernatants were collected, spun down, and mixed with 10x loading buffer. These samples were then analyzed at 140 volts (V) via agarose DNA gel electrophoresis [0.8% agarose, Gel Start DNA-detecting dye (Lonza)]. Gels were imaged with Gel-Doc EZ Imager (BioRad).

STATISTICAL ANALYSES

All statistical analyses and graphing were done using PRISM, version 6.0f.

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