Universidade de Trás-os-Montes e Alto Douro

Neutrophil Extracellular Traps in Bacterial Meningitis

A novel therapeutic target for treatment.

Dissertação de Mestrado em Genética Molecular Comparativa e Tecnológica

José Francisco Pereira Cardoso

Orientador: Adam Linder

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I hereby take full responsability for the ideas presented in this dissertation.

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Resumo

Meningite causada por *Streptococcus pneumoniae* é actualmente uma doença com forte impacto global e uma percentagem de mortalidade de 30% cujos sobreviventes são muito propensos a sequelas. Meningite bacteriana é uma infecção do sistema nervoso central, que resulta na inflamação das meninges que envolvem o cérebro e a espinal medula. O diagnóstico para a meningite bacteriana é um problema devido à dificuldade de a distinguir da meningite viral durante a fase inicial da doença.

Os neutrófilos desempenham um papel importante na resposta do organismo contra agentes patogénicos. O processo de formação de "neutrophil extracellular trap" (NET), também designado NETosis, é um processo no qual os neutrófilos libertam uma estrutura em forma de rede para o espaço extracelular. Estas estruturas são compostas por uma base de DNA e proteínas antimicrobianas, de forma a capturar e eliminar bactérias. Experiências anteriores realizadas pelo nosso grupo identificaram NETs exclusivamente em meningite bacteriana, dentro dos diferentes tipos de meningite. Uma vez que os NETs são reconhecidos pela captura e eliminação de agentes patogénicos, foi formulada a hipótese de que os NETs são importantes no líquido cefalorraquidiano (LCR) para eliminar agentes infecciosos durante meningite.

Para averiguar o papel da NETosis *in vivo* foi desenvolvido um modelo animal de meningite bacteriana. Ratos infectados revelaram um aumento significativo de NETosis comparado com os controlos de soro fisiológico. Para determinar se os NETs afectam a eliminação de agentes patogénicos *in vitro*, uma enzima que dissolve a base de DNA dos NETs (DNase I) foi administrada nas meninges dos animais infectados. A enzima foi capaz de dissolver os NETs presentes no LCR, resultando num decréscimo significativo da carga bacteriana presente no cérebro. O mesmo efeito foi observado na administração de DNase 10 horas após infecção e na administração da enzima por via intravenosa.

Para investigar o mecanismo pelo qual a DNase elimina o agente patogénico foi usado um modelo *in vitro* de NETosis. Primeiro de forma a determinar se a formação de NETs pode ser generalizada a outros tipos de estirpes de meningite bacteriana, nós analisamos a capacidade de diferentes agentes patogénicos para induzir NETosis *in vitro*. A maioria das estirpes de meningite bacteriana foram capazes de induzir NETosis. Para testar se os neutrófilos estariam a usar outros mecanismos para eliminar bactérias ajudando na eliminação de bactérias observadas com DNase, inibimos os processos de fagocitose e

de eliminação por espécies reactivas de oxigénio (ROS). Descobrimos que os inibidores preveniam quase completamente a eliminação das bactérias. A medição do processo de eliminação de bactérias através dos ROS e de fagocitose demonstraram que existe um aumento na sua concentração na presença de DNase.
Os nossos resultados indicam que a formação de NETs dificulta a eliminação da infecção bacteriana nas meninges. Tratamento com DNase promove outros mecanismos dos neutrófilos para a eliminação do agente infeccioso. A DNase como outros compostos capazes de dissolverem NETs poderão vir a ser um alvo para tratamentos terapêuticos. No futuro a detecção de NETosis poderá vir a ser uma excelente maneira de diagnosticar meningite bacteriana numa fase mais inicial.

Palavras-chave: Neutrophil extracellular Traps, meningite bacteriana, DNase I, *Streptococcus pneumoniae*, líquido cefalorraquidiano

Abstract

Streptococcus pneumoniae bacterial meningitis is currently a high impact disease with a mortality rate of 30% and survivors are highly prone to sequelae. Bacterial meningitis is a bacterial infection of the central nervous system (CNS) that results in inflammation of the protective fluid-filled layer, called the meninges, that envelops the brain and spinal cord. The diagnosis of acute bacterial meningitis (ABM) is a major problem due to the difficulty to discern it from viral meningitis (VM) at disease onset.

Neutrophils are the first immune cells to react to an infection and they play a primary role in the organism's response to invading pathogens. Neutrophil extracellular trap (NET) formation, or NETosis, is a process in which neutrophils release a web-like structure to the extracellular space, composed of a DNA backbone and antimicrobial proteins in order to ensnare and kill pathogens. Previous research in our group found that NETs are present specifically in bacterial meningitis patient samples but not in other forms of meningitis or trauma. Since NETs are known to trap and kill bacteria we therefore hypothesize in this study that NETs in the cerebrospinal fluid (CSF) influence bacterial killing during meningitis.

In order to inspect the role of NETosis *in vivo*, we developed a rat model of bacterial meningitis using a clinical isolate of *S. pneumoniae*. Rats with *S. pneumoniae* meningitis showed a significant increase of NETosis compared to the saline vehicle controls. To determine whether NETs affect bacterial killing *in vitro* DNase I, an enzyme that dissolves the DNA backbone of NETs, was infused into the meninges of infected rats. DNase I was able to clear NETs from the CSF and also resulted in a significantly decreased bacterial load in the brain. This effect was observed even if DNase I was administered 10 hours after the infection and even if it was administered intravenously.

To study the mechanism of this bacterial killing by DNase, we used an *in vitro* model of NETosis in isolated human neutrophils. First, to determine whether the formation of NETs can be generalized to other types of bacterial meningitis we analyzed the ability of different meningeal pathogen strains to induce NETosis *in vitro* and determined that most clinically isolated meningeal pathogens were able to induce NETosis. To test whether innate neutrophil killing mechanisms play a role in DNase-mediated bacterial killing, we inhibited neutrophil phagocytosis and oxidative burst using specific inhibitors and found that this almost completely prevented bacterial killing. Assays

measuring neutrophil oxidative burst and phagocytosis also indicated that these processes are increased in the presence of DNase.
Our results indicate that NET formation hinders the clearance of bacterial infection. Treatment with DNase I promotes other innate neutrophil bacterial killing mechanisms. DNase I as well as other NET disrupting compounds might be a potential target for therapeutic treatment of bacterial meningitis. In the future detection of NETosis could be a good way to diagnose early bacterial meningitis discerning it from viral meningitis.

Keywords: Neutrophil extracellular Traps, Acute Bacterial Meningitis, DNase I, *Streptococcus pneumoniae*, cerebrospinal fluid

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List of Abbreviations

4-ABAH	Myeloperoxidase inhibitor 4-Aminobenzoic hydrazide
ABM	Acute bacterial meningitis
AMP	Antimicrobial protein
BBB	Blood-Brain barrier
B-CSFB	Blood-CSF barrier
BHI	Beef heart infusion
CbpA	Choline-binding protein A
CFU	Colony forming units
CNS	Central nervous system
CR3	Complement receptor 3
CSF	Cerebrospinal Fluid
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DPI	Diphenyl iodonium chloride
FBS	Fetal bovine serum
GlpO	α-glycerophosphate oxidase
H_2O_2	Hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HBP/CAP37	Heparin binding protein/azurocidin
HClO	Hypochlorous acid
IL	Interleukin
IPD	Invasive Pneumococcal Disease
I.V	Intravenous
LB	Luria bertani
LR	Laminin Receptor
MMP	Matrix metalloprotease
MOI	Multiplicity of Infection
MPO	Myeloperoxidase
MRSA	Methicillin-resistant Staphylococcus aureus
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NanA	Pneumococcal neuraminidase A
NB	Neuroborreliosis
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NGAL	Neutrophil gelatinase-associated lipocalin
NLR	NOD-like receptor
OD	Optical density
PAD4	Peptidylarginine deiminase 4
PAF	Platelet-activator factor
PAFr	Platelet activating factor receptor
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCho	Phosphorylcholine
PCR	Polymerase Chain Reaction
PECAM-1/CD31	Platelet endothelial cell adhesion molecule – 1
pIgR	Polymeric imunoglobin receptor

PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
SH	Subarachnoid hemorrhage
SLE	Systemic Lupus Erythematosus
TSB	Tryptic soy broth
TLR	Toll-like receptor
VM	Viral Meningitis

1. Introduction

Chapter 1.1 –Meningitis

1.1.1 – History of the Disease

Meningitis is a disease that has been present and was characterized throughout human history (Christodoulides, 2013; Tyler, 2010). Meningitis symptoms have been observed and described in a simple way at first by Hippocrates in the *Corpus Hippocratium* and until the Renaissance era by identifying that these patients commonly exhibited headeaches, neck stiffness, vomiting and fever (Christodoulides, 2013; Tyler, 2010). These symptoms are still observable nowadays, however they aren't sufficently sensitive to accurately diagnose meningitis (Brouwer *et al.*, 2012; Dorsett and Liang, 2016). As technology and science advanced along the years it became possible to observe that pus and infflamation are present in the meninges of these patients (Christodoulides, 2013; Tyler, 2010). Later techniques were developed to allow the collection of cerebrospinal fluid (CSF) by lumbar puncture (Tyler, 2010; Christodoulides, 2013) followed by a blood culture and genetic analyses of the pathogen by polymerase chain reaction (PCR) (Tyler, 2010; Bottomley *et al.*, 2012). This provided the means to identify the pathogens responsible for the infection and to observe the host immune response to these pathogens (Christodoulides, 2013).
Nowadays meningitis is described as an inflammation of the membranes that envelop the brain and the spinal cord, known as meninges (Hoffman and Weber, 2009).

1.1.2 – Different Types of Meningitis

Inflammation of the meninges can be due to meningeal pathogens (Doran *et al.*, 2016; Swanson and McGavern, 2015; Gottfredsson and Perfect, 2000) that invade the central nervous system (CNS) and can also occur in conditions such as trauma, cancer (Fields, 2013; Chamberlain, 2012; Clarke, 2012) or autoimmune diseases like systemic lupus erythematosus (SLE) (Baldwin and Zunt, 2014), neuro-Behçet disease (Miller *et al.*, 2014) and neurosarcoidosis (Fritz *et al.*, 2016). Meningeal pathogens can invade the CSF via infection sites proximal to the brain (nasopharyngeal) (Doran *et al.*, 2016;

Iovino *et al.*, 2016a; Scheld *et al.*, 2002) or through the bloodstream (Doran *et al.*, 2016; Scheld *et al.*, 2002).

Meningitis can thus be classified as bacterial meningitis (Parikh *et al.*, 2012; Doran *et al.*, 2016), viral meningitis (Swanson and McGavern, 2015), fungal meningitis (Parikh *et al.*, 2012), parasitic meningitis (Riddell and Shuman, 2012; Honda and Warren, 2009), neoplastic meningitis (Fields, 2013; Chamberlain, 2012) and aseptic meningitis.

Viral meningitis is the term used when the pathogen responsible for the inflammation of the meninges is a virus (Swanson and McGavern, 2015). Enteroviruses are examples of viruses capable of invading the CNS and infecting the meninges (Swanson and McGavern, 2015; Klein *et al.*, 2016).

When the pathogen responsible for the infection of the CNS is a fungus the case is termed fungal meningitis (Gottfredsson and Perfect, 2000). The most commun fungal pathogen is the *Cryptoccocus neoformans* usually associated with cryptococcal meningitis (Mathur *et al.*, 2012).

Parasitic meningitis occurs when a parasite is able to infiltrate the CNS (Honda and Warren, 2009). The most common parasites are *Angiostrongylus cantonensis* (Riddell and Shuman, 2012; Ramirez-Avila *et al.*, 2009) and *Gnathostoma spinigerum* (Ramirez-Avila *et al.*, 2009).

Neoplastic meningitis or malignant meningitis is the term used for when cancer cells spread to the meninges (Chamberlain, 2012; Clarke, 2012).

Aseptic meningitis is when inflammation on the meninges exists without the ocurrence of infection, for example in trauma patients (Christodoulides, 2013), SLE (Baldwin and Zunt, 2014), neuro-Behçet disease (Miller *et al.*, 2014) and neurosarcoidosis (Fritz *et al.*, 2016). Although some of the reported cases of aseptic meningitis may be due to the fact that we still lack the ability to identify all of the strains responsible for meningitis (Saleem and Macdonald, 2013).

Chapter 1.2 – Bacterial Meningitis

1.2.1 – Pathogenesis

Bacterial meningitis is caused by bacteria capable of invading and colonising the meninges (Doran *et al.*, 2016; Scheld *et al.*, 2002; Hoffman and Weber, 2009). The

most common cases of bacterial meningitis are caused by *Streptoccocus pneumoniae*, *Haemophilus influenzae* and *Neisseria Meningitidis* (Christodoulides, 2013; Doran *et al.*, 2016; Hoffman and Weber, 2009).

These pathogens frequently establish colonies on mucosal surfaces by binding their adhesins to extracellular matrix proteins such as laminin, fibronectin and collogen, facilitating their attachment to the host cells (Doran *et al.*, 2016; Dando *et al.*, 2014). This binding of bacteria to the host cells may lead to a tighter bacterial attachment or to the internalization of the pathogen into the host cell due to signal transduction (Doran *et al.*, 2016; Dando *et al.*, 2014). This first step is important for invading the CNS through the bloodstream (Doran *et al.*, 2016). After the bacteria attach to the host cells they need to be able to evade the immune cells of the host present in the bloodstream (Doran *et al.*, 2016). Bacteria have developed two ways to avoid phagocytosis (Doran *et al.*, 2016). They can express a protective capsule, which protects them (Henriques-Normark and Tuomanen, 2013) until they are able to cross the layer of cells protecting the CNS known as blood-brain barrier (BBB) (Doran *et al.*, 2016). Alternatively they can enter the neutrophils and the macrophages (Dando *et al.*, 2014) and persist against their internal defenses such as antimicrobial proteins and peroxide and oxygen radicals (Doran *et al.*, 2016).

This first phase of the bacterial meningitis pathogenesis which is the invasion of the CNS by bacteria can be done through the bloodstream as described above or through neighboring infected tissues forming a more direct route for the bacteria (Dando *et al.*, 2014; Doran *et al.*, 2016).

Independently of how the first phase is achieved by the bacteria all the pathogens need to breach the BBB and the blood-CSF barrier (B-CSFB) in order to access the host's brain (Doran *et al.*, 2016; Dando *et al.*, 2014). Bacteria can cross these barriers through a paracellular (between cells) or transcellular (through the cells) route depending on their virulence traits (Doran *et al.*, 2016; Dando *et al.*, 2014).

Bacteria possessing cytolytic toxins are able to damage the host cells leading to a disruption in the barrier, opening up spaces for paracellular invasion (Doran *et al.*, 2016; Los *et al.*, 2013).

The transcellular route depends on the ability of the bacteria to intracellularly invade the cells by exploitation of the signal platforms and pathways of the host cell (Doran *et al.*, 2016; Dando *et al.*, 2014).

Once the bacteria breach the barrier and reach the CNS, their bacterial components bind to and activate the local immune cells (Doran *et al.*, 2016). The CNS has two types of immune cells: the microglia and the astrocytes (Mook-Kanamori *et al.*, 2011; Doran *et al.*, 2016). When these cells are activated they attract other immune cells from the bloodstream like neutrophils, granulocytes and monocytes and they infiltrate the infected meninges (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011).

The antimicrobial immune response in the meninges from the invading immune cells, that in normal conditions are not present in the CSF, is considered by many researchers as overwhelming to the surrounding tissues and not well coordinated in the removal of the pathogen (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011; Gerber and Nau, 2010). This response leads to neuronal damage and death (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011; Gerber and Nau, 2010). Even if the host is able to survive the infection there is a high chance that they will suffer from sequelae due to the damage exerted by the immune response (Doran *et al.*, 2016; Putz *et al.*, 2013; Mook-Kanamori *et al.*, 2011). Such sequaelae include deafness and some forms of mental retardation (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011).

1.2.2 – *Streptoccocus pneumoniae*

Streptoccocus pneumoniae is generally a colonizer of the nasopharynx and can cause conditions such as sinusitis and otitis media or more life-threatning situations like pneumonia, bacteremia and meningitis (Doran *et al.*, 2016; Henriques-Normark and Tuomanen, 2013).

Streptoccocus pnemoniae is one of the most common agents of bacterial meningitis worldwide (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011).

Bacterial meningitis caused by *S.pneumoniae* inflicts a wide array of complications for the host including brain edema, increasead intracranial pressure and cerebral ischemia (Doran *et al.*, 2016). Survivors from this disease are highly susceptable to sequelae such as deafness and cognitive impairment (Doran *et al.*, 2016; McGill *et al.*, 2016). Pneumolysin, an exotoxin protein produced by *S.pneumoniae*, and the amino acid/neurotransmitter glutamate are responsible for long term neurological sequelae (Doran *et al.*, 2016; Wippel *et al.*, 2013). These compounds cause focal or diffuse axonal injury and synaptotoxicity as well as dendritoxicity (Doran *et al.*, 2016; Wippel *et al.*, 2013).

1.2.2.1 – Invasion and dissemination

S.pneumoniae enters the host via the respiratory tract and needs to be able to escape the mucus defenses (Doran *et al.*, 2016; Dando *et al.*, 2014).

As referred before the bacteria now have two approaches to reach the CNS either by translocating to the bloodstream, leading to invasive pneumococcal disease (IPD), or by causing a sinusitis or mastoiditis and penetrating the skull by spreading locally along vessels and through skull defects (Doran *et al.*, 2016; Putz *et al.*, 2013).

In order to invade the CNS through the bloodstream from the respiratory mucosa bacteria, including *S.pneumoniae*, use different virulence factors including polysaccharide capsule, cell wall and surface proteins (Putz *et al.*, 2013; Doran *et al.*, 2016). This common strategy used by major meningeal pathogens to invade the CNS is designated innate invasion because it counteracts the innate immune mechanisms and uses molecular mimicry in order to promote invasion (Doran *et al.*, 2016; Thornton *et al.*, 2010). Innate invasion starts when bacteria bind to the respiratory endothelium (Doran *et al.*, 2016). Choline-binding protein A (CbpA), an adhesin, binds to the polymeric immunoglobin receptor (pIgR) to initiate the translocation of the bacteria across the nasopharyngeal epithelium (Doran *et al.*, 2016; Iovino *et al.*, 2016b).

In the cerebrovascular endothelium CbpA, platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and the lectin like domain of the pneumococcal neuraminidase A (NanA) are able to bind to the laminin receptor (LR) to promote pneumococcal attachment to the BBB endothelial cells (Doran *et al.*, 2016; Iovino *et al.*, 2016b).

1.2.2.2 – Translocation into the CNS

The innate invasion proccess allows the bacteria to translocate across the BBB and B-CSFB barriers after attachment to epithelial or endothelial host cells (Doran *et al.*, 2016). Phosphorylcholine (PCho), a hydrophilic polar head group of some phospholipids, is present on the surface of most respiratory pathogens and because its structure is similar to the chemokine platelet-activator factor (PAF) it is able to bind to the human platelet activating factor receptor (PAFr) (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011; Dando *et al.*, 2014). This binding allows the uptake of bacteria into a vacuole mediated by the protein clathrin, facilitating intracellular bacterial translocation into the CNS

(Doran *et al.*, 2016; Dando *et al.*, 2014; Mook-Kanamori *et al.*, 2011). Some researchers have also described the use of vitronectin-αvβ3 integrin complex by *S.pneumoniae* to invade epithelial and endothelial cells (Doran *et al.*, 2016).

Paracellular access to the CNS is gained by the pneumococcus by disrupting the BBB barrier (Doran *et al.*, 2016; Dando *et al.*, 2014). The disruption of the BBB is mediated by the cholesterol-dependent cytolysin called pneumolysin and by α-glycerophosphate oxidase (GlpO) that cause apoptosis of brain microvascular endothelial cells by creating H_2O_2, a well known reactive oxygen species (ROS) (Doran *et al.*, 2016).

Meningitis can also be caused by direct invasion from neighboring infected tissues without resorting to a sustained bacteremia in the bloodstream (Doran *et al.*, 2016; Putz *et al.*, 2013). Pneumococcal invasion of the CNS can start in the nasopharynx and a pneumococcal carriage via retrograde axonal transportation along the olfactory neurons will lead to the infection of the CNS (Doran *et al.*, 2016).

Once bacteria gain access to the meninges, they rapidly multiply in the CSF due to the very limited host defense mechanism in the CNS (Doran *et al.*, 2016; Putz *et al.*, 2013).

1.2.2.3 – Global Impact and Current Therapeutics

Acute bacterial meningitis (ABM) is a life-threatening disease with a major impact worldwide (Linder *et al.*, 2011). After the introduction of vaccines in the late 90s major pathogens which contributed to the majority of the reported cases of meningitis began to decline, however vaccines were not able to cover every type of strain from these pathogens (McGill *et al.*, 2016). The use of vaccines changed the epidemiology of bacterial meningitis worldwide (McGill *et al.*, 2016).

The mortality rate of ABM was around 90% before the introduction of antibiotics (Swartz, 2004). After antibiotics were developed there was a decline in the mortality rate of meningitis pathogens (Swartz, 2004). Nowadays major pathogens like *S.pneumoniae* have a mortality rate of approximately 30%, however around 24.7% of the survivors are prone to develop neurologic sequelae (McGill *et al.*, 2016; Linder *et al.*, 2011).

Diagnosis and treatment remains challenging due to the difficulty of discerning between bacterial and viral meningitis in a rapid and efficient way (Linder *et al.*, 2011). This results in the administration of antibiotics of a broad-spectrum of action in patients that present lymphocytic pleocytosis, a general early characteristic for these two types of

meningitis (Linder *et al.*, 2011). However, this era is becoming progressively marked by the alarmingly fast development of antibiotic resistance by bacteria (Tomasz, 1999). Pleocytosis is considered to be a flawed way of discerning between viral and bacterial meningitis and bacterial detection by CSF Gram stain, blood or CSF culture, another diagnostic method for meningitis, is quite slow and many patients have already been prescribed with medication before the collection of CSF samples (Linder *et al.*, 2011). Therefore a faster and more sensitive way of detecting ABM could improve diagnosis and outcome.

Chapter 1.3 – Immune Response in the Brain

1.3.1 – Immune activation

During multiplication, bacteria release components called pathogen-associated molecular patterns (PAMPs) (Doran *et al.*, 2016). PAMPs are recognized by pattern recognition receptors (PRRs) that are present on the surface of antigen-presenting cells which are present in small numbers in the CSF (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011). After recognition of the bacterial components these cells become active and produce a wide range of pro-inflammatory cytokines and chemokines (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011). This leads to a strong inflammatory response leading to the recruitment of leukocytes to the CSF and subsequent BBB disruption (Doran *et al.*, 2016; Dando *et al.*, 2014). Increased intracranial pressure observed in meningitis is caused by vascular deregulation, occlusion of vessels and vasculitis (Doran *et al.*, 2016; Henriques-Normark and Tuomanen, 2013).

The most significant PRRs that are responsible for the detection of *S.pneumoniae* in the CSF are from the Toll-like receptor (TLR) family, namely TLR2, TLR4 and TLR9, and NOD2 from the NOD-like receptor (NLR) family(Doran *et al.*, 2016; Koppe *et al.*, 2012). TLR2 is responsible for detecting pneumococcal cell wall components, lipoteichoic acid and lipoproteins (Koppe *et al.*, 2012). TLR4 recognizes pneumolysin and TLR9 detects the bacterial DNA released during autolysis (Doran *et al.*, 2016; Koppe *et al.*, 2012). Intracellular NOD2 senses muramyl peptides from pneumococcal peptidoglycan (Doran *et al.*, 2016; Koppe *et al.*, 2012).

Interestingly, inflammation in the CNS can be triggered by components of the bacterial cell alone, in the absence of live bacteria (Doran *et al.*, 2016).

1.3.2 – Inflammatory response

The inflammatory response to pneumococcus activates different signaling cascades which results in the production of pro-inflammatory mediators responsible for orchestrating an efficient immune response against the pathogen (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011). In pneumococcal meningitis cases, high levels of pro-inflammatory cytokines like tumor necrosis factor alpha, interleukin (IL) 1 beta, interferon gamma, IL-2, IL-6 and IL-12 can be observed in the CSF as well as the anti-inflammatory cytokines IL-10 and transforming growth facter beta and the IL-8, macrophage inflammatory protein 1 alpha and monocyte chemoattractant protein 1 chemokines (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011). Secreted chemokines work together with other chemoattractants like PAF and reactive oxygen and nitrogen species as well as the complement system in order to attract highly activated neutrophils to the CSF (Doran *et al.*, 2016). Neutrophils cross the BBB through the tight junctions of the endothelial cells that form this barrier by a multistep process that involves integrins and selectins and leads to pleocytosis in the CSF (Doran *et al.*, 2016). Matrix metalloproteases (MMPs) that are produced by neutrophils, glia cells, endothelial cells and neurons during infection of the CSF are important to promote the BBB breakdown and leukocyte invasion of the CSF since they lyse the subendothelial basement membrane (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011). Since CSF is a leukocyte free environment during normal conditions this overwhelming influx of leukocytes to the CSF during the inflammatory response of the host also comes at a cost to the homeostasis of the organism (Mook-Kanamori *et al.*, 2011). Activated immune cells inside the brain like the microglia, astrocytes, microvascular endothelial cells as well as infiltrating leukocytes during infection amplify the production of pro-inflammatory cytokines and cytotoxic agents leading to tissue damage in cortical and subcortical structures due to their overwhelming presence in the CNS (Mook-Kanamori *et al.*, 2011; Doran *et al.*, 2016). It is also known that invading leukocytes in the CSF are not efficent at phagocytising *S. pneumoniae*, contributing to the pathogenesis of pneumococcal meningitis as well as to survivor's sequelae events (Doran *et al.*, 2016).

1.3.3 – Neutrophils

Neutrophils are the most abundant cells in our immune system and are the first cells to react to infection, playing a primary role in the host response to invading pathogens (Moorthy *et al.*, 2016; Zhang *et al.*, 2016; Li *et al.*, 2010). The disruption of the BBB complex due to this response against invading pathogens like *S.pneumoniae* and other meningeal pathogens allows the neutrophils to access the CSF more promptly (Doran *et al.*, 2016; Mook-Kanamori *et al.*, 2011; Henriques-Normark and Tuomanen, 2013).

Neutrophils are key regulators of the inflammation process capable of releasing pro inflammatory substances like chemokines and cytokines and anti-inflammatory molecules (Zhang *et al.*, 2016; Martinod *et al.*, 2015).

Neutrophils are also able to phagocytose microbial pathogens (Mantovani *et al.*, 2011). In this process the microbes are ingested into a phagosome by a neutrophil (Mantovani *et al.*, 2011; Nauseef and Borregaard, 2014). The phagosome then becomes a phagolysosome by acquiring lysosomal characteristics through fusion with the neutrophil's primary and secondary granules (Doran *et al.*, 2016). The antimicrobial proteins (AMPs) (Doran *et al.*, 2016; Nordenfelt and Tapper, 2011; van Kessel *et al.*, 2014), the hypochlorous acid (HClO) produced by MPO (Nauseef, 2014; van Kessel *et al.*, 2014; Nordenfelt and Tapper, 2011), lactoferrin (van Kessel *et al.*, 2014) and neutrophil gelatinase-associated lipocalin (NGAL) (van Kessel *et al.*, 2014) contained in the granules kill bacteria or deprive their growth leading to the elimination of the captured microbes (van Kessel *et al.*, 2014).

The primary granules of neutrophils, also called azurophilic granules, contain many proteins with antimicrobial activity such as myeloperoxidase (MPO) (van Kessel *et al.*, 2014; Cowland and Borregaard, 2016), serine-proteases like neutrophil elastase (NE) (Cowland and Borregaard, 2016; van Kessel *et al.*, 2014; Nordenfelt and Tapper, 2011), cathepsin G (van Kessel *et al.*, 2014; Cowland and Borregaard, 2016) and proteinase 3 (van Kessel *et al.*, 2014; Cowland and Borregaard, 2016), and AMPs such as α-defensins (van Kessel *et al.*, 2014; Cowland and Borregaard, 2016) and azurocidin (also known as heparin-binding protein or HBP) (Cowland and Borregaard, 2016). The secondary granules mostly contain lactoferrin (Faurschou and Borregaard, 2003). Tertiary granules mainly contain gelatinase, collagenase and MMPs (Faurschou and Borregaard, 2003).

Neutrophils are also capable of phagocytosis-independent killing either through a non-oxidative mechanism (Korkmaz *et al.*, 2008) or through a oxidative mechanism (Segal, 2005). The non-oxidative mechanism is mediated by degranulation of the granules and secretory vesicles, releasing their antimicrobial proteins into the extracelullar milieu where they bind to microbes to neutralize and eliminate them (Korkmaz *et al.*, 2008). The oxidative mechanism is mediated by a oxidative burst and the rapid release of ROS to the extracellular space due to the activity of NADPH oxidase. ROS are then converted to HClO by the MPO killing the bacteria (Winterbourn and Kettle, 2013). This process also occurs inside the phagolysosomes to help degrade the internalized microbes (Winterbourn and Kettle, 2013).

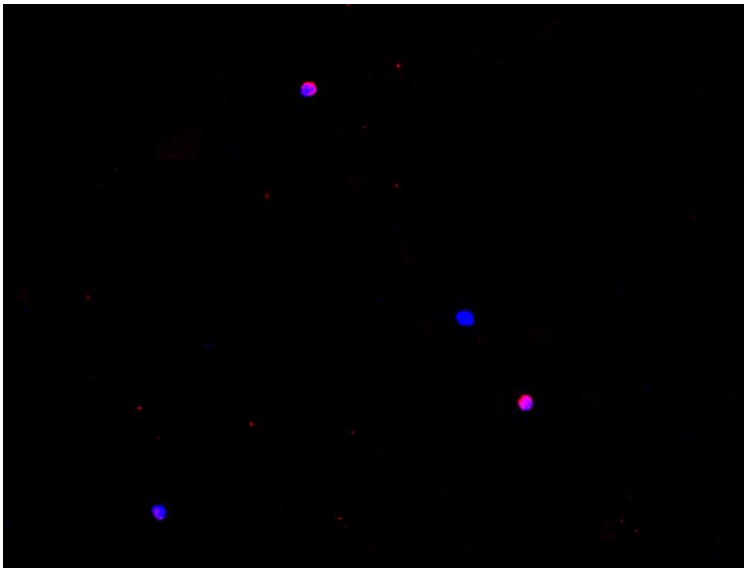

Figure 1 – Immunofluorescence microscopy of a Rat CSF sample where it is possible to observe neutrophils co-staining with DAPI (blue) and rat myeloperoxidase (red) other type of cells can also be seen (staining only with DAPI) in the field.

1.3.3.1 – NETosis

Neutrophils are also capable of killing extracelullar pathogens by undergoing a novel process termed NETosis due to the formation of neutrophil extracellular traps (NETs) (Yipp and Kubes, 2013; Desai *et al.*, 2016). NETs are composed of a decondensed chromatin DNA backbone bound to antimicrobial proteins forming a weblike framework that is released in response to pathogens, ensnaring and killing them

(Buchanan *et al.*, 2006; Mohanty *et al.*, 2015). The most abundant proteins in NETs are histones such as H2A, H2B, H3 and H4, composing the NET backbone and accounting for around 70% of all the NET-associated proteins (Dabrowska *et al.*, 2016). NET-bound proteins include NE, S100 proteins like S100A8 and S100A9 (Dabrowska *et al.*, 2016), lactoferrin (Dabrowska *et al.*, 2016), HBP (Urban *et al.*, 2009), cathepsin G (Dabrowska *et al.*, 2016), MPO (Dabrowska *et al.*, 2016), proteinase 3 (Dabrowska *et al.*, 2016), lysozyme (Dabrowska *et al.*, 2016), actin (Dabrowska *et al.*, 2016) and catalase (Dabrowska *et al.*, 2016). The release of azurophilic granule proteins such as HBP (Fuchs *et al.*, 2010; Urban *et al.*, 2009), MPO and NE and even histones is tightly regulated (Fisher and Linder, 2017; Tapper *et al.*, 2002) and their substantial presence in the extracellular environment is linked to disease severity (Martinod *et al.*, 2015; Chen *et al.*, 2014). In fact, HBP serves as a biomarker with high sensitivity and specificity for predicting organ dysfunction in severe infectious disease (Chen *et al.*, 2014; Linder *et al.*, 2009) including ABM (Linder *et al.*, 2015).

The two currently recognized pathways of NET formation are NETosis and "Vital" NETosis (Yipp and Kubes, 2013; Desai *et al.*, 2016). NETosis is a slow process that can be either dependent or independent of NADPH oxidase and results in the death of the cell (Yipp and Kubes, 2013). NADPH oxidase-dependent NETosis requires chromatin decondensation followed by disintegration of the nuclear envelope and mixing of granule proteins to nucleic acids inside of a vast intracelullar vacuole (Yipp and Kubes, 2013; Desai *et al.*, 2016; Remijsen *et al.*, 2011). After intracellular assembly of the NETs they are released into the extracelullar milieu through perforation of the plasma membrane and cell lysis (Yipp and Kubes, 2013). NETosis is a process that is also possible through a NADPH oxidase independent pathway, in fact the role of NADPH oxidase in NET formation is still unknown (Yipp and Kubes, 2013). Chromatin decondensation in both of this processes is achieved through the activity of NE, MPO and peptidylarginine deiminase 4 (PAD4) (Yipp and Kubes, 2013). During NETosis NE translocates to the nucleus from the granules and iniatiates chromatin degradation by cleaving histones (Yipp and Kubes, 2013). MPO also contributes to the decondensation of the nuclear DNA through an unknown mechanism that is independent of its enzymatic function (Yipp and Kubes, 2013). PAD4 is vital for the decondensation of chromatin in NETs (Lewis *et al.*, 2015). PAD4 citrullinates histones in neutrophils and inhibition of PAD4 disables the formation of NETs (Lewis *et al.*, 2015). "Vital" NETosis is a recently discovered concept in which NETs are released but the host

neutrophil remains active as an anuclear cytoplast capable of performing phagocytosis, leukocyte recruitment and chemotaxis, unlike normal NETosis that results in cell death (Yipp and Kubes, 2013; Brinkmann *et al.*, 2004). "Vital" NETosis is a faster process than normal NETosis and the release of NETs to the extracelullar space occurs via nuclear envelope blebbing and exportation by vesicles and so it preserves the integrity of the plasma membrane (Yipp and Kubes, 2013). This process requires the interaction of the toll-like receptor 4 (TLR4) from activated platelets with the microbial pathogens, thus allowing a rapid activation of the neutrophils mediated by both complement receptor 3 (CR3) and TLR2 (Yipp and Kubes, 2013; Brinkmann *et al.*, 2004).

Recent studies have suggested that the formation of NETs as a host immune response is both a beneficial and harmful process (Mohanty *et al.*, 2015; Kaplan and Radic, 2012; Wong *et al.*, 2015; Fadini *et al.*, 2016). As mentioned before, tissue injury caused by meningitis in the CNS has been demonstrated to be caused both by toxic bacterial products as well as agressive host inflammatory responses, particularly those initiated by neutrophils (Doran *et al.*, 2016; Remijsen *et al.*, 2011).

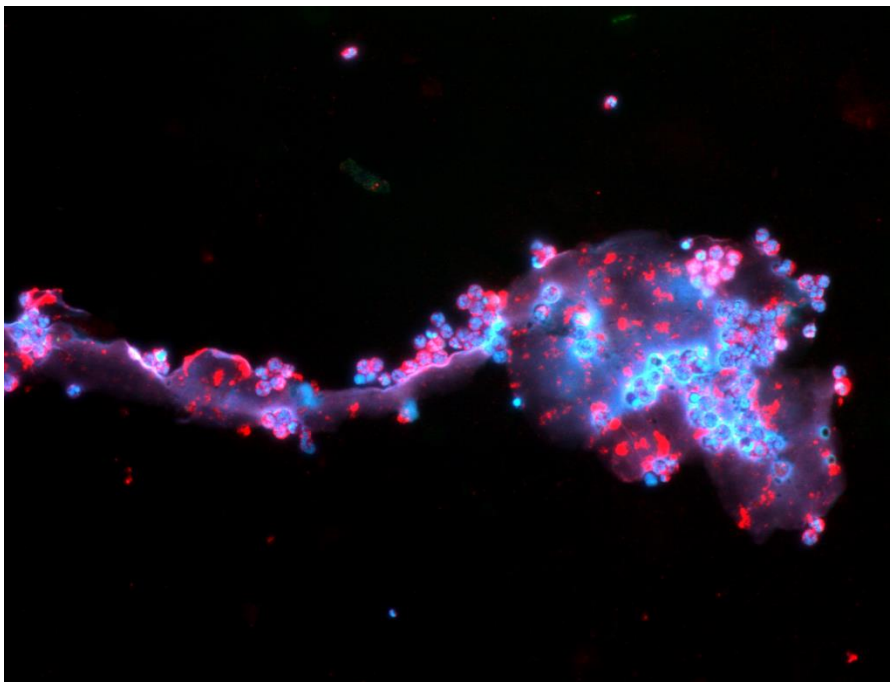

Figure 2 - Immunofluorescence microscopy of a Rat CSF sample where it is possible to observe a NET structure due to the co-staining of DNA (blue) and rat myeloperoxidase (red). This massive structure is released to the extracellular space by the neutrophil to trap and kill bacterial pathogens.

Chapter 1.4 – Preliminary Data

Prior to the start of this project, our lab group assessed whether neutrophil extracellular traps were exclusive in CSF of patients with bacterial meningitis or if they were present in other types of meningitis as well. Tirthankar Mohanty collected CSF samples (n=16) from individuals afflicted with pneumococcal ABM, viral meningitis (VM), neuroborreliosis (NB) or subarachnoid hemorrhage (SH) were analyzed. The data obtained showed that ABM patients displayed distinctly higher neutrophil counts and protein content in the CSF than any of the other patients (Appendix III, Supplementary Table 1). Immunofluorescence microscopy analysis revealed that in ABM patients large areas of decondensed extracellular DNA was co-localizing with NE (mean value = 77.36%) but the same was not observed in CSF samples from patients with VM (mean value = 1.076%), NB (mean value = 0.07171%) and SH (mean value = 2.398%) (Fig. 1a). Treatment of CSF samples from ABM patients with agents that disrupt NETs resulted in the degradation of the DNA structures co-localized with NE, confirming that these structures were NETs (Fig. 1b). DNase I reduced the percentage of NETs in the sample from a mean value of 68.62% to 1.032% while heparin reduced the percentage of NETs in the samples to a mean value of 6.375%.
Therefore we observed that NETs are exclusive to ABM compared to other meningitis forms and trauma. These findings formed the basis to the start of this dissertation project.

Chapter 1.5 – Hypothesis and Specific Aims

We hypothesize that NETs in the CSF influence bacterial killing during meningitis. In order to evaluate this hypothesis we first estabilished a rat model of meningitis using a clinical strain of *S.pneumoniae*. Afterwards we evaluated the importance of NETs in the host response to bacterial meningitis by removing NETs with DNase I treatment in the rat model. Lastly we investigated the mechanism of DNase I induced bacterial killing by *in vitro* inhibition of innate neutrophil killing pathways.

2. Materials and Methods

2.1 – Detection of NETs

In a preliminary study CSF samples (n=16) were fixed with an equal volume of 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at 4°C. The samples were then cytocentrifuged on to glass slides (Thermo Fisher). Samples were permeabilized with 0.5% triton-X-100 (Sigma-Aldrich) and blocked with 5% goat serum (BioWest). Some samples from ABM patients (n=6) were also treated with either heparin (Leo, 1 Unit/mL), or DNase I (Abcam, 5U/mL) for (20 min at 37°C) prior to fixing and cytocentrifugation.

Human samples (n=110) were stained with rabbit-anti-human neutrophil elastase (Dako), and detected with Alexafluor 594-labelled secondary goat-anti-rabbit Fab antibody fragment (Life Technologies). Rat samples (n=66) were stained with anti-mouse myeloperoxidase, validated to cross react with rat myeloperoxidase(Ge *et al.*, 2015) (Novusbio), and detected with Alexafluor 594-labelled secondary goat-anti-rabbit Fab antibody fragment (Life Technologies). Coverslips (diameter - 14 mm, Menzel Gläser) were mounted on top of the samples with mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies) and visualized with a Nikon Ti-E microscope. Images were acquired Andor Neo/Zyla camera and NIS elements advanced research software (Nikon).

2.2 – Induction of NETs *in vitro*

Neutrophils from humans and rats were isolated using polymorphprep (Axis-Shield) according to the manufacturer's directions and resuspended to the desired number in HBSS with calcium and magnesium (Life technologies). Coverslips were washed with PBS and incubated in 24-well plates with 0.01% poly-L-lysine (Sigma-Aldrich) in sterile PBS overnight at 37 °C. Coverslips were washed once in PBS and 200 µL of 3.5 x 105 PMNs/ml and bacteria with a multiplicity of infection (MOI) of 1:10 were added to each well. Human samples (n=10) and rat samples (n=10) were then fixed and immunostaining was performed on the samples as mentioned above. Neutrophils in HBSS (with calcium and magnesium) only were used as non-stimulated controls.

2.3 – Quantification of NETs

For detection of NETs in patient CSF and animals, 3 to 5 random images at 20X or 10X magnification were used for quantifying each condition. NETs were defined as a localization of DNA and neutrophil elastase and expressed as % NETosis of field.
For *in vitro* NET formation, 3 to 5 random images at 20X or 10X magnification, with a minimum of 175 cells in total were used for quantification for each condition. The DAPI channel was used to identify nuclei. The elastase positive area in non-stimulated cells with normal polymorphonuclear morphology as described by Mohanty et al (Mohanty *et al.*, 2015). An increase in elastase-positive area of 33% was used as a cut-off to eliminate non-activated cells and detect NET formation in the samples. Image analysis was performed with the public domain software (Fiji - ImageJ).

2.4 – Bacterial culture

Clinical isolates of various bacteria were collected from either blood or CSF from patients after being diagnosed with meningitis at the clinic for infectious diseases at Skåne University Hospital in Lund, Sweden.
For survival assays, animal infection and NET-induction, all *S. pneumoniae* strains were cultured overnight on blood agar (5% Sheep's blood) and then the colonies from the entire plate were transferred into a total of 7mL of Todd-Hewitt medium with 0.5% yeast extract (BD Biosciences) supplemented with 10% choline chloride (Sigma-Aldrich). Upon reaching an optical density (OD) of 0.4 they were washed and resuspended in phosphate buffered saline solution (Sigma-Aldrich) at an appropriate dilution for further experiments.
All *S. aureus* strains were cultured in tryptic soy broth (TSB) overnight, washed and used for NET induction. For survival assays, bacteria were cultured from an overnight culture until the bacteria reached mid log phase, washed with PBS and set to the desired OD.
All other bacteria were cultured overnight: N. meningitidis in GC broth with 10% FBS, *L. monocytogenes* in beef heart infusion (BHI), *A. baumannii* and *E. coli* in Luria bertani (LB), *S. capitis*, *S. oralis* and *S. epidermidis* in Todd-Hewitt medium with 0.5%

yeast extract. They were then washed with PBS and resuspended to the desired OD. All media were purchased from BD biosciences.

2.5 – Rat model of meningitis

The local Ethical Committee for Animal Research (M80-14) approved the experimental protocol. Adult Sprague Dawley male rats (Taconic, 350-370g) were used. Animals were treated in accordance with the National Institutes of Health for the Care and Use for Laboratory animals. Anaesthesia was induced with pentobarbital (30 mg/kg). Body weight was recorded and body temperature, measured rectally, was maintained at 37°C. All surfaces, instruments, and the head of the rat were cleaned with 70% ethanol before the procedure. The head was fixed on a stereotactic device and the skull was exposed. A hole was drilled in front of the lamboid suture and to the left of the sagittal suture using an automated hollow drill (5mm diameter). The piece of bone inside the drilled area was lifted and set aside. The durum and arachnoid membranes were carefully punctured with a needle (27G) and a catheter (32G) was inserted into the subarachnoid space. Either 20µL of bacterial solution (3 x 106 bacteria), or the same bacterial solution containing 10 Units of recombinant human DNase I (Abcam), or sterile physiological saline solution (Fresenius Kabi), was injected into the subarachnoid space using a syringe pump at a flow rate of 2µL/min. After the infusion was finished, the cathether was left in place for 1-2 minutes more to minimize backflow upon removal of the catheter. The catheter was removed and the piece of bone was cleaned with ethanol and replaced to keep out unwanted bacteria and sealed with histoacryl (Braun). The incision was sealed and wiped with ethanol.

After 24 hours, anaesthesia was induced with pentobarbital as above. Weight was recorded and temperature measured rectally. The head was fixed in the stereotactic device and the incision reopened and the piece of bone removed to expose the meninges. A needle (27G) was inserted into the subarachnoid space and approximately 10µL of CSF was aspirated and diluted with 200µL of physiological saline. NETs were visualized in CSF samples as above.

Blood samples were collected via the left femoral artery. Rats were killed by decapitation and organs were removed and placed in saline (brain - 2ml, lungs and spleen – 800µL).

2.5.1 – 10-hour DNase treatment in rats

Recombinant human DNase I (Abcam) was infused subarachnoidally 10 hours after bacterial infection. In rats receiving treatment at 10 hours, anaesthesia was induced by isofluorane gas. The skull was re-exposed and the previously replaced piece of bone was again removed to expose the brain. A catheter was placed subarachnoidally as described above and 10 Units of DNase was infused at a flow rate of 2μL/min. The piece of bone was replaced and sealed with histoacryl and the wound was again closed.

2.6 – Intravenous DNase treatment in rats

Bacteria or saline vehicle control was infused subarachnoidally in rats as described above. For intravenous administration, a catheter was inserted into the jugular vein (outer diameter 1.19mm, Silastic) and secured to the back of the neck. Six hours after bacteria or vehicle infusion, a bolus dose of 3500 units of DNase (Worthington) or equal volume saline vehicle solution was infused intravenously using a syringe pump. Then a continuous infusion of 780 units/hour of DNase or equal volume saline vehicle solution at a flow rate of 0.05mL/hour was initiated. This infusion continued until the rats were sacrificed after 24 hours as described above.

2.7 – Bacterial counts in rat organ homogenates

The brain, lungs and spleen were homogenized using silicone beads in a TissueLyser (Qiagen) and 20μL of fresh homogenate was plated onto blood agar plates and incubated at 37°C overnight. Colony forming units were counted and confirmed to be *S. pneumoniae* based on colony characteristics. The rest of the homogenate was flash frozen in liquid nitrogen for 10 minutes and then stored at -80°C.

2.8 – Bacterial killing after DNase treatment

NETs were induced in human neutrophils as above, with the simultaneous addition of DNase I (5 Units) or equal volume of saline solution. To determine the killing

mechanism, the following inhibitors were added 30 minutes after addition of DNase I and bacteria: 10μM Cytochalasin D (Calbiochem), 10μM myeloperoxidase inhibitor 4-Aminobenzoic hydrazide (4-ABAH) (Calbiochem) or 10μM NADPH oxidase inhibitor Diphenyl iodonium chloride (DPI) (Calbiochem). Samples were incubated for 2.5 hours after addition of inhibitors. NETs were detected as above using immunofluorescence. Samples were then diluted and 20 μL of a ten-fold dilution of each sample was streaked on blood agar plates and colony-forming units of *S. pneumoniae* were determined as above.

2.9 – Myeloperoxidase (MPO) activity assay

After 30 minutes of *in vitro* NET induction in the presence and absence of DNase I as described above, samples were centrifuged and the cell-free supernatant analyzed for MPO peroxidation and chlorination activity using EnzChek MPO Assay Kit (Molecular Probes) according to the manufacturer's directions. Due to the high background peroxidise activity of plasma, it was excluded from all stimulation conditions. We verified that bacterial killing in the presence and absence of DNase I occurred to a similar extent under these conditions.

2.10 – Gentamicin assay

SP001 was cultured in THY medium supplemented with choline chloride as described previously. The bacteria were then added at MOI of 10 to 1 x 106 neutrophils in HBSS with 10% plasma alone, or in presence of DNase I (5U) for 15 minutes at 37 degrees with shaking. Gentamicin (10μg/mL) was added for 30 minutes at 37 degrees to kill extracellular bacteria. Cells were washed thrice with HBSS and lysed with sterile water. The bacteria were then plated out at 10X or 100 dilutions and colony forming units (CFUs) were counted to assess survival.

2.11 – Visualization of phagocytosis

SP001 strain was labelled with Oregon green 488-X succinimidyl ester (20μM, Life Technologies) in PBS for 30 minutes in the dark at room temperature. The labelled

bacteria were then washed thrice in PBS to remove excess unbound dye. Bacteria were then added to neutrophils in HBSS with 10% autologous plasma (MOI 1:10) alone or in the presence of DNase I (5U) for on shaking for 1 hour at 37 degrees in the dark. Samples were then fixed, cytocentrifuged onto slides and processed for immunocytochemistry as described previously on the methods for detection of NETs. Neutrophils were probed with rabbit human anti-elastase and DNA was visualized with DAPI.

3. Results

3.1 – Meningitis Patient Samples Analysis

Immunofluorescence microscopy analysis revealed that in ABM patients (n=6) large areas of decondensed extracellular DNA was co-localizing with NE (mean value = 77.36%) but the same was not observed in CSF samples from patients with VM (n=3) (mean value = 1.076%), NB (n=3) (mean value = 0.07171%) and SH (n=4) (mean value = 2.398%) (Fig. 3a). Treatment of CSF samples from ABM patients (n=3) with agents that disrupt NETs resulted in the degradation of the DNA structures co-localized with NE, confirming that these structures were NETs (Fig. 3b). DNase I reduced the percentage of NETs in the sample from a mean value of 68.62% to 1.032% while heparin reduced the percentage of NETs in the samples to a mean value of 6.375%. We tested whether other meningeal pathogens were able to cause NET formation *in vitro* on human neutrophils. All bacteria strains were able to induce NETs significantly within an hour *in vitro* with the exception of *L. monocytogenes* (Appendix III, Supplementary Fig.1).

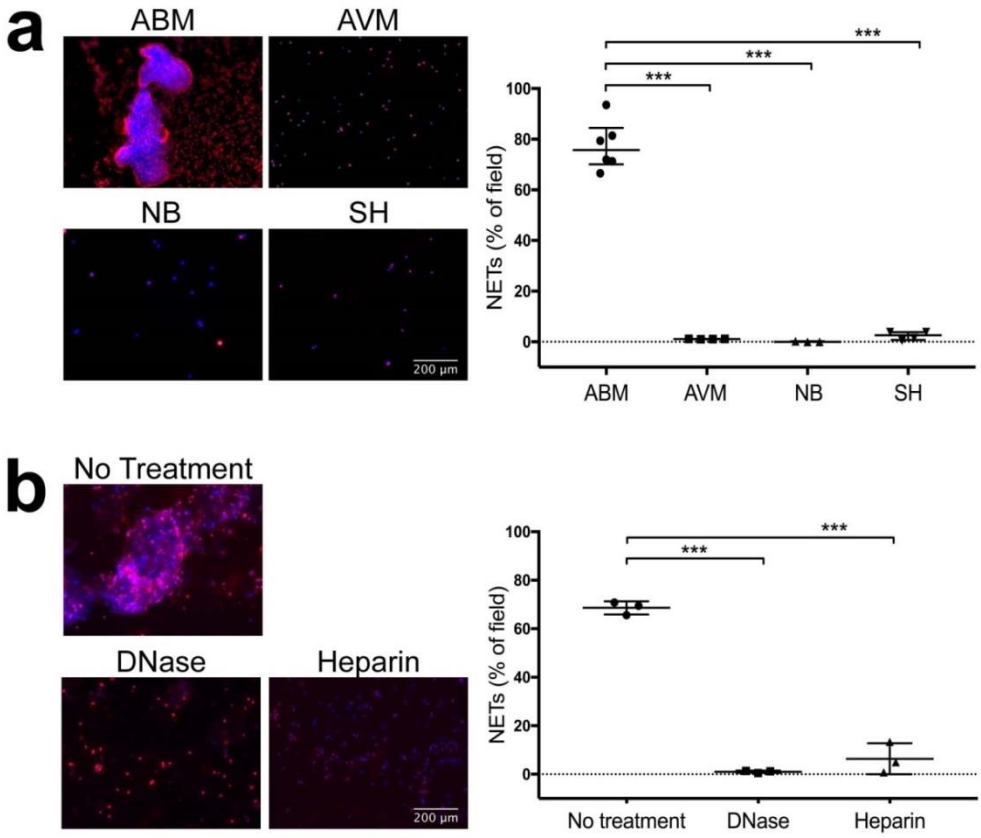

Figure 3 – Neutrophil extracellular traps (NETs) are found in pneumococcal acute bacterial meningitis patients. a) NETs in human CSF samples from patients with acute bacterial meningitis (ABM; n=6), acute viral meningitis (AVM; n=4), neuroborreliosis (NB; n=3), or subarachnoid hemorrhage (SH; n=4) were visualized by immunofluorescence against human neutrophil elastase (red) and DNA (blue). Areas of red and blue co-localization represent NETs. The amount of NETs as a percentage of the total area was determined using Fiji Image J. All groups were compared to ABM by one-way ANOVA followed by Sidak's multiple comparisons test. b) Cerebrospinal fluid samples from some of the ABM patients (n=3) were treated with DNase or heparin prior to visualization by immunofluorescence against neutrophil elastase (red) and DNA (blue). Areas of red and blue co-localization represent NETs. The amount of NETs as a percentage of the total area was determined using Fiji Image J. All groups were compared to the group with no treatment by one-way ANOVA followed by Sidak's multiple comparisons test.

3.2 – Animal Model

We used different *S.pneumoniae* clinical isolates strains of known meningeal pathogens like *S. pneumoniae*, *Neisseria meningitides*, *Listeria monocytogenes*, *Acinetobacter baumanii*, *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus capitis*, and *Streptococcus oralis* (Appendix III, Supplementary Fig. 1). Since our laboratory was not equipped to work with *N. Meningitidis* the pneumococcal strain SP001 isolated from an ABM patient was selected because it was the second best at inducing maximal NETosis in purified human (Appendix III, Supplementary Fig. 1)

and rat (Appendix IV, Supplementary Fig. 2) neutrophils, and was therefore subsequently chosen for the animal model *in vivo* experiments. Our rat models (n=16) were infused subarachnoidally with pneumococci or a saline vehicle control. A higher percentage of NETs were detected in the CSF of infected animals (n=9) (mean value = 71.76%) compared to saline vehicle controls (n=7) (mean value = 1.504%) after 24 hours, as observed by immunofluorescence microscopy, which is similar to amounts observed before in human ABM (Fig. 4a). In order to assess the role of NETs during ABM, DNase I was infused subarachnoidally in the rats (n=15) at two time points. DNase I was infused either at the time of infection (n=9) in the interest of preventing NET formation and at 10 hours after infection (n=6) to simulate a post infection treatment.

NETs were reduced in the CSF of DNase I-treated animals at both time points. Animals (n=9) treated with DNase I simultaneously to the infection (0 hours) had a higher percentage of NETs (mean value = 6.625%) than animals (n=6) treated with DNase I after a 10 hour period (mean value = 2.186%) (Fig. 2a). Bacterial load values in the brains of DNase I-treated animals at both time points showed a significant reduction of infection compared to untreated animals. Infected animals (n=9) had higher value of bacterial load (mean value = 7707) than DNase I-treated animals at 0 hour time point (mean value = 27.04) and 10 hour time point (mean value = 16.39) (Fig. 4b). This indicates that DNase I treatment greatly reduces the CNS infection. Since NETs can prevent bacterial dissemination from the site of infection, it is expected that by clearing them it will allow bacteria to spread. We investigated the bacterial load values of organs where bacteria are known to spread during a brain infection and the blood of infected animals and DNase I-treated animals. Bacterial load values in lungs of DNase I-treated animals (n=5) was significantly reduced at 0 hour time point (mean value = 0) and 10 hour time point (n=6) (mean value = 21.78) compared to infected animals (n=5) (mean value = 194). The spleen of treated animals also showed a significant reduction at 0 hour time point (n=5) (mean value = 0) and 10 hour time point (n=6) (mean value = 18.17) than infected animals (n=5) (mean value = 257.3). The analysis of blood further confirmed that there was no spreading of the bacteria in the treated animals. Blood collected from treated animals (n=9) had a significant reduction in bacterial load when compared to infected animals (n=3) (mean value = 77.78) both at the 0 hour time point (n=3) (mean value = 0) and 10 hour time point (n=6) (mean value =0.9444). Surprisingly, lungs, spleen and blood of DNase-treated animals displayed a reduction in

viable bacteria (Fig. 4b), indicating that DNase treatment somehow was also apparently preventing bacterial dissemination.

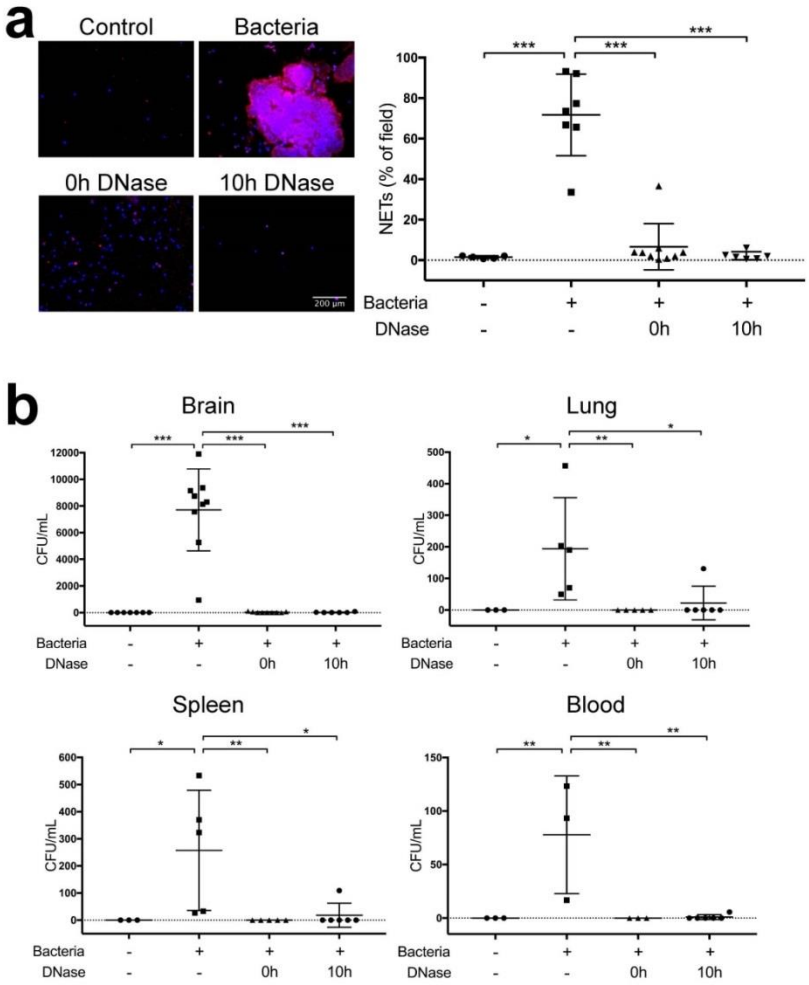

Figure 4 - Neutrophil extracellular traps (NETs) are formed in a rat model of pneumococcal meningitis and dissolution of NETs by DNase results in bacterial clearance – a) To determine the effect of intrathecal DNase treatment, infected rats either received a subarachnoid infusion of 10 units of DNase simultaneously (0h) or 10 hours (10h) after the infection, or they received an equal volume of saline vehicle solution simultaneously to the infection. To determine the effect of intravenous DNase treatment, infected rats either received an intravenous bolus dose of 3500 units of DNase 6 hours (6h) after the infection, followed by intravenous infusion of 780 units/hour over the next 18 hours, or they received an equal volume of saline vehicle control in the same manner. In all cases, uninfected (control) rats received an equal volume of saline vehicle control either intrathecally or intravenously as indicated. All rats were sacrificed 24 hours after the infection. Cerebrospinal fluid was collected and NETs were visualized by immunofluorescence against rat myeloperoxidase (red) and DNA (blue). Areas of red and blue co-localization represent NETs. The amount of NETs as a percentage of the total area was determined using Fiji Image J. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparisons test. b) After the rats were sacrificed, the brain, the right lung, and the spleen were collected and homogenized immediately. The blood was collected centrifuged to obtain plasma. Organ homogenates and blood plasma samples were spread onto agar plates and resulting bacterial colonies were counted after 24 hours. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparisons test (* $P < 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$).

Since intravenous injection in a clinical setting is a more convenient route of administration than intrathecal injection, we investigated the effects of intravenous (I.V) DNase I treatment in our rat model. We injected saline vehicle control or *S.pneumoniae* followed by I.V infusion of either saline or DNase I. The saline vehicle control samples (n=6) displayed a low amount of NETs (mean value = 2.331%) and the infected animals (n=6) displayed a high amount of NETs (mean value = 60.49%) as expected. I.V administration of DNase I was able to clear the NETs from the CSF of infected animals (n=6) showing a significant reduction of NETs observed (mean value = 14.84%) (Fig. 5a). Bacterial loads in the brain, lungs, spleen and blood were also reduced after I.V administration of DNase I. (Fig. 5b). Brain bacterial load values of I.V DNase I treated animals (n=5) (mean value = 364.2) were significantly reduced compared to infected animals (n=6) (mean value = 2926). The mean bacterial load value observed in the lungs of DNase I treated animals (2.5) was significantly lower than the load observed in infected animals (491.1). The spleen of DNase I treated animals also showed a significant reduction of bacterial load (mean value = 1.111) from infected animals (mean value = 171.9). Blood of DNase I treated animals displayed a significant clearance of the bacterial load (mean value = 0) compared to infected animals (mean value = 157.8). These results suggest that I.V. DNase I facilitates bacterial clearance in our meningitis model with the same efficiency as intrathecal injection.

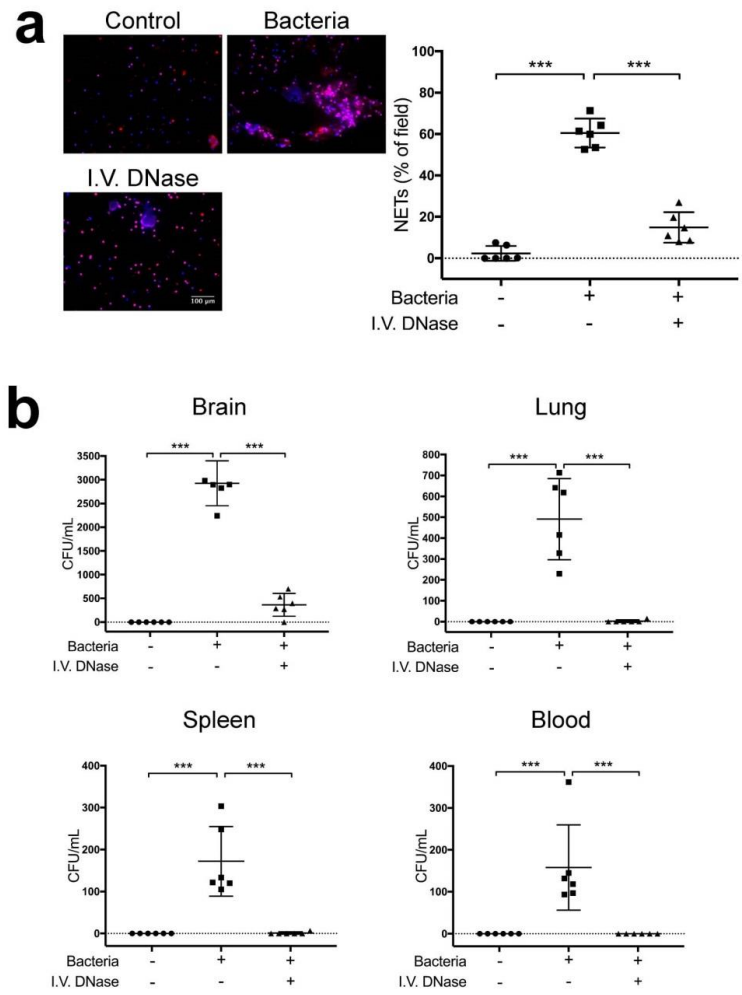

Figure 5 – Intravenous treatment with DNase I. To determine the effect of intravenous DNase I treatment, infected rats either received an intravenous bolus dose of 3500 units of DNase 6 hours (6h) after the infection, followed by intravenous infusion of 780 units/hour over the next 18 hours, or they received an equal volume of saline vehicle control in the same manner. In all cases, uninfected (control) rats received an equal volume of saline vehicle control either intrathecally or intravenously as indicated. All rats were sacrificed 24 hours after the infection. Cerebrospinal fluid was collected and NETs were visualized by immunofluorescence against rat myeloperoxidase (red) and DNA (blue). Areas of red and blue co-localization represent NETs. The amount of NETs as a percentage of the total area was determined using Fiji Image J. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparisons test. b) After the rats were sacrificed, the brain, the right lung, and the spleen were collected and homogenized immediately. The blood was collected centrifuged to obtain plasma. Organ homogenates and blood plasma samples were spread onto agar plates and resulting bacterial colonies were counted after 24 hours. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparisons test (* $P < 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$).

3.3 – Antimicrobial Activity Analysis

NETs were induced *in vitro* by co-incubating purified human neutrophils with *S.pneumoniae* and with control saline or DNase I in order to see if DNase I affects neutrophil-mediated bacterial killing. We observed that NETs and bacteria were significantly reduced in DNase I-treated samples compared to control samples within 30 minutes of stimulation and maximal killing was observed at 3 hours (Fig. 6a). Increased killing by neutrophils in presence of DNase I was observed with several strains of antibiotic resistant pneumococci and methicillin resistant *Staphylococcus aureus* (MRSA) (Appendix V, Supplementary Fig. 3). We also observed that DNase I alone in the absence of neutrophils did not increase bacterial killing (Appendix VI, Supplementary Fig. 4).

Since DNase I didn't have a direct effect on bacteria we hypothesized that one of the major neutrophil killing mechanisms such as phagocytosis followed by MPO dependent hypochlorite production, may be hindered because of the neutrophils also being trapped to NETs and unable to reach NET-trapped bacteria. NETs were induced with *S.pneumoniae* as a positive control and in order to determine the role of the phagocytosis-oxidative burst antimicrobial axis NETs were cleared with DNase I and phagocytosis and oxidative burst inhibitors were added 30 minutes after treatment with DNase I. Neutrophils incubated with bacteria had a higher bacterial load (mean value = 1056) and controls with DNase I clearance of NETs had a lower bacterial load (mean value = 83.98) as expected. We observed that inhibition of phagocytosis through the use of cytochalasin D (mean value = 907.1) significantly decreased bacterial killing. The inhibition of the 2 vital steps in the oxidative burst mechanism through NADPH oxidase inhibition using diphenyleneiodonium (DPI) (mean value = 791.4) or MPO inhibition using a myeloperoxidase inhibitor (MPOI) (mean value = 1081) also significantly decreased bacterial killing (Fig. 6b). We also noticed that bacterial load detected inside the cells after the use of the bactericidal antibiotic gentamicin was higher in the samples with DNase I treatment over a period of 30 minutes (mean value = 36.67) than in samples without treatment (mean value = 8.267) (Fig. 6c). We further analyzed the samples through immunofluorescence microscopy and observed a marked increase of intracellular bacteria within neutrophils in DNase I treated samples, suggesting increased phagocytosis (Fig. 6d). An analysis of peroxidation and chlorination activity, through an MPO activity assay, showed a significant increase in the MPO activity in the

supernatant of neutrophils treated with DNase I (Fig. 6e). DNase I treated samples showed a significantly higher peroxidation (mean value = 7514) and chlorination (mean value = 569121) activity compared to the peroxidation (mean value = 3648) and chlorination (mean value = 145457) activity of samples without treatment. This analysis further corroborated with our speculations that the removal of NETs by DNase was enhancing neutrophil killing mechanisms via the phagocytosis-oxidative burst axis.

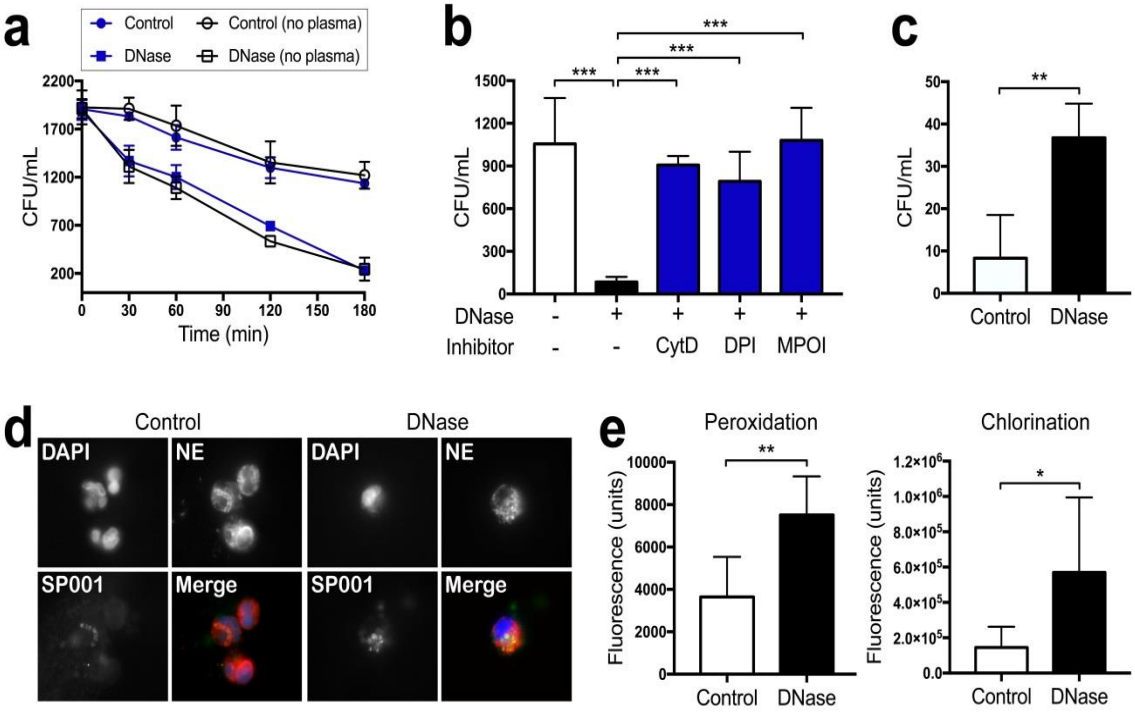

Figure 6. Mechanisms of DNAse-mediated enhancement of bacterial killing

a) Isolated human neutrophils were stimulated with the SP001 strain of *S. pneumoniae* with or without DNase in the presence or absence of plasma. The resulting number of viable colony forming units (CFU) of bacteria was determined after several time points. b) Isolated human neutrophils were stimulated with the SP001 strain of *S. pneumoniae* with or without DNase. Either saline control or the phagocytosis inhibitor Cytochalasin D (CytD) or the NADPH oxidase (ROS production) inhibitor diphenyleneiodonium (DPI) or the myeloperoxidase inhibitor 4-aminobenzoic acid hydrazide (MPOI) was added at the same time. After 3 hours, the number of viable colony forming units of bacteria in the samples was determined. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparison test. c) Isolated human neutrophils were stimulated with the SP001 strain of *S. pneumoniae* with or without DNase. Gentamicin was added to the samples to kill extracellular bacteria and the number of viable colony forming units within the cells, representing phagocytosed bacteria, was determined. Groups were compared by unpaired t-test d) Isolated neutrophils were stimulated with the SP001 strain of *S. pneumoniae*, which was labeled with Oregon Green dye. NETs were then visualized by immunofluorescence against DNA (DAPI, blue) and human neutrophil elastase (NE, red). Areas of red and blue co-localization represent NETs. e) Isolated human neutrophils were stimulated with the SP001 strain of *S. pneumoniae* with or without DNase. Peroxidation and chlorination activity, in arbitrary fluorescence units, was measured in the supernatant by a myeloperoxidase activity assay. Groups were compared by unpaired t-test. Welch's correction was applied to analysis of the chlorination graph because of unequal variance between the groups (as determined by F-test). * $P < 0.05$ ** $P \leq 0.01$. Plasma was excluded from MPO activity assays due to background signal from its inherent peroxidase activity. (* $P < 0.05$ ** $P \leq 0.01$ *** $P \leq 0.001$).

4. Discussion

Our results indicate that different clinical strains of meningeal pathogens are able to promote NETosis, these aligns with a previous report(von Kockritz-Blickwede *et al.*, 2016) and supports that ABM-causing bacteria are the primary NET-inducers in ABM. Our finding of the exclusive and extensive presence of NETs in the ABM cases and the absence of such immunological response in other non-bacterial meningitis highlights a previously unrecognized host defense contribution of NETs *in vivo* during bacterial CNS infections. Our results offer several novel insights into the function and relevance of NETs and NET-associated extracellular DNA during inflammation of the CNS in ABM. It's thought that NETs may have evolved as a mode of defense to immobilize, contain and transfer microbes for later destruction in the reticuloendothelial system during systemic infections(Kolaczkowska *et al.*, 2015). It's known that the CNS environment is more sensitive to damage caused by the inflammation process since it has a less developed lymphatic drainage than in most other organs(Iliff and Nedergaard,

2013). We therefore suggest that NETosis may not be advantageous to the host in ABM. NETosis might make the CNS more susceptible to damage from the inflammation process since it might physically disrupt lymphatic drainage in the CSF. Some bacteria are known to be able to escape these structures making them more of a liability than an immunological asset. Future studies to take into consideration in bacterial meningitis include the detailing of normal and pathological roles of NETs and NET-associated molecules such as HBP(Bentzer *et al.*, 2016; Herwald *et al.*, 2004) and histones(Chen *et al.*, 2014) in ABM. Our data demonstrates that targeting NET-associated extracellular DNA in the CNS with DNase I administered either intrathecally or intravenously significantly increases bacterial clearance in a rat model of pneumococcal meningitis. These results corroborate with a study from 1959 in which Tillett and coworkers suggested a 26 % reduction in mortality by using DNase as an adjunct to penicillin therapy to treat pneumococcal meningitis in human patients(Johnson *et al.*, 1959). The researchers didn't provide a mechanism of action or the source of extracellular DNA at that time due to the lack of tools to identify NETs but they postulated that degradation of DNA somehow exposed invasive bacteria to host defenses and/or antimicrobial therapy. Our results further extend these findings by Tillett and coworkers by identifying the source of extracellular DNA in the CSF as the active release of NETs by neutrophils in response to bacteria. Our findings of enhanced phagocytosis following DNase treatment suggest that NETs might be hindering other immunological cells mechanism of response. We propose that NETs in the CNS might hinder clearance of bacteria since they might also entrap other immunological cells along with the bacteria they are targeting and that DNase I treatment might facilitate processes such as phagocytosis by unmasking bacteria trapped in NETs. Furthermore we hypothesize that the dissolution of NETs catalyzed by DNase releases antimicrobials such as short DNA fragments(Halverson *et al.*, 2015; Bhongir *et al.*, 2017) and NET-bound cationic proteins that then act upon the bacteria. Our collective data leads us to theorize that the overall effect of DNase I allows other modes of neutrophil defense to take over. Our results indicate that an enhanced killing of antibiotic resistant bacteria by neutrophils in presence of DNase I could be a good alternative to antibiotics since DNase I does not directly kill bacteria, making it less likely for bacteria to develop resistance to DNase I. This is an important breakthrough in this era where antibiotic resistant bacteria are progressively having more impact on our health.

In this study we did not identify the pathways of how NETosis is activated and if clearance of NETs in the CSF will be helpful to lessen the tissue damage exerted by the immune response and prevent post infection sequelae. Although we did not check whether DNA fragments coupled with NET-bound cationic proteins occur after DNase I treatment, we clearly show that neutrophil phagocytosis and oxidative burst are elevated after treatment.

Understanding how NETs are formed and their relevance in different types of infections is a great step towards comprehending this immunological reaction of neutrophils and towards the development of new therapeutics. NETs have not only been detected in infections but have also been detected in cancer(Olsson and Cedervall, 2016; Najmeh *et al.*, 2017), autoimmune diseases(Apostolidou *et al.*, 2016; Baldwin and Zunt, 2014; Miller *et al.*, 2014; Fritz *et al.*, 2016) and neurodegenerative diseases like Alzheimer(Pietronigro *et al.*, 2017; Zenaro *et al.*, 2015). Many of these cases show similarities in the onset of the disease either being unable to form NETs or being unable to clear them. SLE patients may hold the key to uncover more important data on NETs. These patients showcase two types of cells on their organism that resemble two steps of either NET formation or clearance(Yipp and Kubes, 2013). Future studies on these diseases may uncover the pathways through which NET formation is regulated.

5. Conclusion

Our findings reveal a novel way to discern between bacterial and viral meningitis based upon the presence of NETs in the CSF. Our data suggest that NETs have a harmful role in pneumococcal meningitis, and that DNase I represents a novel non-antibiotic therapeutic against ABM. Future studies include a continuation of this project with more animal models to evaluate the role of NETs on the damage inflicted to the CNS during the inflammation process in order to infer if treatment with DNase I may prevent this damage and subsequent sequelae in ABM survivors.

Clinical assays on human patients are probably still a distant future despite the pioneering experiment realized by Tillett and his group. Nowadays it's more difficult to be able to leap from animal assays to clinical trials as we have become more aware of the difference between an animal model and a patient. As we come closer to understanding NETs and their role in bacterial clearance, we may soon unlock novel therapeutics to improve outcomes in bacterial meningitis.

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APPENDICES

APPENDIX I - Supplementary table 1 – patient details

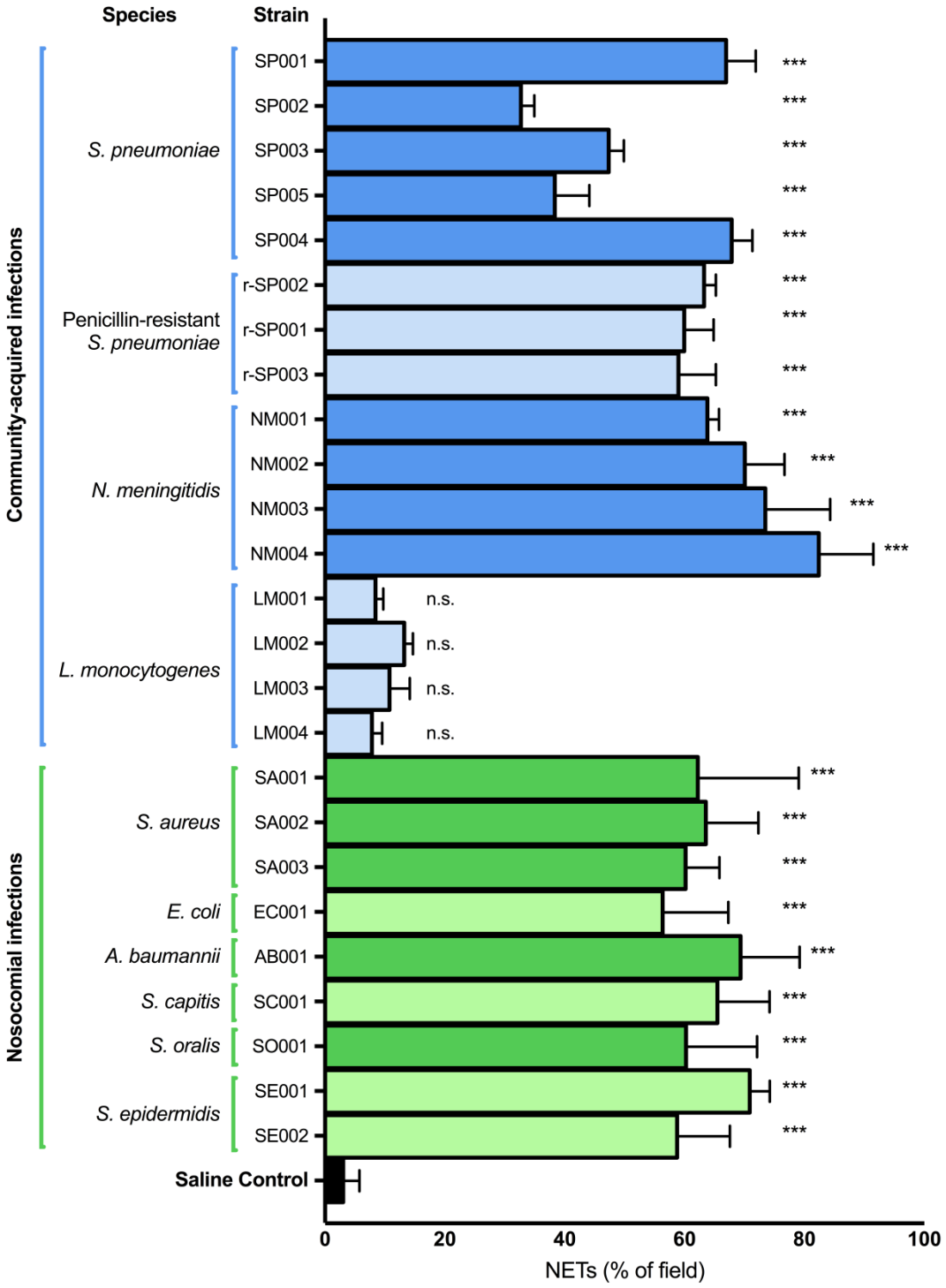
	ABM (n=6)	**VM (n=3)**	**NB (n=3)**	**SH (n=4)**
Male (n, %)	3, 50%	1, 33%	2, 66%	1 (25%)
Age *(mean ± SD)*	46 ± 24	29 ± 13	57 ± 10	48 ± 23
Pathogen (n, %)	*S. Pneumoniae* (6, 100%)	Enterovirus (2, 66%) UNS, 1 (33%)	*B. burgdorferi*, (3, 100%)	None, (4 100%)
Identification method				
Blood culture (n, %)	6, 100%	-	-	-
CSF culture (n, %)	1, 17%	-	-	-
CSF PCR (n, %)	5, 83%	2, 66%	-	-
Serology (n, %)	-		3, 100%	-
Laboratory variables in CSF (mean ± SD)				
Neutrophils ($x10^6$/L)	2240 ± 2090	61 ± 47	7 ± 13	283 ± 324
Monocytes ($x10^6$/L)	211 ± 233	154 ± 148	121 ± 151	374 ± 619
Erythrocytes ($x10^6$/L)	475 ± 585	<300	<300	141000 ± 130000
Lactate (mmol/L)	7.0 ± 4.4	2.3 ± 0.6	2.2 ± 0.6	4.0 ± 1.1
Glucose (mmol/L)	5.3 ± 3.2	4.7 ± 1.4	3.4 ± 0.1	4.3 ± 0.8
Albumin (g/L)	824 ± 1167	394 ± 87	640 ± 267	759 ± 712
Protein (g/L)	2.3 ± 2.4	0.59 ± 0.11	0.97 ± 0.40	1.58 ± 1.45

APPENDIX II - Supplementary table 2 – Spectral counts of NET-associated proteins in CSF

		ABM		NB		AVM		SH	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Pellet	**MPO**	49.0	34.9	0.0**	0.0	5.3*	7.6	4.3**	8.5
	NE	13.0	5.3	0.0**	0.0	1.0**	1.0	1.3**	1.9
	PR3	7.3	2.3	0.3**	0.6	1.7**	2.1	1.3**	1.5
	LL-37	9.7	2.1	0.0**	0.0	0.3**	0.6	0.5**	1.0
	MMP-9	17.7	3.2	0.0**	0.0	2.0*	3.5	0.8**	1.5
	HBP	9.0	7.2	0.0*	0.0	0.0	0.0	0.5*	1.0
	NGAL	16.7	9.9	0.3**	0.6	2.0*	3.5	2.0**	3.4
	S100A8	34.0	26.9	5.3	6.7	5.7	4.9	5.3	3.4
	S100A9	119.7	17.8	9.7**	12.5	11.3**	11.0	7.8**	7.9
	H1.2	1.0	1.0	0.0	0.0	1.7	2.1	0.0	0.0
	H1.4	1.7	1.5	0.0	0.0	0.3	0.6	0.0	0.0
	H4	14.7	16.7	2.7	2.5	8.0	6.6	4.0	1.4
Soluble	**MPO**	14.7	3.2	0.0	0.0	2.7	1.2	0.7	1.2
	NE	5.0	3.6	0.0	0.0	1.3	0.6	0.0	0.0
	PR3	2.0	1.0	0.3	0.6	1.7	0.6	0.3	0.6
	LL-37	5.7	3.8	0.3*	0.6	0.3*	0.6	0.3*	0.6
	MMP-9	19.3	12.1	0.0**	0.0	0.0**	0.0	0.0**	0.0
	HBP	2.7	2.1	0.0	0.0	0.0	0.0	0.0	0.0
	NGAL	14.0	4.0	1.0*	1.7	1.0*	1.0	0.0*	0.0
	S100A8	12.7	4.2	1.0	1.7	3.7	3.8	1.7	2.1
	S100A9	20.7	13.7	2.3	4.0	4.0	6.9	3.0	4.4
	H1.2	0.7	0.6	0.0	0.0	0.3	0.6	0.0	0.0
	H1.4	1.0	1.0	0.3	0.6	0.0	0.0	0.0	0.0
	H4	4.0	3.6	2.0	2.6	2.7	0.6	1.0	1.7

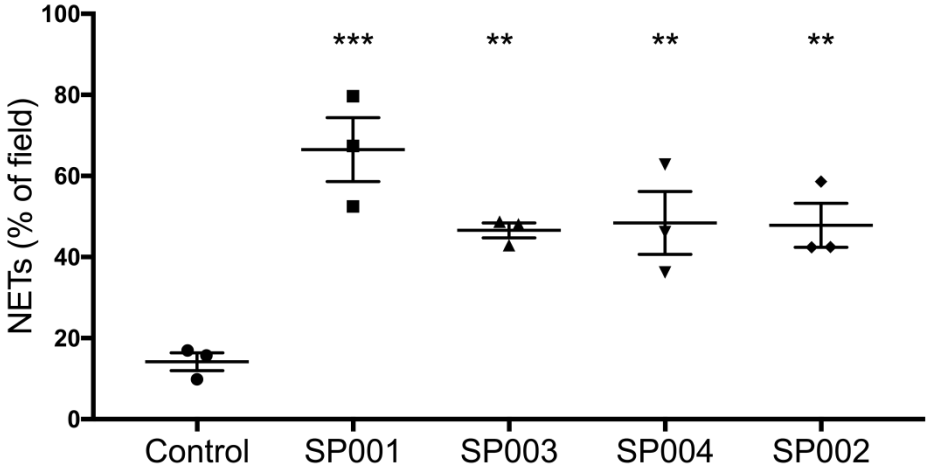
*$p<0.05$, **$p<0.01$ compared to ABM by one-way ANOVA followed by Sidak's multiple comparison test to adjust for the number of comparisons in each group and Bonferroni-adjusted for the number of proteins analyzed.

APPENDIX III - Supplementary Figure 1 – Detection and quantification of NETs *in vitro* by bacteria isolated from patients with acute bacterial meningitis



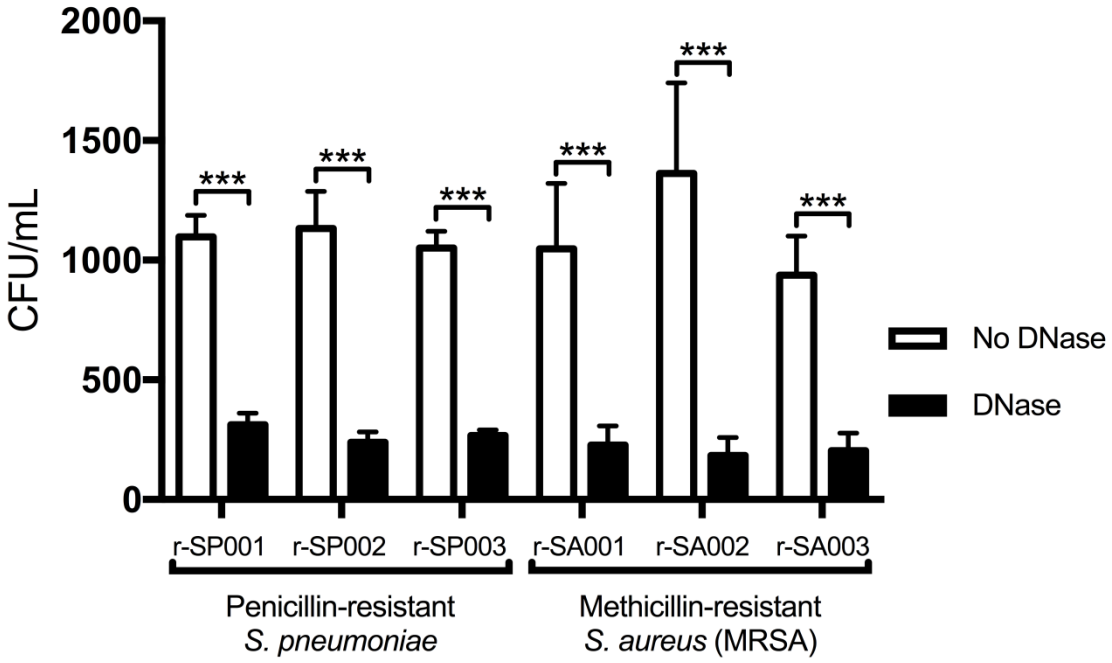
Neutrophils were stimulated with several bacterial strains isolated from either the CSF of blood of patients with acute bacterial meningitis. After 3 hours, NETs were visualized by immunofluorescence against human neutrophil elastase and DNA. The amount of NETs as a percentage of the total area was determined using Fiji Image J. All groups were compared to the saline control group by one-way ANOVA followed by Sidak's multiple comparisons test (*** $P \leq 0.001$, n.s. not significant).

APPENDIX IV - Supplement figure 2. NET formation by rat neutrophils



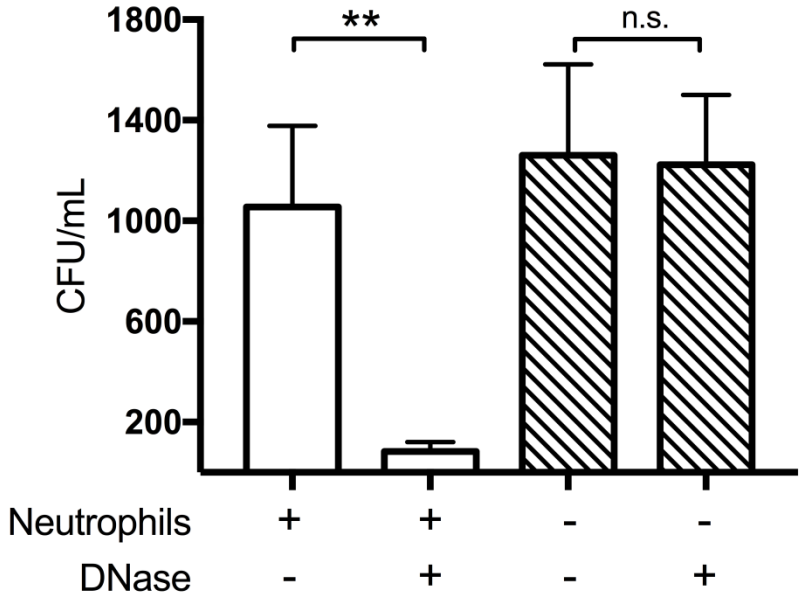
Neutrophils isolated from rats were stimulated with various strains of *S. pneumoniae* or saline control. NETs were visualized by immunofluorescence against rat myeloperoxidase and DNA and the relative amount of NETs was quantified using Fiji Image J. All strains were compared to the control by one-way ANOVA followed by Sidak's multiple comparison test (*** $p<0.001$, ** $p<0.01$).

APPENDIX V - Supplement figure 3. DNase increases bacterial killing of antibiotic resistant strains of *S. pneumoniae* and MRSA.



Neutrophils were challenged with several strains of either penicillin resistant *S. pneumoniae* or MRSA in the presence or absence of DNase. The resulting number of viable colony forming units (CFU) of bacteria was determined after 3 hours of stimulation. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparison test (*** $p<0.001$)

APPENDIX VI - Supplement figure 4. The presence of neutrophils is required for DNase-enhanced killing of bacteria



The SP001 strain of *S. pneumoniae* was stimulated with or without DNase in the presence or absence of neutrophils. The number of viable colony forming units of bacteria was determined after 3 hours of stimulation. Indicated groups were compared by one-way ANOVA followed by Sidak's multiple comparison test (** $p<0.01$).