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Molekulare Mechanismen der NET Bildung in vivo

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1. Introduction

1.1 Neutrophils in inflammation

Elias Metchnikoff first described neutrophils in the late nineteenth century[1]. Neutrophils are the most abundant circulating leukocytes and play a key role in innate immune response. Neutrophils are crucial for defense against invading microorganism[2]. Patients with neutropenia are susceptible to bacterial and fungal infections, demonstrating the importance of neutrophils in host defense[3]. Microorganisms that escape the physical barrier of defense created by the skin and mucous membranes are eliminated by neutrophils that rapidly migrate from the circulation to the site of infection[4].

Neutrophils are the main players in inflammation. Inflammation is the body's protective response to microbial invasion and tissue injury[5]. The primary goal of inflammation is to eliminate the cause of injury as well as clear the damaged tissue[6]. Inflammation delivers neutrophils to the site of injury where they are activated leading to the elimination of the offending substance in one or the other mechanism[7].

The early stages of inflammation are describes as acute inflammation. Typically, the acute inflammatory response is short-lived and leads to tissue regeneration. However, if the cause of inflammation persists or if inappropriately triggered, it will result in tissue injury. The products of activated neutrophils do not distinguish between foreign molecules and host molecules. Hence, a strong reaction against infection may lead to collateral damage to host tissue and prolonged inflammation[8].

Acute inflammation will progress into chronic inflammation, if unresolved[9]. Although neutrophils are the hallmark of acute inflammation, recent studies show that neutrophils play a role in chronic inflammatory diseases including neurodegenerative diseases atherosclerosis, tumour development, chronic obstructive pulmonary disease and arthritis[10,11]. In some cases, they are the key effectors of chronic inflammation due to their direct pro-inflammatory effects, such as the release of reactive oxygen species and neutrophil extracellular traps (NETs), and neutrophil-derived proteolytic activity[12]. In other cases, they support the function of other cells such as platelets monocytes, dendritic

cells (DCs) and lymphocytes[12]. Therefore, the mechanism of inflammation needs to be tightly regulated to minimize damage.

1.1.1 Production and release of neutrophils

Neutrophils are short-lived cells with a circulating half-life of only 6–8 hours. Neutrophils are produced in large numbers every day in the bone marrow. A healthy adult human generates 1 – 2 $x10^{11}$ neutrophils per day at a steady state[13]. The neutrophil population in the bone marrow is subdivided into the stem cell pool, the mitotic pool, and the post-mitotic pool. The stem cell pool consists of undifferentiated hematopoietic stem cells (HSCs), the mitotic pool consists of committed granulocytic progenitor cells, and the mitotic pool consists of fully differentiated mature neutrophils[14]. The release of mature neutrophils is a multistep process controlled by the endogenous cytokine granulocyte colony stimulating factor (G-CSF), expressed by endothelial cells, monocytes, macrophages, and other immune cells.

G-CSF is a 25 kDa glycoprotein that is responsible for the commitment of progenitor cells to the myeloid lineage, the proliferation of granulocyte precursors and release of mature cells from the bone marrow. It regulates the production of neutrophils to meet the requirement of the host during infection[15]. Endogenous plasma levels of G-CSF are elevated during infections due to the need for a higher number of neutrophils[16]. G-CSF also influences the function of mature neutrophils and is therefore increasingly accepted as a regulator of immune responses[17].

G-CSF exerts its effect through a G-CSF receptor (G-CSFR) to carry out its functions. Studies show that mice that lack the G-CSFR are neutropenic[18]. All neutrophils, their precursor cells and primitive haemopoietic stem cells express the G-CSFR[17]. G-CSFR stimulates more than one signal transduction pathways including the Ras/Mek/Erk1/2 pathway, the Src-related kinases Lyn and Hck, the serine/threonine kinase Akt, and the Syk tyrosine kinase and STAT1, 3, and 5 transcription factors[19]. These pathways eventually transmit signals that induce granulocytic proliferation and differentiation[20].

Neutrophils migrate across the bone marrow endothelium to enter circulation in a process called as transcellular migration. Mature neutrophils express CXC chemokine receptor 4 (CXCR4), a G-protein coupled receptor. The ligand for CXCR4 is the stromal-derived factor (SDF-1), produced by bone marrow stromal cells. The interaction between CXCR4 and SDF-1 causes retention of neutrophils within the bone marrow[21]. Mice with a *Cxcr4* deletion in the myeloid lineage, and treatment with CXCR4 antagonists results in increased neutrophil production[22,23]. G-CSF expression induces neutrophil release as it inhibits the CXCR4-SDF-1 interaction[24]. The rate of apoptosis of neutrophils also regulates the production of neutrophils. Macrophages digest the apoptotic neutrophils as well induce the production of cytokines IL 23 and IL 17 that stimulate G-CSF production by immune cells[25,26].

G-CSF is popularly used in clinical medicine as a therapeutic agent[27]. Neutropenia is a significant side effect of many cytotoxic chemotherapies used to treat cancers, predisposing patients to severe infections and compromising the delivery of treatments on schedule and at full dosage. G-CSF administration also reduced the incidence of inflammation[28]. Although G-CSF therapy is generally well tolerated by patients in clinical settings, recent reports link G-CSF administration with vascular complications and exacerbation of underlying inflammatory conditions[29].

1.1.2 Neutrophil granules and granular proteins

As the name suggests, granulocytes are abundant in cytoplasmic granules filled with antimicrobial proteins. These granules form during the transition from myoblasts to promyelocytes[30]. Fixation and staining techniques resulted in the classification of granulocytes into eosinophils, basophils, and neutrophils. Acidic dyes stain eosinophils, and basic dyes stain basophils. A mixture of basic, as well as acidic dyes (neutral dyes) stain neutrophils. Neutrophil granules are classified into four subtypes based on their protein composition, namely azurophilic (primary) granules, specific (secondary) granules, gelatinase (tertiary) granules and secretory vesicles. Each granule contains a characteristic type of proteins. Azurophilic granules contain myeloperoxidase (MPO), defensins as well as neutrophil serine proteases (NSPs). Specific and gelatinase granules include lactoferrin, metalloproteinases and lysozyme; and secretory vesicles which can kill bacteria and fungi[31]. Granular contents except for secretary granules form during

neutrophil maturation. Primary, secondary and tertiary granules are formed in this order. Secretory granules, on the other hand, form through endocytosis in the end stages of neutrophil maturation, and therefore they contain plasma-derived proteins such as albumin[32].

With granules filled with an arsenal of antimicrobial proteins, neutrophils are well equipped to elicit an inflammatory response and eliminate the infectious agents. At the site of infection, they mobilize these granules, exposing granular protein on the cell surface and the extracellular environment. This enables the degradation and the reorganization of the extracellular matrix, favouring neutrophil migration[33]. Alternatively, neutrophils internalize the microbe by phagocytosis, the granules fuse with the phagosome and destroys the microbe by exposing it to a high concentration of antimicrobial proteins[34].

1.1.3 Activation of neutrophils

Neutrophils are activated in a two-stage process involving a "priming step" and an "activation step". Resting neutrophils have a short life as they undergo apoptosis within 6-8 hours, followed by removal by macrophages. Activated neutrophils, on the other hand, have undergone molecular changes that extend their lifespan and enhance their function such as phagocytosis, reactive oxygen species generation, degranulation and formation of neutrophils extracellular traps (NETs)[35]. Exposure of neutrophils to stimuli such as cytokines and chemokines lead to the partial activation or priming of the cell, enhancing its abilities to respond to secondary stimuli[36]. Priming results in the mobilization of intracellular granules, sending pre-formed receptors to the plasma membrane. Thus, increasing the number of surface-expresses receptors. Primed neutrophils enter into a fully activated stage, by upregulation of transcription factors that trigger the *de novo* expression of molecules (receptors and cytokines). Primed neutrophils have increased lifespan, and enhance neutrophil function, as well as the increased potential to release cytokines which further contribute to the inflammatory process[37,38]. Neutrophil priming in chemotaxis is required for the cell to respond to other chemokines, such as MIP1α, allowing for maximal recruitment to the site of inflammation. GM-CSF released at inflammatory sites primes neutrophils to activate receptors present on the cell surface, such as CCR5[39].

1.1.4 Neutrophil receptors

Neutrophils express the various surface receptor. There are surface receptors involves in transendothelial migration (selectins and integrins) and those involved pathogen detection and killing (PRRs, opsonic receptors, G-protein coupled receptors).

1.1.4.1 Receptors involved in transendothelial migration

In response to chemotactic agents, neutrophils exit the circulation and travel to the site of inflammation in a multistep process called transendothelial migration (TEM) or diapedesis. The two significant groups of neutrophil receptors involved in TEM are selectins and integrins. Selectins are a type of cell adhesion molecules (CAM). They are single-chain glycoproteins that recognize carbohydrate moieties and mediate interactions between neutrophils and the vessel wall[40]. There are three subsets of selectins, i.e. P, E, and L selections. P-Selectin is expressed on platelets and in Weibel-Palade bodies of endothelial cells, E-selectin is expressed on endothelial cells during inflammatory responses, and L-selectin is expressed on leukocytes. The interaction between selectins and their ligands on neutrophils initiates the rolling phase of TEM. P- and E-selectins on endothelial cells interact with the P- selectin glycoprotein protein-ligand 1 (PSGL-1) on leukocytes[41]. Integrins are heterodimeric transmembrane glycoproteins present on most mammalian cells. For neutrophils, the most important integrins belong to the β2 integrin family such as LFA-1 and Mac-1, which both bind to endothelial ICAM-1 and are involved in different phases of TEM[42].

1.1.4.2 Receptors involved in neutrophil pathogen detection and killing

Three types of receptors are involved in neutrophil activation to enable pathogen detection and killing. This includes pattern recognition receptors (PRRs), opsonic receptors and G-protein coupled receptors (GPCRs).

a) Pathogen recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs). PAMPs include microbe-specific molecules, bacterial DNA and bacterial cell wall components. DAMPs are host-derived proteins/peptides, such as necrotic cell matter. DAMPs occur during sterile inflammatory episodes such as in burns or hypoxia. Most of PRRs resulting in

phagocytosis are C-type lectin receptors, such as Dectin-1[43]. The non-phagocytic PRRs are the Toll-like receptors (TLRs) which recognize both host-derived and pathogenic molecules such as lipids, carbohydrates peptides, DNA and RNA[44]. TLRs are present on the cell surface so that they can sense products of extracellular and ingested microbes. The engagement of TLRs activates neutrophils, carry out their microbial killing functions, enabling the production of microbicidal substances and cytokines as well as reduces the rate of neutrophil apoptosis[45].

b) Opsonic receptors are critical for neutrophil-mediated pathogen killing. Microorganism maybe coated with antibodies, complement proteins, and lectins. The most efficient way that facilitates rapid uptake and strong stimulation of neutrophil killing mechanisms is the coating of particles with IgG antibodies and components of the complement system such as C3. These are recognized by high-affinity FCγ receptor of phagocytosis called FCγRI and type1 complement receptor (CR1) respectively[46].

c) G Protein-coupled receptors bound neutrophils have diverse downstream signalling outcomes. Ligands that can bind include bacterial peptides containing N-formyl methionyl residues and endogenous molecules such as the chemokine interleukin-8 (IL-8/CXCL8) and breakdown complement products such as C5, platelet activating factor, and leukotrienes that results in neutrophil chemotaxis. Other GPCR signalling results in neutrophil activation which endows the cell with the ability to kill invading microbes[47].

Neutrophil activation may occur as a receptor-independent mechanism. NADPH oxidase complex is activated by a number of non-physiological agents including calcium ionophore ionomycin and diester phorbol 12-myristate 13-acetate (PMA)[48,49]. Shortly after PMA stimulation, the components of NADPH oxidase assemble on the plasma membrane as well as the granule membrane, resulting in the generation of reactive oxygen species. Thus reactive oxygen species (ROS) production occurs both extracellularly and intracellularly[50].

1.1.5 Neutrophil-mediated microbial killing

Neutrophils eliminate microbes via more than one effector mechanism. Based on the strength and type of stimuli, neutrophils decide the appropriate effector mechanism.

1.1.5.1. Phagocytosis

Phagocytosis is a receptor-mediated process of pathogen engulfment by neutrophils into special compartments called phagosomes[51,52]. Phagocytosis occurs through the binding of Fc receptors and complement receptors expressed on the neutrophils[53]. Bacteria that have been opsonized by antibody or complement molecules bind to FcγRII or complement receptors respectively[54]. Phagocytosis involves the activation of Src-tyrosine kinases which in turn trigger various signaling pathways[52]. Within the cell, bacteria is destroyed by the merging of vesicles containing microbicidal molecules with the phagosome. In addition to this, the generation of reactive oxygen species (ROS) occurs within the phagosomal space resulting in a "respiratory burst," and killing the engulfed microbes[35]. ROS is generated by NADPH oxidase, that includes membrane components gp91phox, g22 phox, and the GTPase Rac1 and cytosolic components p47 phox, p67 phox, and p40 phox, which when assembled become the functional oxidase. NADPH-oxidase initiates the electron transfer to oxygen resulting in superoxide (O_2^-) generation. Superoxide dismutase (SOD-2) converts superoxide to hydrogen peroxide (H_2O_2). Finally, H_2O_2 is converted to hypochlorous acid (HOCl) by myeloperoxidase or to water by the enzyme catalase[55,56,57]. HOCl is a powerful antimicrobial agent. The first known function of ROS in neutrophils was its microbicidal activity. Patients with the chronic granulomatous disorder (CGD) show severe neutrophils killing defect due to the absence of respiratory burst[58]. CGD is a genetic disease as a result of dysfunction of NADPH oxidase[59]. During neutrophil phagocytosis of large foreign particles, ROS and cytolytic content may get released into the extracellular environment. This process is known as "frustrated phagocytosis" and can cause damage to other cells[60].

1.1.5.2. Degranulation

Neutrophils produce an array of microbicidal proteins including hydrolytic enzymes and antimicrobial peptides (AMPs) capable of digesting pathogens and their products via non-oxidative pathways[61]. As mentioned earlier, there are three types of granules in the neutrophils namely, the primary, secondary and the tertiary granules. Activation of the neutrophils involves the mobilization of their granules[31]. At the site of inflammation, these granules may fuse with the plasma membrane and release their contents into the extracellular space. Degranulation contributed to the generation of an inhospitable

environment for the microbe[62]. Not much is known about the mechanism that regulates neutrophil degranulation. All evidence about degranulation is only through *in vitro* experiment. Whether this process occurs during an *in vivo* inflammatory response is not well known[31].

1.1.5.3. Neutrophil extracellular traps (NETs)

NET formation is a novel form of extracellular bacterial killing. In 2004, Brinkmann and colleagues described a process by which neutrophils extrude a meshwork of chromatin fibers decorated with granule-derived antimicrobial proteins called NETosis. It was observed that when *in vitro* neutrophil were overlaid with bacteria, they became activated and rapidly ejected their DNA into the extracellular spaces to trap the passing bacteria. This results in the formation of a complex web-like structure, primarily comprised of the long strands of decondensed DNA, decorated with histones, and over 30 neutrophilic proteins that disarm a variety of microbes[63]. The following section will elaborate on NETs.

1.2 Discovery of NETs

Neutrophils elastase (NE) an antimicrobial protein present in neutrophil granules has been shown to degrade virulence factors of various bacterial pathogens. During the microscopic analyses of extracellular NE, Brinkmann and colleagues found that NE is localized on an unconventional extracellular structure formed of DNA and histones. Through this discovery, they described for the first time that nuclear material plays a role in fighting infections. The complex web-like structure of NETs creates a physical barrier to prevent the dissemination of the microbes[64].

1.2.1 Composition and architecture of NETs

High-resolution scanning electron microscopy showed that NETs are made of fine thread-like structures. The major portion of these structures is smooth with a diameter of 15-17 nm that are decorated with globular structures with a width of 25 nm. The smooth

structures aggregate to form thicker, more stable structures resulting in a mesh-like network that can trap pathogens[64,63].

NETs are primarily composed of double-stranded DNA and histones. This was evidenced by the digestion of NETs upon treatment with DNases, as well as staining with DNA intercalating dyes. The nucleosomal complex is made of histones and facilitates the folding of DNA and condensation of the chromatin. Histones account for 70% all proteins found on NETs. Four major types of histones, ie. H2A, H2B, H3, and H4 form the core histones [65]. These histones form dimers and act as a spool around which DNA is wound. Histone H1 is the linker histone that binds to the short DNA stretch present between two nucleosomal complexes called the linker DNA, further supporting the folded chromatin. Studies show that only trace amounts of histone H1 are found in NETs, indicating the presence of decondensed DNA[63].

Granular components make up for 20% for the protein composition of NETs (Urban et al. 2009a). They contain proteins from azurophilic/primary granules, i.e., the neutrophil serine proteases (NE, Cathepsin G and Proteinase 3) and MPO. They also contain specific granule contents such as lactoferrin, lysozyme, antimicrobial LL-37, and pentraxin as well as gelatinase from tertiary granules. All the proteins on NETs maintain their catalytic activity[63,66].

1.2.2 Formation of NETs

NET formation was initially described as an active cell death process resulting in the extrusion of unfolded chromatin and hence was named NETosis, resembling apoptosis and necrosis[67,68]. Recent studies have now described two major mechanisms involved in NET formation namely suicidal NETosis and vital NETosis (Scheme 1). Both mechanisms are similar in their requirement of neutrophil activation and several components involved but, differ in the timing, origin of NET-DNA, composition of NETs and fate of NET forming neutrophils[69].

1.2.2.1 Suicidal NETosis.

Suicidal NETosis is characterized by plasma membrane rupture and cell death. It is a multistage process characterized by distinct morphological changes that last from 2 to 4

hours[70]. It is an NADPH oxidase-dependent process initiated by the engagement of specific cell surface receptors and the activation of the Raf-MEK-ERK pathway[67]. Naive neutrophils have a spherical morphology. Upon stimulation, they become flat and display several cytoplasmic granules. Next, the nucleus loses its characteristic lobulated morphology. A few minutes later the granular membranes begin to disintegrate allowing the granular proteins to translocate into the nucleus. This is followed by a visible decondensation of the chromatin and disintegration of the nuclear membrane. The chromatin continues to unwind after nuclear envelope breaks down, occupying all the available space within the cytoplasm. During this process, de-condensing chromatin is loaded with granular and cytoplasmic components. Finally, after this intracellular assembly of NETs the plasma membrane ruptures, allowing for the NETs to be ejected into the extracellular space[67,71]. Suicidal NETosis has proven to be useful when the conventional functions of neutrophils have been compromised or insufficient[72].

Suicidal NETosis is different from apoptosis and necrosis (Table 1). Apoptosis, defined as programmed cell death, is a silent form of cell death. It is designed to limit the exposure of intracellular components to the extracellular environment[73]. It is characterized by the exposure of phosphatidylserine (PS) on the plasma membrane. PS is usually localized to the inner leaflet of the cell membrane but flips to the outer leaflet in apoptotic cells. PS acts as an "eat-me-signal" and mediates the phagocytosis of apoptotic cells by macrophages[74]. Necrosis is a cell death process characterized by the plasma membrane disintegration and exposure of intracellular components[75]. Necrosis is different from NETosis as the decondensation of the chromatin occurs after the perforation of the plasma membrane. Therefore the extracellular chromatin is not loaded with granular or cytoplasmic proteins[76, 64].

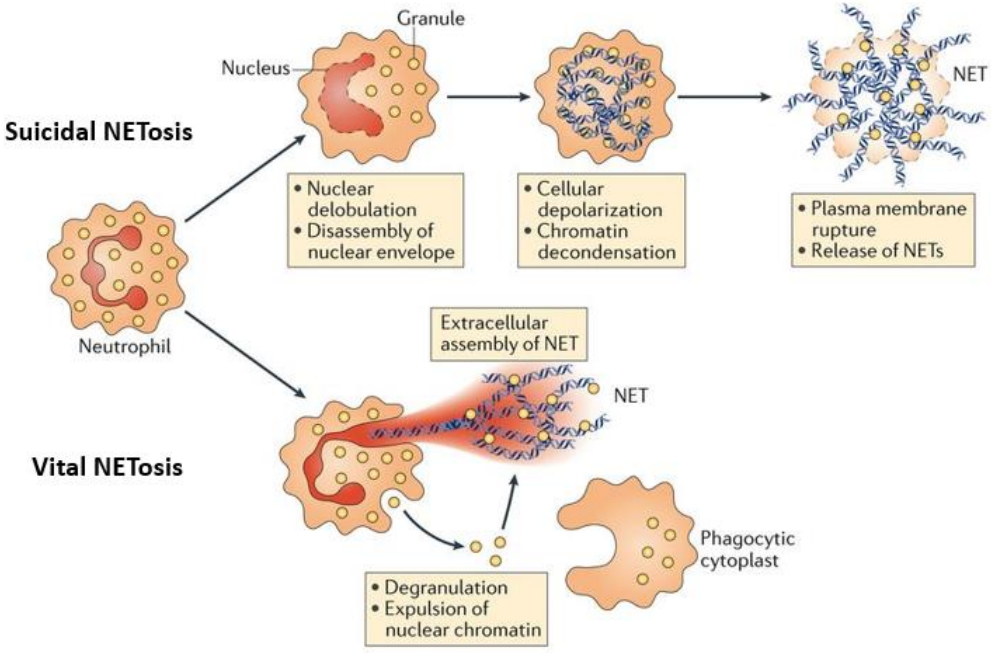
NETosis	Apoptosis	Necrosis
Programmed cell death	Programmed cell death	Cellular damage causing release of intracellular contents
Chromatin decondensation	Chromatin condensation without nuclear membrane disintegration	Cellular swelling and bursting

Phosphatidylserine not exposed	Phosphatidylserine exposed	Phosphatidylserine exposed during early steps of necrosis
No DNA fragmentation	DNA fragmentation	DNA fragmentation
Independent of caspases and RIP 1 kinase	Dependent on caspase and RIP 1 kinase	Dependent on caspase and RIP 1 kinase
Vacuolization	Membrane blebbing	Membrane and organelle disintegration

Table 1 : Difference between NETosis, apoptosis and necrosis[76].

1.2.2.2 Vital NETosis

Vital NETosis is a rapid process that lasts between 5 to 50 minutes wherein the viability and functionality of the neutrophil are retained following NET release[77]. It is characterized by the vesicular trafficking of DNA from the nucleus to the extracellular space. Electron microscopy analysis shows that this is made possible by a phenomenon known as nuclear budding. The nuclear membrane remains intact as the decondensed chromatin is packed into functional vesicles. These vesicles are transported across the cytoplasm. Next, they fuse with the plasma membrane and are exocytosed by the cell, leaving the plasma membrane intact, and the cell alive. This process continues until the nucleus is entirely exteriorized and an anuclear cytoplast remains[78,77]. However, it is currently not clear how granular components assemble with DNA in vital NETosis. It is hypothesized that the NET-components could be assembled in the extracellular space, as granular content is released by classical degranulation. A subtype of vital NETosis may also be a result of the release of mitochondrial DNA[79,80]. Consequently, mitochondria-derived NETs lack histones. Neutrophils being the terminally differentiated cell with low transcriptional activity, the loss of nuclei does not incapacitate the cell. Thus, vital NETosis allows for the co-existence of NET release and conventional neutrophil host defense. Studies have shown that these neutrophils after releasing NETs are still capable of phagocytosis and degranulation[76,81].



Scheme 1: Suicidal and Vital NETosis: NETs form via a suicidal or a vital pathway. The suicidal pathway begins with nuclear delobulation and breakdown of nuclear membrane followed by chromatin decondensation and plasma membrane rupture. The vital NETosis, involves the expulsion of decondensed chromatin that is accompanied by the release of granule proteins through degranulation. These components assemble extracellularly leaving enucleated cytoplasts that continue to ingest microorganisms**.** (Scheme adapted from V. Pappayanopoulous, Nature Reviews Immunology, 2017[82])

1.2.3 Induction of NETosis

Many stimuli are reported to induce NETosis including sterile as well as infectious stimuli (Table 2). For *in vitro* studies the most frequently used stimulus is phorbol myristate acetate (PMA), a plant-derived organic compound. It is a well-known activator of the ubiquitous signal transduction enzyme proteins kinase C (PKC). Concentrations ranging from 5 nm to 100µM have been reported to cause NETosis within a timeframe of 10 minutes to 24 hours[83]. *E.coli, P.aeruginosa, C.albicans,* and *M.bovis* have also been described as potent inducers of NETosis that subsequently get trapped in NETs[84]. In addition to this, bacterial products such as LPS have been investigated[63,85]. Studies show that a high concentration of glucose (20nM-30nM) that resemble a hyperglycaemic environment appear to induce NETosis while low concentration (5-10nM) do not. Other potent inducers of NETs include calcium ionophores A23187, ionomycin, IL-8, TNF-alpha and ROS[84].

Many stimuli require cell surface receptors to be recognized by neutrophils. The engagement of TLRs that sense microbes or microbial compounds, Fc-receptors bind immunoglobulins, complement or cytokines receptors are implicated in the induction of NETosis[85,86]. TLRs are an important group of pathogen recognition receptors (PRRs), belonging to the IL-1R/TLR family of receptors, that regulate innate immune response[87]. There are 13 TLRs identified in mice and 10 in humans[44]. Neutrophils express most of the TLRs that respond to a range of pathogen- associated molecules. This includes LPS, lipoproteins and peptidoglycans, bacterial DNA leading as well as host immune complexes. TLR activation leads to an increase in cytokine and chemokine production[47]. Platelets express TLR4 through which they bind to neutrophils and induce NETosis[85].

The TLR signal transduction pathway is mediated by recruitment of the myeloid differentiation primary response 88 (MyD88) adapter[88]. Stimulation of the MyD88 pathway leads to activation of the MAPK and NF-κB signalling pathways leading to production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12, IL-6, and IL-8[89]. The importance of MyD88 in inducing NETosis is highlighted by studies where mice deficient in MyD88 are highly susceptible to infection with *Staphylococcus aureus***,** *Pseudomonas aeruginosa*, and *Toxoplasma gondii*. These mice exhibit deficient neutrophil recruitment following infection compared to wild-type controls[90,91].

The binding of G-CSF to its receptor leads to internalization of the receptor and immediate activation of neutrophils enhances the phagocytic activity and induces degranulation[92]. Neutrophils of mice receiving daily G-CSF as well as neutrophils of mice bearing G-CSF-producing cancer cells are predisposed to generate NETs after activation[93]. This suggests that G-CSF is a potent inducer of NETosis.

Inducer	**Concentration**	**Induction time**
PMA	4-50nM	10min-16h
H_2O_2	100-1000µM	4h
Growth factors/Platelets		
IL-8	1-250 ng/ml	10min-5h
IL-1ß	50ng/ml	2h
TNF-α	100ng/ml	2h
Activated platelets	2 x10^5-5 x 10^5	1h
Calcium		

A23187	0.2-25µM	20min-4h
Ionomycin	0.9-7µM	30min-4h
MSU crystals	100-200µg/ml	3-5h
Glucose	20-30nm	2h
Glucose oxidase	100mU/ml	1-4h
Bacterial/ fungal products		
LPS	0.1-10µg/ml	15min-18h
ß-Glucan	200 µg/ml	15min-240min
Bacteria/ Fungi		
S.aureus	0.03-50 MOI	30min-24h
S.pneumonia	10 MOI	10min -24h
P.aeuruginosa	10-100 MOI	10min-24h
A.fumigatus	750 CFU / 50µl	2h
C.albicans	0.2-4.2 MOI	5min-4h
M.bovis	10 MOI	4h
E.coli	3-50 MOI	10min-24h

Table 2: Selected inducers of NETs[84].

1.2.4 Regulation of NETs

The molecular mechanism of suicidal NETosis is best understood. The hallmarks of suicidal NETosis is the activation of neutrophils, followed by the delobulation of the nucleus, decondensation of chromatin and the eventual rupture of the plasma membrane releasing NETs into the extracellular space. Three regulatory mechanisms involving ROS, NE and peptidyl arginine deiminase 4 (PAD4) describe the process of chromatin decondensation[70,94]. Recent studies show that pore forming protein gasdermin d (GSDMD) is responsible for plasma membrane rupture[95].

1.2.4.1 Reactive oxygen species, neutrophil elastase and gasdermin D

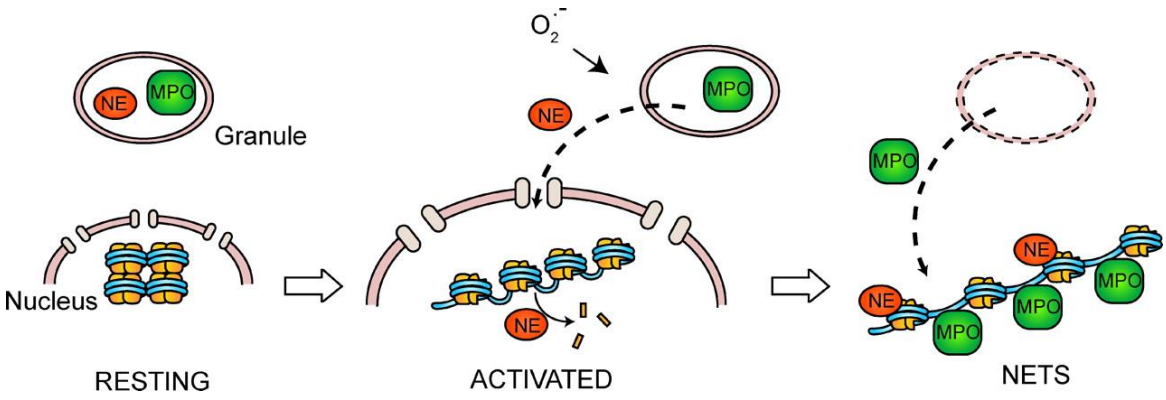
NETs formation requires an NADPH oxidase-dependent process called the "respiratory burst"[96]. NADPH oxidase is a multimeric protein that assembles during activation on the cytoplasmic membrane and membranes of specific granules. The enzyme catalyzes the electron transfer to oxygen resulting in the formation of superoxide. Superoxide dismutase dismutase catalyses conversion of superoxide into hydrogen peroxide. These primary ROS ($O_2^{\cdot}$ - and H_2O_2) undergo further transformation by MPO producing

hypochlorous acid. Hypochlorous acid has a strong microbicidal effect[97]. NETosis is severely impaired in neutrophils from patients with mutations in NADPH oxidase and MPO. The signal pathways leading to NADPH oxidase activation includes Raf/MEK/Erk, PKC[98] and Rac2 (a small GTPase of the Rho-family)-mediated pathway[99] and any defect in these proteins also interfere with ROS production and NETosis.

The involvement of ROS in NET formation is best illustrated by neutrophils of patients with CGD. CGD patients suffer from recurrent infections life-long as their neutrophils do not form NETs. However, it was shown that treatment of neutrophils of CGD patients with H_2O_2 restores the ability to release NETs[96].

Upon activation of neutrophil, the ROS species generated by NADPH oxidase, and MPO triggers the release of NE, a serine protease that is present in the primary granules of naive neutrophils , into the cytoplasm[100]. The cytoplasmic NE then translocates to the nucleus guided by a nuclear localization signal. It cleaves core histones and H1. MPO translocated to the nucleus as well, and facilitates the fragmentation of histone and unfolding of the chromatin[70]. A key event in NETosis is the unfolding of the chromatin (Scheme 2).The degradation of histone by NE is the reason why histones in NETs is smaller in size[65]. NE may be inhibited by an endogenous inhibitor SerpinB1, which locates to the nucleus during NETosis and thereby limits the activity of NE[101].

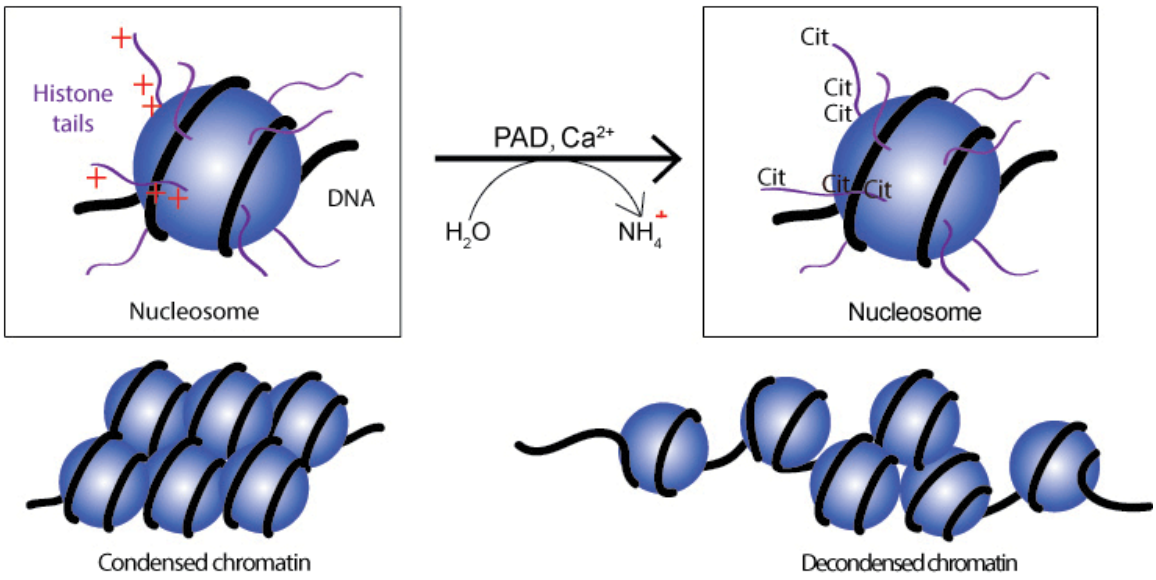
The mechanism by which neutrophil plasma membrane ruptures and NETs are released was poorly understood. Recent studies have demonstrated that NET formation is dependent on the cleavage of GSDMD[95,102]. GSDMD is a pore-forming protein that executes pyroptosis. Under normal conditions, the full-length GSDMD is inactive as the C-terminal domain inhibits the pore-forming capacity of the N terminus [103]. In neutrophils, GSDMD is cleaved by NE, releasing the N-terminal domain to perform its pore forming activity and causing lyses and cell membrane rupture. GSDMD, in turn, forms pores in the granule membrane, thus enhancing NE release into the cytoplasm and allowing further GSDMD cleavage in a feed-forward loop. There GSDMD has two functions in NETosis. Firstly, to release NE allowing it to translocate to the nucleaus and cleave histones. Secondly, to form pores in the plasma membrane to release NETs [95].



Scheme 2: NE and MPO are stored in the azurophilic granules of resting neutrophils. In activated neutrophils, ROS production causes NE release. NE translocate to nuclease and cleaves histones. MPO promotes decondensation. (Scheme adapted from V. Pappayanopoulous *J. Cell Biol.*, 2010[70]).

1.2.4.2 Peptidyl arginine deiminase 4

PADs are a family of 5 calcium-dependent enzymes present in human (PAD1-4, PAD6). Among the five PAD enzymes encoded in humans, PAD4 is especially important in neutrophil biology. This enzyme was cloned from a human myeloid leukemia cell line (HL-60 cells) that were induced to differentiate into granulocytes [104]. PADs are expressed in various cell types including granulocytes and cancer cells. The primary function of this enzyme is to catalyze the post-translational modification, converting arginine residues to citrulline (non-ribosomally encoded). Citrulline is a non-essential amino acid that is generate in proteins by post-translational conversion of arginine residues to peptidyl citrulline. This process is called citrullination or deamination. During NETosis PAD4 citrullinate histones, both core histones as well as the linker histone. Citrullination reduces the net positive charge of histone and increases hydrophobicity (Scheme 3). The loss of charge weakens their interaction with the negatively charged DNA and promotes the disassembly of histone-DNA complex. Citrullination is a key event during NETosis[105,106,107]. Citrullinated histones are associated with many pathogenic states such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS), sepsis, and thrombosis[108]. As mentioned earlier PADs are calcium-dependent and may be activated with increased concentration of calcium in the cytosol.



Scheme 3: PAD 4 mediates histone tail citrullination leading to chromatin decondensation. (Scheme adapted from S.Mohanan, *Biochem. Res. Int.* 2012[109])

1.2.5 Clearance of NETs

The clearance of NETs from inflamed tissue is poorly understood. NETs create a locally high concentration of active molecules which in addition to being harmful to the invading microbe may also pose a threat to host tissue. Therefore the timely removal of NETs is crucial to maintaining host homeostasis and prevent systemic inflammation and autoantibody production.

Human and murine serum contains two endonucleases namely DNASE1 and DNASE1-LIKE 3 (DNASE1L3) that degrade extracellular DNA. Both enzymes belong to the DNASE1 family of proteins, are Ca^{2+}/Mg^{2+}-dependent and cleave double-stranded DNA. DNASE1 is a secretory enzyme and is secreted by a variety of endocrine and exocrine glands, especially those of the gastrointestinal and urogenital tract. DNASE1L3 is secreted in the spleen macrophages, thymus, lymph nodes intestines as well as hepatic Kupffer cells. NETs are degraded by serum DNASE1[110], although a study shows that serum DNASE1 is not able to degrade NETs at a physiological concentration[111]. DNASE1 preferentially digests naked DNA and is able to do so only in the presence of

proteases. DNASE1L3 cleaves nuclear DNA or chromatin without the proteolytic help[112, 113, 114].

NETs may be phagocytosed by DCs. DNA can be sensed by some extra and intracellular receptors. Extracellular DNA, activates TLR-9 present in the phagosomes of monocytes and dendritic cells. DNA complexed with LL37 or HMGB1 form stable structure that activates DCs[115]. NETs are rich in histones. It's been reported that histones activate TLR2 and TLR4 suggesting that NETs serve as innate immune activators through different receptors[116]. Human monocyte-derived macrophages are able to engulf NETs[111]. NETs were also shown to be opsonized by C1q a component of the classical complement pathway and cleared away by macrophages. Finally, inflammation is often associated with pus formation. NETs structure were found in the pus indicating the route of NET clearance.

1.2.6 Antimicrobial function of NETs

NETs were first discovered during *in vitro* infection with the bacteria *Shigella flexneri*[63]. Since then NETs have demonstrated a broad effectiveness against different pathogens, including bacteria, viruses, fungi, and parasites. Experiments indicate that NETosis is triggered by specific microorganisms[117].

Bacteria:

Many gram positive and gram negative bacteria have been reported to induce NETosis. Whole bacteria, as well as cell surface components of bacteria, lipoteichoic acid and LPS, and breakdown products of prokaryotic proteins, such as fMLP, may act as the stimuli[105]. Important examples of bacteria that induce NETs include *S.aureus*, *Streptococcus spp.*, *H. influenza*, *K. pneumoniae*, *P.aeruginosa*, *L. monocytogenes*, *M. tuberculosis*, and *S. flexneri*. Containment of microbial pathogens to sites of initial infection may be an important function of NETs. However various bacteria express virulence factors that degrade extracellular traps and may free the bacterium from NET chromatin[118]. *S. aureus* expresses pore-forming virulence factors neutralizing neutrophils by inducing necrosis at the expense of NETosis[119]. Methicillin-resistant *S. aureus* (MRSA) expresses extracellular nucleases for biofilm dispersal and degradation of NETs. *S. pneumoniae*

escapes NETs in a passive manner through its polysaccharide capsule reducing NET binding[120]. *S. pneumoniae, S. pyogenes* and *V. cholerae* express nucleases which facilitates the escape by degrading NETs. Some strains of *S. pyogenes* resist extracellular killing be by the sequestration and neutralization of the LL37 , a neutrophil antimicrobial peptide[121, 122, 123]. Therefore, it is now established that microbes may develop adaptations to resist NET-mediated killing.

Viruses:

A limited amount of study has been carried out to understand the role of NETs in viral infections. Recent studies have investigated the role of NET formation in response to viral infection. HIV-1 nucleic acids have been shown to induce neutrophils through TLR7 and TLR8, generating ROS and eventually inducing NETosis. NETs capture the HIV virions and expose them to MPO and alpha-defensins[124]. Some studies have also shown the activation of neutrophils by Influenza A via PAD4. The NET-associated α-defensin-1 blocks its replication of the virus by blocking protein kinase C pathway. NETs have also been shown to be induced by dengue virus and respiratory syncytial virus[125]. In most cases virus induces NETs to exert a dual protective and pathogenic role[126]. Hantaviruses rodent-borne human pathogens strongly induce NET. Hantaviruses replicate in endothelial and epithelial cells in the without any cytopathic effects. Studies have shown that. Hantavirus induces high systemic levels of NETs in the circulation of infected patients. It induces ROS production and NET formation through the β2 integrin receptor signaling[127].

Fungi:

C.albicans is a fungus that develops long hyphae that is impossible to phagocytose. The long and sticky strands of DNA in NETs provide an ideal strategy to trap these long structures and prevent the spread of fungal infection. Furthermore, calprotectin in NETs is a major anti-fungal agent. It can chelate magnesium and zinc ions requested for *Candida* growth[65]. Other fungi such as *A.fumigatus, A.candida* and *C.neoformans* can also induce NET formation[128,129].

Parasites:

NETs have also been implicated in controlling protozoan parasites. Elevated NETs in circulation was during *P.falciparum Leishmania* and *T.gondii* infections. NETs entangle and immobilize parasites, decreases their viability and controls infection[130,131]. Other animal parasites such as *E.bovis* and *B.besnoiti*, have been reported to induce NET formation[117].

1.3 Neutrophils and NETs in diseases

1.3.1 Neutrophilia

Neutrophilia is defined as the higher than the usual number of neutrophils in circulation. The normal neutrophil count in circulation is 2.5 to 7.5 x 10^9 / l in healthy humans[132]. In healthy adults, a neutrophil number greater than 7.5 x 10^9/ l is clinically diagnosed as neutrophilia. It is caused by an increased production and mobilization of neutrophils from bone marrow to the bloodstream. Neutrophilia is in itself asymptomatic. Healthy neonates and pregnant women have an increased number of neutrophils without any pathologies[133, 134]. In most cases, it is a secondary condition underlying more severe conditions such as infections, chronic inflammation, cancer, etc. Neutrophilia may be categorized as primary neutrophilia, caused due to abnormalities in the regulation of bone marrow, or secondary/reactive neutrophilia, which is a response to ongoing processes, such as infection, inflammation, smoking, stress, medication or malignancy (Table 3)[135,136.]

Primary neutrophilia
Congenital neutrophilia • Hereditary neutrophilia • Chronic idiopathic neutrophilia • Down syndrome • Leukocyte adhesion deficiency
Acquired Myeloproliferative disease • Chronic myelogenous leukemia

• Polycythemia vera
Reactive/Secondary neutrophilia
Chronic inflammation • Rheumatic disease • Inflammatory bowel disease • Granulomatous disease • Chronic hepatitis
Nonhematologic malignancy • Cytokine-secreting tumors (lung, tongue, kidney, urothelial tumors) • Marrow metastasis (myelophthisis)
Marrow stimulation • Hemolytic anemia, immune thrombocytopenia • Recovery from marrow suppression • Recombinant cytokine administration
Post splenectomy
Cigarette smoking
Stress
Drug induced • Corticosteroids • β-agonists • Recombinant cytokine administration

Table 3: Major causes of neutrophilia[136].

1.3.2 Thrombosis

Neutrophils and NETs have been shown to play a pivotal role in the pathogenesis of vascular diseases such as thrombosis and stroke. Thrombosis is the formation of blood clots within intact blood vessels. The activation of endothelium, secretion of von Willebrand factor (vWF), tissue factor (TF) and adhesion molecules recruit and activate neutrophils and trigger NETosis[137]. Endothelial vWF interacts with DNA and histones and thus potentially immobilizes NETs onto the vascular endothelium[138]. NETs contribute to thrombosis by immobilizing platelets, RBCs and plasma components. Locally high concentrations of neutrophil proteases and histones causing a sustained

vicious cycle leading to chronic inflammation. The histones perforate endothelial cell and platelets and the increase calcium influx further activates platelets[139]. The same happens in the RBCs, inducing the exposure of PS that promote RBC aggregation and promote thrombosis. NETs can activate the intrinsic as well as the extrinsic pathways. The NE is NETs degrades TFPI and promotes thrombin generation via the intrinsic pathway. On the other hand, NETs activate FX II, and initiates the extrinsic pathway[137].

The prothrombotic properties of NETs have been implicated in the disease activity in patients with TMA. TMA are a group of life threatening heterogeneous diseases that are characterized by vascular occlusions, due to thrombus formation[140]. TMA is associated with haemolytic anaemia with evidence of schistocytes in the blood, thrombocytopenia and ischemic end-organ damage[141]. TMAs include thrombotic thrombocytopenic purpura (TTP), which is associated with sever deficiency of ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13). It also includes typical haemolytic uremic syndrome (HUS), cause due to an infection by enterohemorrhagic *E.coli* producing shigatoxin[141]. The ineffective clearance of NETs can contribute to the development of TMA. High levels of NET markers such as DNA/histone complexes, myeloperoxidase and S100A8/A9, are identified in the plasma from patient with TMA[142]. Therefore, a timely and effective clearance of NETs is required to prevent thrombus formation.

Early phases of atherogenesis also involve neutrophils recruitment by upregulated vWF and adhesion molecules on the dysfunctional endothelial surface. NETs are identified in atherosclerotic lesions and arterial thrombi in both human samples and atherosclerotic animals[143,144].

1.3.3 Sepsis

Sepsis is defined as a systemic inflammatory syndrome resulting in deleterious and non-resolving inflammatory response leading to organ failure. Sepsis and its associated complications is a serious problem in medicine worldwide[145]. It is a major cause of death in critically ill patients in intensive care units. Neutrophils play an essential role in the innate immune response and NETs are an important antimicrobial mechanism. NETs are beneficial to the host during the early stages of infection as they trap and kill pathogens.

However, growing evidence demonstrates that during systemic infection, NETs play a role in the pathogenesis of sepsis[146,147].

Studies demonstrate that during sepsis, neutrophils are reprogrammed, resulting in impaired migration and impaired neutrophils function[148]. They may accumulate in vital organs and induce deleterious effects[149]. Fibrin formation and deposition is a crucial step in sepsis to prevent the dissemination of microbes[150]. Studies have shown that NETs contribute to thrombus formation by stimulating platelet adhesion and coagulation. They form an additional scaffold for the thrombi and cause platelets and RBCs adhesion[151]. Therefore the activation of coagulation in sepsis may lead to disseminated intravascular coagulation (DIC) and acute organ failure, increasing mortality due to sepsis[152].

During sepsis, platelets activate neutrophils to form NETs that trap bacteria in circulation. However, NET components induce collateral damage to the endothelial cells. Additionally, thrombocytopenia occurs as a result of platelets binding to neutrophils to form NETs[153]. NETs are an important source of histones that play a role in stimulating coagulation. Extracellular histones are cytotoxic and contribute to endothelial damage, organ failure and death during sepsis. High levels of citrullinated histone H3 is associated with disease severity and mortality in a murine model of sepsis[154] In addition, histones can trigger platelet activation as well as play a role in thrombin generation[155]. Studies show that antibodies against histones H4 reduce the mortality in the different murine sepsis models[156,157].

In patients, the quantification of the aberrantly elevated level of NETs and their components in the plasma act as prognostic markers that may predict the disease outcome during sepsis[158]. A pilot study showed that a high level of NETs in plasma of trauma patients correlated with high risk of secondary inflammation and sepsis. NETs are also reported as markers for the diagnosis of septic arthritis[159]. Therefore, extracellular histone is a biomarker to predict disease progression and mortality during sepsis.

1.3.4 Inflammatory diseases

Cytotoxicity of NETs

NETs are cytotoxic and may aggravate disease outcomes during sterile as well as infectious conditions[64]. The dual protective and cytotoxicity of NETs are best illustrated during an overwhelming infection. Neutrophils help trap the bacteria by forming NETs, but the toxic granular proteins, histones as well as ROS don't specifically targeted the microbes and hence induce collateral damage to the host cells. NE degrade the extracellular matrix and destabilizing the host tissue[160,155]. Histones bind to the phospholipids on the plasma membranes of host cells and perforate the membrane[161]. The cytotoxicity of histones is of importance in sepsis, an infection of the blood. This was confirmed as the neutralization of histones during sepsis reduces the organ damage[64].

NETs are present in the lung of patients suffering from cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), transfusion-related acute lung injury (TRALI) and asthma[162]. In Cystic Fibrosis (CF), the build-up of thick, sticky mucus in the airways facilitates infection. The neutrophils recruited to the airway form NETs to contain the infection. However, the NETs associated proteases destabilized the tissue[163]. Furthermore, the extracellular DNA from NETs contributes to the viscosities of the sputum. Hence, CF patients are treated with antibiotics as well as pulmozyme (DNASE 1) that degrade the extracellular DNA, reducing the viscosity of the sputum[164].

NETs in autoimmune diseases

The discovery of NETs has bridged the gaps between innate, adaptive and autoimmunity. Systemic autoimmune diseases are a result of a multistep process resulting in a defect in the discrimination of self and non- self-components causing the cellular damage and exposing immune cells to autoantigens. The immune system recognizes the host tissue as foreign molecules and attacks. Several studies now show that NETs are key players in the initiation of autoimmune diseases. Neutrophil products act as both targets as well as mediators of autoimmune diseases. Many of the proteins found in NETs are integral autoantigens that are associated with systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and small vessel vasculitis

(SVV)[117] and autoinflammatory diseases such as gout and inflammatory bowel diseases (IBD)[165,117,166.]

SLE is a chronic autoimmune disease where the body's immune system attacks its own cells and tissue[167]. The presence of autoantibodies against host components such as DNA, granulocyte proteins and complement proteins is a hallmark of SLE. The mechanism behind the generation of these autoantibodies was a mystery until the discovery of NETs[168,169]. A distinct subpopulation of neutrophils, i.e. LDNs is dominant in patients with SLE[170]. Patients with SLE have impaired NET clearance[171]. This is because SLE patients either have a DNASE1 inhibitor or can generate anti-NET antibodies that protect NETs from digestion by serum DNase[110]. RA is an autoimmune disease where the immune system attacks self-tissue, primarily in the joints. It is characterized by the swelling of joints and synovial inflammation causing severe destruction of cartilage and bone. The synovial fluid is rich in activated neutrophils. RA is characterized by the production of anti-citrullinated proteins/peptides antibodies (ACPA) is produced in RA, representing specific disease markers[172]. NETs is a primary source of citrullinated autoantigens. ACPAs can in turn trigger NETosis. ACPAs on NETs delays the clearance of NETs generating a reservoir of citrullinated autoantigens resulting in a sustained inflammatory response[110]. SVV is also characterized by the production of circulating auto-antibodies against neutrophils specific antigens, particularly PR3 and MPO. These auto-antibodies are collectively referred to as antineutrophil cytoplasmic antibodies (ANCA)[173, 174,175].

Gout is an autoinflammatory disease that is characterized by the deposition of Monosodium urate (MSU) crystals in the joints[165]. The accumulation of these crystals recruits leukocytes, particular neutrophils, and macrophages to the site of MSU depositions. Crohn´s disease (CD) and Ulcerative colitis (UC) are autoinflammatory diseases characterized by the inflammation of the gastrointestinal tract and form the IBD. The elevated ROS in CD subsequently increases the local neutrophil concentration that is prone to NETosis[117]. The increased NETs and NET-associated proteins in circulation is one of the disease markers of UC[166].

NETs in cancer

NETs are new players in cancer and have a potential role as biomarkers of disease outcome or as therapeutic targets. Neutrophils have a dual and opposite role in tumour progression. The tumor associated neutrophils (TANs) may develop the N1 phenotype which displays proinflammatory and antitumourigenic functions, or the N2 phenotype which is protumorigenic[176]. The interaction between neutrophils and tumour cell-induced NETosis and NETs influence tumour growth, progression, angiogenesis, and metastasis. Demers et al showed that that priming of neutrophils by G-CSF stimulates the NET release and therefore promotes tumour progression[177,178]. Studies show that patients with intratumoral NETs experienced a poorer prognosis[179]. NETs formation within capillaries may provide a scaffold for metastasizing tumour cells. Therefore.

1.4 Low density neutrophils

In the last decade there has been growing evidence on the heterogeneity of neutrophils. Neutrophils are a cell population with subsets capable of different physiological condition and pathological responses. A subpopulation drawing the most attention in recent years are the low-density neutrophils (LDNs)[180]. They were discovered by Hackbarth *et al* in 1986. By generating a density gradient preparation of whole blood from patients with SLE, RA, and ARF, they observed the presence of low buoyant neutrophils that contaminated the PBMC fraction. They propose that the humoral factors present in the plasma were responsible for this phenotype[181]. Later, studies by Bennett *et al* showed that LDNs were immature neutrophils present in the PBMC fraction. Neutrophilia induced as a result of acute inflammation accelerates neutrophil recruitment from the bone marrow. Due to increased demand, functionally competent but morphologically immature neutrophils are released. This is observed in many pathological conditions[182].

LDNs are defined by a reduced buoyant density compared to high density neutrophils (HDNs). When peripheral blood is layered on top of a discontinuous density gradient medium and centrifuged, cells with different densities will fractionate in different bands depending on their densities. HDNs present in healthy blood sediment just above the RBC

pellet. LDNs which are enriched in various pathological conditions co-purify with the lower density PBMCs[180,183].

Since their initial discovery, LDNs have been observed in a variety of physiological and pathological conditions. Although the origin and pathological roles of LDNs is not fully understood, they are implicated in the pathogenesis of SLE, cancer, human immunodeficiency virus (HIV), sepsis, asthma and development of organ damage due to their enhanced pro-inflammatory response. LDNs have several distinct features compared to control neutrophils. They have an enhanced capacity to synthesize type I IFNs, decreases ability to phagocytosed bacteria, but they have a strikingly enhanced capacity for firm NETs[170,184]. Since NETs act as autoantigens and have the potential to activate the adaptive immune system, LDNs may play a role in the pathogenesis of the various diseases, through the formation of NETs[185].

1.5 Discrepancy in mice and human neutrophils

Laboratory mice are the prevalent research tool of choice in cell biology. Mice mirror human biology well. The sequencing of mouse and human genome revealed that only about 300 genes are unique to both species. Despite the conservation there are significant differences between mice and human immune system, development and immune response. This discrepancy indicates that findings in mice may not translate to humans making mice and an inadequate model to study human diseases. Several animal models of sepsis have been described. Many agents for the treatment of sepsis, improved survival in mice models. However, the same treatments when these compounds were ineffective in human trials[186].

Mice evolved in a different environment and are also much smaller and have significantly shorter life spans. They are exposed to different antigens and new pathological challenges from microorganisms, therefore their immune system evolved in a different way[187]. Approximately 70 million years of evolution has given rise to significant intrinsic differences in the biology and balance between humans and mouse neutrophils[188]. Human blood is abundant in neutrophils (50-70% of total leukocytes) with a mean neutrophil

count of $5x10^9$/l in adults, whereas neutrophils in mouse blood are scarce (10-25%of total leukocytes)[189]. The four commonly used mouse strains (129S1/SvlMJ, BALB/cJ, FVB/NJ and C57BL6/6J) have a mean neutrophil count of 1×10^9/l[132]. Neutrophils are produced in the bone marrow. In mice, the bone marrow reserve of neutrophils is about 120 million cells with < 2.5 million in circulation[190].

To study the role of neutrophils *in vivo* and *in vitro*, it is required to have a mouse model with increased neutrophils in circulation. G-CSF is commonly used in clinics to treat neutropenia. It is the key cytokine that stimulates the production and mobilization of neutrophils from the bone marrow. Studies show that injection of 2.5µg of G-CSF, once a day for up to 15 days induces an 8 fold increase in the number of neutrophils in circulation[191]. Murine *Csf3* can be stably expressed as a transgene in the hepatocytes by hydrodynamic tail vein injection. This over-expression of G-CSF in mice leads to the development of mice with an elevated number of neutrophils in circulation. Therefore, G-CSF treatment is a promising methods to overcome the discrepancy between human and mouse neutrophils. In conclusion, mice will continue to be the stand up *in vivo* model for human immunology and will remain an important model for continued progress in the understanding of immune system function in health and disease. Therefore, novel strategies to develop murine models that resemble human diseases are required.

1.6 Challenges in NETs research

Several studies highlight the importance of NETs in innate immunity and in their association with several diseases. However, the growing body of knowledge has given rise to several fundamental questions as well as skepticism about the physiological relevance of NETs. The following section describes the reasons that gives rise to challenges in NETs research (Scheme 4).

1. NETs are diverse in content and stimuli

Originally the defining feature of NETs was the presence of DNA, histones, and neutrophil granular proteins. However, in the last 14 years, the definition of NETs has suffered several modifications. The granular protein content of NETs, requirement of

NADPH oxidase and citrullination of histones for NETosis vary in some settings[69,192, 193]. One group showed that neutrophils can release DNA from mitochondria and that these NETs lack histones[80]. Extracellular DNA, the only component always present in NETs, need not arise from neutrophils[194].

The broad definition of NETs has resulted in a list of stimuli and pathway that induce NETs. It´s not just microbes that trigger NETosis. NETs are formed under sterile conditions and some neutrophils make NETs in autoimmune conditions. Now, NETs is used as an umbrella term that describes the nuclear and cytoplasmic events and the dramatic changes in the morphology of cell resulting in the extrusion of decondensed DNA. However, it is required to establish a firm definition as those based on morphology is subjective and nonspecific. This has led to the misclassification of all biological processes that involve the release of neutrophil DNA as NETs[195].

2. Extracellular traps are formed by various other cell types

Eosinophils, mast cells, macrophages basophils and even endothelial cells have been shown to form extracellular traps[196]. There are at least two mechanisms of NET production, either suicidal NETosis or vital NETosis, where mitochondrial DNA may be the source of NETs[77]. Although all the neutrophils have the capacity to release DNA decorated with granular protein, not all of them respond to stimuli by underdoing NETosis. It's unclear if NETosis is age-related or restricted to a particular subset of neutrophils[197].

3. NETs are linked to a broad range of biological events

In addition to their antimicrobial activities, they may simultaneously induce tissue damage. NETs are implicated in a plethora of pathological conditions, including a variety of infectious diseases, autoimmune diseases, autoinflammatory diseases, thrombosis and cancer[198]. The protein content of NETs varies from disease to disease[199]. Thus the diversity has made NETs controversial and challenging to study and given rise to skepticisms about whether they are an active physiologically relevant mechanism of host defence. The variability and diversity of NET formation suggest that NETosis is either a

process with extraordinary modulation or an unspecific event associated with neutrophil death[197].

A biological classification of the NET-associated proteins in different diseases is necessary. Regardless of how NETs are made, it is clear that NETs induce severe damage when uncontrolled. Such classification of NET-associated proteins helps in the identification of ways to inhibit and manipulate NETosis leading to the development of a therapeutic target. It will also be essential to understanding which diseases benefit from NETosis and which benefit from the inhibition of NETosis[195].

4. Knowledge gaps in the molecular mechanism of NETosis

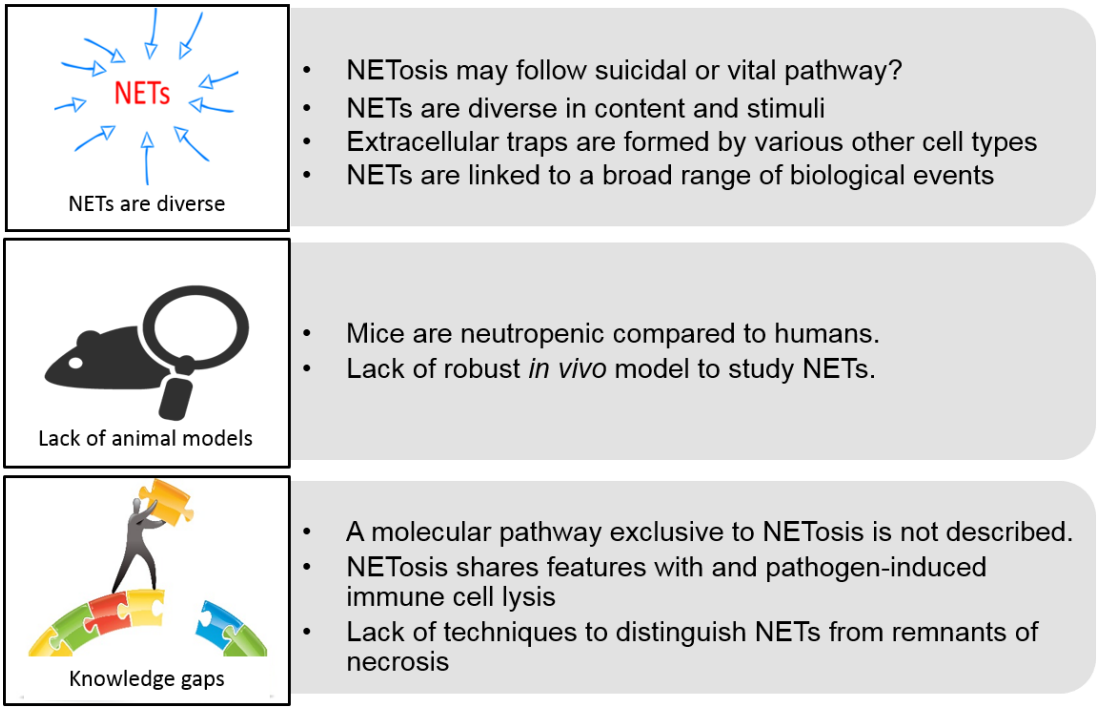
The molecular mechanism involved in NET formation needs to be better understand to critically understand this biological process. It is necessary to identify biochemical markers that are unique to NETs. Some studies have shown that NETs is a regulated form of cell death which dependent on the citrullination of histones by PAD4, generation of reactive oxygen species by NADPH oxidase and degradation of histone by neutrophils elastase. However, in many settings NETs are formed even in the absence of NADPH oxidase and PAD4 citrullination. eg. Influenza injection[200]. Hence it is instrumental to identify a NET specific gene or a NET specific pathway. It is essential to understand and distinguish between the different form of NETs such as suicidal NETosis and vital NETosis[197].

5. NETosis shares features with and pathogen-induced immune cell lysis

The avalanche of research that followed the initial discovery of NETs described the phenomenon in numerous and diverse settings. Some scientists interpret NETs as the fortuitous fate of neutrophils undergoing necrosis and are not convinced that the phenomenon is a host defence strategy, but rather just the chance trapping of microbes in neutrophil-DNA meshwork[199]. Others define NETosis as a form of programmed cell death to trap invading pathogens, suggesting that neutrophils in circulation, do not chase after bacteria, but have acquired the ability to lay traps to ensnare them[197].

6. Approaches that are currently used to measure NETs *in vivo* are not optimal

The presence of extracellular chromatin aggregates decorated with granular proteins has been linked to necrosis[201]. Measurement of cell-free DNA and neutrophil granule protein MPO does not serve as specific markers of NETs. Plasma of healthy humans always contains circulation cell-free DNA which is elevated during pregnancy, trauma, infection, and cancer. MPO is also present in the plasma and is particularly elevated during trauma or infection. The coincidental presence of DNA and MPO is insufficient evidence to measure NETs. Identification of components of NETs *in vivo* is needed. It is important to establish elements that unambiguously distinguish NETs from remnants of necrotic cells[197].



Scheme 4: Challenges in NETs research.

In conclusion, if NETs are formed in more than one way it is important to note that all forms of DNA may not have the same capacity to fight infections, trigger human diseases or be inhibited by targeting a single biochemical pathway.

1.7 Objective of this study

Although the involvement of neutrophils and NETs is becoming increasingly prominent, it remains to be thoroughly understood. The focus of NETs research is shifting to what signalling pathways induce NETs and how these pathways can be inhibited or manipulated. It is important to distinguish between the diseases that may benefit from inducing or enhancing NETosis and those that benefit from inhibiting NETosis. The overall aim of this thesis was to contribute to the current understanding of the role of neutrophils and NETs in inflammatory diseases. We aimed to develop an animal models of chronic neutrophilia and sepsis that can be used to study the biology of NETs *in vivo* and allow us to characterize the molecular mechanism of NETosis. We achieve these aims by setting the following specific objectives. The work described in this thesis had the following specific objectives.

1. Development of a mouse model of neutrophilia.
2. Analyses of host factors that degrades NETs *in vivo*.
3. Molecular mechanism of *in vivo* NETosis.
4. Development of a robust technique to track NETs *in vivo.*

2. Materials and methods

2.1 Animals

All mice were on a C57BL/6 genetic background. The previously described *Dnase1*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice[202,203] were crossed to generate *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice. The *Dnase1*$^{-/-}$ was reported to have an off-target mutation in the *Dnase1* overlapping gene *Trap/Hsp75*, which encodes a mitochondrial chaperone[204]. Hence, an alternative *Dnase1*$^{-/-}$ strain[205] was used to generate *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice for control experiments. The *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice were again crossed with single knock out mice *Pad4*$^{-/-}$, *Nox2*$^{-/-}$ and *Myd88*$^{-/-}$ to generate *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Nox2*$^{-/-}$, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88*$^{-/-}$ triple knock out mice respectively.

2.2 Genotyping

Genetically modified mice were genotyped using polymerase chain reaction (PCR). Genomic DNA was extracted from the tail biopsies using the peqGold Micro Spin Tissue DNA Kit (Peqlab) or the Kappa DNA isolation kit. The master mix used for the PCR reactions contains 12.5µl of 2 Kapa2G Fast HS ready mix (Kapa Biosystems), 1µl of each primer (10µM,) and 5.5µl water. 5µl of genomic DNA was added to the PCR master mix.

2.3 Development of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Gsdmd*$^{-/-}$ using CRISPR/Cas9.

sgRNAs flanking and targeting exon 3 (2nd coding exon) of gasdermin D were designed and templates for transcription were derived by PCR using Q5-Polymerase (Biolabs). Transcription was performed using the HiScribeT7 kit (Biolabs, E20140S) with subsequent purification of the transcripts with the MEGAClear^{TM} kit (Fisher Scientific, AM1908), both according to the manufacturer's instructions. Electroporation into single cell stage embryos derived from superovulated *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice using 300 ng/µl of each sgRNA and 500 ng/ml Cas9 protein (IDT) in OptiMEM medium (Gibco) with the help of the NEPA 21 electroporator. Settings used as described by Remy et al[206]. Injected embryos were implanted into F1 foster mothers (C57BL6/J x CBA). Genotyping of mice was performed using PCR.

2.4 Murine blood, plasma, and tissue collection

Blood was collected by submandibular puncture or by the retro orbital sinus. Blood was collected into 200µl serum tubes (Monovette; Sarstedt, Germany) and 200µl EDTA tubes (GK 150, KABE labortechnik, Germany). Plasma was obtained by collecting the supernatant of blood after centrifugation at 3000 * g for 15 minutes. Serum was obtained by allowing the blood to clot for 1 hour at room temperature followed by centrifugation for 15 minutes at 3000 * g. Serum and plasma samples were stored in aliquots at −20°C until further use. For organ analysis, mice were perfused by intracardiac infusion of PBS. Organs were collected and fixed for 24 hours in 4% paraformaldehyde PFA at 4°C. Fixed organs were embedded in paraffin.

2.5 Blood and plasma analyses

To quantify neutrophils by flow cytometry, EDTA-anticoagulated whole blood was incubated on ice for 15 minutes with 0.2 µg of phycoerythrine-labeled anti-mouse CD11b (M1/70, Biolegend, Germany) and 0.5 µg of Alexa Fluor 488-labeled anti-mouse Ly6G (1AB, Biolegend, Germany). The blood was then diluted with 0.5 ml of PBS and analyzed with a FACS Calibur (BD Bioscience, USA). Total leukocytes, red blood cells, platelets and hemoglobin in EDTA-blood was quantified by an automated hemocytometer (Idexx ProCyte Dx Hematology Analyzer, Netherlands). Blood smears of EDTA-anticoagulated blood were prepared on polylysine-coated slides (Hecht Assistant, Germany). After air-drying, they were incubated for 1 minute in methanol supplemented with 1µM SytoxGreen on dry ice. Blood smears were also stained with Giemsa using a commercial kit (In Vitro Diagnostikum, Germany).

Mouse G-CSF was quantified with a Quantikine ELISA Kit (R&D, United Kingdom). L dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine in plasma were quantified by using standardized kits (Biotron Diagnostics, CA, and USA) following the manufacturer instructions.

2.6 Preparation of in vivo expression vectors

The pLIVE plasmid (Mirus Bio, USA) was used to express proteins in mice. This vector enables a long-lasting and hepatocyte-specific expression of proteins. pLIVE plasmids with murine Csf3, Dnase1, and Dnasel13 was generated. The generation of the pLIVE vector containing the cDNA of Csf3 was outsourced (Eurofins Genomics, Germany). The cDNA of Csf3 (Genbank Accession Number BC120761) was inserted in the MCS between restriction sites SalI and XhoI. For murine Dnase1, a PCR of the cDNA (Genbank Accession Number NM010061) was performed using the pair of primers Dnase1-F 5´-GTCGACATGCGGTACACAGG-3´ and Dnase1-R 5´-CTCGAGTCAGATTTTTCTGAGTGTCA-3´ containing SalI and XhoI restriction sites. For Dnase1l3, a PCR of the cDNA (Genbank Accession Number AF047355) was performed using the pair of primers Dnase1l3-F 5´-GAAGTCCCAGGAATTCAAAGATGT-3´ and Dnase1l3-R 5´-GCGTGATACCCGGGAGCGATTG-3´ containing BamHI and SacI restriction sites. T4 ligase (New England Biolabs, Germany) was used to clone both cDNAs into the multi-cloning site (MCS) of the pLIVE vector, after digestion with appropriate enzymes. The pLIVE vector containing Dnase1l3 was subjected to site-directed mutagenesis with the pair of primers mutDnase1l3-F 5´-AGTCGACTCCCGGCCACCATGTCCCTGCA-3´ and its complementary mutDnase11l3-R 5´-TGCAGGGACATGGTGGCCGGGAGTCGACT-3´ in order to match the consensus Kozak sequence and optimize protein expression. Double-stranded DNA sequencing was done to confirm all the sequence of all the generated vectors. The parental pLIVE plasmid without additional inserts was used as a control. All plasmids were purified using PureLink HiPure Plasmid Maxiprep Kit and potential contaminations of endotoxin were removed using High Capacity Endotoxin Removal Spin Columns (both Thermo Scientific).

2.7 Animal experiments

2.7.1 In vivo gene expression

The pLIVE-plasmids containing *Csf3*, *Dnase1*, *Dnase1l3*, or empty control plasmids were administered to mice via hydrodynamic tail vein injection (HTV). In brief, 50 µg of

plasmid were diluted in 0.9% saline in a volume equivalent to 10% of the body mass of the mouse. Mice were anaesthetized with isoflurane and the plasmid solution was then injected intravenously over 5 - 8 seconds via the tail vein. In rare cases, mice did not fully recover from the injection within the first 24 hours and these animals were excluded from the study. For co-expression studies, 50µg of the *Csf3*-plasmid were mixed with 50µg of the empty control plasmid, or the plasmids containing *Dnase1* and *Dnase1l3*. The solution containing both plasmids was administered via hydrodynamic tail vein injection.

2.7.2 Chronic neutrophilia in wild-type and knock out mice

Wild-type neutrophilic mice:

Female mice at 4 weeks and 8-12 weeks of age were injected with 50µg of the pLIVE-plasmid containing *Csf3* to induce G-CSF expression and neutrophilia. Mice were monitored every day after injection. Body weight and temperature was measured in the perianal area by a contactless infrared-thermometer (Etekcity, Germany).To study the effect of elevated G-CSF over time, the mice were divided into four groups depending on the period of *Csf3* expression, i.e. 3 days, 1 week, 2 weeks, and 4 weeks. At the end of the experiment, each group of mice was euthanized for biosample collection.

Knock out mice

Female mice at 8-12 weeks of age were injected with 50µg of the pLIVE-plasmid containing *Csf3* to stably express G-CSF and induce neutrophilia. Mice were monitored every 8 hours during the first week after injection, and every day afterward. Temperature was measured in the perianal area by a contactless infrared-thermometer (Etekcity, Germany). For survival studies, mice were euthanized and scored as "non-surviving" if the animals showed signs of distress (no spontaneous movement, closed eyes, occasional gasping). In all cases, these signs of distress were accompanied with a rapidly progressing and severe hypothermia, defined as decrease in body temperature of ≥4°C compared to the body temperature before the plasmid injection, and with hematuria. Non-hypothermic mice did not show any signs of distress and were euthanized at the end of the experiment. For organ, blood, and urine collection, we analysed four groups of three *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ mice. Mice in each group were injected with a mixture of *Csf3* with empty

plasma, *Dnase1*, or *Dnase1l3*. Each group of mice was euthanized for biosample collection when the first animal of the group showed signs of distress, severe hypothermia, and hematuria. This time point was defined as "exitus" and occurred within 3 to 6 days after the injection.

2.7.3 Platelet depletion

Mice were injected intraperitoneally with 2µg/g of rat monoclonal antibodies against CD42b (product number R300, Emfret Analytics, Germany) to deplete platelets. Control mice received 2µg/g non-immune rat IgG (product number C301, Emfret Analytics). According to the manufacturer, the dose of anti-CD42b antibodies depletes > 95% of platelets from circulation. We confirmed the efficiency of the platelet depletion in WT mice. After 2 days, mice treated with anti-CD42b showed a platelet count of less than 99%, when compared to mice treated with non-immune rat IgG. Therefore, the treatment was begun 24 hours after the injection of *Csf3* and repeated every 48 hours until completion of the experiment.

2.7.4 Thrombin inhibition

Powdered dabigatran etexilate (Pradaxa, Boehringer Ingelheim, Germany) was mixed with normal chow powder (Altromin, Germany) at a dose of 40 mg/g. Pellets were prepared by mixing the powder with distilled water and allowed to dry at room temperature. The feeding of dabigtran was started 24 hours after the injection of *Csf3*.

2.7.5 Preparation of bacteria for sepsis

Escherichia coli (XEN 14, Perkin Elmer) was grown overnight in lysogeny broth media containing 50 µg/ml kanamycin. Bacteria were pelleted by centrifuging at 4000 * g for 10 minutes, washed with and resuspended in PBS. Aliquots of $1.5 * 10^9$ bacteria/ml were incubated at 70°C for 15 minutes to heat-kill the bacteria. Aliquots were stored at -20°C until further use.

2.7.6 Sepsis

In preliminary experiments, we tested daily intraperitoneal injections of 1 µg/g of LPS from *Salmonella enterica* serotype *thyphimurium* (product number L6511, lot number 025M4042V, Sigma-Aldrich) in 0.9% saline. Additionally, mice received an intravenous injection of $1.5 * 10^7$ heat-killed *E.coli*/g along with the third LPS injection. The shown survival time indicates the time after the injection of *E.coli.* Blood and organs were collected at the time of euthanasia. Mice were euthanized and scored as "non-surviving" if the animals showed signs of severe distress (unresponsiveness to touch). All non-surviving mice showed hematuria and paleness of extremities. All surviving mice were euthanized and scored as "surviving" 24 hours after the intravenous injection of heat-killed *E.coli*.

2.7.7 BrdU labelling of mouse neutrophils

Mice were injected with 50µg of the pLIVE-plasmid containing *Csf3* to induce G-CSF expression and neutrophilia. Following this, mice were injected intraperitoneally with 2 mg 5-Bromo-2-deoxyuridine (BrdU,Sigma), two times, every day for 2 weeks. Neutrophils were purified by negative selections (Neutrophil isolation kit, Miltenyi Biotec). BrdU positive neutrophils were detected by immunofluorescent labelling using anti-BrdU antibody (ab6326, Abcam).

2.8 In vitro experiments

2.8.1 Detection of DNASE1 and DNASE1L3 by DPZ

Sodium dodecyl sulfate (SDS)-polyacrylamide gels were prepared with 4% (v/v) stacking gel without DNA and 10% (v/v) resolving gel containing 200µg/ml of salmon testes DNA (Sigma-Aldrich, Germany). DNASE1 was detected by mixing 0.5µl of murine serum with 14.5 µl of water and 5µl of SDS gel-loading buffer (BioRad, Germany). The mixture was boiled and loaded onto the gels. Electrophoresis was carried out at 120 V in Tris/glycine electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.7). Following this, the proteins were refolded by incubating the gels overnight at 37°C in a re-folding buffer (5% (w/v) milk powder, 10mM Tris/HCl pH 7.8, 3mM $CaCl_2$, 3mM $MgCl_2$, 100U/ml penicillin, and 100µg/ml streptomycin). The gels were then incubated

for 24 hours at 37°C in a refolding buffer without milk powder. SYBR Safe (Thermo Scientific, Germany) was used for fluorescent labelling of DNA. Fluorescent images of gels were recorded with a fluorescence scanner (Molecular Imager FX, BioRad, Germany).

DNASE1L3 was detected by mixing 2 µl of serum with 12 µl of water, 5 µl SDS gel-loading buffer, and 1 µl of beta-mercaptoethanol (BME, Sigma-Aldrich). BME reduces the disulfide bridges of DNASE1, which causes its inactivation[114]. The mixture was boiled for 5 minutes and loaded onto the gels. Electrophoresis was carried out as described for DNASE1. The gels were washed with 10 mM Tris/HCl pH 7.8 for 30 minutes at 50°C twice to remove SDS. The proteins were refolded by incubating the gels for 48 hours at 37°C in a refolding buffer containing 10 mM Tris/HCl pH 7.8, 1 mM BME, 100 U/ml penicillin and 100 µg/ml streptomycin. The gels were then incubated for an additional 48 hours at 37°C in refolding buffer supplemented with 3 mM $CaCl_2$ and 3 mM $MnCl_2$. The addition of Mn^{2+} is required to enable the efficient degradation of protein-free DNA by DNase1L3[114]. DNA was labeled and imaged as described for DNASE1.

2.8.2 Detection of total DNase activity by single radial enzyme diffusion (SRED) assay

DNase activity was measured by dissolving 55 µg/ml DNA from salmon testes in a buffer containing Mn^{2+} (20 mM Tris-HCl pH 7.8, 10 mM $MnCl_2$, 2 mM $CaCl_2$, and 2 * SYBR Safe). The DNA solution was heated at 50°C for 10 minutes and mixed with an equal volume of 2% ultra-pure agarose (Thermo Scientific). The mixture was poured into plastic trays and stored at room temperature until solidification. Two microliters of murine serum were loaded into wells of 1.0 mm diameter. Gels were incubated for 4 hours at 37°C in a humid chamber. The DNA fluorescence of the gels was recorded with a fluorescence scanner.

2.8.3 Neutrophil isolation from human blood

EDTA blood was layered onto histopaque 119 (Sigma Aldrich). After centrifugation for 20 minutes at 800g, the neutrophil-rich layer was collected. Cells were washed with Hanks-buffered salt solution without divalent cations (HBSS-, Life technologies. Cergy

Pointoise, France) supplemented with 5mM EDTA and0.1% BSA. Washed cells were fractionated on discontinuous percoll gradient (GE healthcare). After centrifugation for 20 minutes at 800g, the neutrophils-rich layer was washed with 0.1% BSA in HBSS-. Neutrophil viability was greater than 98% as determined by trypan blue (Sigma-Aldrich) exclusion.

2.8.4 Neutrophil isolation from mouse blood

1ml of EDTA blood was diluted in PBS containing 1% (w/v) bovine serum albumin (BSA) 15mM EDTA to a final volume of 6 ml. Diluted blood was fractionated on a freshly prepared discontinuous sucrose gradient. 3 ml of sterile-filtered sucrose 1.119 g/ml was added to the bottom of a 15 ml conical polypropylene centrifuge tube. 3 ml of sterile-filtered sucrose 1.077 g/ml on top of the 1.119 g/ml layer. Thereafter, 6 ml of diluted blood was layered on top of the 1.077 g/ml layer and centrifuges at 700 x g for 30 min at RT without brake. High-density neutrophils (HDN) are found as a white-to-red ring at the interface between the 1.119 g/ml and the 1.077 g/ml layers (around the 3 ml mark), while the low-density leukocytes are found in a white ring at the interface between the 1.077g/ml layer and the BSA-containing PBS. Cell in the different fractions were harvested. Neutrophils isolation was continues using the negative selection method in the neutrophil isolation kit (MACS Mitenyi).

2.8.5 In vitro NET degradation assay

NET degradation was analysed as previously described[142]. Purified human neutrophils were seeded onto sterile 96-well plates (Falcon, BD Technologies, Germany) coated with 0.001% polylysine (Sigma-Aldrich) in serum free DMEM at a concentration of 5 x 10^4 cells per well. NET formation was induces by activating neutrophils with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 4 hours at 37°C with 5% CO_2 and humidity. We added phosphate buffered saline (PBS) and stored the 96-well plates overnight at 4°C. NETs were washed with PBS, treated for 5 minutes with PBS containing 0.5% Triton X-100, and washed with PBS again. NETs were incubated with 10% murine serum 10 μM PPACK (Santa Cruz, Heidelberg, Germany) in HBSS with divalent cations (HBSS+; Thermo Scientific). NETs were allowed to be degraded for 3 hours at 37°C with 5% CO_2 and humidity. Supernatant was removed and NET degradation was stopped by

adding 2% PFA in PBS for 1 hour at room temperature. PFA was discarded and non-degraded NETs were labeled fluorescently by adding 2 µM of the fluorescent DNA dye SytoxGreen (Thermo Scientific) in PBS. Images of fluorescently stained NETs were acquired with an inverted fluorescence microscope (Axiovert 200M, Objective: LD Plan-Neofluar 20x/0.4, Zeiss, Germany). We quantified non-degraded NETs by recording the fluorescence intensity with a plate reader (Excitation: 485 nm; Emission: 535 nm) or by measuring the area coverage of NETs in microscopy pictures using ImageJ software[207].

2.8.6 Generation of NET clots in vitro

Mouse neutrophils were purified by negative selections (Neutrophil isolation kit, Miltenyi Biotec). Neutrophils were seeded at 1 * 10^7 cells/ml in DMEM supplemented with 2% BSA. The cells were stimulated with 25µM calcium ionophore A23187 (Sigma) for 4 hours at 37°C, 5% CO_2 with humidity and rotating conditions (300rpm). We used unstimulated neutrophils and neutrophils activated with calcium ionophore A23187 in the presence of 10 U/ml recombinant DNase1 (dornase alpha, Roche, Germany) as controls. NET clots were fixed with 2% PFA overnight at 4°C. Fixed NET clots were embedded in paraffin and sections were stained for NETs as described in section 2.9.2.

2.8.7 Western blot analysis for GSDMD

Mouse bone marrow cells were flushed from femur and tibia. Cell lysate was prepared by lysing cells with RIPA lysis buffer (Thermo Fischer Scientific) plus phosphatase inhibitors and Complete Protease Inhibitor Cocktail (Roche, Brighton, MA). Protein concentrations were determined by the BCA protein assay kit (Thermo Ficher Scientific), and equal amounts of proteins were subjected to SDS-PAGE gels (12%). Proteins were transferred onto nitrocellulose membranes and incubated with GSDMD antibody (1:1,000, ab209845, Abcam, Cambridge, MA) overnight at 4°C, followed by a 1-hour incubation with secondary goat anti-rabbit IgG (1:5,000, A21109, Thermo Fisher Scientific). The results were visualized using the Bio-Rad ChemiDoc.

2.9 Histology

2.9.1 Immunohistochemistry

Paraffin-embedded sections were de-waxed, and subjected to heat-induced antigen retrieval for 25 minutes at 100°C in citrate buffer (10 mM sodium citrate, 0.1% Tween, pH 6). Following antigen retrieval, sections were blocked for 30 minutes with 2.5% normal horse serum (Vector, United Kingdom). The sections were incubated overnight at 4°C with 2 µg/ml of the primary antibody against neutrophil elastase (ab68672, Abcam). After washing, sections were stained using the anti-rabbit IgG-AP kit, (ImmPRESS, Vector, United Kingdom) according to manufacturer's instruction. Sections were counterstained with hemalum (Merck, Germany) and mounted with Neo-Mount media (Merck). Images of stained sections were acquired with an inverted microscope (Axiovert 200M, Objective: Plan-Apochromat 20x/0.8, Zeiss).

2.9.2 Immunofluorescence stainings

Paraffin-embedded sections were de-waxed, rehydrated, and subjected to heat-induces antigen retrieval for 25 minutes at 100°C in citrate buffer (10 mM sodium citrate, 0.1% Tween, pH 6).Sections were blocked for 30 minutes with 2.5% normal goat serum (Vector, United Kingdom) followed by incubation with a "mouse-on-mouse" blocking kit (Vector, United Kingdom) for one hour. The sections were then incubated over night at 4°C with 2 µg/ml of the primary antibodies. The antibodies have been applied: anti-MPO (A0398, Agilent, Germany), anti-CRAMP (PA-CRLP-100, Innovagen, Sweden), and anti-citrullinated histone 3 (ab5103, Abcam, United Kingdom), anti-fibrin [clone 59D8], anti-histone H2A/H2B/DNA-complex[208] [anti-chromatin] and anti-BrdU (ab6326, Abcam). Sections were incubated with anti-rabbit and anti-mouse IgG antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 555 (all Thermo Scientific) for 1 hour. After washing, DNA was labeled with 1 µg/ml DAPI for 2 minutes. To quench the auto fluorescence, the sections were incubated with Sudan Black (0.1% in 70% ethanol) for 20 minutes. Next, the sections were mounted with Fluoromount G (Southern Biotech, USA) and mages of fluorescently labeled sections were acquired with an inverted fluorescence microscope (Axiovert 200M, Objective: LD Plan-Neofluar 20x/0.4,

Zeiss) or a confocal microscope (TCS SP5, Objective: HC PL APO 63x/1.40 Oil CS2, Leica, Germany).

3. RESULTS

PART 1: Development of murine neutrophilia model

3.1 Stable hepatic expression of G-CSF induces chronic neutrophilia in mice.

The low abundance of neutrophils in mice compared to humans represents a major limitation to basic research on neutrophil-driven inflammation. We aimed to stably increase the number of blood neutrophils in mice. In mice, subcutaneous injection of 2.5µg of G-CSF, once a day for up to 15 days induced an 8 fold increase in the neutrophil counts in circulation[191]. A therapy with recombinant G-CSF is expensive, and G-CSF has a short half-life *in vivo.* Thus, we hypothesized that the expression of *Csf3* which encodes for G-CSF, as a transgene in mice would induce an increase in neutrophil counts in circulation. To accomplish this aim, we used pLIVE, a liver-specific expression vector that causes sustained long-term gene expression in mouse liver through 8 months post-injection[209]. In brief, we designed a pLIVE vector to stably express G-CSF by inserting the *Csf3* cDNA. We injected 50 µg of the vector into wild-type mice via hydrodynamic tail vein injection (HTVI). HTVI is an effective technique to deliver foreign DNA to the liver[210] that involves rapidly injecting a large volume of liquid intravenously. This technique facilitates the direct uptake of the vector by the hepatocytes[210]. As a control, we injected wild-type mice with the parental pLIVE vector without *Csf3*. Two weeks post-injection, we sacrificed these mice and collected EDTA-anticoagulated blood.

We quantified the number of neutrophils in circulation by flow cytometric analyses. CD11b and Ly6G are commonly used markers to identify neutrophils. CD11b is a marker for all myeloid cell types. It is a member of the integrin family expressed primarily on granulocytes, monocytes, dendritic cells, NK cells and subsets of T and B cells[211]. Lymphocyte antigen six complex locus G (Ly6G) is a small GPI-linked protein expressed on mouse neutrophils[212]. The use of fluorescent cell-specific antibodies facilitates the identification of the cell population of interest by FACS. Staining for CD11b+ cells that express Ly6G is a well-established gating strategy to identify neutrophils[213]. We labelled whole blood from control and *Csf3* treated mice by incubating it with anti-CD11b and anti-Ly6G. Whole blood cells were separated based the forward scatter (FSC) represented on the Y-axis which is a measure of size, and sideward scatter (SSC) represented on the

X-axis, a measure of cell granularity. Granulocytes appear farthest to the right on the scatter plot. We isolated the granulocyte population by gating on the FSC/SSC plot. Neutrophils are double-positive for CD11b and Ly6G. Wild-type mice that expressed the control vector showed 0.5 ± 0.1 x 10^9 cells/l, (N = 4) (Figure 1 A). In comparison, wild-type mice expressing *Csf3* showed 19 ± 5.18 x 10^9 cells/l, (N = 5) (Figure 1 B). *Csf3* expression caused a 20 fold increase in CD11b-Ly6G double positive cells in circulation (Figure 1 C). Thus, we demonstrated that the stable of G-CSF induces an increase in neutrophils count in circulation.

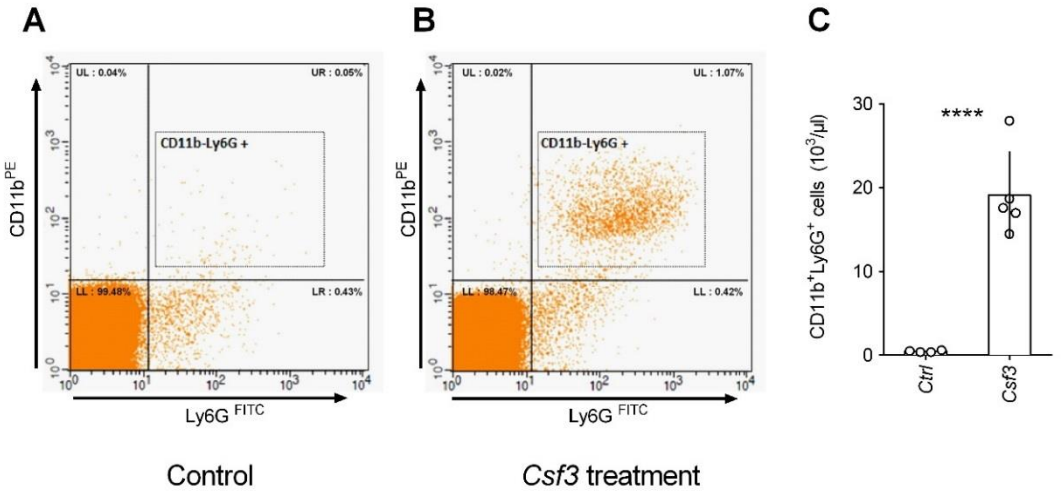


Figure 1: Stable hepatic expression of G-CSF induces an increase in neutrophils in circulation. Whole blood from wild-type mice expressing G-CSF-expression plasmid and the control plasmid was labeled with antibodies against CD11b and Ly6G. FACs analyses were done to quantify the percentage of CD11b-Ly6G double positive cells. (A) CD11b-Ly6G double positive cells in blood from a control mouse. Percentage of cells in UL: 0.04%, UR: 0.05%, LL: 98.48%, LR: 0.43% (N = 4). (B) CD11b-Ly6G double positive cells in *Csf3* expressing mouse. Percentage of cells in UL: 0.02%, UR: 1. 07%, LL: 98.47%, LR: 0.42% (N = 5). (C) Blood neutrophil ($CD11b^+Ly6G^+$) counts of wild-type mice expressing *Ctrl* vector (N = 4) and *Csf3* (N = 5). Statistics: (C) Student's *t*-test; **** P < 0.0001.

To assess the outcome of G-CSF expression over time, we set up a time course experiment. We injected four-week-old wild-type mice with the *Csf3*-expression vector and characterized them at 3, 7, 4, and 28 days after injection. At every time point, we collected blood via retro-orbital bleeding into EDTA treated tubes to determine the blood cell counts, as well as to prepare blood smears. In addition to this, we isolated the plasma from the EDTA-anticoagulated blood to quantify the plasma level of G-CSF by ELISA. Firstly, we measured the level of G-CSF by ELISA in the plasma of wild-type mice at

baseline (without any treatment), wild-type mice treated with *Csf*3-expression vector for increasing time points and wild-type mice treated with the control vector. Plasma from mice at baseline and control mice has low G-CSF. On the other hand, the mice treated with *Csf3*-expression vector showed chronically elevate concentrations of G-CSF in the plasma (Figure 2 A). Next, we quantified the number of circulating neutrophils at each time point by FACS after labeling whole blood with anti-CD11b and anti-Ly6G antibodies. Our results show that mice at baseline, and mice treated with control vector had an average of 0.5 ± 1.6 x10^3 $CD11b^+Ly6G^+$ cells /µl (N = 5) and 0.4 ± 0.5 x 10^3 $CD11b^+Ly6G^+$ cells /µl (N = 4) respectively. The number of $CD11B^+Ly6G^+$ steadily increased with time in mice treated with the *Csf3*-expression vector (Figure 2 B).

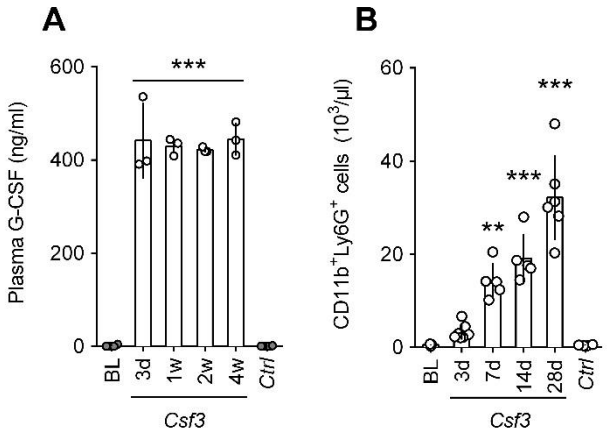


Figure 2: G-CSF expression induces chronic neutrophilia. Four-week-old wild-type mice were injected with *Csf3*-expression plasmid or with a control plasmid (*Ctrl*). **(A)** G-CSF levels in plasma (N = 3-6 mice). BL: baseline, before injection; *Ctrl:* mice expressing empty control plasmid for 2 weeks (N = 4-5 mice). **(B)** Blood neutrophil ($CD11b^+Ly6g^+$) count of wild-type mice expressing *Csf3* for indicated times and *Ctrl* for 14 days (N = 4-7). Statistics: (A) and (B) one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test, *** $P < 0.001$ versus BL.

3.2 Sub-population of neutrophils release NET-like structures.

G-CSF not only stimulates the production of neutrophils but also activates them[214]. The G-CSF receptor (G-CSFR) is expressed primarily on neutrophils and its precursor cells. In mature neutrophils, G-CSF binds to G-CSFR, and activates downstream signal transduction pathways leading to the stimulation of neutrophils[215]. Neutrophils from mice bearing G-CSF producing cancer cells as wells as neutrophils treated with G-CSF are

predisposed to generate spontaneous NETs [93]. Therefore, we analysed blood smears and detected the presence of NET-like structure in the mice treated with *Csf3*-expression vector. We quantified the number of NET-like structure in the blood smears of mice at baseline, 3 days, 7 days and 14 days after treatment, as well as control mice. The number of these spontaneous NET-like structures were few or none in baseline and control treated mice, but significantly increased with time in *Csf3* treated mice (Figure 3). In conclusion, our findings support the involvement of G-CSF in NET formation.

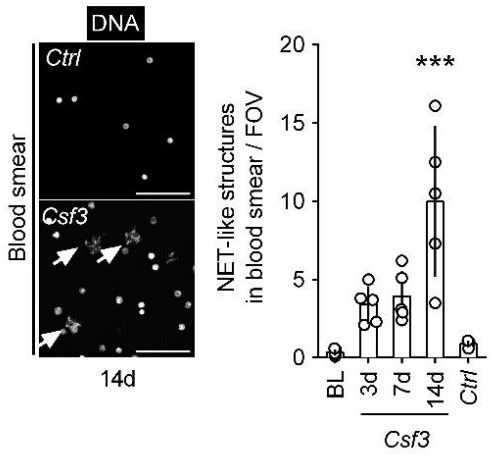


Figure 3: Sub-population of neutrophils release NET-like structures. NET-like structures (arrows) in DNA-stainings of blood smears from WT mice expressing *Csf3* for indicated times or *Ctrl* for 14 days (N = 5). Scale bar: 50 µm. Statistics: one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test, *** $P < 0.001$ versus BL.

3.3 Wild-type mice stably expressing G-CSF are healthy.

To do a systemic characterization of the effects of chronic neutrophilia we sacrificed the mice and harvested the vital organs at different time points. Mice treated with *Csf3*-expression vector had splenomegaly. The spleen size and weight increased with increase in neutrophil counts in circulation. The average spleen weight of untreated and control mice were 93.8 ± 9.2 mg, (N = 5) and 95.6 ± 19.4 mg, (N = 4) respectively, whereas the average spleen weight of treated mice after 4 weeks was 985 ± 111.3 mg, (N = 6) (Figure 4 A). The high number of neutrophils in circulation prompted us to check for the increased sequestration of neutrophils in the tissue of *Csf3* treated mice. We analyzed the histology of the *Csf3* treated mice by immunohistochemistry staining for neutrophil elastase (NE).

NE is present within the cytoplasmic granules of neutrophils. Sections of the lungs, kidneys, and liver of the *Csf3* treated mice, as well as control mice, were incubated with an antibody against NE. Immunohistochemistry (IHC) staining revealed increased infiltration of NE positive cells in the tissue from *Csf3* treated mice in comparison to the control treated mice. Bright field microscope was used to take images of lung, kidney and liver sections. Using ImageJ, we counted the number of neutrophils. Lung sections of *Csf3* treated mice had 420.6 ± 42.25 (N = 3) neutrophils, whereas sections of control mice had only 81 ± 39.3 (N = 3), per field of view. The number of neutrophils in the kidneys and liver were also comparatively higher in *Csf3* treated mice than in control mice (Figure 4 B). Therefore, we conclude that the hepatic expression of G-CSF results in an increased infiltration and deposition of neutrophils in the tissue.

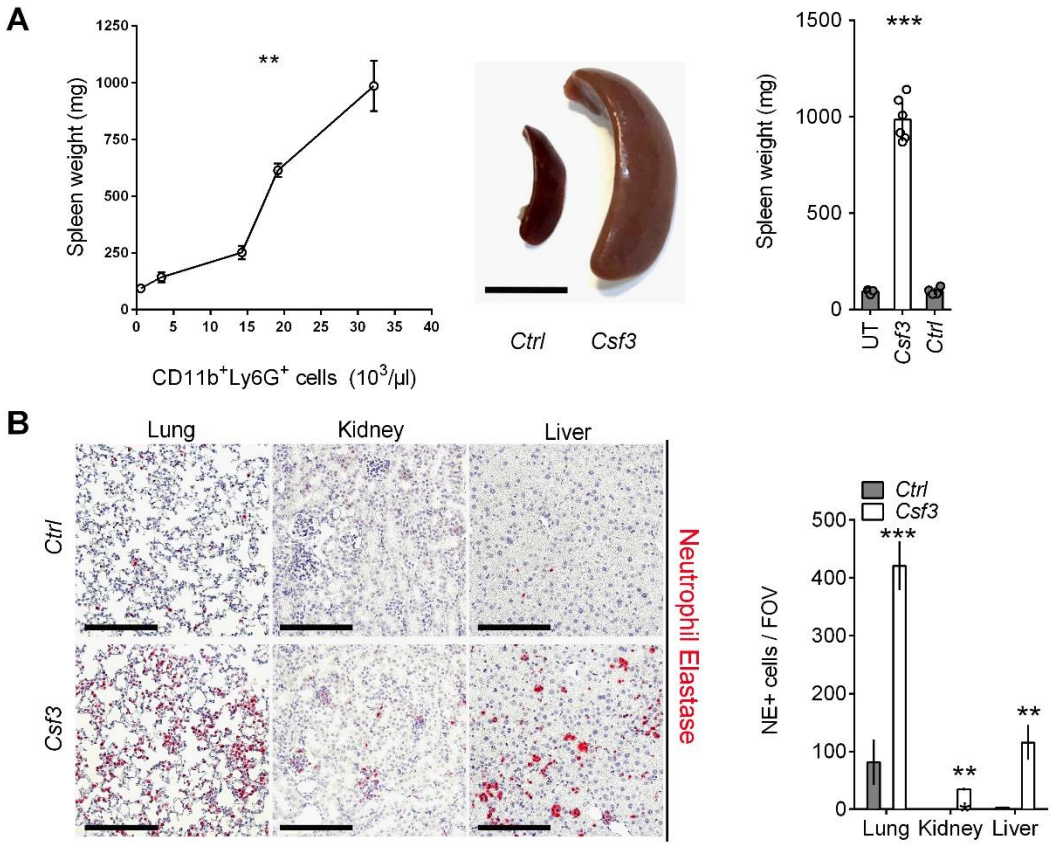
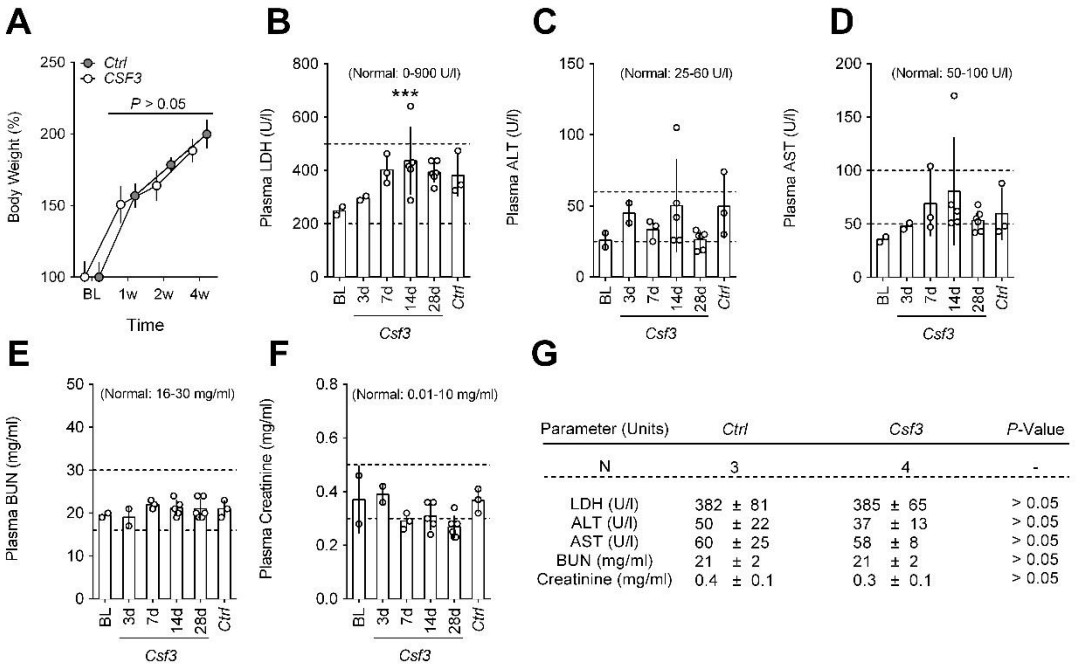


Figure 4: G-CSF expression causes splenomegaly and increased neutrophil infiltration in organs. Spleen, lung, liver, and kidney were harvested from mice injected with *Csf3*-expression plasmid and empty control plasmid. **(A)** Correlation between spleen weight and circulating neutrophils ($CD11b^{+}Ly6G^{+}$ cells), representative spleen images and weights of the spleen of untreated, *Csf3* treated, and *Ctrl* treated mice (N

= 5-6). Scale bars: 1 cm. **(B)** Immunostaining for neutrophils elastase (NE, red) and quantification NE+ cells in lungs, kidneys, and livers (N=3). Scale bars: 200µm. Statistics: (A) Pearson's correlation coefficient and one-way ANOVA followed by Bonferroni's multiple comparisons post hoc test, (B) multiple *t*-test; $^{**}P < 0.01$*** $P < 0.001$ versus UT.

Neutrophils and NETs are linked to tissue damage and injury. Since the *Csf3* treatment induces neutrophilia, we tested these mice for indication of abnormal behaviour and organ damage. First, we compared the relative change in the body weight of mice from the treated and control groups. The mice in both groups exhibited a similar growth rate, suggesting that the *Csf3* treated mice continued to grow normally (Figure 5 A). Next, we measured the level of physiological parameters in the plasma to detect the presence of organ damage. LDH is a cytoplasmic enzyme, expressed in RBCs and various organs (e.g., heart, muscle, liver, and brain). It catalyzes the conversion of lactate into pyruvic acid. Tissue damage, such as haemolysis releases LDH. Thus, LDH levels in the plasma is a measure of the amount of cell damage[216]. ALT and AST are cytosolic enzymes found in high concentration in the liver and are markers of hepatocellular injury[217]. BUN is the primary metabolite derived from dietary protein and tissue protein turnover and creatinine is the product of muscle creatinine catabolism. High levels of BUN and creatinine signifies improper clearance by the kidneys[218]. In heathy C57BL/6 mice, the level of LDH, ALT, AST, BUN and creatinine are LDH: 0-900 U/l, ALT: 25-60U/l, AST: 50-100 U/l, BUN: 16-30 mg/ml and creatinine: 0.01-10 mg/mlq [219,210,211]. We tested the level of these enzymes in the plasma from *Csf3* treated mice as well as the control mice. We show that the levels of all enzymes were within the normal range and there was no significant difference between the two groups (Figure 5 B, C, and D). Therefore, we concluded that the expression of *Csf3* in wild-type mice generates normal neutrophilic mice.



Parameter (Units)	*Ctrl*	*Csf3*	*P*-Value
N	3	4	-
LDH (U/l)	382 ± 81	385 ± 65	> 0.05
ALT (U/l)	50 ± 22	37 ± 13	> 0.05
AST (U/l)	60 ± 25	58 ± 8	> 0.05
BUN (mg/ml)	21 ± 2	21 ± 2	> 0.05
Creatinine (mg/ml)	0.4 ± 0.1	0.3 ± 0.1	> 0.05

Figure 5: G-CSF expression generated healthy mice with chronic neutrophilia. The body weight representing the rate of growth as well as levels of plasma parameters of organ damage were compared between the mice injected with G-CSF-expression plasmid or with an empty control plasmid (*Ctrl*). **(A)** Body weight of mice at indicated times (N = 3-5). **(B)** Plasma levels of LDH at indicated times after the injection. **(C), (D)** Plasma levels of ALT and AST at indicated times after the injection. **(E), (F)** Plasma levels of BUN and creatinine at indicated times after the injection. **(G)** Plasma levels of LDH, ALT, AST, BUN, and creatinine two weeks after the injection. Statistics: (A) two way (B, C, D, E, F,) and one way ANOVA followed by Bonferroni's multiple comparisons post hoc test, *** $P < 0.001$ versus BL.

PART 2: Development of an in vivo NETs model.

3.4 Serum DNASE1 and DNASE1L3 degrade NETs in vitro.

NETs contribute to a variety of diseases[222]. The observation that wild-type mice remain healthy, in spite of intravascular NETosis indicates the presence of a mechanism that prevents the pathological effects NETs. Two DNA-degrading enzymes DNASE1 and DNASE1-LIKE 3 (DNASE1L3) are present in circulation[112]. We hypothesized that the DNA degrading activity of these DNases is the fundamental mechanism that prevents disease development in wild-type neutrophilic mice. To test this hypothesis we generated knockout mice with a single deficiency of DNASE1 (*Dnase1*$^{-/-}$) and DNASE1L3 (*Dnase1l3*$^{-/-}$) as well as mice with a combined deficiency of both DNASE1 and DNASE1L3 (*Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$).We detected the DNases in the serum from *Dnase1*$^{-/-}$, *Dnase1l3*$^{-/-}$ *and Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ using different activity assays. Firstly, we used the serum from wild-type mice, *Dnase1*$^{-/-}$, *Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ in a denaturing PAGE zymogram (DPZ), a method that allows for the simultaneous detection of both nucleases. We detected the activity of both DNASE1 (37 kDa) and DNASE1L3 (34 kDa) in serum from wild-type mice. The serum from *Dnase1*$^{-/-}$ mice only showed the presence of DNASE1L3, and the serum from *Dnase1l3*$^{-/-}$ mice only showed the presence of DNASE1. Secondly, we used the serum in the single radial enzyme detection (SRED) assay to detect the total DNASE activity. As expected the serum from wild-type mice, *Dnase1*$^{-/-}$, *Dnase1l3*$^{-/-}$ showed nuclease activity, and the serum from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ showed no activity (Figure 6 A).

Next, we aimed to test the *in vitro* NET degrading activity of serum DNASE1 and DNASE1L3. To generated *in vitro* NETs, we isolated neutrophils from human blood. We induced NET formation by activating them with phorbol 12-myristate 13-acetate (PMA). PMA is a well-described stimulant of NETosis as it is a specific activator of Protein Kinase C (PKC) resulting in the generation of reactive oxygen species (ROS) [223]. NETs generated *in vitro* were incubated with the sera from wild-type, *Dnase1*$^{-/-}$, *Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ mice. The amount of degradation were detected by staining NETs with the fluorescent DNA dye Sytox Green and analyzed using fluorescence microscopy. We acquired images and quantified the non-degraded NETs by measuring the

fluorescence intensity (Figure 6 B, C). Serum from wild-type, *Dnase1*$^{-/-}$, *Dnase1l3*$^{-/-}$ mice efficiently degraded NETs. Whereas, NETs treated with the serum from *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ remained intact. In conclusion, serum DNASE1 and DNASE1L3 degrade NETs *in vitro.*

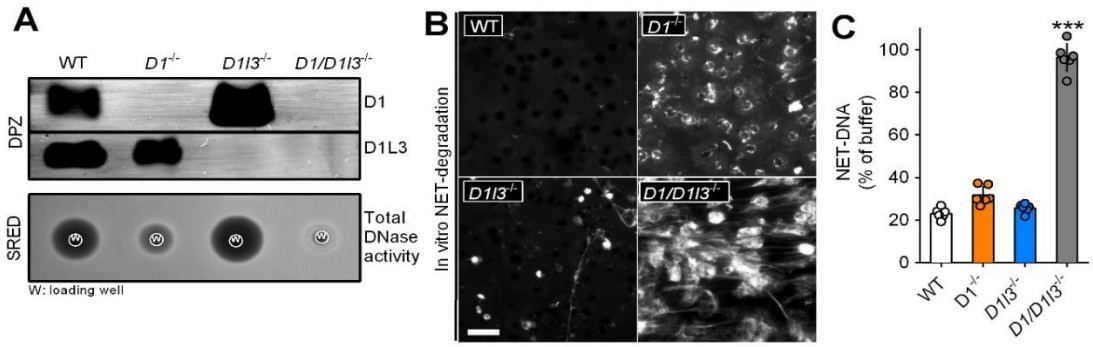


Figure 6: DNASE1 and DNASE1L3 in circulation degrade NETs in vitro. Characterization of DNA-degrading activity of sera from WT, *Dnase1*$^{-/-}$ (*D1*$^{-/-}$), *Dnase1l3*$^{-/-}$ (*D1l3*$^{-/-}$), *Dnase1*/*Dnase1l3*$^{-/-}$ (*D1*/*D1l3*$^{-/-}$) mice. (**A**) Detection of DNase1 (D1), DNase1L3 (D1L3), and total DNase activity by the zymographic assays DPZ and SRED. (**B**) Images and (**C**) quantification of DNA-stainings of NETs generated *in vitro* after incubation with sera from indicated genotypes (N = 6). Scale bar: 50µm. Statistics (C) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test, *** $P < 0.001$ versus all other groups.

As a second approach to test the *in vitro* NET degradation by serum DNases, we restored the DNASE1 and DNASE1L3 activity in *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice. The hepatocyte-specific expression plasmid in conjunction with hydrodynamic gene delivery stably expresses the cDNA of *Dnase1* and *Dnase1l3.* The parent plasmid was injected as a control. DNASE1 and DNASE1L3 are secretory enzymes and hence this approach restores the capacity of the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice sera to degrade NETs[112]. Denaturing PAGE zymogram (DPZ) assay detected DNASE1 and DNASEL3 activity in the serum from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ that was injected with *Dnase1* and *Dnase1l3* cDNA respectively. Total DNASE activity in the serum was tested using the SRED assay. As expected the serum from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated with control vector had no activity (Figure 7 A). Next, we tested the NET degrading activity in the serum. *In vitro* NETs generated from human neutrophils was incubated with buffer and the serum from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated with a control plasmid remained intact. Serum from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated *Dnase1* and *Dnase1l3* cDNA degraded

NETs. Collectively, these results indicate that DNASE1 and DNASE1L3 degrade NETs *in vitro* independent of each other (Figure 7 B, C).

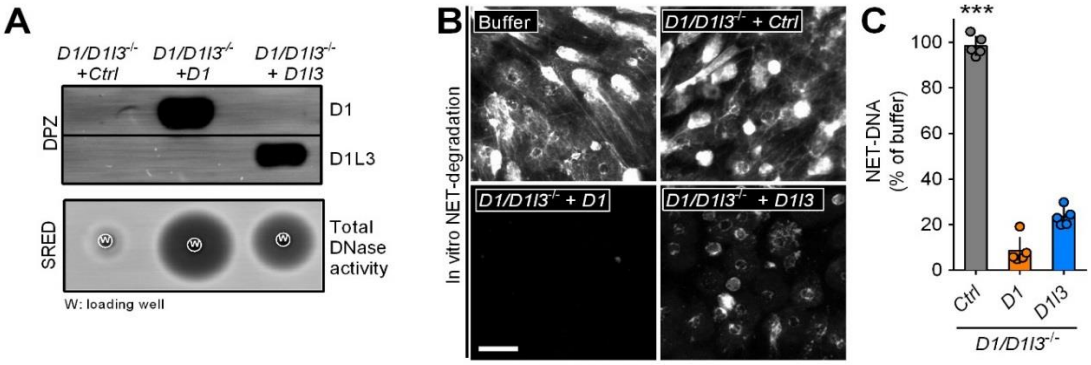


Figure 7: DNASE1 and DNASE1L3 in circulation degrade NETs in vitro. (A) DPZ and SRED analysis of sera from *D1/D1l3*$^{-/-}$ mice stably expressing a plasmid with *Dnase1* (*D1*), *Dnase1l3* (*D1l3*), or a control plasmid (*Ctrl*) for 7 days. (**B**) Images and (**C**) quantification of DNA-staining of NETs generated *in vitro* after incubation with buffer or sera from *D1/D1l3*$^{-/-}$ mice expressing *D1*, *D1l3*, or *Ctrl* (N = 5). Scale bar: 50 µm. Statistics (C) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test, *** P < 0.001 versus all other group**s.**

3.5 DNASE1 and DNASE1L3 degrade NETs in vivo.

Improperly cleared NETs in circulation are pathological. Neutrophils, at high concentrations, become activated to form spontaneous NETs in blood. Based on the *in vitro* data, we hypothesized that DNASE1 and DNASE1L3 degrade NETs in circulation to prevent adverse effects. To test this hypothesis *in vivo*, we induced chronic neutrophilia in *Dnase1*$^{-/-}$, *Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice by injecting the *Csf3*-expression plasmid. As a control, we injected *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice with the parent plasmid. The *Dnase1*$^{-/-}$ and *Dnase1l3*$^{-/-}$ mice developed chronic neutrophilia, but showed no signs of distress and were indistinguishable from wild-type mice with chronic neutrophilia. We euthanized the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ within 3 to 6 days after *Csf3* treatment as they developed a fatal phenotype. In brief, the mice developed a rapidly progressing hypothermia indicated by a sudden drop in body temperature of ≥ 4°C within 8 eight hours. Hypothermia is accompanied by hematuria, indicated by reddish urine. They lacked any spontaneous movements and had partially closed eyes. Notably, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice that received the empty control vector remained healthy (Figure 8 A).

In conclusion, these data show that DNASE1 or DNASE1L3 is required to tolerate G-CSF induced neutrophilia.

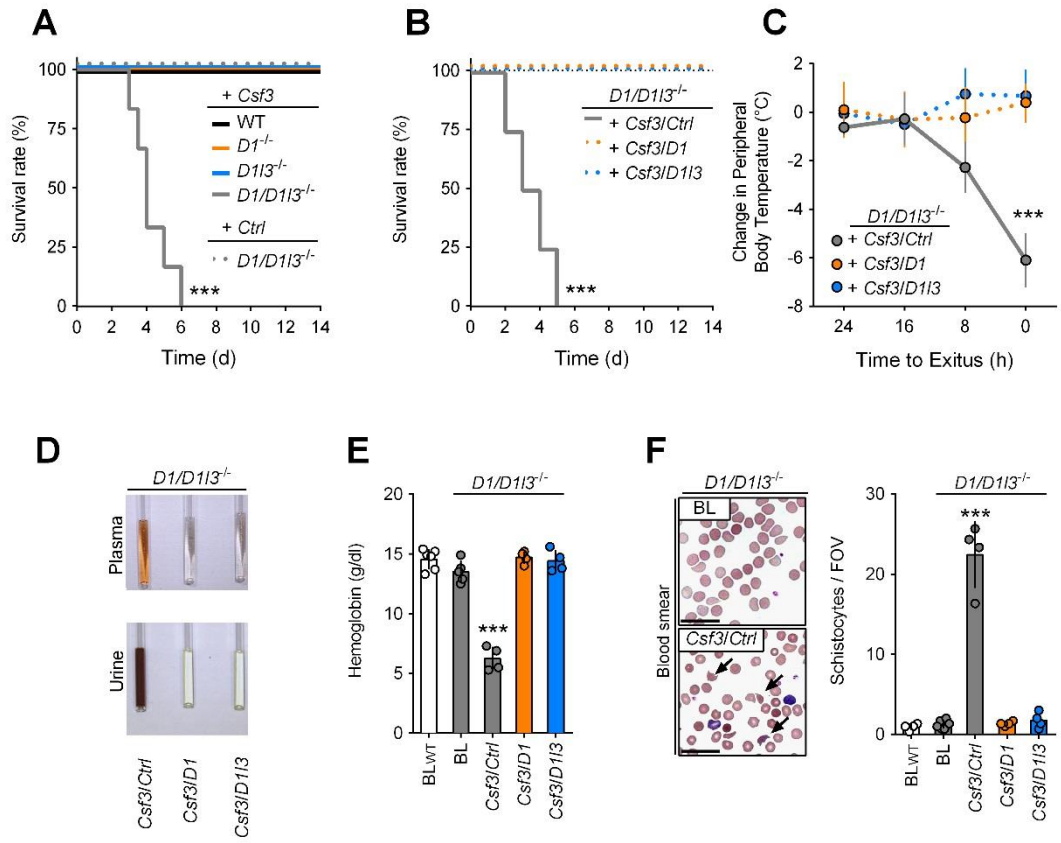


Figure 8: DNASE1 or DNASE1L3 is required to tolerate chronic neutrophilia. Chronic neutrophilia was induced by injection of a G-CSF-expression plasmid (*Csf3*). Controls received an empty plasmid (*Ctrl*). (A) Survival of WT (N = 7), *Dnase1*$^{-/-}$ (*D1*$^{-/-}$, N = 6), *Dnase1l3*$^{-/-}$ (D1l3$^{-/-}$, N = 6), *Dnase1*/*Dnase1l3*$^{-/-}$ mice (*D1*/*D1l3*$^{-/-}$, N = 6) injected with *Csf3* or *Ctrl* (N = 4). (B) Survival of *D1*/*D1l3*$^{-/-}$ mice co-expressing *Csf3* with *Dnase1* (*Csf3*/*D1*, N = 5), *Dnase1l3* (*Csf3*/*D1l3*, N = 6), and controls (*Csf3*/*Ctrl*, N = 4). (E to I) Characterization of mortality during chronic neutrophilia (N = 4). (C) Change in peripheral body temperature. (D) Photographs of plasma and urine. (E) Concentration of hemoglobin in blood. (F) Images and quantification of schistocytes in blood smears. Arrows point to schistocytes. Scale bars: 20µm. Statistics: (A, B) log rank test, (C) two way and (E, F) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test; *** $P < 0.001$ versus BL_{WT}.

In the next experiment, we genetically restored DNASE1 and DNASE1L3 activity in *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice to rule out any functions of DNASE1 and DNASE1L3 other than degrading extracellular DNA[224]. We co-expressed *Dnase1* and *Dnase1l3* cDNA along with *Csf3*. This approach induces neutrophilia and NETs and simultaneously

restores DNASE1 and DNASE1L3 in circulation. Empty plasmid injection in *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ generated the control group of mice. In line with our previous results, we found that the expression of DNASE1 or DNASE1L3 was sufficient for the survival of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice with chronic neutrophilia (Figure 8 B). The co-expression of the *Csf3* with a control plasmid, however, was fatal in the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice. These mice develop a rapidly progressing hypothermia indicated by a sudden drop in body temperature and haematuria as observed by reddish urine (Figure 8 C). The mice expressing DNASE1 or DNASE1L3 maintained a normal body temperature and showed no signs of haematuria and sacrificed two weeks after treatment. We collected EDTA-anticoagulated blood, plasma and harvested the lungs, liver, kidneys and the spleen for histological analyses from all the groups of mice. We observed that the plasma from the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice appeared red indicating hemolysis whereas the plasma from the mice expressing either DNASE1 or DNASE1L3 appeared normal (Figure 8 D). We measured the hemoglobin levels in the blood and revealed that *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ had decreased hemoglobin in comparison to the wild-type mice at baseline, *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ mice at baseline, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ expressing DNASE1 or DNASE1L3 (Figure 8 E). To support these results, Wright-Giemsa staining of blood smear showed an increased amount of schistocytes in the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice (Figure 8 F). Schistocytes are severed or fragmented RBCs, typically present at triangular or helmet shaped with pointed edge. Schistocytes formation causes haemolysis[225]. A schistocytes count for healthy individual in <0.5 % of total RBCs. A normal higher than normal count is suggestive of DIC and TMA[226]. The fibrin strands generated in DIC and TMA sever RBCs as they move past a thrombus, thereby generating schistocytes[226]. It is conceivable that extracellular DNA strands of NETs are similar to fibrin strands and cause the formation of schistocytes.

3.5.1 DNASE1 and DNASE1L3 prevent multiple organ damage

Dnase1$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated with *Csf3* expression vector develop a fatal phenotype. To detect organ damage, we measured the levels of LDH, ALT, AST, BUN and creatinine in plasma. *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated with a control vector had elevated levels of plasma LDH (Figure 9 A) indicating tissue damage. Elevated plasma levels of ALT and AST, indicate liver damage (Figure 9 B), while high levels of BUN and creatinine in

plasma suggest kidney failure (Figure 9 C). Collectively, the data suggests that DNASE1 and DNASE1L3 protect from multiple organ failure.

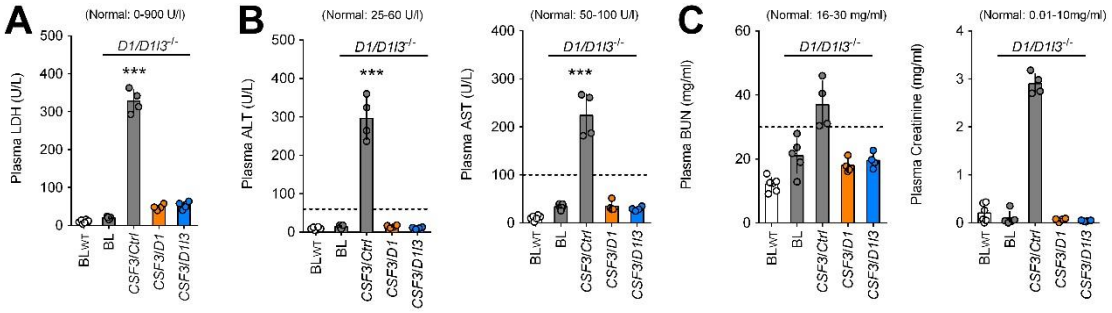


Figure 9: DNASE1 and DNASE1L3 prevent multiple organ damage. The concentration of indicators of organ damage in the plasma was measured. **(A)** LDH concentration in plasma. BL: baseline (N = 5-6). **(B)** Plasma levels of alanine and aspartate aminotransferases (ALT, AST). **(C)** Plasma levels of blood urea nitrogen (BUN) and creatinine. Statistics: (A, B, C) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test; *** $P < 0.001$ versus BL_{WT}.

3.5.2 DNASE1 and DNASE1L3 prevent vascular occlusions.

To document the cause for multi-organ failure, we analysed lungs, liver, and kidneys histologically. In brief, we prepared paraffin section of the lungs, liver, and kidney of wild-type mice at baseline, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice at baseline *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ treated with a control vector and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ treated with the *Dnase1* or *Dnase1l3* cDNA. Haematoxylin and eosin (H&E) staining is a histopathology technique used regularly to recognize various tissue types and morphological changes. Haematoxylin has a deep blue-purple colour and stains nucleic acids. Eosin is pink and stains the cytoplasm, extracellular matrix and proteins non-specifically[227]. Light microscopy analyses of H&E stained sections revealed the presence of several intravascular hematoxylin-rich clots that occluded blood vessels in the lungs, liver, and kidneys of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$. The clots were abundant in a light purple stain indicating decondensed DNA, interspersed with deep blue-purple spots, representing the individual leukocyte nuclei (Figure 10). The clots trapped and immobilized erythrocytes. In conclusion, the clots are primarily composed of DNA trapping leukocytes.

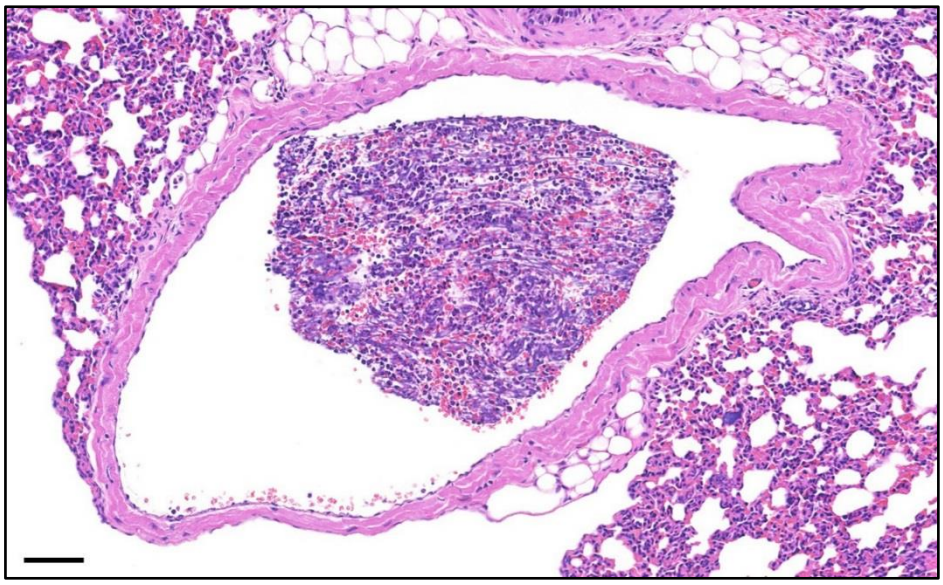


Figure 10: Representative image of an intravascular hematoxylin-rich clot in the lungs. Blood vessel of *D1/D1l3*$^{-/-}$ mice + *Csf3/Ctrl* shows a hematoxylin-rich clot with entrapped erythrocytes and few leukocyte nuclei. Scale bar: 25µM.

The sections from wild-type mice at baseline, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice at baseline, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice that expressed either DNASE1 or DNASE1L3 had no occluded blood vessels. We quantified the number of fully and partially occluded vessels in five adjacent fields of view and showed that *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated with the control vector had a high number of occluded blood vessels (Figure 11 A, C, E).

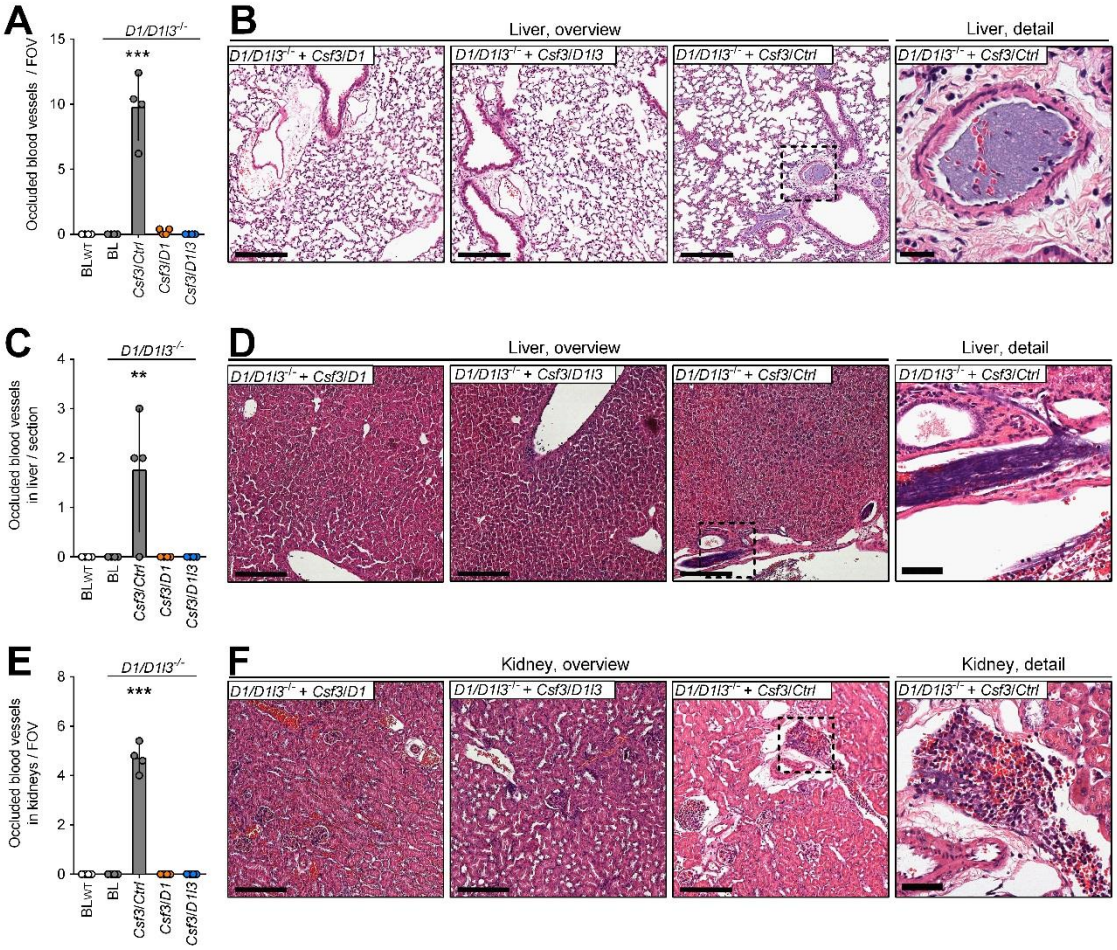


Figure 11: DNASE1 and DNASE1L3 prevent vascular occlusion by NET clots during chronic neutrophilia. Histological analysis of *D1/D1l3*$^{-/-}$ co-expressing *Csf3* with *Dnase1* (*Csf3*/*D1*, N = 4), *Dnase1l3* (*Csf3*/*D1l3*, N = 4), or a control plasmid (*Csf3*/*Ctrl*, N = 4). **(A)** Quantification of blood vessels in lungs occluded by hematoxylin-positive clots per field of view (FOV). Baseline WT mice (BL_{WT}, N = 4), baseline *D1/D1l3*$^{-/-}$ mice (BL; N = 4). Scale bars: 500 µm (Overview), 25 µm (Detail). **(B)** Hematoxylin and eosin stainings (H & E) of lungs. Blood vessel of *D1/D1l3*$^{-/-}$ mice + *Csf3*/*Ctrl* shows a hematoxylin-rich clot with entrapped erythrocytes and few leukocyte nuclei. **(C)** Quantification of intravascular hematoxylin-rich clots in livers per section. **(D)** Representative H & E stainings of livers. Scale bars: 500 µm (overviews), 50 µm (detail). **(E)** Quantification of blood vessels in kidney occluded by hematoxylin-rich clots per FOV. **(F)** Representative H & E stainings of kidney. Scale bars: 500 µm (overviews), 50 µm (detail). Statistics: (A, C, E) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test;** $P < 0.01$, *** $P < 0.001$ versus BL_{WT}.

Furthermore, we analyzed the lung sections from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ with chronic neutrophilia by using fluorescent double-stranded DNA-intercalating dyes and antibodies against chromatin. A robust staining for both DNA and chromatin confirmed that DNA was the major component of these vascular occlusions (Figure 12). In summary, these

data suggest that mice lacking DNASE1 and DNASE1L3 died of multi-organ damage induced by systemic intravascular DNA-clots comprising NETs.

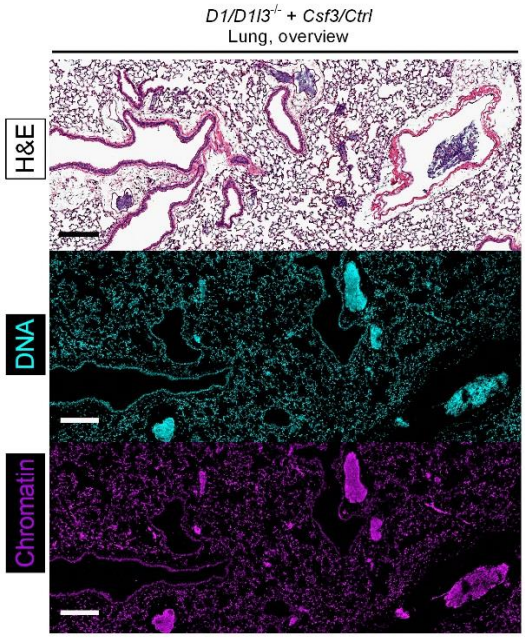


Figure 12: Hematoxylin-positive clots stain robustly for double-stranded DNA and chromatin. Analysis of lungs from *D1/D1l3*$^{-/-}$ mice with chronic neutrophilia (*D1/D1l3*$^{-/-}$ + *Csf3*/*Ctrl*). Staining with hematoxylin and eosin (H & E), for DNA (cyan), and for chromatin (purple) of consecutive lung sections. Scale bars: 200 µm.

3.5.3 NETs form intravascular occlusions in the absence of DNASE1 and DNASE1L3

Dnase1$^{-/-}$*Dnase1l3*$^{-/-}$ mice were treated with *Csf3* that induces chronic neutrophilia. Therefore, we speculated that the DNA rich vascular occlusion is composed of neutrophil DNA from NETs. To test this, we co-stained these clots with antibodies against chromatin and as well as neutrophils granule-derived enzymes. Cathelicidin-related antimicrobial peptides (CRAMP) a family of polypeptides and, MPO are proteins stored in neutrophils[228]. The co-localization of the chromatin signal, with MPO and CRAMP showed that the DNA in these clots come from neutrophils (Figure 13 A, B).

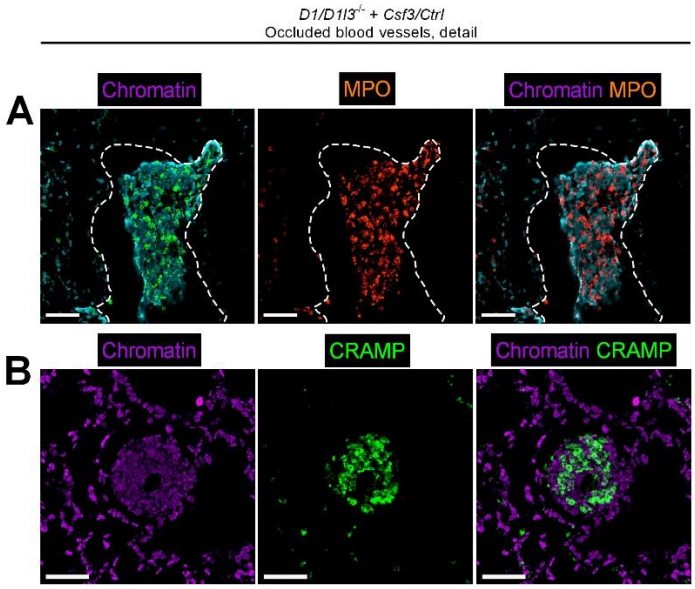


Figure 13: DNA clots are composed of NETs. Lungs from *D1/D1l3*$^{-/-}$ mice with chronic neutrophilia (*D1/D1l3*$^{-/-}$*+Csf3/Ctrl*) were stained with chromatin and neutrophil and NET markers. **(A)** Immunostaining of occluded blood vessels for chromatin (cyan) and the neutrophil-marker MPO (red). Scale bars: 50 µm. **(B)** Immunostaining of occluded blood vessel for chromatin (purple) and CRAMP (green). Scale bars: 50 µm. Images are representative of four mice.

Citrullinated histones are a marker of NETosis[192]. The co-localization of the chromatin signal with the signal from NET-surrogate marker citrullinated histones (citH3) indicated that the clots were composed of NETs (Figure 14).

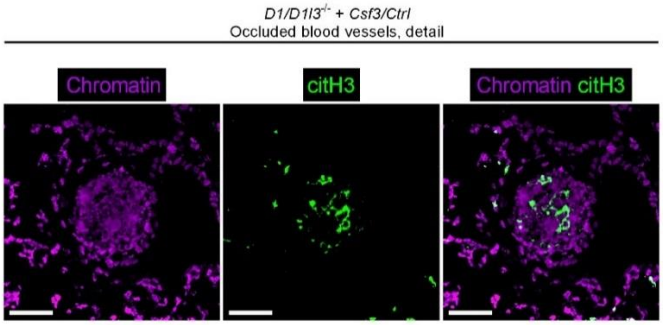


Figure 14: DNA clots are composed of NETs. Lungs from *D1/D1l3*$^{-/-}$ mice with chronic neutrophilia (*D1/D1l3*$^{-/-}$*+Csf3/Ctrl*) were stained with chromatin and NET markers. Immunostaining of occluded blood vessel for chromatin (purple) and citH3 (green). Scale bars: 50 µm. Images are representative of four mice.

3.5.4 Intravascular NET clots formed independent of platelets and fibrin.

Platelet activation and fibrin generation results in a canonical thrombi. We aimed to test the involvement of platelets and fibrin in the formation of in travascular NET clots. In the first approach, we stained lung sections from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ with chronic neutrophilia for vWF and fibrin. vWF is a blood glycoprotein stored in the secretory vesicles of platelets[229]. Fibrin is an insoluble protein formed from fibrinogen during the clotting of blood[230]. Injury to the vessel wall exposes collagen from the subendothelial matrix, and tissue factor that activate platelets[231,232]. Activated platelets modulates thrombus formation by triggering the contact pathway in a Factor XII dependent manner[233, 234]. This results in the formation of thrombin, subsequently converting inactive fibrinogen into the insoluble protein fibrin. NET clots vary in their composition of vWF and fibrin. The percentage of vWF was 45.7 ± 27.1% (N = 4) and the percentage of fibrin was 3.4 ± 4.4% (N = 4) (Figure 15 A). Importantly, 9.6 ± 8.4 % (N = 4) of NET clots showed no signs of vWF and fibrin (Figure 15 B). Collectively these data shows that NET-clots create a scaffold to trap and immobilize platelets and erythrocytes and occlude blood vessels *in vivo*, resulting in organ damage.

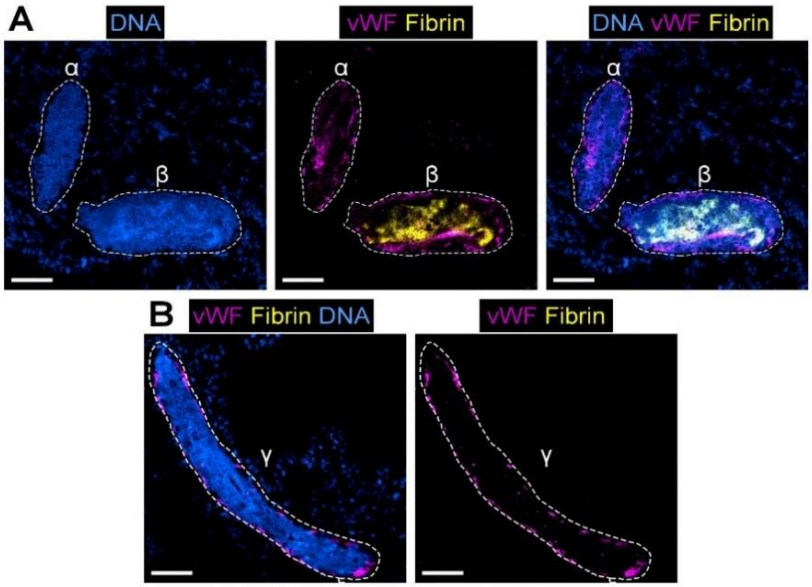


Figure 15: NET clots independent of vWF and Fibrin. Lungs from *D1*/*D1l3*$^{-/-}$ mice with chronic neutrophilia (*D1*/*D1l3*$^{-/-}$ + *Csf3*/*Ctrl*) were stained for components of canonical thrombi, vWF and Fibrin. **(A)** Immunostaining for von Willebrand factor (vWF, pink), and **(B)** Fibrin (yellow), and DNA (blue).

NET clots comprise vWF or fibrin or lack these components (α, $vWF^{+}/fibrin^{-}$: 65.3 ± 24.5 %; β, $vWF^{+}/fibrin^{+}$: 25.1 ± 30.8 %; γ, $vWF^{-}/fibrin^{-}$: 9.6 ± 8.4 %; Mean ± SD, N = 4 mice).

In the second approach to detect the presence of components of canonical thrombi, we used two antithrombotic treatments. Firstly, we depleted platelets in the circulation of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice with chronic neutrophilia by treating them with an antiplatelet antibody. IgG-treated mice formed the control group. After 2 days, mice treated with anti-CD42b showed a platelet count of less than 99%, when compared to mice treated with non-immune rat IgG (Figure 16 A). Both, control as well as platelet-depleted mice, developed rapid hypothermia and haematuria and had to be sacrificed within six days after *Csf3* expression (Figure 16 B). Secondly, we treated mice with dabigatran, which is a pharmacological inhibitor of thrombin[235]. Dabigatran was included in the food eaten by the mice. The control group of mice were fed with regular chow. WT mice fed with the dabigratan diet for 1 day showed a 6.48 ± 1.19-fold (Mean ± SD, N = 4) increased activated partial thromboplastin time when compared to mice receiving normal chow without dabigratan (Figure 16 E). Similar to the platelet depletion experiment, both control group, as well as dabigatran, treated mice developed rapid hypothermia, and hematuria and had to be sacrificed within eight days after *Csf3* expression (Figure 16 F). Furthermore, histological analyses by staining the lung section from these mice with H&E revealed the presence of several occluded blood vessels. We quantified the number of the blood vessel in adjacent fields of view and showed that the control groups of mice and the platelet depleted or thrombin inhibited mice showed no significant difference (Figure 16 C, D, G, and H**)**. Our results show that neither of the antithrombotic treatment was sufficient to protect these mice and prevent the formation of occluded blood vessels during chronic neutrophilia. In conclusion, intravascular NET clots is independent of the components of canonical thrombi.

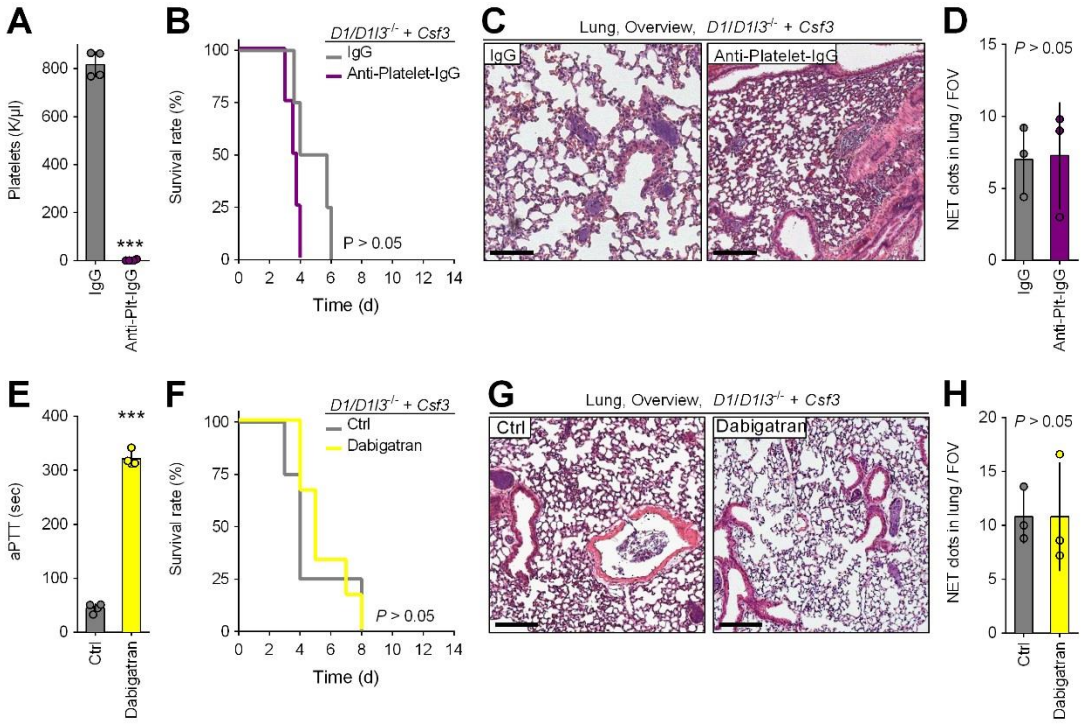


Figure 16: Anti-thrombotic treatment is not sufficient to prevent mortality in neutrophilic Dnase1/Dnasel3-/- mice. (A) Blood platelet counts in WT mice 48 hours after injection of 2 µg/g non-immune IgG or Anti-Platelet (Plt)-IgG (N = 4). **(B)** Survival curve of neutrophilia in *Dnase1*/*Dnase1l3*$^{-/-}$ mice (*D1*/*D1l3*$^{-/-}$, N = 5) derived from an alternative *Dnase1*$^{-/-}$ strain. Mice were treated non-immune IgG or Anti-Platelet-IgG. **(C)** Representative H & E stainings of lungs show hematoxylin-rich clots in IgG or Anti-Platelet (Plt)-IgG treated neutrophilic *Dnase1*/*Dnase1l3*$^{-/-}$ mice. Scale bars: 200 µm. **(D)** Quantification of intravascular hematoxylin-rich clots in lungs per field of view [(FOV), N = 3]. **(E)** Activated partial thromboplastin time (aPTT) of WT mice fed with a dabigatran diet for 1 day (Dabigatran) and WT mice fed with regular chow (Ctrl). (F) Survival curve of neutrophilia in *Dnase1*/*Dnase1l3*$^{-/-}$ mice treated with Dabigatran (N = 6) and Ctrl (N = 4). **(G)** Representative H & E stainings of lungs show hematoxylin-rich clots neutrophilic *Dnase1*/*Dnase1l3*$^{-/-}$ mice treated with Dabigatran and Ctrl. Scale bars: 200 µm. (H) Quantification of intravascular hematoxylin-rich clots in lungs per field of view [(FOV), N = 3]. Statistics: (A, D, E, H) Student's *t*-test, *** $P < 0.001$; (B, F) log-rank test, $P > 0.05$.

3.5.5 DNASE1 and DNASE1L3 prevents intravascular NETs in sepsis

Increased G-CSF levels have been reported in patients with various malignancies, including chronic myelogenous leukaemia (CML) inflammatory and autoimmune disease[236]. Particularly in inflammatory arthritis[237], experimental allergic encephalomyelitis[238], and uveoretinitis[239]. Induction of chronic neutrophilia by the hepatic expression of G-CSF provides a model to study the outcome of elevated G-CSF levels. However this set up has some limitations as it is no physiological. The next aim of this thesis was to analyze the outcome of DNase deficiency in a second physiological model that is independent of *Csf3* induced neutrophilia.

Sepsis or endotoxemia is a full body infection, caused due to bacterial infection in the blood. Sepsis induces an acute inflammatory response. Bacteria in circulation activates neutrophils to form NETs[240]. Several studies show that sepsis rapidly triggers the formation of intravascular NETs[241,160]. We hypothesized that the absence of DNases would aggravate the outcome of sepsis. To test our hypothesis, we generated a mouse model of sepsis by injecting mice with lipopolysaccharides (LPS) and heat-killed bacteria (*E.coli*). LPS is a major component of the outer membrane of gram-negative bacteria[242]. To mimic the inflammatory response that occurs in humans during sepsis, we injected wild-type mice, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice expressing control plasmid and *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ mice expressing DNASE1 or DNASE1L3 mice with a sublethal dose of LPS for three days consecutive days and a single IV injection of heat-killed bacteria. We observed that all groups of mice show reduced activity accompanied by apparent cachexia. Upon injection of the heat-killed bacteria, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ expressing control plasmid but not wild-type or *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ expressing either DNASE1 or DNASE1L3 developed severe hypothermia with accompanying hematuria. The wild-type mice and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ expressing either DNASE1 or DNASE1L3 recovered (Figure 17 A). Our results showed that *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ with sepsis developed a phenotype similar to what we observed in the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ with neutrophilia. They developed hemolytic anemia indicated by the drop in hemoglobin levels in the plasma **(**Figure 17 B), and hematuria indicated by the presence of reddish-brown urine and plasma (Figure 17 C). The blood smears revealed the presence of an elevated number of schistocytes suggesting the hemolysis due to RBC fragmentation by DNA strand from

NETs (Figure 17 D). In addition to this, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ showed an elevated LDH concentration in the plasma indicating organ damage (Figure 17 E).

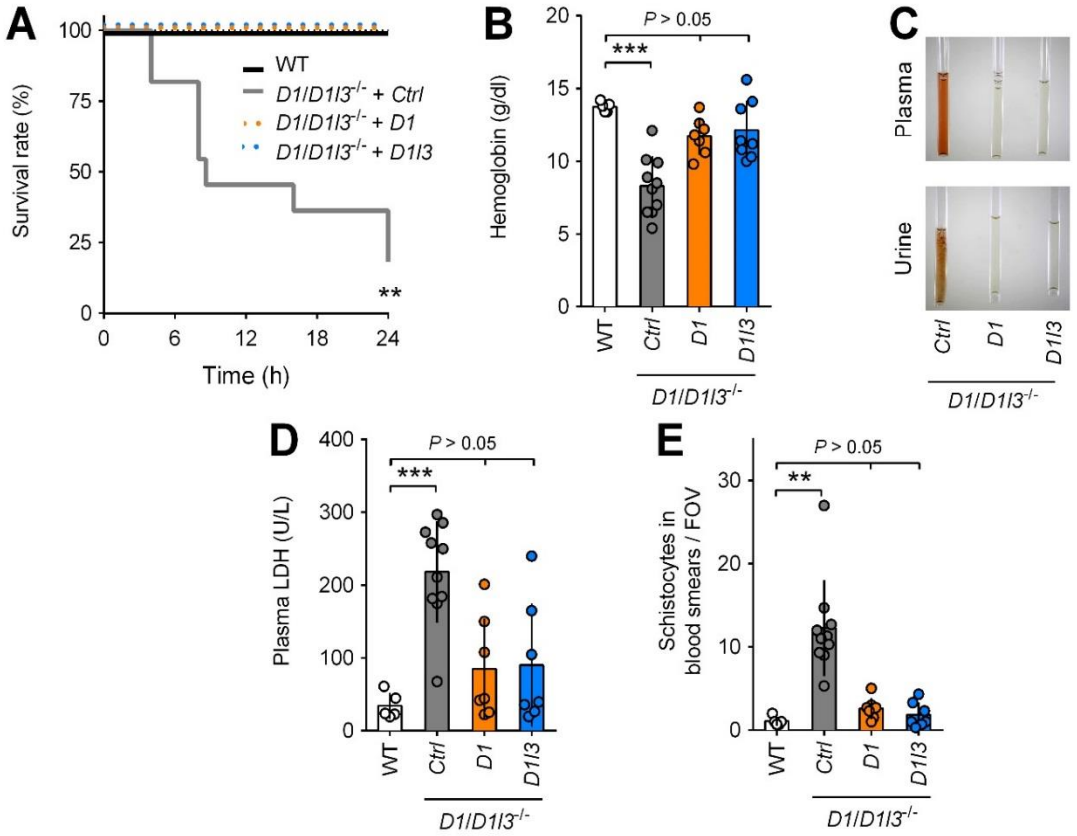


Figure 17: DNASE1 and DNASE1L3 protect against host injury in septicemia. WT mice (N = 5) and mice with a combined deficiency in DNASE1 and DNASE1L3 (*D1/D1l3*$^{-/-}$) expressing *Dnase1* (*D1*, N = 7), *Dnase1l3* (*D1l3*, N = 8), or a control plasmid (*Ctrl*, N = 11) were treated with LPS and heat-killed *E.coli* to induce septicemia. (**A**) Survival time of septic mice. (**B**) Concentration of hemoglobin in the blood. (**C**) Representative photographs of plasma and urine. (**D**) LDH concentration in plasma. (**E**) Quantification of schistocytes in blood smears per field of view (FOV). Statistics: (A) log-rank test, ** $P < 0.01$ versus all other groups; (B, D, E) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test;** $P < 0.01$, *** $P < 0.001$ versus all other groups.

Furthermore, we analyzed the lung section from sepsis mice following H&E staining, to look for vascular occlusions. As expected, we found that the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ expressing control plasmid mice had several vascular occlusion in the lungs (Figure 18 B). In line with our observations in the chronic neutrophilia, these occlusions comprised of hematoxylin rich clots (Figure 18 B, C). Wild-type mice and hepatic expression of

DNASE1 or DNASE1L3 in *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice prevented the formation of NET clots during sepsis (Figure 18 A, D).

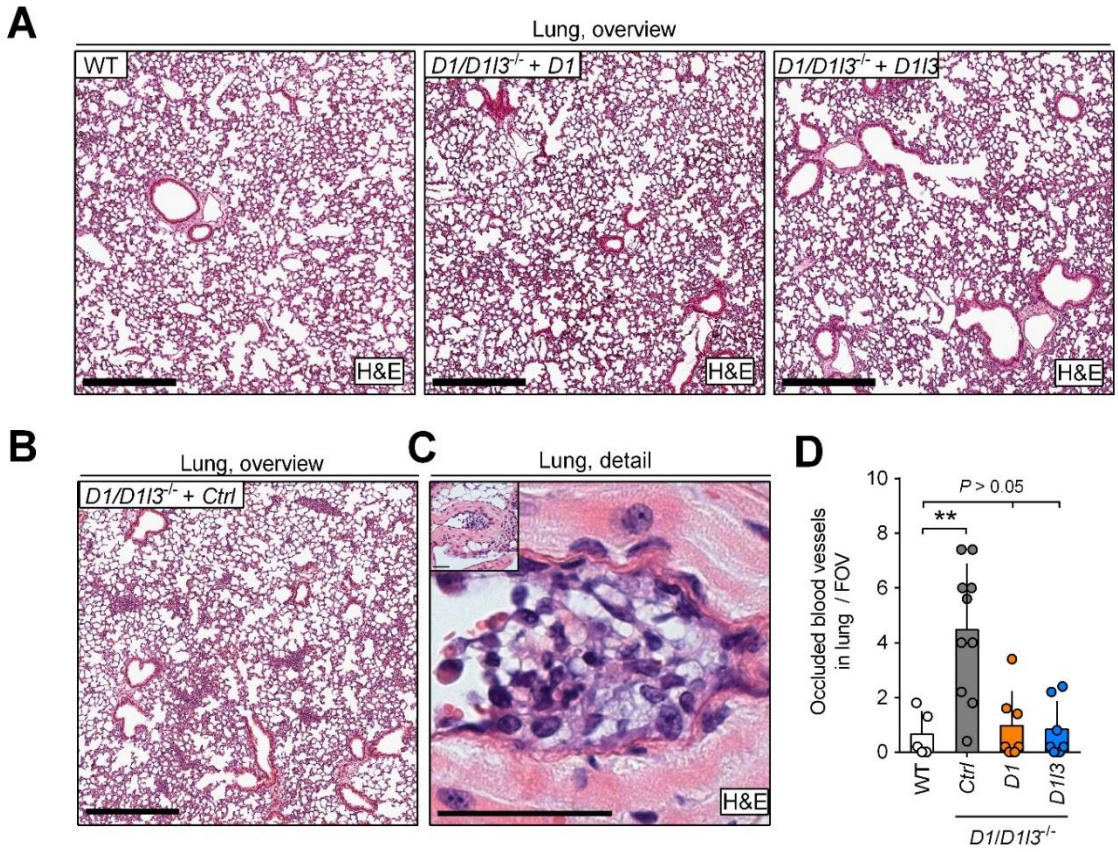


Figure 18: DNASE1 and DNASE1L3 prevent vascular occlusion by NETs in septicemia. Lung sections from *D1/D1l3*$^{-/-}$, expressing *Dnase1*, *Dnase1l3* or a control plasmid were treated with lipopolysaccharide and heat-killed *E.coli* to induce septicemia were treated with LPS and heat-killed *E.coli* to induce septicemia. **(A)** Representative hematoxylin and eosin stainings (H & E) of lungs of WT mice and *D1*/*D1l3*$^{-/-}$ mice expressing *D1* or *D1l3*. **(B)** Representative H & E staining of lungs of *D1*/*D1l3*$^{-/-}$ mice expressing the control plasmid. Scale bars: 500 µm. **(C)** Representative H & E staining of the occluded blood vessel. Inset is the overview. Scale bars: 50µm. **(D)** Quantification of occluded blood vessels in lungs per FOV. Statistics: (D) one way ANOVA followed by Bonferroni's multiple comparisons post hoc test;** $P < 0.01$, versus all other groups.

In summary, our data from the chronic neutrophilia model as well as the sepsis model describe a novel non-canonical mechanism for vascular occlusion by NETs. Host enzymes DNASE1 and DNASE1L3 independently degrade intravascular NETs. In the absence of both host enzymes, intravascular NETs clots occlude blood vessels leading to organ damage.

PART 3: Molecular mechanism of in vivo NET formation

NETs are implicated in many pathological conditions. However, limited insights into the molecular mechanism in *in vivo* NETs formation has made the identification of potential therapeutic target challenging. The reason for this is the lack of an appropriate preclinical animal model that provides a robust readout for NETs *in vivo*. In this study, we developed the first murine model with a robust intravascular NETosis due to a combined deficiency of circulatory DNASE1 and DNASE1L3. We now used this model to investigate the molecular mechanism of NETosis *in vivo.*

Firstly, we aimed to test the function of the canonical pathways of NETosis *in vivo* using our novel model of vascular occlusion by NETs in mice with chronic neutrophilia and sepsis. In brief, canonical pathways describe three enzymes that regulate the process of chromatin decondensation, i.e. PAD4, Nox2, and NE (Figure 18). PAD4 is required for the citrullination of histones. Histone citrullination creates a charge shift from positive to neutral, which facilitates chromatin decondensation[107]. Nox2 is a multimeric protein that is assembled during activation and is required for the generation of reactive oxygen species[67]. Finally, NE, which enters the nucleus and degrades histones, subsequently causing the chromatin to decondense[70]. Recent studies indicate that GSDMD lysis the plasma membrane cause the release of NETs[243]. NE cleaves inactive GSDMD, liberating the N terminal domain which has the capacity to form pores in membranes. Secondly, we aimed to understand the role of MyD88, which plays a role in TLRs signalling. Given that NETs are a cause of mortality in mice with dual deficiency in DNASE1 and DNASE1L3, this approach will help identify a key players of intravascular NETosis.

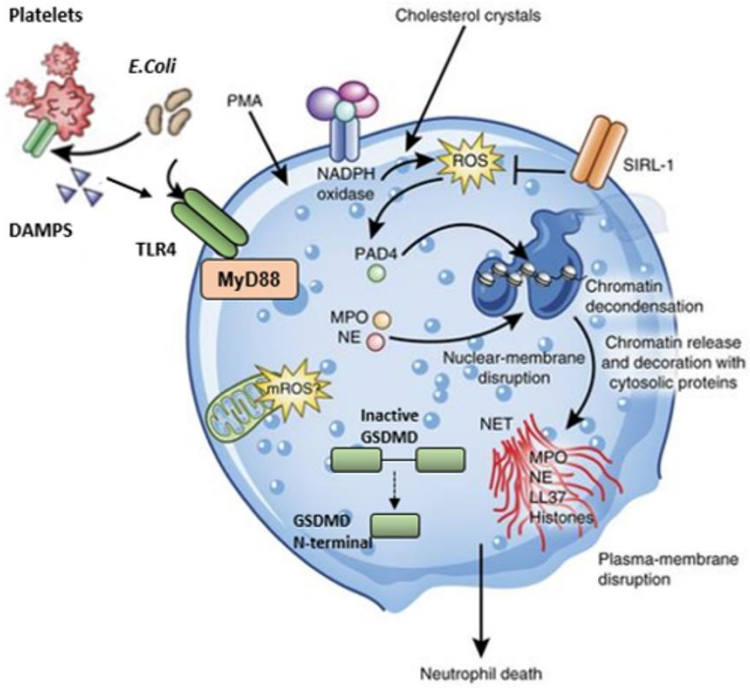


Figure 19: Mechanism of NETosis. Various stimuli induce NETosis. Activation of NADPH oxidase produces ROS, which activates PAD4 resulting in chromatin decondensation. Later, neutrophil elastase NE and MPO are translocated into the nucleus to promote further unfolding of chromatin resulting in nuclear membrane disruption. Chromatin is released into the cytosol, where it becomes decorated with granular and cytosolic proteins. Finally, NETs are released through disruption of the plasma membrane, by GSDMD. (Figure modified from S.K.Jorch, P.Kubes, *Nature medicine*, 2017 [244]**).**

We generated mice with a triple-deficiency of DNASE1, DNASE1L3, and the enzyme whose function we wanted to study, i.e., PAD4, Nox2, GSDMD and MyD88 (Figure 20). We aimed to comprehensively phenotype the triple knockout mice *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Nox2*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88*$^{-/-}$ at baseline, in G-CSF induced chronic neutrophilia and in LPS induced sepsis. We analyzed the mice as per the experiments described above. The analyses included our previously established protocols of macroscopic and behavioral characterization, blood and urine analyses and histological staining of multiple organs. This will provide us with a robust characterization of canonical pathways in NETosis *in vivo*.

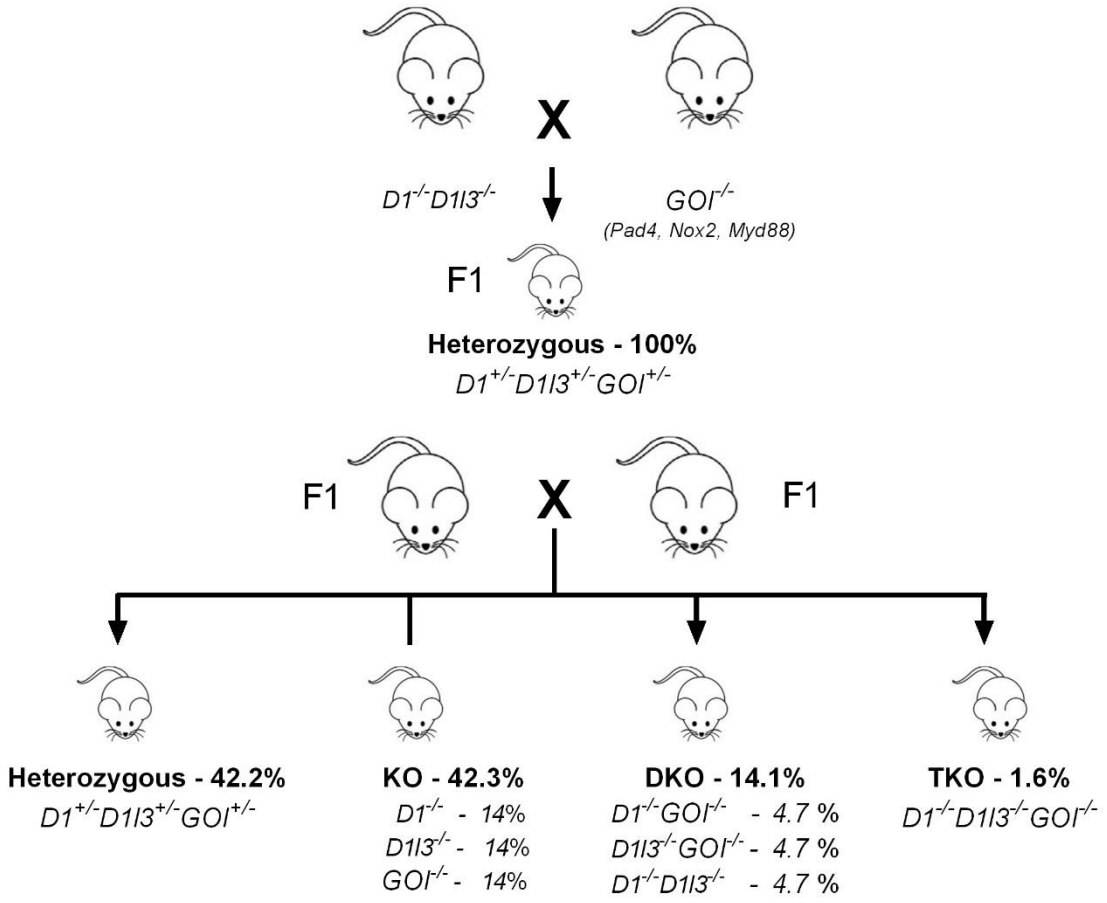


Figure 20: Generation of triple knock out mice. *D1/D1l3*$^{-/-}$ were crossed with *PAD4* $^{-/-}$, *Nox2* $^{-/-}$ and *Myd88*$^{-/-}$ mice to produce heterozygous F1 generation. Genotyped F1 mice were bred to generate homozygous mice.

3.6 Intravascular NET formation is independent of PAD4

Mice with a dual deficiency in DNASE1 and DNASE1L3 develop intravascular NET clots composed of chromatin and citrullinated histones. Thus, PAD4 is activated during *in vivo* NETosis. Therefore, we hypothesized that PAD4 is a promising therapeutic target to inhibit NET formation. To test the role of PAD4 in intravascular NET formation, we injected the *Csf3* cDNA in WT mice, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$, to induce chronic neutrophils. In line with our previous experiments, the wild-type mice show no signs of distress and remain healthy, and the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ died within four days as a result of intravascular NET-clot formation. Importantly, the *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$, also died within six days after *Csf3 i*njection (Figure 21 A). Similar to

the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$mice developed a rapidly progressing hypothermia accompanied by haematuria (not shown) (Figure 21 B). Therefore, the PAD4 deficiency is not protective during G-CSF induced chronic neutrophilia in the absence of DNASE1 and DNASE1L3.

In the next experiment, we restored the DNASE1 and DNASE1L3 activity in *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$ mice. We co-expressed *Csf3* along with a mixture of *Dnase1-Dnase1l3*, or a with empty pLIVE plasmid. In line with our previous result, the expression of the DNASES was sufficient to for the survival of the *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$ mice. The mice co-expressing empty control plasmid along with *Csf3* died within 6 days (Figure 21 C).

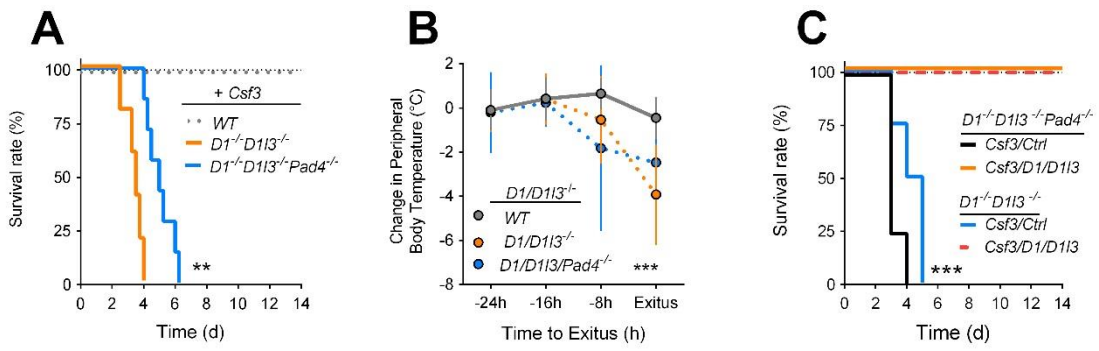


Figure 21: Absence of PAD4 is not protective in mice with double DNase deficiency during chronic neutrophilia. WT, *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ were injected with *Csf3*-expression plasmid to induce chronic neutrophilia. **(A)** Survival of wild-type (N=6), *D1/D1l3*$^{-/-}$ (N=5) and *D1/D1l3/Pad4*$^{-/-}$ (N=7). **(B)** Change in peripheral body temperature. **(C)** Survival of *D1/D1l3*$^{-/-}$ (N=7) co-expressing *Csf3*/*D1+D1l3*, (N = 3) and controls *Csf3*/*Ctrl* (N = 4), and *D1/D1l3/Pad4*$^{-/-}$ (N = 8) co-expressing *Csf3*/*D1+ D1l3* (N = 4) and controls *Csf3*/*Ctrl* (N = 4). Statistics: (A) (C) log-ranks test, (B) two way ANOVA followed by Bonferroni's multiple comparisons post hoc test;** $P < 0.01$, *** $P < 0.001$.

Furthermore, we analyzed the lung sections and showed the presence of several occluded blood vessels that stained positive for hematoxylin indicating the presence of DNA clots, in both *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$ mice expressing *Csf3*, but not wild-type mice (Figure 22 A, B).

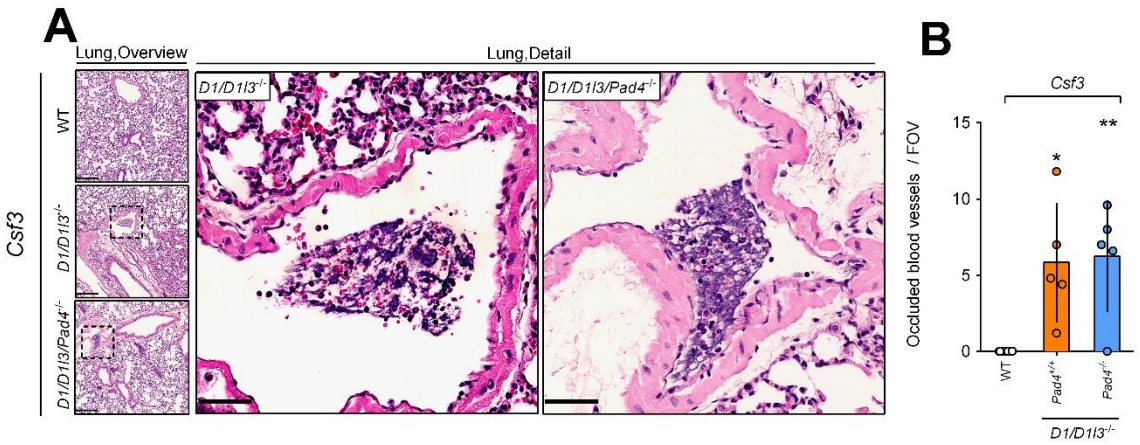


Figure 22: Hematoxylin-positive clots are formed in the absence of PAD4. Lung section from WT, *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ injected with *Csf3*-expression plasmid were stained with Hematoxylin and eosin. **(A)** Blood vessels of *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ expressing *Csf3* show hematoxylin-rich clots. Scale bar 500µm (overview), 25µm (detail). **(B)** Quantification of blood vessels occluded by hematoxylin-positive clots per FOV. WT (N = 6), *D1/D1l3*$^{-/-}$ (N = 5), *D1/D1l3/Pad4*$^{-/-}$ (N = 5). Statistics: (B) two way ANOVA followed by Bonferroni's multiple comparisons post hoc test;** P < 0.01, *** P < 0.001 versus all other groups. Statistics: (B) one way ANOVA followed by Dunnett´s multiple comparisons test. ;*P < 0.1, ** P < 0.01 versus WT.

We confirmed that the DNA clots were composed of neutrophil DNA and NET components by staining lung cross section for chromatin and neutrophil marker MPO **(**Figure 23).

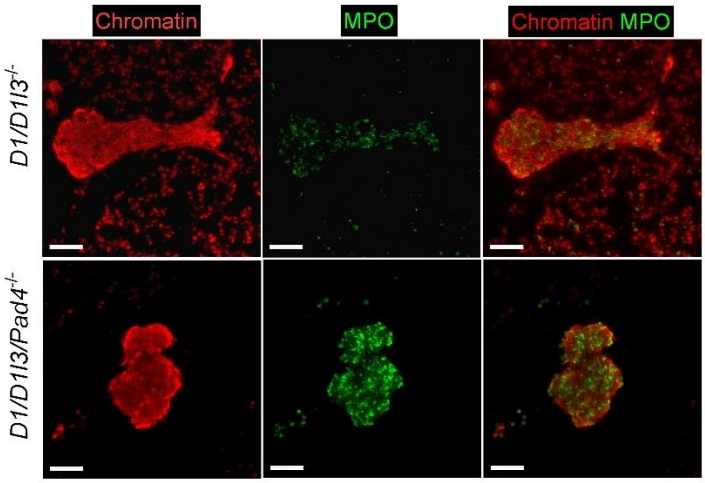


Figure 23: Vascular occlusions composed of neutrophil DNA. Immunostaining of occluded blood vessels for chromatin (red) and neutrophil marker myeloperoxidase (MPO, green) in lung section from *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ mice expressing *Csf3*. Scale bars: 25µM

Finally, the lung sections were stained for citrullinated histone. The lack of citH3 signal in the tissue from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$ confirms that the *in vivo* NET clots are formed even in the absence of histone citrullination (Figure 24). Thus we conclude that the formation of *in vivo* NETs during chronic neutrophilia is independent of activation of PAD4.

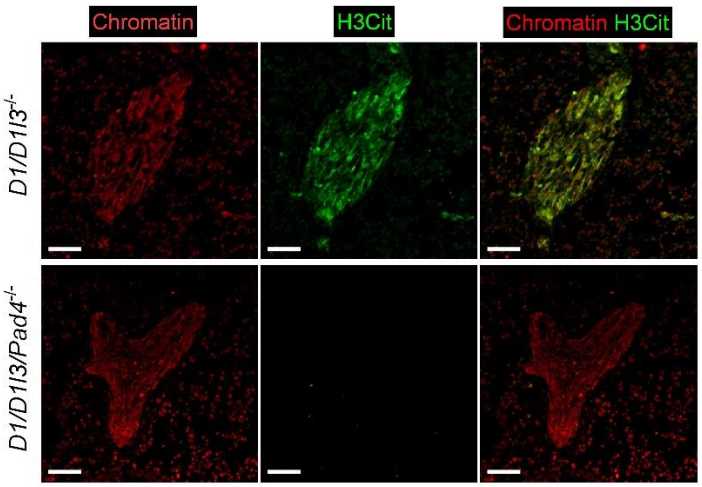


Figure 24: Vascular occlusions formed in the absence of histone citrullination by PAD4. Immunostaining of occluded blood vessels for chromatin (red) and citrullinated histone 3 (green) in lung section from *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ mice expressing *Csf3*.Scale bars: 25µM

Next, we aimed to test the requirement of histone citrullination by PAD4 in the sepsis model. We injected wild-type, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$ mice with a sublethal dose of LPS for three days and an IV injection of heat-killed *E.coli* along with the third dose of LPS. In line with results of chronic neutrophilia, the *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Pad4*$^{-/-}$ succumbed to the effect of sepsis. We sacrificed most of the mice within 4 days after injection of heat-killed *E.coli* as they developed hypothermia and hematuria (Figure 25 A). H&E staining of lung sections showed the presence of several occluded blood vessels in both *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$ but not wild-type mice (Figure 25 B, C). The lung sections were stained for citrullinated histone. The lack of citH3 signal in the tissue from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$ confirms that the *in vivo* NET clots are formed even in the absence of histone citrullination (Figure 25 D). Collectively, these data suggest that the formation

of intravascular NETs in independent of PAD4 during *Csf3* induced chronic neutrophilia and LPS induced sepsis.

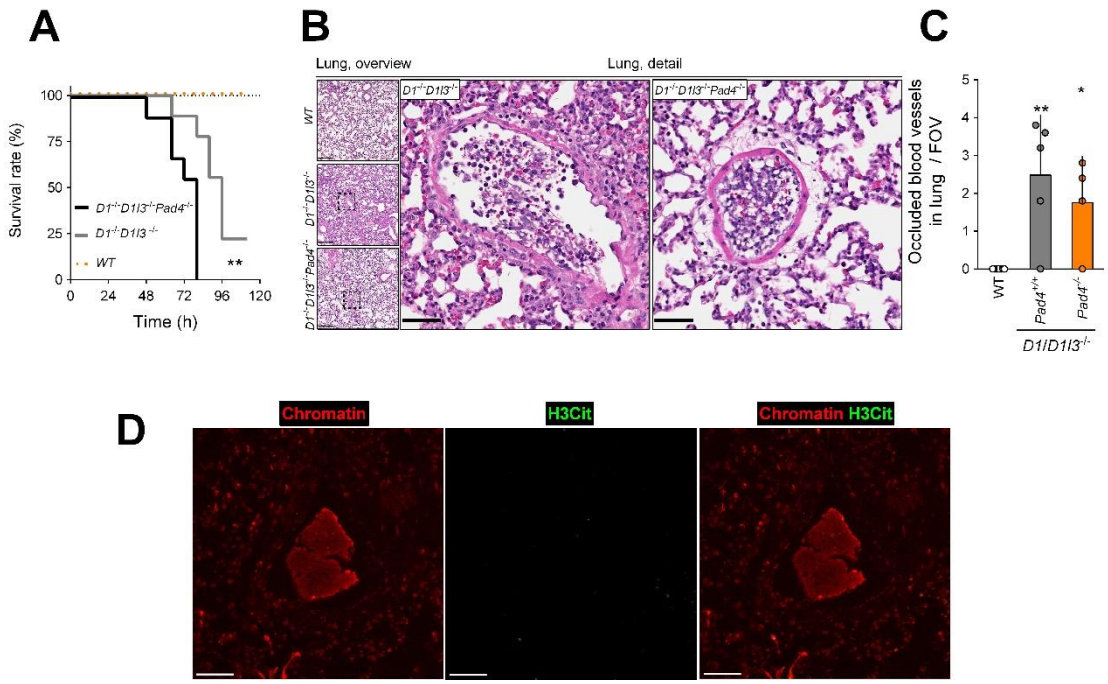


Figure 25: Absence of PAD4 does not protective in double DNase deficient mice during sepsis. Wild-type, *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ were treated with lipopolysaccharide and heat-killed *E.coli* to induce sepsis **(A)** Survival of wild-type (N = 7), *D1/D1l3*$^{-/-}$ (N = 9) and *D1/D1l3/Pad4*$^{-/-}$ (N = 9). **(B)** Lung section from wild-type, *D1/D1l3*$^{-/-}$, *D1/D1l3/Pad4*$^{-/-}$ showing blood vessels with hematoxylin-rich clots. Scale bar 500µm (overview) 25µm (detail)**. (C)** Quantification of blood vessels occluded by hematoxylin-positive clots per FOV. WT (N = 9), *D1/D1l3*$^{-/-}$ (N = 5), *D1/D1l3/Pad4*$^{-/-}$ (N = 4). **(D)** Immunostaining of occluded blood vessels for chromatin (red) and citrullinated histone 3 (green) in lung section from *D1/D1l3/Pad4*$^{-/-}$ mice. .Scale bars: 25µM. Statistics: (A) log-ranks test, (C) one way ANOVA followed by Dunnetts's multiple comparisons test;*P < 0.1, ** P < 0.01 versus WT.

Next, we confirmed that NET formation is in independent of PAD4 by setting up an *in vitro* experiment. We isolated blood neutrophils from wild-type (WT) mice and *Pad4*$^{-/-}$ mice. Purified neutrophils were seeded in DMEM medium and stimulated with calcium-ionophore (A23187). Cell were incubated at 37°C, with shaking, to mimic the environment in circulation. Macroscopic NET clots were formed within 4 hours upon stimulation. We observed no such aggregates in the presence of DNASE1 or in untreated neutrophils (Figure 26 A). Furthermore, we detected a robust histone citrullination within NET clots in WT neutrophils, but not *Pad4*$^{-/-}$ neutrophils (Figure 26 B). Therefore,

activation of PAD4 and histone citrullination occurs during NETosis, but is not required for in NETosis *in vitro.*

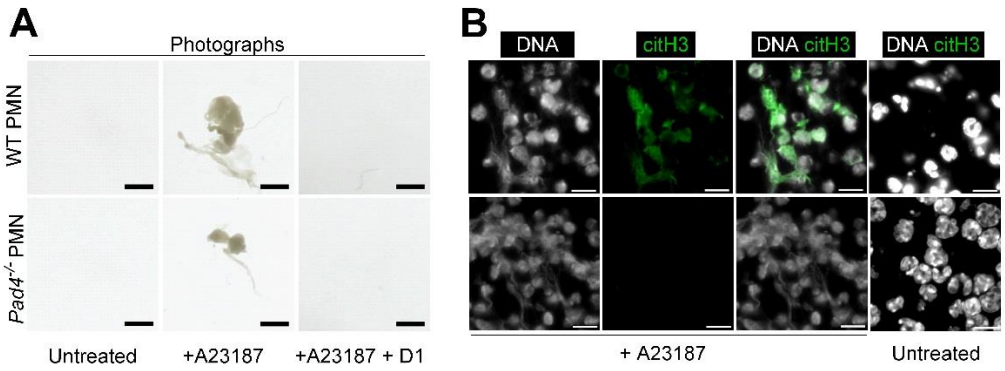


Figure 26: In vitro NET formation is independent of PAD4 activation. (A) Photographs of macroscopic NET clots generated by A23187 activation of wild-type and *Pad4*$^{-/-}$ neutrophils *in vitro*. Untreated neutrophils and treated in the presence of recombinant human DNase1 serve as controls. Scale bars 2.5mm **(B)** immunostainings of A23187-activated murine neutrophils. Scale bars: 25µm.

3.7 Intravascular NET formation is independent of Nox2.

Nox2 is required for the generation of reactive oxygen species (ROS). ROS mediates the release of granular enzymes such as neutrophil elastase into the cytoplasm. Therefore, we hypothesized that Nox2 is a promising therapeutic target to inhibit NET formation. To test the functions of Nox2 in the formation of intravascular NETs, we treated *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ *and Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$ mice with *Csf3*-expression vector to induce chronic neutrophilia. The *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice treated with *Csf3* expression vector acts as controls in this experiment as the phenotype and outcome has been well established in the previous experiments. As expected, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ died after *Csf3* treatment. Interestingly, we also found that most of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$ mice (5 out of 7 mice) developed a phenotype similar to *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ and died after *Csf3* treatment. Two *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$ remained healthy after *Csf3* treatment. In line with our previous experiments, the non-surviving the mice developed rapidly progressing hypothermia, indicated by a sudden drop in peripheral body temperature, and hematuria indicated by red urine and plasma (Figure 27 A, B).

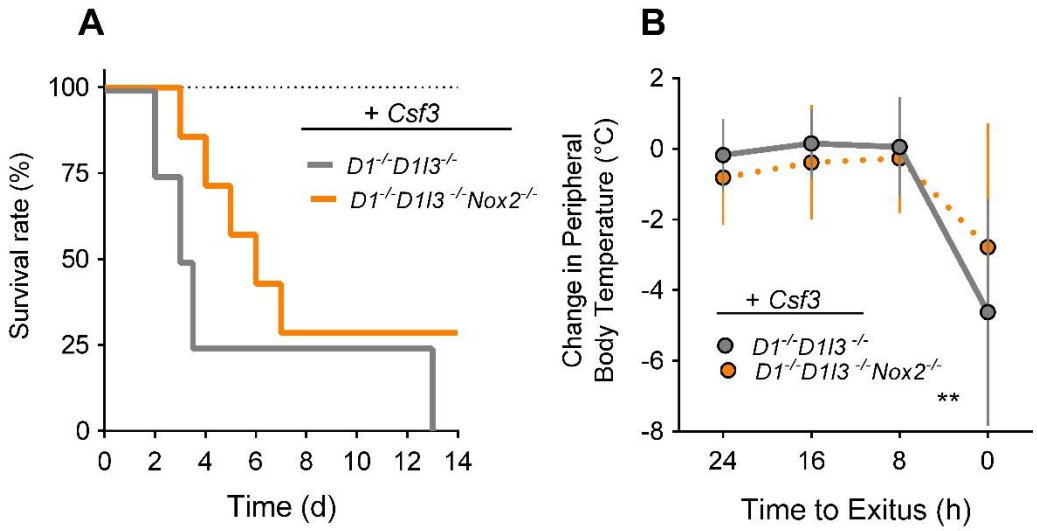


Figure 27: Absence of Nox2 is not protective in double DNase deficient mice during chronic neutrophilia. *D1/D1l3*$^{-/-}$, *D1/D1l3/Nox2*$^{-/-}$ mice were injected with *Csf3*-expression plasmid to induce chronic neutrophilia. **(A)** Survival of *D1/D1l3*$^{-/-}$ (N = 4) and *D1/D1l3/Nox2*$^{-/-}$ (N = 7). **(B)** Change in peripheral body temperature. Statistics: (A) log-ranks test, (B) two way ANOVA followed by Bonferroni's multiple comparisons post hoc test;** $P < 0.01$.

We analyzed the lung sections of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$ and found the presence of several hematoxylin rich clots occluding the blood vessels in all the mice that died. The two *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$ mice that survived has no occluded blood vessels (Figure 28 A, B). The lung sections were stained for citrullinated histone. The presence of H3Cit signal in the tissue from *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$ mice indicates that activity of PAD4 (Figure 28 C). Taken together, these results indicate that intravascular NET formation is independent of Nox2 in chronic neutrophilia.

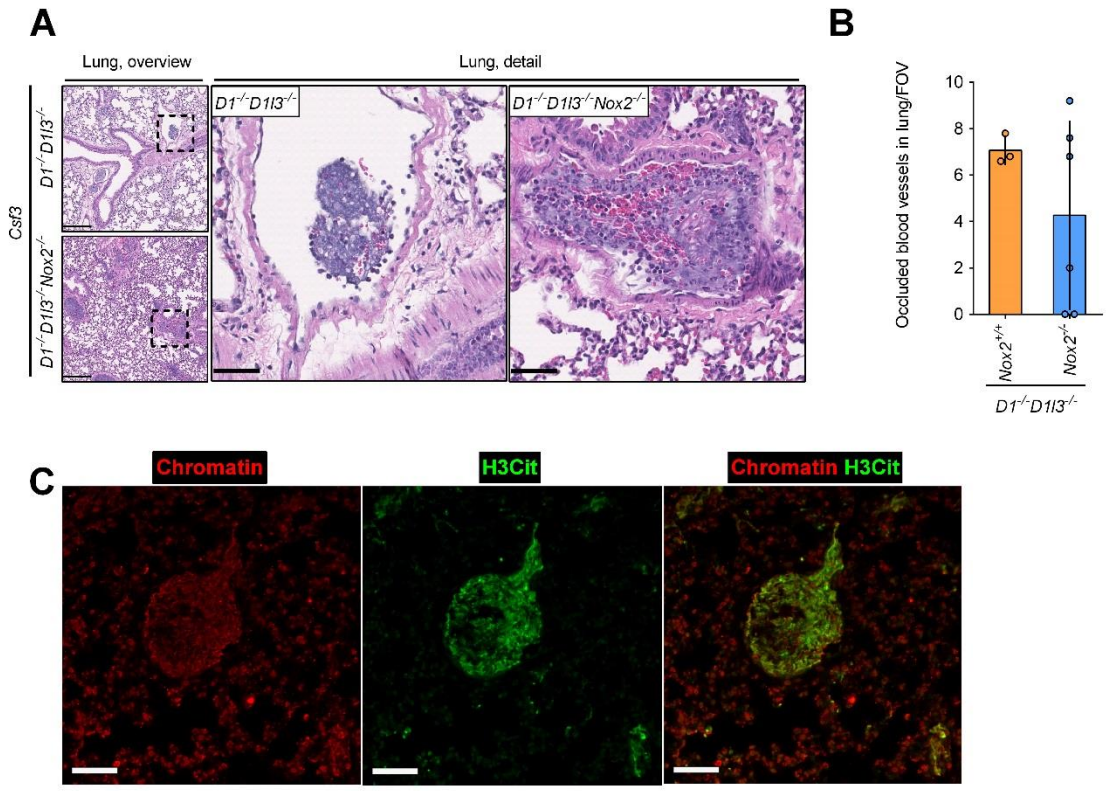


Figure 28: Intravascular NET clots are formed even in the absence of Nox2. Lung section from *D1/D1l3*$^{-/-}$, *D1/D1l3/Nox2*$^{-/-}$ injected with *Csf3*-expression plasmid were stained with hematoxylin and eosin. (A) Blood vessels of *D1/D1l3*$^{-/-}$, *D1/D1l3/Nox2*$^{-/-}$ show hematoxylin-rich clots. Surviving *D1/D1l3/Nox2*$^{-/-}$ mice showed no occluded blood vessels. Scale bars 500µm (overview), 25µm (detail). (B) Quantification of blood vessels occluded by hematoxylin-positive clots per FOVin *D1/D1l3*$^{-/-}$ (N=3) *D1/D1l3/Nox2*$^{-/-}$ (N=6) (C) Immunostaining of occluded blood vessels for chromatin (red) and citrullinated histone 3 (green) in lung section from *D1/D1l3/Nox2*$^{-/-}$ mice expressing *Csf3*.Scale bars: 25µM. *D1/D1l3*$^{-/-}$(N = 3), *D1/D1l3/Nox2*$^{-/-}$ (N = 6). Statistic: (B) Student's *t*-test.

To test the role of Nox2 in *in vitro* NET formation, we isolated blood neutrophils from WT mice and *Nox2*$^{-/-}$ mice. We induced the formation of NET clots with calcium-ionophore (A23187). Macroscopic NET clots were formed within 4 hours upon stimulation. We observed no such aggregates in the presence of DNASE1 or in untreated neutrophils (Figure 29 A). Furthermore, we detected a robust histone citrullination within NET clots in WT neutrophils, and *Nox2*$^{-/-}$ neutrophils (Figure 29 B) indicating that PAD4 is active. In conclusion, *in vivo* and *in vitro* NETosis is independent of Nox2 during G-CSF induced neutrophilia.

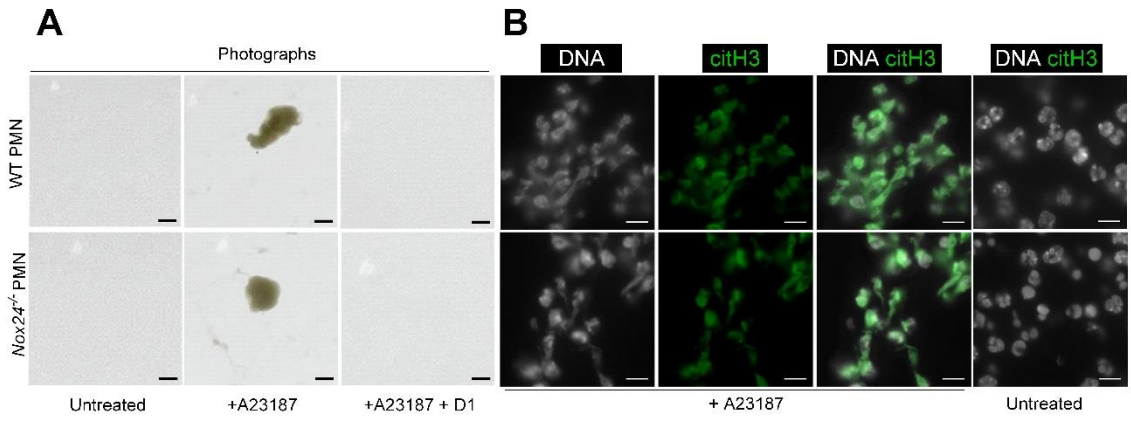


Figure 29: In vitro NET formation is independent of NADPH oxidase. (A) Photographs of macroscopic NET clots generated by A23187 activation of wild-type and *Nox2*$^{-/-}$ neutrophils *in vitro*. Untreated neutrophils and treated in the presence of recombinant human DNase1 serve as controls. Scale bar 2.5mm. **(B)** Immunostainings of A23187-activated murine neutrophils. Scale bars: 25µm.

3.8 Intravascular NETosis is independent of GSDMD

GSDMD is a pore forming protein, described as the effector molecule of pyroptosis, which is a highly inflammatory programmed cell death pathway. GSDMD is localized in the cytoplasm in an inactive state. During NETosis, different proteases cleave inactive GSDMD to liberate its N terminal domain which has pore-forming capacity[95,102]. In PMA induced NETosis, NE cleaves GSDMD. GSDMD in turn enhancing NE release into the cytoplasm by forming pores in granules[103]. In response to LPS, caspase 11 cleaves inactive GSDMD. Importantly, GSDMD disintegrates plasma membrane, releasing NETs[95]. Based on this we aimed to test the role of GSDMD in the formation of intravascular NETs during chronic neutrophilia and sepsis.

We generated triple knock mice with a combined deficiency of both DNASE1 and DNASE1L3, as well as GSDMD. We used the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/ CRISPRassociated9) system to cause the GSDMD mutation. CRISPR/Cas9 system is a fast and efficient method to develop genetically engineered mice. The system uses component from bacteria and archaea that protect them against viruses. In principle, double stranded DNA breaks are caused by a single guide RNA (sgRNA) bound to a nuclease Cas9 from the bacteria *Streptococcus pyogenes*. This

creates random nucleotide mutations (insertions and deletions) in the target site as a result of the error-prone non-homologous end joining repair pathway[245, 246].

CRISPR/Cas9 mutations was introduced directly in the fertilized oocyte from *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ through the use of a guide RNA, endonuclease activity, and error-prone non-homologous end joining repair mechanisms. The subsequent embryo was transferred into pseudo-pregnant animals to produce a viable heterozygous founder mice (Figure 30 A). The heterozygous founder mice were bred with mice *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ to generate F1 offspring with targeted mutations. The genotyped F1 mice were bred to generate homozygous mice (Figure 30 B).

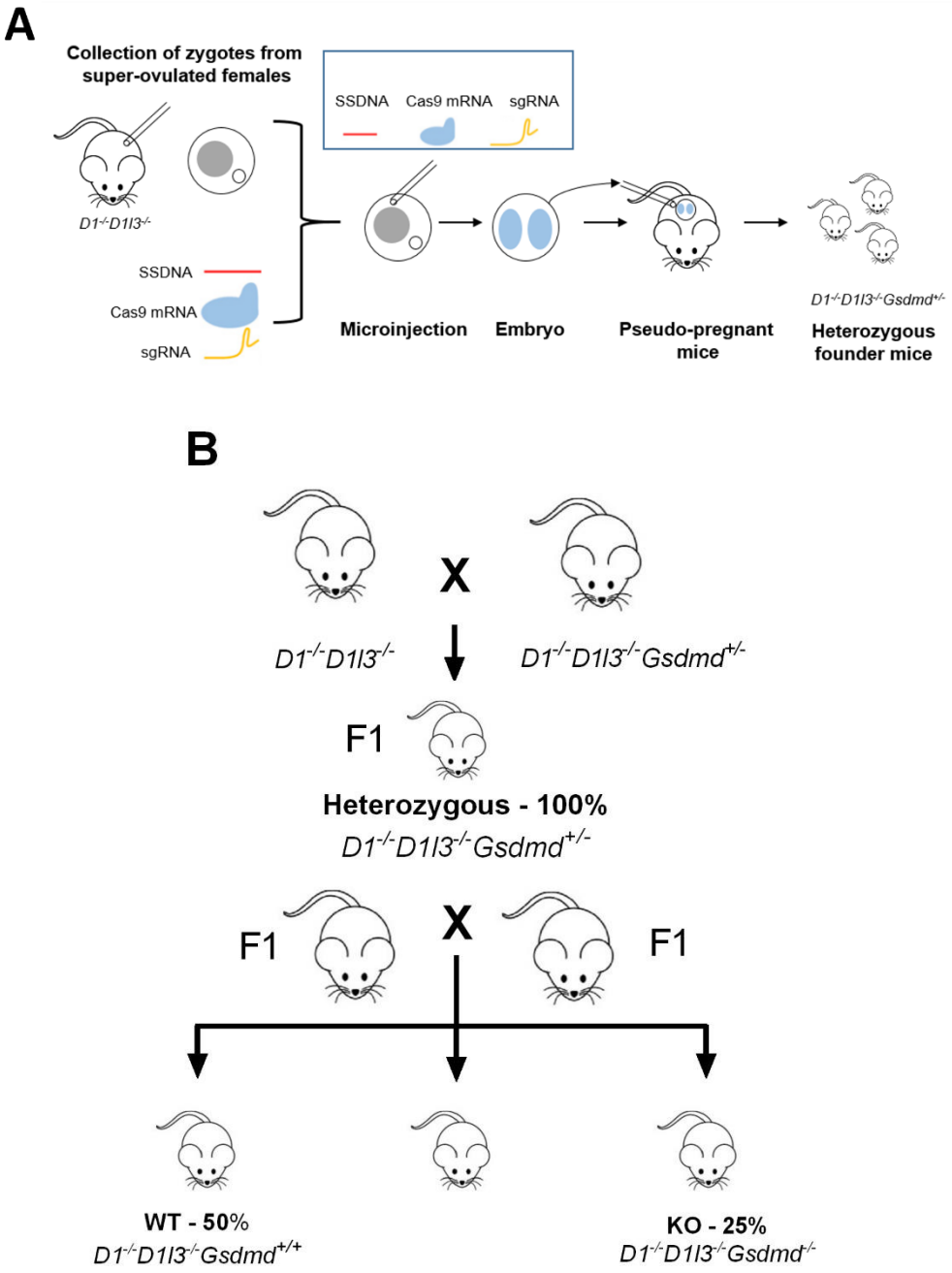


Figure 30: Generation of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$ using CRISPR/Cas9. (A) *In vitro* prepared targeting template, Cas9 mRNA, sgRNA are microinjected into zygotes from super-ovulated D1/D1l3-/- females. The CRISPR/Cas9 system changes targeted DNA in embryos by NHEJ. Developed embryos from injected zygotes are implanted into pseudo-pregnant mice to produce heterozygous founder mice (F0). **(B)** Heterozygous founder mice are crossed with D1/D1l3-/- mice to produce heterozygous F1 generation. Genotyped F1 mice were bred to generate homozygous mice.

The GSDMD deficiency was confirmed by western blot. We prepared bone marrow cell lysates from wild-type mice, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$. Equal amounts of proteins were subjected to SDS-PAGE gels. GSDMD consists of two domains, the N-terminal (GSDMD-N) and C-terminal (GSDMD-C) domain. We detected full-length GSDMD (54 kDa), GSDMD-N (36 kDa) and GSDMD-C (25 kDa) in the bone marrow cell lysate of wild-type and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice, but not in *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$ (Figure 31 A).

To test the role of GSDMD in NETosis *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Gsdmd*$^{-/-}$ were treated with *Csf3* expression vector to induce chronic neutrophilia. Mice from both groups developed hypothermia and were sacrificed within 14 days of *Csf3* expression (Figure 31 B, C). In the next experiment, we restored the DNASE1 and DNASE1L3 activity in *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$ mice. We co-expressed *Csf3* along with a mixture of *Dnase1-Dnase1l3*, or a with empty pLIVE plasmid. The expression of the DNASES was sufficient to for the survival of the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$ mice. The mice co-expressing empty control plasmid along with *Csf3* died within 6 days (Figure 31 D).

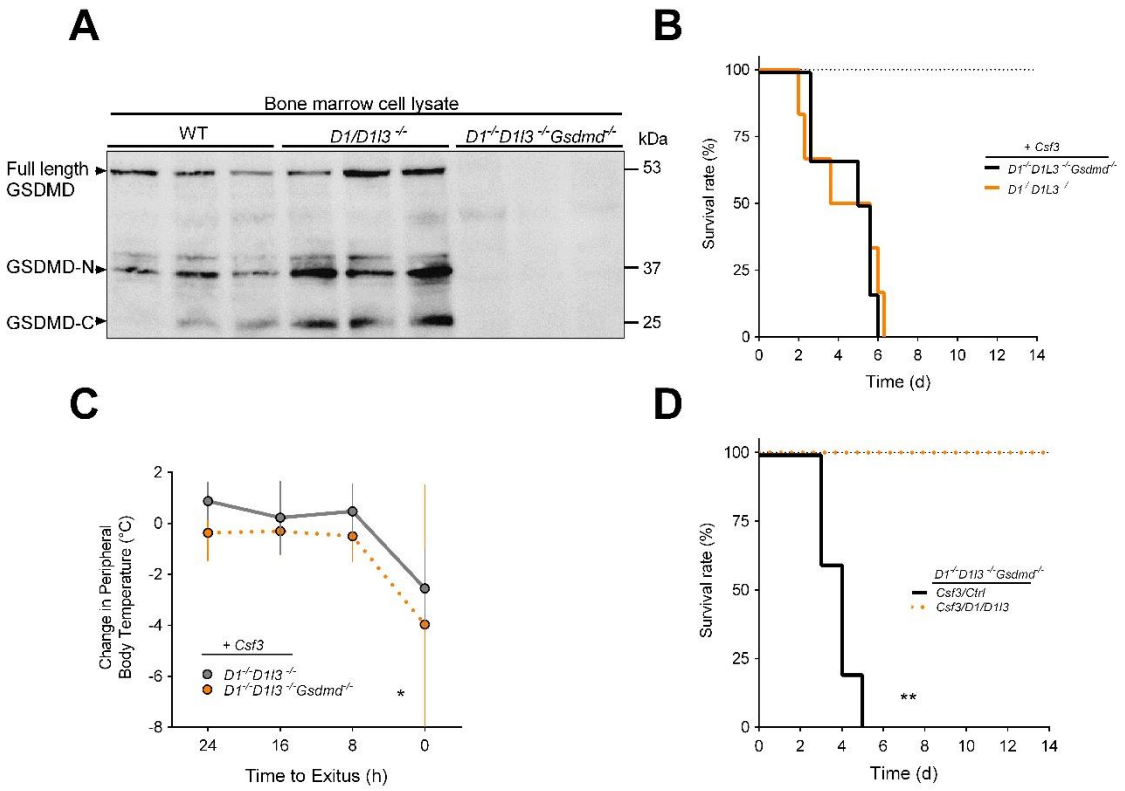


Figure 31: Absence of GSDMD is not protective in mice with double DNase deficiency during chronic neutrophilia. *D1/D1l3*$^{-/-}$, *D1/D1l3/Gsdmd*$^{-/-}$ mice were injected with *Csf3*-expression plasmid to induce chronic neutrophilia. **(A)** Western blot for GSDMD in the bone marrow cell lysates from WT, *D1/D1l3*$^{-/-}$, *D1/D1l3/Gsdmd*$^{-/-}$. **(B)** Survival of *D1/D1l3*$^{-/-}$ (N = 4) and *D1/D1l3*$^{-/-}$*/Gsdmd*$^{-/-}$ (N = 4). **(C)** Change in peripheral body temperature. **(D)** Survival of *D1/D1l3/Gsdmd*$^{-/-}$ (N = 7) co-expressing *Csf3*/*D1+D1l3*, (N = 5) and controls *Csf3*/*Ctrl* (N = 5). Statistics: (B), (D) log-ranks test, (C) two way ANOVA followed by Bonferroni's multiple comparisons post hoc test;* P < 0.1, *** P < 0.01.

Analyses of the lung sections of *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Gsdmd*$^{-/-}$ showed the presence of several hematoxylin rich clots occluding the blood vessels. These DNA clots stained positively for citrullinated histone indicating that PAD4 is active. These results indicate that intravascular NET formation is independent of GSDMD in chronic neutrophilia (Figure 32).

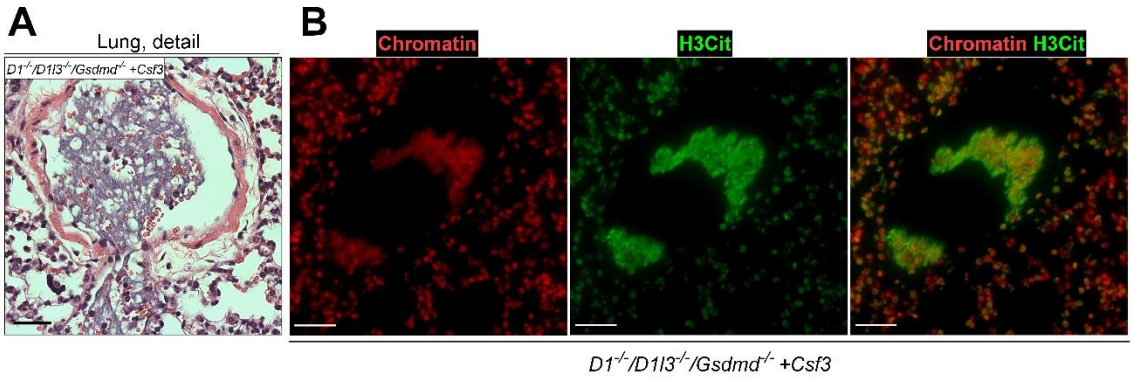


Figure 32: Hematoxylin-positive clots are formed in the absence of GSDMD. (A) Representative H&E staining of occluded blood vessel in the lungs. Scale bar: 25µM. (B) Immunostaining of occluded blood vessels for chromatin (red) and citrullinated histone 3 (green) in lung section from *D1/D1l3/Gsdmd*$^{-/-}$ mice expressing *Csf3*.Scale bars: 25µM

Next, we aimed to test the requirement of GSDMD in the sepsis model. We injected wild-type, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Gsdmd*$^{-/-}$ mice with a sublethal dose of LPS for three days and an IV injection of heat-killed bacteria along with the third dose of LPS. In line with results of chronic neutrophilia, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Gsdmd* $^{-/-}$ succumbed to the effect of sepsis (Figure 33). Collectively, these data suggest that the formation of intravascular NETs in independent of GSDMD during *Csf3* induced chronic neutrophilia and LPS induced sepsis.

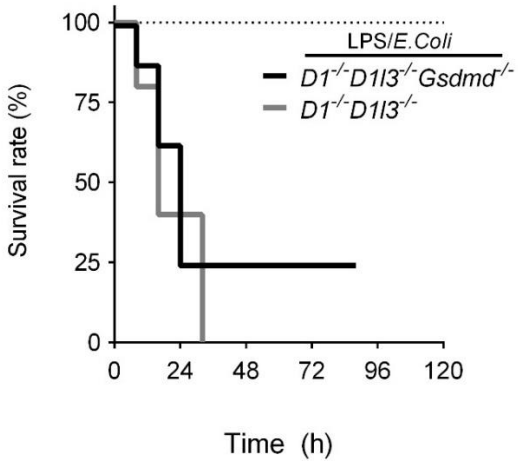


Figure 33: Absence of GSDMD is not protective in mice with double DNase deficiency during sepsis. *D1/D1l3*$^{-/-}$, *D1/D1l3/Gsdmd* $^{-/-}$ were treated with lipopolysaccharide and heat-killed *E.coli* to induce sepsis. Survival of *D1/D1l3*$^{-/-}$ (N = 6) and *D1/D1l3/ Gsdmd* $^{-/-}$ (N = 8). Statistics: log-ranks test.

3.9 Intravascular NET formation is independent of MyD88 in neutrophilia

MyD88 is a universal adaptor protein present downstream of the TLR and IL-1 family of receptors. MyD88 recruits the downstream proteins of the TLRs signaling pathway giving rise to an inflammatory response. It plays an important role in the activation of the canonical inflammasome. In response to danger signals, signaling through TLRs-Myd88 leads to the activation and translocation of transcription factor NF-kB into the nucleus[247]. NF-kB regulates the transcription of the components of the inflammasome, pro-IL-1ß and pro- IL-18 and pro-GSDMD[248,249]. The inflammasome proteolytically activates GSDMD and the pro-inflammatory cytokines, IL-1β and IL-18, leading to the recruitment and the activation of other immune cells, such as neutrophils[250].

MyD88-deficient mice have been used extensively as a model for TLR deficiency and are susceptible to a large variety of bacterial pathogens or parasites[251]. We aimed to test if the activation of neutrophils during chronic neutrophilia is a MyD88 dependent pathway. *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$, and *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88*$^{-/-}$ were treated with *Csf3* expression vector to induce neutrophilia. Mice from both groups developed hypothermia, hematuria (not shown) and were sacrificed within 8 days of *Csf3* expression. (Figure 34 A, B).

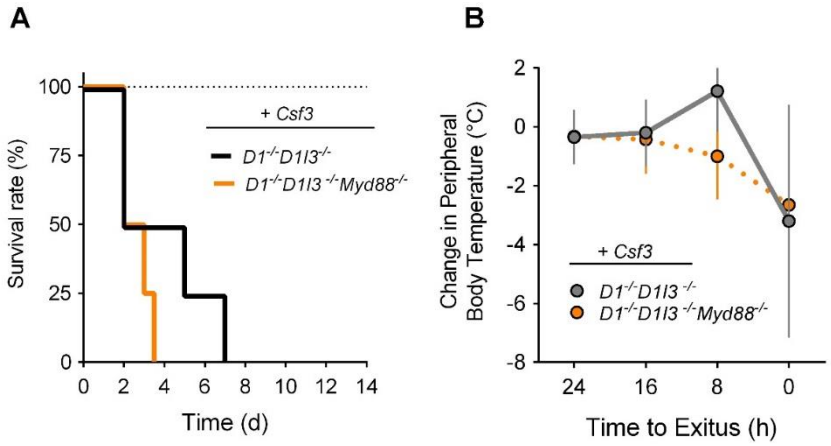


Figure 34: Absence of Myd88 is not protective in mice with double DNASE deficiency during chronic neutrophilia. *D1/D1l3*$^{-/-}$, *D1/D1l3/Myd88*$^{-/-}$ mice were injected with *Csf3*-expression plasmid to induce chronic neutrophilia. **(A)** Survival of *D1/D1l3*$^{-/-}$ (*N = 4*) and *D1/D1l3/Myd88*$^{-/-}$ (*N = 4*). **(B)** Change in

peripheral body temperature. Statistics: (A) log-ranks test, (B) two way ANOVA followed by Bonferroni's multiple comparisons post hoc test.

Histological analyses revealed that both groups had occluded blood vessel in the lungs (Figure 35 A, B). These DNA clots stained positively for citrullinated histone indicating that PAD4 is active. Therefore we concluded that the activation of neutrophils and formation of intravascular NETs in chronic neutrophilia is a MyD88-independent pathway.

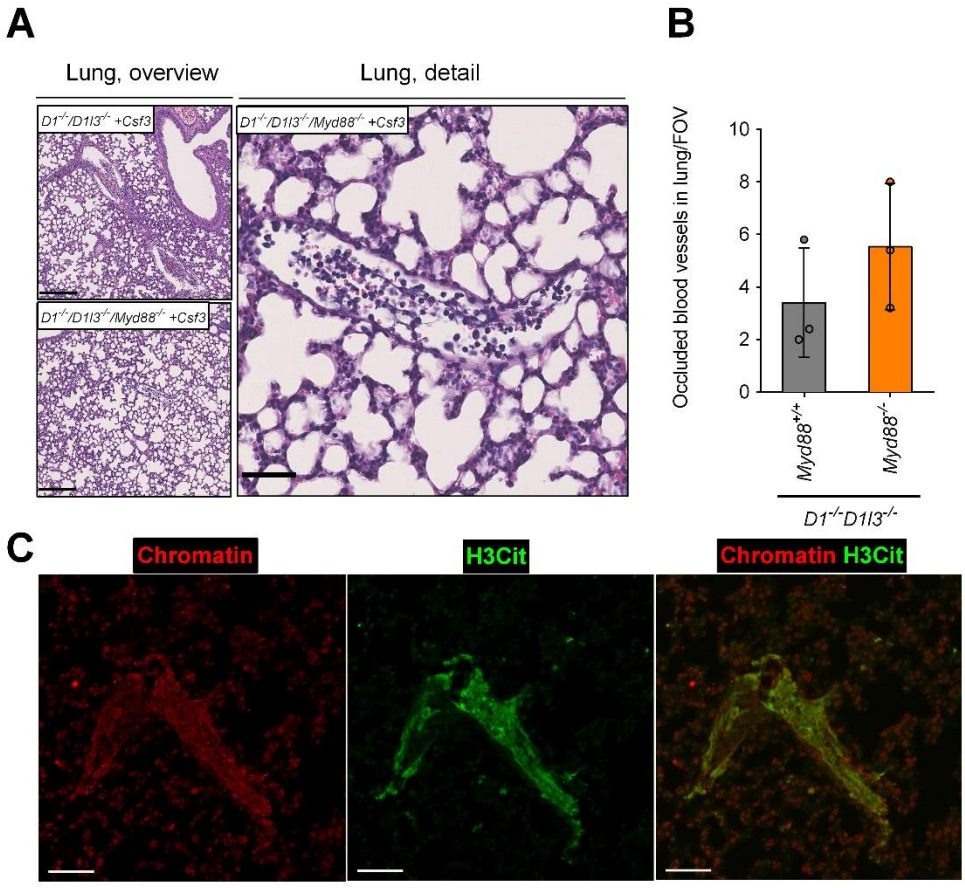


Figure 35: Hematoxylin-positive clots are formed in the absence of MyD88. Lung section from *D1/D1l3*$^{-/-}$, *D1/D1l3/Myd88*$^{-/-}$injected with *Csf3*-expression plasmid were stained with hematoxylin and eosin. **(A)** Blood vessels in the lungs of *D1/D1l3*$^{-/-}$, *D1/D1l3/Myd88*$^{-/-}$show hematoxylin-rich clots. Scale bar 500µm. **(B)** Quantification of blood vessels occluded by hematoxylin-positive clots per FOV. *D1/D1l3*$^{-/-}$ (N = 3), *D1/D1l3/Myd88*$^{-/-}$ (N = 3). **(C)** Immunostaining of occluded blood vessels for chromatin (red) and citrullinated histone 3 (green) in lung section from *D1/D1l3/Myd88*$^{-/-}$ mice expressing *Csf3* Statistics: (B) Unpaired t-test.

3.10 Intravascular NET formation during sepsis is a MyD88 mediated pathway

Earlier studies have shown that MyD88 deficient mice are protected from the effects of LPS and sepsis[252]. We expected that LPS is not sensed in the absence of My88. We induced sepsis in *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88* $^{-/-}$ mice by injecting sublethal dose of LPS for three days and, an IV injection of heat-killed bacteria. The *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice, showed a rapidly declining body weight and lower body temperature. Importantly, all *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88* $^{-/-}$ mice recovered from the sepsis. The *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88* $^{-/-}$ maintained the normal body temperature and weight during the LPS treatment. After the treatment with heat-killed bacteria, the *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ mice developed hypothermia and haematuria and were sacrificed. The *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88*$^{-/-}$ on the other hand recovered (Figure 36 A, B and C). These findings confirm that MyD88 is required to respond to LPS.

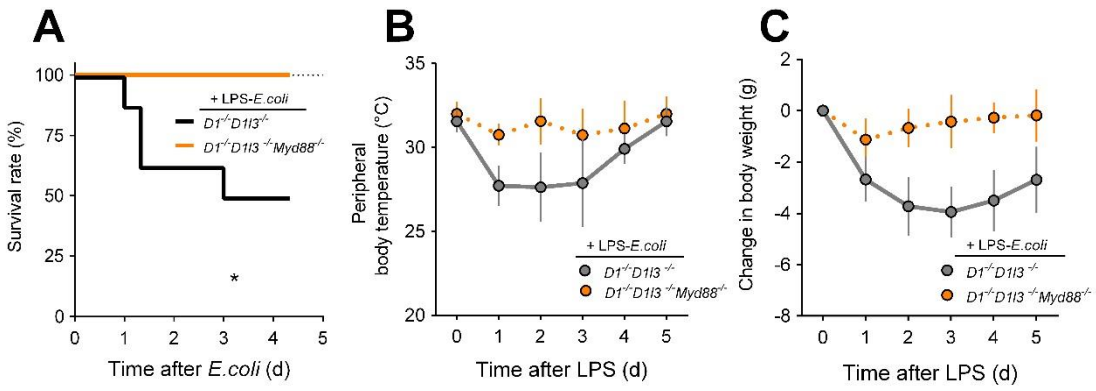


Figure 36: Absence of Myd88 is protective in mice with double DNASE deficiency during sepsis. *D1/D1l3*$^{-/-}$, *D1/D1l3/Myd88*$^{-/-}$ mice were treated with LPS/*E.coli* to induce sepsis **(A)** Survival of *D1/D1l3*$^{-/-}$ (N=5) and *D1/D1l3/Myd88*$^{-/-}$ (N = 5). **(B)** Change in peripheral body temperature **(C)** Change in body weight. Statistics: (A) log-ranks test, (B, C) two way ANOVA followed by Bonferroni's multiple comparisons post hoc test;* P < 0.1.

3.11 Summary of molecular mechanism of in vivo NET formation

G-CSF induces chronic neutrophilia and LPS induced sepsis in double DNase deficient mice lead to the development of two *in vivo* NETs models. We used these models to test the functional requirement of the different proteins implicated in the regulation of *in vivo* NETosis, in response to G-CSF and LPS/*E.coli*. Our results show that, in the chronic

neutrophilia, PAD4, Nox2, GSDMD and MyD88 are not the key drivers of intravascular NETosis. In the sepsis model, intravascular NETosis is independent of PAD4 and GSDMD, but dependent on MyD88 (Table 5).

Proteins	**Function in NETosis**	**In vivo NETosis Model 1 : Neutrophilia**	**In vivo NETosis Model 2:Sepsis**
PAD 4	Citrullination of histones	NETs formation independent of PAD4	NETs formation independent of PAD4
NADPH oxidase	Generation of ROS	NETs formation independent of ROS	-
Gasdermin D	Plasma membrane disintegration	NETs formation independent of GSDMD	NETs formation independent of GSDMD
MyD88	LPS sensing	NETs formation independent of MyD88	NETs formation requires MyD88

Table 5: Functional requirement of PAD4, Nox2, GSDMD and MyD88 in chronic neutrophilia and sepsis

PART 4: Development of a technique to track in vivo NETs

3.12 Intravascular NETosis in response to G-CSF likely involves LDNs

Neutrophils are a heterogeneous cell populations. LDNs are a subset of neutrophils that are engaged in an unexplored pathway of NETosis. HDNs are the classical neutrophils with lobulated nucleus and many granules. LDNs defined by a reduced buoyant density compared to normal/high density neutrophils[180]. When peripheral blood is layered on top of a discontinuous density gradient medium and centrifuged LDNs co-purify with the lower density PBMCs and HDNs sediment just above the RBC pellet (Figure 37). Importantly, LDNs counts are increases in a variety of pathological conditions. These cells have pro-inflammatory profile and have enhanced capacity for firm NETs [183,253,254].

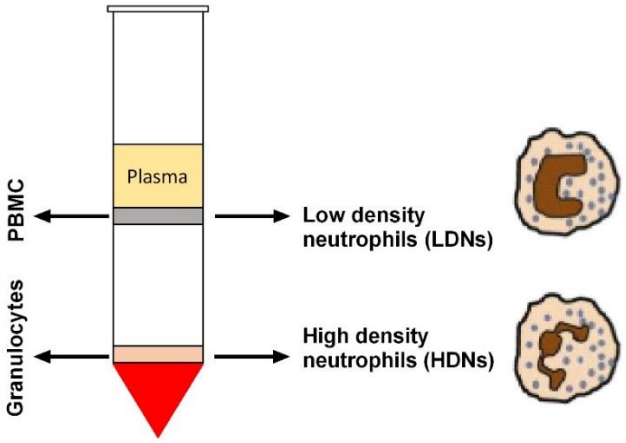


Figure 37: Neutrophil subsets can be separated through density gradient centrifugation. The normal or high density neutrophils are denser and sediment at the bottom of the tube above the RBC pellet. HDNs are the classical neutrophils with lobulated nucleus and many granules. The LDNs are found in the PBMC fraction and comprise immature neutrophils with a less lobulated nucleus.

We hypothesized that LDNs play a role in NETosis in response to G-CSF expression. We prepared blood smears of whole blood from mice that were treated with G-CSF to induce chronic neutrophils. A Wright-Giemsa staining of these blood smears showed the presence of two distinct types of neutrophils. The cell that stains lighter represent the LDNs and the cells that stain darker represent the HDNs. Furthermore the LDNs showed the formation of spontaneous NET like structure (Figure 38 A).

Next, we purified the neutrophils in circulation from mice treated with G-CSF to induce chronic neutrophilia. Whole blood was layered on to a histopaque gradient. After centrifugation, neutrophils were purified from the PBMC/LDN fraction as well as the HDN fraction. Purified neutrophils from both fractions were counted on a hemocytometer. We found that the LDNs were highly enriched in comparison to the HDNs (Figure 36 B). This suggests that intravascular NETs in response to G-CSF involves the LDNs.

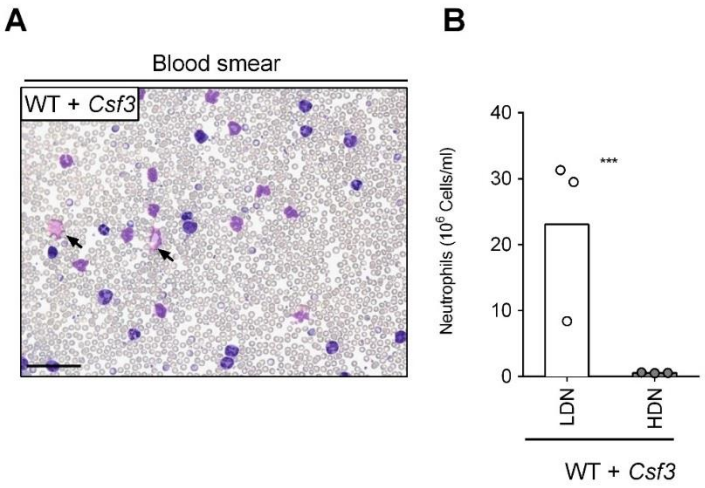


Figure 38: G-CSF induces neutrophilia generates LDNs. Blood smears were prepared from mice treated with G-CSF. **(A)** Wright-Giemsa staining shows the presence of two distinct neutrophil population. The cell with light purple nucleus represents LDNs and dark purple nucleus represents HDNs. Arrows: LDNs forming spontaneous NET-like structures. **(B)** Quantification of purified neutrophils from the LDNs (N =3) and HDNs (N =3) showed that LDNs were highly enriched in blood from G-CSF treated WT mice. Statistics: (B) Student's *t*-test, *** $P < 0.001$.

3.13 BrdU labeled neutrophils provide a robust technique to track NETs

A major technical problem in the field of NETosis is the detection of NETs in tissue. In the absence of DNases, NETs can be detected in the tissue as long strands occluding blood vessels. When DNases activity is present NETs are digested into soluble fragments. Therefore we need a technique to track the fate of NETs and its degradation products.

To detect NETs specifically, we established a method based on the 5-bromo-2-deoxyuridine (BrdU). BrdU is a synthetic analogue of thymidine that incorporates into DNA during S-phase in proliferating cells. We treated mice with *Csf3* to induced chronic neutrophilia, and repeatedly dosed them with BrdU. BrdU gets incorporated into the proliferating neutrophils**.** Next, we purified neutrophils from blood of neutrophilic mice using negative selection. To test the percentage of BrdU labelled neutrophils, we prepared smears of the purified cells, and stained them for DNA, CRAMP and BrdU. The percentage of BrdU+ cells were quantified using fluorescent microscopy. We found that >97% of cells were BrdU positive.

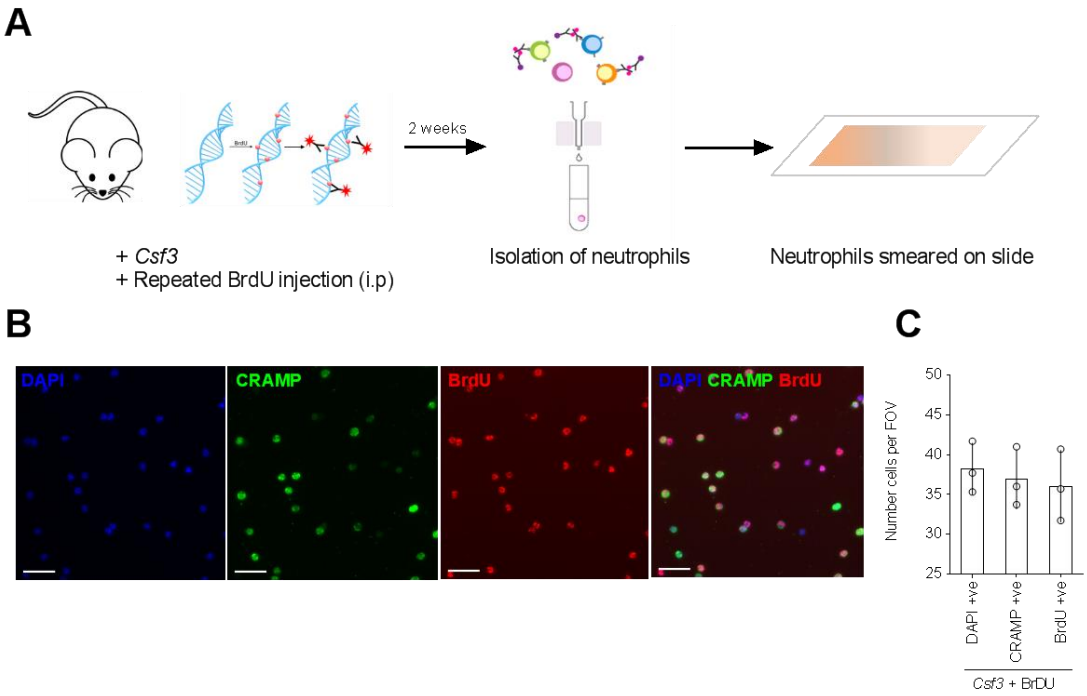


Figure 39: BrdU labelling of neutrophils. (A) WT mice were treated with Csf3 to induce neutrophilia and dosed repeated with BrdU. Neutrophils were isolated after two weeks, and pure neutrophil smears were prepared. **(B)** Immunostaining of neutrophil smears for DAPI, CRAMP and BrDU Scale bars: 25µm. **(C)** Quantification of number of DAPI+, CRAMP+ and BrDU+ cells per FOV.

BrdU labeled neutrophils have a specific marker in the nucleus. A monoclonal antibody against BrdU can be used for the detection of NETs *in vivo*. We propose to use this technique in future experiments, to track the formation and deposition of NETs in recipient mice of different diseases models. Therefore, the BrdU labelling of neutrophils provide a robust technique to track NETs *in vivo*.

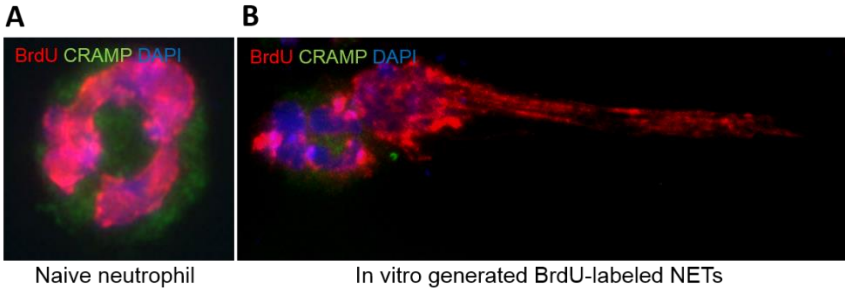


Figure 40. BrdU labelled neutrophils provide a robust technique to track NETs. (A) BrdU-staining of a naïve neutrophil. (B) Staining of NETs from a BrdU-labeled murine neutrophil

4. Discussion

4.1 Neutrophilic mice - A model for developing drugs against NETs

Neutrophils represent 50-70% of blood leukocytes and are essential effector cells of the innate immune system. Neutrophils are generated in large numbers in the bone marrow, and are recruited into circulation in baseline numbers[255]. The release of neutrophils is tightly controlled and only 1 or 2% of all neutrophils in the body are found in the blood under normal homeostatic conditions[256]. In response to infection or injury, the number of circulating neutrophils rapidly increases through inflammation. As the first line of defense against invading microorganisms, neutrophils control infection by acting as phagocytes, releasing lytic enzymes from their granules, produce reactive oxygen species or generate NETs[257,258]. Neutrophils are also key regulators of both acute and chronic inflammation. By releasing proteases and secreting cytokines such as IL-1ß, IL-18, and TNF-alpha, neutrophils can amplify inflammation[259,260].

Defects in neutrophils quantity and function lead to severe and potentially life-threatening disease in humans, underscoring the important role of the neutrophils in the immune system[261, 262]. An increase in the absolute number of neutrophils in circulation refers to as neutrophilia. Primary neutrophilia results from abnormalities in the regulation of bone marrow neutrophil production. Secondary or reactive neutrophilia occurs in response to ongoing processes such as acute infections, and chronic inflammation such as rheumatoid arthritis and inflammatory bowel disorders[261, 263]. NETs are particularly important in the amplification of inflammation. Although NET formation is an essential event in innate immunity, it has adverse effects on the hosts. The release of intracellular components, such as DNA, histones, MPO, and NE, induce injury to the endothelial cells and thrombosis. In the case of persistent NETs *in vivo*, autoantibodies against NET components are produced[264,265]. Hence, impaired regulation of NETs is associated with the pathogenesis of autoimmune diseases such as SLE, vasculitis, RA and gout [222,82, 266]. Several studies show that NETs play a role in the pathogenesis of sepsis[160]. Indeed, the NET components act as a prognostic marker of sepsi [267].

More than a decade of research has uncovered the destructive functions of neutrophils and NETs in inflammatory diseases. However, there has been little progress in explicitly targeting them for therapeutic purpose. There are several experimental challenges in studying NETs, including their short life, isolation of naive neutrophils, stimulus used to activate neutrophils and the detection of NETs in tissue[260]. For better understanding of the relation of NETosis with human diseases, a simple and quantitative method to evaluate NETs is required. The use of knockout mice helped uncover the role of various proteins and ROS in disease. However, due to the lack of tools to visualize neutrophils it has made it challenging to show that neutrophils are the source of these proteins[260,268,269].

An important challenge is the low number of circulating neutrophils in mice. Laboratory mice are the leading model system for biomedical research[189]. Many mouse strains are widely used to develop *in vivo* disease models, to test drugs, and to develop novel therapeutic strategies[270]. Despite the high conservation in the genome between mice and humans, there exist intrinsic differences in the biology in neutrophils between the two species. Human blood is abundant in neutrophils (50-70% neutrophils) with a mean neutrophil count of $5x10^9$/l in adults, whereas neutrophils in mouse blood are scarce (10-25%)[189]. This discrepancy between murine and human neutrophils indicates that findings in mice may not translate to humans. Establishing an optimal mouse model to understand NETs holds the potential to further elucidate the role of NETs in normal and pathologic processes as well as therapeutic targeting.

To study the role of neutrophils *in vivo* and *in vitro*, we developed a mouse model with increased neutrophils in circulation by transgenic G-CSF expression. G-CSF is commonly used in clinics to treat neutropenia[215]. It is the key cytokine that stimulates the production and mobilization of neutrophils from the bone marrow[271,190]. The level of G-CSF is increased during infection, to facilitate for the need for greater number of neutrophils[20]. G-CSF also activates mature granulocytes. Binding of G-CSF to its receptor prolongs the lifespan of granulocytes and enhances the phagocytic activity and induces degranulation[272,214]. Mature neutrophils stay in the bone marrow through the function of chemokine receptors, CXCR2 and CXCR4. Bone marrow stromal cells and osteoblasts produce CXCL12, and that retains the CXCR4-expressing neutrophils in the bone marrow. G-CSF interferes with the CXCR4-CXCL12 interaction and induces

neutrophil exit from the bone. Furthermore, when neutrophils need to be mobilized into the blood, the ligands for CXCR2, are expressed by endothelial cells outside the bone marrow. G-CSF induces the upregulation of CXCR2 ligands, reduces the expression of CXCL12 by bone marrow stromal cells and reduced expression of CXCR4 on neutrophils[273, 274,24]. Therefore, G-CSF treatment is a promising method to overcome the discrepancy between human and mouse neutrophils

Using the pLIVE hepatocyte-specific expression vector, we stably expressed G-CSF as a transgene in the hepatocytes by hydrodynamic tail vein injection. This artificial expression of G-CSF in mice lead to the development of mice with an elevated number of neutrophils in circulation. These mice can mimic humans with regards to neutrophils numbers in circulation. In humans, chronic neutrophilia is characterized by splenomegaly. In line with this, we showed that G-CSF treated mice developed splenomegaly. The spleen weight and size increased with the neutrophil counts in circulation. In addition to this, histological analyses showed the infiltration of neutrophils in vital organs. Spontaneous NET-like structure that stained positively for the DNA specific fluorescent dye Sytox green were present in the neutrophilic mice. This observation suggests that neutrophils in circulation become activated to form NETs, when present at high concentrations. Surprisingly, the neutrophilic mice remained healthy, showed normal behavior, and a normal rate of growth. Measurement of plasma parameters of tissue damage (LDH), liver damage (ALT and AST) as well as renal dysfunction (Creatinine and BUN), indicated no organ damage. In conclusion, we developed a mouse model, exhibiting the characteristic features of chronic neutrophilia in humans, thereby creating a bridge in the immune system between both species. This animal model provides a valuable tool to understand the role of neutrophils and NETs *in vivo*.

It is well established that NETs in circulation are cytotoxic, yet these mice remain healthy[275]. Taken together, we hypothesized that the wild-type mice remained healthy despite the formation of NET-like structures in circulation because they are equipped with the machinery to clear NETs in circulation.

4.2 DNASE1 and DNASE1L3 provide a therapy to degrade intravascular NETs

The detection of NETs *in vivo* has been challenging. When purified neutrophils are stimulated *in vitro*, NETs can be detected by a DNA staining, and appear as a complex meshwork of long DNA strands decorated with antimicrobial proteins. However, several scientists still question the physiological relevance of NETs due to limited *in vivo* data[276]. Although NETs are implicated in several diseases, some scientists interpret NETs as a fortuitous fate of neutrophils undergoing necrosis and are not convinced that the phenomenon is a host defence strategy[277]. The morphological characterization and interaction of NETs with vascular endothelium and the cellular components of circulation such as platelets and other leukocytes is required for the complete understanding of the role of NETs in diseases[276,278].

Diverse methods have been used to identify NETs. The most popular method is the measurement of soluble NET remnants such as cell-free DNA and neutrophils granule protein such as MPO and NE in serum by enzyme-linked immunosorbent assay (ELISA)[279,280,281]. However, the presence of extracellular chromatin aggregates decorated with granular proteins has been linked to necrosis[201]. Also, the plasma of healthy humans always contains circulation cell-free DNA, which is elevated during pregnancy, trauma, infection, and cancer. MPO is also present in the plasma and is particularly elevated during trauma or infection[279]. Therefore, the coincidental presence of cell free DNA and neutrophil proteins do not reflect *in vivo* NETs accurately[277]. In order to understand the impact of NETs and the mechanism of NETosis, it is important to develop methods for the unambiguous and robust detection of NETs. In this study, we established a mouse model that allows for the objective detection of NETs *in vivo.*

DNASE1 is a circulating endonuclease that digests extracellular DNA and aids its effective removal from circulation[110]. Although DNASE1 is considered the central nuclease, Napirei et al. showed the presence of another nuclease in the serum, i.e., DNASE1L3. DNASE1L3 belong to the DNASE1 family of proteins. DNASE1 degrades chromatin efficiently in the presence of proteases. In other words, it preferably digests naked DNA. DNASE1L3, on the other hand, can digest protein-coated DNA even in the absence of proteases. Both enzymes have an N-terminal signal peptide for their

translocation in the endoplasmic reticulum. The DNASE1L3 has two nuclear localization signals. They concluded that the two enzymes might substitute each other or work in cooperation to clear extracellular DNA[114,112].

A reduced DNASE1 level in the plasma is linked to impaired NET degradation, but the role of DNASE1L3 in NET degradation was poorly understood[142]. To test the function of DNASE1 and DNASE1L3 in NET degradation, we used mice that lacked either one or both DNases. While serum from wild-type mice and mice lacking one of DNases efficiently degraded NETs *in vitro,* the serum from the double DNase deficient mice left them intact. Furthermore, the NET-degrading potential was reestablished by expressing either one of the DNases in the livers of double DNase deficient mice. Therefore, we showed that either DNASE1 or DNASE1L3 is sufficient to restore that NET-degrading capacity in the serum.

Next, we tested the function of the DNases in the degradation of NETs in circulation. We induced chronic neutrophilia in mice by stably expressing G-CSF. Upon development of chronic neutrophilia, wild-type mice and mice expressing either one of the DNases remained healthy and showed normal behavior and phenotype. However, mice with a combined deficiency of both enzymes did not tolerate the neutrophilia. Double DNase deficient mice quickly developed hypothermia and hematuria. Further analyses showed that the mice developed systemic autoimmunity, characterized by haemolysis and occlusion of blood vessels in the peripheral organs. The vascular occlusions were positive for hematoxylin and had a characteristic-staining pattern of decondensed chromatin, which is the hallmark of NETosis. They are interspersed with dark spots indicating individual leukocyte nuclei. Furthermore staining for NETs specific markers such are chromatin-MPO, chromatin-H3Cit and Chromatin-CRAMP, confirmed that the clots were composed of NETs. The formation of NET clots in *Dnase1*$^{-/-}$ *Dnase1l3*$^{-/-}$ mice shares features with infection-induced thrombotic microangiopathies (TMAs) and disseminated intravascular coagulation in patients[282].

Neutrophils and NETs have been shown to play a pivotal role in the pathogenesis of vascular diseases such as thrombosis and stroke[198]. NETs contribute to thrombosis by forming a scaffold that immobilizes platelets, RBCs, and plasma component[283].

Components of NETs are implicated as activators of coagulation. Histones perforate endothelial cells and platelets and increased calcium influx that further activates platelets[139]. The NE in NETs degrades TFPI and promotes thrombin generation. NETs also bind and activate FX II to the extrinsic pathway[137]. Considering the involvement of NETs in thrombus formation, we aimed to test the involvement of the components of canonical thrombi in the formation of these clots. In a canonical thrombus formation, the clot is formed of fibrin and involves the activation of platelets and endothelial cells and the secretion of vWF[284]. Although some clots showed the presence of fibrin and vWF, there were other clots that had no signal for any of the components of the canonical thrombi. Additionally, the formation of NETs clots in platelet-depleted and dabigatran (thrombin inhibitor) treated mice ruled out the requirement of platelets and thrombin for NET clot formation. In summary, we concluded that NETs are sufficient to form vascular occlusions in vital organs, causing severe organ damage. Therefore, this study describes a novel mechanism by which NETs can occlude blood vessels during inflammation.

NET-clots are not exclusive to *Csf3* induced neutrophilia but form in other diseases with elevated neutrophils in circulation. Sepsis is an infection in the blood. It induces a severe acute inflammatory response[285]. Sepsis is the leading cause of critical illness and mortality in humans. Neutrophils are the primary effector cells of innate immunity during sepsis. Despite the protective role of NETs in the initial stages of sepsis, excessive NET formation induces thrombosis and multiple organ failure in murine sepsis models[286]**.** Coagulation in sepsis leads to DIC and acute organ failure, increasing mortality due to sepsis[152].

We induced sepsis in mice by consecutive intraperitoneal injection of LPS an intravenous injection of heat-inactivated *E.coli*. LPS is a component of the cell wall of gram-positive bacteria[287]. Corresponding to our observations in mice with chronic neutrophilia model, the wild-type and mice expressing either of the DNases tolerated the sepsis and eventually recovered. The double DNase deficient mice, on the other hand, develop hypothermia, hematuria and systemic NET-clots. Therefore we concluded that DNASE1 or DNASE1L3 prevent the formation of vascular occlusions by NETs during sepsis.

NETs during a severe inflammatory disease are a leading cause of disease progression[288]. Thrombotic microangiopathies (TMA) is a group of diseases where the characteristic

feature in the formation of systemic microvascular occlusion[289]. Patients with TMA show several features that we observed in mice that developed intravascular NET clots including elevated LDH, hemolysis, hematuria, schistocytes, and organ damage[290].

Therefore, by inducing chronic neutrophilia and sepsis in the double DNase deficient mice, we have developed two animal models where NETs can be detected *in vivo*, forming intravascular occlusions. Importantly, we have described a previously unexplained method of vascular occlusion by NETs, and show that DNASE1 and DNASE1L3 are redundant enzymes that degrade intravascular NETs. We show for the first time the direct causality of NETs in disease, and provide proof that NETs are physiologically relevant structures that need to be adequately regulated in order to be protective. DNase infusion is a potential acute and prophylactic treatment strategy in patients that are predisposed to form intravascular NETs. Our *in vivo* NETs model provides a tool to investigate therapeutic strategies.

4.3 Intravascular NET formation independent of PAD4, Nox2, GSDMD and MyD88.

NETosis involves unique cellular changes that result in the extrusion of decondensed chromatin[96,68,80,291]. NETosis was originally described as a mechanism that results in the death of the neutrophils following NETs release[208]. Later studies showed that neutrophils can induce a faster and vital form of NETosis, where the neutrophil remains viable and can perform its phagocytic function[77]. There is considerable diversity in the stimuli and the mechanism of NETosis, indicating the presence of multiple pathways resulting in NETs[292]. Stimuli such as PMA, autoantibodies or cholesterol induces suicidal NETosis which occurs within 4 hours after stimulation[244]. In response to PMA, activation of NADPH oxidase generates ROS, which in turn activates PAD4, the enzyme that citrullinated histones and causes chromatin decondensation. ROS also induces the release of NE and MPO, which translocates to the nucleus to promote further unfolding of the chromatin. NETs are eventually released by the disintegration of plasma membrane[67,70,100]. The pore forming protein GSDMD has been implicated in the disintegration of plasma membrane[95,102]. Vital NETosis occurs within minutes, in response to bacteria or bacterial products and complement proteins via TLR2, TLR4 or complement receptors. Similar to suicidal NETosis, PAD4, NE and MPO translocate to the nucleus to aid with chromatin decondensation. The NETs assembled intracellularly

are then packed into vesicles and exocytosed from the cell, leaving the neutrophil alive for phagocytosis[77,244]. NETosis generated by different pathway may vary in their capacity to fight infection, cause cellular damage or be in inhibited by targeting a single biochemical pathway[195].

We have developed two *in vivo* NETs models, by inducing chronic neutrophilia by G-CSF expression, and by inducing sepsis by LPS and heat killed *E.coli* treatment. Since the stimuli appear to be important in determining the pathway involved in NETosis[293], these animal models provide a tool to test the functional requirement of different enzymes in response to G-CSF or LPS and heat killed *E.coli*. This model system will help identify new genetic pathways that can be therapeutically targeted to inhibit NETosis.

PAD4 is an enzyme that catalyzes the citrullination of histone. It is expressed by immune cell and localizes in the nucleus and cytoplasmic granules[108,294]. PAD4 is implicated as an important regulator of NET formation. The citrullination of H3 by PAD4 result in weakening of DNA–histone binding, thereby facilitate chromatin decondensation[107,106, 94].

Earlier studies report that the role of PAD4 in NETosis is conflicting. It is commonly accepted that the function of PAD4 is required for chromatin unfolding and the formation of NETs. Several studies presented evidence that citrullination is key for NETosis[295, 296]. This resulted in the interest on PAD4 as a promising therapeutic target to inhibit NETosis, particularly in SLE, in which NETs are believed to play a pathogenic role[297]. However, many other more recent studies now show that PAD4 might not be crucial for the process of NETosis. PAD4 deficient mice or treatment with PAD4 inhibitors such as Cl-amidine, caused reduced NETs in PMA and LPS induced NETosis from murine neutrophils, but not human neutrophils[195, 298, 299]. A recent study be Kenny et al. showed that the inhibition of PAD4 does not influence NETosis despite reduced citrullination of H3[292]. Another study, showed the presence of NET-like structures in lung tissue during *Klebsiella pneumoniae* infection in PAD4 deficient mice[298] . Therefore, the relative importance of PAD4 in NETosis has been controversial.

Most of the studies investigating PAD4 inhibitors rely on the quantification of citrullinated histone H3 to identify NETs[297]. Therefore, studies using PAD4 deficient mice and PAD4 inhibitors, lack a proper read readout to confirm the presence or absence NETs. It is worth noting that, the studies concluding that PAD4 is required for NETosis used stimuli that caused a calcium influx. However, calcium influx causes the hyper-activation of PAD4 and hypercitrullination of histones, gives rise to a form of neutrophil cell death known as leukotoxic hypercitrullination (LTH) which is not antimicrobial but a bacterial strategy to kill neutrophils[195]. Histone citrullination is also observed in other forms of cell death such as apoptosis and is therefore not a suitable indicator of NETosis[300, 301].

A23187 also known as calcium ionophores, causes a calcium influx, thereby activating PAD4. In this study, first we show that stimulation of WT neutrophils *in vitro* with A23187 causes the activation of PAD4 and *in vitro* NETosis. Furthermore, we found that neutrophils from PAD4 deficient mice, when stimulated with A23187 were able to form NETs *in vitro* even in the absence of H3 citrullination. These results provided us with the first evidence that PAD4 may not be required for NETosis.

In the earlier studies, we showed that double DNase deficient mice die as a result of the formation of intravascular NET-clots[282]. These double DNase deficient mice are a valuable tool to test role of PAD4 for the formation of *in vivo* NETs upon stimulation by G-CSF or LPS and heat killed *E.coli*. We subjected triple deficient mice, lacking both DNases and PAD4 to chronic neutrophilia and sepsis. We showed that the absence of PAD4 was not protective in the neutrophilia and the sepsis mice. Our results show that, PAD4 is not universal for the formation of NETs. Although the citrullination of histones by PAD4 occurs during NETosis, it is not required for the formation of NETs in response to G-CSF or LPS and heat killed *E.coli*.

Several studies have shown that NADPH oxidase (Nox2) is essential for NET formation. In fact, the generation of ROS is considered a hallmark of NETosis[67]. Also, ROS such as superoxide, HOCl, H_2O_2 can directly induce NETs in neutrophils[97]. Most of the current understanding of the molecular mechanism driving NETosis is based on the studies using PMA to activate neutrophils[302,76]. PMA directly activates the PKC pathway, which phosphorylates components of Nox2 inducing superoxide formation. Pharmacologically

inhibiting Nox2, as well as mice with a defect in the gene for Nox2, showed suppressed NET formation in response to PMA or bacteria[303,304]. Patients with a CGD have a mutation in the gene for Nox2 and are unable to generate ROS. Neutrophils isolated from CGD patients failed to undergo NETosis in response to PMA. Therefore it has become widely accepted that NETosis is ROS and Nox2 dependent process[305]. However, few studies now show that the NETosis occurs in response to stimuli that are ineffective inducers of ROS, such as calcium ionophores, GM-CSF, TNFα, or IL-1β. Therefore, the requirement of ROS in NETosis must be reinvestigated. Although production of ROS CGD patients was sufficient to restore NETosis, ROS restoration did not induce NETosis in neonates. This suggests that NETosis does not universally require Nox2 but depends on the stimuli and is disease specific[306,71,307].

Keeping this in mind, we tested the requirement of Nox2 for *in vitro* NETosis in response to A23187. Neutrophils from Nox3 deficient mice formed macroscopic NET clots that stained for citrullinated histones, indicating that *in vitro* NETs in response to A23187 is independent of Nox2. We then subjected triple deficient mice, lacking both DNases and Nox2 to chronic neutrophilia. Mice lacking Nox2 along with the DNases developed systemic autoimmunity and intravascular NET clots. Therefore, the absence of Nox2 was not protective during chronic neutrophilia. In conclusion, Nox2 plays an important role in regulating NETosis but is not the key driver of G-CSF induced intravascular NETosis.

Pyroptosis is an inflammatory caspase-dependent programmed cell death occurring in innate immune cells[308]. Similar to NETosis, pyroptosis is characterized by cell lysis, therefore releasing the components of the cell. Pyroptosis is mediated by the formation of a multimeric protein complex is formed known as the inflammasome. An inflammasome complex is comprised of a sensor, an adaptor, and a zymogen pro-caspase. In response to danger signals, the inflammasome complex assembles and activates inflammatory caspase through proximity-induced self-cleavage. The inflammatory caspases induce the maturation of pro-inflammatory cytokines by proteolytic cleavage of pro-IL-1ß and pro-IL-18. Ultimately, cell lysis facilitating the release of active IL-1ß and IL-18 into the extracellular space[309,310,311]. The molecular mechanism of cell lysis was unknown until recently. Chen at al., 2016 showed that the pore formation by GSDMD is the effector mechanism of pyroptosis. GSDMD is pore forming protein and a substrate of

inflammatory caspases (caspase-1,-4, -5 in humans and caspase-1,-11 in mice)[312]. GSDMD consists of two domains, a 31kDa N-terminal domain and a 22 kDa C-terminal domains. Under normal conditions full-length GSDMD is in an inactive state. Inflammatory caspases in the inflammasome cleave GSDMD in the cytoplasm, liberating the N-terminal fragment of GSDMD that has pore-forming capacity. The N-terminal domain localizes to the plasma membrane and causes cell lysis[313].

The cleavage of GSDMD may occur through the canonical (caspase-1-dependent) or a non-canonical (caspase-1-independent) inflammasome pathway. In the canonical pathway, the recognition of danger signals by inflammasome sensors causes the assembly of inflammasome subsequently activating caspase-1. In the non-canonical pathway, caspase-4,-5 and-11 are activated by directly binding LPS secreted by gram-negative bacteria in the host cytosol[314,315,314].

Pyroptosis and NETosis are mechanistically different pathways but have similar morphological outcomes. After the assembly of intracellular NETs, the plasma membrane ruptures to release NETs [76]. The molecular mechanism of NET release was not well understood. Recent studies have implicated GSDMD as a common executioner protein in pyroptosis and NETosis. Distinct from pyroptosis, which employs inflammatory caspases to cleave GSDMD, NETosis uses different proteases activated to cleave GSDMD depends on the stimuli. In response to classical stimuli such as PMN, NETosis proceeds independently of caspase activity. It involves the ROS dependant activation of NE that also cleaves GSDMD[95,102].

In classical NETosis, GSDMD has two functions. Firstly, cleaved GSDMD forms pores in the plasma membrane, causing NET release. Secondly, it forms pores in granule membrane, enhancing NE release in to the cytoplasm that further cleaves GSDMD by engaging in a feed forward mechanism[95]. Alternatively, in response to LPS and gram-negative bacteria, caspase-11 is directly activated in a non-canonical inflammasome pathway, and cause GSDMD cleavage independent of NE[102].

Based on these findings, we aimed to characterize the role of GSDMD in *in vivo* NETosis during chronic neutrophilia and sepsis. We subject triple deficient mice to lacking GSDMD and the two DNases to G-CSF induced chronic neutrophilia and LPS induced

sepsis. The absence of GSDMD was not protective in both models and did not prevent the formation of intravascular NETs. In conclusion, the involvement of GSDMD in intravascular NETs depends on the stimuli. Further studies to elucidate the function of GSDMD in NETosis, in response to other stimuli is required. Understanding the function of GSDMD in NETosis during different inflammatory diseases could reveal new therapeutic targets.

The family of TLRs help the innate immune system detect microorganisms and initiate the host defense mechanism and induce innate inflammatory responses[316]. MyD88 is a universal adaptor protein that presents downstream of TLR and IL-1 receptors as well as interferon (IFN) -γ receptor that recruits the other proteins of the signaling pathway giving rise to an inflammatory response[317]. MyD88 adaptor protein mediates numerous biologically important signal transduction pathways in innate immunity[318].

The MyD88-mediated pro-inflammatory signaling is activated during bacterial infection. Earlier studies showed that MyD88 gene silencing in primary human cells prevents staphylococcal enterotoxin and lipopolysaccharide (LPS) induced inflammatory response[319]. It was also shown that bone marrow neutrophils isolated from MyD88 deficient mice had a decreased ability to form NETs when treated with nontypeable haemophilus influenza (NTHI)[319]. This suggests that MyD88 mediated signaling is important for NET formation.

Furthermore, Myd88 plays an important role in the assembly of an inflammasome[320]. The formation of an inflammasome is a two-step process involving inflammasome priming followed by inflammasome assembly[320]. In the first step, extracellular danger signals such as LPS (signal 1) initiates inflammasome priming by binding to TLRs and activating the MyD88 pathway. Signaling through TLR-MyD88 leads to the activation of NF-kB which in turn regulates the transcription of the components of the inflammasome, pro-IL-1ß and pro- IL-18 and pro-GSDMD[321]. In the second step, the assembly of the inflammasome is triggered in response to intracellular signals (signal 2). The active inflammasome cleaves inactive GSDMD, and causes the maturation and releases pro-inflammatory cytokine IL-1β and IL-18, eventually promoting inflammation[320,250].

During classical NETosis, NE proteolytically cleaves GSDMD[95]. The engagement of the TLR-Myd88 pathway causes the generation of ROS, which in turn is required for the release of NE from neutrophils granules[322]. Based on this we attempted to understand the potential role of MyD88 in *in vivo* NETosis during to chronic neutrophilia and sepsis. We subject triple deficient mice to lacking Myd88 and the two DNases to chronic neutrophilia and sepsis. Our results show that, under the chronic neutrophilia, the absence of MyD88 does not affect the induction NETosis and formation of intravascular NET clots. There the TLR-MyD88 signaling is not crucial for NETosis in response to G-CSF. However, in the sepsis model, all MyD88 deficient mice were protected. Our results provide supporting evidence that LPS initiates the inflammatory response through TLRs in a MyD88 mediated pathway.

The identification of molecular mechanism exclusive to NETosis has therapeutic potential. Although the initiation components and molecular pathway leading to NETs is poorly understood, NETosis involved unique cellular changes that result in the extrusion of decondensed chromatin[82]. Inhibition of chromatin decondensation and neutrophil inflammasome actives seemed like possible therapeutic strategies[323].

In this study, we showed that the formation of intravascular NETs in mouse models of G-CSF induced chronic neutrophilia and LPS/*E.coli* induced sepsis is independent of PAD4, Nox2, and GSDMD. Although MyD88 was not crucial for NETosis in response to G-CSF, it mediated intravascular NETosis in response to LPS.

We conclude that PAD4, Nox2, and GSDMD have an important role in NETosis, but neither appears to be crucial for NETosis in response to G-CSF and LPS. Based on these findings, firstly we question if the functions of PAD4, Nox2 and GSDMD are redundant. This can be addressed using triple knock out mice lacking GSDMD or Nox2 along with the two DNases, in combination with PAD4 inhibitors. Secondly, we question the presence of other unexplained mechanisms that maybe inducing NETosis in the two *in vivo* NETs model of chronic neutrophilia and sepsis. We speculate that the presence of low density neutrophils (LDNs), causes spontaneous NETosis in response to G-CSF and LPS.

4.4 BrdU labeled neutrophils provide a robust technique to track in vivo NETs

Neutrophils are a cell population with subsets capable of different functions. LDNs were discovered in density gradient preparation of whole blood from patients with SLE, RA, and ARF. Bennett *et al* described that LDNs were immature neutrophils present in the PBMC fraction. An increased demand for neutrophils results in the release of functionally competent, but morphologically immature neutrophils. This is observed in many pathological conditions[182].

LDNs have an enhanced pro-inflammtory profile and aa strikingly enhanced capacity for firm NETs[170,184]. Since NETs act as autoantigens and have the potential to activate the adaptive immune system. LDNs are implicated in the pathogenesis of SLE, cancer, human immunodeficiency virus (HIV), sepsis, asthma and development of organ damage due to their enhanced pro-inflammatory response LDNs may play a role in the pathogenesis of the various diseases, through the formation of NETs[185].

Sepsis patients exhibit neutrophil dysfunction and morphological alterations that has been correlated with the severity of infection[241]. Morisaki et al reported the presence of LDN in sepsis. They shows that the density gradient centrifugation of whole blood from patients with severe infection had 8 ± 6% of HDNs while 40 ± 10% of neutrophils were LDNs. Neutrophils from sepsis patients shows low β-glucoronidase activity and reduced chemotactic response towards bacterial products that correlate with decreased intracellular enzymatic activity and chemotactic ability of LDNs[324].

In this study, we detected the presence of LDNs in circulation in G-CSF induces neutrophilia. Wright-Giemsa staining of whole blood smears from neutrophilic mice showed the presence of two distinct subpopulation of neutrophils. One type of cells where the nucleus was stained light purple represented the LDN and the cell with a dark purple nucleus represented the normal density neutrophils. Furthermore, the LDN fraction was highly enriched in comparison to the HDN fractions. These finding correlate with the fact that LDN are elevated during pathogenic conditions. Therefore, we conclude that *in vivo* NETosis in response to G-CSF may be due to the elevated levels of LDNs.

NETs are commonly measured using markers for DNA and citrullinated histones. However, recent studies from us and other group's shows that citrullination of histones is not universal to NETosis[201,195]. Other methods include measurement of soluble NET remnants such as cell-free DNA and neutrophils granule protein such as MPO and NE in serum by ELISA. This is not optimal as cell-free DNA decorated with granular proteins has been linked to neutrophil necrosis. Healthy plasma always contains circulation cell-free DNA, which is elevated during pregnancy, trauma, infection, and cancer[281]. Therefore we need a technique to explicitly detect NETs *in vivo* and distinguish them from remnants of necrosis.

We showed that, in the absence of both DNASE1 and DNASE1L3, NETs *in vivo* are not degraded and form vascular occlusion that can be easily detected by histological analyses. However, NETs are implicated in several diseases where the DNase activity is not completely absent. In these diseases models such as SLE, vasculitis, RA and gout, DNases in circulation, may chop down NETs into soluble fragments. However, the NETs fragments trigger autoimmunity and amplify inflammation. Therefore we require a technique to track the fate of NETs *in vivo*.

Neutrophils can be labelled by injecting BrdU into donor mice. BrdU is an analogue of thymidine and is incorporated into newly synthesized DNA in proliferating cells. BrdU is commonly used in the detection of proliferating cells in living tissue. In the case of neutrophils, BrdU will be incorporated into proliferating precursor cells that retain BrdU staining when differentiating into mature neutrophils. The incorporated BrdU can be stained using specific anti-BrdU fluorescent antibodies. When mice with *Csf3* induced chronic neutrophilia received repeated injection of BrdU, the neutrophils become labelled with BrdU. For future experiments, we propose to purify the BrdU labelled neutrophils. Adoptive transfer of BrdU-labeled neutrophils into recipient mice will help us track the fate of NETs their degradation products during different diseases.

5. Abstract:

Inflammation is a protective response to microbial invasion and tissue injury. Persistent inflammation is pathological. Neutrophils are the predominant cell types of inflammation. At the site of injury, neutrophils eliminate microbes and damaged tissue by phagocytosis, degranulation or by neutrophil extracellular traps (NETs) formation. NETs are lattices of DNA-filaments decorated with toxic protein. NETs are important for host defence, but improper NETs clearance is harmful due to their cytotoxic, pro-inflammatory and pro-thrombotic properties. Although NETs are implicated in a number of diseases, therapeutic targets to treat NETs are not well established.

Due to the lack of robust *in vivo* NETs model, the molecular pathways involved in NETosis is poorly understood. The detection of NETs *in vivo* has been challenging due to the low abundance of neutrophils in mice. Standard laboratory mice (C57BL/6) contain only 10–25% neutrophils in circulation, in contrast to humans, who have 50–70% neutrophils in circulation. In this thesis, we developed a murine neutrophilia model by the stable hepatic expression of granulocyte colony stimulating (G-CSF), a cytokine that stimulates neutrophil production in recipient mice. Furthermore, we observed that two DNA-degrading enzymes, DNASE1 and DNASE1-LIKE 3 (DNASE1L3), degrade NETs in circulation. Importantly, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ develop vascular clots of NETs during G-CSF driven neutrophilia and sepsis. NET clots obstructed blood flow and cause organ damage and death. Thus, this mouse model provides direct link between NETs and disease.

Next, we used this mouse model as a tool to study the molecular mechanism of intravascular NETosis. NET formation is regulated by peptidyl arginine deiminase 4 (PAD4), NADPH oxidase (Nox2), gasdermin d (GSDMD) and myeloid differentiation primary response 88 (MyD88). We tested the role of these proteins in intravascular NETosis by inducing chronic neutrophilia and sepsis in triple knock out mice i.e. *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Gsdmd*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Myd88*$^{-/-}$. The triple knock out mice were not protected from death due to intravascular NET clots during G-CSF induced neutrophilia. In contrast to this, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88*$^{-/-}$ mice, survived sepsis. We confirm that intravascular NETosis in

sepsis is a MyD88 mediated signalling pathway and that neutrophils engage different pathways of NETosis under sterile and infectious conditions. Furthermore we found that a subset of neutrophils, known as low density neutrophils (LDNs), were highly enriched in the G-CSF treated mice. In conclusion, G-CSF may induce NETosis via a non-canonical pathway involving LDNs. Finally, we established a specific method to track NETs *in vivo* using 5-bromo-2-deoxyuridine (BrdU) a synthetic analogue of thymidine. BrdU labelled neutrophils provide a tool to explicitly study the formation and deposition of NETs in different diseases models.

Zusammenfassung:

Entzündungsprozesse dienen als Schutzmechanismus vor pathogenen Mikroorganismen und Gewebeschäden. Eine anhaltende Entzündung kann pathologische Auswirkungen haben. Während einer Entzündung sind Neutrophile der vorherrschende Zelltyp. Neutrophile eliminieren Mikroorganismen und Gewebeschäden am Entzündungsherd mithilfe von zellulären Mechanismen wie Phagozytose, Degranulation oder der Bildung von Neutrophil Extracellular Traps (NETs). NETs bestehen aus langen DNA Strängen, die mit toxischen Proteinen dekoriert sind. NETs spielen eine wichtige Rolle in der Wirtsabwehr, allerdings kann ein unvollständiger Abbau oder eine unangemessene Freisetzung eine Reihe von zytotoxischen, pro-inflammatorischen und pro-thrombotischen Prozessen auslösen. Obwohl NETs an der Pathogenese verschiedener Krankheiten beteiligt sind, existieren wenige NET-spezifische Targets.

Die molekularen Signalwege der NETose sind weitestgehend unerforscht, was sich vor allem auf den Mangel von geeigneten präklinischen Tiermodellen zurückführen lässt. In Labormäusen sind Neutrophile nicht häufig, was das Detektieren von NETs *in vivo* erheblich erschwert. Der Labormausstamm C57BL/6 hat 10-25% Neutrophile im Blut, demgegenüber hat der Mensch 50-70% Neutrophile in der Blutzirkulation. In dieser Arbeit konnten wir ein chronisches Neutrophilie Modell in Labormäusen etablieren. Mithilfe eines Leberspezifischen Expressionsvektors war es uns möglich den Granulocyte Colony Stimulating Factor (GCS-F), ein Cytokin das die Bildung von Granulozyten anregt, stabil zu exprimieren. Darüber hinaus konnten wir zwei DNA-abbauende Enzyme finden, DNASE1 und DNASE1-LIKE 3 (DNASE1L3), die NETs in der Blutzirkulation abbauen können. Zudem konnten wir feststellen, dass die Doppel-Knockout-Linie, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ nach G-CSF-gesteuerter Neutrophilie und Sepsis, vaskuläre NET-Okklusionen bildete. NET-Okklusionen behindern den Blutfluss, verursachen Gewebeschäden und können zum Tod führen. Folglich konnten wir ein Mausmodell etablieren, das eine direkte Verbindung zwischen NETs und Pathogenese zeigt.

Anschließend konnten wir mithilfe des Mausmodells zur weiteren Erforschung der molekularen Signalwege bei der intravaskulären NETose beitragen. NETose wird durch

Peptidyl Arginine Deiminase 4 (PAD4), NADPH Oxidase (Nox2), Gasdermin D (GSDMD) und Myeloid Differentiation Primary Response 88 (MyD88) reguliert. Wir testeten daraufhin den Einfluss dieser Proteine in der intravaskulären NETose, indem wir chronische Neutrophilie und Sepsis in den folgenden Dreifach-Knockout (K.O.) Mäusen auslösten: *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Pad4*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Nox2*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Gsdmd*$^{-/-}$, *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$ *Myd88*$^{-/-}$. Die Dreifach-K.O. Mäuse bildeten intravaskuläre NET Aggregate nach der G-CSF induzierten Neutrophilie infolge derer sie verstarben. Im Gegensatz dazu, überlebten die *Dnase1*$^{-/-}$*Dnase1l3*$^{-/-}$*Myd88*$^{-/-}$ die ausgelöste Sepsis. Wir konnten zeigen, dass die intravaskuläre NETose während einer Sepsis MyD88 abhängig ist und dass Neutrophile unter sterilen oder infektiösen Konditionen unterschiedliche Signalwege nutzen. Des Weiteren konnten wir einen Subtyp von Neutrophilen, bekannt als „low density neutrophils“ (LDN), beschreiben, der in den G-CSF Mäusen deutlich erhöht war. Zusammenfassend kann man sagen, dass G-CSF NETose vermutlich über einen nicht-kanonischen Signalweg mit Beteiligung von LDNs auslöst. Letztlich konnten wir eine spezifische Methode etablieren, um NETs *in vivo* unter Zuhilfenahme eines synthetischen Thymidin-Analogons, 5-bromo-2-deoxyuridine (BrdU), nachzuweisen. Mit BrdU markierte Neutrophile dienen als Werkzeug, um die Bildung und Ablagerung von NETs in unterschiedlichen Krankheitsmodellen darzustellen.

6. List of Abbreviation:

ACPA	**Anti-citrullinated peptide antibody**
ADAMTS13	**A disintegrin and metalloproteinase with thrombospondin type 1 Motifs, member 13**
ALT	**Alanine Transaminase**
AMPs	**Antimicrobial peptides**
ANCA	**Ani-neutrophil cytoplasmic antibodies**
AST	**Aspartate Aminotransferase**
B cells	**Bone marrow or Bursa derived cells**
BALB/c	**Bagg Albino**
BrdU	**5-bromo-2'-deoxyuridine**
BUN	**Blood urea nitrogen**
C3	**Complement 3**
C5	**Complement 5**
C57BL6/6J	**C57 Black 6/6J**
CAM	**Cell adhesion molecules**
CD	**Chrohn´s disease**
CD11b	**Cluster of differentiation molecule 11b**
CF	**Cystic fibrosis**
cfDNA	**Cell free deoxyribonucleic acids**
CFU	**Colony forming units**
CGD	**Chronic granulomatous disease**
citH3	**Citrullinated histone 3**
COPD	**Chronic obstructive pulmonary disease**

CR1	**Complement receptor 1**
CRAMP	**Cathelecidin-related antimicrobial peptides**
CRISPR	**Clustered regularly interspaced short palindromic repeats**
CXCL-8	**C-X-C motif chemokine ligand 8**
CXCR4	**C-X-C chemokine receptor type 4**
CXCR5	**C-X-C chemokine receptor type 5**
DAMPs	**Damage-associated molecular patterns**
DCs	**Dendritic cells**
DIC	**Disseminated intravascular coagulation**
DNA	**Deoxyribonucleic acid**
DNase1	**Deoxyribonuclease 1**
Dnase1L3	**Deoxyribonuclease 1-Like 3**
DPZ	**Denaturing polyacrylamide gel electrophoresis zymography**
EDTA	**Ethylenediaminetetraacetic Acid**
ELISA	**Enzyme-linked immunosorbent assay**
FACS	**Fluorescence-activated cell sorting**
Fc R	**Fragment crystallisable Receptor**
FSC	**Forward scatter**
FVB/NJ	**Friend Virus B NIH Jackson**
G-CSF	**Granulocyte Colony stimulating Factor**
GM-CSF	**Granulocyte –macrophage colony stimulating factor**
GPCR	**G-protein coupled receptor**
GPI	**Glycosylphosphatidylinositol**
GSDMD	**Gasdermin D**

GTPase	**Guanosine triphosphates**
H&E	**Hematoxylin & Eosin**
HDN	**High density neutrophils**
H2A	**Histone 2A**
H2B	**Histone 2B**
H_2O_2	**Hydrogen peroxide**
H3	**Histone 3**
H4	**Histone 4**
HIV-1	**Human immunodeficiency virus**
HL-60	**Human Leukemia - 60**
HMGB1	**High-mobility group protein B1**
HOCl	**Hypochlorous acid**
HSCs	**Hematopoietic stem cells**
HTV	**Hydrodynamic tail vein**
HUS	**Hemolytic uremis syndrome**
IBD	**Inflammatory bowel disease**
I-CAM 1/2	**Intercellular Adhesion Molecule 1/2**
IFN	**Interferron**
IgG	**Immunoglobulin G**
IHC	**Immunohistochemistry**
IL-1b	**Interleukin -1 beta**
IL17	**Interleukin 17**
IV	**Intravenous**
JAM	**Junction adhesion molecule**

LDGs	**Low density granulocytes**
LDH	**Lactate dehydrogenase**
LDN	**Low density neutrophils**
LFA-1	**Lymphocyte function-associated antigen 1**
LPS	**Lipopolysaccharide**
Ly6G	**Lymphocyte antigen 6 complex, locus G**
Mac-1	**Macrophage-1 antigen**
MIP1 alpha	**Macrophage Inflammatory Protein 1 alpha**
MMP-8	**Matrix metalloproteinase**
MOI	**Multiplicity of infection**
MPO	**Myeloperoxidase**
MS	**Multiple sclerosis**
MSU	**Monosodium urate**
MyD88	**Myeloid Differentiation Primary Response 88**
NADPH	**Nicotinamide adenine dinucleotide phosphate hydrogen**
NDN	**Normal density neutrophils**
NE	**Neutrophil elastase**
NETs	**Neutrophils extracellular traps**
NF-Kb	**Nuclear factor kappa-light-chain-enhancer of activated Bcells**
NK cells	**Natural killer cells**
Nox2	**NADPH oxidase**
NSPs	**Neutrophil serine proteases**
PAD4	**Peptidyl arginine deaminase4**
PAF	**Platelet activating factor**

PAGE	**Polyacrylamide gel electrophoresis**
PAMPs	**Pathogen associated molecular patterns**
PBS	**Phosphate buffered saline**
PFA	**Paraformaldehyde**
PKC	**Protein kinase C**
PMA	**Phorbol myristate acetate**
PR3	**Proteinase 3**
PRRs	**Pathogen recognition receptors**
PS	**Phosphatidylserine**
PSGL-1	**P-selectin glycoprotein ligand-1**
RA	**Rheumatoid arthritis**
RBCs	**Red blood cells**
ROS	**Reactive oxygen species**
SDF-1	**Stromal derived factor**
SLE	**Systemic lupus erythematosus**
SOD-2	**Superoxide dismutase**
SRED	**Single radial enzyme diffusion**
SSC	**Sideward scatter**
SVV	**Small vessel vasculitis**
T cells	**Thymocyte cells**
T1DM	**Type 1 Diabetes Mellitus**
TAN	**Tumor associated neutrophils**
TEM	**Transendothelial migration**
TF	**Tissue factor**

TFPI	**Tissue factor pathway inhibitor**
TLRs	**Toll like receptors**
TNF-alpha	**Tumor necrosis factor**
TMA	**Thrombotic microangiopathies**
TRALI	**Transfusion related acute lung injury**
TTP	**Thrombotic thrombocytopenia purpura**
UC	**Ulcerative colitis**
VEGF	**Vascular endothelial growth factor**
vWF	**Von willebrandt factor**
WPB	**Wiebel Palade bodies**

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8. Statement of contribution by others

The experiments listed below and presnted in this thesis were performed by Miguel Jiménez-Alcázar.

1. Detection of DNASE1 and DNASE1L3 by DPZ
2. Detection og total DNase activits by SRED assay
3. Neutrophil isolation from human blood
4. *In vitro* NET degradation assay.

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10.Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

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Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....