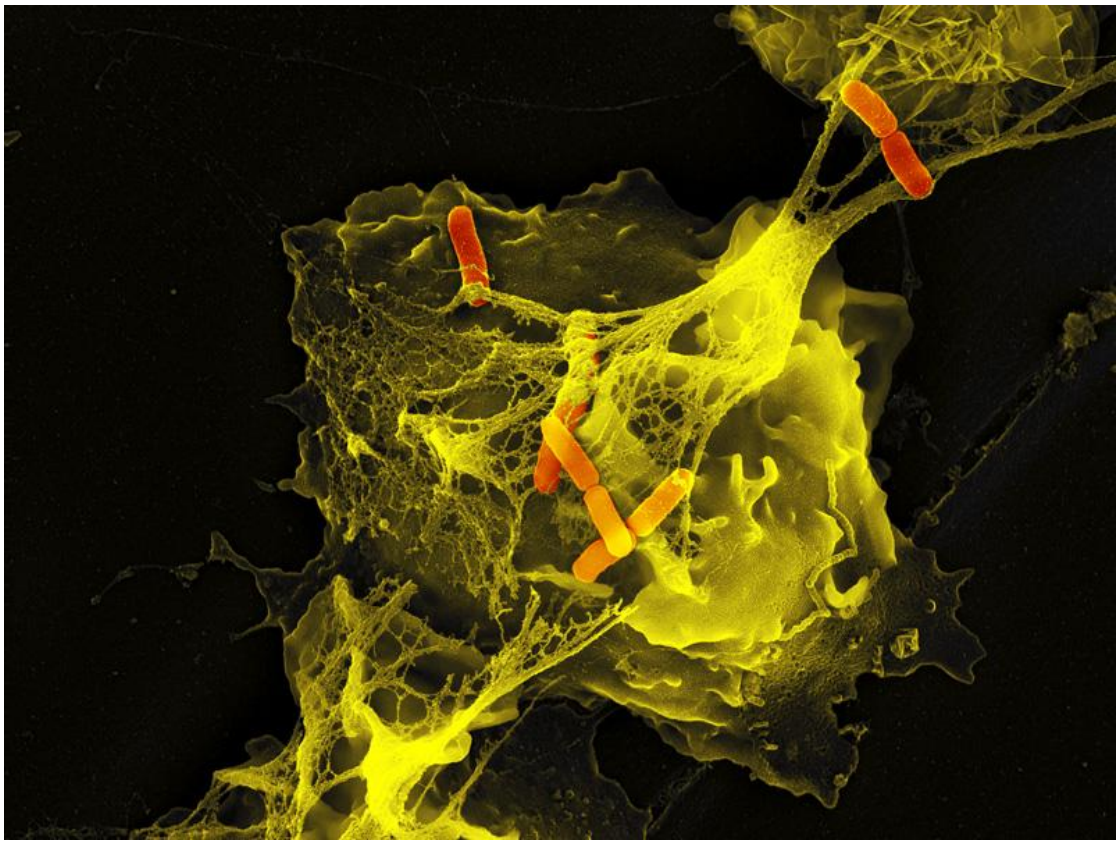
The Truth about NETs



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The Truth about NETs

Master Thesis

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About the cover: Activated neutrophils entrap Shigella flexneri in NETs[1].

Introduction

Neutrophils are considered to be the main cells responding to the invasion of bacteria. Attracted by chemotaxis, neutrophils migrate to the site of infection. Here, bacteria get engulfed by neutrophils and killed within the phagosome. During phagocytosis, activated neutrophils release their proteases, stored in cytoplasmic granules, into the phagosome. Simultaneously, neutrophils undergo a respiratory burst and produce enormous amounts of hydrogen peroxide, essential for microbial killing[2, 3]. Neutrophils of patients with chronic granulomatous disease (CGD) lack the ability to undergo these metabolic changes and are more susceptible to fungal and bacterial infections[4]. CGD is caused by impaired function of an electron transporter, the NADPH oxidase complex, located at the phagosomal membrane. Normally, NADPH oxidase generates superoxide which subsequently results in the production of oxygen radicals and their reaction products, the so called reactive oxygen species (ROS). In addition, myeloperoxidase, an enzyme highly present in granules, is capable to consume hydrogen peroxide for the oxidation of halides, which thereafter can destroy microorganisms (reviewed in 5). Therefore, for a long time, investigators thought that generation of ROS and myeloperoxidase halogenation were the mechanisms of microbial killing in phagosomes[6, 7]. However, later studies showed that mice deficient for proteases, like neutrophil elastase, can not eradicate Gram-negative bacteria as efficient as normal mice[8]. Moreover, mice lacking both elastase and cathepsin G are more susceptible to fungal infections[9]. These studies assume that in addition to ROS dependent killing, proteases are essential to kill microorganisms intracellularly.

Reeves *et al*. investigated this phenomenon and demonstrated that the antimicrobial activity of neutrophils is dependent on a potassium flux which activates the proteases. After phagocytosis massive amounts of potassium is pumped in, to compensate the negative charge within the vacuole, caused by the NADPH oxidase activity. This consequently results in an elevation of pH. At the same time, proteases dissolve from the anionic sulphated proteoglycan matrix of the granules due to the high potassium levels and become soluble and active. Moreover, the activity of the proteases is optimal at the elevated pH[10]. Finally, the ingested microorganisms are killed by the active proteases.

After 30 minutes, neutrophils exploit a second strategy to eradicate pathogens (reviewed in 11). At the site of infection, neutrophils get activated by inflammatory stimuli and release their antimicrobial peptides, stored in granules, in the surroundings. The antimicrobial peptides have diverse mechanisms to eliminate bacteria (reviewed in 12, 13). To ensure neutrophil degranulation of antimicrobial peptides can induce bacterial killing, high protein

level is required. However, *in vivo* the antibacterial effect of these peptides were disappointing (reviewed in 13, 14).

Recently, Brinkmann *et al.* discover a novel mechanism of microbial killing by neutrophils. In response to activating stimuli, neutrophils extrude their content leading to the formation of fiber like structures, so called neutrophil extracellular traps (NETs) (figure 1). The bacteria are caught and thereafter killed extracellularly by NET constituents. These observations resulted in a new field of research to unravel the processes behind the formation of NETs[15].

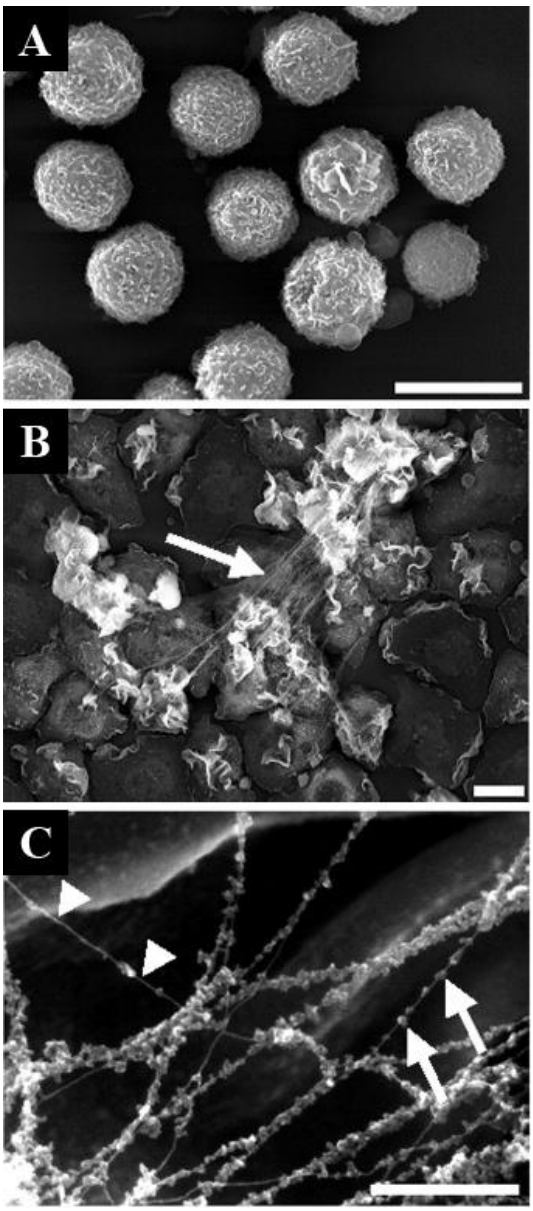

Figure 1 | Electron microscopy images of (A) resting neutrophils, (B) PMA stimulated neutrophils with NET formation and (C) NET fibers[15].

In this review, a complete overview is given about the knowledge of neutrophil extracellular killing so far. The composition of the NETs, even as the process underlying the formation of these structures will be discussed. Additionally, owing to the finding of NETs, some human diseases like CGD can be explained in a different way. Finally, to confirm the existence of NETs - in evolutionary point of view - bacteria should be adapted to evade extracellular killing by neutrophils to enhance their survival.

Neutrophil extracellular traps

NETs are fiber like structures particularly composed of DNA, but contain a lot of other components from the nucleus and azurophilic granules, like histones and elastase (figure 2), and surprisingly lack cytoplasmic proteins (table 1). NETs of activated neutrophils are completely degraded after treatment with protease free deoxyribonuclease (DNase)[15].

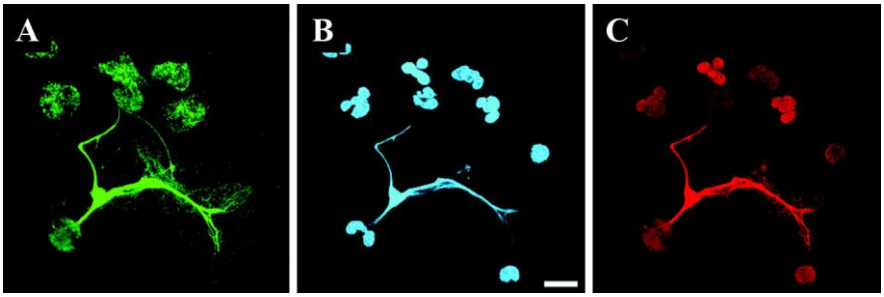

Figure 2 | Immunostaining of (A) neutrophil elastase, (B) DNA, (C) histone complexes in a IL-8 activated neutrophil culture[15].

Fuchs *et al*. showed that the formation of NETs occurs after activation of a new mechanism of cell death. Live-cell imaging of activated neutrophils, stimulated with PMA for 240 minutes, revealed this new process. During the first 220 minutes of stimulation, only the lobular structure of the nucleus disappeared. Then suddenly, the membrane of the neutrophil ruptured, visualized by loss of cytoplasmic content and positive phosphatidylserine staining. Simultaneously, at this same time point, also NETs got visible (figure 3). Electron microscopy images of neutrophils illustrated the loss of lobular nuclei and the heavily decondensing of chromatin within 60 minutes of activation. After 120 minutes vesicles were formed from the nuclear membrane and after 180 minutes only small vesicles, derived from the nuclear envelope, were visible in the cytoplasm (figure 4). Finally, all nuclear and granular membranes were lost, resulting in the gathering of chromatin with cytoplasmic and granular substances. Moreover, after extrusion of NETs, the majority of elastase remains localized on the chromatin fibers. Apparently, PMA activated neutrophils had no defragmented DNA and showed different morphology then apoptosis induced neutrophils. Additionally, neutrophils incubated with pore-forming toxins of *Staphylococcus aureus* showed typical necrotic morphology and lack the capacity to form NETs. These observations demonstrate that NET extrusion is a novel mechanism preserved for dying neutrophils different from apoptic and necrotic neutrophils[16].

Table 1 | Constituents of NETs (adapted from Brinkmann *et al.*)[15].

Components	**Cellular localization**	**NET inclusion**
▪ DNA	nucleus	+ (major component)
▪ histones (H1, H2A, H2B, H3, H4)	nucleus	+
▪ elastase	azurophilic granules	+
▪ myeloperoxidase	azurophilic granules	+
▪ cathepsin G	azurophilic granules	+
▪ bactericidal permeability increasing protein	azurophilic granules	+
▪ lactoferrin	specific granules	+
▪ gelatinase	tertiary granules	+
▪ cd63	granule membrane	-
▪ annexin I	cytoplasm	-
▪ β-catenin	cytoplasm	-
▪ α-tubulin	cytoplasm	-
▪ f-actin	cortex	-
▪ cytochrome c	mitochondria	-

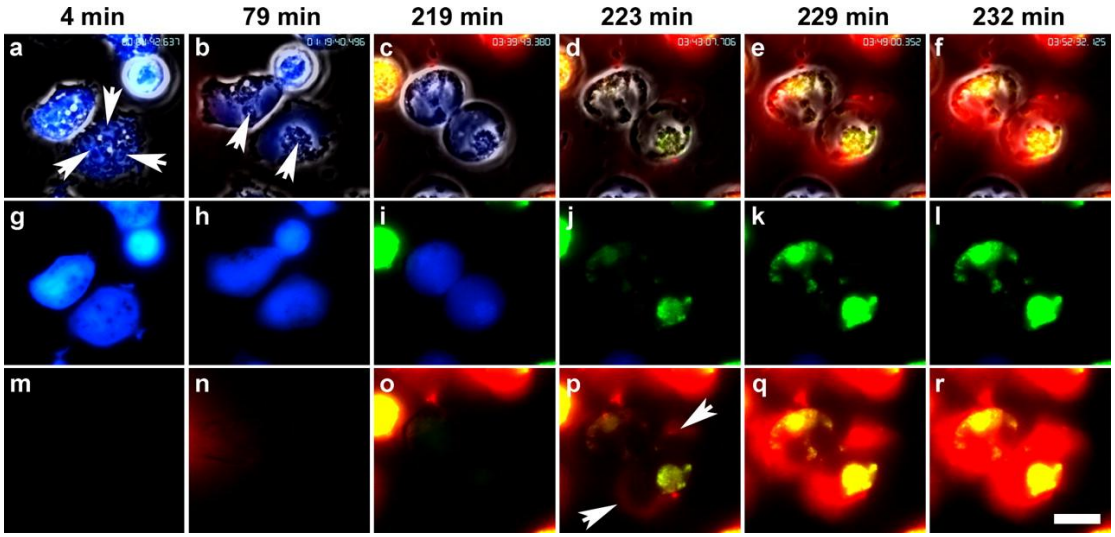

Figure 3 | Live-cell imaging of PMA activated neutrophils in time, stained for cytoplasmic content (blue), phosphatidylserine (green) and histone complexes (red). (a-f) Merge images of all stainings inclusive phase-contrast. (g-l) Merge images of cytoplasmic content and phosphatidylserine. (m-r) Merge images of phosphatidylserine and the histone-DNA complexes[16].

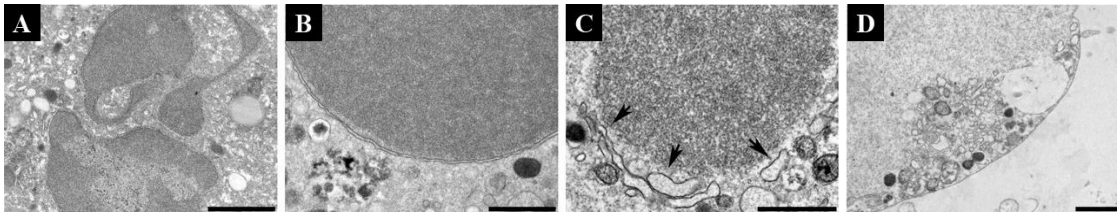

Figure 4 | Electron microscopy images of a PMA activated neutrophil. (A) 0 minutes of activation, normal lobular nuclear structure. (B) 60 minutes of activation, less lobulated structure and thinner nuclear membrane. (C) 120 minutes of activation, vesicle formation at the site of the nuclear membrane. (D) 180 minutes of activation, loss of nucleus, only vesicles visible in cytoplasm[16].

Nevertheless, the underlying mechanism of NET formation remains to be established. Stimulation of neutrophils with glucose oxidase, generating membrane permeable superoxide exogenously, PMA or *S. aureus* resulted in the production of ROS, which consequently resulted in neutrophil death. Inhibition of NADPH oxidase with diphenylene iodonium (DPI) ceased the ROS production in PMA or *S. aureus* stimulated neutrophils. Since glucose oxidase acts downstream of NADPH oxidase, glucose oxidase stimulated neutrophils generated ROS at the same level with or without DPI. Moreover, almost no neutrophils die after simultaneously incubation with DPI and stimulation with PMA or *S. aureus*. Additionally, NET formation of PMA activated neutrophils was blocked by catalase, converting superoxide in water and oxygen, and DPI, but was stimulated by an inhibitor of endogenous catalases, 3-amino-1,2,4-triazole, suggesting that the formation of NETs, and thereby neutrophil death, is depended on the production of ROS. Clinical investigations showed results parallel to this conclusion. Patients suffering from CGD did not generate ROS upon neutrophil activation and did not show the morphological changes characteristic for NET formation. In addition, these neutrophils only formed NETs upon stimulation with

glucose oxidase and not after stimulation with *S. aureus* or PMA (figure 5)[16]. NET formation is also impaired in both premature and term neonates. Even upon glucose oxidase stimulation of neonatal neutrophils no NETs were observed, suggesting that additional processes, next to the production of ROS are requested to obtain NETs[17]. In short, formation of NETs is dependent on ROS production and a still unknown mechanism.

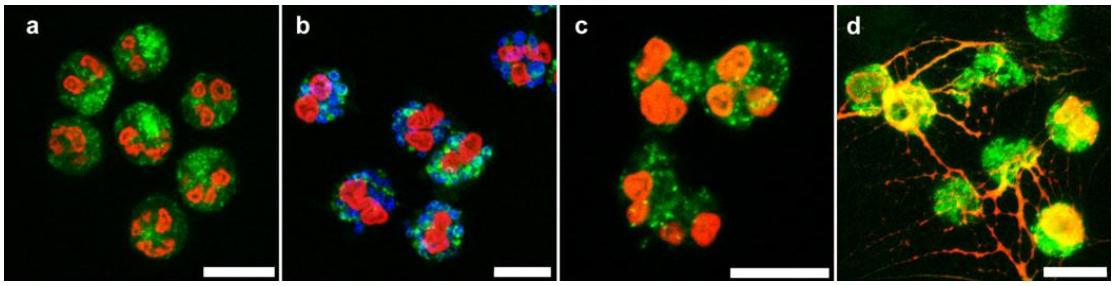

Figure 5 | Immunofluorensce images of different stimulated neutrophils of chronic granulomatous disease patients stained for histone complex (red), neutrophil elastase (green) and *Staphylococcus aureus* (blue). (a) Unstimulated neutrophils. (b) *S. aureus* stimulated neutrophils. (c) PMA stimulated neutrophils. (d) Glucose oxidase stimulated neutrophils generating NETs[16].

Neutrophils can be directly activated by different stimuli, like IL-8, LPS, PMA and bacteria, to generate NETs. Furthermore, Clark *et al.* demonstrated that indirectly activating neutrophils by platelets can lead to NET formation as well. Under flow conditions, comparable with the shear within sinusoids and capillaries of liver and lung, LPS stimulated platelets can bind and activate neutrophils to form NETs. In addition, septic blood, which induces adhesion of platelets to neutrophils, causes NET formation. However, also an adverse effect of NET formation is shown. Inflamed endothelium results in improved binding of LPS stimulated platelets to neutrophils contributing to damage on endothelial cells. Additionally, LPS treated endotoxemia mice show occlusions of sinusoids and increased tissue damage[18].

That NET formation can be harmful, is observed in patients with small-vessel vasculitis (SVV). SVV patients have circulating antineutrophil cytoplasm autoantibodies that can bind to proteinase-3 and myeloperoxidase, and activates neutrophils to form NETs without bacterial infection. Kidney biopsies of SVV patients show chromatin fibers with proteinase-3, meyoloperoxidase and other granular enzymes at the site of neutrophil infiltration[19].

NETs and bacteria

NETs trap both Gram-positive, *S. aureus*, as well as Gram-negative bacteria, *Salmonella typhimurium* and *Shigella flexneri* (figure 6). When bacteria were bound, constituents of NETs, like elastase, degrade virulence factors such as α toxin and IpaB of *S. aureus* and *S. flexneri* respectively. Even when phagocytosis of neutrophils is blocked with cytochalasin D,

an inhibitor of actin polymerization, bacterial virulence factors are degraded[15]. Mouse neutrophils express soluble peptidoglycan recognition protein (PGRP-S), which is stored in granules. Murine PGRP-S bind to the bacterial peptidoglycan layer and subsequently inhibits the growth of only Gram-positive bacteria[20]. However, recombinant human PGRP-S can bind Gram-positive, *S. aureus*, as well as Gram-negative bacteria, *Escherichia coli*, and inhibits their growth. In addition, PGRP-S together with lysozyme, which targets as well, stops the growth of *E. coli* completely, whereas lysozyme only has no effect. Surprisingly, both PGRP-S and lysozyme colocalize within NETs (figure 7)[21]. It seems that these fibrous structures of NETs are a web to assemble antimicrobial molecules in high concentrations, which can kill pathogens locally[15].

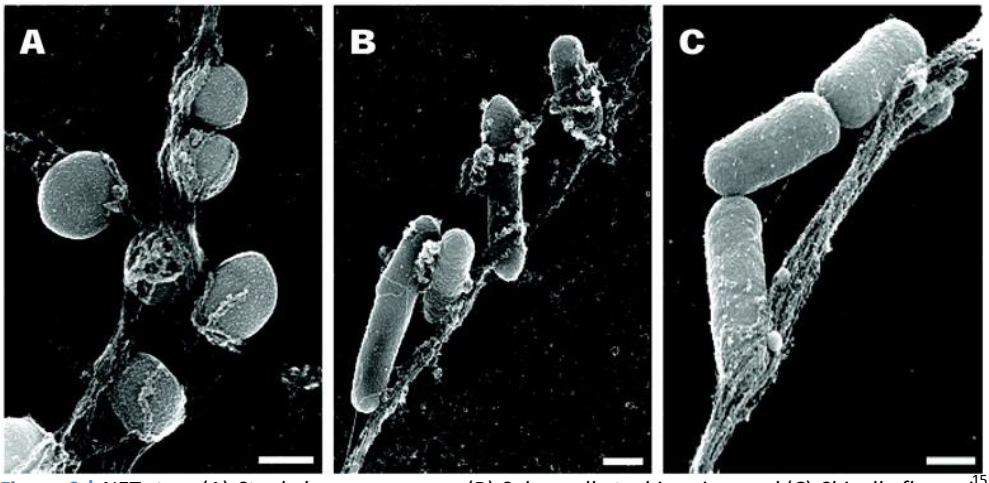

Figure 6 | NETs trap (A) *Staphylococcus aureus*, (B) *Salmonella typhimurium* and (C) *Shigella flexneri*[15].

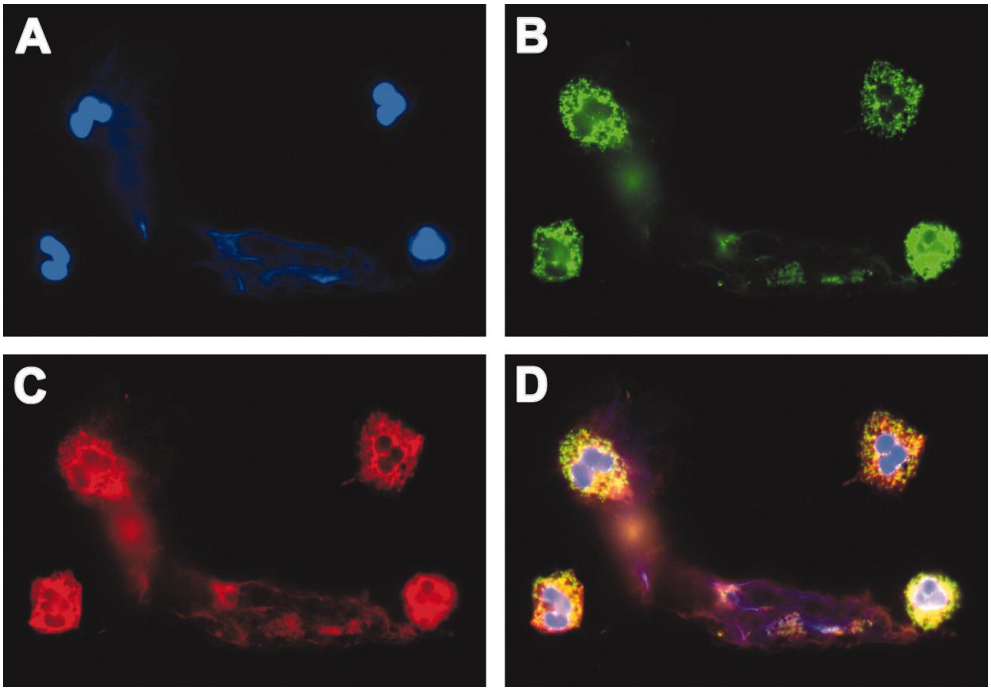

Figure 7 | PMA stimulated neutrophils stained for (A) DNA, (B) soluble peptidoglycan recognition protein (PGRP-S) and (C) lysozyme. (D) Merge image of (A), (B) and (C) showing colocalization of PGRP-S and lysozyme in NETs[21].

Naïve neutrophils kill *S. aureus* in particular by phagocytosis. During neutrophil activation, neutrophils clear bacteria more by extracellular NET killing and less by phagocytic killing. Obviously, this is also impaired in CGD patients[16]. The NET dependent killing is about 30% in neutrophil cultures infected with *S. flexneri* and *S. aureus*. After treatment with DNase and thereby dismantling NETs, no killing is observed. In addition, treatment with antibodies against histone-DNA complexes results also in negligible killing[15].

NET evasion

Activated neutrophils can kill bacteria extracellularly by NETs. Escaping from these killing NETs is a matter of life and death for bacterial pathogens. Logically, bacteria have developed several mechanisms to disintegrate and inactivate NETs or even better to interfere with NET formation.

One of these mechanisms is the expression of DNases. Group A *Streptococcus* (GAS) strain MGAS5005 express three different extracellular DNases, spd, spd3 and sda1. An *in vitro* GAS killing assay reveals that polymorphonuclear leukocytes (PMNs) can kill MGAS5005 and the triple DNases mutant strain efficiently. However, when PMNs are preincubated with dihydrocytochalasin B (DHCB), blocking phagocytosis, PMNs are less capable in killing MGAS5005, whereas the triple mutant strain is killed as efficient as without DHCB. Apparently, NETs are degraded by DNases expressed by GAS, which has an enormous effect on the killing capacity of neutrophils[22]. Noninvasive serotype M49 isolate of GAS and nonpathogenic Gram-positive bacterium *Lactococcus lactis* both transformed with a plasmid containing sda1 (pSda1), show similar characteristics as WT M1 GAS. *In vitro* both bacteria degrade DNA efficiently and evade extracellular killing of neutrophils. *L. lactis* transformed with pSda1 decrease the formation of NETs tremendously[23]. Parallel to the expression of DNases by GAS, *Streptococcus pneumoniae* (pneumococci) express EndA, a surface endonuclease and homolog of spd and sda DNases. EndA is important in uptake of DNA[24-26], but can also disintegrate NETs when neutrophils were infected with pneumococci[27]. Loss- and gain-of-function studies corroborate that extracellular DNases like sda1 and EndA contributes to the bacterial virulence of GAS and pneumococci *in vitro*.

To interfere with the formation of NETs, bacteria can express proteases which can eliminate activating signals for neutrophils. SpyCEP, an IL-8 proteases[28, 29], is also encoded by GAS strains. Besides retardation of IL-8 dependent neutrophil endothelial transmigration[28, 30], SpyCEP inhibits bacterial killing by neutrophils. In addition, SpyCEP can not improve bacterial viability in whole blood and acts therefore only at the site of infection. More

importantly, GAS inhibits the IL-8 dependent production of NETs via the expression of SpyCEP[30].

Moreover, Group B *Streptococcus* (GBS) regulates neutrophil function *in vitro*. GBS has a capsular polysaccharide composed of sialoglycans, which interact with an inhibitory protein, Siglec-9, expressed on neutrophils. GBS binding to Siglec-9 results in the inhibition of ROS production in the phagolysosomes of neutrophils as well as the formation of NETs (figure 8)[31]. Additionally, β proteins expressed by some GBS strains, bind to Siglec-5 and can also negatively regulate neutrophils. A β protein mutant strain does not decrease the oxidative burst of neutrophils and enhances the extrusions of NETs. Moreover, neutrophils, incubated with anti Siglec-5 antibody, clear less bacteria[32].

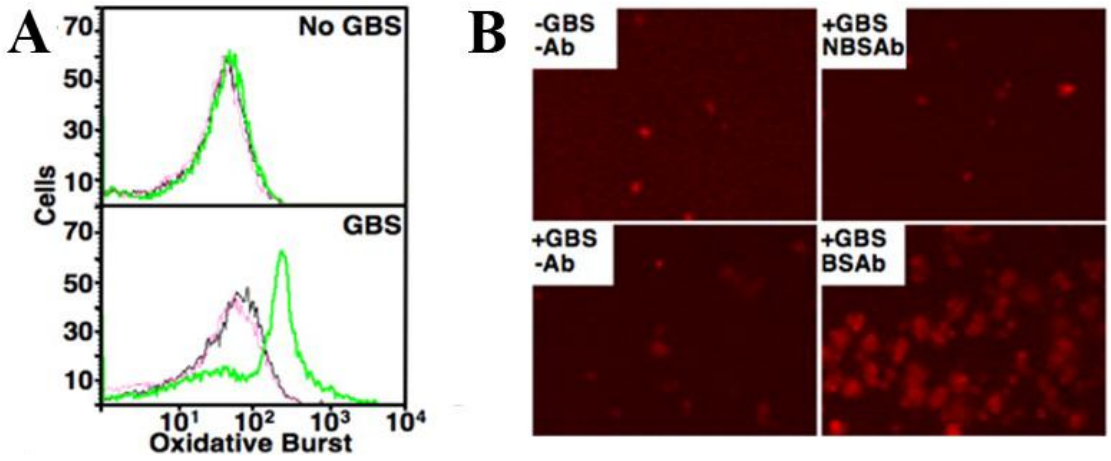

Figure 8 | (A) Reactive oxygen species production upon Group B *Streptococcus* (GBS) stimulation of neutrophils with blocked Siglec-9 receptor (green, BSAb), no blocking (black, -Ab) or blocked Siglec-9 at non GBS binding site (pink, NBSAb). (B) NET formation after Siglec-9 depended GBS binding stained for DNA (orange)[31].

In vivo evidence

The existence of NETs can be heavily debated due to possible *in vitro* artifacts, but confirmed by *in vivo* data. In case of experimental shigellosis in rabbits, *Shigella* is closely associated with DNA and histones, which are even harmful for bacteria in a lower concentration compared to granular enzymes in NETs[15]. Also mice treated with LPS show NETs within their long capillaries (figure 9). In addition, these mice trap a greater amount of *E. coli* in their sinusoids, sites where platelets bind neutrophils, compared to untreated mice[18]. Furthermore, mice infected with *S. pneumonia* inducing pneumonia form NETs localized in alveoli of lung tissue (figure 10)[27]. More significant, NETs are shown in human, namely in a section of neutrophil exudate in spontaneous appendicitis (figure 11) and within a lung blood vessel during acute interstitial pneumonitis[15, 18].

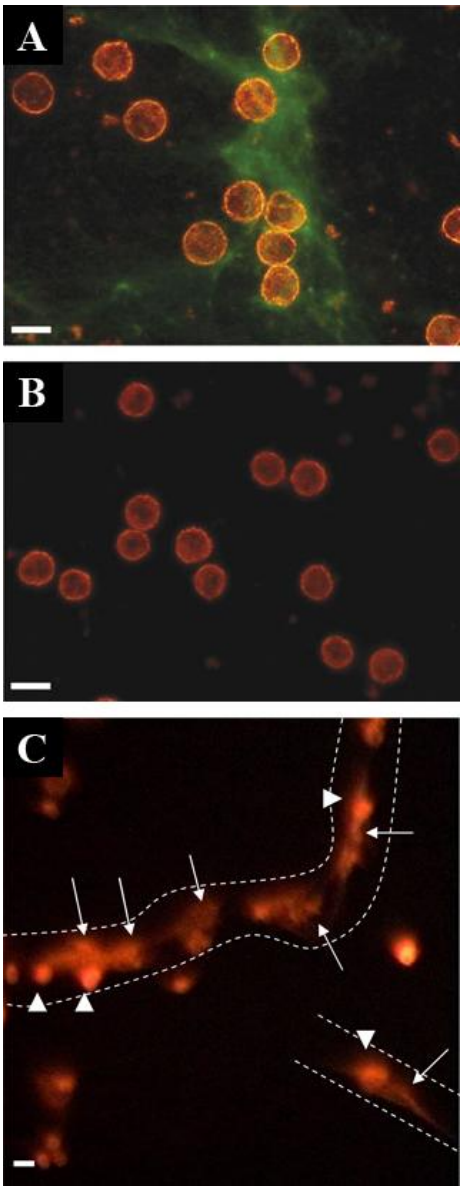

Figure 9 | (A) Neutrophils (orange) incubated with septic blood forming NETs (green). (B) Neutrophils incubated with blood of healthy individuals. (C) LPS treated mice showing NETs (orange) in pulmonary capillaries[18].

Besides NET formation *in vivo*, bacterial NET evasion strategies are investigated in mouse models. Nude mice, injected subcutaneously with a triple DNases mutant GAS strain, lacking spd, spd3 and sda1, have an enhanced survival compared to MGAS5005 infection. Only the single sda1 mutant strain shows a comparable fraction of nude mice with lesions compared to mice injected intraperitoneal with the MGAS5005 strain. However, the triple mutant strain shows almost no lesions. In addition, infection of MGAS5005 in a non-primate model of GAS pharyngitis results in erythema contrary to triple mutant strain infected monkeys showing no severity, indicating that extracellular DNases are involved in GAS pathogenesis[22]. Moreover, mice infected with M1 GAS strain lacking sda1 trap bacteria within NETs (figure 12). Furthermore, subcutaneous injection in a murine model of GAS necrotizing fasciitis with M49 GAS transformed with pSda1 increases the lesion area and enhances the number of bacteria per lesion[23]. Mice infected with a mutant EndA *S. pneumonia* strain have a higher life expectancy than mice infected with a WT strain. Furthermore, EndA is not so important during the initial infection in upper respiratory tract, but even more imperative for pneumococcal spread into the lungs and bloodstream. Not surprisingly and logical, type 1 pneumococci have a less invasive nature, but also a diminished potential to degrade NETs compared to other pneumococcal types[27].

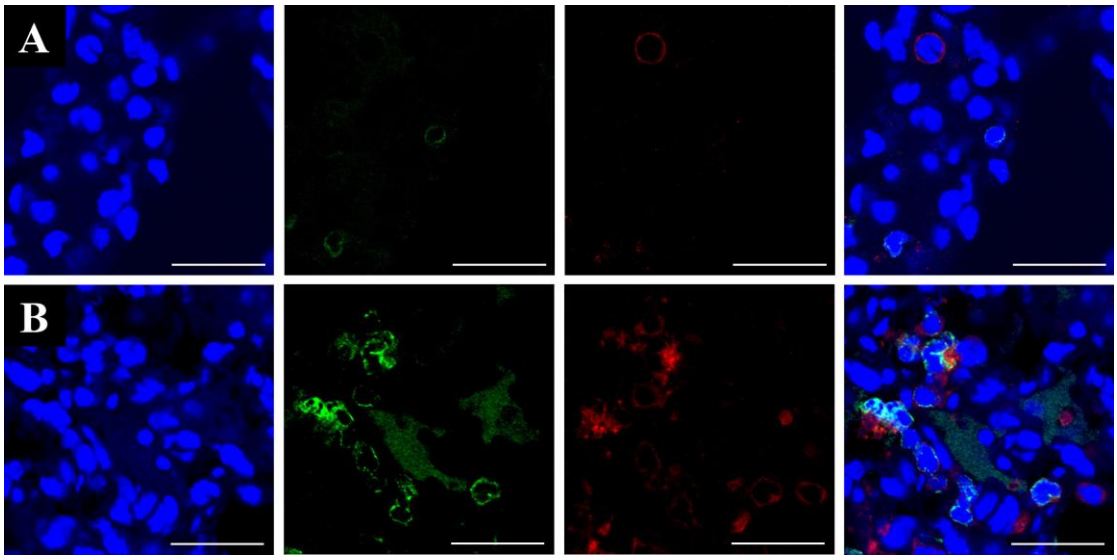

Figure 10 | Lungs of mice infected with (A) bacterial growth medium or (B) *Streptococcus pneumoniae* stained for DNA (blue), histones (green) and neutrophils (red). (B) Merge image shows NETs[27].

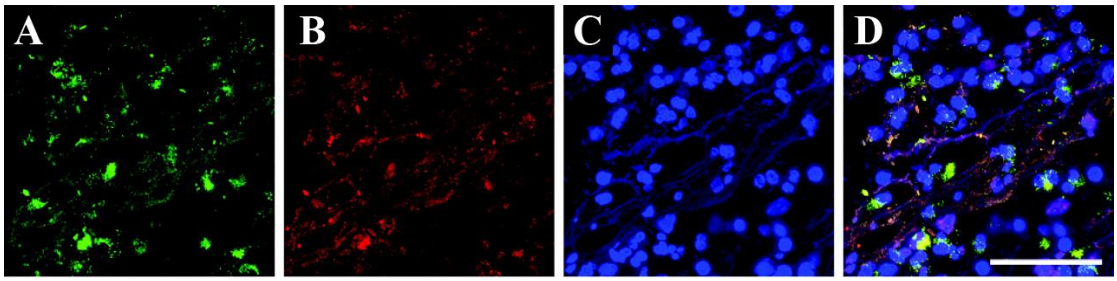

Figure 11 | Tissue at the site of neutrophil infiltration of spontaneous human appendicitis stained for elastase (green), histones (red) and DNA (blue). (D) Merge image of (A), (B) and (C) showing colocalization of elastase and histones in NETs[15].

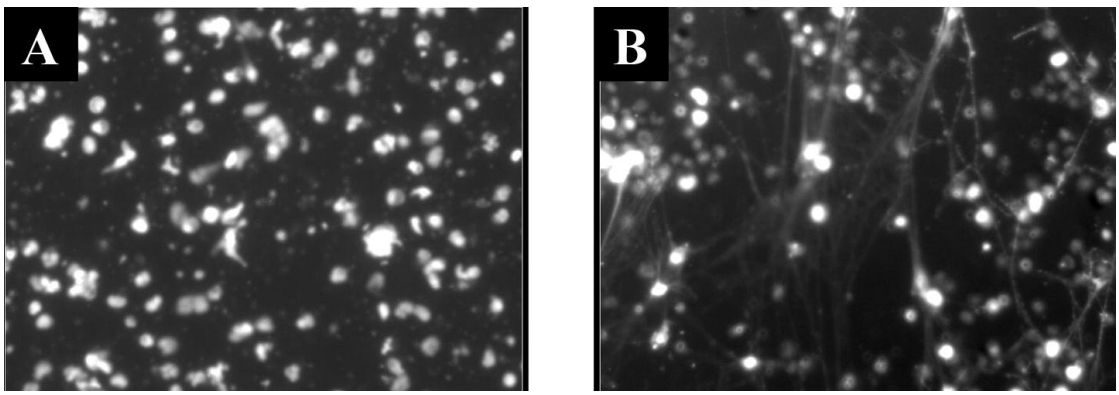

Figure 12 | Tissue of abscess exudates of mice infected with (A) wt M1 Group A *Streptococcus* (GAS) and (B) sda1 mutant strain of M1 GAS stained for DNA[23].

Discussion

This review gives an overview of investigations done to reveal the truth about NETs. Since the major finding of Brink *et al.* many groups studied extracellular neutrophil killing to corroborate the existence of NETs. Sumby *et al.*, the first who support NET formation upon

bacterial infection, showed that GAS express DNases to disintegrate the web of chromatin fibers and to increase their viability[22]. Besides GAS, also GBS and pneumococci have developed a mechanism to evade the extracellular killing by neutrophils and enhance their survival within the host[27, 30-32]. Consequently, two different mechanisms can be described to influence the killing by NETs. Because DNA is the major component of NETs, bacteria can arm themselves with DNases, endonucleases or other DNA degrading enzymes to disintegrate the NETs. Obviously, also shielding to the granular enzymes can be a way to protect them for the noxious enzymes. However, in this way bacteria will still be trapped and can not invade the host effectively. Another way to interfere with NETs is to suppress their formation by stimulating inhibitory receptors on neutrophils or by removing the activating stimuli. Evidently, bacteria have obtained mechanisms during evolution to evade the extracellular killing by neutrophils.

Possible *in vitro* artifacts of NETs are excluded by mice studies. In LPS treated mice, neutrophils get activated by platelets and generate NETs within long capillaries[18]. In addition, lung of mice with pneumococcal pneumonia is filled with activated and NET extruded neutrophils[27]. *In vitro* studies showed that bacterial virulence factors have the capacity to disintegrate NETs. Mice infected with bacteria lacking these factors have an enhanced life expectancy and show smaller lesions[22, 27, 30]. Furthermore, more NETs are present when mice were infected with the mutant strain[23]. Moreover, human tissues are investigated to ensure the existence of NETs during human diseases. In tissue of spontaneous human appendicitis as well as in tissue of acute interstitial pneumonitis NETs were identified[15, 18]. *Ex vivo* experiments with neutrophils from term and preterm newborns, and from CGD patients clearly illustrate the lack of NETs due to mutations or unknown causes in the mechanism behind NET formation[16, 17].

This review shows that in response to bacterial stimuli, neutrophils get activated and form NETs to trap and kill the invader. Beside the normal way of clearing pathogens by phagocytosis and exposure to proteases this novel mechanism of neutrophil extracellular killing plays an important role in primary host defense. Moreover, the knowledge about bacterial adaptation to evade the immune system can be used in practice. For instance, DNases inhibitors can be used as potential therapeutic to prevent degradation of NETs by GAS DNases[23]. In the future, therapeutics depended on the maintenance of NETs can be used to clear bacterial infections. All in all, without doubts, the existence of NETs is evident.

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