Intracellular Radicals in Neutrophils

Processing and Functional Implications

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Cover illustration: Human blood neutrophils.

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ABSTRACT

Neutrophils are the most abundant leukocyte in human blood and essential components of our defense against microbial pathogens. These cells can neutralize microbial pathogens by phagocytosis, which involves engulfment and degradation of microbes intracellularly, as well as by the formation of neutrophil extracellular traps (NETs), which are structures released from neutrophils made up of DNA and proteins that capture microbes extracellularly. One characteristic of neutrophils is that they can produce massive amounts of reactive oxygen species (ROS) upon activation of a specialized enzyme system, the NADPH oxidase. The ROS can be produced at different cellular sites, inside the phagosome, intracellularly inside granules, and at the plasma membrane leading to the release of ROS extracellularly. Whereas ROS produced inside phagosomes are crucial for microbial killing, much less is known about intracellular ROS produced inside granules, which is therefore in focus in this thesis.

Neutrophils contain multiple types of granules that are storage organelles for soluble proteins, receptors, and effector molecules. Part of the NADPH oxidase is found in granule membranes and upon activation, ROS can be produced inside granules where they may be processed by myeloperoxidase (MPO) to yield other types of ROS. In **paper I**, MPO-processing of intracellular ROS was shown to be dependent on phospholipase A_2 (PLA_2) activity. However, PLA_2 was not directly involved in the processing but rather indirectly by mediating the fusion of different granule types, which enables the ROS and MPO to meet inside the cell. It has previously been suggested that the autoinflammatory disorder SAPHO syndrome, characterized by neutrophil dermatosis and typically sterile inflammation of the bone, is associated with neutrophils lacking the production of intracellular ROS. In **paper IV,** four patients with SAPHO syndrome were investigated with respect to ROS production and other neutrophil functions. All patients, however, produced normal amounts of intracellular ROS demonstrating that decreased intracellular ROS production is not a general feature of SAPHO syndrome.

In **paper II** and **III,** the role of intragranular ROS for the formation of NETs was studied. **Paper II** demonstrates that intragranular ROS are essential to drive active NET formation and that intracellular processing of these ROS by MPO is a critical step. **Paper III** shows that NETs are not only the result of an active process but can also be induced by alternative means, e.g., by cytotoxic peptides released from bacteria. Unlike the process described in the literature and in **paper II**, this type of NET formation was not dependent on ROS or MPO.

In conclusion, the processing of intracellularly produced ROS in neutrophils has been characterized and both production and processing were found to be essential for active NET formation. Further, an alternative mechanism of NET formation was described that is independent of ROS production.

SAMANTEKT Á ÍSLENSKU (SUMMARY IN ICELANDIC)

Mannslíkaminn er undir stöðugu áreiti frá örverum í umhverfi okkar. Ónæmiskerfið er varnarkerfi líkamans sem vinnur að því að losa okkur við óæskilegar örverur og laga skemmdan vef. Þetta varnarkerfi er mjög öflugt sem sést best á því að þó að við séum stöðugt í návist örvera þá verðum við sjaldan mikið veik.

Ónæmiskerfið byggist á hvítum blóðkornum, það eru sérhæfðar frumur af nokkrum gerðum sem hafa mismunandi hlutverk í ónæmissvarinu. Þegar ónæmiskerfið er virkjað vegna sýkingar þá myndast svokallað bólgusvar. Flestir kannast við einkenni bólgumyndunar en þau eru roði, bjúgumyndun, hiti, verkur, og hugsanlegt tap á virkni þess hluta líkamans sem er bólginn. Þetta eru einkenni þess að ónæmiskerfið er að störfum og myndast vegna breytinga í æðakerfinu nálægt upphafsstað sýkingarinnar og áhrifa frá ónæmisfrumum sem þangað fara.

Þó svo að bólgusvarið sé nauðsynlegt til að verja okkur gegn örverum þá þarf að stjórna því ítarlega því of mikið bólgusvar getur leitt til ýmissa sjúkdóma, svo sem gigt, psoriasis, og þarmabólgusjúkdóma.

Þessi doktorsritgerð fjallar um daufkyrninga (e. neutrophils) sem er fjölmennasta tegund hvítra blóðkorna. Þessar frumur eru aðallega í blóðrásinni en bregðast fljótt við ógnum og ferðast þá frá blóðrás inn í vef þar sem þær mæta örverum. Daufkyrningar eru átfrumur sem þýðir að þær geta gleypt örverur og drepið þær inn í sérstökum innfrumukornum. Inni í kornum daufkyrninga eru örverudrepandi efni, til dæmis hvarfgjarnar súrefnissameindir (e. reactive oxygen species; ROS) sem myndast í miklu magni inn í daufkyrningum. Þessar hvarfgjörnu súrefnissameindir hafa einnig áhrif á stjórnun bólgusvarsins, en lítið er vitað um hvernig þær hafa áhrif það.

Í þessu doktorsverkefni hafa hvarfgjarnar súrefnissameindir framleiddar af daufkyrningum verið rannsakaðar. Áhrif þeirra á ferla inn í frumunum hafa verið skoðuð, bæði í heilbrigðum einstaklingum og einstaklingum sem þjást af bólgusjúkdómum.

SAMMANFATTNING PÅ SVENSKA (SUMMARY IN SWEDISH)

Den mänskliga kroppen är konstant utsatt för mikrober från vår miljö. Immunsystemet är kroppens försvarssystem som arbetar för att befria oss från oönskade mikroorganismer och reparera skadad vävnad. Detta försvar är mycket effektivt vilket framgår av det faktum att även om vi är ständigt i närvaro av mikroorganismer så blir vi sällan allvarligt sjuka.

Immunsystemet består av vita blodkroppar, de är specialiserade celler av olika typer som spelar diverse roller i immunförsvaret. När immunförsvaret aktiveras på grund av infektion kommer det att finnas inflammation. De flesta människor känner igen symtomen på inflammation, rodnad, svullnad, feber, smärta och funktionsnedsättning av den inflammerade kroppsdelen. Detta är tecken på att immunförsvaret arbetar och orsakas av förändringar i kärlen nära det initiala infektionsstället och effekter från immunceller som åker dit.

Även om inflammation är nödvändig för att skydda oss mot mikroorganismer måste det regleras eftersom för mycket inflammatoriskt svar kan leda till olika sjukdomar, såsom artrit, psoriasis och inflammatoriska tarmsjukdomar.

Denna avhandling handlar om neutrofiler som är den vanligaste typen av vita blodkroppar. Dessa celler cirkulerar i blodet men reagerar snabbt på hot och migrerar då från blodet in i vävnaden där de möter mikroorganismer. Neutrofiler är fagocyter, vilket innebär att de kan svälja bakterier och döda dem. Inne i neutrofila granule finns antimikrobiella medel, såsom reaktiva syreradikaler som produceras i stora mängder i neutrofiler. Dessa reaktiva syremolekyler har också en inverkan på inflammatoriskt reglering, men lite är känt om hur detta går till.

I denna doktorsavhandling har reaktiva syreradikaler som produceras av neutrofiler undersökts. Deras inverkan på processer i neutrofilen har studerats både hos friska individer och personer som lider av inflammatoriska sjukdomar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Björnsdottir H, Granfeldt D, Welin A, Bylund J, Karlsson A. Inhibition of phospholipase A2 abrogates intracellular processing of NADPH-oxidase derived reactive oxygen species in human neutrophils. Experimental Cell Research 2013. 319: 761-74.

II. Björnsdottir H, Welin A, Michaëlsson E, Osla V, Berg S, Christenson K, Sundqvist M, Dahlgren C, Karlsson A, Bylund J. Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Submitted manuscript.*

III. Björnsdottir H, Welin A, Stylianou M, Christenson K, Urban C, Forsman H, Dahlgren C, Karlsson A, Bylund J. Cytotoxic peptides from Staphylococcus aureus induce ROS-independent neutrophil cell death with NET-like features. *Manuscript.*

IV. Wekell P*, Björnsdottir H*, Björkman L, Sundqvist M, Christenson K, Osla V, Berg S, Fasth A, Welin A, Bylund J, Karlsson A. Neutrophils from patients with SAPHO syndrome show no signs of aberrant NADPH-oxidase dependent production of intracellular reactive oxygen species. *Submitted manuscript. *Joint first authorship*

The following paper is also referred to in the text:

Appendix A

Bylund J, Björnsdottir H, Sundqvist M, Karlsson, A, Dahlgren C. Measurement of respiratory burst products, released or retained, during activation of professional phagocytes. Methods in Molecular Biology 2014. 1124: 321-328.

CONTENT

ABBREVIATIONS

CGD	chronic granulomatous disease
CL	chemiluminescence
CR	complement receptor
DAMPs	damage-associated molecular patterns
ecROS	extracellular ROS
GPCR	G-protein coupled receptor
icROS	intracellular ROS
LPS	lipopolysaccharide
MPO	myeloperoxidase
NE	neutrophil elastase
NETs	neutrophil extracellular traps
nphROS	non-phagosomal intracellular ROS
PAD4	protein arginine deiminase 4
PAMPs	pathogen-associated molecular pattern
PFAPA	periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis
PHPA	p-hydroxyphenyl acetic acid
phROS	phagosomal intracellular ROS
PKC	protein kinase c
PLA2	phospholipase A2
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
PSM	phenol soluble modulin
ROS	reactive oxygen species
SAPHO	synovitis, acne, pustulosis, hyperostosis, osteitis
SOD	superoxide dismutase

INTRODUCTION

Inflammation is the body's reaction to harmful stimuli such as pathogens, damaged cells, or irritants. It is a complex biological response that involves many players of the immune system, both cells and soluble mediators that in cooperation have the goal to clear the initiating stimulus and heal the tissue. Inflammation is thus a vital process that fights invading pathogens and repairs damaged tissue. However, the inflammatory response is very powerful and sometimes inflicts damage to our own tissue. The response must therefore be controlled accurately; uncontrolled inflammation can lead to a variety of different diseases, such as rheumatoid arthritis, psoriasis, gout, and inflammatory bowel disease.

Our cells have evolved the ability to recognize (and respond to) conserved structures on microbes, so-called pathogen-associated molecular patterns (PAMPs), and structures that are indicative of tissue damage, known as damage-associated molecular patterns (DAMPs). When such structures are recognized, cells close to the infection or injury respond to these cues by release of soluble mediators, such as cytokines and chemokines, which alerts the blood leukocytes and direct them from the blood stream to the affected tissue. Neutrophils are phagocytic leukocytes that are key players in inflammatory responses and they are also the first cell type to arrive at the affected tissues.

This thesis deals with the life and death of neutrophils and how these events can have an impact on human health and disease.

Neutrophils are the most common white blood cells found in human blood and they comprise 50-70% of the circulating leukocytes. Neutrophils are found in two pools in circulation. Around 50% float in the blood stream, accounting for the neutrophils acquired in a blood sample, and the other half is loosely attached to the vascular endothelium, known as the marginating pool [1].

Neutrophils along with basophils and eosinophils comprise the group polymorpohonuclear leukocytes (PMN). Due to the fact that neutrophils outnumber basophils and eosinophils by large, the designation PMN, however, often refers to this specific cell type. PMN have morphologically distinct nuclei with multiple lobules, which explain the term polymorphonuclear. Additionally, the term granulocyte is often used for PMN due to the fact that they contain an extensive amount of intracellular vesicles, more commonly referred to as granules.

Function

The main function of neutrophils is to eradicate microbial threats and help in the healing of damaged tissue. The invasion of microbes usually does not occur directly in the blood, but at sites that are in direct contact with the outer world, e.g., the epithelial layers. Such threats are rapidly sensed by the surrounding tissue that calls for help from neutrophils and other immune cells through secretion of cytokines and chemokines. Neutrophils then rapidly leave the blood stream and move to the site of infection, a process called transmigration. Once in the infected tissue, the threats are most often neutralized by the vast array of antimicrobial functions that the neutrophils possess. These processes will be described in more detail below.

Neutrophil cell biology

All blood cells are produced in the bone marrow, and a large proportion of the blood forming activity is directed towards myelopoiesis, the production of neutrophils and monocytes. In a human adult, around 1 - 2 x 10^{11} neutrophils are produced every day [2].

Neutrophils are formed and allowed to mature in the bone marrow in a process that takes approximately 14 days [3]. There are several maturation phases where the distinct granule types (described below) are formed in an orderly process [4,

5]. After completion, fully mature neutrophils leave the bone marrow and enter circulation. Neutrophils have some features that are distinct compared to most other leukocytes, e.g., regarding energy production, protein biosynthesis, and granule storage of cell components (all discussed below).

Energy production

Neutrophils have stores of glycogen and generate energy almost solely through glycolysis, unlike other leukocytes that generate energy predominantly through oxidative phosphorylation in the mitochondria [6, 7]. This oxygen-independent energy production in neutrophils is believed to be beneficial as it allows the cells to function at sites that are low in oxygen, such as in inflamed deep tissue. Still, oxygen consumption by neutrophils can be tremendously increased upon activation, however not due to mitochondrial respiration but instead through the activity of the NADPH oxidase that catalyzes the formation of superoxide anion from molecular oxygen (the respiratory burst, discussed below). Although neutrophils do not primarily use mitochondria for energy metabolism they contain mitochondria, but much less than e.g., peripheral mononuclear leukocytes [8]. The precise function of mitochondria in neutrophils has not been intensely explored, but they have been shown to be important for apoptotic signaling [9, 10].

Protein biosynthesis

Neutrophils are considered to synthesize only very limited amounts of protein after they have left the bone marrow, even though some *de novo* synthesis, e.g. of cytokines, may occur when neutrophils are activated [11, 12]. Most of the proteins that neutrophils need in order to fulfill their functions are synthesized during maturation in the bone marrow and stored in granules of mature cells. These granules are membrane-enclosed vesicles that are centrally involved in most neutrophil effector functions.

Neutrophil granules

There are at least four distinct types of granules and intracellular vesicles that differ in their content (Figure 1) and are formed at different times of neutrophil maturation in the bone marrow [13]. The purpose of having these distinct granule populations is for the cell to be able control when and where the components of each granule are used. The granules contain both soluble proteins and membrane receptors that can be translocated to the plasma membrane upon degranulation. This will increase the number of receptors, rendering the neutrophils in a so-called primed state that is associated with increased responsiveness to stimulation [14].

Azurophil granules

The azurophil (or primary) granules are formed earliest in the maturation process. They contain a number of cytotoxic molecules and are involved in the killing of microbes. The azurophil granules are lysosome-like organelles that mainly fuse with the phagosome to form the phagolysosome [15] and are rarely released extracellularly [16]. The characteristic protein of azurophil granules is myeloperoxidase (MPO) that is involved in the processing of reactive oxygen species (ROS; **paper I**) and thereby contribute to oxygen-dependent microbial killing [15, 17]. MPO also participates in the formation of neutrophil extracellular traps (NETs; **paper II**), as will be described in detail in the following chapters. There are also several directly microbicidal proteins found in these granules, including alpha-defensins, bactericidal/permeability-increasing protein, and serine proteases with microbicidal activity (proteinase-3, cathepsin G, and neutrophil elastase (NE); [15]). The fact that azurophil granules contain many cytotoxic and/or proteolytic substances is presumably the reason for that these granules are not easily released extracellularly since that would have damaging effects also on the surrounding host tissue.

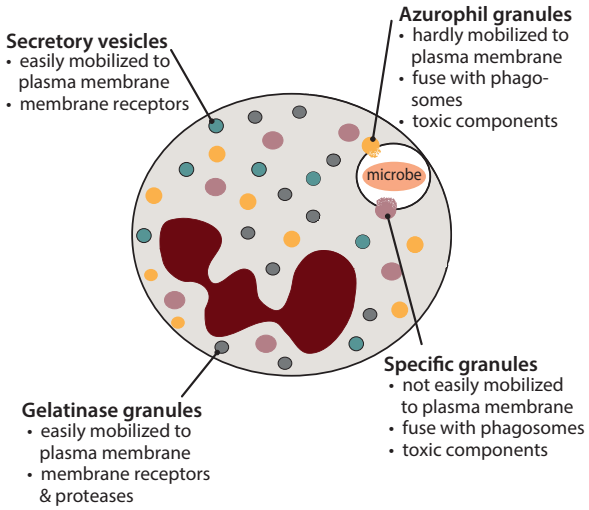

Figure 1. Neutrophil granules. Schematic drawing of the four distinct granule/vesicle populations in neutrophils and their properties and content.

Specific and gelatinase granules

The next granules to be formed during neutrophil maturation are the specific (secondary) granules and then the gelatinase (tertiary) granules. Specific granules contain antimicrobial substances that are mainly delivered to the phagosome but that can also be mobilized to the extracellular space. Proteins used as markers

for specific granules include the bactericidal proteins lactoferrin and neutrophil gelatinase-associated lipocalin [15, 18].

Gelatinase granules on the other hand store fewer antimicrobial substances but contain membrane receptors that are important for the extravasation toward the site of infection, and proteases that help the cells to degrade the extracellular matrix in order to make way. These granules are named after the protease gelatinase, which they contain [19]. Gelatinase granules are more easily mobilized to the plasma membrane than the specific granules [14].

Both specific and gelatinase granules contain the membrane-bound part of the NADPH oxidase [20] that is responsible for the production of ROS, which will be dealt with in detail in the following chapters (and in **papers I, II,** and **IV)**.

Secretory vesicles

The last vesicle type that is formed in more or less mature neutrophils is the secretory vesicle. These organelles are formed through endocytosis [21] as opposed to the granules that are formed from the Golgi [22]. Their membrane thus contains plasma membrane components, while their matrix is filled with plasma proteins picked up from the extracellular fluids of the bone marrow. Secretory vesicles are very easily mobilized to the plasma membrane to expose their reservoir of membrane receptors that are needed for the first steps of an inflammatory response [15].

Limitations in studying neutrophil function

There are several limitations when studying neutrophil function that are related to their cellular biology. These cells are terminally differentiated when leaving the bone marrow and they do not divide in culture. This means that genes cannot be manipulated to overexpress proteins or to knock down the biosynthesis of proteins, which is commonly done in biological research to examine the function of specific proteins in a given setting.

Neutrophil-like cell lines are in many instances useful for overexpression and knockdown of genes. However, the cell lines available, e.g., HL-60 cells, are not phenotypically identical to primary neutrophils and have an immature granule composition, making them limited as models for functional studies [23].

Animal models, most commonly using mice, are widely used in experimental immunology. There are, however, significant differences between neutrophils from mice and men; neutrophils make up only 10-25% of the circulating leukocytes in mice compared to 50-70% in humans [24, 25]. Also, murine neutrophils completely lack defensins [26] and contain markedly lower concentrations of MPO as compared to human cells [27]. The granule composition of mouse neutrophils is not entirely clarified, reflected e.g. in the

inability of these cells to produce intragranular oxygen radicals [28]. These differences taken together make mouse models of limited use with regards to functional studies of (human) neutrophils.

Hence, to study the importance of individual molecules and their interactions in a given cellular process, neutrophil researchers are at large forced to rely on experiments using pharmacological inhibitors (**paper I**, **II**, and **III**) and/or cells from donors with genetic defects (**paper II**, **III**).

Life of neutrophils

Neutrophils are the first cells recruited to sites of inflammation, either caused by invading pathogens or by presence of damaged tissue. As they are almost solely found in blood in the absence of a threat, neutrophils must rapidly transmigrate to the infected/damaged site (Figure 2) when called for by chemokines. The body constantly experiences minor threats that are readily taken care of by neutrophils without causing any noticeable symptoms to the host, a process (limited in time and space) that can be thought of as 'physiological inflammation'. In the case of bigger threats, the response is enhanced and extended and results in the cardinal signs of inflammation, i.e., redness, edema, increased temperature, pain, and loss of function.

When infection arises or the tissue is damaged, the local surroundings are alarmed by PAMPs from microbes or DAMPs from the damaged tissue. Epithelial cells, as well as resident leukocytes such as macrophages and mast cells, start to secrete cytokines and chemokines, which work to increase the local permeability of the blood vessels. The endothelium of nearby vasculature is activated and begins to express cell adhesion molecules that start interacting with neutrophils flowing by. The neutrophils sense chemokines that are attached to the endothelium as a result of inflammatory activation of the nearby tissue, slow down, and start rolling along the vessel wall [29]. This leads to neutrophil activation characterized by cytoskeletal rearrangements and degranulation of the most easily mobilized granules/vesicles [2, 30].

The degranulation results in upregulation of granule-stored receptors (e.g., adhesion and chemotactic receptors) to the cell surface making the neutrophil more responsive (primed) and simultaneously the early adhesion molecule L-selectin is shed from the surface. Measurements of changes in cell surface receptors are commonly used to determine the activation status of neutrophils (**paper I** and **IV**). Circulating neutrophils are in a quiescent state (i.e., no degranulation has occurred) in healthy individuals, whereas degranulation has typically taken place in neutrophils isolated from inflamed tissues [31]. Primed neutrophils in circulation have been reported during severe systemic inflammation such as sepsis [32]. In **paper IV** we found that during

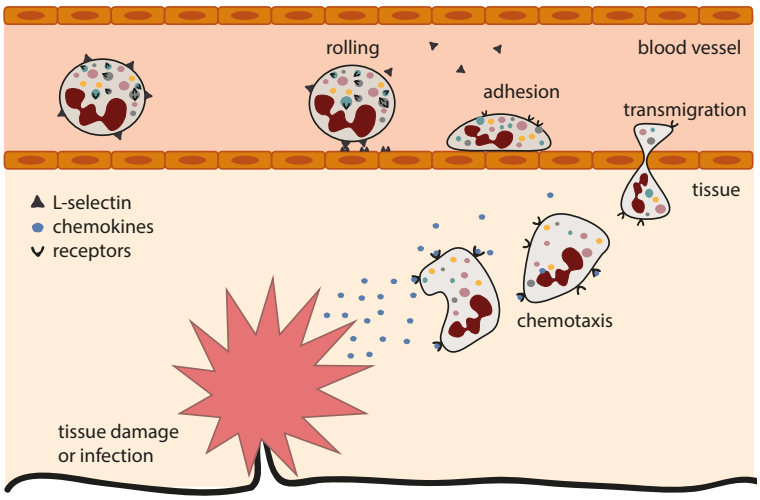

Figure 2. Neutrophil transmigration from blood to tissue. Upon tissue damage or infection, leukocytes in the tissue activate vascular endothelium that signal to neutrophils to slow down. First neutrophils attach to endothelium through L-selectin and neutrophils start to roll along the endothelium. This is followed by degranulation in neutrophils that leads to increased surface receptors and allows a firm binding. The L-selectin is cleaved and the neutrophils cross the endothelium and migrate towards the site of infection/damaged tissue guided by chemokines and substances released from the damaged site.

inflammatory episodes in patients with the autoinflammatory disease SAPHO (discussed further under 'Deficiencies in neutrophil ROS production'), blood neutrophils were more prone to degranulate (even though it had not happened yet) compared to neutrophils from healthy controls. This suggests that the neutrophils in these patients were not entirely quiescent in the blood, but existed in a "pre-primed" state.

After degranulation of secretory vesicles and loose attachment to the endothelium, the neutrophils finally stop rolling and attach firmly with the help of integrin receptors [30, 33]. When leaving the vessels they must traverse the endothelium and the basal membrane. The endothelial transmigration either occurs in a paracellular manner (between endothelial cells) or in a transcellular manner (neutrophil passes directly through an endothelial cell) [34]. The passage through the basal membrane is most probably supported by degradation of the extracellular matrix by neutrophil proteases released from the gelatinase granules [13, 35]. Once in the tissue, more potent chemotactic factors (e.g., formylated peptides originating directly from bacteria or complement factor C5a resulting from activation of the complement cascade) than those found in the endothelial surroundings guide neutrophils to the site of infection. Well arrived at the inflammatory site, the neutrophils are ready to exert their antimicrobial actions [30, 36], discussed further under 'Antimicrobial actions of neutrophils' below.

Death of neutrophils

Neutrophils are short-lived cells and have a half-life of only hours to days in circulation [37]. However, their life span may increase considerably under inflammatory conditions due to exposure to cytokines and/or microbial components [38, 39]. If the neutrophils are not called for by the tissue to fight microbes, they will senescence in circulation and undergo spontaneous apoptosis (programmed cell death, see below). As apoptotic cells, they will be removed from circulation by resident macrophages in the liver, spleen, and bone marrow [1, 40, 41]. Neutrophils that have engulfed microbes after transmigration to the tissue may also undergo apoptosis and need to be cleared by other phagocytes, mainly resident and infiltrating macrophages [42].

Whereas apoptosis is a physiological way for a cell to die, there are other modes of cell death that are violent and more pathological in nature, such as necrosis and NETosis (discussed below). Yet other types of cell death have been defined in immune cells, such as pyroptosis and pyronecrosis. These are cell death mechanisms that are known to occur in monocytes/macrophages, involving multi-protein complexes, inflammasomes, which also are involved in cleaving inactive pro-inflammatory cytokines to their active form [43]. Although neutrophils contain inflammasomes that are involved in the cleavage of cytokines, it seems like neutrophils do not die via these pathways [44, 45].

Non-violent cell death (apoptosis)

Apoptosis is a regulated cell death process where the dead cells maintain their membrane integrity and thus spare the surrounding tissue from damage and further immune activation that can be induced by extracellular release of intracellular constituents (DAMPs). Nearly all cells can be induced to undergo apoptosis, but aged neutrophils undergo spontaneous apoptosis if not called for duty in the tissues [4].

During apoptosis, cells undergo numerous morphological changes where internal structures are disintegrated and the cells become non-functional [46]. The phospholipid phosphatidylserine, located on the inside of the plasma membrane in viable cells, flips to the outside of the cells and serves as a signal for other phagocytes to ingest the dying cell [47]. When macrophages ingest apoptotic neutrophils they start to secrete anti-inflammatory cytokines that contribute to resolving the inflammation [48].

Thus, if neutrophils are removed rapidly after they become apoptotic, the surrounding tissue will not be harmed by substances released from the dying cells. However, if they are not cleared promptly, the cellular membrane integrity will eventually collapse, leading to an uncontrolled release of (normally intracellular) DAMPs and proteolytic enzymes.

Violent cell death

Violent cell death of neutrophils is accompanied by broken cell membranes and, as opposed to apoptosis, is regarded as a pro-inflammatory process. Violent cell death may result from a passive process, such as physical damage leading to necrosis, or an active process, such as the induction of NETosis.

Necrosis

Cell death by necrosis is destructive and characterized by plasma membrane rupture and leakage of internal constituents. These components include proteases that can directly damage the tissue, cytokines that activate the immune system, as well as DAMPs that contribute to prolongation of the inflammation.

Cells can die by destructive necrosis e.g. after physical damage [49] or exposure to microbial toxins [50, 51]. However, as will be discussed below, microbial toxins can also induce "alternative" NETosis (see below and **paper III**), which might be hard to distinguish from necrosis due to the fact that the membrane integrity is also disrupted during this process.

Necrosis can also occur in apoptotic cells that have not been cleared. Apoptotic cells are very sensitive to membrane disturbing factors, such as the cathelicidin LL37 and the *Staphylococcus aureus*-derived peptides PSMα, that both selectively induce necrosis in already apoptotic cells [52-54]. Some phagocytosed bacteria can also accelerate the process from apoptosis to necrosis [55, 56].

NETosis

A spectacular and violent form of neutrophil cell death, NETosis, was initially described by Brinkmann and co-workers in 2004 [57]. NETosis was then stated to be a novel defense mechanism used by neutrophils to neutralize microbes from a distance by the formation of so-called NETs. This is a process where neutrophils release their nuclear DNA, decorated with numerous intragranular proteins, to the extracellular space, where these structures can trap and kill microbes extracellularly. It is generally agreed that the formation of NETs most often leads to the death of neutrophils, but there are reports of 'vital NETosis' [58]. This will be discussed in 'Mechanisms behind NET formation' below, where NET formation is described in more detail.

During NETosis the nuclear envelope disintegrates, the nuclear content blends with granular and cytoplasmic material, and the cytoplasmic organelles disappear [59]. These features are distinct from those of necrosis where the nuclear membrane remains intact [59]. However, NETs can also be released after addition of *S. aureus*-derived cytotoxic peptides to neutrophils (**paper III**) and this suggests that the line between necrosis and NETosis is probably not as clear-cut as originally proposed.

ROS PRODUCTION BY NEUTROPHILS

Reactive oxygen species (ROS) are highly reactive oxygen-derived molecules that can interact with (and alter) a wide variety of bio-molecules. All cells produce ROS as a side product from mitochondrial respiration, but ROS can also be derived from other cellular sources, such as the NADPH oxidases (Nox1-5) [60] and the xanthine oxidase [61]. Cellular ROS participate in intracellular signaling by reversibly reacting with proteins and changing their activity [60], but too much ROS can have damaging effects on cells/tissue. Thus, a balance in ROS production is vital for normal cell function and this is promoted by the expression of antioxidants. The human body contains an array of antioxidants, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and thioredoxin [62].

A main feature of neutrophils is that they produce vast amounts of ROS through the Nox2-containing NADPH oxidase (a.k.a., the phagocyte oxidase). The ROS production in neutrophils is different from the ROS that participate in regular cytoplasmic redox reactions in that the levels are much higher, they are not produced within the cytoplasm, and the production typically occurs as a distinct burst (classically, the respiratory burst) after cellular activation. Neutrophil ROS are primarily aimed for killing microbes in the phagosome, but are increasingly also recognized to take part in other cellular processes such as intracellular signaling. As ROS can have damaging effects on tissues, neutrophil derived ROS, which are produced in large quantities, have generally been thought of as pro-inflammatory and destructive molecules. However, in recent years, evidence has appeared showing that ROS also have anti-inflammatory effects [63-66].

The NADPH oxidase

The NADPH oxidase in neutrophils is a multicomponent enzyme that catalyzes the reduction of oxygen on the non-cytosolic side of membranes by transporting electrons over the membrane from cytoplasmic NADPH. The reduced oxygen (superoxide anion; O_2^-) is formed extracellularly or within intracellular compartments (such as granules and phagosomes) as is shown in Figure 3.

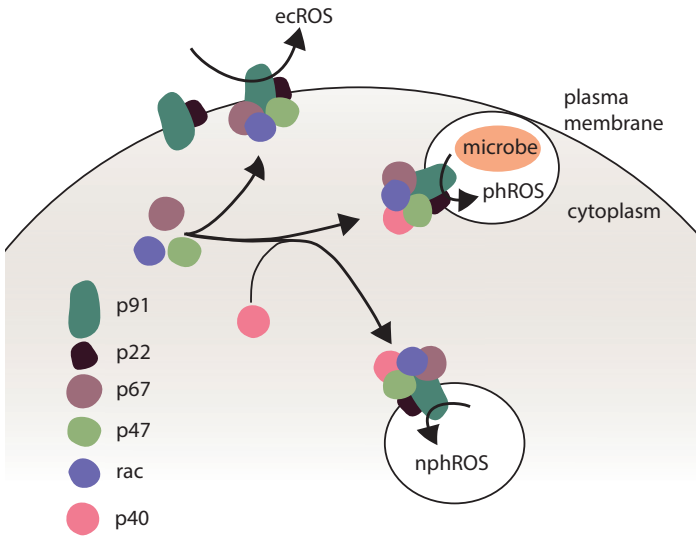

Figure 3. Different sites of ROS production in neutrophils. The NADPH oxidase mediates ROS production in neutrophils. This is a multi-component enzyme that in a resting state is distributed in the membrane and cytoplasm. Upon activation the components in the cytoplasm translocate to the membrane bound parts found in the plasma membrane, the phagosome, or in granules, leading to ecROS, phROS, and nphROS, respectively. The active enzyme transports electrons through the membrane to oxygen on the other side yielding superoxide anion and subsequently other types of ROS.

In resting cells, the different components of the NADPH oxidase are separated between membrane and cytosol. The membrane-bound part consist of $gp91^{phox}$ (Nox2) and $p22^{phox}$ that together form the flavohemoprotein cytochrome b_{558}, which is the electron-transporting element of the oxidase [67]. The subunits $p47^{phox}$, $p67^{phox}$, and the GTPase Rac are found in the cytosol and translocate to cytochrome b_{558} –containing membranes upon stimulation of the cell, forming an active enzyme [68]. The cytosolic subunit $p40^{phox}$ is special in the way that it seems to be a part of the active NADPH oxidase in intracellular membranes only, but not at the plasma membrane [64, 69, 70].

The primary product of the NADPH oxidase, superoxide anion, is a short-lived molecule that spontaneously dismutates to H_2O_2 (hydrogen peroxide), a reaction that can also be catalyzed by SOD. Superoxide and hydrogen peroxide are designated primary ROS, and these can be further processed to secondary ROS, e.g. hypochlorous acid (HOCl), by the azurophil granule enzyme MPO.

Subcellular sites of ROS production

The NADPH oxidase can assemble both in the plasma membrane and in internal membranes harboring cytochrome b_{558}. The enzyme is assembled and

activated at different subcellular sites by different stimuli and the resulting ROS likely have distinct functions depending on where they are generated.

Extracellular ROS (ecROS)

Assembly of the NADPH oxidase at the plasma membrane leads to ROS being released extracellularly (ecROS). The exact function of ecROS is unknown but they have been shown to directly damage external microbes [71] and inactivate virulence factors [72]. However, to what extent this contributes to the neutrophil microbial killing *in vivo* is unclear. ROS released extracellularly can also serve as signaling molecules to surrounding cells and they have been shown to suppress adaptive immunity against cancer [73-75] and in rheumatoid arthritis [66, 76, 77]). These immunosuppressive effects of ecROS on adaptive immune cells can therefore be both bad (inhibits cancer immunity) and good (inhibits activity of cells promoting rheumatoid arthritis). As usual in biological processes, it is likely a matter of balance, where the appropriate amount of ecROS produced may vary depending on setting and purpose.

Assembly of the NADPH oxidase at the plasma membrane occurs e.g. after activation of chemotactic G-protein coupled receptors (GPCRs) by microbial factors, such as formylated peptides [78, 79], or endogenous chemokines, such as IL8 [79]. The activation of NADPH oxidase through GPCRs leads almost exclusively to ecROS production, whereas other activators may lead to production both of ecROS and intracellular ROS (icROS).

Intracellular phagosomal ROS (phROS)

After phagocytosis of a prey, specific granules (containing cytochrome b_{558}) and azurophil granules both fuse with the phagosome to form a phagolysosome. In the phagolysosome, azurophil granule constituents, including MPO, are mixed with specific granule components. The granule membranes become part of the phagolysosomal membrane after fusion, meaning that the cytochrome b_{558} from the specific granules will be positioned in the membrane surrounding the engulfed prey and enable ROS production directly into the phagosome (phROS). In the presence of MPO, the phROS will form even more toxic microbicidal ROS, such as hypochlorous acid [17]. This process appears to be designed to optimize microbial killing while protecting the host cell by keeping these toxic substances separated from each other in the resting cell.

Intracellular non-phagosomal ROS (nphROS)

The majority of the membrane bound cytochrome b_{558} is located in the membranes of specific and gelatinase granules [20]. It is becoming increasingly clear that the NADPH oxidase can be activated in granule membranes also in the absence of phagosome formation [80]. These intracellular non-phagosomal

ROS will hereafter be referred to as nphROS. In **paper I** and **IV** they are simply called icROS and in **paper II** they are referred to as intragranular ROS.

There are several findings that support the view that the neutrophil NADPH oxidase can be assembled in non-phagosomal granule membranes. That a proportion of the oxidative burst takes place at an intracellular location inaccessible to large extracellular scavengers also when triggered by soluble stimuli has been known for a long time [81]. Further, NADPH oxidase assembly and ROS production can be induced in specific granules isolated from neutrophils [82], and ROS-producing granules have been identified by electron microscopy in intact neutrophils [83]. In further support of the view that nphROS are formed in specific and/or gelatinase granules are findings that no nphROS can be triggered in the neutrophil-like cell line HL-60 that is devoid of specific granules [84], or cytoplasts where all granules have been experimentally removed [85, 86]). Both HL-60 cells and cytoplast are fully competent to form ecROS [87, 88]. That nphROS really are derived from the NADPH oxidase is clear from the observation that neutrophils from oxidase-deficient individuals (CGD patients; see 'Deficiencies in ROS production') do not produce any ROS, including nphROS, when activated by soluble stimuli ([87] and our unpublished observations).

There are a few studies that indicate that nphROS in neutrophils could have regulatory roles on the inflammatory responses; neutrophils from patients with hyper-inflammation have been found to produce altered levels of nphROS [64, 89, 90]. There is no clear model for how nphROS in neutrophils could impact regulation of inflammation *in vivo*, but the findings that link altered levels of nphROS to inflammatory disease will be discussed further below. As for direct cellular consequences of nphROS production, **paper II** of this thesis is the first study to demonstrate that nphROS production is critical for the formation of NETs in human neutrophils. These findings will be discussed in detail below (see 'Mechanisms behind NET formation').

Production of nphROS can be induced by various soluble and particulate stimuli. Direct activation of protein kinase C with phorbol myristate acetate (PMA) or diacylglycerol, potently induces nphROS (**paper I, II**, and **IV**) as well as ecROS production [87, 91]. Galectin-1, -3, and -8 are endogenous inflammatory mediators that induce both nphROS and ecROS in a receptor-dependent manner [92-95]).

Exclusive nphROS production can also be seen when neutrophils are stimulated with Ca^{2+} ionophores [96] such as ionomycin (**paper I**). Also, crosslinking of complement receptor (CR) 3 by pansorbins [97] results in selective nphROS production. As for more physiologically relevant stimuli, pneumolysin, which is an important virulence factor of *Streptococcus pneumonia*, released during autolysis

of the bacteria, potently triggers nphROS formation [98], as does cell surface interactions with outer membrane protein A-deficient *Escherichia coli* [99].

Granule-granule fusion

One of the techniques to measure intracellular ROS (phROS as well as nphROS) is luminol-enhanced chemiluminescence (CL; see 'Measuring nphROS production in neutrophils' below and **Appendix A**). The luminol reaction with ROS is absolutely dependent on the presence of an active peroxidase. Thus, enzymatically active MPO needs to be present at the site of ROS production; neutrophils lacking MPO do not generate any intracellular CL even though they produce at least the same amount of ROS as measured by other (peroxidase-independent) methods (**Paper II** and [100, 101].

As the ROS-producing NADPH oxidase is not found in membranes of azurophil granules, whereas MPO is only found in these organelles, the question arises how the CL reaction, depending on both, can take place in the absence of phagosome formation. Clearly, the nphROS and MPO must meet intracellularly in order to produce CL signals. One possibility for such a meeting to take place would be that ROS diffuses from the specific/gelatinase granules to the azurophil granules. However, cytoplasmic scavengers would likely consume ROS before they reach the azurophil granules. Another, more plausible mechanism that would lead to colocalization of ROS and MPO is through heterotypic granule fusion, i.e., that azurophil and specific/gelatinase granules fuse with one another. Heterotypic granule fusion events are known to occur in many types of leukocytes during endocytosis, phagocytosis, and lysosomal maturation [102, 103]. Also neutrophils have the capacity to undergo compound exocytosis, which is a form of secretion where granule-granule fusion precedes exocytosis [104].

The lipid messenger arachidonic acid is formed from membrane lipids by the action of phospholipase A_2 (PLA_2) and has been shown to be involved in the production of eicosanoids [105], activation of the NADPH oxidase [106], and in membrane fusion events [107]. There are several isoforms of PLA_2 that can mediate different functions in the same neutrophil [108] and in **paper I** we show that a group of inhibitors of PLA_2 specifically block nphROS detection by luminol-amplified CL whereas the extracellular CL response is unaffected. The inhibitors did not have an effect on nphROS production *per se* (measured with a MPO-independent method) and the inhibitors did not affect the enzymatic MPO activity. These data suggest that PLA_2 is involved in the fusion of specific/gelatinase granules and azurophil granules when nphROS are produced.

Deficiencies in neutrophil ROS production

One way to understand the function of a biological process or a molecule of interest is to study cells and/or individuals that are deficient in the entity. With regards to the NADPH oxidase, patients with a total deficiency or partial defect in enzyme activity have been of outmost importance for our understanding of the involvement of phagocyte ROS in antimicrobial killing and inflammatory processes.

Chronic granulomatous disease

The importance of the phagocyte NADPH oxidase in microbial defense is seen most clearly in individuals that have defects in the proteins that make up the oxidase; mutations in genes that encode the NADPH oxidase components lead to a rare condition called chronic granulomatous disease (CGD). Patients with CGD are highly susceptible to infections from particular types of fungi as well as bacteria that are resistant to non-oxidative killing [63, 109]. However, CGD patients are not only susceptible to infections but also suffer from inflammatory conditions [109]. The fact that CGD phagocytes are hyperinflammatory *in vitro* [110-112] suggests that NADPH oxidase-produced ROS also are involved in regulation of the immune response.

In the majority of CGD patients the component affected is $gp91^{phox}$, which is encoded by the *CYBB* gene located on the X chromosome (thus also called X-linked CGD). Consequently, predominantly male patients suffer from this ROS deficiency [109, 113]. Since $gp91^{phox}$ is the electron transfer component of the NADPH oxidase and thus vital for the ROS producing capacity of the enzyme, mutation in *CYBB* will give the most pronounced symptoms, both with regards to susceptibility to infections and hyperinflammation [109].

Mutations in the *NCF2* and *NCF1* genes that encode $p67^{phox}$ and $p47^{phox}$, respectively, also lead to CGD. Although these patients suffer from a serious disease their symptoms are often not as pronounced as in $gp91^{phox}$ CGD patients [114]. This is possibly explained by residual amounts of ROS that are formed even in the absence of $p67^{phox}$ or $p47^{phox}$ [114, 115].

The $p40^{phox}$ subunit translocates specifically to cytochrome b_{558} in intracellular membranes (Figure 3) and is dispensable for ecROS production [64]. Murine studies indicate that $p40^{phox}$ is important for ROS production in the phagosome and $p40^{phox}$ knockout mice are more susceptible to bacterial infections [69, 70]. Only one patient with a mutated $p40^{phox}$ has so far been described; this patient's neutrophils showed impairment in killing *S. aureus* but the major clinical manifestation was chronic inflammation of the gastrointestinal tract (and not increased susceptibility to infection) [64]. Interestingly, polymorphisms in the gene coding for $p40^{phox}$, *NCF4*, have been associated with Crohn's disease, a

chronic inflammatory gastrointestinal disease [116]. These data suggest that intracellular ROS in neutrophils might be of importance in controlling inflammatory responses.

nphROS in autoinflammatory disease

Apart from the one patient described with a $p40^{phox}$ deficiency, where neutrophils display normal generation of ecROS, but defective generation of intracellular ROS [64], there are other studies indicating a role for nphROS in controlling the inflammatory response. Neutrophils from a patient with the autoinflammatory syndrome SAPHO (synovitis, acne, pustulosis, hyperostosis, osteitis; presenting with sterile bone inflammation accompanied by dermatological complications, such as severe acne, palmoplantar pustulosis, or psoriasis [117]), was found to produce aberrantly low levels of nphROS whereas ecROS was intact [89]. This finding inspired the authors to suggest that deficient nphROS production is associated with the apparently dysregulated inflammation seen in patients with SAPHO syndrome. The findings of this publication were followed up in **paper IV** where neutrophils from four patients with SAPHO syndrome were studied. However, in contrast to the paper by Ferguson et al, neutrophils from all of the studied patients produced normal amounts of nphROS (as well as ecROS). Two of the patients were examined both when the disease was active and in remission, and the nphROS response was actually higher during the inflammatory phase. Hence, our data show that decreased nphROS production in neutrophils is not a general feature of SAPHO syndrome, but indicate that levels of nphROS may vary during different phases of disease .

The increase in neutrophil nphROS production associated with inflammatory flares in SAPHO syndrome (**paper IV**) is well in line with an earlier finding in another autoinflammatory disease, PFAPA (periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis). In this pediatric periodic fever syndrome, the neutrophil phenotype varies from being normal with regard to nphROS production in afebrile periods to being increased during fever flares [90].

There are thus reports associating elevated inflammation with decreased [64, 89], as well as increased ([90] and **paper IV**) nphROS production. It is hard to explain how both too low and too high nphROS production can contribute to the same outcome. The perhaps obvious explanation is that balance is of great importance; both too high and too low amounts of nphROS may result in disturbed intracellular signaling and thereby inducing cellular imbalance, leading to hyperinflammation.

As will be described below, measuring of nphROS requires close attention to methodological detail, and ROS production at different cellular sites is not typically distinguished in standard clinical immunology laboratories. It is

possible that aberrant generation of nphROS is under-diagnosed and may be an important parameter also in other diseases.

MPO deficiency

Lack of MPO function, i.e., MPO deficiency, is not a deficiency in ROS production *per se*, but alters the processing of ROS and thus leads to a deficiency in certain secondary ROS molecules. The first reports of MPO deficiency, in the 1970s, indicated that the defect led to increased susceptibility to fungal infections [17, 118]. With more sensitive detection techniques it became clear that (at least partial) MPO deficiencies are quite common, ranging from one in 2000 in North America [119] to one in 57000 in Japan [120], and that affected individuals are generally healthy and surprisingly do not display increased susceptibility to infections [17, 121].

Neutrophils from MPO-deficient individuals show reduced microbial killing *in vitro* against certain pathogens, but also seem to have enhanced alternative antimicrobial systems such as increased capacity to produce nitric oxide [122], possibly as an adaptation to the lack of proper ROS processing [17]. The enzymatic activity of MPO is not only involved in phagosomal killing of microbes but also participates in the induction of NETosis (**paper II**). The ability to form NETs has earlier been shown to depend on MPO but by examining several MPO deficient individuals it was found that low residual peroxidase activity, which is found in partially MPO-deficient individuals, is enough for cells to undergo NETosis to a certain degree [123]. In contrast, neutrophils from completely MPO-deficient individuals do not undergo NETosis (**paper II**; [123]). The concentration of MPO within azurophil granules is very high and it is possible that residual MPO activity is enough to ensure normal neutrophil function.

The observation that MPO deficiencies do not give rise to more distinct (immunosuppressive) phenotypes is puzzling. It has been speculated that other, oxygen-independent, microbicidal actions can make up for defective ROS processing, and also that modern man is less exposed to major pathogens due to better sanitation as well as increased access to antibiotics. According to this view, MPO might have been more important in the past, or is in less developed parts of the world [17].

Measuring nphROS production in neutrophils

Measuring nphROS in neutrophils requires careful methodological considerations [124]. ROS are short-lived and reactive molecules that can spontaneously transform from one type of ROS to another, processes that may

be affected by enzymatic antioxidants. They may of course also interact with other molecules in the cell, forming additionally complex radicals [125].

Cell permeable probes need to be used to directly detect nphROS. One such probe is the aforementioned luminol that upon reaction with superoxide anion in the presence of a peroxidase is excited and, when returning to ground state, emits light that can be measured in a luminometer. Extracellular and intracellular ROS are both detected with luminol, but with the addition of scavengers that remove all extracellular ROS, the nphROS can be measured specifically ([126]; **paper I, II, IV**).

Flow cytometry probes have gained popularity in the recent years. These probes (e.g. DCHF and DHR) have in common that they diffuse into cells and upon oxidation by ROS become fluorescent. There are some drawbacks in using these probes; the technique is not directly quantitative and cannot be used to observe the kinetics of a response, it is often not known exactly which ROS the probes react with, and the uptake of the probe might be a limiting factor [124].

The production of nphROS can also be measured indirectly by the cell impermeable molecule p-hydroxyphenyl acetic acid (PHPA) that upon reaction with H_2O_2 in the presence of a peroxidase becomes fluorescent. The NADPH oxidase produces O_2^- that spontaneously dismutates to H_2O_2 and can be measured by PHPA extracellularly. Intracellular H_2O_2 can traverse membranes and travel from intracellular locations to the extracellular space. However, before reaching the extracellular space, such ROS are normally consumed by endogenous antioxidants (e.g., MPO and/or cytoplasmic catalase). Thus, if these scavengers are inhibited (e.g. by azide) the intracellularly produced H_2O_2 can leak out of the cell and be detected extracellularly. The intracellular H_2O_2 production can then be calculated as the difference between the PHPA response without and with azide (**paper I** and **II**).

These methods are described in more detail in the methodological paper found in **Appendix A** of this thesis.

ANTIMICROBIAL ACTIONS OF NEUTROPHILS

Neutrophils have many antimicrobial tools but the most pronounced killing occurs through the engulfment and intra-phagosomal destruction of the microbes (phagocytosis). Another antimicrobial mechanism is the ensnaring of microbes in NETs, which is the topic of **papers II** and **III** of this thesis. Both these killing mechanisms are described further below. Other defense mechanisms have been described, such as extracellular inactivation of microbial factors by ROS [72] and secretion of microbicidal ectosomes (cell-derived microvesicles) [127]. However, it is unknown to what extent these contribute to the antimicrobial effect of neutrophils and these will not be discussed further here.

Phagocytosis

Phagocytosis, first described late in the 19th century by Metchnikov [128, 129], is a process whereby foreign particles, such as microbes, are taken up by cells. From the perspective of evolution, this process was likely first used as a mechanism for intake of nutrients in unicellular organisms, but in higher organisms the process is of importance for tissue homeostasis and remodeling including the use by specialized phagocytes to engulf invading pathogens [130]. Specialized phagocytosing cells are found in many multicellular organisms. In humans, neutrophils along with macrophages, mast cells, and dendritic cells are professional phagocytes that through an active process recognize and engulf microbes (Figure 4). These cells have surface receptors that recognize microbes directly, or indirectly when the microbes are opsonized with antibodies or complement factors [129, 131].

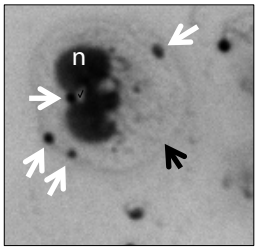

Figure 4. Phagocytosis. Micrograph of a neutrophil phagocytosing bacteria. Nucleus, n; neutrophil membrane, black arrow; bacteria, white arrows.

After recognition, the microbe is engulfed into a phagosome that subsequently fuses with azurophil and specific granules, creating a phagolysosome. The fusion of azurophil granules leads to a release of defensins, proteases and other enzymes such as MPO into the phagolysosome. Specific granules also fuse with the phagosome and provide the cytochrome b_{558} component of the NADPH

oxidase. The combined actions of toxic ROS and anti-microbial peptides and proteins ensures efficient killing of the engulfed prey [17, 125, 132].

Neutrophil extracellular traps (NETs)

Neutrophils can in a somewhat peculiar way neutralize microbes also extracellularly, through the formation of NETs. These are cobweb-like structures of DNA (Figure 5) clad with intracellular proteins that neutrophils discharge extracellularly in a process first described roughly a decade ago by Brinkmann and co-workers [57].

The backbone of NETs is nuclear DNA and the intracellular proteins that attach to the backbone are mainly derived from granules, but a few nuclear and cytosolic proteins can also be found in NETs [133]. The NETs can capture bacteria (**paper III** and [57], fungi (**paper III** and [134]), parasites [135], and viruses [136], and are suggested to help preventing dissemination of infection. Furthermore, the NETs contain proteases as well as antimicrobial proteins and peptides, proposed to directly kill the microbes that are entangled in the NETs [133, 135]. There is, however, skepticism about whether NETs actually kill microbes as opposed to just trapping them to prevent spreading [137].

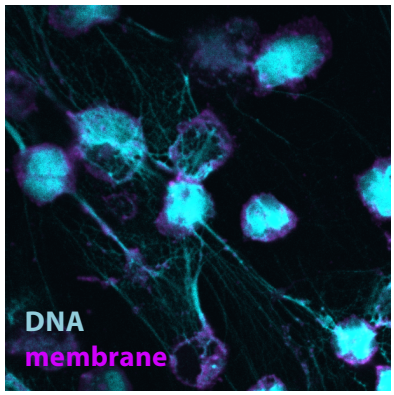

Figure 5. Neutrophil extracellular traps. Neutrophils that have undergone NETosis. NET structures can be seen by staining the DNA (cyan) and the neutrophil membrane is seen in purple.

Most reports of microbial killing by NETs have utilized standardized viable count methodologies, but just counting the colony forming units of trapped bacteria risk to be confounded as the NETs induce aggregation of microbes, which could lead to an underestimation of viable bacteria. To circumvent this problem, we instead measured the ability of NETs to inhibit growth of *S. aureus* and the fungus *Candida albicans* by metabolic activity (**paper III)**. The data showed that *C. albicans* growth was indeed inhibited by NETs (see also [133]) while *S. aureus* growth was not. The microbicidal effect of NETs might well depend on the type of microbe that is attacked; the lack of killing of *S. aureus* is likely due to secretion of DNase, which is a prominent feature of these bacteria [138]. It has long been known that many bacteria secrete nucleases [139, 140], but the benefit of such secretion has not been fully understood. One advantage for bacterial virulence could of course be to degrade and escape from the NETs, and this has been shown to occur for DNase-secreting bacteria ([141, 142], and

paper III). The expression of DNase might therefore have evolved in the bacteria as an evasion strategy against NET-induced microbial clearance.

A substantial amount of research has been performed on NETs *in vitro* but investigating the phenomenon *in vivo* has proven more difficult. Using animal models, it has been shown that NETs can be observed *in vivo* [143, 144] and that they have beneficial effects for the host during infection [145, 146]. It is harder to study *in vivo* NET formation in humans, but microscopical data exist showing NET formation in human tissues [58, 147, 148].

MECHANISMS BEHIND NET FORMATION

The formation of NETs was originally described as a cell death process, NETosis [149], which differs from apoptosis and necrosis by that DNA is actively released extracellularly [59]. Microorganisms, cytokines, and other factors have been shown to induce NET formation [150] but activation of protein kinase C (PKC) by PMA is the most commonly used stimulation in experimental studies of NET formation (**paper II** and [59, 134, 143, 151, 152]). Typically, NET formation (e.g., induced by PKC activation) is preceded by a coordinated series of cellular events, a process that in this text will be referred to as active NETosis (Figure 6). However, NETs can also be the result of a non-typical process, e.g., after stimulation of neutrophils with bacterial toxins or activated platelets (Figure 6). This is referred to as alternative NET formation below.

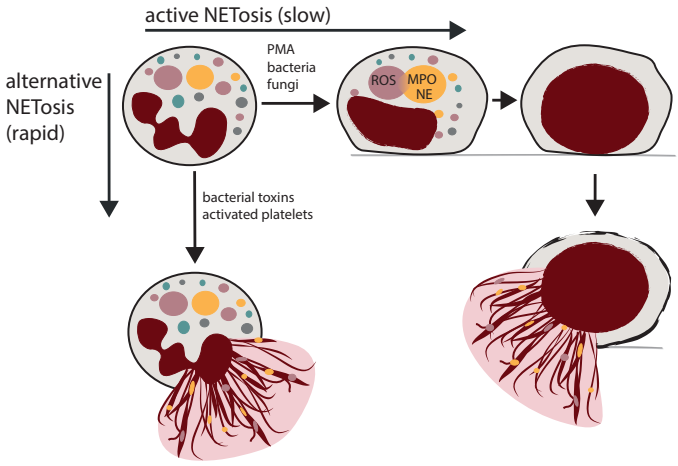

Figure 6. Active and alternative NETosis. Neutrophils can be induced to active NETosis by e.g. PMA, bacteria, and fungi, in a slow process (takes hours). First after stimulation, the cells are activated to produce ROS and attach to a surface. Granule contents translocate to the nucleus that decondenses before the membrane ruptures and DNA covered in proteins is released. Alternative NETosis is a much more rapid process (takes minutes) that can be induced by e.g. bacterial toxins and activated platelets. This process is not as well described but the nuclei are not decondensed, at least not when induced by bacterial toxins, before the NETs are released.

Active NETosis

The process of active NETosis has been described to occur after stimulation of neutrophils with PMA [59], bacteria [59], and fungi [134]. After stimulation, the cells undergo specific morphological changes that are dependent on various cellular processes and/or components.

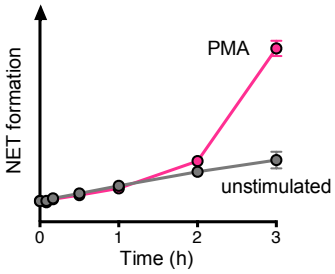

Figure 7. PMA-induced NET formation. Neutrophils can be induced to form NETs with PMA. The extracellular DNA is measured and it can be seen that the process takes several hours and NETs cannot be detected until after 2 h incubation.

Morphological changes during active NETosis

During active NETosis, the neutrophil rapidly attaches to a surface, taking on a flattened morphology and forming intracellular vacuoles [59, 153]. During the next hour the cell's nucleus loses its lobular appearance and expands as the chromatin decondenses to fill up a larger space in the cell [59]. The nuclear membrane subsequently disintegrates and the granules disappear, resulting in contact of chromatin with cytoplasmic and granular components. Finally, the cell membrane ruptures and the NETs are released [59]. This process typically takes several hours; after PMA stimulation, NETs can first be observed after around 2h (Figure 7). The exact intracellular mechanisms that lead to NET formation are not defined but several components are known to be needed for the process to occur.

ROS and MPO as a basis for NET formation

After activation by PMA or microbes, neutrophils start to produce vast amounts of ROS (**paper I**, **II**, and **IV**), and ROS are known to be essential in order for neutrophils to form NETs. This has been shown by that neutrophils from patients with CGD do not undergo active NETosis *in vitro* [59, 154] and our unpublished observations), a finding that has also been confirmed in knockout animal models [155, 156]. Active NETosis can also be inhibited by DPI, a potent inhibitor of the NADPH oxidase (**paper II** and [59, 151]).

As discussed above, neutrophils can produce ROS at multiple cellular sites, and although it has been long established that ROS in general are required for active NETosis, **paper II** is the first study to investigate where the NETosis-triggering ROS production takes place. We found that ecROS were only of minor importance for NET formation but that nphROS were absolutely essential.

As described, nphROS are processed by MPO (otherwise they would not be detected by luminol-enhanced CL) and we found that such intracellular ROS

processing is critical for active NETosis (**paper II**). Earlier studies have indicated that MPO plays a role in active NETosis; neutrophils from individuals with a complete MPO deficiency did not undergo active NETosis [123]. It has been suggested that the mere presence of MPO protein, but not its enzymatic activity, is needed for the process [143], even though MPO activity was shown to ensure the translocation of active NE into the nucleus where it degrades histones resulting in chromatin decondensation [157]. One reason for this somewhat confused view of the role of MPO for active NETosis is the relative inefficiency of pharmacological MPO inhibitors to inhibit NET formation [151, 158, 159].

The participation of MPO in active NETosis was the subject of **paper II** in this thesis. We show that the enzymatic activity of MPO is indeed crucial for PMA-induced NET formation and that the efficiency of MPO inhibitors to inhibit NETosis correlates with the inhibitory concentrations needed to neutralize MPO activity inside granules. In further support for an intracellular action of MPO, extracellular addition of purified MPO to completely MPO-deficient neutrophils did not restore NET formation from these cells. We finally used luminol, the cell-permeable CL substrate used for measuring ROS (described above and in **Appendix A**), as a means to specifically neutralize MPO-processed nphROS and found that it was able to completely block NET formation. In fact, inhibition was as potent as that seen when using DPI to abolish all NADPH oxidase activity. Our data on pharmacological inhibition of MPO activity are in line with results from MPO deficient individuals, where only cells that completely lack MPO fail to form NETs whereas partial deficiency leads to NET formation, although to a lesser extent [123]. It should be noted that MPO is one of the most abundant proteins in neutrophils, constituting 5% of neutrophil dry weight [160], and therefore it is not surprising that supremely potent inhibitors are needed to block its activity inside granules. Also, inhibitors must be cell permeable to reach the intracellular site where MPO is active.

Other suggested components involved in NET formation

The early morphological events leading to NETosis include decondensation of chromatin. This decondensation is believed to be mediated through the enzymatic activity of peptidylarginine deiminase 4 (PAD4) and NE. PAD4 is a nuclear enzyme that modifies histones by citrullination, which then in turn leads to decondensation of the chromatin [161]. The serine protease NE has been shown to translocate from the azurophil granules to the nucleus after PMA stimulation and ROS production [59, 143]. Neutrophils lacking enzymatically active NE (e.g., those from patients with Papillon-Lefèvre syndrome that lack active serine proteases) fail to form NETs in response to PMA [162], indicating that protease activity is indeed crucial for active NETosis.

Alternative processes leading to NET formation

There are a few reports on the formation of NETs where the results do not fit with the previously described mechanism. These have in common that the process is much more rapid – the NETs are observed within minutes instead of hours (Figure 6).

The first example of rapid NET formation is when neutrophils were exposed to platelets activated with lipopolysaccharide (LPS). This stimulation induced NET formation within 5 min, whereas platelets or LPS alone did not have any effect [163]. This was proposed to be an effect that only occurs under extreme conditions such as during sepsis where the NETs capture bacteria in the narrowest vessels but at the same time cause damage to the surrounding tissue [163].

Secondly, *S. aureus* bacteria and toxins secreted by *S. aureus* induce rapid NET formation in a process that apparently does not lead to death of the neutrophils [58, 164]. This vital NETosis was claimed to involve budding off of vesicles filled with DNA that were suggested to capture and kill microbes while the anuclear neutrophils could continue to crawl and perform their duties afterwards [58, 164]. Rapid NET formation was not dependent on ROS production [164], but another study showed that a different type of toxin, derived from *Mannheimia heamolytica* induced rapid NET formation in bovine neutrophils that was dependent on ROS production [165].

Yet another study showed that the *S. aureus* leukotoxin GH caused non-specific neutrophil damage and cell death that resulted in NET formation [166]. In **paper III** of this thesis we report that yet another type of bacterial toxin, the phenol soluble modulin (PSM) α, peptides secreted by highly pathogenic and antibiotic resistant strains of *S. aureus* also induce violent cell death at high concentrations. This cell death was very rapid and resulted in the formation of widespread NETs in a manner independent of ROS and MPO. Although the NETs were formed very rapidly they contained the common markers of NETs, such as DNA, histone, NE, and MPO (**paper III**) but the nuclear morphology was not decondensed as occurs during active NETosis. The PSMα peptides have previously been found to cause membrane disturbance at the same concentration range that we used to induce NETs [167], suggesting that membrane perturbation may be the mechanism behind this alternative NET formation.

The dark side of NET formation

As for other inflammatory processes the balance of the response is of utmost importance, and though many claim NET formation to be of significance for

fighting infections, the (excessive) formation of NETs has been linked to various diseases that are of non-infectious origin.

Autoimmune diseases

During NET formation, proteins as well as DNA that are normally kept inside cells get exposed extracellularly which can lead to the formation of autoantibodies. There are several known autoantibodies linked to autoimmune diseases that are directed towards neutrophil proteins. These include antibodies against MPO and proteinase-3, and are often found in patients with vasculitis and thought to be directly involved in disease pathogenesis [147]. Patients with the autoimmune disease systemic lupus erythematosus (SLE) often have autoantibodies against DNA, histones, and/or neutrophil proteins [168, 169]. Extracellular DNA would be degraded *in vivo* by DNase-1 and a subset of SLE patients has been found to degrade NETs less efficiently than healthy persons due to the presence of autoantibodies against DNase-1 [170]. Similarly, SLE patients with high titers of autoantibodies against NET epitopes had impaired ability to degrade NETs [171]. Impairment in NET degradation has also been linked with a higher risk of developing nephritis in SLE patients [170].

Intracellular antigens can also be exposed extracellularly, e.g. during necrosis or conventional degranulation, thus, NETs are not the only instance at which these antigens could be exposed. However, the formation of NETs could potentially be a platform for recurrent exposure of these antigens that leads to immune reactivity. Flares in autoimmune diseases are often associated with infections that might lead to NET formation with subsequent exposure of intracellular antigens extracellularly [172] [173].

Other diseases

Apart from autoimmune diseases NETs have been found in a number of other pathological conditions. These include thrombus formation, as NETs stimulate and act as a scaffold for the formation of thrombi [174, 175]. NETs have also been reported in the sputum of cystic fibrosis patients [176] where they would contribute to the increased viscosity of the sputum that decreases lung function in these patients [177]. Additionally, NETs have been shown to be involved in cancer progression by promoting metastasis [178].

The list of diseases where NETs have been observed is constantly expanding but it is hard to imagine that NETs would have considerable pathological effects in all these different diseases. Whether NETs actively contribute to pathology or simply are found in the tissue as bystanders of inflammation remains to be thoroughly investigated. Further understanding of their roles in diseases could be of clinical importance where targeting NETs therapeutically might prove useful.

Roles of NET formation *in vivo*

NETs have been shown to capture and kill microbes *in vitro* and data from various animal models support the view that NET formation is an important defense mechanism in humans. However, individuals whose neutrophils lack MPO or NE, making the cells unable to undergo active NETosis *in vitro* have also been defined (**paper II**, [123, 162]). Surprisingly, these individuals do not suffer from an increased susceptibility to infections, which contrarily would suggest that NET formation is not a crucial defense mechanism in humans [179].

However, the experiments that were done to test the capacity of NET formation in the MPO- and NE-deficient neutrophils included only stimuli that induce active NETosis [123, 162] and as discussed above (and in **paper III)**, there are alternative mechanisms that also lead to the formation of NETs. In **paper III** we show that NETs that are formed in response to the cytotoxic PSMα peptides, are not dependent on ROS or MPO. This suggests that cells deficient in these components may in fact still form NETs in response to certain stimuli, i.e., alternative NETosis may make up for an inability to undergo active NETosis.

The outcome of both active and alternative NETosis is the catapulting of DNA together with attached proteins to the extracellular space. How the catapulting is carried out is unknown and even though extracellular DNA extrusions have been described also for other types of leukocytes [180-182], it is a phenomenon mostly ascribed to neutrophils. Other cell types, e.g., a melanoma cell line, were also killed by PSMα peptides, but death was not associated with the formation of widespread DNA structures outside of the dead cells (**paper III**). It thus seems as if neutrophils have specific features that make the catapulting of DNA feasible. One characteristic that distinguishes granulocytes from other cells is their nuclear morphology. The nucleus is segmented into 3-4 lobes and has been shown to be very malleable, a feature that is thought to be of benefit during transmigration [183]. These characteristics might also make it easy for neutrophils to transfer their nuclei to the exterior of the cell and this might occur via different processes, such as active and alternative NETosis.

For many biological processes there are often redundant mechanisms that can make up for the loss of one particular system. The formation of NETs might well be an antimicrobial defense system, but adequate phagosomal killing could perhaps compensate in such a way that "NET-deficient" individuals with deficiencies in MPO or NE do not become overly susceptible to infections. Interestingly, both MPO and NE are thought to actively participate in the killing of microbes inside phagosomes too. Clearly, our view of how neutrophils actually kill microbes, inside phagosomes or through NET formation, is still not completely understood.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It has been known for long that neutrophils are phagocytes of main importance for the killing of invading microbes and that the remarkable amounts of ROS that they are able to produce by activation of the NADPH oxidase are central for this killing. Additionally, although the knowledge that the granule protein MPO can catalyze a variety of reaction that include ROS is fairly old, it is not clear to what extent MPO-processing of phagosomal ROS contributes to microbial elimination. The observation that individuals deficient in MPO are not notably susceptible to infections, at least not nearly as immunocompromised as CGD patients that cannot produce ROS at all, indicates that our understanding of how (and which) ROS really kill microbes is incomplete.

It has gradually become apparent that neutrophil ROS can impact human health not only when produced in the phagosome aimed at microbial killing, but also in other situations and locations that suggest other functions. For instance, neutrophil ROS released extracellularly can inactivate other immune cells that upon situation can be harmful, facilitating cancer progression, or useful, keeping (overly active) immune cells inactive. Furthermore, neutrophils can produce ROS intracellularly, inside non-phagosomal granules, a phenomenon that has not previously been studied in great detail. The nphROS are processed by MPO and these two entities presumably meet intracellularly through granule-granule fusion. In **paper I** we show that PLA_2 is indirectly involved in the processing of ROS by facilitating heterotypic fusion of granules, allowing the ROS and MPO to colocalize within the neutrophil upon stimulation. The exact nature of the intracellular ROS-producing granules awaits further characterization. As for the function of nphROS, there may be several but they are clearly essential for active NETosis (**paper II**). That neutrophils have additional means to neutralize microbes (apart from phagocytosis), and that killing may occur extracellularly is a rather new knowledge. It is, however, not entirely clear how important NETs are to fulfill proper immune defenses. The processing of nphROS by MPO inside granules is an essential factor in the process of active NETosis (**paper II**). Exactly how MPO-processed nphROS drive active NETosis is an interesting venue for future investigations. The tools that were discovered to block active NETosis in **paper II** could potentially be of use for such studies aiming to clarify this issue.

Whereas ROS and MPO are clearly indispensible for active NETosis, we describe in **paper III** an alternative process leading to NET formation that is independent of both ROS and MPO. This type of NET formation results from membrane disturbing peptides derived from bacteria, and although the mechanism behind this process differs from active NETosis the outcome is the same; widespread DNA fibers, covered in intracellular proteins and capable of trapping microbes, are released extracellularly. The *in vivo* effect of such alternative NET formation is unknown but presumably this type of NETosis can occur in parallel to active NETosis, and perhaps be of increasing importance in individuals with neutrophils that fail to undergo active NETosis.

Neutrophils have for long been described as brainless soldiers of the immune system taking care of "simple" tasks without having any regulatory effects on inflammation. It is however becoming increasingly clear that neutrophil dysregulation can have dramatic effects, leading not only to increased susceptibility to infections but also to inflammatory complications. Although we could not confirm previous findings that ROS formation is aberrant in patients suffering from the autoinflammatory SAPHO disease (**paper IV**), we could show that ROS production is enhanced during inflammatory flares, possibly as part of the disease regulation mechanisms. There are many examples showing that neutrophil ROS production may be involved in regulation of inflammatory processes *in vitro,* but if and how dysregulated ROS production directly contributes to inflammatory disease is still unknown.

Despite the fact that phagocytes such as neutrophils have been known since the days of Metchnikov, and that a lot of research has been performed on these cells since then, we still have a lot to learn about their roles in health and disease.

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