Storage organelles that are distinct from the classical granules in human neutrophils

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Scanning electron micrograph of human neutrophils.
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STORAGE ORGANELLES THAT ARE DISTINCT FROM THE CLASSICAL GRANULES IN HUMAN NEUTROPHILS

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ABSTRACT

The human neutrophil is a crucial participant in acute inflammation. Appropriate immune response is dependent on rapid direction of phagocytic cells through the tissue, towards the inflammatory focus. Such movement is governed by chemoattractants, e.g., interleukin-8 (IL-8/CXCL-8). Circulating neutrophils are packed with granules that contain effector molecules used for different cell activities. Upon activation, the neutrophil mobilizes the granules to the plasma membrane and the forming phagolysosome, thereby exposing new receptors and releasing substances to the extracellular milieu or into the phagolysosome. The classical neutrophil granules are well-defined and one of them, the secretory vesicle, has been suggested to contain the CXCL-8 of resting neutrophils. However, using fractionation techniques and immunogold labeling, we show that neutrophils store CXCL-8 in an organelle distinct from the granules and secretory vesicles. In neutrophil cytoplasts, we found partial colocalization of CXCL-8 and calnexin, a marker for the endoplasmic reticulum (ER), suggesting that a proportion of CXCL-8 is localized to the ER or ER-like structures in the neutrophil.

The identification of specific markers for individual subcellular compartments is crucial to neutrophil research. HLA class I (HLA-I) has been proposed as an ideal marker for the plasma membrane, much due to the fact that it is un-influenced by stimulation. By the use of detailed fractionation protocols, we found that HLA-I not only colocalizes with the plasma membrane but is also present in other organelles of slightly higher densities. Moreover, the mixed enzyme-linked immunosorbent assay (MELISA), used to detect the β_2-microglobulin (β_2m)/HLA-I complex, proved to be negatively affected by uncomplexed β_2m, making it difficult to use HLA-I as a marker during, for example, phagolysosome formation.

The involvement of the ER in macrophage phagocytosis is a matter of debate. The classical dogma that mature neutrophils are poor producers of protein and that they contain ER of very limited amounts has essentially precluded these cells from the discussion. However, neutrophils do produce proteins, such as CXCL-8, upon stimulation, suggesting a functional ER in these cells. We studied calnexin and CXCL-8 in the context of phagocytosis, using the promyelocytic cell line HL-60, known to carry out phagocytosis in much the same way as neutrophils do. CXCL-8 and calnexin did not colocalize during phagocytosis, and calnexin was not detected on the phagosomal membrane. We conclude that phagocytosis does not involve ER fusion in HL-60 cells and neutrophils, and that these cells differ from macrophages in this respect.

Key words: neutrophil, HL-60 cells, cytoplasts, granules, CXCL-8, ER, phagocytosis, HLA-I, subcellular fractionation, immunogold

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PREFACE

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Pellmé S, Mörgelin M, Tapper H, Mellqvist U-H, Dahlgren C and Karlsson A. Localization of human neutrophil interleukin-8 (CXCL-8) to organelle(s) distinct from the classical granules and secretory vesicles
Journal of Leukocyte Biology (2006); 79:564-73

II. Pellmé S, Dahlgren C and Karlsson A. The two neutrophil plasma membrane markers alkaline phosphatase and HLA Class I antigen do not co-localize completely in granule deficient cytoplasts. An ideal plasma membrane marker in human neutrophils is still lacking.
Submitted for publication

III. Pellmé S, Nordenfelt P, Lönnbro P, Johansson V, Dahlgren C, Karlsson A and Tapper H. Phagosomes form and mature without involvement of the endoplasmic reticulum in neutrophil-like HL-60 cells
In manuscript

Till Agnes och Siri

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ABBREVIATIONS

AML	acute myeloid leukemia
ALP	alkaline phosphatase
β_2m	β_2-microglobulin
BPI	bactericidal/permeability-increasing protein
CFU	colony forming unit
CR	complement receptor
CXCL-8	CXC chemokine ligand 8 (IL-8)
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
fMLF	*N*-formyl-methionyl-leucyl-phenylalanine
FPR	formyl peptide receptor
FPRL1	formyl peptide receptor-like 1
HBP	heparin-binding protein
HLA	human leukocyte antigen
ICAM-1	integrin cell adhesion molecule-1
IF	immunofluorescence
IL	interleukin
IP	inositol phosphate
LAMP	lysosome-associated membrane glycoprotein
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MELISA	mixed enzyme-linked immunosorbent assay
MLC	multilaminar compartment
MPO	myeloperoxidase
MVB	multivesicular body
NADPH	nicotinamide adenine dinucleotide phosphate
NGAL	neutrophil gelatinase-associated lipocalin
PAF	platelet activating factor
PCAM-1	platelet endothelial cell adhesion molecule-1
PLC	phospholipase C
PSGL-1	P-selectin glycoprotein ligand -1
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
Vit. B_{12} b.p.	vitamin B_{12} binding protein

INTRODUCTION

Neutrophils are the most abundant of the leukocytes in human blood. These cells are crucial participants in the innate immune reaction as they are readily directed to sites of infection, where they display an impressive battery of actions in order to combat an intruder during the early stages of inflammation. The most important tools in the finely tuned process of neutrophil activation are the intracellular granules, with which the cells are packed. The granule types differ in morphology, content, and mobilizability and are used in a strict order during the various stages of neutrophil activation, starting with rolling and adherence of the cells to the blood vessel wall and ending with engulfment of the intruder into a phagosome for final degradation.

Unlike the blood cells of the adaptive immune system, the neutrophils do not rely on gradual maturation in order to perform their task during the inflammatory process. Instead, the prevailing view is that the neutrophil is a fairly static cell type, leaving the bone marrow after myelopoiesis, already equipped with the required proteins and peptides, receptors and effector molecules, and stored in the preformed granules and vesicles. In accordance with this scheme, the endoplasmic reticulum (ER) and Golgi apparatus are not major structures of the mature, circulating neutrophil. However, over the past decade, it has become evident that neutrophils are capable of *de novo* protein synthesis, and as the ER has emerged as a potential participant in the process of phagocytosis, it has become a subcellular structure of considerable interest in neutrophil research.

This thesis will focus on the subcellular compartments of the neutrophil, with emphasis on structures that are distinct from the classical granules and the secretory vesicles.

THE NEUTROPHIL

Direction of neutrophils by chemoattractants

The recruitment of neutrophils to inflammatory foci is of fundamental importance in the response of the body to infection and acute inflammation. This is a rapid process that is initiated and directed by the exposure of the cells to a biochemical gradient of a chemoattractant, towards which the leukocytes migrate. Chemoattractants include products of the complement cascade (e.g., C5a), bacterial substances, such as formylated peptides (e.g., *N*-formyl-methionyl-leucyl-phenylalanine (fMLF)), an array of cytokines, which are commonly referred to as chemokines (e.g., IL-8/CXCL-8), and lipid metabolites, such as platelet activating factor (PAF) and leukotriene B_4 (LTB_4).

Not only do these chemoattractants orchestrate cellular movement, but they can also activate the neutrophil NADPH-oxidase, leading to oxygen radical production. Furthermore, chemoattractants (and other proinflammatory molecules, such as tumor necrosis factor-α (TNF-α)[1] and bacterial lipopolysaccharide (LPS)) induce granule mobilization [2, 3], exposing intracellularly stored membrane molecules on the cell surface. *In vivo*, the direction of the neutrophil from the bloodstream out into the inflamed tissue is an exceptionally complex process, relying on a finely tuned interplay between the leukocyte and its surroundings. Primarily, there is a shift in neutrophil morphology, in that as the cell becomes polarized, its leading edge points in the direction of the highest concentration of the chemoattractant. Movement of the phagocyte is then propagated by actin polymerization at the leading edge of the cell, concomitant with depolymerization at the opposite end of the cell [4].

The intricate process of diapedesis, during which the neutrophil traverses the endothelial cell layer of the blood vessel, is dependent upon concomitant activation of the endothelial cells. Cytokines and complement factors induce the endothelium to upregulate rapidly its surface expression of P-selectin, which in turn interacts with carbohydrate structures (P-selectin glycoprotein ligand -1, PSGL-1, and L-selectin) on the plasma membrane of the circulating neutrophil [5-7]. This low-affinity interaction between the two cell types leads to rolling of the neutrophil along the vessel wall. At this stage, the neutrophil is activated by e.g., CXCL-8 or PAF [8, 9] produced by the endothelial cells. This causes the rolling neutrophil to attach more firmly to the endothelium, as it responds by upregulating surface integrins (e.g., CR3 and CD11b/CD18), which bind with high affinity to their endothelial counterparts (e.g., integrin cell adhesion molecule-1 (ICAM-1)). Integrin binding induces the release of heparin-binding protein (HBP) [10] from the neutrophil secretory vesicle [11], eventually leading to contraction of the endothelial cells and allowing the activated neutrophil to pass or extravasate. Platelet endothelial cell adhesion molecule-1 (PECAM-1),

which is located on both the neutrophil and endothelium, is another crucial molecule in transmigration across the blood vessel wall [7].

In the tissue, a chemotactic gradient developed from the focus of infection/inflammation then draws the neutrophils to the infected site, yielding high concentrations of neutrophils in a specific area and thereby increasing the ability of the innate immune system to clear the tissue of foreign material.

Phagocytosis

Upon reaching the site of infection, the primary tasks for the neutrophil, phagocytosis and elimination of the intruder, begin. Phagocytosis is a phylogenetically conserved activity, the purposes of which range from nutrient uptake in unicellular organisms to a highly complex series of events in the immune system of mammals, leading to the engulfment of prey and subsequent degradation in the phagolysosome.

In the case of neutrophil phagocytosis, the pathogen must initially be attached to the surface of the phagocyte. This neutrophil-pathogen interaction may be induced by the recognition of opsonins on the bacterial surface or by the presentation of antibodies or complement factors to Fc or complement receptors [12], which are clustered on the plasma membrane of the exudated neutrophil [13]. Microbial lectins that bind directly to glycoconjugate receptors on the phagocyte (or *vice versa*), particularly at sites where complement factors or antibodies are scarce (e.g., in the urinary tract) [14, 15], may also initiate intracellular signalling, leading to phagocytosis of the pathogen. Following occupation of the neutrophil surface receptors, actin rearrangement is initiated and pseudopodial extensions appear. The prey is wrapped by the extensions and is eventually completely enclosed in a phagocytic vacuole, which is composed predominately of the plasma membrane [16]. However, a recently proposed new model for phagocytosis suggests that instead of the plasma membrane being the primary source of the early phagosomal membrane, the ER is the major contributor to this nascent organelle [17, 18]. Phagosome formation is, according to this model, the result of particles sliding into the ER *via* an opening at the base of the phagocytic cup. There is an ongoing discussion as to whether this ER-mediated phagocytosis actually occurs, and this model awaits verification [19-21].

The involvement of the ER in phagocytosis has been studied almost exclusively in human macrophages, probably because these phagocytes contain far more ER than mature neutrophils. Nevertheless, it is clear that neutrophils are capable of *de novo* protein production, implying a more prominent role for the ER and Golgi in these cells than has previously been thought. Thus, if ER-mediated phagocytosis is taking place, it is possible that neutrophils also utilize this route of particle uptake.

Regardless of the type of membrane that forms the early phagocytic vacuole, it is generally accepted that other intracellular compartments deliver additional

membrane to the forming phagolysosome. Newly formed phagosomes are immature organelles that are incapable of killing and degrading the ingested prey. Therefore, a maturation process (referred to as phagolysosome biogenesis [22, 23]), in which the phagosome acquires its microbicidal functions, is required. Early endosomes fuse with the phagocytic cup, probably contributing to the pseudopod extensions [24, 25]. Further fusion of endosomes with the phagocytic vacuole takes place early after sealing, ensuring proper ingestion of the prey [26]. The lysosomal counterparts in neutrophils, the azurophil granules, fuse with the phagosome at a later stage, releasing hydrolytic enzymes, peroxidase (MPO), and antimicrobial substances, such as cathepsin G, bactericidal/permeability-increasing protein (BPI), and defensins, into the phagolysosome that holds the prey. A second species of neutrophil granules, very much involved in the killing of the target, includes the specific granules, which transport the membrane-bound NADPH-oxidase to the phagolysosome. This enzyme system catalyses the conversion of oxygen into reactive oxygen species, a process often referred to as the respiratory burst [27]. In the phagolysosome, the vast amounts of superoxide anion and hydrogen peroxide produced by the oxidase are further converted (in reactions catalysed by MPO, which is delivered from azurophil granules) into hypochloric acid, which is highly toxic for the phagocytosed intruder [28]. The combination of the oxygen-independent antimicrobial machinery and the latter, oxygen-dependent antimicrobial functions provides the neutrophil with superb artillery in the early combat of infection.

A proposed model for phagosome maturation is the “kiss and run” hypothesis. This model, although predominantly studied in macrophages, describes membrane interactions between endosomes and the forming phagosome, which is moving along the cytoskeleton. Upon close encounter of the organelles, transient membrane interactions lead to the induction of a fusion pore [23, 29]. This fusion pore allows exchange of soluble compounds between the organelles, contributing to the gradual maturation of the phagosome. However, according to this model, complete organelle fusion is not accomplished, and as the fusion pore closes, the organelles separate and may become available for additional rounds of fusion events.

Despite the harsh treatment of ingested microorganisms inside the phagosome, certain pathogens have developed mechanisms of escaping this fate. *Listeria monocytogenes* is one such example [30]. This Gram-positive bacterium, which causes listeriosis manifested by gastroenteritis, infections of the central nervous system, and in some cases, mother-to-child propagated *in utero* infections, actually promotes its own phagocytosis [31]. However, after uptake, *L. monocytogenes* lyzes the phagosomal membrane and multiplies in the cytosol of the phagocyte. While in the cytosol, the bacterium polymerizes the host actin to enable its own movement towards the plasma membrane. From this location, *L. monocytogenes* invades adjacent cells *via* protrusions or cell-to-cell spread [30]. Another way of escaping phagolysosome degradation is exerted by the Gram-

negative *Francisella tularensis*. This pathogen has recently come to attention for its potential use in the development of biological weapons. If transmitted *via* the aerosol route, *F. tularensis* is extremely virulent and only a few bacteria may cause fatal pneumonia. The bacterium is readily taken up by neutrophils and enclosed in a forming phagolysosome. However, following uptake of this bacterium, NADPH-oxidase assembly in the phagosomal membrane is altered, which explains why the phagocytosis of *F. tularensis* does not provoke a respiratory burst. Thus, this bacterium is not eliminated by the neutrophil, and during late infection, *F. tularensis* escapes the phagosome and replicates in the cytosol [32]. *Mycobacteria, Salmonella* and *Leishmania* species are other examples of pathogens that are capable of escaping destruction in the phagosome by interfering with phagolysosome biogenesis [29].

Myelopoiesis

The professional phagocytes of the human body comprise monocytes, macrophages, and granulocytes. The granulocyte population can be further divided into eosinophils, basophils, and neutrophils, the latter being the dominant cell type, making up 95% of the granulocyte population and 64% of all white blood cells. The neutrophil is a polymorphonuclear leukocyte that is easily recognizable microscopically by its characteristic multilobular nucleus, a feature of great significance for cellular function.

The production of neutrophils, as well as of all other blood cells (myelopoiesis) occurs in the red bone marrow. The different blood cells originate from the same progenitor cells, which differentiate into specific lineages, one of which eventually gives rise to the mature neutrophil [33]. The development of neutrophils is a 12-14-day process, during which the maturing cell passes through six morphologically distinct developmental stages. Changes in cell size are accompanied by the appearance of primary (azurophil) and secondary (specific/gelatinase) granules. In the early myeloid precursors, the ER, Golgi, and mitochondria are apparent, and the nucleus assumes its characteristic shape. The transformation from myelocyte to metamyelocyte marks the end of the mitotic phase, and thereafter, the cells no longer divide, but instead move into the phase of maturation [34].

Under normal circumstances, the turnover of neutrophils is about one billion cells/kg/day, although production may increase 10-fold during infection. End-stage, not yet fully mature, neutrophils are then released from the bone marrow and the result, a tremendous number of circulating neutrophils, is a prominent clinical sign of infection. Neutrophils are short-lived cells, surviving 8-20 hours in the circulation. However, in tissues they may persist for a several days, after which the senescent cells undergo apoptosis and are eventually cleared by macrophages [35].

Granulopoiesis and sorting by timing

As stated above, the phase of neutrophil maturation, during which the cells are transformed from myeloblasts into promyelocytes, is also when granules begin to form [33, 36]. This is an ongoing process that continues through the metamyelocyte and band cell stages, all the way to the segmented cell (Fig. 1). Azurophil granules, or primary granules, are formed first, followed by the secondary granules (the specific and gelatinase granules), and upon leaving the bone marrow, the mature neutrophils are packed with granules that are ready to be mobilized under the appropriate circumstances.

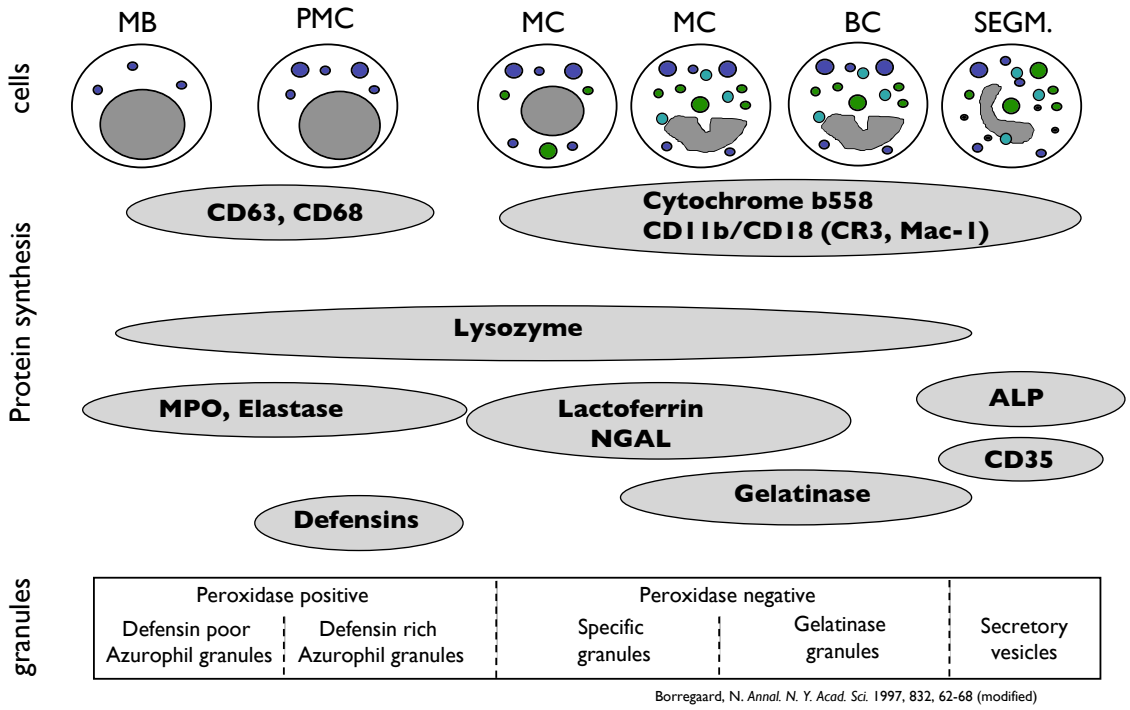


Figure 1. Granulopoiesis is governed by the timing of protein synthesis. MB, myeloblast; PMC, promyelocyte; MC, myelocyte; MMC, metamyelocyte; BC, band cell; SEGM, segmented cell.

Granule formation is governed at the transcriptional level, ensuring the expression at the appropriate time of the mRNAs for the various granule constituents [37]. The resulting mature neutrophils are thus equipped with their characteristic granules, with each group being created within a certain time span of neutrophil maturation [36] and packed with proteins that are synthesized during the same period. The view that proteins formed at the same time during myelopoeisis are sorted into the same granule subset is commonly referred to as the sorting or targeting-by-timing hypothesis [38-40]. Granules are formed as immature transport vesicles, packed with concentrated granule proteins, bud off

from the *trans*-Golgi network and undergo homotypic fusion [41]. Sorting of proteins to either the constitutive secretion pathway or to regulated secretory pathways, i.e., into granules, is a prerequisite for this to work properly.

In clinical situations, such as acute myeloid leukemia (AML), the normal differentiation program is disturbed, resulting in abnormal protein expression and as a consequence, altered granule packing/formation. To understand the molecular sorting and regulation of granulopoeisis is thus of great relevance, and a number of studies have been conducted on this subject. Azurophil granules, the most extensively studied granule type in the context of protein sorting [42, 43], are well characterized, and it is evident that these granules are not just randomly arranged stores. Early azurophil granules appear as large, peroxidase-negative organelles [33], while in mature neutrophils, the granule population is largely made up of electron-dense, peroxidase-positive granules [44], which seem to be the products of the final packaging [45]. The intra-granular distributions of MPO and defensins differ between the subgroups of azurophil granules, and it seems that the sorting of proteins according to appearance over time is accompanied by rearrangement of the granule contents during granule maturation, as well as after the initial packaging [40].

The segregation of granule proteins into different compartments may not only serve the purpose of conferring upon the neutrophil the ability to display different receptors and effector molecules at specific time-points in the process of activation, but it may further provide the cell with the option of segregating proteins that simply cannot co-exist from a safety point-of-view. For example, in the azurophil granules, MPO, serine proteases, and antibiotic peptides are all terminally processed and potentially active [46]. In contrast, specific granules store antibiotic peptides and metalloproteases in their inactive proforms, and these peptides are not activated until degranulation has occurred. Therefore, a consequence of degranulation is that catalytically active proteases from the azurophil granules encounter proform peptides from the specific granules, transforming the latter into active effector molecules. Another important example of separate storage is the segregation of MPO (stored in azurophil granules) from NADPH-oxidase (stored in the specific granules). Upon specific granule mobilization, the enzyme complex becomes incorporated into the phagosomal membrane. The reduced oxygen species generated are transferred to highly bactericidal metabolites when the azurophil granules are delivered to the phagosome, providing MPO for the reaction [27, 47]. Thus, even though a variety of substances are stored separately in the resting neutrophil, they work together at specific sites in a process that is stringently regulated by the degranulation process.

In the case of aberrant sorting, during which specific granule proteins could end up in the azurophil granules, there would be an obvious risk of degradation, although the catalytically active proteases should be blocked by binding to the azurophil granule matrix. However, under normal circumstances, aberrant sorting

seems to occur only to a minor extent, which indicates that stringent control of the expression of proteins over time ensures proper storage [36].

Ultrastructure of the mature neutrophil

Mature neutrophils have been the subject of many microscopical studies using a variety of different techniques. Transmission electron microscopy (TEM) (preferably using thin sections of around 60-nm thickness) reveals the stunning complexity of this cell type (Fig. 2A). In combination with immuno-gold labelling, this technique allows precise mapping of protein distribution and distinction between different subcellular compartments within the cell.

The diameter of the mature neutrophil is between 5 and 10 μm. Scanning electron microscopy (SEM) images display a very ruffled cellular surface (i.e., plasma membrane) that is capable of forming deep clefts as well as large protrusions (Fig. 2B).

The cytoplasm (cytosol) of the neutrophil is rich in soluble proteins, and is also packed with granules and vesicles (Fig. 2C) that contain both matrix proteins and membrane-bound proteins (see below). The mature neutrophil contains few mitochondria, and only small amounts of the ER and Golgi apparatus. Thus, *de novo* protein synthesis has historically been regarded as being of minor importance when studying the

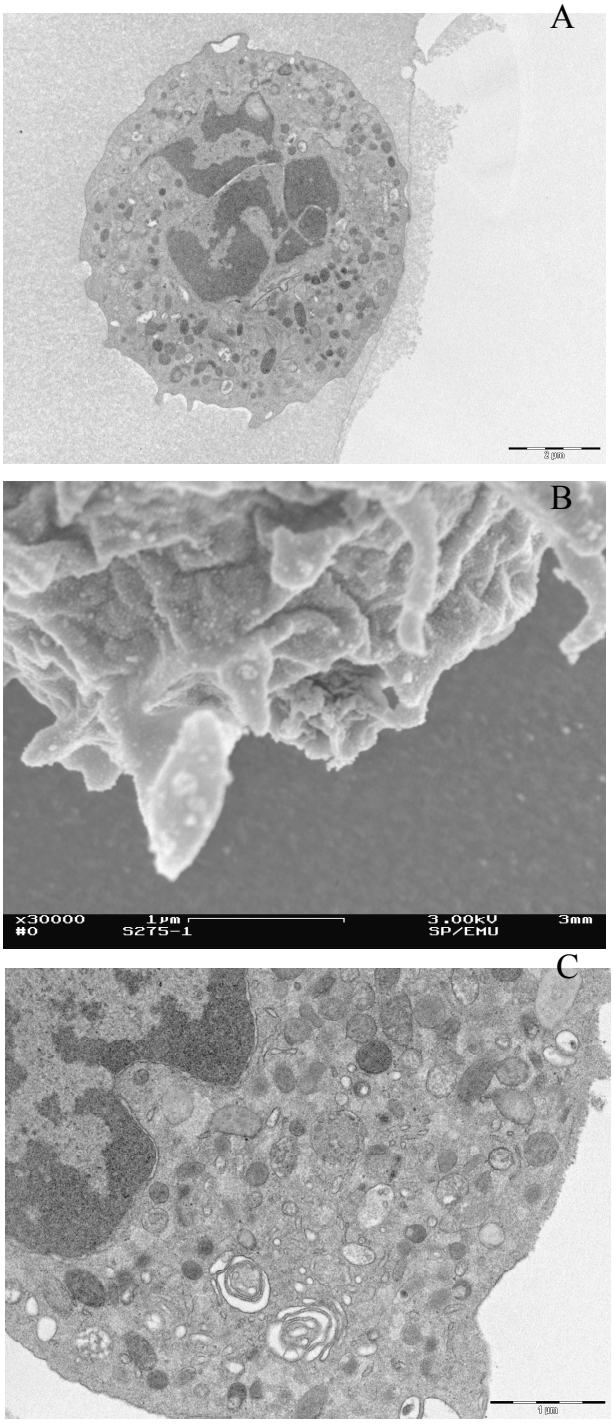


Figure 2. (A, C) Standard TEM of mature, resting, human neutrophil. Thin section (60 nm), (A) magnification x15,000 and (C) magnification ×25,000. (B) SEM of resting, human neutrophil. Magnification x30,000. (S. Pellmé 2005)

physiology of circulating neutrophils. However, over the past decade, it has become evident that neutrophils are capable of protein production. Cytokines, such as interleukin-8 (IL-8/CXCL-8) [48-54], and possibly also IL-1β, IL-1 receptor antagonist (IL-1ra) [55], and IL-6 [56] are produced upon stimulation with inflammatory stimuli, such as LPS. The view of the mature neutrophil as a rather static cell that is destined to exocytose its preformed granule proteins in appropriate situations has been partly re-evaluated. It seems likely that the neutrophil ER, even though rudimentary in the mature cell, is more functionally important than was previously thought.

NEUTROPHIL GRANULES

Granules may be classified on the basis of their morphology, size, electron density, and perhaps most commonly, their content of specific proteins or markers. However, it should be taken into account that granules form a continuum from azurophil granules to gelatinase granules. This is due to the fact that even though proteins are sorted into their specific granule types according to the targeting-by-timing hypothesis, there will always be overlaps in protein expression and thus, to some extent, sharing of proteins between the granule subsets. Therefore, the distinction of granule subsets should not be regarded as absolute. Nevertheless, there are some proteins that are more suitable as specific markers for particular subsets (Fig. 3), as will be discussed below.

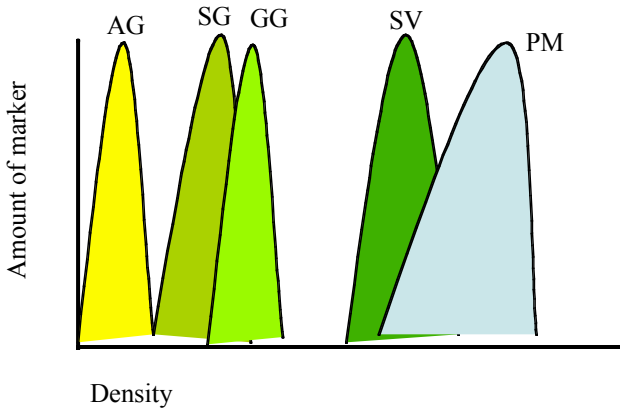


Figure 3. Schematic diagram of granule/marker distribution in subcellular fractions, following Percoll gradient cetrifugation of desintegrated, mature neutrophils.
AG: azurophil granules, primary marker MPO; SG: specific granules, primary marker vitamin B_{12} binding protein (Vit. B_{12} b.p); GG: gelatinase granules, primary marker gelatinase; SV: secretory vesicles, primary marker latent alkaline phosphatase (ALP); and PM: plasma membrane, primary marker non-latent ALP.

Azurophil granules

The first type of granules to be formed and recognized during neutrophil maturation are the azurophil, or the primary granule, which are acquired at the myeloblast and promyelocyte stages [33]. Making up around 30% of the total granule population, azurophil granules were first identified as peroxidase-positive granules, as they contain large amounts of MPO. In addition to MPO, which is the primary marker of azurophil granules, these granules contain a variety of lysosomal enzymes, such as sialidase and lysozyme. Proteinase 3, elastase, defensins (comprising 30-50% of the granule content), cathepsin G, and bactericidal permeability-increasing protein (BPI) [57-60] are also found in the matrix of azurophil granules [61]. These substances are needed during the late stages of neutrophil action and are involved in the microbicidal processes associated with the degradation of phagocytosed material.

Azurophil granules were originally thought of as the lysosomes of the neutrophil. However, this is a view that has been re-evaluated, as the azurophil granules no longer meet the definition of lysozomes. Lysosomes are defined as acidic organelles that contain high concentrations of lysosome-associated membrane glycoproteins (LAMPs), a variety of dephosphorylated lysosomal enzymes, but no mannose-6-phosphate receptor. The azurophil granules deviate from this definition in at least two aspects; they contain neither LAMPs nor dephosphorylated enzymes [62].

Until a few years ago, very little was known about the membranes of the azurophil granules, with CD63 [63] and CD68 [64] being the only identified components. Recently, proteomic studies have revealed the complexity of the various functions of the azurophil granule, apart from its bactericidal activities. In a recent study, we identified lipid rafts as components of the azurophil granule membrane. When characterized with regard to protein content, these domains were suggested to serve as functional platforms, raising the possibility that azurophil granules participate in regional signaling within the neutrophil cytosol [65].

Specific and gelatinase granules

Secondary (specific) granules appear during the myelocyte-metamyelocyte stages of neutrophil maturation. They are part of the peroxidase-negative granule population, a group of granules that could be described as a continuum, ranging from the most dense and least-mobilizable granules, which contain high concentrations of lactoferrin and Vit. B_{12} b.p. but hardly any gelatinase (defined as specific granules), to the light and readily mobilizable granule subtype, which is rich in gelatinase but contains very low levels of lactoferrin and Vit. B_{12} b.p. (referred to as the gelatinase granules). The protein constitution of each granule within this group thus reflects the time of biosynthesis according to the sorting-by-timing hypothesis described above. Specific granules are smaller, somewhat

lighter, and more readily mobilized than the dense azurophil granules. Their membranes host a variety of functional proteins, e.g., complement receptor 3 (CR3), formyl peptide receptor (FPR), and the NADPH-oxidase component cytochrome b_{558} [66]. These are all incorporated into the plasma membrane as a result of mobilization of specific granules to the cell surface, but perhaps more important, they become part of the phagosomal membrane as specific granules fuse with the phagosomal vacuole. Assembly of the NADPH-oxidase in the plasma membrane and phagosomal membrane enables the neutrophil to produce toxic oxygen radicals both intracellularly and extracellularly, providing different avenues for microbial destruction. However, the extracellular release of radicals that accompanies phagosomal production of toxic oxygen species is also highly destructive to the surrounding tissue, which is why activation of the oxidase needs to be tightly regulated during inflammation.

In addition to lactoferrin and vit. B_{12} b.p. (the latter is used in this thesis as a marker for this granule subset), neutrophil gelatinase-associated lipocalin (NGAL) [67], collagenase, and β_2-microglobulin (β_2m) are other prominent matrix components of the specific granules. Moreover, it has recently been discovered that in addition to the mature defensins that are stored in the azurophil granules, specific granules contain a more easily mobilized pool of unprocessed pro-defensins [68]. It remains to be elucidated whether these seemingly inert pro-molecules are capable of carrying out biological functions of their own following specific granule mobilization.

Gelatinase granules

Gelatinase granules, which are often referred to as tertiary granules, appear a little later than the specific granules during neutrophil maturation, i.e., during the band cell stage [38]. Similar to the specific granules, the gelatinase granules are peroxidase-negative and constitute about 25% of the peroxidase-negative granule population [69]. Being slightly lighter than the specific granules, gelatinase granules can be distinguished from the other granule subtypes by subcellular fractionation techniques [70], enabling the characterization of the granule membrane and matrix.

Although specific granules and gelatinase granules share numerous proteins, they differ in certain respects. The gelatinase granules contain more tissue-degrading enzymes and adhesion-regulating molecules, indicating that these granules are, to a greater extent than specific granules, involved in the migration of neutrophils into tissues. Twenty percent of the cellular contents of CR3 and cytochrome b_{558} are located in the gelatinase granule membrane and upon mobilization of the granule subset, these molecules become incorporated into the plasma membrane [70]. At the same time, substantial amounts of gelatinase are released, and this is important for neutrophil diapedesis and migration into tissues, as it plays a role in degrading the type IV collagen of the basal membrane that surrounds the blood vessel endothelium [71]. Moreover, specific and

gelatinase granules differ in their responses to inflammatogenic stimulation. Gelatinase granules are more readily mobilized to the cell surface [72, 73], and thus it is possible for the neutrophil to fine-tune the exocytosis of different granules within the peroxidase-negative subpopulation, i.e., to mobilize gelatinase granules without concomitant upregulation of specific granules.

Plasma membrane and secretory vesicles

The most easily mobilized subcellular neutrophil compartment is the secretory vesicle. This organelle is formed through endocytosis during the late stages of maturation [74]. Thus, the secretory vesicles contain material from the extracellular milieu of the bone marrow, i.e., plasma proteins, such as albumin. Important features of the secretory vesicle membrane are the chemoattractant receptors FPR and FPRL-1[3], which are used very early in the process of neutrophil activation, complement receptors 1 and 3 (CR1 and CR3 (CD11b/CD18)), and the NADPH-oxidase component cytochrome b_{588} [75]. The secretory vesicle is a light membrane structure that colocalizes with the plasma membrane in density gradients. This feature, together with the fact that the vesicle membrane is derived from the plasma membrane, makes it difficult to distinguish biochemically these two structures.

Stimulation of resting neutrophils with nanomolar concentrations of fMLF results in mobilization of the secretory vesicles to the plasma membrane, a phenomenon that makes distinction between the two structures even more challenging. The neutrophil secretory vesicle was originally described as a tetranectin-containing compartment [76] that could be identified by the presence of alkaline phosphatase (ALP) activity [77]. However, in density gradients of disrupted neutrophils, secretory vesicle-derived ALP can be detected only in the presence of detergent, i.e., latent ALP [78], in contrast to ALP that is localized in the plasma membrane, which is readily detected without detergent treatment (non-latent ALP). Under circumstances where neither azurophil, nor specific or gelatinase granules are mobilized, the secretory vesicles are fused with the plasma membrane, resulting in translocation of ALP to the cell surface and release of tetranectin and other plasma proteins [79].

Alternative markers for the plasma membrane

The identification of optimal markers for the plasma membrane and secretory vesicles has proven to be a complex problem. An ideal marker should be specific for the compartment in question, unaffected by stimulation, and the assay used for detection should be easy to perform and reproducible. Various assays for the detection of plasma membrane-specific proteins, such as the receptors for PAF [80] and C5a [81] have been used, but due to methodological disadvantages, they have been abandoned.

HLA-I

The human leukocyte antigen class I (HLA-I) molecules of the immunoglobulin superfamily are integral membrane proteins of the plasma membranes of all nucleated cells [82]. The HLA-I complex is a heterodimer of one light chain, β_2–microglobulin (β_2m), linked to a heavy chain, HLA-I, which contains the transmembrane part of the protein. In the search for an alternative neutrophil plasma membrane marker, Bjerrum and Borregaard designed a sandwich technique for the detection of HLA-I [83]. This mixed enzyme-linked immunosorbent assay (MELISA) uses anti-β_2m catching antibodies and then detects the HLA-I complex with an antibody directed towards the HLA-I part of the complex. The HLA-I complex is present in the plasma membrane and its distribution is not influenced by secretagogues. Therefore, it is regarded as a better marker for the plasma membrane than ALP, the latter being greatly affected by neutrophil stimulation due to its presence in the easily mobilized secretory vesicles. Furthermore, ALP is linked to inositol phosphate (IP) [84] and is cleaved from the cell surface by phospholipase C (PLC), making it less reliable as a plasma membrane marker.

However, only 15% of the neutrophil content of β_2m is located in the plasma membrane, in complex with HLA-I. The remaining β_2m is uncomplexed and located in either the specific granules (65%) or secretory vesicles (20%) [85]. As the initial step of the MELISA uses a β_2m-specific catching antibody, this part of the assay is greatly affected by the amount of free β_2m in the sample to be measured (Paper II). Moreover, the data presented in Paper II suggest that additional HLA-I-containing organelles, distinct from the plasma membrane, are present in the light membrane fractions when neutrophils are fractionated on ordinary Percoll gradients. However, these organelles can be discerned and analyzed first when the granules/secretory vesicles have been removed from the cells, as demonstrated in Paper II, in which neutrophil cytoplasts were fractionated and the distribution of HLA-I in each fraction was determined. As the distribution profile of HLA-I in these seemingly empty cells did not coincide with that of the plasma membrane, we conclude that HLA-I is not a specific marker for the plasma membrane in neutrophils. If used as a marker, it should be combined with assays of ALP activity.

GPI-80

Another alternative marker for the neutrophil plasma membrane is glycosylphosphatidyl-inositol (GPI)-anchored glycoprotein-80 (GPI-80), which is involved in the regulation of β_2-integrin-dependent adhesion of the neutrophil to the endothelium during extravasation/diapedesis [86]. The exposure of GPI-80 on the cell surface is increased with weak stimulation, which suggests that in addition to its presence in the plasma membrane, GPI-80 is also stored intracellularly, probably within the secretory vesicles [87]. This finding and the

fact that GPI-80 (IP-linked in the same way as ALP) can be cleaved from its lipid anchor by externally added PLC support the use of GPI-80 and ALP as markers for the plasma membrane and/or secretory vesicles. One can assume that in activated neutrophils that have mobilized their secretory vesicles, GPI-80 and ALP are equally useful as markers for the plasma membrane.

In conclusion, it seems that neither ALP nor GPI-80 nor HLA-I are optimal markers for the plasma membrane, and the search for better markers should continue.

Granule mobilization

Exocytosis of granule contents is a consequence of granule membranes fusing with the plasma membrane, which leads to the incorporation of granule membrane proteins into the plasma membrane. Intact granule membranes are organized in an inverted fashion in order to ensure the correct functional orientation of membrane-associated molecules (e.g., receptors) after merging with the plasma membrane. In most cell types, this membrane is then rapidly retrieved for re-use, although this is not the case in neutrophils. Instead, granule mobilization seems to be a tool that is used by the neutrophil to change dramatically its surface appearance, which exposes new membrane structures (proteins and lipids) to the surroundings, thereby facilitating new interactions [66].

One way of defining the subcellular localization of a specific protein in the neutrophil is to stimulate the cell *in vitro* and study the release or exposure of the protein in relation to other, known granule markers. Proteins that are mobilized together can be assumed to localize together in the resting neutrophil, even though such studies should always be complemented with microscopical evaluations or at least subcellular fractionation, in order to confirm the localization of the protein in the granule subsets.

Neutrophil granules are mobilized in the reverse order of formation. The first organelle to be mobilized to the cell surface upon external stimulation is the light secretory vesicle. The membrane of this vesicle is particularly rich in receptors [88, 89], the translocation of which to the plasma membrane transforms the neutrophil from a passively circulating cell to a β_2-integrin-presenting, highly responsive cell that is ready for migration into the tissue. Following the secretory vesicles in order of mobilization are the gelatinase granules, the specific granules, and finally, the azurophil granules. Even though the strict hierarchical order in which the granules are used in the inflammatory process is well established, the molecular mechanism underlying granule mobilization is largely unknown. An attractive explanation for this phenomenon is that all the granules and vesicles are linked to the microtubular system, and when exposed to a stimulus (such as LPS or chemoattractants, the latter inducing elevated intracellular calcium), they are propagated along the microtubuli towards the cell surface and/or phagosome. The lighter and smaller the granule, the faster its

movement would be completed [79] and the faster the granule content would be exocytosed or displayed on the neutrophil surface.

Table 1. Contents of neutrophil granules and secretory vesicles. Except when indicated otherwise in the table, the references are found in [93], from which the table is adapted.

AZUROPHIL GRANULES	SPECIFIC GRANULES	GELATINASE GRANULES	SECRETORY VESICLES	MVB	MLC
Membrane					
CD63 CD66 CD68 Dysferlin[90] Flotillin[90] Presenilin 1 V-type H^+-ATPase Stomatin[90]	CR3 CD15 antigens CD66a (CEACAM1) [91] CD66b (CEACAM8) Cytochrome b_{558} FPR Fibronectin-R G-protein$_\alpha$-subunit Laminin-R NB 1 antigen 19- kDa protein 155-kDa protein Rap1, Rap2 SCAMP SNAP-23, -25 Stomatin[90] Thrombospondin-R TNF-R uPA-R VAMP-2 Vitronectin-R	CD66 CR3 Cytochrome b_{558} Diaglycerol-decylating enzyme FPR FPRL-1[3] Leukolysin NRAMP-1 SCAMP SNAP-23,-25 Stomatin[90] uPA-R VAMP-2 V-type H^+-ATPase	ALP CD10, CD13 CD14 CD16 CD45 CR1 CR3 Cytochrome b_{558} $C1_q$-R DAF GPI-80 [87] FPR FPRL-1[3] Leukolysin Stomatin[90] uPA-R VAMP-2 V-type H^+-ATPase	LAMP-1 [62] LAMP-2 [62] Mannose-6-P-receptor [92]	LAMP-1[62] LAMP-2[62]
Matrix					
Acid β-glycerophosphatase Acid -Mucopolysaccharide α_1-Antitrypsin α-Mannosidase HBP BPI β-Glycerophosphatase β-Glucuronidase Cathepsins Defensins Elastase Lysozyme MPO glucosaminidase Proteinase-3 Sialidase Ubiquitin-protein	β_2-m Collagenase CRISP-3 (SGP-28) Gelatinase hCAP-18 Histaminase Heparanase Lactoferrin Lysozyme NGAL prodefensins[68] uPA Sialidase Vit. B_{12}-bp	Acetyltransferase β_2-m CRISP-3 Gelatinase Lysozyme	Plasma proteins (including tetranectin)		

ALTERNATIVE STRUCTURES AND ORGANELLES

MVB/MLC - the true lysosomes in neutrophils?

During granulopoiesis, large amounts of lysosomal enzymes are synthesized in the neutrophil precursors. On their route from the ER to the Golgi, these enzymes are modified to incorporate mannose 6-phosphate (Man 6-P), which binds to receptors in the Golgi complex. This binding directs the enzyme to acidic endosomes, in which the receptor-ligand complex dissociates [94]. The receptors are then either recycled back to the Golgi complex or translocated to the plasma membrane, while the enzymes reach the lysosome and readily become dephosphorylated. Under normal conditions, lysosomes contain most of the dephosphorylated enzymes of the neutrophil, which defines one of the criteria for lysosome distinction (see above). Enzymes (e.g., MPO) that are sorted into azurophil granules during granulopoiesis are still phosphorylated. These features and the fact that the azurophil granules have been proven not to contain LAMPs in significant quantities [62, 95], disqualify these organelles from being defined as lysosomes.

Based on this outcome, thorough investigations of the intracellular distributions of Man 6-P and LAMP-1and LAMP-2 have been undertaken [92]. Using subcellular fractionation of mature neutrophils, Man 6-P and MPO were found to colocalize in the dense fractions, while LAMPs were detected mainly in fractions of lower density, corresponding to the specific and gelatinase granules, but also to some extent in yet lighter fractions, which contain the secretory vesicles and plasma membrane. However, thin sections of neutrophils revealed that LAMPs were in fact not present in either azurophil granules or in any of the other known granule types, but were instead distributed in the membranes of two other types of vesicles, i.e., multivesicular bodies (MVB) and multilaminar compartments (MLC) (Fig. 4) [92]. This subcellular localization of LAMP is confirmed in Paper I. The LAMPs associate with the mature phagosome in neutrophils that have engulfed a prey [62, 92, 95], supporting the hypothesis that MVBs/MLCs are the true lysosomes of the neutrophil.

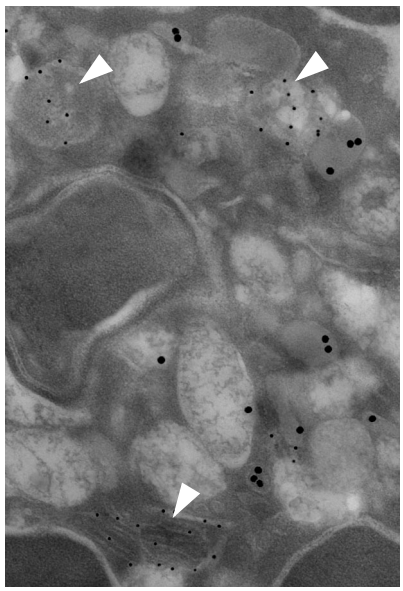


Figure 4. TEM of neutrophil. Arrowheads indicate the presence of MVB (top) and MLC (bottom). Magnification x 30,000. (M. Mörgelin, 2003).

MVBs in particular are described in various other cell types, such as dendritic cells [96] and other antigen presenting cells [97]. MVBs in these cells are believed to be filled with exosomes, small vesicles bound for secretion. A prominent function of such exosomes is to get rid of membrane proteins which have fullfilled their purposes and are no longer used by the cell, an example being the removal of transferrin receptors from mature erythrocytes [98]. Hence, exosomes may be an alternative to lysosomal degradation, possibly used to degrade proteins that are resistant to, or cannot be reached by, lysosomal proteases. Antigen presentation and T-cell stimulation are other *in vitro* features ascribed to exosomes of dendritic cell origin [99]. However, these findings await validation *in vivo*.

In the case of MLC function, it is tempting to speculate that they may provide membrane to the forming phagosome, a theory that would be supported by the finding that LAMPs translocate to the neutrophil phagosome, as described above.

The IL-8/CXCL-8-containing organelle

Interleukin-8 (IL-8/CXCL-8) is a cytokine that is produced by macrophages, monocytes [100, 101], endothelial cells [102] and fibroblasts [103], as well as by activated neutrophils [48, 104, 105 and Paper I]. The prominent feature of CXCL-8 is its ability to act as a chemokine, i.e., to attract neutrophils to sites of infection. Thus, CXCL-8 is regarded as being highly important in the inflammatory process.

Even though soluble CXCL-8 in the blood and other bodily fluids is considered to be a good marker for predicting the outcome or severity of various clinical conditions (e.g., sepsis, bronchitis, severe burns or multiple organ failure following injury, as well as asphyxic conditions in the preterm baby [106, 107]), a large proportion of the CXCL-8 in whole blood is cell-associated [108], being bound to Duffy antigen on the surfaces of erythrocytes [109]. However, CXCL-8 also binds with high specificity to high-affinity receptors (CXCR-1 and CXCR-2) on leukocytes [110, 111]. Both CXCR-1 and CXCR-2 are G protein-coupled receptors that are expressed predominantly on neutrophils, and CXCL-8 is the primary agonist for CXCR-1. The binding of agonist (CXCL-8) to neutrophil CXCR-1 mobilizes calcium stores and induces degranulation, leading to chemotaxis of the neutrophil up the CXCL-8 gradient. Moreover, CXCL-1 binding activates the neutrophil NADPH-oxidase [112], provoking the respiratory burst *in vitro*. This effect is not observed for CXCL-2, and this receptor seems to be more important in regulating the chemotactic processes.

Circulating neutrophils contain very low levels of intracellular CXCL-8. However, exudated neutrophils have been shown to be desensitized to CXCL-8 [113, 114], which suggests that this cytokine is involved in the accumulation of neutrophils at inflammatory sites. Under such conditions, neutrophils may themselves contribute to the amount of tissue-localized CXCL-8. The latter notion is supported by the fact that neutrophils synthesize considerable amounts

of cytokines upon stimulation with certain inflammatory components or mediators. *In vitro*, the amount of CXCL-8 increases significantly over background levels when neutrophils are activated, e.g., by G protein-coupled receptors [48, 51, 115], by intracellular calcium increase [50], during phagocytosis [116], and by bacterial lipopolysaccharide [49, 117-119]. All these agonists/pathways induce both transcriptional and translational activation, resulting in increased levels of intracellular CXCL-8 with concomitant release of the cytokine to the surrounding milieu.

In Paper I [54], neutrophils were incubated with *E. coli* LPS in order to induce CXCL-8 production. In accordance with previous work, the cells produced large amounts of the cytokine, and about 40% of the cellular CXCL-8 was spontaneously released 8 hours after the start of incubation. However, only in resting cells, secretion of CXCL-8 could be induced by the addition of secretagogues. In neutrophils that produced CXCL-8 *de novo* in response to LPS, no such effects on secretion were noted, regardless of the secretion protocol applied. Therefore, we speculate that regardless of the compartment(s) responsible for the storage of CXCL-8, either its responsiveness to external secretagogues is lost during LPS incubation or the newly produced cytokine is stored in a different intracellular compartment.

Based on subcellular localization experiments using Percoll gradients, previous work by Kuhns et al. has implicated secretory vesicles as possible stores for CXCL-8 in resting neutrophils [105]. However, the fact that stimulated neutrophils retain CXCL-8 intracellularly under conditions in which the secretory vesicles are clearly mobilized to the cell surface, led us to challenge that conclusion and study the subcellular localization of CXCL-8 more thoroughly. In Paper I [54], we show that CXCL-8 in resting and LPS-stimulated neutrophils is stored in a compartment that is distinct from the classical granules, the secretory vesicles, the MVB/MLC, and the TIMP-containing organelle (see below). Using neutrophil cytoplasts, which are "empty" cell ghosts that are devoid of granules and vesicles, to create a reductionistic system in which to study CXCL-8 localization, we found partial colocalization of CXCL-8 with the ER protein calnexin. However, we could not draw the conclusion that CXCL-8 is stored in the ER of neutrophils. The distribution patterns of CXCL-8 and calnexin in HL-60 cells during phagocytosis (Paper III) suggest that these two proteins are stored separately in the neutrophil, or at least are directed differently during the phagocytic process.

Whether the CXCL-8-containing organelle fits the pattern of synthesis and mobilization that is applicable to the other neutrophil granule types is currently unknown. If CXCL-8 is to be used early in the inflammatory process, it should, according to the formed-first-released-last model, be stored in an easily mobilized vesicle formed late in the differentiation process. CXCL-8 in resting peripheral blood neutrophils is indeed stored in an easily mobilized organelle, but when it is first formed remains to be determined. On the other hand, CXCL-8 may be involved during the later phases of the immune response, e.g., during

phagocytosis or in attracting a second wave of neutrophils to an inflammatory site when the present cells are primed by bacterial and host components. Furthermore, late secretion of CXCL-8 may desensitize the cells to additional chemotactic stimulation by the same chemokine, resulting in arrest of the cells in a specific area. In this type of scenario, one would expect CXCL-8 to be produced in large quantities and to be constitutively secreted, in a process that we have shown to be uninfluenced by secretagogues (Paper I [54]). It is however possible that this large pool of CXCL-8 is mobilized when provided with the appropriate stimuli under the right conditions.

The TIMP organelle

An intracellular compartment that co-fractionates with light membranes (plasma membrane/secretory vesicles) in density gradients and, just like the CXCL-8-containing organelle, differs from the defined granule populations, has recently been described [120]. This organelle contains the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) but is devoid of markers for the secretory vesicle, thus making it a possible candidate for the CXCL-8-storing organelle. However, during our studies of fractionated cytoplasts, it became evident that the TIMP-1- and CXCL-8-containing organelles differed in at least one aspect, i.e., the cytoplasts contained very low amounts of TIMP-1 as compared to the intact cells, while the content of CXCL-8 was in the same range as that of neutrophils (Paper I [54]). This suggests that the TIMP-1-containing organelle leaves the neutrophil along with the granules and vesicles during cytoplast preparation, while the CXCL-8-containing organelle remains.

Neutrophil ER and Golgi

Newly produced proteins are processed and sorted in the ER and Golgi apparatus before they are routed to their intracellular destinations or delivered to the plasma membrane. Therefore, ER/Golgi function is crucial in cellular biology, and the mechanisms that govern protein processing within the ER have been extensively studied in most cell types. However, in the mature neutrophil, the ER is very sparse, which goes some way towards explaining why this has been a neglected area of research to date. Nonetheless, over the past few years, attention has been focused on the ER in the contexts of phagocytosis and antigen expression. Thus, the neutrophil ER has also attracted interest.

Molecular chaperones are highly important features of the ER, as these molecules co-operate to aid protein processing and folding. Moreover, the chaperones prevent misfolded proteins from escaping prematurely from the ER. A very common modification of proteins within the ER is the addition of N-linked glycans, and one of the best-characterized chaperone systems is the calnexin-calreticulin cycle, which co-ordinates this type of glycosylation process [121-123]. The major proteins of this machinery are calnexin, which is an

integral 90 kDa membrane protein, and calreticulin, which is the 60 kDa, soluble, lumenal partner of calnexin [124]. In neutrophil precursors and HL-60 cells, these two proteins are known to participate in the biosynthesis of MPO [125, 126].

In order to study the subcellular localization of the ER in mature neutrophils, we used calnexin as the primary marker. In density gradients, calnexin and calreticulin were found in light membranes that were distinct from both the secretory vesicles and plasma membrane, as shown in Paper III.

THE INVOLVEMENT OF THE ER IN PHAGOCYTOSIS

The conventional view of phagocytosis is that of the phagocyte enclosing the ingested prey into a phagocytic vacuole, which is made up almost exlusively of the plasma membrane [16]. However, over the past few years, an alternative mechanism for phagocytosis by macrophages has evolved, challenging the old paradigm in that it describes phagosomal formation as a result of recruitment of the ER to the site of particle entry [17]. This phenomenon is referred to as ER-mediated phagocytosis [18] and its validity is strongly debated [19-21]. Whether or not the proposed theory applies to human neutrophils has not been resolved, probably due to the scarcity of ER in these cells. However, given that mature neutrophils are capable of protein synthesis, the neutrophil ER may well possess more functions than previously thought. Therefore, we found it of significant interest to evaluate the possible involvement of the ER in neutrophil phagocytosis.

To address this task, we chose to study the presence of the ER protein calnexin in neutrophil-like HL-60 cells, which are developmentally arrested in the promyelocytic stage. Thus, these cells presumably contain more ER than mature neutrophils (see below). In Paper III, HL-60 cells were allowed to phagocytose bacteria or latex beads and the distribution of calnexin was studied and compared to those of azurophil granule markers, i.e., CD63 and MPO, thereby defining the presence of phagolysosomes. Using an immunofluorescence (IF) technique, we were able to locate phagosomes that were characterized by a distinct circular pattern of CD63 staining, accompanied by staining for the content marker MPO. However, under no circumstances could we detect calnexin staining in the phagosomal membranes, why we conclude that HL-60 cells do not use the ER as a membrane source during phagocytosis, and this presumably also applies to mature neutrophils.

HL-60 cells

The human leukemic cell line HL-60 was originally derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia [127]. These

cells correspond to neutrophil precursors and are developmentally arrested at the promyelocyte stage of myelopoeisis. In culture, HL-60 cells are thus predominantly promyelocytes, although in the presence of dimethylsulfoxide (DMSO) [128] or all-*trans* retinoic acid (ATRA) [129] they differentiate into myelocytes, metamyelocytes, band cells, and eventually, segmented neutrophils [130]. During differentiation, HL-60 cells mature without developing all the granules and vesicles of the neutrophil. In fact, azurophil granules are the only granules that are found in these cells, and it seems that differentiated HL-60 cells are unable to transcribe specific and gelatinase granule proteins or to form storage granules [39, 130]. The process of phagocytosis *in vitro* is very similar in neutrophils and HL-60 cells (Tapper et al., unpublished observations), making HL-60 cells a suitable model cell in which to study phagocytic uptake of, for example, bacteria. The fact that HL-60 cells display far more ER than the mature equivalent cells encouraged us to use this cell line in the studies described in Paper III.

The cytoplast as a model cell

Neutrophil cytoplasts are defined as enucleated cells that are depleted of granules and vesicles. These virtually empty cells, although artificial, are very useful in studying subcellular structures that are distinct from the classical granules, since they represent a reductionistic system. In several experiments with neutrophil cytoplasts, we noted that despite their harsh treatment during transformation from neutrophils into cytoplasts (see below), the cytoplasts contained intracellular structures that did not correspond to any of the classical granules or vesicles (Fig. 5). Previous findings have demonstrated that cytoplasts retain many neutrophil functions, e.g., phagocytosis (as demonstrated in Paper III) and killing of certain bacteria, chemotaxis, and metabolic activities, such as radical production following stimulation [131]. Although similar to neutrophils in some aspects, morphologically, cytoplasts and their parent cells differ significantly. The surface area of a cytoplast is about 30% of that of the neutrophil plasma membrane. Moreover, the cytoplast plasma membrane is smoother than the neutrophil surface, displaying fewer protrusions and clefts, as is evident in the SEM images in fig. 6. The volume of a cytoplast is approximately 25% of the neutrophil volume, and its diameter is about half the diameter of the neutrophil [131].

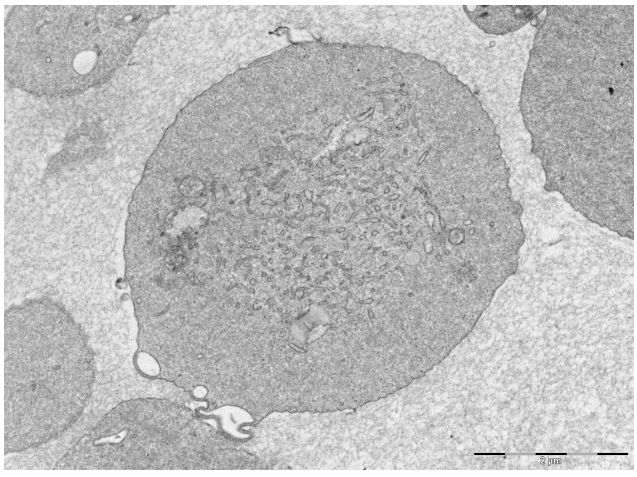


Figure 5. Neutrophil cytoplasts prepared for standard TEM. Thin section (60 nm), magnifycation ×15,000 (S. Pellmé, 2005).

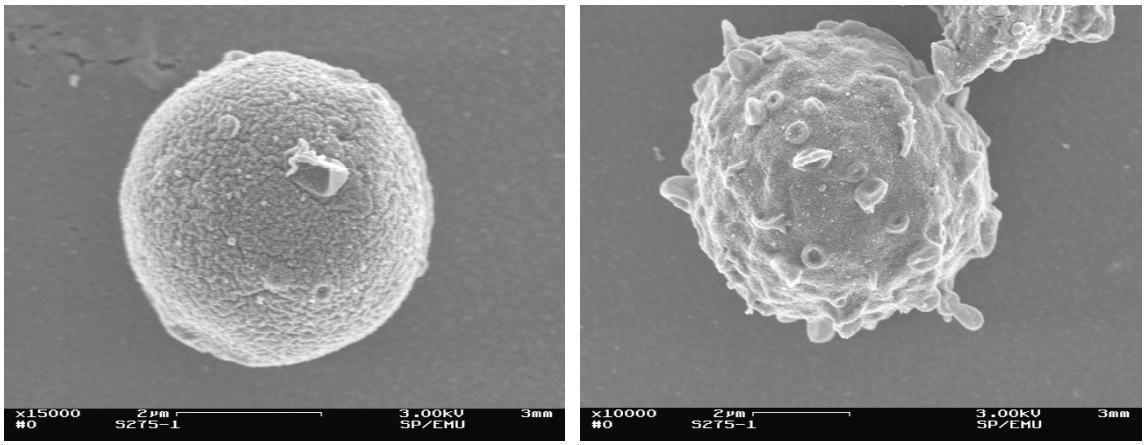


Figure 6. SEM images of a cytoplast (left) and a neutrophil (right).

In the present work, cytoplasts were used for several reasons. The cytoplasts facilitated observations of subcellular structures that could not with certainty be analyzed in the presence of granules (Paper II). It is clear that cytoplasts contain intracellular membrane structures, even though they are considered to be completely emptied with respect to granule content. The amount of CXCL-8 in cytoplasts was found to equal that in neutrophils, which suggests that the CXCL-8-containing organelle is not removed together with the granules. This finding may prove to be useful in our future investigations into the identity of the CXCL-8 organelle (Paper I).

COMMENTS ON THE TECHNIQUES USED FOR STUDYING THE INTRACELLULAR COMPARTMENTS OF THE NEUTROPHIL

Subcellular fractionation

Subcellular fractionation of cavitated human neutrophils represents an excellent tool for the isolation and biochemical characterization of intracellular structures; currently, the protocols for these experiments are highly elaborate and efficient. Protocols for subcellular fractionation using sucrose as the gradient medium have also been described. However, these protocols suffer from some disadvantages. First, the relatively high osmolality of sucrose generates hypertonic gradients, in which the organelles are likely to shrink [132]. Second, the traditional gradient protocols often involve high concentrations of heparin in order to prevent aggregation of granules [133].

A method that is commonly used is one that is based on nitrogen cavitation to disrupt the neutrophils, with subsequent separation of the organelles on discontinuous Percoll gradients. The original protocol was described by Borregaard and coworkers in 1983 [66] and have the advantage over sucrose-based techniques in that it satisfactorily preserves the organelles and their contents for subsequent marker analysis. Another advantage of the Percoll protocol is the use of relaxation buffer, which significantly reduces aggregation of the granules after nitrogen cavitation [66], without interfering with the separation of the different organelles. We have used Percoll gradients throughout the work for this thesis, in order to isolate pure subcellular organelles in as gentle a manner as possible.

Marker analysis — choosing the appropriate markers

A prerequisite for proper analysis of subcellular fractions is the identification of suitable markers for the compartment of interest. In the case of neutrophil granules, an array of different markers for the classical granules and vesicles is available and widely used. An ideal marker should be compartment-specific, easy to detect in reproducible assays and in the case of plasma membrane markers, preferably not influenced by cellular activation. For this work we chose ALP as a marker for the plasma membrane on the basis that it could be used to verify the separation by detergent treatment of the secretory vesicles (also containing ALP) from the plasma membrane.

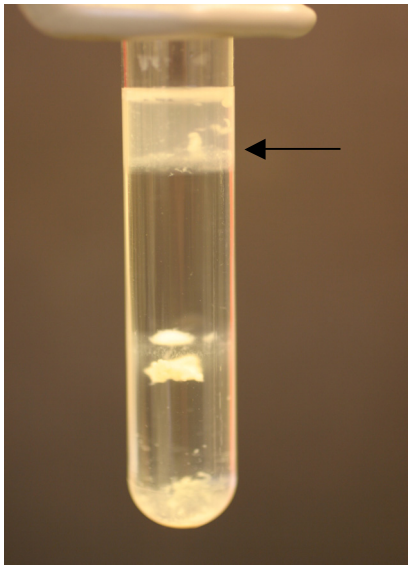
It is important to point out that in a gradient, no matter how carefully it is prepared, one will always see some overlapping of marker distributions. This is due, at least in part, to the fact that the formation of granules during neutrophil maturation is a continuum, always generating granules that co-store proteins not

normally present together in the granules that meet the definition of the classical granules. In order to be conclusive, the biochemical data obtained from fractionation studies should, if possible, be combined with the findings generated by immunogold electron microscopy , which clearly show colocalization/separate location of different subcellular markers.

Neutrophil cytoplasts as a model system

In an attempt to isolate subcellular structures that are distinct from the granules and vesicles of the neutrophil, the use of ghost neutrophils, i.e., cytoplasts, appears to be a valid approach. Since the cell remnants are depleted of nuclei, granules, and secretory vesicles, the cytoplasts are seemingly empty and thus provide a reductionist system.

During the preparation of cytoplasts, as originally described by Roos [131], the neutrophils are treated with cytochalasin B in order to disrupt the cytoskeleton. The cells are then layered on top of a prewarmed Ficoll gradient and centrifuged at high speed. The neutrophils move down the gradient, and as this happens, the granules and nuclei are mechanically removed. Upon reaching the most-dense layer of Ficoll, the neutrophils are emptied, and the so-formed cytoplasts, now with a much lower density, float and are easily recovered at the interface of the 17% and 12.5% Ficoll layers (Fig. 7).



Under normal circumstances, the yield in cytoplast preparations is approximately one cytoplast per neutrophil. Thus, this is a powerful tool for subcellular fractionation experiments, in that it acquires a large number of cells. As described in this thesis, cytoplasts are applicable as models in fractionation studies and as possible tools for the isolation of non-classical structures. However, it should be noted that cytoplasts are artificial “cells”, and the results achieved in studies performed on these entities must be evaluated accordingly.

Figure 7. Cytoplast gradient. Order of layers from the bottom of the gradient: nuclei and granules, cell debris, cytoplasts (indicated by the arrow).

Electron microscopy

Transmission electron microscopy and immunogold labeling are valuable techniques in cell biology, allowing the visualization of structures and proteins that are otherwise not detectable with high-resolution microscopy techniques. However, as amazingly complex and stunning as the resulting images appear,

they require careful examination and evaluation in order to be conclusive. Large sample areas need to be studied to ensure representative images and the choice of antibodies is crucial to rule out non-specific staining and false positives.

A drawback of immunogold labeling is the difficulty associated with choosing the right fixative for sample preparation. Fixation of the cells prior to embedding and sectioning may interfere with antibody-mediated detection by altering the antigenicity; generally, the use of a low percentage of glutaraldehyde is preferred. On the other hand, this type of fixative may result in the leakage of granule proteins, in particular those of low molecular weight [134]. In double-labeling experiments, the binding of the first label may interfere with the binding ability of the second label, which is a problem that can be overcome by labeling the sections in the reverse order.

During the generation of all the EM images presented in this thesis, we have taken every precaution to provide the reader with conclusive data. Nevertheless, EM is and should be used as a complement to other biochemical studies.

CONCLUDING REMARKS

During my studies of the subcellular structures of the neutrophil, the complexity of this cell type has become increasingly evident. To the well-described and characterized classical granules and secretory vesicles, we can now add organelles of hitherto unknown origin and function, such as the CXCL-8-containing organelle and the TIMP-I-containing organelle. Moreover, in conflict with the historically accepted dogma, the ER and Golgi of mature neutrophils appear to have prominent functions in neutrophils. Excitingly, this opens up a new field of neutrophil research.

In Paper I of this thesis, the subcellular localization of naïve and *de novo* produced CXCL-8 was described. Using biochemical markers for the neutrophil granules in density gradients and immunogold labeling of TEM preparations, we discovered that CXCL-8 is not stored in any of the classical granules or vesicles of either circulating or activated neutrophils. In accordance with this finding, the pattern of secretion of CXCL-8 did not follow that of CR3, which serves as marker of classical degranulation. In the search for alternative stores of CXCL-8, the potential colocalization of this chemokine with the proposed lysosomes of the neutrophil, the MVBs and MLCs, was investigated. Since LAMP, which is a marker for MVBs and MLCs, did not colocalize with CXCL-8, these compartments were excluded as possible CXCL-8 stores. However, when studying the distribution of ER-associated proteins in the subcellular fractions, CXCL-8 was found to colocalize in part with calnexin, which is an integral component of the ER membrane. However, their patterns of distribution were however not perfectly coincinding, Therefore, we hypothesized that either CXCL-8 is stored in a subsection of the small, yet present, ER of mature neutrophils or it is stored elsewhere, in a location separate from the known intracellular structures.

The focus in Paper II was to evaluate HLA-I as a marker for the neutrophil plasma membrane. Previous work suggested that HLA-I in complex with β_2m might be a better marker of the plasma membrane than, for example, ALP, due mainly to the fact that HLA-I is unaffected by neutrophil activation and degranulation. However, during the analyses of fractionated neutrophils and cytoplasts, we obtained some anomalous results, leading us to question the specificity of HLA-I as a plasma membrane marker. After further fractionation studies, we conclude that not only is HLA-I present in the plasma membrane of mature neutrophils, but also in other light compartments, fractionating at somewhat higher densities in gradients. These HLA-I-containing structures were not discernible in the presence of granules, probably because they are masked by

granule-derived uncomplexed β_2m in the samples, permitting the detection of plasma membrane HLA-I only. We conclude, in contrast to what was claimed in the original publication [83], that uncomplexed β_2m indeed affects the accuracy of MELISA, which is the sandwich ELISA used to detect the β_2m/HLA-I complex.

As described in Paper I, the intracellular stores of CXCL-8 in human neutrophils are located in organelles that are distinct from the classical granules and vesicles but present in light membrane fractions after subcellular fractionation. They are retained in cytoplasts, in which they may be separated from the plasma membrane using flotation gradients. In these types of gradients, CXCL-8 partly cofractionated with the ER marker calnexin. Furthermore, CXCL-8, calnexin, and HLA-I were all found in membrane fractions that were denser than the plasma membrane. These results suggest the ER as well as the CXCL-8 organelle as possible storage depots for the HLA-I complex.

In Paper III, the subcellular distribution of the ER in mature neutrophils was determined. As in the neutrophil cytoplasts, the ER was localized to the light membrane fractions, in agreement with previous reports regarding Golgi localization. Mature human neutrophils are historically regarded as poor producers of proteins, due to the sparse distribution of ER and Golgi in these cells. However, given the finding (Paper I) that neutrophils do indeed produce proteins, such as CXCL-8, upon stimulation, it is obvious that the ER plays a role also in neutrophils. In the light of the ongoing debate regarding the involvement of the ER in macrophage phagocytosis, we decided to investigate the role of the neutrophil ER in the process of phagocytic uptake. Using the neutrophil-like HL-60 cell line, which comprises promyelocytic cells that contain more ER than mature neutrophils, we achieved calnexin staining of sufficient magnitude to allow certain conclusions to be drawn. Under none of our applied phagocytic conditions could we detect calnexin staining in the phagosomal membrane, which suggests that HL-60 cells, and presumably neutrophils, do not use the ER as a source of membrane components during phagosome formation, but rather apply the conventional mechanism of plasma membrane-mediated phagocytosis.

In conclusion, it is evident that human neutrophils contain, in addition to the classical granules and vesicles, yet other subcellular structures of unknown origin. In a subgroup of these structures, CXCL-8 is stored in manner that is time- and stimulation-dependent. We cannot rule out the possibility that neutrophil CXCL-8 is, at least partly, stored in the ER. However, the distribution patterns of CXCL-8 and calnexin in HL-60 cells during phagocytosis suggest that these two proteins are stored separately in the neutrophil, or are directed differently during the phagocytic process. The role of CXCL-8 in phagosome formation and microbial degradation is intriguing and should be the subject of further studies. Moreover, as neutrophils are capable of producing proteins, even

after leaving the bone marrow, it seems likely that the ER and Golgi exert more functions in these cells than was previously thought possible, opening up a new avenue of neutrophil research.

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