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Mitochondria in Neutrophil Apoptosis

B.J. van Raam

Mitochondria in Neutrophil Apoptosis

Bram van Raam

2008

The studies described in this thesis were performed at the department of Blood Cell Research, Sanquin Research and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

Mitochondria in Neutrophil Apoptosis

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Mitochondria in Neutrophil Apoptosis

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Outline and Scope of the Thesis

Introduction

Neutrophils are the most abundant phagocytes of the human immune system, with a primary function in phagocytosing invading micro-organisms and killing them by means of a rapid, NADPH-dependent, respiratory burst. Neutrophils have a very short life-span of up to 24 hours in the circulation and several more days in the tissues, after which they are programmed to die by apoptosis. The energy that neutrophils require for their functions is mostly derived from glycolysis¹, whereas most other cells derive a substantial part of their energy from oxidative phosphorylation (OXPHOS) by the respiratory chain, a series of four respiratory complexes (I-IV) within the mitochondria. In fact, it has long been thought that neutrophils do not possess any, or very few, functional mitochondria, since these organelles were hard to identify on electron micrographs and the cells did not seem to respond to OXPHOS inhibitors^{1,2}. Several years ago, the introduction of novel fluorescent probes and refined microscopy techniques led to the identification of a complex mitochondrial network in neutrophils^{3,4}.

The function of mitochondria is not restricted to metabolism. These organelles also play an important role in apoptosis. A number of proteins contained within the mitochondria, such as cytochrome *c*, Smac/DIABLO, AIF and Omi/Htra2, rapidly accelerate the process of apoptosis upon release^{5,6}. Although neutrophils contain very little cytochrome *c*, the other cell-death proteins are clearly present in their mitochondria^{4,7}. Since the mitochondria in neutrophils have no obvious role in cellular metabolism, it has been suggested that their function is restricted to the regulation of apoptosis⁴. However, neutrophil mitochondria do maintain a membrane potential ($\Delta\psi_m$), normally indicative of mitochondrial respiration and OXPHOS-activity^{3,4}.

Scope of the Thesis

The scope of this thesis is to elucidate the functions and properties of mitochondria in neutrophils, as well as to address the role these organelles play in neutrophil metabolism and apoptosis, respectively. **Chapter 1** provides an overview of the earlier literature on neutrophil mitochondria in apoptosis. In **chapter 2**, the metabolic properties of neutrophil mitochondria are described. Neutrophils appear to maintain $\Delta\psi_m$ by respiratory chain complex-III activity only, while respiratory supercomplexes, which are required for efficient electron-transfer along the respiratory chain, are mostly absent from neutrophils. **Chapter 3** addresses the functional consequences of this mitochondrial defect for neutrophils. It appears that, while neutrophil mitochondria produce a relatively large amount of reactive oxygen species (mROS) due to their inherent metabolic defect, these mROS do not induce apoptosis but fulfill an important role as signaling intermediates in neutrophil survival. **Chapter 4** places the metabolic properties of neutrophil mitochondria in the context of Barth syndrome (BTHS), a genetically determined mitochondrial disease associated with neutropenia. BTHS neutrophils produce elevated amounts of mROS and have a lowered $\Delta\psi_m$, but display normal survival in the presence of enhanced phosphatidyl serine (PS) exposure, normally a hallmark for apoptosis. The results described in this chapter indicated that neutrophil mitochondria might play an important role in the regulation of the intracellular Ca^{2+} homeostasis. **Chapter 5** discusses the role of intracellular free Ca^{2+} and calpains, Ca^{2+} -activated cysteine proteases, in neutrophil apoptosis. It appeared that the intracellular free Ca^{2+} levels rise steadily during neutrophil apoptosis, a process that accelerates apoptosis via calpain-mediated degradation of XIAP. The growth factor G-CSF prevented this rise in Ca^{2+} levels and thus prevented calpain activation and slowed down apoptosis. **Chapter 6** discusses a gene array study with neutrophils from healthy volunteers treated with G-CSF and dexamethasone, a common method for mobilizing neutrophils for granulocyte transfusion. From this study, it

appeared that G-CSF also inhibits calpains by enhancing the expression of calpastatin, the endogenous inhibitor of calpains. Finally, **chapter 7** discusses the role of the mitochondria as Ca^{2+} buffers and the functional association of the mitochondria with the ER, the main intracellular Ca^{2+} storage. The anti-apoptotic Bcl-2 family member Bfl-1 appeared to have a role in the dissociation of these organelles during apoptosis, but the exact nature of this role has not yet been elucidated.

In sum, this thesis demonstrates the complex and essential role that neutrophil mitochondria have, both in the process of apoptosis and in mediating survival as well as in the continued functioning of the cells. These findings imply that mitochondria play a more complex role in cell death and survival than previously assumed.

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Introduction: Mitochondria in neutrophil apoptosis

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Abstract

Central in the regulation of the short life span of neutrophils are their mitochondria. These organelles hardly contribute to the energy status of neutrophils but play a vital role in the apoptotic process. Not only do the mitochondria contain cytotoxic proteins that are released during apoptosis and contribute to caspase activation, but they also act as sensors of the metabolic and redox state of the cell and as scavengers of free Ca^{2+} . The balance of the expression and activity of the pro- and anti-apoptotic members of the Bcl-2 family of proteins determines the life span of neutrophils, since these proteins are essential for the formation of a permeability transition pore in the mitochondria and control the release of Ca^{2+} from the endoplasmic reticulum.

Introduction

Neutrophils are short-lived cells which are cleared by apoptosis within approximately 48 hours after recruitment from the bone marrow. The turnover of neutrophils in humans is high, at about 1.6×10^9 cells/kg body weight per day¹. Tight regulation of the neutrophil life span is crucial, since an extended life span may lead to chronic inflammation, whereas a shortened life span can lead to neutropenia, rendering the body prone to infections²⁻⁸.

Although both pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF- α), and bacterial products, such as lipopolysaccharide (LPS), may extend the life of neutrophils, these cells are rapidly cleared after resolution of the infection⁸⁻¹⁸. Neutrophil apoptosis may be triggered either extrinsically through, for example, Fas ligation or ligation of the TNF-related apoptosis-inducing ligand (TRAIL) receptor on senescent neutrophils, or intrinsically^{12,19-21}. The exact mechanism of intrinsically regulated or spontaneous neutrophil apoptosis is still under debate, although it has become clear that mitochondria play a crucial role in the regulation of this process²².

Caspases, cysteine proteases that are involved in both apoptosis and inflammation, are activated during both intrinsically-regulated and death-receptor-mediated apoptosis of neutrophils^{23,24}. This review will mainly focus on the process of spontaneous neutrophil apoptosis and the role of mitochondria therein.

Mitochondrial functions in neutrophils

It has been assumed for a long time that mature neutrophils possess no or only very few functional mitochondria, and that these organelles play no role in the cellular functions. This assumption was based on the fact that these organelles cannot be readily identified in electron micrographs of mature neutrophils, while the few mitochondria that are found are small, with poorly defined cristae and overall morphology²⁵. Furthermore, treatment of neutrophils with respiratory chain inhibitors such as antimycin A, sodium azide or cyanide does not affect the phagocytic ability of the cells *in vitro* and neither does it seem to affect intracellular ATP levels or oxygen consumption of either phagocytosing or resting neutrophils^{26,27}.

Studies on neutrophil metabolism have shown that these cells can easily rely on glycolysis alone for their energy requirements^{26,28}. This ability allows neutrophils to operate in an inflammatory environment where oxygen may be scarce, while the cells themselves convert all the available oxygen into reactive oxygen species to eliminate invading pathogens. In recent years, however, it has become clear that neutrophils do possess mitochondria that can be stained with various mitochondria-specific dyes and form a complex tubular network in the cells^{22,25}.

Neutrophil mitochondria maintain a membrane potential which can be disrupted by chemical uncouplers of electron transport, and also drops during the initiation of apoptosis^{22,25}. After staining with the fluorescent dye JC-1,

which forms red fluorescent j-aggregates in hyperpolarized (high membrane potential) mitochondria but emits green fluorescence when the mitochondrial membrane potential is low, the mitochondrial network in neutrophils stained both red and green on fluorescence micro-graphs²⁵. A similar pattern of hyperpolarized and depolarized patches in a mitochondrial network has been reported in other cell types and has been suggested to reflect an uneven distribution of proton circuits, ATP synthesis, respiration or localized Ca^{2+} inside mitochondria²⁹. Another indication for the imperfect function of neutrophil mitochondria is the fact that these mitochondrial networks can be stained with dihydro-rhodamine-1,2,3 (DHR) after about 15 minutes of incubation²⁵. DHR is colourless, but becomes fluorescent upon oxidation by, for example, H_2O_2 . This indicates that within the neutrophil mitochondrial network rather large amounts of reactive oxygen species (ROS) are generated, indicative of imperfect respiration. However, disruption of the mitochondrial membrane potential does not impede the rapid respiratory burst generated by phagocytosing or phorbol 12-myristate 13-acetate (PMA)-treated neutrophils, but it does disrupt chemotaxis, even at low concentrations of the uncoupler.²⁵ Thus, mitochondria-derived ROS and ATP do not seem to be required in the generation of a functional respiratory burst, but mitochondrial ROS may be important signalling mediators in the initiation of apoptosis³⁰.

Upon induction of apoptosis pro-apoptotic proteins such as Smac/DIABLO (Second mitochondria-derived activator of caspase/Direct IAP-binding protein with low pI), Omi/HtrA2 (Omi/High temperature-requirement protein A2) and cytochrome *c* have been shown to be released from neutrophil mitochondria.^{9,22,31,32} The pro-apoptotic Bcl-2 family members Bad (Bcl2-antagonist of cell death), Bax and tBid (truncated BH3-interacting domain death agonist) were also shown to translocate to the mitochondria of apoptotic neutrophils, where they collaborate to form a permeability transition pore (PTP) that facilitates the release of the previously mentioned pro-apoptotic proteins^{9,33,34}. The anti-apoptotic Bcl-2 family members Bcl-X_L (Bcl X-linked), Bfl-1/A1 and Mcl-1, on the other hand, have been shown to promote neutrophil survival by preventing the formation of a PTP in neutrophil mitochondria^{10,21,34,35}.

However, death seems to be inevitable for neutrophils. While apoptosis can be delayed by growth factors such as granulocyte colony-stimulating factor (G-CSF) or granulocyte/macrophage colony-stimulating factor (GM-CSF), the process of cell death can never be prevented in neutrophils^{9,31,33,36}.

Bcl-2 family members in neutrophil apoptosis

The Bcl-2 family of proteins represents a major checkpoint in the regulation of neutrophil apoptosis. These proteins reside either in the mitochondria or in the cytosol and dimerize after activation, forming the PTP and releasing the contents of the mitochondrial intermembrane space into the cytosol. This includes cytochrome *c*, Smac/DIABLO and apoptosis-inducing factor (AIF), which accelerate the process of apoptosis by inducing caspase activation, as will be discussed below. Some 20 members of the Bcl-2 family have thus far been identified, of which some are anti-apoptotic and others are pro-apoptotic³⁷⁻³⁹.

The Bcl-2 family members are classified by sequence homology in four alpha-helical segments, called BH1-BH4. The anti-apoptotic members, which are highly conserved, contain all four BH domains, of which the BH1-BH3 domains structurally form a pocket in which the BH3 domain of the other members can bind. The pro-apoptotic members on the other hand can be subdivided by the number of BH domains they possess. Bax, Bak and Bok possess the BH1-3 domains while the other pro-apoptotic members (*e.g.* Bid, Bad and Bim) only possess the BH3 domain. Generally, the BH3-only members act in concert with Bax, Bak or Bok to form the PTP^{38,39}.

The formation of the PTP is Ca^{2+} dependent and is believed to require docking of the pro-apoptotic Bcl-2 family members to cardiolipin in the mitochondrial membrane^{30,39}.

It has been shown that Bax, Bak and tBid, the truncated and activated form of Bid, not only localize to the mitochondria upon induction of apoptosis, but also to the endoplasmic reticulum (ER)⁴⁰⁻⁴². Since the ER is the main storage of Ca^{2+} in cells, release of Ca^{2+} from the ER by Bax, Bak and tBid may contribute to the formation of a mitochondrial PTP. The release of Ca^{2+} from the ER has recently been shown to be controlled by the anti-apoptotic Bcl-2 family member Bcl-X_L. Bcl-X_L associates with the carboxyl terminus of the inositol 1,4,5-trisphosphate receptor (IP₃R) and Ca^{2+} channel in the ER⁴¹. This association renders the receptor more sensitive to IP₃, which results in a steady release of Ca^{2+} from the ER. Bax and tBid were shown to antagonize this association, resulting in an increased Ca^{2+} concentration in the ER and leading to a sudden, uncontrolled release of Ca^{2+} in response to increased concentrations of IP₃.

When the local concentration of Ca^{2+} released from the ER is high enough, Ca^{2+} can enter the nearby mitochondria through the voltage-dependent anion channel (VDAC), which eventually results in apoptosis^{43,44}. The relevance of these mechanisms for neutrophil apoptosis remains to be shown, although local Ca^{2+} signalling is certainly relevant in neutrophil apoptosis^{5,7,45-47}.

The action of the pro-apoptotic Bcl-2 family members can be counteracted by phosphorylation. Both Bax and Bad were shown to be phosphorylated, and thereby inactivated, in neutrophils as a result of stimulation by, for example, Toll-like receptor (TLR) agonists or GM-CSF^{10,33}. Phosphorylation was dependent on the Phosphoinositide 3-kinase/Protein kinase B (PI-3K/PKB) pathway. However, activation of this pathway also leads to increased expression of the anti-apoptotic Bcl-2 family members Mcl-1 and Bfl-1 in neutrophils, leading to a further decrease in apoptosis^{9,10,48,49}.

Forkhead transcription factors (FOXO) are also involved in the regulation of the expression and actions of the Bcl-2 family members in neutrophils. Neutrophils stimulated with the bacteria-derived chemoattractant formyl-Met-Leu-Phe (fMLP), for example, showed an increased expression of Mcl-1 and concomitant phosphorylation of FOXO1, FOXO3a and FOXO4 through activation of PI-3K/PKB and mitogen-activated kinases (MAPKs). Phosphorylated FOXO1 was furthermore shown to be associated in a complex containing Mcl-1, presumably regulating its activity.⁵⁰ FOXO3a regulates the expression of the BH3-only protein Bim in T-lymphocytes and macrophages, but whether Bim is expressed in neutrophils is unknown⁵¹. However, FOXO3a does regulate the expression of Fas ligand (FasL) in neutrophils after stimulation with TNF α and interleukin-1 β (IL-1 β) by inhibiting its expression and thus preventing apoptosis during inflammation in a mouse model of rheumatoid arthritis⁵².

Thus, the Bcl-2 family members play an important role in controlling neutrophil apoptosis. Activation of these proteins eventually leads to the release of pro-apoptotic proteins from the mitochondria. Their role in the activation of caspases, which are the executioners of apoptosis, will be discussed in the next section.

Caspase activation in neutrophil apoptosis

Caspases are expressed as inactive zymogens and require processing for activation.⁵³ Activation of the extrinsically activated caspase-8 and -10 is initiated by ligation of a trans-membrane death receptor of the tumour-necrosis factor type-1 superfamily, such as Fas⁵⁴. One of the down-stream targets of caspase-8 is the Bcl-2 family member Bid, which, after truncation by caspase-8, triggers the release of pro-apoptotic factors from the mitochondria as has been mentioned in the previous section. Molecules such as Smac/DIABLO and Omi/HtrA2, which are released from the

mitochondria, initiate apoptosis by binding to the baculovirus IAP repeat (BIR) on the inhibitors of apoptosis (IAPs), thus preventing their inhibitory activity^{22,55-58}.

IAPs are normally associated with both caspase-9 and -3 and are thought to continuously target these caspases for degradation in the proteasome⁵⁵. Cytochrome *c*, which is also released from the mitochondria, activates caspase-9 by initiating the formation of the apoptosome, a multi-protein complex wherein caspase-9 is activated⁵⁹. Even though neutrophil mitochondria contain very low amounts of cytochrome *c*, this amount is probably sufficient to activate the apoptosome in neutrophils⁶⁰. Activated caspase-9 can cleave and activate the executioner caspases, which in their turn cleave a number of target proteins, resulting in apoptosis of the cell. Although caspase-8 can also activate caspase-3 directly, mitochondrial amplification seems to be required for effective apoptosis in most cells, including neutrophils²².

Reactive oxygen species and neutrophil apoptosis

The primary function of neutrophils is to ingest and kill micro-organisms by means of an NADPH-dependent respiratory burst. Their metabolism is adapted for this role: a high rate of glycolysis guarantees a steady supply of NADPH and allows the cells to survive and function under anaerobic conditions²⁶. While bacterial products such as LPS are known to prolong neutrophil survival, phagocytosis and killing of pathogens seem to induce apoptosis^{16-18,49,61}. These two mechanisms guarantee that neutrophils remain alive and active when bacteria are present, while neutrophils that have fulfilled their task and are loaded with killed bacteria are rapidly cleared.

The mechanisms of phagocytosis-induced apoptosis are not quite clear although a role for ROS, NADPH-oxidase derived or not, has been proposed¹⁸. Watson *et al.* tested the effect of different antioxidants on *E. coli*-induced neutrophil apoptosis and found that the $\cdot\text{OH}$ scavenger dimethyl sulfoxide (DMSO) and the H_2O_2 scavengers glutathione (GSH) and *N*-acetylcysteine (NAC) inhibited this form of apoptosis, while they had no effect on spontaneous neutrophil apoptosis¹⁸.

In most cell types an increase in ROS leads to DNA damage as well as to oxidation of polyunsaturated fatty acids in the cell membrane. DNA damage induces the activation of Poly(ADP-ribose) polymerase 1 (PARP-1), an enzyme involved in DNA repair. Overactivation of PARP-1, on the other hand, mediates the release of AIF from the mitochondria through an as yet unclear mechanism, causing a form of caspase-independent cell death⁶². However, PARP-1 is not expressed in neutrophils. AIF is present in the mitochondria of neutrophils but is not translocated to the nucleus upon induction of apoptosis²².

The mitochondrial translocation sequence at the N-terminus of AIF has to be cleaved off by calpain-1, a cysteine protease not related to the caspases, to release this protein from the mitochondria. Cleaved AIF reveals a nuclear translocation sequence and is consequently shuttled to the nucleus, where it becomes involved in peripheral chromatin condensation and large-scale fragmentation of DNA⁴⁷. Calpain-1 is activated in a Ca^{2+} - and ROS-dependent manner and has been shown to play an important role in neutrophil apoptosis, since inhibition of calpain reduces the rate of spontaneous neutrophil apoptosis^{5,7}. Calpain presumably enters the mitochondria through the PTP formed by tBid and Bax⁴⁷. However, its targets in neutrophils do not appear to include AIF, but calpain-1 is apparently involved in the degradation of the X-linked inhibitor of apoptosis (XIAP) and in the activation of Bax^{5,7}. This would suggest that either PARP-1 activity is required for the calpain-1-mediated cleavage of AIF, or that calpain-1 is unable to enter the mitochondria of neutrophils.

Mammalian AIF is not only involved in apoptosis but also contains a flavin adenine dinucleotide (FAD)-binding domain that is not required for the apoptotic function of AIF. AIF has both NAD(P)H oxidase and monohydroascorbate reductase activity in the mitochondria^{62,63}. Mice deficient in AIF have no defects in apoptosis but instead die during embryogenesis, while harlequin mice, which are AIF hypomorphs, are more instead of less sensitive to the induction of apoptosis⁶⁴. This suggests that the primary function of AIF is not in apoptosis but in the regulation of cellular metabolism. Whether and how AIF contributes to neutrophil metabolism is as yet unknown.

Another mitochondrial protein with a dual agenda is Omi/HtrA2. This protein was originally identified as a serine protease released from the mitochondria in response to oxidative stress that targets the IAP proteins. Omi/HtrA2 does not compete with caspases for binding of the IAP BIR domain, as Smac/DIABLO does, but degrades IAPs by virtue of its serine protease activity, causing increased caspase activation^{65,66}. In the absence of caspase activation, the serine protease activity of Omi/HtrA2 can also contribute to cell death, although its targets have not been identified⁵⁷. Omi/HtrA2, which is well conserved from bacteria through humans, has a role in the maintenance of mitochondrial homeostasis under normal conditions as well, but this role has not been clearly defined yet⁶⁷.

The intramitochondrial serine protease activity of Omi/HtrA2 has been shown to be required for caspase-independent cell death of neutrophils. In the presence of the pan-caspase inhibitor zVAD, neutrophils that are stimulated with TNF α still undergo a form of cell death, as described by Maiani *et al.* and Liu *et al.*^{68,69}. The ROS scavenger NAC and the respiratory chain inhibitor rotenone provided partial protection against this form of cell death but it was completely abrogated by inhibition of Omi/HtrA2^{57,68,69}. In neutrophils from patients with chronic granulomatous disease (CGD), which lack a functional NADPH oxidase, this form of cell death could also be induced, whereas it did not occur in mitochondria-deficient cytoplasts⁶⁸. This suggests that TNF α signals directly to the mitochondria, where it activates Omi/HtrA2, which is somehow responsible for the generation of ROS that enhance neutrophil cell death.

The potential cytotoxic effects of zVAD itself, as suggested by Cowburn *et al.*⁷⁰, are still unexplained, however, as is the role of Omi/HtrA2 in this process. The protective role of Omi/HtrA2 in the maintenance of mitochondrial homeostasis in neutrophils has thus far not been addressed at all. Since the protein has been suggested to respond to redox stress, and neutrophils usually expose themselves to a high level of redox stress during the respiratory burst, Omi/HtrA2 may have an important role in preventing necrosis in neutrophils and favouring apoptosis instead as a response to excessive redox stress⁶⁷.

Finally, neutrophils cultured under hypoxic or anoxic conditions show a delay in apoptosis. This effect is dependent on the activity of hypoxia-induced factor-1 (HIF-1) and NF- κ B p65^{71,72}. The transcription factor HIF-1 comprises two subunits (α and β), of which the β -subunit is stably expressed while the α -subunit is constantly degraded after hydroxylation. Low oxygen tension or an increase in succinate levels due to failing respiratory chain activity leads to stabilization of the α -subunit and consequent activation of HIF-1^{73,74}. The activity of HIF-1 leads to the expression of a large number of genes, including genes involved in glycolysis, angiogenesis and cell survival. Interestingly, the gene for Mcl-1, a protein that is very important for neutrophil survival, as mentioned above, has been identified as a putative HIF-1 target gene⁷⁵.

In conclusion, the redox and the metabolic state of the neutrophil clearly contribute to determining the cell's survival. Paradoxically, low mitochondrial respiration seems to prolong neutrophil survival, whereas a similar state would induce apoptosis in other cell types. Apparently, neutrophils are uniquely equipped to deal with these conditions.

Conclusion

Spontaneous neutrophil apoptosis is a complex process regulated at many different levels. However, all of the pathways that lead to neutrophil apoptosis converge at the mitochondria. These organelles not only act as containers for cytotoxic proteins but also as sensors for the metabolic and redox state of the cell and collectors of free Ca^{2+} , as summarized in figure 1^{22,30,43,71,72}. All of these functions seem to be required to safely guide the neutrophil into the apoptotic process without accidentally triggering the release of the toxic contents of the cell. It seems, in fact, not surprising that neutrophils become spontaneously apoptotic, but it is surprising that they remain alive long enough to perform their duties. It would, therefore, be interesting to investigate the functions of mitochondrial proteins such as AIF and Omi/HtrA2 in healthy neutrophils. Recent evidence also points towards a role for the Bcl-2 family of proteins outside the process of apoptosis^{42,67,76}. Especially Bid and Mcl-1, both oddballs in the family and highly expressed in neutrophils, are interesting targets for further investigation. The emerging role of HIF-1 in innate immunity in general and in neutrophil apoptosis in particular demonstrates that although we have learned much about neutrophil apoptosis in the past decades, we do not yet fully appreciate the complexity of the process.

Figure 1. Overview of the signalling pathways involved in neutrophil survival and apoptosis.

Growth factors such as G-CSF promote neutrophil survival by activating PI-3K that governs the activity of FOXO and HIF-1, promoting neutrophil survival by controlling the activity and expression of survival factors such as Mcl-1. A state of hypoxia and ROS, generated by the mitochondria, also contribute to this effect. Ligation of death receptors or intrinsically triggered apoptosis, leads to caspase cleavage, activation of Bid, the sudden release of Ca^{2+} from the ER and cell death. Central in these processes are the mitochondria (Mito).

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Mitochondrial membrane potential in human neutrophils is maintained by complex III activity in the absence of supercomplex organisation

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Abstract

Background

Neutrophils depend mainly on glycolysis for their energy provision. Their mitochondria maintain a membrane potential ($\Delta\psi_m$), which is usually generated by the respiratory chain complexes. We investigated the source of $\Delta\psi_m$ in neutrophils, as compared to peripheral blood mononuclear leukocytes and HL-60 cells, and whether neutrophils can still utilise this $\Delta\psi_m$ for the generation of ATP.

Methods and Principle Findings

Individual activity of the oxidative phosphorylation complexes was significantly reduced in neutrophils, except for complex II and V, but $\Delta\psi_m$ was still decreased by inhibition of complex III, confirming the role of the respiratory chain in maintaining $\Delta\psi_m$. Complex V did not maintain $\Delta\psi_m$ by consumption of ATP, as has previously been suggested for eosinophils. We show that complex III in neutrophil mitochondria can receive electrons from glycolysis via the glycerol-3-phosphate shuttle. Furthermore, respiratory supercomplexes, which contribute to efficient coupling of the respiratory chain to ATP synthesis, were lacking in neutrophil mitochondria. When HL-60 cells were differentiated to neutrophil-like cells, they lost mitochondrial supercomplex organisation while gaining increased aerobic glycolysis, just like neutrophils.

Conclusions

We show that neutrophils can maintain $\Delta\psi_m$ via the glycerol-3-phosphate shuttle, whereby their mitochondria play an important role in the regulation of aerobic glycolysis, rather than producing energy themselves. This peculiar mitochondrial phenotype is acquired during differentiation from myeloid precursors.

Introduction

Neutrophils are the main phagocytic cells of the human immune system. These cells are the first to arrive at a site of infection, where they kill ingested microorganisms by producing superoxide through a rapid, NADPH (nicotinamide adenine dinucleotide phosphate, reduced) oxidase-mediated, respiratory burst, in combination with an arsenal of proteolytic enzymes and anti-microbial peptides^{1,2}. Neutrophils derive most of the energy required for these and other functions from a high rate of glycolysis³. This ensures that neutrophils can function in an inflammatory environment where the oxygen tension may be low or even absent³⁻⁵.

Neutrophils contain relatively few mitochondria, which form a complex network inside the cell^{6,7}. It has been assumed that these organelles do not produce energy by respiration, as they do in other cells^{3,7}. However, neutrophil mitochondria can be stained with various mitochondria-specific dyes and do maintain a transmembrane potential ($\Delta\psi_m$), which is normally associated with respiratory chain activity and oxidative phosphorylation (OXPHOS) of adenosine diphosphate (ADP)^{6,7}.

$\Delta\psi_m$ is generated by a series of four protein complexes (complex I-IV, respectively) in the mitochondrial inner membrane, which transfer electrons derived from the oxidation of reduced nicotinamide adenine dinucleotide (NADH) by complex I (NADH-ubiquinone oxidoreductase) or from the oxidation of succinate by complex II (succinate-ubiquinone oxidoreductase). Complex I, complex III (ubiquinol-cytochrome *c* oxidoreductase) and complex IV (cytochrome *c* oxidase) are the proton exchangers that maintain $\Delta\psi_m$ by transferring electrons to a

subsequent complex or to molecular oxygen, with a concomitant transfer of protons out of the mitochondrial matrix. The final complex of the OXPHOS machinery, complex V (F_1/F_0 -ATPase), is a very efficient molecular motor that allows the protons to flow back through its subunit F_0 while utilizing the proton gradient for the phosphorylation of ADP to adenosine triphosphate (ATP) in the F_1 subunit^{8,9}.

$\Delta\psi_m$ can also be maintained by the adenosine nucleotide transporter (ANT) in collaboration with complex V. ANT is responsible for transporting ADP into the mitochondrial matrix in exchange for ATP. However, in the absence of respiration, ANT can transport ATP^{4-} into the matrix in exchange for ADP^{3-} and thus maintain $\Delta\psi_m$ by a net influx of negative charges⁹⁻¹². This mechanism is only functional if the F_1 -ATPase provides the necessary ADP by hydrolysing ATP, but it is independent of the proton exchanger F_0 .

Recent evidence has indicated that respiratory chain complexes can be organized in larger supercomplexes in the mitochondrial inner membrane^{13,14}. Supercomplexes of different composition have been described in various organisms, including yeast and mammals. In bovine heart and human skeletal muscle, these supercomplexes appear to be composed of a complex I monomer, a complex III dimer and up to four copies of complex IV, representing the largest forms of OXPHOS complex assemblies, also termed ‘respirasomes’^{13,14}. The inclusion of complex IV in the supercomplex significantly enhances the activity of complex I and complex III¹³. Thus, the efficiency of electron transfer along the respiratory chain is largely dependent on the composition of these respiratory supercomplexes.

Resting neutrophils consume little or no oxygen, and classical inhibitors of respiration, such as cyanide (KCN), an inhibitor of complex IV, do not affect neutrophil functions^{3,7}. In addition, neutrophil mitochondria contain very little cytochrome *c*, which would indicate a very low complex IV content and inefficient respiration^{7,15}. However, prolonged incubation with oligomycin (longer than 2 hours), an inhibitor of the F_0 -ATPase of the respiratory chain, does affect both NADPH oxidase activity and neutrophil chemotaxis⁶. This suggests that complex V is active and may play an important role in maintaining neutrophil functions.

In this report, we investigated how neutrophils maintain $\Delta\psi_m$ and whether their mitochondria still play a role in the regulation of their energy metabolism. We show that $\Delta\psi_m$ in neutrophils is maintained by residual respiratory chain activity, even though this activity is apparently hardly coupled to ADP phosphorylation. In addition, we show that the respiratory chain complexes in neutrophils lack supercomplex organisation, while these supercomplexes are present in peripheral blood mononuclear cells (PBMC) and the myeloid cell line HL-60. During differentiation to neutrophil-like cells, HL-60 cells lose mitochondrial supercomplex organisation, while gaining neutrophil-like abilities. We suggest that the lack of mitochondrial supercomplex organisation and the consequent lack of respiration is a requirement to maintain a high rate of aerobic glycolysis in order to fulfil the primary human neutrophil functions.

Results

Neutrophils retain some respiratory chain complex activity

To establish whether the respiratory chain could be responsible for maintaining $\Delta\psi_m$ in neutrophils, we determined the activity of the OXPHOS complexes in the isolated mitochondria of neutrophils, PBMC, and HL-60 cells. Although the mitochondrial content, as expressed by citrate synthase activity, was significantly reduced in neutrophils as compared to the other cells (Figure 1A), neutrophils still displayed respiratory chain enzyme activity. Complex II and complex V (F_1) displayed normal levels of activity, while some complex I and complex III activity was detected as well (Figure 1B). The activity of complex IV was severely reduced in neutrophils as compared to the control cells.

These results are in line with the observation that neutrophil mitochondria contain very little cytochrome *c*, an essential component of complex IV^{7,15}.

Even though complex I, III and IV displayed very little enzymatic activity, Western blotting showed the presence of the cornerstone subunits of these complexes in neutrophil mitochondria lysates, although the total amount of the OXPHOS proteins that could be detected on blot was significantly less than in the control cells (Figure 1C).

Figure 1. Mitochondrial content and respiratory chain enzyme activity in neutrophils, PBMC and HL-60 cells.

A) Neutrophils (PMN) contain significantly less mitochondria as expressed per unit citrate synthase activity. Data represent the mean ($\pm$ SD) of 13 (PMN), 6 (HL-60) or 16 (PBMC) different experiments performed in duplicate.

B) Neutrophils retain full complex II and V activity while the activity of the remaining complexes is significantly reduced. Data represent the mean ($\pm$ SD) of 10 different experiments performed in duplicate. Values were corrected for mitochondrial protein content and citrate synthase activity.

C) Crucial subunits of the respiratory chain can be detected in neutrophil lysates on Western blot. 20 μ g of mitochondrial protein was separated by SDS-PAGE and immuno-detected with anti-complex I (20 kDa), complex II (30 kDa), complex III (Core 2, 47 kDa), complex IV (COX II, 26 kDa) and complex V (α , 55 kDa) subunit antibodies. Blots exposed for 15 sec and 5 minutes are representative of three independent experiments.

Respiratory chain complexes in neutrophil mitochondria are not organised in supercomplexes

To visualise the organisation of the respiratory chain complexes in neutrophils and the control cells, isolated mitochondria from these cells were solubilised with digitonin before subjecting the samples to blue native(BN)-PAGE. The BN-PAGE gels were subsequently processed by denaturing SDS-PAGE to resolve the (super)complexes into their respective subunits, before visualising the essential subunit of each of the five OXPHOS complexes by immunoblotting.

In PBMC, complex III appears to form three distinct supercomplexes with complex IV of ~1.5 MDa, ~600 kDa and ~470 kDa (Figure 2A). Fully assembled complex I is only present in the largest of these three supercomplexes (~1.5 MDa), but a subcomplex of about 370 kDa of complex I in association with complex IV is also demonstrable. Complex II is not present in any supercomplex with the other respiratory chain complexes, as expected. Complex V showed apparent molecular masses of ~1.5 MDa (dimer), ~600 kDa (monomer), 470 and 370 kDa (F_1 subcomplexes), and even less (Figure 2A).

Complex III and complex IV appear outside any supercomplex in neutrophils, having apparent molecular masses of ~230 and ~370 kDa, respectively (Figure 2B). The level of expression of complex I and II was below the detection limit on the neutrophil blot (Figure 2B). In neutrophils, complex V was present in four super- and subcomplexes with molecular masses ranging from 1.5 MDa to 370 kDa, but the level of expression of these complexes was significantly less than in PBMC.

Figure 2. Supercomplex organisation of OXPHOS complexes in PBMC and neutrophils.

A-B) Mitochondria isolated from PBMC (**A**) and neutrophils (**B**) were solubilized in digitonin, and 200 μ g of mitochondrial protein was separated by BN-PAGE. Strips from the first dimension were excised and used for second dimension SDS-(12%)PAGE, transferred to PVDF membrane and immuno-detected with anti-complex I (20 kDa), complex II (30 kDa), complex III (Core 2, 47 kDa), complex IV (COX II, 26 kDa) and complex V (α , 55 kDa) subunit antibodies. Blots are representative of three independent experiments. High molecular weight purified proteins were used as molecular mass markers in the first dimension BN-PAGE: thyroglobulin, 669 kDa; ferritin monomer, 440 kDa; catalase 232 kDa; lactate dehydrogenase, 140 kDa.

Activity of complex III but not V is required for maintaining $\Delta\psi_m$ in neutrophil mitochondria

The fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide) was used to determine $\Delta\psi_m$ in freshly prepared cells. JC-1 forms red fluorescent J-aggregates when the positively charged dye accumulates at high concentration in functional mitochondria, while the monomeric form is green fluorescent at lower concentrations. Thus, changes in the ratio between the intensity of the red (Fl-2) to green (Fl-1) fluorescence indicate changes in $\Delta\psi_m$.

Because complex V displayed normal activity in neutrophils and represents the only remaining active complex capable of maintaining $\Delta\psi_m$ in neutrophil mitochondria, complex V was inhibited with increasing concentrations of either the F_0 -ATPase inhibitor oligomycin or the F_1 -ATPase inhibitor aurovertin B. No decrease in $\Delta\psi_m$ was observed in either neutrophils or PBMC (Figures 3A and 3B). However, oligomycin did cause a collapse of $\Delta\psi_m$ at concentrations above 1 μ M in both cell types, with or without glucose. This is probably due to an aspecific effect of the inhibitor at high concentrations in intact cells, since even the PBMC, which have a normal respiratory chain, were affected. Generally, titration with the complex V inhibitors induced a rise in $\Delta\psi_m$, as expected in cells with respiring mitochondria. After all, complex V normally lowers $\Delta\psi_m$ by allowing protons to flow back into the mitochondrial matrix along with the gradient produced by the other complexes.

Inhibition of complex III by antimycin A annihilated the $\Delta\psi_m$ in both cell types, with neutrophils slightly more sensitive to low concentrations of this irreversible inhibitor than PBMC (Figure 3C). The higher sensitivity of neutrophils to this inhibitor can be explained by the fact that neutrophils contain relatively small amounts of complex III as compared to PBMC (Figure 2). This observation indicates that complex III, although showing little cytochrome *c*-reducing activity in neutrophils, still plays an important role in maintaining $\Delta\psi_m$ in these cells.

Inhibition of complex I by titration of rotenone also reduced the $\Delta\psi_m$ in both neutrophils and PBMC in the absence of glucose, although the inhibitory effect was larger in PBMC (Figure 3D). In the presence of glucose, increasing concentrations of rotenone had a slightly protective effect on $\Delta\psi_m$ in neutrophils, whereas in PBMC rotenone did reduce $\Delta\psi_m$, albeit not as pronounced as in the absence of glucose. These diminished effects are probably due to a combination of complex II activity compensating for the loss of electron flux from complex I and of ATP hydrolysis by complex V.

Figure 3. Mitochondrial membrane potential in neutrophils and PBMC.

A-D) Neutrophils (■, □) and PBMC (●, ○) were incubated with (■, ●) or without (□, ○) 5 mM glucose. After 2 hours of pre-incubation at 37°C, the cells were incubated for an additional 2 hours in the presence of various concentrations of respiratory chain enzyme inhibitors. Afterwards, the cells were stained with the fluorescent dye JC-1, and fluorescence in FI-1 and FI-2 was determined with by flowcytometry. The ratio (FI-2/FI-1) represents the coupling efficiency ($\Delta\psi_m$) of the mitochondria as expressed as a percentage of the control (2 hour incubation without inhibitor). The average initial value for neutrophils was 3.32 ± 0.85 (SD) and for PBMC 3.41 ± 0.75 (SD). The F_0 inhibitor oligomycin (**A**) and the F_1 inhibitor aurovertin B (**B**) had a protective effect in all cells, demonstrating that reversal of complex V does not maintain $\Delta\psi_m$ in neutrophils. The complex III inhibitor antimycin A (**C**) completely reduced $\Delta\psi_m$ in all cells, while the complex-I inhibitor rotenone (**D**) did not affect neutrophils cultured with glucose. Data represent the means ($\pm$ SEM) of five independent experiments performed in duplicate.

Figure 4. ATP levels in neutrophils and PBMC after treatment with respiratory chain inhibitors.

A-F) Neutrophils (■, □) and PBMC (●, ○) were incubated with (■, ●) or without (□, ○) 5 mM glucose. After 2 hours of pre-incubation at 37°C, the cells were incubated for an additional 2 hours in the presence of various concentrations of the mitochondrial uncoupler CCCP (**A**), or inhibitors for the OXPHOS complexes I-V(F₁), rotenone (**B**), 3-nitropropionate (3NP; **C**), antimycin A (**D**), KCN (**E**), or aurovertin B (**F**), respectively. ATP levels were determined with a luciferase-based assay. Data represent

Neutrophil mitochondria control lactate production but hardly contribute to ATP levels

To investigate the role of respiratory chain enzyme activity in maintaining the ATP levels of intact neutrophils and PBMC, cells were incubated with or without 5 mM glucose in the presence of increasing concentrations of specific OXPHOS inhibitors. After 2 hours of starvation, followed by a 2-hour incubation period with the inhibitor, the cells were lysed and ATP and lactate levels were determined in the neutralised lysates (Figures 4 and 5, respectively).

ATP levels in the control cells, i.e. PBMC cultured without glucose, proved particularly sensitive to the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Figure 4A), to rotenone (Figure 4B), to antimycin A (Figure 4D) and to aurovertin B (Figure 4F). These compounds affected the ATP levels of the PBMC at a concentration of 100 nM or less. The complex II inhibitor 3-nitropropionate (3NP, Figure 4C), which was preferred over tenoyltrifluoroacetone (TTFA) in intact cells owing to the aspecific effects of the latter (data not shown), and the complex IV inhibitor KCN (Figure 4E) were only effective in reducing the ATP levels of the PBMC at concentrations

of $>100\ \mu\text{M}$. This is probably due to the hydrophilic nature of these inhibitors.

In neutrophils, the ATP levels remained largely unaffected by any of the inhibitors tested. Only CCCP (Figure 4A) caused a complete loss of ATP in neutrophils at a concentration of $10\ \mu\text{M}$. However, at this concentration the plasma membrane potential is also affected by this uncoupling agent (results not shown and ¹⁶). This may lead to compensation by the Na/K-exchangers and rapid consumption of ATP. At $100\ \text{nM}$ CCCP, the ATP levels in neutrophils incubated without glucose were reduced by about 30%, whereas this concentration did not affect the plasma membrane potential (results not shown). This suggests only a partial requirement for $\Delta\psi_m$ in maintaining ATP levels in neutrophils, since $\Delta\psi_m$ is completely lost at this concentration of CCCP (not shown). Similarly, the inhibitors KCN (Figure 4E) and aurovertin B (Figure 4F) decreased ATP levels in neutrophils by about 30% at concentrations above $100\ \mu\text{M}$ and $100\ \text{nM}$, respectively. In PBMC, these concentrations were much more effective, leading to a

Figure 5. Lactate levels produced by neutrophils and PBMC after treatment with respiratory chain inhibitors.

A-F) Neutrophils (■, □) and PBMC (●, ○) were incubated with (■, ●) or without (□, ○) 5 mM glucose. After 2 hours of pre-incubation at 37°C, the cells were incubated for an additional 2 hours in the presence of various concentrations of the mitochondrial uncoupler CCCP (**A**), or inhibitors for the OXPHOS complexes I-V(F₁), rotenone (**B**), 3-nitropropionate (3NP; **C**), antimycin A (**D**), KCN (**E**), or aurovertin B (**F**), respectively. Lactate levels were determined with an enzymatic assay. Data represent the means (± SEM) of three independent experiments performed in duplicate.

decrease in ATP to less than 10% of the normal levels. Apparently, the OXPHOS system only partly maintains ATP levels in starving neutrophils.

Cells incubated in the presence of glucose seemed almost impervious to inhibition of the respiratory chain, as indicated by ATP levels. However, lactate production in both cell types increased significantly in response to increased levels of the inhibitors (Figure 5). Even in the absence of glucose (and inhibitors), neutrophils displayed about five-fold higher lactate production than PBMC, indicative of the ability of the former cells to utilize glycogen as an alternative energy source ³. This may also explain why ATP levels in intact neutrophils were not as profoundly affected as in PBMC by mitochondrial inhibition in the absence of glucose (Figure 4).

In response to inhibition of complex I, II, III and V (F₁), the increase in lactate production in the presence of glucose was similar for both neutrophils and PBMC, although the starting level of lactate produced by neutrophils cultured in the presence of glucose was generally higher (Figure 5). However, in response to the complex IV inhibitor KCN, PBMC produced significantly more lactate than did neutrophils, further indicating the strong dependency on the intact OXPHOS system of the former cells in contrast to the latter, which are adapted to low levels of complex IV by shuttling electrons from complex III directly to molecular oxygen, thereby generating superoxide ⁶.

Neutrophils acquire their mitochondrial phenotype during differentiation

HL-60 cells can be differentiated to neutrophil-like cells in 7-9 days by stimulating the cells with 1.25% DMSO in the culture medium ⁷. During differentiation, HL-60 cells gain neutrophil-like morphology and functions. Up to 70% of the cells become double positive for the neutrophil markers gp91^{phox} and EMR3 ¹⁷, respectively (Figure 6A) while the cells also gain the ability to produce a respiratory burst after stimulation with formyl-methionyl-leucyl-phenylalanine (fMLP), the combination of platelet activating factor (PAF) and fMLP or phorbol myristate acetate (PMA) (Figure 6B). Before differentiation (Day 0), the supercomplex organisation in the mitochondria of HL-60 cells is similar to the organisation found in PBMC (Figure 6C). After 9 days of differentiation, HL-60 cells have lost some of this organisation. In particular, complex I expression is significantly reduced in the large 1.5 MDa supercomplex. In the 370 kDa supercomplex, complex I is no longer detectable while a complex I spot appears around the same molecular weight as complex II (~250 kDa). The expression of complex IV is also reduced during HL-60 differentiation. Especially the large 1.5 MDa supercomplex displays reduced levels of complex IV after differentiation. Similarly, expression of complex III is reduced in differentiated HL-60 cells, especially from the 600 kDa supercomplex.

While HL-60 cells lose supercomplex organisation, they gain resistance to the complex-I inhibitor rotenone (Figure 6D) and the complex-III inhibitor antimycin A (Figure 6E). In the presence of glucose, both differentiated and undifferentiated HL-60 cells are resistant to rotenone and antimycin A, but in the absence of glucose higher concentrations of these inhibitors is required to affect ATP levels in the differentiated cells than in the undifferentiated cells (Figures 6D and E). Differentiated HL-60 cells incubated in the presence of glucose respond to inhibitor titration by producing increasing amounts of lactate, just like neutrophils.

Figure 6. HL-60 cells lose supercomplex organisation and acquire neutrophil-like properties during differentiation. HL-60 cells were differentiated to neutrophil-like cells in 9 days. To demonstrate that HL-60 cells gain a neutrophil-like morphology and abilities during differentiation, the expression of the neutrophil markers gp91^{PHOX} and EMR3 was determined by flowcytometry (**A**) and their ability to produce a respiratory burst was determined by an Amplex Red assay (**B**). On day 9 of differentiation, approximately 60% of the cells stained positive for both neutrophil markers, while responding to all three stimuli (PMA, fMLP and the combination of PAF and fMLP) by producing a respiratory burst. Plots are representative of four independent experiments. **C**) Supercomplex organisation in HL-60 mitochondria before (Day 0) and after (Day 9) differentiation. Respiratory chain complexes were detected on Western blots of 2D-native/reduced SDS-PAGE gels with anti-complex I (20 kDa), complex II (30 kDa), complex III (Core 2, 47 kDa), complex IV (COX II, 26 kDa) and complex V (α , 55 kDa) sub-unit antibodies. Blots are representative of four independent experiments. During differentiation, HL-60 cells gain the metabolic properties of neutrophils (**D** and **E**). Undifferentiated (■, □) and differentiated (●, ○) HL-60 cells were incubated with (■, ●) or without (□, ○) 5 mM glucose. After 2 hours of pre-incubation at 37°C, the cells were incubated for an additional 2 hours in the presence of various concentrations of the complex-I inhibitor rotenone (**D**) or the complex-III inhibitor antimycin A (**E**) as indicated. Afterwards, ATP (left panels) and lactate (right panels) levels were determined. Data represent the means ($\pm$ SEM) of three independent experiments performed in duplicate.

Neutrophils can maintain $\Delta\psi_m$ by oxidation of glycerol-3-phosphate

Neutrophils have a relatively high rate of glycolysis, and this process seems thus to provide all the energy needed for phagocytosis and the respiratory burst³. During this process, NAD^+ is reduced, requiring NADH to be reoxidised to keep the glycolytic pathway running. This can be done by either reducing pyruvate to form lactate, which normally occurs under anaerobic circumstances, or by reducing dihydroxyacetone phosphate (DHAP) to form glycerol-3-phosphate (G3P). G3P can diffuse into the mitochondria, where it is reoxidised to DHAP by mitochondrial flavin adenine dinucleotide (FAD)-dependent glycerol phosphate dehydrogenase (mGPD) on the outer surface of the inner mitochondrial membrane. Electrons from G3P are subsequently transferred to complex III of the respiratory chain via ubiquinol.

In this respect, the response of both cell types to complex III inhibition was particularly striking, with a three-fold increase in lactate production (Figure 5D), as would be expected since the G3P shuttle delivers electrons directly to complex III. Isolated mitochondria from neutrophils can be energised by glycerol phosphate, as demonstrated by staining with tetramethylrhodamine-methylester (TMRM), proving that the mGPD is present and functional (Figure 7). The $\Delta\psi_m$ generated with glycerol phosphate as a substrate was significantly higher ($P < 0.05$) than with the complex I substrates glutamate/malate, the complex II substrate succinate or in the absence of substrate (Figure 7C).

Figure 7. Membrane potential of isolated neutrophil mitochondria.

Isolated neutrophil mitochondria were analysed by flow cytometry after staining with Mitotracker Green, to differentiate intact mitochondria from cellular debris, and TMRM to determine $\Delta\psi_m$.

A) Scatter plot displaying intact mitochondria, defined as Mitotracker-Green-positive events (FITC channel) with high side scatter in Q2. **B)** Intact mitochondria gained TMRM staining ($\Delta\psi_m$, PE channel) after incubation with mitochondrial substrates. The complex-I substrates glutamate/malate (green) and the complex-II substrate succinate (yellow) induced a lower $\Delta\psi_m$ than glycerol phosphate (blue). Graphs are representative of three independent experiments. **C)** Graphic representation of the pooled experiments. Relative $\Delta\psi_m$ is expressed as a percentage of the mean fluorescence intensity (MFI) in the PE channel of the mitochondria population (Q2 in Figure 7A). The MFI of glycerol phosphate (GlycP) treated mitochondria was set at 100% while the MFIs of the untreated (C-), glutamate/malate (Glut/Mal) and succinate (Succ) treated cells were expressed as a percentage of this value. The average original value for C- was 1701 ± 498 (SEM), for Glut/Mal 4442 ± 2689 (SEM), for Succ 5481 ± 3106 (SEM) and for GlycP 5838 ± 3139 (SEM). Bars represent the means ($\pm$ SEM) of three independent experiments.

Discussion

Even though neutrophils retain some activity of the respiratory chain complexes involved in OXPHOS, the present study shows that this activity is mostly required to maintain $\Delta\psi_m$. In PBMC and HL-60 cells, which have an active respiratory chain and do depend on OXPHOS activity to maintain their ATP levels, the respiratory chain is organised in supercomplexes. During differentiation of HL-60 cells to neutrophil-like cells, this supercomplex organisation is partly lost while the cells gain the metabolic properties of neutrophils. The inefficiency of neutrophil mitochondria to couple $\Delta\psi_m$ to OXPHOS can be explained by a decreased expression of the respiratory complexes I, III and IV, and the lack of their ability to assemble into supercomplexes.

Neutrophils possess a reduced copy number of mtDNA compared to HL-60 cells and PBMC, a defect that arises during differentiation⁷. Since most subunits of the respiratory chain complexes - and all subunits of complex II - are encoded in the nucleus, but several key proteins are still encoded by mitochondrial DNA (mtDNA), a decreased expression of all OXPHOS complexes except for complex II is not difficult to envision, but the consequences are far reaching. It has been shown in several human disorders that a defect in mtDNA leads to a different composition of the respiratory chain complexes and supercomplexes in skeletal muscle^{18,19}. However, assembly and stability of complex I not only depend on the correct information in the nuclear and mitochondrial genome, but also on whether respirasomes can be formed with complex III^{20,21}. While fully assembled complex I has a molecular mass of about 900 kDa, seven distinct complex I assembly intermediates ranging from 200 to 650 kDa have been identified in patients with complex I deficiency²². In addition, critical amounts of complexes III and IV are required for respiratory supercomplexes to assemble and to provide mitochondrial functional complementation¹⁹. Moreover, the expression of two forms of a supercomplex consisting of complex III and IV, namely III_2IV_2 and III_2IV_1 , and free complex III depends on the growth conditions of the cells¹⁴. In neutrophils, only free complex III was demonstrable. The importance of the remaining complex III is stressed by our finding that lactate production, while being already high from the beginning, still increases when this complex is inhibited, and the fact that $\Delta\psi_m$ in neutrophils is lost upon incubation with antimycin A. Complex IV showed only residual activity in neutrophils, although very high concentrations of the complex IV inhibitor KCN ($\geq 500 \mu M$) did affect the ATP levels in starved neutrophils. Complex IV did not form supercomplexes with other respiratory chain complexes. As a consequence of the minor activity of complex IV, the electrons that flow from the proximal end of the respiratory chain will remain in the Q-cycle of complex III unless they are transferred on a one-to-one basis to molecular oxygen. While we have shown that the residual components of respiratory chain are not important for the production of ATP, maintenance of the $\Delta\psi_m$ in neutrophils will thus be at the cost of a relatively high mitochondrial superoxide production⁶. The majority of the required energy is provided by the G3P shuttle via mGPD as indicated by our finding that inhibition of complex III, but not so much of complex I or II, leads to a significant increase in lactate production in neutrophils. This mechanism also explains the fact that rotenone only affects $\Delta\psi_m$ in neutrophils in the absence of glucose. Under such starvation conditions, pyruvate will not be converted to lactate, but its full energy content will be utilized to produce NADH and $FADH_2$ by which the remainder of complex I can play a (modest) role in maintaining $\Delta\psi_m$.

It is quite striking that the PBMC displayed such remarkable sensitivity for the mitochondrial inhibitors in the absence of glucose, as far as their ATP levels are concerned. Apparently, the absence of extracellular glucose is a strong stimulus for PBMC to utilize their mitochondria for their energy production, while aerobic glycolysis seems to be inhibited under these conditions. Starving cells may switch to fatty acid metabolism, which is completely

dependent on mitochondria. As a consequence, the cells become more sensitive to mitochondrial inhibition, as shown in figures 4 and 5. The ATP levels in undifferentiated HL-60 cells are affected by starvation alone, as shown in figure 6D and E. Apparently, these cells are not selected for the ability to use substrates other than glucose very efficiently. On the other hand, starvation alone is not sufficient to affect the ATP levels in the differentiated cells. This might indicate that differentiation stimulates the cells to store glucose in the form of glycogen.

Complex I was not detectable on the BN/SDS-PAGE of neutrophil mitochondria (Figure 2B), but only on a (denaturing) Western blot (Figure 1C). Its protein expression was estimated to be only about 1% of that present in HL-60 cells and about 6% of that in PBMC. In the latter two cell types, complex I apparently assembles into two supercomplex forms, of about 1.5 MDa with complex III and IV, and of about 370 kDa with complex IV only (Figures 2A and 6C). Since a fully assembled complex I has an apparent molecular mass of about 900 kDa, the smaller supercomplex in HL-60 and PBMC must, therefore, contain an assembly intermediate of complex I as found in complex-I-deficient patients²². The subcomplex II is a likely candidate, since it contains the subunit NDUF5 of 20 kDa to which the reference antibody was directed (Figures 2A and 6C) and has the proper apparent molecular mass of 230 kDa. We seem to detect this assembly intermediate around 230 kDa in differentiated HL-60 cells (Figure 6C), where it is not associated with either complex III or complex IV.

In neutrophils, the activity of complex V was comparable to that in PBMC and HL-60 cells, but its relative protein expression is 50% less than in PBMC and about 3-fold less than in the myeloid precursor HL-60. Besides the monomeric (~600 kDa) and dimeric (~1.5 MDa) forms, various subcomplexes of complex V were detected (Figure 2) including the assembly intermediates of about 370 and 470 kDa in the three cell types under study, and even smaller complexes still containing subunit α in PBMC and HL-60 cells. Such subcomplexes of complex V are indicative for mitochondrial biosynthesis disorders¹³. In that respect, neutrophils in particular resemble the mitochondrial DNA depletion syndrome¹⁸, in accordance with the low mtDNA copy number found earlier by us⁷. In such patients, subcomplexes of complex V lack the F_0 -subunit¹³. This generally leads to less efficient coupling of respiration to ADP phosphorylation^{13,18}. Recent evidence indicates that complex V has a strong influence on the shape and architecture of the mitochondria due to its size and the tendency of F_0 to form dimers in the mitochondrial membrane^{23,24}. A decreased expression of such dimers in neutrophil mitochondria might explain the peculiar tubular shape of these mitochondria^{6,7}, and their inability to couple $\Delta\psi_m$ to ATP synthesis.

In summary, we show for the first time that the $\Delta\psi_m$ in neutrophils is maintained by complex III of the respiratory chain in the absence of detectable complex IV activity and not by complex V, as has been suggested for eosinophils²⁵. This complex III activity does not contribute significantly to the energy status of the cells, while the energy for maintaining $\Delta\psi_m$ is mainly derived from the G3P shuttle (Figure 7). Thus, the role of neutrophil mitochondria is not restricted to apoptosis, but is also important in the regulation of glycolysis. Neutrophil mitochondria do not contain respirasomes and, as a consequence, they do not couple $\Delta\psi_m$ to efficient respiration and ATP synthesis. This particular phenotype probably arises during maturation from myeloid precursor cells, since we found that the mitochondria from HL-60 cells do contain respirasomes, while they lose some supercomplex organisation during differentiation to a neutrophil-like phenotype (Figure 6). These findings demonstrate the diversity of mitochondrial organisation within the human organism, and the implications of this organisation for cellular functions. In addition, they provide insight in the nature and consequences of mitochondrial biosynthesis disorders as well as indicating neutrophils as a model system for these disorders. The implications of these findings for neutrophil

survival warrant further investigation.

Materials and Methods

Primary cell isolation and culture

Neutrophils and PBMC were isolated from heparinised blood of healthy volunteers, after informed consent had been obtained, by density gradient centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) and erythrocyte lysis²⁶. PBMC were defined as the cells in the interface above the Percoll layer. Cells were cultured in Hepes-buffered saline solution (HBSS, 132 mM NaCl, 20 mM Hepes, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄, 1 mM CaCl₂, pH 7.4; chemicals from Merck) at a concentration of 10⁷ cells/mL in polypropylene round-bottom tubes of 14 mL (Falcon; BD; Franklin Lakes, NJ, USA). The medium was supplemented with 5 mM glucose where indicated. The incubation volume was never larger than 2.5 mL. All incubations were performed in a shaking water bath at 37°C.

Culture and differentiation of HL-60 cells

HL-60 cells were cultured in Iscove's modified Dulbecco's medium (BioWhittaker, Brussels, Belgium) supplemented with 10% heat-inactivated foetal calf serum (Gibco BRL, Paisley, United Kingdom), 100 IU/mL penicillin (Gibco BRL), 100 µg/mL streptomycin (Gibco BRL), 300 µg/mL L-glutamine (Gibco BRL) and 50 µM β-mercaptoethanol (Gibco BRL), at 37°C, 10% CO₂, until use.

Cells were differentiated in the same medium, supplemented with 1.25% dimethyl sulfoxide (DMSO; J.T. Baker BV, Deventer, The Netherlands) at a concentration of 0.5*10⁶ cells/mL. Every other day, the cells were counted and diluted to 0.5*10⁶ cells/mL in IMDM + 1.25% DMSO. On day 0 and 9 of the differentiation the cells were harvested. To determine the differentiation state, cells were tested for the expression of the neutrophil markers EMR3 (clone 3D7, biotinylated F(ab')₂ fragments, a kind gift from Dr. J. Hamann at the Academic Medical Centre in Amsterdam, The Netherlands¹⁷) and gp91^{phox} (clone 7D5, Sanquin, Amsterdam, The Netherlands). Labelling was performed in HBSS on ice for 20 minutes with the primary antibodies, after which the cells were washed and incubated with APC-labelled streptavidin (BD) to detect EMR3 and Alex Fluor-488-labelled goat-anti-mouse-IgG (Molecular Probes, Eugene, OR, USA) to detect gp91^{phox}. Fluorescence was detected with an LSRII flowcytometer (BD). Quadrant settings were based on the background staining of non-differentiated cells. In addition, morphology of the cells was routinely checked by microscopy of cytopspins stained with May-Grünwald-Giemsa stain.

NADPH-oxidase Activity

Activity of the NADPH-oxidase in differentiated HL-60 cells was determined with an Amplex Red assay (Molecular Probes) after stimulation with 1 µM fMLP, the combination of 1 µM fMLP and 1 µM PAF, added simultaneously, or 100 ng/mL PMA, as described²⁷.

Isolation of Mitochondria and Oxidative Phosphorylation Enzyme Activities

Cells were resuspended at a concentration of 5 mg/mL protein in mito buffer (0.2 mM EDTA, 0.25 mM sucrose, 10 mM Tris.HCl, pH 7.8) supplemented with a protease inhibitor mixture (PIM) of two Complete tablets per 50 mL (Roche Diagnostics, Almere, The Netherlands), 1 mM 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride (Pefabloc SC, Roche) and 2 mM diisopropyl fluorophosphate (DFP; Fluka Chemica, Steinheim, Switzerland), freeze/thawed in liquid nitrogen and, after the addition of 10 mM triethanolamine and 0.1 mg/mL digitonin, incubated for 10 min on ice, homogenised in a pre-cooled glass-Teflon Potter-Elvehjem homogenizer, and centrifuged at 1000xg for 10 min at 4°C. The supernatant was saved, the pellet resuspended in the same volume of mito buffer supplemented with 0.1 mg/mL digitonin, homogenised and centrifuged once again. The combined supernatants were centrifuged at 12000xg for 15 min at 4°C, and the mitochondria-rich pellet was resuspended in mito buffer at the desired protein concentration.

OXPHOS complexes were determined according to Birch-Machin & Turnbull²⁸ with some essential modifications. The spectrophotometric methods were scaled down by the use of sub microcell quartz cuvettes (Hellma GmbH, Müllheim, Germany) of 100 µL, by which the concentration of cells could be increased without requiring large amounts of blood. Complex I activity was determined as the rotenone-sensitive oxidation of NADH with ubiquinone₁ as electron acceptor (100 µM final concentration) at 340 nm, with 380 nm as the reference wavelength²⁹. To assess complex II activity the extinction difference between 600 and 520 nm was used to follow the ubiquinone₂-coupled TTFA-sensitive reduction of 2,6-dichlorophenolindophenol, with succinate as the substrate. Complex III was measured as the change in extinction difference between 550 and 540 nm for the antimycin-A-sensitive reduction of cytochrome *c* by ubiquinol₂. Complex IV was determined by following the oxidation of reduced cytochrome

c at 550 nm, with 540 nm as the reference wavelength in the presence of n-dodecyl- β -D-maltoside. Complex V was measured as oligomycin-sensitive Mg-ATPase activity immediately after brief sonication of the neutrophils according to Rustin *et al.*³⁰. All inhibitors and substrates were obtained from Sigma (St. Louis, MO, USA).

The OXPHOS complex activities were expressed per mg protein as determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Veenendaal, The Netherlands) with BSA as a standard, or normalized to the activity of citrate synthase determined according to Srere³¹. All assays were performed at least in duplicate.

Blue-Native PAGE and Western-blotting

Mitochondria were incubated in lysis buffer (50 mM NaCl, 5 mM aminocaproic acid, 50 mM imidazole, pH 7.0) supplemented with PIM and digitonin (4 g/g mitochondrial protein) for 10 min on ice, and 200 μ g of mitochondrial protein was further processed for first dimension BN-PAGE according to Schagger³² on a 5-13% acrylamide gradient gel. Strips from the first dimension BN-PAGE were then excised, reduced for one hour at room temperature in 1% SDS containing 4 μ L/mL tributylphosphine, and used for second dimension SDS-(12%)PAGE.

Western blotting was performed in a fully submerged Mini Trans-blot system (Bio Rad) using CAPS buffer (10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, pH 11, 10% methanol) on PVDF membrane, after which the cornerstone subunit of each of the five OXPHOS complexes were visualized with the OXPHOS Western blotting kit (MS601; Mitosciences), biotinylated anti-mouse IgG (RPN1001, Amersham Biosciences), streptavidin-biotinylated horseradish peroxidase complex (RPN1051; Amersham) and SuperSignal West Femto (Pierce). Images were acquired using a calibrated densitometer (GS-800; Bio-Rad) and the PDQuest software package (version 6.2.1; Bio-Rad).

Determination of $\Delta\psi_m$

To assess $\Delta\psi_m$ in neutrophils and PBMC, cells were incubated at 10^7 cells/mL in HBSS with or without 5 mM glucose. After 2 hours of pre-incubation at 37°C the cells were incubated for an additional 2 hours in the presence of various concentrations of the indicated inhibitors in a shaking incubator. All inhibitors were obtained from Sigma and dissolved in dimethyl sulfoxide (DMSO). The total amount of DMSO added to the cells was 1% (v/v) for all incubations.

After incubation, the cells were stained by addition of 0.5 μ M of the fluorescent dye JC-1 (all probes from Molecular Probes, Eugene, OR, USA) for an additional 15 minutes at 37°C. To determine a background value for $\Delta\psi_m$, cells were incubated with 1 μ M CCCP (Calbiochem, La Jolla, CA, USA), which is sufficient to completely abrogate $\Delta\psi_m$. Red (Fl-2) and green (Fl-1) fluorescence was subsequently determined on a BD FACScan flowcytometer. The red-to-green ratio was determined by dividing the geometric mean fluorescence in Fl-2 by that in Fl-1. This value was corrected by subtracting the value obtained from the CCCP incubation and expressed as a percentage of the DMSO control. All assays were performed in duplicate.

ATP and lactate assays

Incubation conditions were the same as mentioned above for $\Delta\psi_m$ determination. After incubation, the cells were lysed by the addition of 1.5% HClO₄ for 10 minutes on ice. After centrifugation the supernatant was neutralized with an equimolar amount of K₂CO₃. Insoluble KClO₄ was precipitated by quick centrifugation and the supernatant was stored at -80°C.

ATP levels were determined by adding 100 μ L of the firefly luciferase based adenosine 5'-triphosphate (ATP) assay mix (Sigma) diluted 1:10 in ATP-assay buffer (5 mM MgSO₄, 100 μ M EDTA, 100 μ M NaN₃, 4 mM DTT [DL-dithiothreitol, Sigma], in 25 mM TrisHCl, pH 7.8) to 10 μ L of the supernatant of the neutralized lysate in a white 96-well plate (Corning Life Sciences, Corning, NY, USA). Samples were compared with an ATP standard (Sigma) treated in the same way as the samples. Luminescence intensity was determined immediately on a Spectra Fluor Plus spectrophotometer (Tecan, Zurich, Switzerland).

To determine lactate levels produced by the cells, 20 μ L of the supernatant of the neutralized lysate was mixed with 100 μ L of lactate assay solution (Trinity Biotech plc, Wicklow, Ireland). After 15 minutes of incubation at room temperature, absorbance was measured at 540 nm in the spectrophotometer.

Analysis of $\Delta\psi_m$ in isolated mitochondria

The mitochondrial fraction from $\sim 150 \times 10^6$ neutrophils was resuspended in 2 mL of mitochondria assay buffer (120 mM KCl, 5 mM KH₂PO₄, 1 mM EDTA, 1 mM MgCl₂, 3 mM Hepes, pH 7.8, 1% (v/v) human serum albumin; Sanquin, Amsterdam, The Netherlands) and divided in 100- μ L portions that were either left unsupplemented or supplemented with either 10 mM *sn*-glycerol 3-phosphate, the combination of 10 mM glutamate and 2 mM malate, or 10 mM succinate.

After 1 hour of incubation at 37°C, the mitochondria were stained for an additional 15 minutes with 1 μ M Mitotracker

Green, to differentiate intact mitochondria from cellular debris, and 100 nM TMRM to determine $\Delta\psi_m$. Samples were analysed on a LSRII flow cytometer (BD) with FACSDiva software. To detect small particles, forward scatter (FSC) was set to 600 and side scatter (SSC) to 550. Intact mitochondria were defined as having high side scatter (SSC) and high Mitotracker staining (FITC channel). Compensation between PE and FITC was set at 15%. At least 10,000 positive events were collected for each sample.

Statistical analysis and image processing

Graphs were drawn and statistical analysis was performed with Prism 4.03 (GraphPad Software). The results are presented as the mean $\pm$ SEM or SD, as indicated. Data were evaluated by paired, one-tailed student's *t*-test, where indicated. The criterion for significance was $P < 0.05$ for all comparisons.

Images were processed in Adobe Photoshop CS (Adobe Systems Inc.) and CorelDRAW 11 (Corel Corporation).

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Mitochondria-derived reactive oxygen species are required for the pro-survival effect of
Tumor Necrosis-Factor- α in human neutrophils

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Abstract

Neutrophils, the most abundant phagocytes of the human immune system, have a brief life-span and are constitutively cleared by apoptosis. Neutrophils do not rely on their mitochondria for their energy provision, and their mitochondria have a disturbed respiratory supercomplex organization. They can maintain a membrane potential ($\Delta\psi_m$) by complex-III activity, but produce little or no ATP. In this study, we demonstrate that neutrophil mitochondria produce reactive oxygen species (mROS). These mROS do not contribute to apoptosis, but play an important role in the survival of neutrophils as signaling intermediates instead. Inhibition of the respiratory chain by addition of specific inhibitors led to increased mROS production and increased neutrophil survival, while these effects were abrogated by the mitochondria-targeted mROS scavenger MitoQ. In addition, the pro-survival effect of the potent pro-inflammatory factor tumor-necrosis factor- α (TNF- α), also appeared to depend on mROS production and was also abrogated by MitoQ. These findings potentially have important implications for the use of mROS scavengers in the treatment of inflammatory diseases.

Introduction

Neutrophils, the most abundant phagocytic cells of the human immune system, have a very short life-span of up to 24 hours in the circulation. Neutrophil homeostasis is controlled by constitutive, spontaneous apoptosis as well as by receptor-mediated apoptosis. Neutrophils kill invading micro-organisms by means of a rapid NADPH-dependent respiratory burst as well as with an array of proteolytic and bactericidal enzymes. Proper control of neutrophil cell death and clearance is essential to prevent tissue damage due to the release of the neutrophil's toxic contents. It has previously been shown that mitochondria play an important role in neutrophil apoptosis, even though cytochrome *c*, the main mitochondria-derived inducer of cell death, is scarce in these cells¹⁻³. Furthermore, a state of hypoxia has been shown to delay constitutive neutrophil apoptosis in a hypoxia-inducible factor 1 (HIF-1) and nuclear factor κ B (NF- κ B)-dependent manner⁴. This indicates that oxygen metabolism and/or sensing is vital for the induction of neutrophil apoptosis.

In most cells, mitochondria contribute significantly to the energy balance. Within the mitochondria, electrons are transferred through a chain of 4 protein complexes, the respiratory chain. Electrons are transferred via the electron-carriers ubiquinone (complex I and II to complex III) and cytochrome *c* (complex III to IV). Complex II is an essential enzyme complex within the tri-citric acid cycle, while complexes I, III and IV are the proton pumps that maintain the mitochondrial membrane potential ($\Delta\psi_m$). This $\Delta\psi_m$ is utilized by a fifth protein complex, complex V or the F_1/F_0 -ATPase, to generate ATP. Complex IV transfers electrons from complex III to molecular oxygen and produces H_2O . If electron transfer to complex IV is obstructed, highly reactive semi-reduced ubiquinone (semi-quinone) in complex III can spontaneously transfer its spare electron to oxygen, which leads to the formation of superoxide and, indirectly, other reactive oxygen species (ROS). In neutrophils, complexes I and IV are practically absent, and complex III activity is mainly responsible for maintaining $\Delta\psi_m$ ². As a result, neutrophil mitochondria are expected to produce a relatively large amount of superoxide in complex III.

Complex-III-derived ROS can damage proteins, lipids, and DNA within the mitochondria but also serve as signaling intermediates, for example in the activation of HIF-1, a potent pro-survival signal for neutrophils^{4,5}. During hypoxia, mitochondrial ROS (mROS) production is increased, which explains the HIF-1-mediated neutrophil survival under hypoxic conditions. In addition, tumor necrosis factor- α (TNF- α) has been shown to enhance mROS

production^{6,7}. This potent pro-inflammatory factor has been reported to have a dual effect on neutrophil cell death. At low doses, TNF- α acts as a potent survival factor for neutrophils, whereas at high doses TNF- α enhances neutrophil apoptosis⁸. Mitochondria-derived ROS may play an important role in the effects of TNF- α on neutrophils.

Recently, mitochondria-targeted ROS-scavenger molecules have been developed to attenuate mROS production without interfering with mitochondrial functions⁹. The most effective of these compounds, MitoQ₁₀, comprises a ubiquinone head group attached to a tail of 10 five-carbon isoprenoid units. Since mROS have been reported to contribute to a number of human pathologies, including ischemia-reperfusion injury, Parkinson's disease, Alzheimer's disease and aging, this compound has great therapeutic potential^{9,10}.

In this study, we investigated the role of mROS in neutrophil apoptosis and in particular TNF- α -induced survival, using the mitochondria-targeted ROS scavenger MitoQ₁₀.

Results

Resting neutrophils produce ROS independent of the NADPH oxidase

Freshly isolated neutrophils from healthy donors or from patients with chronic granulomatous disease (CGD), who have no detectable NADPH-oxidase activity, were loaded with the fluorogenic cell permeable ROS-indicator 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCFDA) and ROS production was monitored for one hour (Figure 1). The rate of ROS production in resting cells was equal in both the controls and the CGD patients, while ROS production was increased almost twofold by treating the cells with the complex-III inhibitor antimycin A. When the cells were stimulated with phorbol myristate acetate (PMA), a potent activator of the NADPH-oxidase-dependent respiratory burst, ROS production in the control cells increased dramatically, while the level of ROS production in the stimulated CGD cells was the same as in the unstimulated cells. This indicates that the ROS production, as determined in resting neutrophils, is NADPH-oxidase independent and most likely mitochondria derived.

Figure 1. ROS production in resting neutrophils is not mediated by the NADPH oxidase.

Freshly isolated neutrophils of healthy controls (white circles/bars) or CGD patients (black squares/bars) were loaded with DCFDA and incubated in the presence or absence of either 1 μ M antimycin A or 100 ng/mL PMA.

A) The cells were incubated at 37°C and the fluorescence increase was determined every minute for up to one hour. Values represent arbitrary fluorescence units. Plots are representative for three independent experiments performed in duplicate. **B)** Bar graphs displaying the average maximum slope in relative fluorescence units (RFU) per minute over at least 20 minutes within a 60-minute interval. Data represent the average ($\pm$ SEM) of three independent experiments performed in duplicate. *** $p < 0.001$.

MitoQ scavenges ROS from resting and antimycin-A-stimulated neutrophils, but not from PMA-stimulated neutrophils

Freshly isolated neutrophils were loaded with DCFDA and incubated in the presence of increasing concentrations of the mROS scavenger MitoQ or the negative control compound decyl-triphenyl-phosphonium (TPP) ⁹. In unstimulated cells, MitoQ inhibited the ROS production in a dose-dependent manner (Figure 2A). However, at concentrations above 1 μ M, both TPP and MitoQ stimulated ROS production (not shown). This effect is most likely due to a disruption of the mitochondria at high concentrations. In cells stimulated with antimycin A, MitoQ inhibited the ROS production to an even greater extent, while the compound had no effect on the PMA-induced ROS production (Figure 2B and C). The complex-I inhibitor rotenone and the complex-II inhibitor 3-nitropropionate (3NP) did not affect mitochondrial ROS production (Figure 2C). This indicates that the majority of the mitochondrial ROS production observed in neutrophils is complex-III derived. Complex III obtains most of its electrons from the glycerol-3-phosphate receptor, as previously demonstrated ².

Figure 2. MitoQ scavenges ROS from resting and antimycin-A-stimulated neutrophils, but not from PMA-stimulated neutrophils.

A) Freshly isolated neutrophils were loaded with DCFDA and incubated in the presence of increasing concentrations of the mitochondria-specific ROS scavenger MitoQ (black circles) or the negative-control compound TPP (grey squares). The fluorescence increase was determined every minute for one hour at 37°C. The maximum slope was determined over a 20-minute interval of linear increase within this period. Data represent the average ($\pm$ SEM) maximum slope expressed in relative fluorescence units/minute (RFU/min) of 3 independent experiments performed in duplicate. **B)** Neutrophils were incubated as in A, with the exception that they were incubated in the presence of either 100 ng/mL PMA (black circles) or 1 μ M antimycin A (grey squares). **C)** Neutrophils were incubated as in A and B, in the presence (black bars) or absence (white bars) of 1 μ M MitoQ and / or 1 μ M of the complex-I inhibitor rotenone, 100 μ M of the complex-II inhibitor 3NP, 1 μ M of the complex-III inhibitor antimycin A or 100 ng/mL PMA, where indicated. Data represent the average maximum slope ($\pm$ SEM) of at least three independent experiments performed in duplicate. MitoQ significantly inhibited the ROS production in resting cells and inhibitor-treated cells, but not in PMA-treated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: Not significant.

Mitochondrial inhibition delays neutrophil apoptosis

Isolated neutrophils were incubated overnight in the presence or absence of the complex-III inhibitor antimycin A or the complex-II inhibitor 3NP. Survival was assessed by fluorescein isothiocyanate-labeled (FITC)-annexin V staining of the cells and by morphology (Figure 3A-C). Complex II (succinate dehydrogenase) is the only respiratory complex that shows normal activity in neutrophils ² and, in addition, when succinate, the substrate for this complex, is not degraded and the cellular levels increase, this serves as a potent activator for HIF-1¹². Both inhibitors significantly decreased the rate of spontaneous neutrophil apoptosis, especially after 24 hours (Figure 3A-C). Incubating the cells in the presence of MitoQ completely abrogated the protective effect of the inhibitors. The non-targeted ROS-scavenger Q₁₀ had no effect on neutrophil apoptosis (not shown). This indicates that at least a low level of mitochondrial ROS

production is required for the protective effect of mitochondrial inhibitors.

Loss of $\Delta\psi_m$ does not accelerate neutrophil apoptosis

Neutrophil apoptosis is associated with a gradual loss of $\Delta\psi_m$ ¹³. Mitochondria-targeted compounds, such as MitoQ, may also decrease $\Delta\psi_m$. To illustrate this, cells were treated with increasing concentrations of MitoQ, the negative control compound TPP or the mitochondrial uncoupler CCCP. As demonstrated by staining with the fluorescent $\Delta\psi_m$ -indicator JC-1, all of these compounds affect $\Delta\psi_m$ (Figure 4A). At 1 μ M, the concentration used in most experiments in this study, both MitoQ and TPP reduced $\Delta\psi_m$ to approximately 50% of the control value. However, neither TPP nor CCCP had a significant effect on neutrophil apoptosis, as demonstrated in Figure 4B. Only MitoQ significantly accelerated spontaneous neutrophil apoptosis during 8 hours of incubation.

Figure 3. Mitochondrial inhibition delays neutrophil apoptosis in an mROS-dependent manner.

Neutrophils were incubated overnight in the absence (white bars) or presence (black bars) of 1 μ M MitoQ and/or 100 μ M of the complex-II inhibitor 3NP or 1 μ M of the complex-III inhibitor antimycin A, where indicated.

A) To determine apoptosis, cells were stained with FITC-labeled annexin V and analyzed by flow cytometry after 8 and 24 hours. Both 3NP and antimycin A significantly delayed neutrophil apoptosis, an effect that was completely abrogated by MitoQ. Data represent the average ($\pm$ SEM) of five independent experiments performed in duplicate. ** $p < 0.01$, *** $p < 0.001$, n.s.: Not significant. **B)** Representative histograms displaying the effect of MitoQ (grey) on spontaneous and 3NP-inhibited neutrophil apoptosis after 24 hours of incubation. Events in P1 were marked as surviving, whereas events in P2 were marked as apoptotic. Annexin-V-FITC staining is expressed on the x-axis as fluorescence intensity. Plots are representative of at least five independent experiments. **C)** Micrographs of May-Grünwald-Giemsa-stained cells displaying the morphology of the cells after 24 hours of incubation at 60x magnification.

TNF- α induces mitochondrial ROS production in human neutrophils, which is vital for its pro-survival effect

The cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and TNF- α , are all potent inhibitors of neutrophil apoptosis¹³⁻¹⁷. To compare the effects of these cytokines on mROS production, DCFDA oxidation was followed in the presence or absence of MitoQ. The mROS production was only increased by TNF- α , an effect that was almost completely blocked by MitoQ (Figure 5A). In addition, the anti-apoptotic effect of TNF- α after 24 hours of incubation, was also completely blocked by MitoQ, while the compound reduced the anti-apoptotic affects of GM-CSF and G-CSF only partially (Figure 5B). After 8 hours of incubation, MitoQ accelerated the rate of apoptosis in all samples. Also under these conditions, the non targeted scavenger Q₁₀ had no effect (not shown).

Figure 4. Loss of $\Delta\Psi_m$ does not accelerate neutrophils apoptosis.

A) Neutrophils were loaded with increasing concentrations of the mROS scavenger MitoQ, the negative-control compound TPP or the mitochondrial uncoupler CCCP for one hour at 37°C. Afterwards, the cells were loaded with the fluorescent $\Delta\Psi_m$ indicator JC-1 and analyzed by flow cytometry. Data are expressed as the mean ($\pm$ SEM) $\Delta\Psi_m$, expressed as percentage of the untreated cells, and represent three independent experiments. **B)** Cells were loaded with 1 μ M of the indicated compounds and incubated for up to 24 hours at 37°C. To determine apoptosis, cells were stained with FITC-labeled annexin V and analyzed by flow cytometry after 8 (white bars) and 24 (black bars) hours. Significance was determined as compared to the untreated cells. Data are expressed as the mean ($\pm$ SEM) of three independent experiments. Only MitoQ-treated cells displayed significantly accelerated apoptosis after 8 hours of incubation. ** $p < 0.01$.

It has previously been reported that the anti-apoptotic effect of TNF- α depends on NF- κ B activation as well as on HIF-1 activity⁴. To control for any effects of MitoQ on the activation of NF- κ B, the degradation of the inhibitor of κ B- α (I κ B α), a reliable indicator for NF- κ B activation, was determined on Western blot after 2 hours of incubation in the presence or absence of MitoQ and TNF- α (Figure 5C). I κ B α was completely degraded in TNF- α -treated neutrophils, while MitoQ did not affect this degradation. During this short incubation, Mcl-1 levels were not significantly affected.

Neutrophils from patients with a NEMO deficiency do not survive with TNF- α

NEMO is an essential subunit of the inhibitor- κ B kinase complex. If this complex does not assemble, I κ B α is not phosphorylated and NF- κ B cannot be activated. Thus, in patients with a confirmed genetic defect in NEMO, NF- κ B activation is severely reduced¹⁸. As a result, neutrophils from NEMO-deficient patients do not show enhanced survival after overnight incubation with TNF- α (Figure 6). Interestingly, neither GM-CSF nor G-CSF had a significant effect on survival either in the neutrophils from NEMO-deficient patients, although all three cytokines significantly delayed apoptosis in control neutrophils. This indicates an essential role for NF- κ B in GM-CSF and G-CSF induced neutrophil survival as well as in the TNF- α -induced survival. When compared to the healthy control cells, the survival effects of GM-CSF and TNF- α were significantly different, but the G-CSF effect was not. These results are very similar to the results with MitoQ treated neutrophils (Figure 5B).

MitoQ prevents the stabilization of Mcl-1

The Bcl-2 family member Mcl-1 is a putative HIF-1-target gene^{19,20} and acts as a potent pro-survival factor for neutrophils^{21,22}. During apoptosis, Mcl-1 is gradually degraded in a proteasome-dependent manner¹⁵. To determine the effect of MitoQ on Mcl-1 degradation, Mcl-1 protein levels were determined on Western blot after 8 hours of incubation in the presence of various survival factors and MitoQ (Figure 7A). The degradation of Mcl-1 during spontaneous neutrophil apoptosis was not significantly accelerated by MitoQ. However, the stabilization of Mcl-1 by survival data (Figure 5), this effect was most pronounced for TNF- α . Neither G-CSF nor TNF- α has been described to increase the mRNA expression of Mcl-1^{23,24}, while the pro-survival effect of GM-CSF is mostly due to an increase in Mcl-1 protein stability¹⁵.

A

B

C

Figure 5. TNF- α -induced mROS production is required for its pro-survival effect.

A) Freshly isolated neutrophils were loaded with the mROS-indicator DCFDA and incubated in the presence or absence of 10 ng/mL GM-CSF, G-CSF, TNF- α and/or 1 μ M MitoQ (black bars). The fluorescence increase was determined every minute for one hour at 37°C. The maximum slope was determined over a 20-minute interval of linear increase within this period. Data represent the average ($\pm$ SEM) maximum slope expressed in relative fluorescence units/minute (RFU/min) of 3 independent experiments performed in duplicate. The mROS production was only increased significantly by TNF- α . ** $p < 0.01$, *** $p < 0.001$. **B)** To determine apoptosis, cells were stained with FITC-labeled annexin V and analyzed by flow cytometry after 8 and 24 hours. All cytokines significantly delayed neutrophil apoptosis, an effect that was at least partially cancelled by MitoQ (black bars). Data represent the average ($\pm$ SEM) of five independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: Not significant. **C)** Representative Western blot showing the expression of I κ B α and Mcl-1. Freshly isolated neutrophils were incubated in the presence (+) or absence (-) of 10 ng/mL TNF- α and/or 1 μ M MitoQ for 2 hours, as indicated. Data represent 3 independent experiments.

Figure 6. Neutrophils from patients with a defect in NF- κ B activation have a similar apoptotic phenotype as MitoQ-treated neutrophils.

Freshly isolated neutrophils from three separate patients with a confirmed NEMO deficiency (black bars) or from healthy controls (white bars) were incubated for 24 hours in the presence or absence of the indicated cytokines. To determine apoptosis, cells were stained with FITC-labeled annexin V and analyzed by flow cytometry after 24 hours. In the controls, all cytokines significantly delayed neutrophil apoptosis, an effect that was severely reduced in the patients. Data represent the average ($\pm$ SEM) of three independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: Not significant.

Whereas Mcl-1 acts upstream of mitochondrial acceleration of apoptosis by inhibiting the translocation of the pro-apoptotic protein Bax²⁵, the X-linked inhibitor of apoptosis (XIAP) acts downstream of the mitochondria by inhibiting caspases-9 and -3²⁶. During neutrophil apoptosis, XIAP is cleaved in a calpain-dependent manner^{13,27}. MitoQ did not have a significant effect on this process during spontaneous neutrophils apoptosis, as demonstrated by Western blot analysis in Figure 7C. All pro-survival factors significantly delayed XIAP cleavage, an effect that was at least partially abrogated by the addition of MitoQ. In case of G-CSF-delayed neutrophil apoptosis, MitoQ had no significant effect on XIAP stability. This can be explained by the fact that G-CSF delays neutrophil apoptosis by the inhibition of calpains, the proteases responsible for XIAP cleavage^{13,28}.

Figure 7. MitoQ prevents the stabilization of Mcl-1.

Freshly isolated neutrophils were incubated for 8 hours in the presence or absence of the indicated growth factors and inhibitors. Afterwards, the cells were lysed and Mcl-1 and XIAP protein expression levels were analyzed on Western blot. **A)** Representative Western blot of three independent experiments. The bands for Mcl-1, full-length XIAP, degraded XIAP and p38 MAPK (loading control) are indicated by the arrows. **B)** Data represent the mean relative integrated intensity ($\pm$ SEM) of the Mcl-1 band as compared to the fresh control (C- 0h) of three independent experiments. **C)** Data represent the relative ratio of the mean integrated intensity ($\pm$ SEM) of the 20-kDa XIAP degradation product divided by the total XIAP signal (full length + 20 kDa) of three independent experiments. All values were corrected for loading by the signal for p38 MAPK. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: Not significant.

Discussion

In this study, we show that neutrophils produce mROS and that these mROS have an important signaling function in neutrophil survival. Neutrophils are short-lived cells and it appears that the survival benefit of mROS outweighs the potential damage done to the cell. It has been shown before that neutrophils display an increased survival under hypoxic conditions, and that this survival is mediated through HIF-1 and NF- κ B⁴. A state of hypoxia is known to induce mROS production in complex III⁵.

Neutrophils hardly use their mitochondria for their energy provision and maintain a high rate of glycolysis^{2,29}. A direct consequence of this metabolic profile is a constant, low-level, production of mROS. Alternatively, this mROS production may also be a requirement to maintain this metabolic profile, since mROS are known activators of HIF-1, while HIF-1 activation is known to induce aerobic glycolysis^{30,31}. In tumor cells, this phenomenon is referred to as the Warburg effect, and tumor cells are known to abuse the oxygen-sensing system to allow their continuous expansion^{32,33}. For a tumor cell, the advantage in switching from mitochondrial respiration to aerobic glycolysis is that energy from glycolysis is more rapidly available. Since neutrophils are end-stage differentiated cells, they do not require aerobic glycolysis for proliferation. Instead, their metabolism seems dedicated to their main function; *i.e.* killing invading micro-organisms by means of an NADPH-dependent respiratory burst³⁴. A high rate of glycolysis seems to be a requirement for their functions²⁹. In addition, the metabolic profile of neutrophils allows them to survive in an inflammatory environment where oxygen tension may be low⁴.

TNF- α is an important pro-inflammatory factor with a dual effect on neutrophil survival^{8,35}. In this study, we show that the pro-survival effect of TNF- α , and to a lesser degree the effects of GM-CSF and G-CSF, depends on mROS production. When mROS, required for the stabilization of HIF-1 α , are scavenged by MitoQ, the survival effect of TNF- α is strongly reduced, as illustrated in Figure 8. Interestingly, whereas HIF-1 has been shown to control the expression of vital components of the NF- κ B activation machinery⁴, NF- κ B has also been shown to be involved in the transcriptional regulation of HIF-1 α ³⁶. Thus, these central pathways in innate immunity and metabolism are thoroughly intertwined. Scavenging mROS with MitoQ resulted in the same apoptotic phenotype in neutrophils as seen in patients with a defect in NF- κ B activation, even though MitoQ did not interfere with the activation of NF- κ B. This suggests that HIF-1 activation, downstream from NF- κ B, is essential for TNF- α -induced neutrophil survival. According to our model, mROS production is essential for the stabilization of HIF-1 α , the regulatory subunit of HIF-1, whereas NF- κ B activation seems to be required to induce the expression of HIF-1 α . Only the activation of both pathways in conjunction leads to sufficient HIF-1 activation to induce neutrophil survival.

Figure 8. Model for the involvement of mROS in TNF- α signaling. TNF- α is a well known activator of NF- κ B signaling. Recent studies have indicated the interdependence of NF- κ B and HIF-1^{4,36}. In this study, we demonstrate the importance of mROS in the pro-survival effect of TNF- α on neutrophils. Although NF- κ B activation (and thus HIF-1 α expression³⁶) is not affected by MitoQ (Figure 5C), mROS are required for the stabilization of HIF-1 α ³⁰. This indicates HIF-1, downstream of NF- κ B, as the main pro-survival effector in neutrophils.

In inflammatory pathologies, such as septicemia or ischemia-reperfusion injury, clearance of neutrophils by apoptosis is essential for the resolution of inflammation^{37,38}. In such pathologies, inflammatory cytokines such as TNF- α are usually responsible for the activation and increased life-span of the neutrophils and the subsequent tissue damage³⁹. However, most attempts to attenuate septicemia by targeting these cytokines have failed in the clinic. Instead, our study indicates that TNF- α -reduced neutrophil apoptosis may be restored by targeting mROS production. The mROS scavenger MitoQ is currently under investigation in clinical trials for the treatment of age-related and neurological diseases such as Alzheimer's and Parkinson's^{9,40}. The present study indicates how the compound may be beneficial for the treatment of inflammatory diseases as well, by canceling the protective effect of TNF- α on neutrophil apoptosis.

In conclusion, mROS produced by neutrophil mitochondria are not harmful for the cell. Instead, they comprise important signaling intermediates in neutrophil survival. Scavenging these mROS with the mitochondria-targeted ROS scavenger MitoQ abrogates the pro-survival effect that TNF- α has on neutrophils. These findings may have important implications for the treatment of inflammatory pathologies.

Materials and Methods

Antibodies and reagents

Mouse monoclonal anti-I κ B α (clone L35A5) and the rabbit polyclonal antibody directed against p38 were obtained from Cell Signaling Technology (Boston, MA, USA). Rabbit anti-Mcl-1 and mouse anti-XIAP (clone 48) were obtained from BD Pharmingen (Franklin Lakes, NJ, USA).

All chemical reagents were obtained from Merck Biosciences (Darmstadt, Germany), unless otherwise indicated. Rotenone and antimycin A were acquired from Sigma-Aldrich (St. Louis, MO, USA), 3-nitropropionic acid was from Fluka (Steinheim, Switzerland), recombinant human TNF- α and GM-CSF were from PeproTech (Rocky Hill, NJ, USA) and clinical grade G-CSF (Neupogen) was acquired from Amgen (Breda, the Netherlands). The mitochondria-specific ROS scavenger MitoQ₁₀ and the negative-control compound decylITPP⁹ were a kind gift from Prof. Michael P. Murphy of the Dunn Human Nutrition Unit, Cambridge, UK. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was obtained from Calbiochem (La Jolla, CA, USA).

Cell isolation and culture

Neutrophils were isolated from the heparinized blood of healthy volunteers or patients after informed consent had been acquired according to the Helsinki declaration. All studies were approved by the local ethical committee. The blood was centrifuged over isotonic Percoll (Pharmacia, Uppsala, Sweden), and the erythrocytes in the pellet were subsequently lysed, as described¹¹. Neutrophil preparations were typically >97% pure, with the contaminating cells being mostly eosinophils. Cells were cultured in Hepes-buffered saline solution (HBSS; 132 mM NaCl, 20 mM Hepes, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄, 1 mM CaCl₂, pH 7.4) supplemented with 1% (v/v) human serum albumin (Cealb; Sanquin, Amsterdam, the Netherlands) and 5 mM glucose at a concentration of 5×10^6 cells/mL in polypropylene round-bottom tubes of 14 mL (BD Biosciences, Franklin Lakes, NJ, USA). Incubations were performed in a shaking water bath at 37°C.

ROS detection

Freshly isolated neutrophils were incubated in HBSS at a concentration of 5×10^6 /mL in the presence of 10 μ M 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCFDA-AM [Molecular Probes]) and the indicated stimuli/inhibitors in a white 96-wells plate (Corning, Corning, NY, USA). The total reaction volume was 125 μ L for all conditions. Increase in fluorescence was determined every minute on a Genios Plus spectrophotometer (Tecan, Zürich, Switzerland) at excitation 488 nm, emission 520 nm, at 37°C for up to one hour.

Flow cytometry

To detect apoptosis, cells were labeled for 10 min on ice with fluorescein-isothiocyanate (FITC)-labeled Annexin V (Bender Med Systems, Vienna, Austria), diluted 1:500 in HBSS, supplemented with 2.5 mM CaCl₂. Annexin-V labeling was followed by a

single wash step with the same medium, whereupon the cells were resuspended in HBSS 2.5 mM CaCl₂ and analyzed by flow cytometry on an LSRII flow cytometer (BD Biosciences).

To detect changes in $\Delta\Psi_m$, the cells were loaded for 15 min at 37°C with 0.5 μ M 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA) in HBSS, containing 1 μ M tetraphenyl boron to facilitate entry of the dye into the cells, and were analyzed immediately afterwards by flow cytometry. Relative $\Delta\Psi_m$ was calculated as a ratio of the fluorescent signal in the red (PE-Texas Red) channel over the green (FITC) channel.

Western blot

Western blot samples were prepared from 5×10^6 cells by washing the cells once in ice-cold phosphate-buffered saline (PBS) before resuspension in cytosol extraction buffer (250 mM sucrose, 70 mM KCl, complete protease inhibitor cocktail mix [PIM; Roche Diagnostics, Almere, The Netherlands] and 2 mM diisopropylfluorophosphate [DFP; Fluka Chemica, Steinheim, Switzerland] in PBS) at a concentration of 100×10^6 cells/mL. Cells were incubated for 15 min on ice, after which they were dissolved in Laemmli sample buffer (LSB; 50 mM TrisHCl, pH 6.8, 10% glycerol (v/v), 5 mM DTT [DL-dithiothreitol, Sigma-Aldrich], 1% 2-mercaptoethanol, 1% sodium dodecylsulphate (SDS) (w/v), 100 μ g/mL bromophenol blue) and boiled for 30 min at 95°C. All samples were stored at -20°C before subjection to SDS-polyacrylamide gelelectrophoresis (SDS-PAGE).

Samples were run on 12%, 1.5 mm, polyacrylamide gels in a protean-3 mini system (Bio-Rad Laboratories, Veenendaal, The Netherlands). The equivalent of 1.5×10^6 cells was loaded in each lane. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-FL; Millipore, Billerica, MA, USA), which were subsequently blocked for 30 min in blocking buffer (5% non-fat dry milk (w/v) [Elk; Campina, Zaltbommel, The Netherlands] in Tris-buffered saline, 0.1% Tween-20 (v/v) (TBST)). Blots were immuno-labeled with specific antibodies against the indicated proteins in blocking buffer containing 2 mM Na₃N, overnight at 4°C. After washing the blots in TBST, they were treated with IRDye-labeled secondary antibodies directed against the primary antibodies (goat anti-rabbit-IgG IRDye 680 or goat anti-mouse-IgG IRDye 800CW; LI-COR Biosciences, Lincoln, NE, USA). Labeling was followed by another round of washing in TBST before detection of the specific signals on an Odyssey Infrared Imager (LI-COR Biosciences).

Statistical analysis and image processing

Graphs were drawn and statistical analysis was performed with GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA USA). The results are presented as the mean $\pm$ SEM, as indicated. Data were evaluated by paired, one-tailed student's *t*-test, where indicated. The criterion for significance was $p < 0.05$ for all comparisons. Images were processed in Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA) and CorelDRAW 11 (Corel Corporation, Ottawa, Ontario, Canada).

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Neutropenia and mitochondrial defects in Barth syndrome

Manuscript in Preparation

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Abstract

Patients with the rare, X-linked, mitochondrial disorder Barth syndrome (BTHS) suffer from moderate to severe neutropenia amongst other symptoms. BTHS is caused by mutations in the *TAZ* gene, encoding tafazzin, which is involved in cardiolipin metabolism. Cardiolipin is a phospholipid restricted to the inner mitochondrial membrane and is required for the ultrastructural curvature of this membrane as well as for docking certain respiratory chain proteins. It has previously been reported that neutrophils from BTHS patients avidly bind annexin V, indicative of phosphatidyl serine (PS) exposure, even though the cells displayed no other signs of apoptosis or differentiation defects. In this study, we monitored the neutrophils of five BTHS patients over time to investigate mitochondrial properties during neutropenic and neutrophil-sufficient periods. We found that the mitochondria in BTHS neutrophils were consistently defective and produced an increased amount of reactive oxygen species (ROS). Independent of the mitochondrial dysfunction, a linear inverse correlation between the absolute neutrophil cell count and their level of annexin-V binding was demonstrated. These findings indicate that BTHS neutrophils may have a higher clearance rate, causing the fluctuating and variable neutropenia in BTHS.

Introduction

Barth syndrome (BTHS; MIM302060) is an X-linked recessive disorder characterized by cardiomyopathy, neutropenia and skeletal myopathy as well as 3-methylglutaconic aciduria^{1,2}. Circulating neutrophil levels in BTHS patients may vary between normal and the near complete absence of neutrophils. Neutrophils isolated from the blood of BTHS patients avidly bind annexin V, which indicates phosphatidyl serine (PS) exposure, normally considered to be a hallmark for apoptosis. However, BTHS neutrophils are not apoptotic and function normally³.

BTHS is caused by mutations in the *TAZ* gene (formerly *G4.5*), encoding tafazzin⁴⁻⁶. Although the function of tafazzin has not been completely elucidated, it belongs to a conserved family of acyl transferases and has been shown to be involved in cardiolipin remodeling⁷⁻⁹. Cardiolipin is a phospholipid that is almost exclusively located in the inner membrane of the mitochondria. There, it plays an import role in maintaining the strong curvature of the mitochondrial inner membrane as well as functioning as a docking lipid for subunits of the respiratory chain complexes^{10,11}. In BTHS patients, cardiolipin levels are strongly reduced and only the monolyso-form of this phospholipid is abundantly present^{12,13}. As a consequence, the mitochondria in BTHS cells lack the characteristic curvature of the mitochondrial matrix, and the respiratory chain complexes in BTHS cells are not organized in supercomplexes, which normally facilitate electron transport and respiration^{10,14-17}.

Healthy neutrophils rely mostly on glycolysis for their energy provision and were long thought not to contain any mitochondria¹⁸. In recent years, it has become clear that these cells do contain mitochondria, which maintain a membrane potential ($\Delta\psi_m$), normally associated with respiratory chain activity^{19,20}. The respiratory chain consists of a series of four electron-transferring protein complexes. The efficient transfer of electrons along this chain of complexes relies on the organization of the complexes in larger supercomplexes¹⁷. However, neutrophils do not express a number of essential, mitochondrial-DNA encoded, subunits of the respiratory chain complexes and, as a consequence, have reduced respiratory chain supercomplex organization²¹.

In the respiratory chain of neutrophils, electrons are not –as in other cells– transferred linearly from complex I and II to complex III and finally to complex IV, in which they are normally bound to molecular oxygen to form H₂O.

Instead, electrons are mostly directly transmitted to complex III from glycolysis via the glycerol-3-phosphate receptor (G3PR). Although complex II, a vital component of the citric-acid cycle, is normally active in neutrophils, complexes I and IV are practically absent²¹. As a consequence, complex III generates a relatively large amount of reactive oxygen species (mROS)²². However, these ROS are not necessarily harmful for the cell since complex-III-derived ROS are known activators of the transcription factor hypoxia-inducible-factor-1 (HIF-1)²³, a potent pro-survival signal for neutrophils²⁴.

For the present study, we monitored a number of BTHS patients in time to characterize the properties of their neutrophil mitochondria. We found an inverse correlation between the absolute neutrophil count in BTHS patients and the degree of annexin-V binding. In this way, we have further addressed the implications of the mitochondrial dysfunction in BTHS-related neutropenia.

Results

Neutropenia in BTHS patients

White cell counts were determined in whole blood of five BTHS patients during the course of the study (Figure 1). Overall, the absolute neutrophil counts were significantly decreased in the patients as compared to the controls (Figure 1A), while monocyte – and to a lesser extent – lymphocyte counts were elevated (Figures 1B and C). Although all patients had shown some neutropenic periods, the neutrophil levels in patients 1, 2 and 3 were normal (*i.e.* above 1.5×10^6 cells/mL) at several time points during the study. Patients 4 and 5 were consistently neutropenic throughout the test period (Figure 1D). No clear pattern of cyclic neutropenia was detected in any of the patients during this study.

Figure 1. White-cell counts in BTHS patients. A-C) White-cell levels as determined in the circulation of BTHS patients and healthy controls, expressed as 10^9 cells/L whole blood. On average, the neutrophil levels were significantly reduced in the BTHS patients. Both lymphocyte and monocyte counts were elevated. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$. D: Neutrophil counts in five BTHS patients over time.

Figure 2. Annexin-V binding is significantly elevated on BTBS neutrophils and correlates with cell counts.

Freshly isolated neutrophils were stained with FITC-labeled annexin V and subjected to flow cytometry. **A)** Annexin-V binding on BTBS neutrophils is, on average, significantly higher than on control neutrophils. ****** $p < 0.01$. **B)** The level of annexin-V binding correlates with the number of circulating neutrophils in BTBS patients. $R^2 = 0.67$.

Annexin-V binding inversely correlates with neutrophil levels in BTBS patients

It has previously been observed that BTBS neutrophils avidly bind annexin V, a marker for phosphatidyl serine (PS) exposure³. With a more sensitive flow cytometer, we determined a wide range in the level of annexin-V binding in freshly isolated cells of BTBS patients when expressed as mean fluorescence intensity (MFI) per cell. We sought to determine whether the level of annexin-V binding correlated with the circulating neutrophil level in BTBS patients. Even though not all BTBS patients displayed elevated annexin-V staining during the course of the study, annexin-V binding in BTBS patients was still significantly increased as compared to the controls (Figure 2A). In addition, an inverse linear correlation was found between cell count and annexin-V binding (Figure 2B). This suggests that increasing levels of PS exposure lead to increased cell clearance, resulting in decreased numbers of circulating neutrophils.

Decreased mitochondrial functions in BTBS neutrophils

Even though neutrophils do not require mitochondrial activity to maintain their energy balance, their mitochondria maintain a measurable $\Delta\psi_m$, which in neutrophils largely depends on complex-III activity of the residual respiratory chain^{20,21}. The $\Delta\psi_m$ in BTBS neutrophils and controls was determined with the fluorescent $\Delta\psi_m$ indicator JC-1. This dye forms red fluorescent J-aggregates in charged mitochondria, while the dye is green fluorescent when $\Delta\psi_m$ is low. The ratio red/green provides a reliable indication for $\Delta\psi_m$. In BTBS neutrophils, $\Delta\psi_m$ was consistently lower than in control neutrophils (Figure 3A). Importantly, the neutrophils from BTBS patients still maintained a certain level of $\Delta\psi_m$, as demonstrated by the fact that $\Delta\psi_m$ was still decreased by the mitochondrial uncoupler CCCP (Figure 3A).

Even though neutrophils do not utilize their mitochondria for ATP production, these organelles still control lactate production by accepting electrons from aerobic glycolysis via the glycerol-3-phosphate shuttle²¹. In BTBS neutrophils, the lactate production was significantly higher than in the controls (Figure 3B). ATP levels in resting BTBS cells were the same as in control neutrophils, while the cells displayed an increased resistance to monoiodoacetic acid (IAA), a potent inhibitor of glycolysis (Figure 3C). These results collectively suggest an increased glycolytic rate in BTBS neutrophils.

BTBS neutrophils produce increased levels of mitochondria-derived ROS (mROS)

Of the respiratory chain, complexes I, III and IV act as the proton pumps that maintain $\Delta\psi_m$. A fifth complex, the F_1/F_0 -ATPase, utilizes this proton gradient to produce ATP. In neutrophils, complex III is responsible for maintenance of the $\Delta\psi_m$ ²¹. Since the mitochondria in BTBS patients are defective, as indicated by a lowered $\Delta\psi_m$, an increased

Figure 3. Mitochondrial functions are decreased in the neutrophils of BTHS patients.

A) Freshly isolated neutrophils were loaded with the $\Delta\Psi_m$ indicator JC-1 and analyzed by flow cytometry. Although $\Delta\Psi_m$ in BTHS neutrophils was significantly lower than in control cells, the addition of 1 μM of the uncoupler CCCP reduced this $\Delta\Psi_m$ even further, to a similar level as in control neutrophils. $\Delta\Psi_m$ is expressed as a percentage of the control, *i.e.* neutrophils from healthy donors. *** $p < 0.001$; n.s., not significant. **B)** Control (white bar) and BTHS (black bar) neutrophils were incubated for two hours at 37°C, after which the amount of lactate was determined, expressed as nmol lactate per 10⁶ cells. Data represent the average ($\pm$ s.e.m.) lactate production of six different patients and controls, determined in duplicates. *** $p < 0.001$. **C)** ATP levels in fresh neutrophils or in neutrophils treated for two hours with 100 μM of the glycolysis inhibitor IAA, expressed as pmol ATP per 10⁶ cells. All data represent the average ($\pm$ SEM ATP levels of six different patients and controls, determined in duplicates. ** $p < 0.01$; n.s., not significant.

Figure 4. Neutrophils and PBMC from BTHS patients display an increased production of mROS.

A-B) Freshly isolated neutrophils and PBMC from BTHS patients or healthy controls were loaded with the mROS indicator DCFDA, after which the increase in fluorescence was measured for one hour at 37°C. Data are expressed as the maximum slope in relative fluorescent units (RFU) per minute, as determined over a 20-minute interval during incubation. * $p < 0.05$; *** $p < 0.001$.

production of mROS was expected. The level of mROS production was determined in freshly isolated neutrophils or PBMC by measuring the increase in fluorescence for up to one hour, after loading the cells with the cell-permeable ROS-indicator DCFDA/AM (Figure 4). Resting neutrophils display no measurable NADPH-oxidase activity under those conditions²². Indeed, the neutrophils from BTHS patients produced elevated amounts of mROS. This effect was less pronounced, albeit still present, in their PBMC.

Increased mROS production does not result in increased extracellular oxidation

The increased production of mROS in BTHS neutrophils could lead to increased oxidation of the cell. Oxidation of phospholipids on the plasma membrane has been suggested as an explanation for the increased annexin-V binding and – as a consequence – a potentially increased clearance of BTHS neutrophils^{3,26}. To determine the oxidation status of the plasma membrane, the cells were labeled with the cell-impermeable AlexaFluor633-conjugated maleimide and analyzed by flow cytometry. Maleimide specifically binds to reduced thiol groups. Therefore, a high signal for maleimide means that the cells are reduced, whereas a low staining indicates oxidation. No significant difference was observed between fresh BTHS neutrophils and controls, as far as maleimide staining was concerned (Figure 5).

During overnight culture the maleimide staining of the cells decreased drastically, indicating increased oxidation of

Figure 5. Both control and BTHS neutrophils have a similar level of reduced thiol groups on their surface. Freshly isolated neutrophils from healthy control donors and BTHS patients were labeled with the AlexaFluor633-conjugated thiol-indicator maleimide and analyzed by flow cytometry. A high staining with maleimide indicates a reduced surface, low staining is indicative for oxidation. No significant difference was observed between the neutrophils from fresh controls and BTHS patients, but apoptotic cells displayed a significant reduction in maleimide staining after 24h of incubation. *** $p < 0.001$; n.s., not significant.

the cells during apoptosis. No correlation was observed between annexin-V binding and maleimide staining in fresh cells of patients and controls (not shown).

Scramblase activity in fresh neutrophils is increased as compared to PBMC

The increased staining with annexin V as observed in BTHS neutrophils, while the PBMC are unaffected, could be explained by a difference in the activity ratio between the phospholipid translocases that maintain the asymmetric distribution of phospholipids in the plasma membrane in both cell types. To determine flippase and scramblase activities, freshly isolated control neutrophils and PBMC were loaded with NBD-labeled PS or PC, respectively, and incubated for the indicated time, after which the uptake of phospholipids was determined by flow cytometry (Figure 6). While flippase activity, as determined by NBD-PS uptake, was equal in neutrophils and PBMC, scramblase activity, as determined by NBD-PC uptake, was higher in neutrophils. As a result, the scramblase over flippase ratio in freshly isolated neutrophils is higher than in PBMC (Figure 6B), albeit still below 1. Thus, a moderate inhibition of the flippase or a moderate activation of the scramblase activity has a greater chance to result in PS exposure in neutrophils than in PBMC.

Figure 6. Scramblase and flippase activities in neutrophils vs. PBMC. A) Activities of the scramblase and the flippase in freshly isolated neutrophils (PMN; squares) and PBMC (circles) from healthy donors were determined by the uptake of NBD-labeled phospholipids (PS (white) for the flippase and PC (black) for the scramblase) on the flow cytometer. B) Ratio of the scramblase over flippase activities in neutrophils (PMN; white bar) vs. PBMC (black bar). Scramblase and flippase activities were determined by calculating the average speed of PC and PS uptake respectively, in the linear part of the graph in (A). Both values were corrected for the theoretical maximum uptake, which is 1 for PS by the flippase and 0.5 for PC by the scramblase. In neutrophils, the ratio is markedly higher, indicative of a relatively greater scramblase activity. All data represent the mean ($\pm$ SEM of three independent experiments. *** $p < 0.001$.

Discussion

It has previously been reported that annexin-V binding in BTHS neutrophils does not correlate with the level of circulating neutrophils in these patients³. However, the use of a more sensitive flow cytometer has allowed us to redefine our previous findings. In the present study, five BTHS patients were monitored over the course of several years. A clear inverse correlation between annexin-V binding and absolute neutrophil count has now been observed. In BTHS neutrophils, the mitochondrial defects correlate with an increased mROS production. However, the intracellular ROS production in BTHS neutrophils did neither lead to an increase in extracellular oxidation nor correlated with the level of annexin-V binding to BTHS neutrophils. It appears that the ability of neutrophils to reduce their cell surface has not been affected in BTHS patients. Therefore, it seems unlikely that the presence of oxidized phospholipids increases annexin-V binding in BTHS neutrophils, and the best explanation for this latter phenomenon seems to be an increased exposure of PS during neutropenia. Although the mitochondrial defects are also present in lymphocytes and monocytes, the levels of these cells in the circulation were somewhat increased in BTHS patients as compared to the healthy controls. However, no clear correlation between a decrease in neutrophil count and an increase in PBMC was observed. The two questions that still remain unanswered are 1) how PS exposure is triggered in BTHS neutrophils in the absence of apoptosis, and 2) how this low-level PS exposure leads to apparent increased clearance of neutrophils.

Recently, significant progress has been made towards understanding the recognition and clearance of PS-positive cells by tissue macrophages^{27,28}. Not a single receptor, but several surface proteins seem to recognize PS on apoptotic cells. In addition, it has also become increasingly clear that macrophages come in different flavors²⁹. Thus, only certain types of macrophages, expressing the right combination of receptors, may be responsible for clearing cells that display PS in the absence of other apoptotic markers. This may of course limit a straight-forward interpretation of any experiment with *in vitro* derived macrophages to investigate the phagocytosis of BTHS neutrophils³. Further phenotyping of tissue macrophages *in vivo*, or other cells that may express PS receptors, will be required before conclusions can be drawn about the possibility of clearance of the BTHS neutrophils.

In normal cells, PS is constantly and passively transported from the inner to the outer leaflet of the plasma membrane in a slow process by a phospholipid scramblase, while a fast-acting ATP-dependent enzyme, called flippase, is constantly transporting PS back to the inner leaflet. The net result of this reaction is that PS is exclusively found in the inner leaflet of the plasma membrane in normal, viable cells³⁰. Protein-kinase-C- δ (PKC- δ) seems to be a primary player in the regulation of PS exposure via the inactivation of the flippase^{31,32}. It has been suggested that PKC- δ activation can be triggered by mROS, via oxidized diacylglycerol³³. Such a mechanism could be present in BTHS neutrophils, since their mitochondria display an increased production of ROS. However, this would not explain why circulating PBMC, which also produce more mROS, as well as the platelets, do not show any change in PS exposure in BTHS patients.

On the other hand, PKC- δ activation can be triggered by Ca^{2+} , via the activation of calpains^{34,35}. Calpains, Ca^{2+} -dependent cysteine proteases, are permanently active in healthy neutrophils³⁶. However, when intracellular Ca^{2+} levels rise during neutrophil apoptosis, calpain activity increases^{36,37}. Mitochondria play an important role in the regulation of the Ca^{2+} homeostasis in neutrophils³⁸. Through interaction with the endoplasmic reticulum (ER), the main intracellular Ca^{2+} store, mitochondria can act as Ca^{2+} -buffering-organelles. They take up the Ca^{2+} that leaks from the ER, before it is re-absorbed by the ER^{39,40}. When the $\Delta\psi_m$ is compromised, the mitochondria can no longer fulfill

this function. As a result, cytosolic Ca^{2+} levels rise and calpain activity increases^{37,38}. Since $\Delta\psi_m$ is lowered in BTHS neutrophils (Figure 3), such a scenario also belongs to the possibilities.

In addition, Ca^{2+} can inhibit the phospholipid flippase directly, while promoting scramblase activity³⁰. An increased concentration of intracellular free Ca^{2+} could therefore lead to a higher scramblase activity, relative to the flippase, resulting in PS exposure. The scramblase over flippase activity ratio is normally higher in neutrophils than in PBMC and this could explain why BTHS patients have PS-positive neutrophils but no PS-positive PBMC (Figure 6). After all, while both cell types receive the same inhibitory and activating signals on these enzymes due to increased mROS and Ca^{2+} , the relative scramblase activity in PBMC may remain too low to lead to net PS exposure, while in neutrophils the balance is slightly tipped towards the scramblase. This hypothesis is summarized in Figure 7, but further research has to be done to confirm this hypothesis.

Figure 7. Hypothetical model for PS exposure in BTHS neutrophils.

In healthy cells (left panel), the activity of the flippase, that transports PS (white circles) to the inside of the cell, is greater than the activity of the scramblase, which randomly distributes PS and PC (black circles). The ER constantly leaks Ca^{2+} into the cytosol, which is mostly taken up by the mitochondria, before it is absorbed by the ER again. The cytosolic Ca^{2+} concentration (indicated by a gray scale) is low. In BTHS neutrophils (right panel), the mitochondria have a reduced $\Delta\psi_m$. As a consequence, Ca^{2+} that leaks from the ER is not sufficiently buffered by the mitochondria and the spill-over into the cytosol is increased. Increased Ca^{2+} levels in the cytosol activate the scramblase while inhibiting the flippase. In addition, Ca^{2+} can activate the flippase-inhibitor PKC- δ via calpain activity, while PKC- δ can also be activated via mROS. Altogether, this results in an increased PS exposure in the BTHS neutrophils.

In conclusion, the increase in annexin-V binding on BTHS neutrophils inversely correlates with cell counts, suggesting enhanced clearance of annexin-V-positive cells. This apparent increase in PS exposure cannot be explained by mROS alone, since the PBMC in BTHS patients produce more mROS than their neutrophils but do not display PS. Moreover, the surface of BTHS neutrophils did not seem to be more generally oxidized. However, the $\Delta\psi_m$ in BTHS neutrophils is considerably lower than in control neutrophils. This could potentially lead to increased localized or

cytosolic free Ca^{2+} levels in BTHS neutrophils, which can trigger PKC-induced PS exposure via calpain activity or directly via inhibition of the flippase and activation of the scramblase. Further study is warranted before definitive conclusions can be drawn about this mechanism. Given the rarity of the disorder and the impossibility of culturing or storing neutrophils, an experimental model system for myeloid cell development and function is required to facilitate more extensive studies on the neutropenia in BTHS.

Materials and methods

Chemical reagents

All chemicals were obtained from Merck Biosciences (Darmstadt, Germany), unless otherwise indicated.

Cell isolation and culture

White blood cell levels were determined in whole blood on an Advia 2120 hematology system (Bayer, Leverkusen, Germany). Neutrophils were isolated from the heparinized blood of healthy volunteers or patients after informed consent had been acquired according to the Helsinki declaration. All studies were approved by the local ethical committee. The blood was centrifuged over isotonic Percoll (Pharmacia, Uppsala, Sweden), and the erythrocytes in the pellet were subsequently lysed, as described²⁵. Peripheral blood mononuclear cells (PBMC) were defined as the cells in the interface above the Percoll layer. Neutrophil preparations were typically >97% pure, with the contaminating cells being mostly eosinophils. Cells were cultured at a concentration of 5×10^6 cells/mL in Hepes-buffered saline solution (HBSS; 132 mM NaCl, 20 mM Hepes, 6 mM KCl, 1 mM MgSO_4 , 1.2 mM K_2HPO_4 , 1 mM CaCl_2 , pH 7.4) supplemented with 1% (v/v) human serum albumin (Cealb; Sanquin, Amsterdam, the Netherlands) and 5 mM glucose in polypropylene round-bottom tubes of 14 mL (BD Biosciences, Franklin Lakes, NJ, USA). Incubations were performed in a shaking water bath at 37°C.

ROS detection

Freshly isolated neutrophils or PBMC were incubated in HBSS at a concentration of 5×10^6 /mL in the presence of 10 μM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (DCFDA/AM [Molecular Probes, Eugene, OR, USA]) and the indicated stimuli/inhibitors in a white 96-wells plate (Corning, Corning, NY, USA). This assay has previously been demonstrated to specifically detect mitochondria-derived ROS in resting neutrophils²². The total reaction volume was 125 μL for all conditions. Increase in fluorescence was determined every minute on a Genios Plus spectrophotometer (Tecan, Zürich, Switzerland) at excitation 488 nm, emission 520 nm, and 37°C for up to one hour.

Flow cytometry

To detect PS exposure, cells were labeled for 10 min on ice with fluorescein-isothiocyanate (FITC)-labeled Annexin V (Bender Med Systems, Vienna, Austria), diluted 1:500 in HBSS, supplemented with 2.5 mM CaCl_2 . Annexin-V labeling was followed by a single wash step with the same medium, whereupon the cells were resuspended in HBSS 2.5 mM CaCl_2 and analyzed by flow cytometry on an LSR II flow cytometer (BD Biosciences). Cells were labeled concomitantly with the AlexaFluor633-conjugated thiol indicator C_5 -maleimide (Molecular Probes), at a concentration of 5 $\mu\text{g}/\text{mL}$.

To measure the changes in mitochondrial membrane potential ($\Delta\psi_m$), the cells were loaded for 15 min at 37°C with 0.5 μM JC-1 (Molecular Probes) in HBSS, containing 1 μM tetraphenyl boron to facilitate entry of the dye into the cells, and were analyzed immediately by flow cytometry. To determine a background value for $\Delta\psi_m$, the cells were incubated with 1 μM CCCP (Calbiochem, La Jolla, CA, USA), which is sufficient to completely abrogate $\Delta\psi_m$. Relative $\Delta\psi_m$ was calculated as a ratio of the fluorescent signal in the red (PE-Texas Red) channel over the green (FITC) channel.

ATP and lactate assays

Freshly prepared neutrophils were incubated for 2 hours at 37°C in HBSS, supplemented with 5 mM glucose. After incubation, the cells were lysed by the addition of 1.5% HClO_4 for 10 minutes on ice. Following centrifugation, the supernatant was neutralized with an equimolar amount of K_2CO_3 . Insoluble KClO_4 was precipitated by quick centrifugation at 4 °C and the supernatant was stored at -80°C.

ATP levels were determined by adding 100 μL of the firefly luciferase-based adenosine 5'-triphosphate (ATP) assay mix (Sigma Aldrich, St. Louis, MO, USA) diluted 1:10 in ATP-assay buffer (5 mM MgSO_4 , 100 μM EDTA, 100 μM NaN_3 , 4 mM

DTT [DL-dithiothreitol, Sigma Aldrich], in 25 mM TrisHCl, pH 7.8) to 10 μ L of the supernatant of the neutralized lysate in a white 96-well plate (Corning Life Sciences). Samples were compared with an ATP standard (Sigma Aldrich) treated in the same way as the samples. Luminescence intensity was determined immediately on a Spectra Fluor Plus spectrophotometer (Tecan).

To determine lactate levels produced by the cells, 20 μ L of the supernatant of the neutralized lysate was mixed with 100 μ L of lactate assay solution (Trinity Biotech plc, Wicklow, Ireland). After 15 minutes of incubation at room temperature, absorbance was measured at 540 nm in a spectrophotometer.

Scramblase and flipase activity

Freshly isolated neutrophils and PBMC were incubated at 37 °C at a concentration of 5×10^6 /mL in HBSS in the presence of 50 nM of the fluorescent PS and PC (phosphatidylcholine) analogues palmitoyl-C₆-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-phosphatidylserine respectively –phosphatidylcholine (NBD-PS/PC; Avanti Polar Lipids, Birmingham, AL, USA). Samples of 100 μ L were taken at the indicated time points and diluted with 200 μ L ice-cold HBSS supplemented with 4% HSA to extract label remaining in the outer leaflet of the plasma membrane or without HSA to determine total fluorescence. Afterwards, the cells were analyzed by flow cytometry on an LSRII flow cytometer (BD). The fraction of the probe incorporated was determined by dividing the mean fluorescence of the sample after HSA treatment (internalized probe) by the mean fluorescence of the untreated sample (total bound probe).

Statistical analysis and image processing

Graphs were drawn and statistical analysis was performed with GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA). The results are presented as the mean $\pm$ SEM, as indicated. Data were evaluated by paired, one-tailed student's *t*-test. The criterion for significance was $p < 0.05$ for all comparisons.

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Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3

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Abstract

Neutrophils have a very short life span and undergo apoptosis within 24 hours after leaving the bone marrow. Granulocyte colony-stimulating factor (G-CSF) is essential for the recruitment of fresh neutrophils from the bone marrow but also delays apoptosis of mature neutrophils. To determine the mechanism by which G-CSF inhibits neutrophil apoptosis, the kinetics of neutrophil apoptosis during 24 hours in the absence or presence of G-CSF were analyzed *in vitro*. G-CSF delayed neutrophil apoptosis for about 12 hours and inhibited caspase-9 and -3 activation, but had virtually no effect on caspase-8 and little effect on the release of pro-apoptotic proteins from the mitochondria. However, G-CSF strongly inhibited the activation of calcium-dependent cysteine proteases calpains, upstream of caspase-3, via apparent control of Ca^{2+} -influx. Calpain inhibition resulted in the stabilization of the X-linked inhibitor of apoptosis (XIAP) and hence inhibited caspase-9 and -3 in human neutrophils. Thus, neutrophil apoptosis is controlled by G-CSF after initial activation of caspase-8 and mitochondrial permeabilization by the control of post-mitochondrial calpain activity.

Introduction

Neutrophils, the primary phagocytic cells of the human immune system, have a very short life span after leaving the bone marrow of approximately 24 hours. Thereafter, they die either by intrinsically or extrinsically induced apoptosis. Extrinsically, neutrophil apoptosis can be activated through the ligation of death receptors, such as Fas/CD95 or other receptors of the tumor necrosis factor alpha (TNF α) family^{1,2}. Intrinsically, neutrophil apoptosis appears to be regulated at the level of their mitochondria and involves the spontaneous clustering of death receptors³ or release of cathepsin D⁴, but many details about intrinsic, or spontaneous, neutrophil apoptosis remain unclear^{5,6}.

A vitally important factor for the recruitment of fresh neutrophils from the bone marrow is granulocyte colony-stimulating factor (G-CSF)^{7,8}. G-CSF has previously been shown to inhibit neutrophil apoptosis both *in vivo*⁹ and *in vitro*¹⁰ and is widely used in a clinical setting to treat various conditions associated with severe neutropenia. Healthy donors are also given G-CSF prior to a peripheral hematopoietic stem cell or granulocyte donation.

Most studies on G-CSF signaling have been done with the murine myeloblast cell line 32D clone 3 (32Dcl3). In this cell line, signal transduction of the G-CSF receptor occurs via the Janus kinase (Jak)/signal transducers and activators of transcription 3 (STAT-3) pathway¹¹⁻¹³. These studies mainly focused on neutrophil differentiation, and little as yet is known about the inhibition of apoptosis by G-CSF in primary human neutrophils.

Previous studies in our lab have indicated that G-CSF primarily inhibits apoptosis by preventing the activation of the executioner of apoptosis, the cysteine protease caspase-3^{10,14}. Caspase-3 is activated downstream of the initiators of apoptosis, caspase-8 and -9. Caspase-8 is activated after death receptor clustering, which occurs spontaneously during neutrophil apoptosis, while caspase-9 activation normally occurs after the release of pro-apoptotic factors from the mitochondria, including Smac. When Smac is released from the mitochondria, it competes with caspase-9 and -3 for binding to the so-called inhibitors of apoptosis (IAPs). The main member of this protein family of IAPs that is present in neutrophils and responsible for the inhibition of both caspase-9 and -3 is the X-linked inhibitor of apoptosis (XIAP)¹⁴⁻¹⁶. Several other IAP family members have been identified in neutrophils and are implicated in neutrophil apoptosis^{17,18}, but it has recently become clear that only XIAP is a true inhibitor of caspase activation¹⁹⁻²¹. Both caspase-9 and caspase-3 can become activated only after they have been released from XIAP by Smac^{22,23}. Caspase-8 controls the release of Smac from the mitochondria via cleavage of the pro-apoptotic Bcl-2

family member Bid. Truncated Bid (tBid) is involved in the activation and mitochondrial translocation of another pro-apoptotic Bcl-2 family member, Bax, and subsequent permeabilization of the mitochondrial outer membrane ²⁴.

Several studies have implicated calpain activity in spontaneous neutrophil apoptosis ^{25,26}. Calpains are a family of calcium-dependent cysteine proteases of which calpain-1 (μ -calpain, calpain I), calpain-2 (m-calpain, calpain II) and the natural inhibitor of calpains, calpastatin, are ubiquitously expressed. Additional, more tissue-specific, isoforms of the calpains have also been identified, but these are not expressed in neutrophils ²⁷. Calpain-1 has been shown to be involved in the activation of the pro-apoptotic Bcl-2 family member Bax in human neutrophils ²⁸. In chronic neutrophilic leukemia, calpain activation and subsequent degradation of XIAP is impaired, leading to increased neutrophil survival ¹⁶. Thus, calpain activation plays an important role in the regulation of neutrophil apoptosis, both by activation of pro-apoptotic factors as well as by the degradation of anti-apoptotic proteins.

For this study, a number of apoptotic parameters at various time points during 24 hours of neutrophil incubation in the absence or presence of G-CSF was monitored. We show that G-CSF inhibits phosphatidyl serine (PS) exposure and the morphologic features of apoptosis but has no effect on the loss of mitochondrial membrane potential and the release of Smac from the mitochondria. Although incubation with G-CSF did inhibit the activation of caspase-9 and -3, activation of caspase-8 was not prevented. We show that G-CSF inhibits the activation of calpains and limits the increase in intracellular Ca^{2+} during neutrophil apoptosis. As an apparent consequence of this inhibition, XIAP degradation is prevented and the downstream activation of caspase-9 is delayed, resulting in inhibition of cell death. Thus, G-CSF controls neutrophil apoptosis downstream of the mitochondria at the level of caspase activation.

Results

G-CSF inhibits neutrophil apoptosis

A common marker for apoptotic cells is PS exposure on the outer leaflet of the plasma membrane. PS exposure was monitored on neutrophils during incubation in the presence or absence of 10 ng/mL G-CSF. In Figure 1A, the percentage of cells is shown that stain negative for both Annexin V (as a marker for PS) and PI (as a marker for membrane-permeable late-apoptotic or necrotic cells). After 6 hours of incubation, PS exposure accelerated rapidly on the control neutrophils. A similar acceleration was not seen in the G-CSF treated neutrophils until after 16 hours of incubation, in all six donors included in this study. Although G-CSF significantly inhibited the loss of mitochondrial membrane potential ($\Delta\psi_m$), associated with the release of pro-apoptotic proteins from the mitochondrial intermembrane space ³⁰⁻³², this process still proceeded gradually in the G-CSF treated cells as in the control cells (Figure 1B). To analyze the morphology of the neutrophils for apoptotic features, cytopsins of the cells were prepared at the indicated time points and stained with May-Grünwald Giemsa stain before analysis by light microscopy. Morphological changes of the neutrophils, such as cell shrinkage and nuclear condensation, matched the exposure of PS, as shown in Figure 1C.

During neutrophil aging, expression of the CXC chemokine receptor 4 (CXCR4) is increased on the outer membrane of the cells (Figure 1D). Neutrophil maturation in the bone marrow is associated with a decrease in CXCR4 expression, which is induced by G-CSF and leads to the release of mature cells into the circulation ³³. During the first 12 hours of neutrophil incubation, G-CSF did not have a significant effect on CXCR4 exposure. However, after 12 hours, G-CSF prevented the further increase in CXCR4 exposure, while the expression of this marker continued to rise on the untreated cells (Figure 1D). Shedding of the Fc γ receptor IIIb (CD16), another hallmark of neutrophil apoptosis

Figure 1. G-CSF inhibits neutrophil apoptosis. Neutrophils were incubated in the absence or presence of G-CSF (10 ng/mL). Samples were taken at various times to determine the indicated hallmarks of apoptosis. **A)** Cells were stained with FITC-labeled Annexin V and PI and analyzed by flow cytometry. The percentage of double negative cells is expressed as survival. **B)** Cells were loaded with the $\Delta\psi_m$ -sensitive dye JC-1 and analyzed by flow cytometry. When JC-1 accumulates in mitochondria with a high $\Delta\psi_m$ it displays red fluorescence (FL-2), while the dye is green fluorescent (FL-1) when $\Delta\psi_m$ is low. The percentage of cells with a high $\Delta\psi_m$ is expressed in this graph, corrected for background staining (*i.e.* the JC-1 fluorescence of cells treated with 1 μ M of the uncoupler CCCP). The average slope for the loss of $\Delta\psi_m$ in the control cells was $-3.53 (\pm 0.1801, \text{SEM}) \%/h$, while the average slope for the G-CSF treated cells was $-2.66 (\pm 0.1927, \text{s.e.m.}) \%/h$. The difference between the slopes is highly significant with a p value of 0.001. **C)** 2×10^5 cells were spotted on microscopy glasses and stained with May-Grünwald Giemsa stain before analysis with a light microscope. Pictures were taken at 60x magnification and are representative of 6 different experiments. **D-E)** Cells were double labeled for CXCR4 and CD16 and analyzed by flow cytometry. Data are expressed as a percentage of the maximum fluorescence for the control sample at 0h for CD16 and 24h for CXCR4.

^{34,35}, was not affected by G-CSF at these time points (Figure 1E). This finding is surprising, since GM-CSF, another potent inhibitor of neutrophil apoptosis, has been described to preserve CD16 expression ³⁶.

G-CSF inhibits cleavage of caspase-3 and -9, but not caspase-8

In healthy cells, caspases are present as inactive zymogens, while active caspases are obligate dimers of identical catalytic subunits ³⁷. After a pro-apoptotic stimulus, caspases first dimerize, after which their p10/p12 domain is cleaved off by autocatalysis, revealing the active sites in the p20/p22 domain. Full activation only occurs after a second cleavage event has released the p20/p22 domain. The fully activated caspase consists of a dimer of two p10/p12 and two p17/p18 subunits. Caspases are activated in sequence, starting with the initiator, caspase-8, which is activated after forced oligomerization at the death receptors. One of the main targets of active caspase-8 is the pro-apoptotic Bcl-2 family member Bid. After Bid has been cleaved by caspase-8 (tBid), it associates with Bax at the mitochondria to form the permeability transition pore, through which the pro-apoptotic proteins that activate caspase-9 are released ²⁴. Finally, caspase-9 activates the executioner caspase-3 and apoptosis ensues.

During spontaneous neutrophil apoptosis, caspase-8, -9 and -3 are rapidly activated. The cleavage products of the active caspases can already be spotted on Western blot after 4 hours (Figure 2). For caspase-8, which has two splice variants in neutrophils, the appearance of the semi-active caspases (p43/p41) is most readily detected with the

Figure 2. G-CSF inhibits the activation of caspase-9 and -3, but not of caspase-8.

Samples of neutrophils incubated in the presence or absence of 10 ng/mL G-CSF were taken at the indicated times, subjected to SDS-PAGE and analyzed on Western blots, stained with antibodies against the indicated caspases. Arrows indicate the full-length, inactive caspases and their cleavage products after activation. Equal amounts of cells (1.5×10^6), dissolved in sample buffer, were loaded in each lane. All blots are representative of at least 4 independent experiments.

antibody used in this study, while the inactive forms (p57/p55) gradually disappear and the appearance of the fully active form can vaguely be detected (p18). For caspase-9, the disappearance of the full-length caspase (p47) is most readily detected. At approximately 35 kDa, a band is visible on the blot that could be the semi active caspase p35, but since this band is also detected in fresh cells, it is not deemed likely that this is a cleavage product of caspase-9. It could be a non-specific band instead. On the caspase-3 blots, both the disappearance of the full length caspase (p32) as well as the appearance of the cleavage product (p17) can be detected. After 12 hours, all caspases are completely cleaved, showing that caspase activation precedes PS exposure and morphological changes. Although G-CSF inhibits the activation of caspase-9 and -3 for at least 12 hours, corresponding with the 12-hour delay in PS exposure, caspase-8 activation was not affected. Thus, G-CSF inhibits neutrophil apoptosis downstream of caspase-8 activation.

G-CSF inhibits neither the translocation of Bax and Bid nor the release of mitochondrial Smac

The pro-apoptotic Bcl-2 family members Bax and Bid are both abundantly present in the cytosol of fresh neutrophils. During apoptosis, both proteins translocate to the mitochondria to initiate the formation of the permeability transition pore in the outer mitochondrial membrane, through which Smac and other pro-apoptotic proteins can be released. A certain amount of Bax is already present on the mitochondria of healthy cells, where it is counteracted by anti-

Figure 3. G-CSF does not inhibit the release of pro-apoptotic factors from the mitochondria during apoptosis.

Untreated neutrophils or neutrophils treated with 10 ng/mL G-CSF were fractionated at the indicated time points. The cytosolic fractions, which contain no mitochondria, and the pellet fractions, which are enriched for mitochondria, were subjected to SDS-PAGE and analyzed on Western blots stained for the indicated proteins. Arrows indicate the full-length proteins and cleavage products in the cases of Bax and Bid. Blots were re-probed for ASC (cytosolic) and MnSOD (mitochondrial matrix) to control for cross contamination and protein loading (lower panels). All blots are representative of at least 4 independent experiments.

apoptotic Bcl-2 family members such as Mcl-1^{38,39}. To detect the translocation of the activated proteins to the mitochondria and the release of Smac from the mitochondria, we extracted the cytosol from neutrophils after permeabilization of the plasma membrane with digitonin at the indicated time points. The remaining pellet, containing the mitochondria, was dissolved in a buffer containing NP40. Both fractions were analyzed on Western blot.

In the untreated cells, an increase in mitochondrial Bax can be seen from 4 hours onwards (Figure 3) in the membrane fraction. Cleaved, active, Bax also appears in the membranes fraction from 4 hours onwards. The increase in mitochondrial Bax occurs concomitantly with a decrease in cytosolic Bax. In the same period, Bid is gradually cleaved and also translocates to the mitochondria-enriched membranes fraction. Smac is exclusively present in the membranes fraction of fresh cells, but translocation of Smac from the mitochondria to the cytosol already occurs after 4 hours. In the G-CSF treated cells, these events occur from 8 hours onwards. Thus, G-CSF delays mitochondrial outer membrane permeabilization for approximately 4 hours, while caspase activation is delayed for at least 12 hours.

To control for cross contamination of the fractions, the blots were stained for either the cytosolic adaptor protein ASC or the mitochondrial matrix protein MnSOD, as shown in the lower panels of Figure 3. Only in the latest time point, MnSOD was also found in the cytosol fraction, indicative of complete degradation of the mitochondria at this time point.

G-CSF delays the gradual rise of intracellular Ca^{2+} during neutrophil apoptosis

Previously, it has been noted that neutrophil apoptosis is associated with a rise in the basal levels of intracellular Ca^{2+} ^{25,40}. Since we observed that most events relevant for apoptosis occur during the first 12 hours of neutrophil culture, we decided to monitor the changes in intracellular Ca^{2+} during this period. In 12 hours, intracellular Ca^{2+} rises to about 1.5 μM in unstimulated cells, while Ca^{2+} levels in G-CSF-stimulated cells did not reach 0.5 μM in the same period (Figure 4A). When the cells were incubated in the absence of extracellular Ca^{2+} , intracellular Ca^{2+} levels did not rise significantly, suggesting that the observed was due to extracellular influx. In addition, prevention of the rise in

Figure 4. G-CSF inhibits the steady increase in cytosolic free Ca^{2+} during neutrophil aging, which is essential for apoptosis. **A)** Neutrophils were loaded with the fluorescent Ca^{2+} indicator Fluo-3 and incubated in the presence or absence of 10 ng/mL G-CSF in the stirred cuvette of a fluorometer, while the increase in fluorescence was measured every minute for 12 hours. As controls, cells were loaded with 10 μM of the cell permeable Ca^{2+} -chelator BAPTA-AM or incubated in the absence of extracellular Ca^{2+} . Data represent the average of 3 independent experiments. **B)** An increase in intracellular free Ca^{2+} is a requirement for the process of neutrophil apoptosis, since treating the cells with an increasing dose of BAPTA-AM induced up to 75% neutrophil survival at 10 μM after 18h incubation. The percentage of surviving cells is expressed as a lack of Annexin V/PI staining determined by flow cytometry. Data represent the mean ($\pm$ s.e.m.) of 3 independent experiments performed in duplicate.

intracellular Ca^{2+} by treating neutrophils with the cell permeable Ca^{2+} -chelator BAPTA-AM (Figure 4A), strongly promoted survival in a concentration-dependent manner after 18h in culture (Figure 4B), as indicated by Annexin V staining. These observations were confirmed on cytopins of the same samples, stained by May-Grünwald Giemsa stain and analyzed by light microscopy (not shown). Thus, the observed rise in intracellular Ca^{2+} seems to be a requirement for neutrophil apoptosis.

G-CSF inhibits calpain activation in neutrophils, upstream of caspase-3

Calpains are cysteine proteases that are activated in a Ca^{2+} -dependent manner and play an important role in neutrophil apoptosis^{16,26}. The observed critical rise in intracellular Ca^{2+} in neutrophils during apoptosis, prompted us to investigate the activation of calpains during neutrophil apoptosis.

Calpain and caspase-3 activities were measured in cell extracts, with fluorescently labeled peptides specific for each protease, although the calpain substrate we used for this study (calpain substrate II) does not distinguish between calpain-1 and -2 activities. Active proteases cleave the peptide whereupon the fluorescent group is released. As a result, the increase in fluorescence is indicative of the protease activity.

During incubation, both calpains and caspase-3 were found to be gradually activated in neutrophils (Figure 5A-B). Caspase-3 activity became detectable after 6 hours and did not reach peak levels in the 8 hours of the experiment, while calpain activity could already be measured from 4 hours onwards reaching peak levels after 6 hours. Both proteases were significantly inhibited by G-CSF (Figure 5), although G-CSF had a more pronounced effect on calpains than on caspase-3 in the time frame of the experiment. Moreover, the specific calpain inhibitor 3 (CI3) inhibited calpain activity completely but also inhibited caspase-3 significantly in whole cells (Figure 5C). In contrast,

Figure 5. G-CSF inhibits calpain activation, upstream of caspase-3. To determine calpain and caspase-3 activation during neutrophil apoptosis, cytosolic extracts of neutrophils, incubated in the presence of 10 ng/mL G-CSF and various inhibitors as indicated, were made at the indicated times (**A and B**) or after 8 hours (**C**). Protease activity was determined by incubating the samples in the presence of a fluorescent peptide, specific for either caspase-3 or calpains. **A-B**) After incubation, the increase in fluorescence was used as an indicator of the relative protease activity in the samples. **C**) To control for inhibitor/substrate specificity, the sample with the highest protease activity (the 8h control) was treated with 20 μM of the pan-caspase inhibitor z-VAD or 20 μM of the calpain inhibitor 3 (CI3). Activity is expressed as a relative to the control (8h, no inhibitors) as corrected for background activity (0h, no inhibitors). All data represent the mean ($\pm$ s.e.m.) of 3 independent experiments performed in duplicate. Significance was determined relative to the control (8h, no inhibitors) by paired, one-tailed students t-test. * $p < 0.05$, ** $p < 0.0001$ and n.s. (not significant) $p > 0.05$.

the pan-caspase inhibitor z-VAD had no significant effect on calpain activity but inhibited caspase-3 to a similar extent as G-CSF.

To verify specificity of the inhibitors, CI3 and z-VAD were added to samples with a high calpain/caspase-3 activity (*i.e.* the extract of untreated neutrophils, cultured for 8 hours). In these samples, CI3 completely inhibited calpain activity but it did not inhibit caspase-3. On the other hand, z-VAD completely inhibited caspase-3 activity in those samples but also prevented the cleavage of the calpain-specific fluorescent peptide to a significant degree. Apparently, z-VAD competes with the calpain peptide when added directly to a sample with active proteases, while it did not have this effect in intact cells. Since the calpain inhibitor completely blocked all activity measured with the calpain substrate, but had little effect on active caspases, we conclude that z-VAD might aspecifically inhibit calpains, rather than that the calpain substrate detects caspase activity. Therefore, we conclude that CI3 itself does not inhibit caspases, but inhibition of calpains leads to caspase-3 inhibition in whole cells. This demonstrates that calpains operate upstream of caspase-3 to regulate its activity.

G-CSF prevents the degradation of XIAP by calpains

Activation of the caspases downstream of mitochondrial permeabilization is controlled by the inhibitors of apoptosis, most notably the X-linked inhibitor of apoptosis (XIAP). XIAP has recently been identified as a target for calpains in neutrophils¹⁶.

XIAP degradation was analyzed on Western blots of whole cell extracts (Figure 6A). In fresh cells, only the full-length, 57-kDa form of XIAP was detectable. During incubation, the cleaved form of the protein became visible in the untreated cells after 8 hours. In G-CSF treated cells, the cleaved form became detectable only after 20 hours. To see whether calpain inhibition could prevent XIAP degradation, neutrophils were incubated overnight with various inhibitors and controls as shown in Figure 6B. After overnight incubation, full-length XIAP was nearly completely degraded while two fragments appeared on the blot, one around 30 kDa and one around 20 kDa. Incubation with G-CSF delayed the cleavage of XIAP. Addition of cycloheximide (CHX, 10 µg/mL), a potent inhibitor of mRNA translation, to either control or G-CSF-treated cells had no effect on XIAP expression or degradation. However, CHX did have an effect on the delay of apoptosis induced by G-CSF after overnight incubation (Figure 6C). Inhibition of calpains by CI3 fully prevented the degradation of XIAP, whereas the addition of the proteasome inhibitor MG132 had no effect, even though this inhibitor did delay neutrophil apoptosis (Figure 6B). The effect of MG132 on neutrophil apoptosis is mainly explained by stabilization of the anti-apoptotic Bcl-1 family member Mcl-1, as previously demonstrated by Derouet *et al*⁴¹ (data not shown). None of the inhibitors by itself could completely prevent caspase-3 activation, as shown in Figure 6B, although caspase-3 activation was clearly reduced in G-CSF-, CI3- and z-VAD-treated cells.

The caspase inhibitor z-VAD only had a partial effect on XIAP degradation. Some degradation did occur, but the lower cleavage product did not appear on blot. This could suggest that XIAP degradation is a two-step process; the first step depending on calpain activity and the second on active caspases. Addition of 5 µM BAPTA-AM to the cells almost completely prevented the degradation of XIAP, thus confirming that the initiation of XIAP degradation indeed depends on intracellular Ca²⁺, which is an essential prerequisite for calpain activity.

Figure 6. G-CSF inhibits the degradation of XIAP in neutrophils.

A-B) Neutrophils were incubated in the absence or presence of G-CSF, cycloheximide (CHX), G-CSF + CHX (G+CHX), the calpain inhibitor CI3 (20 μ M), the proteasome inhibitor MG132 (50 μ M), the caspase inhibitor z-VAD (20 μ M) or the Ca^{2+} chelator BAPTA (5 μ M) for 16 hours. After incubation, whole-cell lysates were prepared, run on SDS-PAGE gel and analyzed by Western blot for XIAP expression (A and B). Arrows indicate full-length XIAP (57 kDa) and its cleavage products (~30 kDa and ~20 kDa). The antibody is directed against a region at the C-terminal site of the protein, including the BIR3 domain. The blot is representative of 3 independent experiments. For B, the blot was re-probed to detect caspase-3. **C)** Survival was determined by Annexin V staining on the flow cytometer. Data are expressed as a percentage ($\pm$ s.e.m.) of Annexin V negative cells and represent 4 different experiments performed in duplicate. Significance was determined relative to the control (20h, no inhibitors) for all samples except G-CSF+CHX, which was compared to G-CSF, by paired, one-tailed students t-test. * $p < 0.05$, ** $p < 0.0001$ and n.s. (not significant) $p > 0.05$.

Discussion

In this report, we demonstrate for the first time that G-CSF inhibits neutrophil apoptosis by the inhibition of calpain activity, which acts upstream of caspase-3. Our results indicate that spontaneous neutrophil apoptosis is accompanied by a gradual increase in intracellular free Ca^{2+} and suggests that neutrophil apoptosis proceeds at an accelerated pace after the intracellular free Ca^{2+} concentration has reached a certain threshold. G-CSF delays the influx of extracellular Ca^{2+} , the subsequent activation of calpains and the downstream activation of caspase-3. Calpain inhibition completely prevents the degradation of XIAP in neutrophils. Apparently, the release of pro-apoptotic proteins from the mitochondria is not enough to overcome the inhibition of caspases by XIAP, since G-CSF hardly delayed the release of mitochondrial Smac. The fact that neutrophil mitochondria contain very little cytochrome *c*^{6,42} might explain this observed inefficiency of Smac to activate caspases, since cytochrome *c* normally activates the apoptosome, the complex wherein caspase-9 is activated. A schematic overview of these processes and the effect of G-CSF is given in Figure 7.

Calpain activation is regulated in several ways. During neutrophil activation, by bacterial ligands for example,

Ca^{2+} levels also increase significantly⁴⁰. Although this may lead to temporal activation of calpains, it does not induce neutrophil apoptosis. Under these conditions, Smac and other pro-apoptotic factors are not released from the mitochondria and caspase-9 remains inactive. Moreover, XIAP is not the only target of calpains in neutrophils. Several isoforms of protein kinase C, for example, form another important target for calpains⁴³. After this kinase has been cleaved by calpains, the free catalytic subunit (protein kinase M, PKM) rapidly phosphorylates its targets. One of the results of PKC activation is the exposure of PS on the outer leaflet of the cell membrane via activation of the phospholipid scramblase responsible for an uneven distribution of PS over the inner and outer leaflet of the plasma membrane⁴⁴. Especially the PKC- δ isoform seems to be involved in neutrophil apoptosis. Whether this enzyme is regulated by caspase-3 or calpains in neutrophils, is not quite clear⁴⁵. Since we show that caspase-3 activation in neutrophils occurs downstream of calpain activation, we may have explained this apparent discrepancy in the literature. In addition, it appears that PKC- δ is involved in the stabilization and upregulation of the Bcl-2 family member Bad, which also plays an important role in neutrophil apoptosis⁴⁶. Thus, calpains regulate several processes involved in neutrophil apoptosis, and their inactivation can delay the process at the different levels where the targets for calpain activity operate.

Figure 7. Schematic representation of the apoptotic pathways in neutrophils. Caspase-8 is activated after death receptor clustering and consequently activates Bid. Bid activates Bax, which multimerizes in the mitochondrial membrane, forming the permeability transition pore, through which Smac and cytochrome *c* are released. Smac competes with caspase-3 and -9 for binding with XIAP, thus removing its inhibitory effect from these caspases. Cytochrome *c* activates caspase-9 through the apoptosome. Finally, caspase-9 activates caspase-3, which executes the process of apoptosis. In parallel, Ca^{2+} levels rise spontaneously, which leads to the activation of calpains. The calpains cleave XIAP, resulting in an increased activation of caspases-9 and -3. G-CSF inhibits this rise in Ca^{2+} , leading to inhibition of calpain activation, preventing the subsequent degradation of XIAP and causing a delay in the activation of caspases-9 and -3.

Similarly of course, it is not to be expected that inhibition of Ca^{2+} influx is the only way by which G-CSF inhibits neutrophil apoptosis and calpain activation. Probably, G-CSF also influences the gene expression pattern in mature neutrophils. Thus far, no detailed studies have been performed on gene expression profiling in mature neutrophils after G-CSF treatment. In the current study, protein translation inhibition by cycloheximide partially prevents the G-CSF-induced delay of neutrophil apoptosis. This suggests that gene expression plays an active role in this process. Further research is required to study the relevant effects of G-CSF on neutrophil gene expression.

As yet, we do not exactly know how G-CSF signaling prevents the spontaneous influx of Ca^{2+} during neutrophil apoptosis. The main intracellular store for Ca^{2+} is the endoplasmic reticulum (ER). It is well known that depletion of the Ca^{2+} store of the ER leads to an influx of extracellular Ca^{2+} , the process of so-called store-operated Ca^{2+} influx⁴⁷. Ca^{2+} storage in the ER is mainly controlled by the interaction of inositol 1,4,5-triphosphate (IP3) with its receptor on the ER⁴⁸. An increase in free intracellular IP3 can thus lead to a release of Ca^{2+} from the ER, resulting in a brief rise of intracellular Ca^{2+} . As a consequence of the depletion of Ca^{2+} from the ER, the store-operated channels for Ca^{2+} are opened in the plasma membrane, leading to a rapid, more steadfast, increase in intracellular free Ca^{2+} . In recent years, it has become clear that the Bcl-2 family of proteins plays an important role in the control of intracellular

Ca^{2+} homeostasis⁴⁹⁻⁵¹ by controlling the sensitivity of the IP3 receptor for its ligand. When the anti-apoptotic protein Bcl-2, for example, associates with the IP3 receptor, it is less sensitive for IP3, while association of the pro-apoptotic Bcl-2 family member Bak increases the sensitivity of the receptor for IP3. Thus, expression and activation of Bcl-2 family members control the influx of extracellular Ca^{2+} . Neutrophils do not express Bcl-2, but, instead, express the antiapoptotic Bcl-2 family members Bcl-x_L, Mcl-1 and Bfl-1⁵²⁻⁵⁴. Bfl-1 has been shown to associate with Bak and to control its activity⁵⁵. In addition, G-CSF has been shown to increase the expression of Bfl-1 in neutrophils⁵⁶. Therefore, it would not seem unlikely that G-CSF controls the influx of extracellular Ca^{2+} via control of Bfl-1, but further research has to be done to clarify this link in neutrophils.

In conclusion, we show that G-CSF controls neutrophil apoptosis via control of calpain activity. Thus, we shed new light on the actions of this clinically relevant cytokine in the control of neutrophil homeostasis. In addition, we emphasize the importance of the control of Ca^{2+} homeostasis in the process of apoptosis. Further research is required to elucidate the exact mechanism of Ca^{2+} control by G-CSF.

Materials and methods

Antibodies and reagents

Antibodies against caspase-3 (#9662), caspase-8 (1C12, #9746), caspase-9 (#9502), Bid (#2002) and Smac/Diablo (#2954) were obtained from Cell Signaling Technology (Boston, MA, USA). Anti-human ASC was obtained from Medical&Biological Laboratories (MBL; Woburn, MA, USA). The anti-Mn-SOD antibody was obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Rabbit anti-human-Bax was obtained from BD PharMingen and mouse anti-human-hILP/XIAP (clone 48) was obtained from BD Transduction Laboratories.

All chemical reagents were obtained from Merck Biosciences (Darmstadt, Germany), unless otherwise indicated.

Cell preparation and culture

Neutrophils were isolated from the heparinized blood of healthy individuals after informed consent had been acquired, by centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) and subsequent lysis of erythrocytes as described²⁹. Neutrophil preparation were typically >97% pure, with the contaminating cells being mostly eosinophils. Cells were cultured in Hepes-buffered saline solution (HBSS; 132 mM NaCl, 20 mM Hepes, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄, 1 mM CaCl₂, pH 7.4) supplemented with 1% human serum albumin (Cealb; Sanquin, Amsterdam, the Netherlands) and 5 mM glucose at a concentration of 5×10^6 cells/mL in polypropylene round-bottom tubes of 14 mL (Falcon; BD Biosciences; Franklin Lakes, NJ, USA). Incubations were performed in a shaking water bath at 37°C. Cells were incubated in the presence of 10 ng/mL clinical grade G-CSF (Neupogen, Amgen, Breda, the Netherlands) where indicated.

Cell fractionation and Western blot

Sub-cellular fractions were prepared of 5×10^6 cells by washing the cells once in ice-cold phosphate-buffered saline (PBS) before resuspension in cytosol extraction buffer (250 mM sucrose, 70 mM KCl, 250 µg/mL digitonin, complete protease inhibitor cocktail mix [PIM; Roche Diagnostics, Almere, The Netherlands] and 2 mM diisopropylfluorophosphate [DFP; Fluka Chemica, Steinheim, Switzerland] in PBS) at a concentration of 100×10^6 cells/mL. Cells were incubated for 10 min. on ice, after which they were centrifuged for 5 min. at 1000xg, 4°C. The supernatant represented the cytosolic fraction, the pellet, containing the mitochondria, was dissolved in mitochondrial lysis buffer (100 mM NaCl, 10 mM MgCl₂, 2 mM EGTA, 2 mM EDTA, 1% (v/v) NP-40, 10% (v/v) glycerol [Sigma Aldrich], PIM, 2 mM DFP in 50 mM TrisHCl buffer at pH 7.5) and incubated for another 10 min. on ice. After incubation, the samples were centrifuged at 10,000xg for 10 min. at 4°C. The supernatant, containing the mitochondrial content, represented the membrane fraction. The insoluble pellet was discarded. Both fractions were dissolved in Laemmli sample buffer (LSB; 50 mM TrisHCl, pH 6.8, 10% glycerol (v/v), 5 mM DTT [DL-dithiothreitol, Sigma Aldrich], 1% 2-mercaptoethanol, 1% sodiumdodecylsulphate (SDS) (m/v), 100 µg/mL bromophenol blue) and boiled for 10 min. at 95°C.

Total cell lysates were prepared by lysing the cells in cell lysis buffer (250 mM sucrose, 70 mM KCl, 0.5% Triton X-100 (v/v), 0.5% β-octylglucoside (v/v), 2 mM NaVO₄, 1 mM NaF, 1 mM EDTA, PIM and 2 mM DFP in PBS) for 30 min. on ice. Afterwards, the samples were dissolved in LSB and boiled for 30 min. at 95°C. All samples were stored at -20°C before subjection

to SDS-polyacrylamide gelelectrophoresis (SDS-PAGE).

Samples were run on 12%, 1.5 mm, polyacrylamide gels in a protean-3 mini system (Bio-Rad Laboratories, Veenendaal, the Netherlands). The equivalent of 1.5×10^6 cells was loaded in each lane. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad), which were subsequently blocked for 30 min. with blocking buffer (5% non-fat dry milk (*m/v*) [Elk; Campina, Zaltbommel, The Netherlands] in Tris-buffered saline, 0.1% Tween-20 (*v/v*) (TBST)). Blots were immuno-labeled with specific antibodies against the indicated proteins in blocking buffer containing 2 mM NaN_3 , overnight at 4°C. After washing the blots in TBST, they were treated with horse-radish- peroxidase-labeled secondary antibodies directed against the primary antibodies (donkey anti-rabbit-IgG or sheep anti-mouse-IgG, Amersham, Little Chalfont, UK). Labeling was followed by another round of washing in TBST before detection of the specific signals with Pierce ECL Western blotting substrate (Pierce, Rockford, IL, USA) on Fuji medical X-ray film (FujiFilm Corporation, Tokyo, Japan).

Flow cytometry

To detect apoptosis, cells were labeled for 10 min. on ice with fluorescein-thiocyanate (FITC)-labeled Annexin V (Bender Med Systems, Vienna, Austria), diluted 1:500 in HBSS, supplemented with 2.5 mM CaCl_2 . Annexin V labeling was followed by a single wash step with the same medium, whereupon the cells were resuspended in HBSS 2.5 mM CaCl_2 containing 1 $\mu\text{g/mL}$ propidium iodide (PI; Sigma). After an additional 5 min. on ice, the samples were analyzed by flow cytometry on a FACScan flow cytometer (BD Biosciences). Surviving cells were defined as the cells in the lower left quadrant that stained negative for both Annexin V and PI.

To detect changes in $\Delta\psi_m$, the cells were loaded for 15 min. at 37°C with 0.5 μM JC-1 (Molecular Probes, Eugene, OR, USA) in HBSS, containing 1 μM tetraphenyl boron to facilitate entry of the dye into the cells, and analyzed immediately by flow cytometry. To determine a background value for $\Delta\psi_m$, cells were incubated with 1 μM CCCP (Calbiochem, La Jolla, CA, USA), which is sufficient to completely abrogate $\Delta\psi_m$. Cells with high $\Delta\psi_m$ were defined as the population of cells with high fluorescence in the FL-2 (red) channel ($10 < \text{fluorescence intensity} < 1000$) that did not include cells that were treated with CCCP.

Surface expression of CXCR4 and CD16 was detected by incubating the cells for 30 min. at room temperature with a phycoerythrin (PE)-labeled antibody directed against CXCR4 (BD Pharmingen) and a FITC-labeled antibody directed against CD16 (Sanquin). Both antibodies were diluted 1:200 in HBSS. After washing, the samples were analyzed on an LSRII flow cytometer (BD Biosciences).

Calpain and caspase assay

Calpain and caspase-3 activities were determined in cytosolic extracts, prepared in the same way as for the subcellular fractions. Samples were stored at 4°C until use. Protein concentration in the samples was determined with the BCA protein assay kit from Pierce. Protease activity was determined by the addition of 100 μL reaction buffer (10 mM Hepes, pH 7.4, 20% glycerol, 5 mM DTT, 1 mM CaCl_2 and 20 μM fluorescent substrate for caspase-3 (Ac-IETD-AMC; Alexis Biochemicals) or Calpain Substrate II (Suc-LY-AMC; Calbiochem) for calpains) to 10 μL of the sample, containing 4 mg protein/mL. The fluorescence increase was measured in a SpectraFluo Plus spectrophotometer (Tecan, Zürich, Switzerland) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm after 2 hours of incubation at 37°. As controls, intact cells or samples with high protease activity (8h, untreated) were treated with 20 μM of the cell permeable pan-caspase inhibitor Z-VADfmk (Alexis Biochemicals) or 20 μM of the broad spectrum Calpain-Inhibitor-3 (CI3, Calbiochem).

Determination of cytosolic free Ca^{2+}

Cytosolic free Ca^{2+} was determined by loading the cells with 1 μM of the Ca^{2+} -indicator fluo-3 acetoxymethyl (AM) ester (Molecular Probes) for 45 min. at 37°C. After loading, the cells were washed once in HBSS, resuspended in HBSS containing 1 $\mu\text{L/mL}$ anti-fluorescein antibody (Molecular Probes) to quench extracellular Fluo-3, at a concentration of 5×10^6 cells/mL, and transferred to the quartz cuvettes of a fluorometer, after which 10 ng/mL G-CSF was added to one of the samples. As controls, samples were measured of cells loaded with 10 μM BAPTA-AM or of cells incubated in medium without Ca^{2+} . The increase in fluorescence was measured every minute for 12 hours under continuous stirring. At the end of a run, the cells were permeabilized by addition of 2 $\mu\text{g/mL}$ digitonin to determine the maximum fluorescence (F_{max}). Minimum fluorescence (F_{min}) was determined after addition of 5 mM EDTA.

Statistical analysis and image processing

Graphs were drawn and statistical analysis was performed with Prism 4.03 (GraphPad Software, San Diego, CA, USA). The

results are presented as the mean $\pm$ SEM or SD, as indicated. Data were evaluated by paired, one-tailed student's *t*-test, where indicated. The criterion for significance was $P < 0.05$ for all comparisons. Images were processed in Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA) and CorelDRAW 11 (Corel Corporation, Ottawa, Ontario, Canada).

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Changes in gene expression of granulocytes during
in vivo G-CSF/dexamethasone mobilization for
transfusion purposes

Submitted for Publication

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Abstract

Treatment of healthy donors with G-CSF and dexamethasone results in sufficient numbers of circulating granulocytes to prepare granulocyte concentrates for clinical purposes. Granulocytes obtained in this way demonstrate relatively normal functional behavior combined with a prolonged life span. To study the influence of mobilizing agents on granulocytes, we used oligonucleotide microarrays to identify genes that are differentially expressed in mobilized granulocytes as compared to control granulocytes. More than 1000 genes displayed a differential expression pattern, with at least a three-fold difference. Among these, a large number of genes was induced that encode proteins involved in inflammation and the immune response, such as C-type lectins and leukocyte immunoglobulin-like receptors. Since mobilized granulocytes have a prolonged life span, we focused on genes involved in the regulation of apoptosis. One of the most prominent among these was calpastatin (CAST), the endogenous inhibitor of calpains, a family of calcium-dependent cysteine proteases recently shown to be involved in neutrophil apoptosis. Messenger-RNA expression of the *CAST* gene was induced by G-CSF/dexamethasone treatment, both *in vivo* and *in vitro*, while the protein expression of CAST was stabilized during culture.

These studies provide new insight in the genotypic changes as well as in the regulation of the immunological functions and viability of mobilized granulocytes used for clinical transfusion purposes.

Introduction

Granulocyte concentrates constitute a promising adjuvant tool in the treatment of neutropenic and immunocompromised patients suffering from life-threatening infections in which the exclusive use of modern antimicrobial drugs and additional growth factors are ineffective¹⁻³. Donor stimulation with a combination of granulocyte colony-stimulating factor (G-CSF) and dexamethasone has become the standard procedure to increase the number of neutrophils in the circulation of the donors, and thus to collect a sufficient amount of cells for the preparation of granulocyte concentrates⁴.

In addition to its important function in granulopoiesis, G-CSF has been reported to modulate a number of granulocyte functions *in vitro*. For instance, G-CSF increases the chemotactic ability, enhances cell adhesion to vascular endothelium, promotes phagocytosis and primes the NADPH-oxidase activity (for a review see Eyles *et al.* 2006)⁵. On the other hand, glucocorticosteroids such as dexamethasone have been suggested to suppress some granulocyte functions, including mobility, adhesion, and microbial killing⁶⁻⁸.

In spite of all the afore-mentioned studies, we and others have found that donor granulocytes, when mobilized for transfusion purposes, show virtually normal functional characteristics *in vitro* in the presence of some minor phenotypic changes, whereas their life span is consistently prolonged^{9,10}.

Both G-CSF and dexamethasone are well established pro-survival factors for neutrophilic granulocytes¹¹⁻¹³ and this effect involves various survival signaling pathways. G-CSF has been shown to increase in neutrophils the mRNA expression of A1/Bfl-1, an anti-apoptotic member of the Bcl-2 family of proteins¹⁴. The pro-survival effect of dexamethasone in neutrophils has been connected to the stabilization of Mcl-1, another anti-apoptotic Bcl-2 family member, during neutrophil culture¹⁵. Furthermore, G-CSF inhibits the mitochondria-dependent activation of caspase-3 in neutrophils via control of the calpain-dependent degradation of the X-linked inhibitor of apoptosis (XIAP)^{16,17}. In these studies, the anti-apoptotic effect of G-CSF was shown to depend on *de novo* protein synthesis.

Calpains form a family of calcium-dependent cysteine proteases of which calpain-1 (μ -calpain, calpain I),

calpain-2 (m-calpain, calpain II) and the natural inhibitor of calpains, calpastatin, are ubiquitously expressed. Several studies have implicated calpain activity in spontaneous neutrophil apoptosis¹⁷⁻¹⁹, and there is a growing number of indications that calpains play an important role in the early phase of programmed cell death^{17,20}.

Although some of the major functional characteristics relevant for host defense seem well preserved in the donor granulocytes, the extent to which other relevant properties of these cells are altered by the *in vivo* pre-activation is as yet unclear. In order to understand how G-CSF and dexamethasone may induce the granulocytes to obtain their increased survival capacity, we performed a comparative study with Agilent microarrays coupled with real-time RT-PCR, flow cytometry and immunoblotting. In the present study, we demonstrate that mobilization of granulocytes with G-CSF and dexamethasone has a considerable impact on gene expression of these cells, with more than 1,000 genes being strongly affected. These changes could be partially mimicked by *in vitro* culture of neutrophils in the presence of G-CSF and dexamethasone. However, more than 75% of changes in gene expression were unique for *in vivo* mobilization. The affected genes encoded proteins involved in cellular transcriptional activity and protein synthesis, immune response and inflammation, as well as cell survival and apoptosis. We show that among the genes involved in control of cell apoptosis, G-CSF and dexamethasone treatment induced an increased expression of calpastatin, the endogenous inhibitor of calpains. This could also be accomplished by culturing neutrophils with G-CSF/dexamethasone *in vitro*. Furthermore, calpains are demonstrated to contribute importantly to neutrophil apoptosis, whereas the increase in calpastatin mRNA and protein levels directly corresponds to the prolonged life-span of neutrophils when treated with G-CSF/dexamethasone *in vivo* or *in vitro*.

Results

G-CSF/dexamethasone mobilization of neutrophils induces global changes in gene expression

Treatment of healthy donors with a combination of a single dose of G-CSF and dexamethasone has been described to result in a rise in the number of circulating neutrophils within 2-4 hours after administration, which peaks within 12-

Figure 1. Effects of G-CSF and dexamethasone on granulocytes. A) Comparison of the granulocyte concentration per mL of blood and the EMR3 expression level in time, after donor treatment with G-CSF and dexamethasone (representative graph). **B)** Changes in granulocyte gene expression after stimulation with G-CSF and dexamethasone. 18-20 hours after stimulation of the donors, or culture of granulocytes with G-CSF and dexamethasone *in vitro*, neutrophil gene expression was determined by Agilent Whole Human Genome microarrays. Numbers refer to differentially expressed genes in neutrophils isolated 18-20 hours after administration of G-CSF and dexamethasone *in vivo*, and/or those cultured overnight in medium supplemented with G-CSF and dexamethasone.

16 hours (Figure 1A) and returns back to normal levels within 48-72 hours²⁵. Granulocytes for transfusion are usually collected during the peak phase of mobilization (*i.e.* after overnight treatment).

We used a microarray approach to analyze gene expression patterns in mobilized cells in comparison to the cells obtained from the same healthy control donors prior to G-CSF/dexamethasone administration. G-CSF/dexamethasone administration results in both mobilization of immature neutrophils from the bone marrow, (as indicated by staining with the late granulocyte marker EMR3 Figure 1A), as well as direct effects on the already circulating cells. In order to estimate the contribution of the latter, we also performed *in vitro* G-CSF/dexamethasone incubations and analyses on neutrophils taken before the *in vivo* treatment. This also allows the evaluation of changes in gene expression associated with the G-CSF/dexamethasone-induced delay in apoptosis.

By the use of Agilent Whole Human Genome microarrays we screened ~32,000 gene transcripts. This revealed that ~1,000 genes were differentially expressed in the cells after *in vivo* administration of G-CSF/dexamethasone, as compared to those isolated before mobilization (Fig 1B and Supplementary Data). Even more genes were differentially expressed after *in vitro* treatment of neutrophils with G-CSF/dexamethasone (Fig. 1B and Supplementary Data). Almost 200 genes were similarly regulated in both experimental systems (*in vivo* versus *in vitro*, Table 1), but still over 800 genes demonstrated a unique expression pattern for the *in vivo* stimulation.

Confirmation of microarray data by RT-PCR and flow cytometry

We used Light cycler RT-PCR to verify the changes in gene expression as identified by the microarray analysis. We selected six representative genes, encoding well-characterized cell surface receptors, from the microarray data set for subsequent confirmation by RT-PCR and flow cytometry (Fig. 2). For these genes there was a strong correlation ($r = 0.94$) between mRNA levels determined by either microarray or RT-PCR analysis. Mobilization of granulocytes resulted in increased expression levels of complement regulatory factors (CD55 and CD59) and a strong increase in the level of CD177 mRNA, as well as the simultaneous downregulation of CLEC2A (CD69), CD52 and EMR3 mRNA levels.

Figure 2. G-CSF/dexamethasone mobilization changes the phenotype of granulocytes

A) Light-cycler confirmation of representative genes. Genes ($n=6$ identified as differentially expressed by Agilent Whole Humane Genome microarrays (white bars) were selected from the overall dataset for confirmation by Light cycler real-time PCR (grey bars) following G-CSF/dexamethasone mobilization. Data represent the mean $\pm$ SD of fold changes in gene expression from the three donors used in microarray experiments. Microarray data are presented as the mean fold change of the three individuals. **B)** Flow cytometric analysis of different neutrophil surface receptors. Neutrophils isolated from control donors (white bars) and G-CSF/dexamethasone treated (black bars) were analyzed for the expression of various surface receptors. Cells were stained with directly labeled monoclonal antibodies and measured by flow cytometry. Results represent the data from three independent experiments (mean $\pm$ SEM), significant differences are indicated by an asterisk (*) when $p < 0.05$.

Table 1: Genes similarly regulated by the treatment with G-CSF/dexamemethasone *in vivo* and *in vitro*

Sequence ID	Primary Sequence Name	Sequence Code	Sequence Description	<i>in vivo</i>	<i>in vitro</i>
1984958	CD177	CR592446	CD177 molecule	24,38	8,50
1519170	GADD45A	NM_001924	Growth arrest and DNA-damage-inducible, alpha	20,49	10,75
1515523	SLC1A3	NM_004172	Solute carrier family 1	16,98	11,45
1523267	FKBP5	NM_004117	FK506 binding protein 5	13,12	7,36
1528485	SORT1	NM_002959	Sortilin 1	12,11	20,42
1534791	SAMSN1	NM_022136	SAM domain, SH3 domain and nuclear localisation signals, 1	11,87	8,34
1506996	ADORA3	NM_020683	Adenosine A3 receptor	10,90	10,49
1525991	TRPS1	NM_014112	Trichorhinophalangeal syndrome 1	9,13	4,45
1538316	ACN9	NM_020186	ACN9 homolog (<i>S. cerevisiae</i>)	9,13	6,87
1508652	KLF9	NM_001206	Kruppel-like factor 9	8,65	7,46
1524454	XBP1	NM_005080	X-box binding protein 1	7,48	6,51
1531325	ORF1-FL49	NM_032412	putative nuclear protein ORF1-FL49	7,04	4,25
1516682	CAST	NM_001750	Calpastatin	6,97	10,64
1506968	ST6GALNAC3	NM_152996	ST6	6,56	4,94
1513738	SPFH1	NM_006459	SPFH domain family, member 1	6,39	6,20
1991986	TRPM2	NM_001001188	Transient receptor potential cation channel, subfamily M, member 2	6,38	13,63
1989099	GRINA	NM_000837	Poly (ADP-ribose) polymerase family, member 10	5,60	11,34
1522658	VNN1	NM_004666	Vanin 1	5,35	4,47
1533591	SIRT5	NM_012241	Sirtuin	4,60	4,50
1521022	HIPK2	AK074291	Homeodomain interacting protein kinase 2	4,28	8,24
1527074	PEX11G	NM_080662	Peroxisomal biogenesis factor 11 gamma	4,06	4,21
1507368	CCND3	NM_001760	Cyclin D3	3,69	5,58
1519571	CDC42EP3	NM_006449	CDC42 effector protein (Rho GTPase binding) 3	3,58	7,45
1531872	TMEM140	AK056910	Transmembrane protein 140	3,48	6,02
1511860	ZDHHC2	NM_016353	Zinc finger, DHHC-type containing 2	3,24	4,21
1530609	QSCN6	NM_002826	Quiescin Q6	3,08	4,22
1518979	CPD	NM_001304	Carboxypeptidase D	3,03	4,63
1506387	MAPK6	NM_002748	Mitogen-activated protein kinase 6	3,03	5,03
1515049	ZNF278	NM_032051	Zinc finger protein 278	-3,05	-4,11
1983183	NDUFB6	NM_182739	NADH dehydrogenase (ubiquinone) 1	-3,10	-4,04
1507683	FANCA	NM_000135	Fanconi anemia, complementation group A	-3,19	-4,79
1985202	IGKC	BC067092	Immunoglobulin kappa constant	-3,21	-5,50
1522438	GLIPR1	U16307	GLI pathogenesis-related 1 (glioma)	-3,25	-12,71
1525398	RFX5	NM_000449	Selenium binding protein 1	-3,25	-5,02
1522187	TOPORS	NM_005802	Topoisomerase I binding, arginine/serine-rich	-3,26	-4,96
1536170	TST	NM_003312	Thiosulfate sulfurtransferase (rhodanese)	-3,28	-6,47
1530334	CST3	NM_000099	Cystatin C	-3,29	-5,23
1534756	PSMB10	NM_002801	Proteasome (prosome, macropain) subunit, beta type, 10	-3,30	-5,60
1519302	STAT1	NM_139266	Signal transducer and activator of transcription 1, 91kDa	-3,39	-10,76
1522898	MVP	AK097472	Major vault protein	-3,51	-4,58

Table 1 Continued

1538148	RAB37	NM_175738	RAB37, member RAS oncogene family	-3,55	-5,17
1986068	RNF43	CR620892	Ring finger protein 43	-3,60	-7,06
1523761	CBR1	NM_001757	Carbonyl reductase 1	-3,61	-4,75
1511505	RABGAP1L	AB007940	RAB GTPase activating protein 1-like Egf-like module containing,	-3,63	-10,78
1511424	EMR3	NM_032571	mucin-like, hormone receptor-like 3	-3,71	-8,61
1513189	TRERF1	NM_033502	Transcriptional regulating factor 1	-3,76	-16,11
1990225	IGF2BP2	AA451676	Insulin-like growth factor 2 mRNA binding protein 2	-3,79	-6,35
1513264	CST5	NM_001900	Cystatin D	-3,82	-5,63
1990830	FAM111A	BU684362	Family with sequence similarity 111, member A	-3,83	-8,95
1534340	CD46	NM_002389	CD46 molecule, complement regulatory protein	-3,83	-6,11
1517555	CYP27A1	NM_000784	Cytochrome P450, family 27, subfamily A, polypeptide 1	-3,88	-7,43
1516190	SAMHD1	NM_015474	SAM domain and HD domain 1	-3,89	-8,86
1528817	MME	NM_007289	Membrane metallo-endopeptidase	-3,92	-8,11
1536250	CCT2	NM_006431	Chaperonin containing TCP1, subunit 2 (beta)	-4,04	-5,43
1515232	POLB	NM_002690	Polymerase (DNA directed), beta	-4,07	-5,28
1527068	PSME1	NM_006263	Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	-4,15	-4,84
1986072	COL27A1	AI382322	Collagen, type XXVII, alpha 1	-4,21	-4,54
1532664	PDLIM1	NM_020992	PDZ and LIM domain 1 (elfin)	-4,25	-5,30
1524915	OR51E1	NM_152430	Olfactory receptor, family 51, subfamily E, member 1	-4,27	-4,44
1507227	P2RY14	NM_014879	Purinergic receptor P2Y, G-protein coupled, 14	-4,28	-12,99
1508056	EGR3	NM_004430	Early growth response 3 Proline rich Gla (G-carboxyglutamic acid) 4	-4,41	-4,50
1515615	PRRG4	NM_024081	(transmembrane)	-4,42	-4,38
1517506	EGR2	NM_000399	Early growth response 2 (Krox-20 homolog, Drosophila)	-4,67	-14,32
1513793	CATSPER2	NM_172097	Cation channel, sperm associated 2	-4,74	-4,19
1533707	RG9MTD2	NM_152292	RNA (guanine-9-) methyltransferase domain containing 2	-4,80	-7,17
1507738	FBP1	NM_000507	Fructose-1,6-bisphosphatase 1	-4,82	-7,32
1535598	NCOA4	NM_005437	Nuclear receptor coactivator 4	-4,87	-21,40
1507851	CTGLF1	NM_133446	Centaurin, gamma-like family, member 1	-4,88	-5,47
1506502	S100A4	NM_002961	S100 calcium binding protein A4	-4,91	-16,73
1522210	TIGD7	NM_033208	Tigger transposable element derived 7	-5,02	-10,17
1522120	MATN1	AK057725	Matrilin 1, cartilage matrix protein	-5,06	-4,48
1519593	IFIT5	NM_012420	Interferon-induced protein with tetratricopeptide repeats 5	-5,07	-11,79
1533259	RBP7	NM_052960	Ubiquitination factor E4B (UFD2 homolog, yeast)	-5,16	-12,38
1532326	DDX58	NM_014314	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-5,17	-14,04
1510042	NHS	NM_198270	Nance-Horan syndrome (	-5,40	-12,90
1983408	ZFP36L1	CD619445	Zinc finger protein 36, C3H type-like 1	-5,41	-4,09
1518324	FAM111A	NM_022074	Family with sequence similarity 111, member A	-5,59	-5,82
1990540	CLU	NM_203339	Clusterin	-5,66	-5,71
1520999	DGKG	NM_001346	Diacylglycerol kinase, gamma 90kDa	-5,71	-5,82
1983869	ADAM28	NM_014265	ADAM metalloproteinase domain 28	-5,76	-7,39
1518750	PARP16	NM_017851	Poly (ADP-ribose) polymerase family, member 16	-5,84	-11,81
1505761	TNFAIP2	NM_006291	beta-amylase	-5,90	-9,81

Table 1 Continued

1534912	CMTM5	NM_181618	Chemokine-like factor superfamily 5	-5,91	-4,63
1508430	SLC25A22	NM_024698	Solute carrier family 25 (mitochondrial carrier: glutamate),	-5,94	-13,23
1525314	S100A10	NM_002966	S100 calcium binding protein A10	-5,94	-6,72
1521056	GP1BB	NM_000407	Septin 5	-5,97	-4,76
1539206	SPECC1	NM_152904	Spectrin domain with coiled-coils 1	-6,24	-4,94
1527296	HERC5	NM_016323	Hect domain and RLD 5	-6,27	-7,29
1506913	MBIP	NM_016586	MAP3K12 binding inhibitory protein 1	-6,28	-21,40
1515002	CYSLTR1	NM_006639	Cysteinyl leukotriene receptor 1	-6,45	-9,25
1512448	ISOC1	NM_016048	Isochorismatase domain containing 1	-6,51	-7,66
1513897	GIMAP5	NM_018384	GTPase, IMAP family member 5	-6,56	-4,68
1520165	AF242507	AF242507	Homo sapiens mTNF-alpha induced protein mRNA,	-6,58	-5,74
1534914	MPEG1	AK074166	macrophage expressed gene 1	-6,60	-5,82
1988054	RAD50	AK128882	RAD50 homolog (S. cerevisiae)	-6,71	-6,23
1513813	G0S2	NM_015714	G0/G1switch 2	-6,74	-10,55
1530332	MX1	NM_002462	Myxovirus (influenza virus) resistance 1,	-6,76	-12,23
1522509	OR2B2	NM_033057	Olfactory receptor, family 2, subfamily B, member 2	-6,88	-4,91
1991493	PARP14	NM_017554	Poly (ADP-ribose) polymerase family, member 14	-7,03	-4,29
1527378	CSF1R	NM_005211	Colony stimulating factor 1 receptor,	-7,08	-8,69
1514500	CEP57	BC039711	Centrosomal protein 57kDa	-7,08	-7,41
1522188	PTGS2	NM_000963	Prostaglandin-endoperoxide synthase 2	-7,22	-90,22
1537251	OR52K3P	AF143328	Olfactory receptor, family 52, subfamily K, member 3 pseudogene	-7,25	-5,96
1514502	CCDC37	NM_182628	Coiled-coil domain containing 37	-7,27	-5,51
1528770	RPGRIPI	NM_020366	Retinitis pigmentosa GTPase regulator interacting protein 1	-7,32	-6,15
1987032	TREX1	NM_016381	Three prime repair exonuclease 1	-7,45	-5,26
1510272	RNASE3	NM_002935	Ribonuclease, RNase A family, 3 (eosinophil cationic protein)	-7,61	-11,42
1991208	TUSC1	NM_001004125	Tumor suppressor candidate 1	-7,62	-5,54
1539129	SDPR	NM_004657	Serum deprivation response (phosphatidylserine binding protein)	-7,80	-5,25
1538745	SGK	NM_005627	Serum/glucocorticoid regulated kinase	-7,90	-5,90
1524314	IFI44	NM_006417	Interferon-induced protein 44	-7,91	-10,63
1989096	SSBP3	NM_001009955	Single stranded DNA binding protein 3	-8,76	-4,70
1520076	MFI2	BC032042	Antigen p97	-8,93	-6,15
1518480	DDHD1	AK091528	DDHD domain containing 1	-9,02	-8,19
1512017	RNASE2	NM_002934	Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	-9,05	-8,41
1536355	LPAL2	AK125910	Homo sapiens cDNA FLJ43922 fis	-9,08	-13,45
1533135	CXCL10	NM_001565	Chemokine (C-X-C motif) ligand 11	-9,10	-8,41
1522417	TRIO	AY358688	Triple functional domain (PTPRF interacting)	-9,21	-10,68
1518425	PSME2	NM_002818	Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	-9,29	-12,60
1512119	KNTC2	NM_006101	Kinetochore associated 2	-9,33	-4,25
1506804	PLD4	NM_138790	Phospholipase D family, member 4	-9,53	-4,93
1520503	NKX3-1	NM_006167	NK3 transcription factor related, locus 1	-9,56	-7,38
1533140	NME5	NM_003551	nucleoside-diphosphate kinase	-9,99	-8,02

Table 1 Continued

1536919	HLA-DMB	NM_002118	Major histocompatibility complex, class II, DM beta	-10,73	-4,66
1538875	AF15Q14	NM_020380	AF15q14 protein	-10,83	-7,68
1519782	LAP3	NM_015907	Leucine aminopeptidase 3	-11,23	-24,38
1529525	LYSMD2	NM_153374	LysM, putative peptidoglycan-binding, domain containing 2	-11,50	-5,09
1516830	P2RY10	NM_014499	Purinergic receptor P2Y, G-protein coupled, 10	-11,67	-4,14
1519975	PRSS33	NM_152891	Protease, serine, 33	-11,84	-5,57
1507811	PMAIP1	NM_021127	Phorbol-12-myristate-13-acetate-induced protein 1	-12,76	-6,44
1506299	MMP12	ENST00000326227	Macrophage metalloelastase precursor	-12,98	-7,44
1536513	IFIT3	NM_001549	Interferon-induced protein with tetratricopeptide repeats 3	-13,08	-9,40
1513158	HRH4	NM_021624	Histamine receptor H4	-13,66	-6,92
1537338	FGL2	NM_006682	Fibrinogen-like 2	-13,76	-31,09
1509838	GBP1	NM_002053	Guanylate binding protein 1, interferon-inducible, 67kDa	-13,91	-11,20
1512112	EPSTI1	NM_033255	Epithelial stromal interaction 1 (breast)	-14,02	-9,27
1522895	GOLPH4	NM_014498	Golgi phosphoprotein 4	-14,30	-12,18
1508419	CD52	NM_001803	CD52 molecule	-14,95	-4,38
1506912	RARRES1	NM_002888	Retinoic acid receptor responder (tazarotene induced) 1	-15,02	-9,44
1509737	IFIT2	NM_001547	Interferon-induced protein with tetratricopeptide repeats 2	-15,06	-31,11
1505505	CCL23	NM_005064	Chemokine (C-C motif) ligand 23	-15,06	-4,22
1518780	AF131788	AF131788	Clone 24875 mRNA sequence	-16,78	-12,00
1522258	ISG15	NM_005101	Interferon, alpha-inducible protein (clone IFI-15K)	-18,16	-4,81
1519399	GBP5	NM_052942	Guanylate binding protein 5	-18,22	-10,65
1531078	RSAD2	NM_080657	Radical S-adenosyl methionine domain containing 2	-18,54	-8,17
1515740	SLC45A3	NM_033102	Solute carrier family 45, member 3	-26,27	-28,60
1517997	IFIT1	NM_001548	Interferon-induced protein with tetratricopeptide repeats 1	-34,47	-35,07
1533457	FCER1A	NM_002001	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	-61,82	-31,20
1532327	CLC	NM_001828	Charcot-Leyden crystal protein	-69,08	-16,00

Table1: Genes similarly regulated by the treatment with G-CSF/dexamethasone *in vivo* and *in vitro*. Results are presented as the mean fold-increase or –decrease of three separate donors (comparison is with freshly isolated neutrophils t = 0)

Since all transcripts selected for the validation of the microarray data by quantitative RT-PCR represented surface receptors expressed on the plasma membrane, we determined the protein expression levels by flow cytometry (Fig. 2B). Both complement regulatory factors CD55 (also known as decay-accelerating factor [DAF]) and CD59 were found to be present on fresh neutrophils. Their expression increased significantly after G-CSF/dexamethasone treatment *in vivo*. Granulocyte mobilization also resulted in the increased expression levels of CD177 (also known as the human neutrophil antigen [HNA]-2a or NB1). Increased expression of CD177 on neutrophils has previously been reported in response to G-CSF treatment for hematopoietic stem cell mobilization²⁶. Control neutrophils clearly expressed the GPI-anchored proteins CD52 and – to a lesser extent – CD69, whereas the surface expression was either diminished (CD52) or had become completely null (CD69) on donor neutrophils mobilized *in vivo*, confirming the microarray data.

The expression of EMR3, a myeloid-specific member of the epidermal growth factor-seven-transmembrane

(EGF-TM7) family of adhesion class TM7 receptors, which has been described as a marker for mature granulocytes²⁷, was also found to be lower on the surface of *in vivo* mobilized cells. This corresponds with the lower maturation state of granulocytes ‘purged’ from the bone marrow by the combined treatment with G-CSF and dexamethasone treatment, as also indicated by the concomitant neutrophilic left shift (data not shown).

Regulation of different biological processes by G-CSF/dexamethasone mobilization

Genes with different expression patterns between the control granulocytes and those isolated after G-CSF/dexamethasone administration fell into six main, functional, gene ontology (GO) categories: 1) transcription and regulation of transcription, 2) signal transduction, 3) immune responses, 4) cell survival and apoptosis, 5) cell adhesion and motility, and 6) cell metabolism (Table 2).

Twenty six genes involved in the regulation of transcription or the process of transcription itself (*e.g.* *CCNA1*, *SMARCA3* and *MEF2A*) were up-regulated after administration of G-CSF and dexamethasone. On the other hand, the expression of over forty of such genes was reduced, with *NR4A1*, *NR4A3* and *ZNF649* being most strongly affected. This may suggest that granulocytes obtained from G-CSF/dexamethasone-treated donors express a transcriptional program that diverges from normal, circulating neutrophils. These data are indicative of coordinated changes in transcription and suggest the existence of both positive and negative regulatory mechanisms in the G-CSF/dexamethasone-induced transcriptional response.

Granulocyte mobilization has an effect on genes involved in inflammation

The expression of dozens of genes encoding molecules involved in the process of inflammation and the immune response was shown to be modulated by the *in vivo* administration of G-CSF/dexamethasone (Table 2). For example, the transcription of the gene *CYBB* was strongly induced. This gene encodes gp91^{phox}, the enzymatic subunit of the NADPH oxidase – the enzyme complex responsible for superoxide production and microbial killing by neutrophils (Table 2).

The expression of several receptors responsible for ligand or pathogen sensing was induced as well, as indicated, for example, by the increase in transcripts for several C-type lectins such as *CLEC5A* – also known as myeloid DAP12-associated protein (MDL-1) and involved in the activation of cells²⁸ – or *CLEC4D* (CLECSF8), which is also expressed on myeloid cells and has been suggested to take part in the process of endocytosis²⁹. Expression of another set of immune receptors that is associated with the activation of cells, *e.g.* *CD48*, *LAIR1* (Leukocyte-associated Ig-like receptor 1), *LILRA5* (LIR9 – a member of the immunoglobulin-like receptor family), was also induced after mobilization.

On the other hand, we observed a significant reduction in the expression of genes traditionally associated with the adaptive immune response (*e.g.* *HLA-DQB2*, *HLA-DRB5*, *HLA-DPA1*, *HLA-DRA*, *HLA-DMB*, and granzymes [Table 2]).

In addition, the transcriptome of mobilized cells was characterized by the modulation of a cluster of genes coding for cytokines/chemokines and/or their receptors. Within the chemokine system, we identified *CCL3*, *CCL4*, *CCL5*, *CXCL1*, *CXCL10*, as well as the stroma-derived factor-1 receptor *CXCR4*, and *CCR3*, as being repressed by G-CSF and dexamethasone treatment at the transcriptional level. Moreover, the transcripts for Annexin-1 and -3 were highly induced by the G-CSF/dexamethasone treatment. Unexpectedly, we also found a strong reduction in IL-8 transcripts after mobilization, whereas no defects in the release of IL-8 from mobilized granulocytes have –to the best of our knowledge– as yet been reported.

Table 2: G-CSF/dexamethasone mobilization of granulocytes modulates changes in expression profile of genes involved in different biological processes.

<i>Sequence ID</i>	<i>Primary sequence name</i>	<i>Sequence code</i>	<i>Sequence description</i>	<i>Fold change</i>
<i>Transcription and regulation of transcription</i>				
1514893	CCNA1	NM_003914	Cyclin A1	21,02
1983249	SMARCA3	BF513730	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	10,44
1513827	MLXIP	AB020674	MLX interacting protein	8,66
1519529	MSL3L1	NM_078629	Male-specific lethal 3-like 1 (Drosophila)	6,75
1521872	MEF2A	NM_005587	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	6,47
1985292	ETS2	BX433326	V-ets erythroblastosis virus E26 oncogene homolog 2	5,59
1525013	BATF	NM_006399	Basic leucine zipper transcription factor, ATF-like	5,59
1537803	ENO1	NM_001428	Enolase 1, (alpha)	5,50
1533218	HIPK2	BC041926	CDNA clone IMAGE:5300349	5,07
1522326	PHTF1	NM_006608	Putative homeodomain transcription factor 1	4,81
1533591	SIRT5	NM_012241	Sirtuin (silent mating type information regulation 2 homolog) 5 (S. cerevisiae)	4,60
1512793	ZNF167	NM_018651	Zinc finger protein 167	4,30
1511846	PLAG1	NM_002655	Pleiomorphic adenoma gene 1	4,26
1512354	ZNF438	AK095436	hypothetical protein LOC220929	4,20
1507417	DACH1	NM_080759	Dachshund homolog 1 (Drosophila)	4,04
1526663	ZNF438	NM_182755	Zinc finger protein 438	4,02
1537859	ZBTB20	NM_015642	Zinc finger and BTB domain containing 20	3,77
1522362	ZNF658	NM_033160	Zinc finger protein 658	3,75
1534837	ZNF254	NM_004876	Zinc finger protein 539	3,73
1507368	CCND3	NM_001760	Cyclin D3	3,69
1509049	VENTX	NM_014468	VENT homeobox homolog (Xenopus laevis)	3,61
1985753	CRTC3	AK090443	CREB regulated transcription coactivator 3	3,58
1529278	XRN2	NM_012255	5'-3' exoribonuclease 2	3,43
1522102	E2F2	NM_004091	E2F transcription factor 2	3,36
1517472	CCNC	NM_005190	Cyclin C	3,07
1535521	KLF6	U51869	Kruppel-like factor 6	3,03
1537902	TSC22D1	NM_183422	TSC22 domain family, member 1	-3,01
1983175	MCM7	NM_182776	MCM7 minichromosome maintenance deficient	-3,02
1529350	SSRP1	NM_003146	Structure specific recognition protein 1	-3,03
1505187	RELB	NM_006509	V-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	-3,04
1537144	DEXI	AK055529	dexamethasone-induced transcript	-3,07
1516882	MEF2C	NM_002397	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	-3,10
1527019	CHAF1A	NM_005483	Chromatin assembly factor 1, subunit A (p150)	-3,24
1535620	NCOR2	NM_006312	Nuclear receptor co-repressor 2	-3,34
1523857	ZNF395	NM_018660	Zinc finger protein 395	-3,34
1512878	ZMYND11	NM_006624	Zinc finger, MYND domain containing 11	-3,35
1991240	HMGAI	NM_145904	High mobility group AT-hook 1	-3,37
1531990	HEY1	NM_012258	Hairy/enhancer-of-split related with YRPW motif 1	-3,39
1533586	ELK1	NM_005229	ELK1, member of ETS oncogene family	-3,50

Table 2 Continued

1510333	THRA	NM_003250	Thyroid hormone receptor, alpha	-3,68
1537025	BTG2	NM_006763	BTG family, member 2	-3,73
1513189	TRERF1	NM_033502	Transcriptional regulating factor 1	-3,76
1511905	JARID2	NM_004973	Jumonji, AT rich interactive domain 2	-3,81
1526826	ATF3	NM_004024	Activating transcription factor 3	-3,82
1523890	RASSF7	NM_003475	Ras association (RalGDS/AF-6) domain family 7	-3,84
1533807	MAFF	NM_012323	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F	-3,89
1525862	JUN	NM_002228	V-jun sarcoma virus 17 oncogene homolog (avian)	-3,94
1507971	NRL	NM_006177	Neural retina leucine zipper	-4,09
1533916	LEF1	NM_016269	Lymphoid enhancer-binding factor 1	-4,22
1506607	ASF1B	NM_018154	ASF1 anti-silencing function 1 homolog B (<i>S. cerevisiae</i>)	-4,36
1509496	AES	NM_198969	Amino-terminal enhancer of split	-4,40
1533535	TAF4	NM_003185	TAF4 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 135kDa	-4,41
1508056	EGR3	NM_004430	Early growth response 3	-4,41
1533262	BRF1	BC016743	BRF1 homolog, subunit of RNA polymerase III transcription initiation factor IIIB (<i>S. cerevisiae</i>)	-4,48
1533571	PHF6	NM_032458	PHD finger protein 6	-4,53
1517506	EGR2	NM_000399	Early growth response 2 (Krox-20 homolog, <i>Drosophila</i>)	-4,66
1989375	SMAD3	NM_005902	SMAD, mothers against DPP homolog 3 (<i>Drosophila</i>)	-4,97
1507856	MYB	NM_005375	V-myb myeloblastosis viral oncogene homolog (avian)	-5,06
1528623	OLIG2	NM_005806	Oligodendrocyte lineage transcription factor 2	-6,11
1524585	GATA1	NM_002049	GATA binding protein 1 (globin transcription factor 1)	-6,64
1991493	PARP14	NM_017554	Poly (ADP-ribose) polymerase family, member 14	-7,03
1510055	PCGF5	BC007377	Polycomb group ring finger 5	-7,57
1510369	ASCL2	NM_005170	Achaete-scute complex-like 2 (<i>Drosophila</i>)	-7,72
1522208	ZNF584	NM_173548	Zinc finger protein 584	-7,93
1523937	RPS6KA5	NM_004755	Ribosomal protein S6 kinase, 90kDa, polypeptide 5	-8,07
1529851	ETV7	NM_016135	Ets variant gene 7 (TEL2 oncogene)	-8,56
1506298	GATA6	NM_005257	GATA binding protein 6	-8,65
1989096	SSBP3	NM_001009955	Single stranded DNA binding protein 3	-8,76
1520503	NKX3-1	NM_006167	NK3 transcription factor related, locus 1 (<i>Drosophila</i>)	-9,56
1522991	ZNF649	NM_023074	Zinc finger protein 649	-11,07
1514700	NR4A1	NM_002135	Nuclear receptor subfamily 4, group A, member 1	-92,91
Signal transduction				
1513543	GPR84	NM_020370	G protein-coupled receptor 84	25,51
1511321	PTGER2	NM_000956	Prostaglandin E receptor 2 (subtype EP2), 53kDa	8,26
1511570	ARHGAP24	AK130576	Rho GTPase activating protein 24	7,28
1523886	PDE4D	NM_006203	Phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 <i>dunce</i> homolog, <i>Drosophila</i>)	6,64
1527053	MYO10	BC041694	Myosin X	6,48
1512860	MS4A4A	NM_024021	Membrane-spanning 4-domains, subfamily A, member 4	6,12
1539087	DLC1	NM_182643	Deleted in liver cancer 1	5,96
1528643	MS4A6A	NM_022349	Membrane-spanning 4-domains, subfamily A, member 6A	5,87
1524040	GPR141	NM_181791	G protein-coupled receptor 141	5,28
1989101	EDARADD	NM_080738	EDAR-associated death domain	4,64
1508615	EXT1	NM_000127	Exostoses (multiple) 1	4,30
1521881	STK3	NM_006281	Serine/threonine kinase 3 (STE20 homolog, yeast)	3,79
1510158	ITPKC	NM_025194	Inositol 1,4,5-trisphosphate 3-kinase C	3,63

Table 2 Continued

1534557	DPYSL3	NM_001387	Dihydropyrimidinase-like 3	3,54
1536781	RPS6KA3	NM_004586	Ribosomal protein S6 kinase, 90kDa, polypeptide 3	3,53
1533999	HHIP	NM_022475	Hedgehog interacting protein	3,50
1512578	GPR97	NM_170776	G protein-coupled receptor 97	3,42
1514738	SLC26A6	NM_022911	Inositol hexaphosphate kinase 2	3,28
1517434	IL4R	NM_000418	Interleukin 4 receptor	3,21
1539209	GABBR1	NM_001470	Gamma-aminobutyric acid (GABA) B receptor, 1	3,19
1511435	STK16	NM_003691	Serine/threonine kinase 16	3,18
1520958	PRKAR2A	BC002763	Inositol hexaphosphate kinase 2	3,12
1506387	MAPK6	NM_002748	Mitogen-activated protein kinase 6	3,03
1523162	ITPK1	NM_014216	Inositol 1,3,4-triphosphate 5/6 kinase	-3,05
1507956	GNGT2	NM_031498	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	-3,09
1514326	TAGAP	NM_138810	T-cell activation GTPase activating protein	-3,09
1530414	GPR171	NM_013308	G protein-coupled receptor 171	-3,16
1527339	P2RY5	NM_005767	Purinergic receptor P2Y, G-protein coupled, 5	-3,17
1517355	ARHGAP25	BC039591	Rho GTPase activating protein 25	-3,23
1520830	KIT	NM_000222	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-3,28
1517660	KLRK1	NM_007360	Killer cell lectin-like receptor subfamily C, member 4	-3,37
1514077	KLRB1	NM_002258	Killer cell lectin-like receptor subfamily B, member 1	-3,43
1518328	GRP	NM_002091	Gastrin-releasing peptide	-3,64
1516849	EPHB1	NM_004441	EPH receptor B1	-3,80
1514753	PRKAG2	NM_016203	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-4,10
1512127	SOCS1	NM_003745	Suppressor of cytokine signaling 1	-4,13
1518864	FLT3LG	NM_001459	Fms-related tyrosine kinase 3 ligand	-4,26
1524915	OR51E1	NM_152430	Olfactory receptor, family 51, subfamily E, member 1	-4,27
1507227	P2RY14	NM_014879	Purinergic receptor P2Y, G-protein coupled, 14	-4,28
1523084	GUCA1A	NM_000409	Guanylate cyclase activator 1A (retina)	-4,37
1525046	PILRA	NM_178273	Paired immunoglobulin-like type 2 receptor alpha	-4,48
1534223	PPP1R12B	NM_032105	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	-4,66
1518048	GPR34	NM_005300	G protein-coupled receptor 34	-4,79
1534320	ARRB1	NM_004041	Arrestin, beta 1	-5,02
1534974	KLRD1	NM_002262	Killer cell lectin-like receptor subfamily D, member 1	-5,16
1525314	S100A10	NM_002966	S100 calcium binding protein A10	-5,94
1530940	PRKAR2B	NM_002736	Protein kinase, cAMP-dependent, regulatory, type II, beta	-5,97
1522410	IL2RB	NM_000878	Interleukin 2 receptor, beta	-6,06
1518652	CD74	NM_004355	CD74 molecule, major histocompatibility complex, class II invariant chain	-6,23
1515002	CYSLTR1	NM_006639	Cysteinyl leukotriene receptor 1	-6,45
1530332	MX1	NM_002462	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	-6,76
1522509	OR2B2	NM_033057	Olfactory receptor, family 2, subfamily B, member 2	-6,88
1984459	PDE4B	AB209081	Phosphodiesterase 4B, cAMP-specific	-6,89
1527378	CSF1R	NM_005211	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	-7,08
1527858	NRGN	NM_006176	Neurogranin (protein kinase C substrate, RC3)	-7,50
1516837	MS4A3	NM_006138	Membrane-spanning 4-domains, subfamily A, member 3	-9,24
1989999	IL5RA	NM_175725	Interleukin 5 receptor, alpha	-9,66
1524637	GPR162	NM_019858	Triosephosphate isomerase 1	-10,10

Table 2 Continued

1516830	P2RY10	NM_014499	Purinergic receptor P2Y, G-protein coupled, 10	-11,67
1518123	CD83	NM_004233	CD83 molecule	-11,67
1517216	KLRF1	NM_016523	Killer cell lectin-like receptor subfamily F, member 1	-12,68
1512228	EBI2	NM_004951	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor)	-13,11
1513158	HRH4	NM_021624	Histamine receptor H4	-13,66
1537338	FGL2	NM_006682	Fibrinogen-like 2	-13,76
1515714	GNG11	NM_004126	Guanine nucleotide binding protein (G protein), gamma 11	-16,57
1527111	GPR44	NM_004778	G protein-coupled receptor 44	-23,51
1528909	RGS1	NM_002922	Regulator of G-protein signalling 1	-32,65
Immune responses				
1531927	CYP1B1	NM_000104	Cytochrome P450, family 1, subfamily B, polypeptide 1	46,19
1984958	CD177	CR592446	CD177 molecule	24,38
1983056	MERTK	U08023	C-mer proto-oncogene tyrosine kinase	16,00
1532739	CLEC5A	NM_013252	C-type lectin domain family 5, member A	13,44
1511835	CLEC4D	NM_080387	C-type lectin domain family 4, member D	12,02
1535646	CST7	NM_003650	Cystatin F (leukocystatin)	11,43
1506996	ADORA3	NM_020683	Adenosine A3 receptor	10,89
1532148	LAIR1	NM_021706	Leukocyte-associated immunoglobulin-like receptor 1	10,45
1506562	HP	NM_005143	Haptoglobin	10,31
1507483	LILRA5	NM_181879	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	7,56
1524454	XBP1	NM_005080	X-box binding protein 1	7,48
1521047	CR1	NM_000651	Complement component (3b/4b) receptor 1 (Knops blood group)	5,87
1520792	MAPK14	NM_139013	Mitogen-activated protein kinase 14	5,75
1535716	CD59	NM_203330	CD59 molecule, complement regulatory protein	5,67
1513648	FCER1G	CB529629	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	5,26
1530114	MAP2K6	NM_002758	Mitogen-activated protein kinase kinase 6	4,87
1533789	SERPINB1	NM_030666	Serpin peptidase inhibitor, clade B (ovalbumin), member 1	4,84
1522193	FCAR	NM_002000	Fc fragment of IgA, receptor for	4,52
1527121	PROK2	NM_021935	Prokineticin 2	4,33
1506853	CHIT1	NM_003465	Chitinase 1 (chitotriosidase)	3,48
1531091	CD55	NM_000574	CD55 molecule, decay accelerating factor for complement	3,39
1515424	CYBB	S67289	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)	3,29
1985998	ALOX5	BQ548663	Arachidonate 5-lipoxygenase	3,16
1514172	CD48	NM_001778	CD48 molecule	3,09
1511452	SERPING1	NM_000062	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	-3,00
1530131	IRF2BP2	BC020516	Interferon regulatory factor 2 binding protein 2	-3,03
1528251	NCR3	NM_147130	Natural cytotoxicity triggering receptor 3	-3,13
1513772	HLA-DRB3	NM_022555	Major histocompatibility complex, class II, DR beta 1	-3,17
1518778	ALOX15	NM_001140	Arachidonate 15-lipoxygenase	-3,24
1524640	OAS3	NM_006187	2'-5'-oligoadenylate synthetase 3, 100kDa	-3,31
1985491	HLA-DRB1	X12544	Major histocompatibility complex, class II, DR beta 1	-3,31
1519302	STAT1	NM_139266	Signal transducer and activator of transcription 1, 91kDa	-3,39
1509654	CTSW	NM_001335	Cathepsin W (lymphopain)	-3,45
1992017	PLAUR	NM_001005377	Plasminogen activator, urokinase receptor	-3,50
1526588	LY75	NM_002349	Lymphocyte antigen 75	-3,51

Table 2 Continued

1515890	FAIM3	NM_005449	Interleukin 24	-3,65
1511424	EMR3	NM_032571	Egf-like module containing, mucin-like, hormone receptor-like 3	-3,71
1534340	CD46	NM_002389	CD46 molecule, complement regulatory protein	-3,83
1537056	GBP3	NM_018284	Guanylate binding protein 1, interferon-inducible, 67kDa	-3,86
1517555	CYP27A1	NM_000784	Cytochrome P450, family 27, subfamily A, polypeptide 1	-3,88
1516190	SAMHD1	NM_015474	SAM domain and HD domain 1	-3,89
1519488	HLA-DQB2	NM_182549	Major histocompatibility complex, class II, DQ beta 2	-3,94
1505541	GZMB	NM_004131	Granzyme B	-4,04
1536691	GZMK	NM_002104	Granzyme K (granzyme 3; tryptase II)	-4,67
1522713	GZMA	NM_006144	Granzyme A	-4,80
1510975	HLA-DRB5	NM_002125	Major histocompatibility complex, class II, DR beta 1	-5,13
1527466	HLA-DPA1	NM_033554	Major histocompatibility complex, class II, DP alpha 1	-5,16
1516918	B2M	AK022379	Beta-2-microglobulin	-5,43
1538818	HLA-DQB1	NM_002123	Major histocompatibility complex, class II, DQ beta 1	-5,87
1521122	SPON2	NM_012445	Spondin 2, extracellular matrix protein	-5,95
1517271	PF4V1	NM_002620	Platelet factor 4 variant 1	-6,48
1508682	HLA-DPB1	NM_002121	Major histocompatibility complex, class II, DP beta 1	-6,67
1529151	OASL	NM_003733	2'-5'-oligoadenylate synthetase-like	-7,10
1987518	CYB561	NM_001017916	Cytochrome b-561	-7,40
1524314	IFI44	NM_006417	Interferon-induced protein 44	-7,91
1533656	DEFA4	NM_001925	Defensin, alpha 4, corticostatin	-9,04
1526794	HLA-DRA	NM_019111	Major histocompatibility complex, class II, DR alpha	-10,12
1536919	HLA-DMB	NM_002118	Major histocompatibility complex, class II, DM beta	-10,73
1525510	PF4	NM_002619	Platelet factor 4 (chemokine (C-X-C motif) ligand 4)	-11,03
1514544	DEFA3	NM_005217	Defensin, alpha 1	-11,85
1520461	CD69	NM_001781	CD69 molecule	-28,65
1528045	IL8	NM_000584	Interleukin 8	-35,22
1533457	FCER1A	NM_002001	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	-61,82
Cell survival & apoptosis				
1519170	GADD45A	NM_001924	Growth arrest and DNA-damage-inducible, alpha	20,49
1525148	ANXA1	NM_000700	Annexin A1	7,39
1533289	ANXA3	NM_005139	Annexin A3	7,33
1516682	CAST	NM_001750	Calpastatin	6,97
1532823	PECR	NM_018441	Peroxisomal trans-2-enoyl-CoA reductase	5,32
1539226	CARD6	NM_032587	Caspase recruitment domain family, member 6	5,15
1531614	ELMO2	NM_022086	Engulfment and cell motility 2	4,67
1512780	LGALS8	NM_006499	Lectin, galactoside-binding, soluble, 8 (galectin 8)	4,24
1521881	STK3	NM_006281	Serine/threonine kinase 3 (STE20 homolog, yeast)	3,79
1526256	DDAH2	NM_013974	Dimethylarginine dimethylaminohydrolase 2	3,75
1522394	TNFRSF10A	NM_003844	Tumor necrosis factor receptor superfamily, member 10a	3,70
1988846	AIM1	NM_001624	Absent in melanoma 1	3,52
1509305	LTB4R	NM_181657	Leukotriene B4 receptor	3,20
1984749	HIP1	BC082970	Huntingtin interacting protein 1	3,19
1534293	LGALS3	NM_002306	Lectin, galactoside-binding, soluble, 3 (galectin 3)	3,03
1535837	SQSTM1	NM_003900	Sequestosome 1	-3,01
1537407	NFKBIE	NM_004556	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	-3,12
1987065	TNFRSF25	NM_148968	Homo sapiens tumor necrosis factor receptor superfamily	-3,36

Table 2 Continued

1516125	PRDX2	NM_005809	Peroxiredoxin 2	-3,40
1531960	GSTP1	NM_000852	Glutathione S-transferase pi	-3,42
1532501	PIM2	NM_006875	Pim-2 oncogene	-3,76
1528041	IL31RA	NM_139017	Interleukin 31 receptor A	-3,91
1533374	PHLDA2	NM_003311	Pleckstrin homology-like domain, family A, member 2	-3,97
1520783	DYRK2	NM_006482	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	-4,29
1530737	LGALS2	NM_006498	Lectin, galactoside-binding, soluble, 2 (galectin 2)	-4,47
1510435	LGALS12	NM_033101	Lectin, galactoside-binding, soluble, 12 (galectin 12)	-4,47
1523074	HSPA9B	NM_004134	Heat shock 70kDa protein 9B (mortalin-2)	-5,16
1532326	DDX58	NM_014314	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	-5,17
1508150	AOC3	NM_003734	Amine oxidase, copper containing 3 (vascular adhesion protein1)	-5,52
1538822	IFI6	NM_022873	Interferon, alpha-inducible protein (clone IFI-6-16)	-5,56
1990540	CLU	NM_203339	Clusterin	-5,66
1538745	SGK	NM_005627	Serum/glucocorticoid regulated kinase	-7,90
1523311	BIRC4BP	NM_017523	XIAP associated factor-1	-9,09
1510278	NGFRAP1	NM_014380	Nerve growth factor receptor (TNFRSF16) associated protein 1	-9,35
1533140	NME5	NM_003551	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	-9,98
1533249	HIG2	NM_013332	hypoxia-inducible protein 2	-15,17
Cell adhesion and motility				
1536096	MMP8	NM_002424	Matrix metalloproteinase 8 (neutrophil collagenase)	97,77
1519153	OLFM4	NM_006418	Olfactomedin 4	23,81
1514453	CD44	NM_000610	CD44 molecule (Indian blood group)	10,05
1532879	EMILIN2	NM_032048	Elastin microfibril interfacier 2	9,85
1521082	CEACAM1	NM_001712	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	5,97
1509526	MMP9	NM_004994	Matrix metalloproteinase 9	5,64
1522658	VNN1	NM_004666	Vanin 1	5,35
1984856	ADAM15	NM_207191	ADAM metalloproteinase domain 15 (metargidin)	3,80
1507356	F5	NM_000130	Coagulation factor V (proaccelerin, labile factor)	3,78
1516175	JAK2	NM_004972	Janus kinase 2 (a protein tyrosine kinase)	3,78
1508488	SPP1	NM_000582	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	3,72
1523771	ITGAM	NM_000632	Integrin, alpha M (complement component 3 receptor 3 subunit)	3,52
1508621	PVRL2	NM_002856	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	3,42
1531024	SIGLEC9	NM_014441	Sialic acid binding Ig-like lectin 9	3,34
1505504	VIM	NM_003380	Vimentin	3,34
1986801	LAMB3	NM_001017402	Laminin, beta 3	3,11
1518582	CEACAM6	BC005008	Carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	-3,07
1506966	SIGLEC8	NM_014442	Sialic acid binding Ig-like lectin 8	-3,16
1534776	CXCL1	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	-3,20
1520341	AZU1	NM_001700	Azurocidin 1 (cationic antimicrobial protein 37)	-3,23
1520433	ITGB7	NM_000889	Integrin, beta 7	-3,59
1505769	TUBB1	NM_030773	Tubulin, beta 1	-4,35
1528734	ICAM2	NM_000873	Intercellular adhesion molecule 2	-5,24
1991393	CXCR4	NM_001008540	Chemokine (C-X-C motif) receptor 4	-5,90
1989016	CCL3L3	NM_001001437	Chemokine (C-C motif) ligand 3-like 3	-5,98

Table 2 Continued

1517696	CCL5	NM_002985	Chemokine (C-C motif) ligand 5	-7,71
1533135	CXCL10	NM_001565	Chemokine (C-X-C motif) ligand 11	-9,10
1526404	CCL4	NM_002984	Chemokine (C-C motif) ligand 4	-15,45
1533611	PPBP	NM_002704	Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	-16,30
1515169	ITGA4	NM_000885	Integrin, alpha 4 (antigen CD49D), alpha 4 subunit of VLA-4 receptor	-16,74
1537942	CCR3	NM_001837	Chemokine (C-C motif) receptor 3	-19,63
Cell metabolism				
1538977	HPGD	NM_000860	Hydroxyprostaglandin dehydrogenase 15-(NAD)	36,26
1989284	PFKFB2	NM_001018053	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	31,40
1986330	ATP9A	NM_006045	ATPase, Class II, type 9A	25,82
1511217	LDHA	NM_005566	Lactate dehydrogenase A	10,11
1527335	PCSK9	NM_174936	Proprotein convertase subtilisin/kexin type 9	7,95
1991955	PLB1	CR600701	Phospholipase B1	7,60
1983476	ATP13A3	AJ306929	ATPase type 13A3	7,56
1513367	DPH5	NM_015958	DPH5 homolog (<i>S. cerevisiae</i>)	6,38
1526202	NMNAT2	NM_015039	Nicotinamide nucleotide adenyltransferase 2	6,31
1536086	DPYD	NM_000110	Dihydropyrimidine dehydrogenase	6,11
1990338	ATP11B	NM_014616	ATPase, Class VI, type 11B	6,09
1515968	ALPL	NM_000478	Alkaline phosphatase, liver/bone/kidney	5,59
1506037	MGAM	NM_004668	Maltase-glucoamylase (alpha-glucosidase)	5,41
1532823	PECR	NM_018441	Peroxisomal trans-2-enoyl-CoA reductase	5,32
1986414	ATP2B4	NM_001001396	ATPase, Ca ⁺⁺ transporting, plasma membrane 4	5,00
1506589	PFKFB3	NM_004566	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	4,75
1512399	AGPAT6	NM_178819	1-acylglycerol-3-phosphate O-acyltransferase 6 (lysophosphatidic acid acyltransferase, zeta)	4,74
1523383	PGD	NM_002631	Phosphogluconate dehydrogenase	4,70
1519383	ATP6V1C1	NM_001695	ATPase, H ⁺ transporting, lysosomal 42kDa, V1 subunit C1	4,11
1520655	SMPDL3A	NM_006714	Sphingomyelin phosphodiesterase, acid-like 3A	4,09
1989848	ACSL4	NM_004458	Acyl-CoA synthetase long-chain family member 4	4,02
1522656	LDLR	NM_000527	Low density lipoprotein receptor	3,63
1538541	DHRS9	NM_005771	Dehydrogenase/reductase (SDR family) member 9	3,55
1514810	ATP1A3	NM_152296	ATPase, Na ⁺ /K ⁺ transporting, alpha 3 polypeptide	3,53
1524666	FABP5	NM_001444	Fatty acid binding protein 5 (psoriasis-associated)	3,38
1538372	ATP1A1	NM_000701	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	3,38
1524650	GNS	NM_002076	Glucosamine (N-acetyl)-6-	3,37
1523432	ACSL1	NM_001995	Acyl-CoA synthetase long-chain family member 1	3,35
1529572	SGSH	NM_000199	N-sulfoglucosamine sulfohydrolase (sulfamidase)	3,29
1538734	ACSL3	NM_004457	Acyl-CoA synthetase long-chain family member 3	3,27
1985998	ALOX5	BQ548663	Arachidonate 5-lipoxygenase	3,16
1527407	GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	3,16
1534240	SULT1B1	D89479	Sulfotransferase family, cytosolic, 1B, member 1	3,10
1983183	NDUFB6	NM_182739	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	-3,10
1525237	ALDH6A1	NM_005589	Aldehyde dehydrogenase 6 family, member A1	-3,22
1518778	ALOX15	NM_001140	Arachidonate 15-lipoxygenase	-3,24
1523761	CBR1	NM_001757	Carbonyl reductase 1	-3,61
1510672	MCCC1	NM_020166	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)	-4,02
1990453	NDUFV2	BI520375	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa	-4,32
1521732	ATP12A	NM_001676	ATPase, H ⁺ /K ⁺ transporting, nongastric, alpha polypeptide	-4,74

Table 2 Continued

1526184	LDHB	NM_002300	Lactate dehydrogenase B	-6,08
1512448	ISOC1	NM_016048	Isochorismatase domain containing 1	-6,51
1989369	METTL7A	BC004492	Methyltransferase like 7A	-6,99
1518627	FA2H	NM_024306	Fatty acid 2-hydroxylase	-7,75
1506804	PLD4	NM_138790	Phospholipase D family, member 4	-9,53
1521949	SORD	NM_003104	Sorbitol dehydrogenase	-10,20
1512822	SULF2	NM_018837	Sulfatase 2	-21,22

Table 2: G-CSF/dexamethasone mobilization of granulocytes modulates changes in expression profile of genes involved in different biological processes. Genes that were found to be differentially expressed after *in vivo* treatment with G-CSF- dexamethasone were analyzed by the OntoExpress program (<http://vortex.cs.wayne.edu/projects.htm#Onto-Express>), and categorized into functional profiles (e.g. transcription, signal transduction, immune response, cell faith & apoptosis, cell adhesion & mobility, metabolism). Results are presented as the mean fold-increase or –decrease of three separate donors (comparison is with freshly isolated neutrophils $t = 0$)

Modulation of genes involved in the apoptotic machinery

Because mobilized granulocytes show delayed apoptosis upon *in vitro* culture^{9,10}, we carefully examined genes involved in the regulation of cell fate or apoptosis (Table 2).

One group of molecules that is known to be regulated at the transcriptional level and is involved in the apoptotic process is the group of galectins, a family of lectins that is defined by their ability to recognize β -galactose and the presence of consensus amino-acid sequences³⁰. Different members of this family have been shown to modulate various steps of the inflammatory response, such as cell-matrix interactions, cell trafficking, cell survival, cell-growth regulation, chemotaxis, and proinflammatory cytokine secretion^{31,32}. We observed a strong induction of galectin-8 (*LGAL8*) and moderate induction of galectin-3 (*LGALS3*), as well as a reduction of galectin-2 and -12 (*LGALS2*, *LGALS12*).

Most striking was the strong induction observed for the transcript of *GADD45A*, a member of the Gadd45 family of genes that have been implicated in stress signaling in response to physiological or environmental stressors, resulting in either cell-cycle arrest, DNA repair, cell survival and senescence, or apoptosis. Hematopoietic cells from Gadd45a-deficient mice are more susceptible to apoptosis induced by UV radiation or genotoxic stress^{33,34}

Expression of the *CAST* gene encoding calpastatins, endogenous inhibitors of calpains, was also strongly induced in mobilized cells. Calpain activity has previously been shown to be involved in neutrophil cell death^{17,20}.

Calpastatin is a well documented, specific, endogenous inhibitor of calpains³⁵. It is encoded by a single gene that produces a number of isoforms via differential splicing or use of promoters. Calpastatins are known to be associated with an anti-apoptotic effect in neutrophils²⁰, and we have recently observed that inhibition of calpain activity by G-CSF strongly affects neutrophil survival¹⁷. Since mobilized cells have been reported to have a better survival capacity, calpastatin was investigated in greater detail.

Induction of calpastatin correlates with a delay in apoptosis

G-CSF causes rapid neutrophilia but is also known to act as a survival factor on mature, circulating neutrophils both *in vitro*^{16,17,36} and *in vivo*⁹. Dexamethasone, although shown to induce apoptosis in various cell types, including lymphocytes, monocytes³⁷ and eosinophils^{15,38}, also acts as a pro-survival factor for neutrophils^{13,39}. This is in line with our observation that, upon *in vitro* culture, granulocytes obtained from donors treated with a combination of the two drugs showed a prolonged life span after overnight culture as compared to neutrophils isolated from the blood of the same donors before treatment (Fig. 3). The same extent of survival was achieved by the *in vitro* addition of G-CSF

Figure 3. Prolonged survival of granulocytes isolated from G-CSF/dexamethasone mobilized donors. Granulocytes were isolated from healthy, control donors (white bars) and from the same donors treated with G-CSF and dexamethasone (black bars), and cultured for 20 hours in HBSS. Afterwards, the cell viability was assessed by Annexin-V/PI staining. Results represents data from 6 independent experiments (mean $\pm$ SD), significant differences are indicated by an asterisk (*) when $p < 0.05$.

and dexamethasone to the culture medium during overnight culture (data not shown). The calpastatin transcripts in neutrophils were significantly induced by G-CSF/dexamethasone treatment, either when applied *in vivo* or *in vitro* (Table 1). First, we used RT-PCR to confirm the increase in transcription of the *CAST* gene that was observed in the microarray analysis. Neutrophils from individuals treated with a combination of G-CSF and dexamethasone, as well as neutrophils cultured with this combination *in vitro*, both displayed an increase in *CAST* gene expression (Fig 4A). In freshly purified neutrophils, several isoforms of calpastatin are expressed. In agreement with previously published work, also in other cells, the molecular sizes of these isoforms were found at 110, 108, 90 and 41 kDa⁴⁰. The most abundant isoforms expressed in neutrophils were the high-molecular-weight (HMW) forms found as 110 and 108 kDa bands. Neutrophils isolated from granulocyte transfusion donors expressed increased levels of HMW calpastatin when compared to the control cells (Fig. 4B).

Next, the CAST protein levels were monitored during neutrophil spontaneous apoptosis in the presence or absence of G-CSF and dexamethasone. Culturing of the cells resulted in reduced levels of all isoforms, but especially the HMW forms of calpastatin were diminished (Fig. 4C), suggesting proteolysis of the protein during apoptosis. Addition of G-CSF and dexamethasone to culture media resulted in an increase in the protein expression after 8 hours of culture, and prevented the absolute loss of the protein after 24 hours in culture. Inhibition of new protein synthesis by the addition of cycloheximide (CHX, 10 μ g/mL), prevented the increase and led to the total loss of protein expression after prolonged culture, similar to the control cells (Fig. 4C). The reduction in CAST protein level was apparently due to degradation by either the calpains that have overcome the inhibitory effect of the calpastatin⁴¹ or by caspases activated via a different, calpain-independent route⁴⁰, since addition of either the cell-permeable calpain inhibitor III (CI3), or the cell-permeable pan-caspase inhibitor zVAD partially prevented the lost of calpastatin during overnight culture (Fig 4D).

Inhibition of calpains prevents neutrophil apoptosis

To confirm the involvement of calpain activity in neutrophil apoptosis, cells were incubated overnight in the presence of the combination of G-CSF/dexamethasone, the cell-permeable calpain inhibitor III (CI3), or the cell-permeable pan-caspase inhibitor zVAD as a positive control, while cell viability was measured by Annexin-V staining and verified by cellular morphology (data not shown). As reported before^{11,15}, the addition of G-CSF/dexamethasone to the culture medium decreased the number of apoptotic cells after overnight culture. This pro-survival effect was abolished by the addition of CHX. In addition, CI3 delayed neutrophil apoptosis to a similar extent (Fig. 4E). Together, these data suggest a direct link between the calpain activity, endogenous calpastatin levels and apoptosis.

Figure 4. Modulation of Calpain/Calpastatin pathway by G-CSF/dexamethasone treatment. **A**) Changes in the expression of the CAST gene after treatment with G-CSF and dexamethasone *in vivo* (grey bar) or *in vitro* (black bar) were measured by the light cycler RT-PCR. Results are presented as the mean $\pm$ SEM from three independent donors. **B**) Different expression of calpastatin after administration of G-CSF and dexamethasone. Samples of neutrophils isolated from control donors (white bars) and donors stimulated with G-CSF and dexamethasone (black bars) were subjected to SDS-PAGE and analyzed on Western blots, stained with antibodies against calpastatin. Equal amounts of cells (1.5×10^6) were dissolved in sample buffer, and loaded in each lane. After staining with fluorescently labeled secondary antibody, the blots were scanned with an Odyssey infrared scanner and analyzed by Licor Odyssey 2.1 software. Signals were normalized for p38 expression. The intensities of the two highest bands recognized by the antibody, which represent two major forms of calpastatin in neutrophils, were analyzed. Results represent data from 5 different donors (mean $\pm$ SEM), significant differences are indicated by an asterisk (*) when $p < 0.05$. A representative blot comparing calpastatin levels in control cells and mobilized neutrophils is presented. **C**) Samples of neutrophils incubated in the presence or absence of G-CSF and dexamethasone were taken at the indicated times and analyzed by Western Blot. When indicated, CHX (10 μ g/mL) was added to prevent new protein synthesis. Results represent data from 5 independent experiments (mean $\pm$ SEM), significant differences are indicated by an asterisk (*) when $p < 0.05$. **D**) Samples of neutrophils incubated in the presence or absence of calpain inhibitor 3 (CI3, 20 μ M) or caspase inhibitor zVAD (20 μ M) were taken at the indicated time points and analyzed by Western Blot. Results represent data from 5 independent experiments (mean $\pm$ SEM), significant differences are indicated by an asterisk (*) when $p < 0.05$. **E**) Inhibition of Calpains prolongs neutrophil viability. Neutrophils were isolated from healthy donors and cultured overnight alone or with the addition of G-CSF (10 ng/mL), CI3 (20 μ M), or zVAD (20 μ M). When indicated, CHX (10 μ g/mL) was added to prevent new protein synthesis. Cells negative for Annexin-V staining were considered to be alive. Results represent data from 6 independent experiments (mean $\pm$ SEM). Significant differences are indicated by an asterisk (*) when $p < 0.05$.

Discussion

Mobilization of granulocytes by the administration of G-CSF and corticosteroids is a well-established procedure to achieve the number of granulocytes required for transfusion to neutropenic patients with severe, non-responsive infections^{2,4}. We studied the effects of G-CSF and dexamethasone administration on the gene expression pattern of granulocytes to be used for transfusion. We found that a high number of genes was differentially regulated after *in vivo* mobilization of granulocytes. Approximately 20% of those genes changed similarly upon *in vitro* culture of granulocytes with the combination of G-CSF and dexamethasone. Still, the expression of more than 700 genes was only changed significantly after *in vivo* administration, and this is perhaps not surprising. It seems reasonable to assume that, within the time frame of the experiment (18-20h), secondary donor factors released upon administration of the mobilizing agents, from either granulocytes themselves or from other cell types, could have modified granulocyte gene expression. In this context it is important to realize that the G-CSF receptor is not only present on

precursor and mature neutrophilic granulocytes, but also on monocytes and platelets, as well as on endothelial cells and on adult neuronal stem cells⁴²⁻⁴⁴. Moreover, the glucocorticoid receptors are ubiquitously expressed, and known to induce or modulate gene transcription in various cell types⁴⁵.

An additional, or perhaps in some cases alternative, explanation may be that the granulocytes recently mobilized from the bone marrow represent a less mature neutrophilic phenotype, as indicated by the lower expression of EMR3, a late marker of granulocytic differentiation²⁷, and the distinct left shift of the mobilized neutrophils. Another supportive feature of the induced neutrophil egress from the bone marrow was found in the increased transcriptional activity of the genes *MMP9* and *MMP8*, both encoding matrix metallo-proteases. The products of these *MMP* genes are important for creating a highly proteolytic environment in the bone marrow, thus facilitating the release of granulocytes from the stromal microenvironment⁴⁶.

The basic effector functions of granulocytes collected for transfusion after administration of G-CSF and dexamethasone, including interaction with endothelial cells, migration, respiratory burst or killing capacity seem to be unaffected^{4,10,47,48}. However, the life span of those cells is prolonged upon *in vitro* culture as well as *in vivo*. In addition, supplementation of the culture medium of previously untreated control cells with G-CSF/dexamethasone also prolongs cell survival. This apparently direct effect depends on gene expression and new protein synthesis, since it is abolished by the addition of cycloheximide.

From the groups of genes differentially regulated in mobilized granulocytes, we focused on the regulatory genes of apoptosis. The most impressive change was observed for calpastatin, the endogenous inhibitor of calpains. Inhibition of calpains with pharmacological inhibitors results in a significant delay of neutrophil apoptosis¹⁹. We have recently found that G-CSF is implicated in the regulation of calpain activity by influencing the intracellular levels of Ca^{2+} upon *in vitro* culture of neutrophils¹⁷, an effect depending on new protein synthesis. It has been proposed that, upon mild stimulation, calpastatin binds to calpains, preventing the proteases from degrading their substrates. However, the intramolecular, autolytic activation of calpains is not prevented by calpastatin binding^{41,49}, and if the level of intracellular Ca^{2+} increase even higher, so stimulation becomes stronger, the protease will degrade its inhibitor and thereafter proceed to cleave other substrates. Calpastatin degradation has been shown to occur during spontaneous neutrophil apoptosis by activated calpains or pro-apoptotic caspases (*e.g.* caspase-3, -7)^{40,20,50}. Calpastatin expression has also been shown to be preferentially induced in neutrophils isolated from cystic fibrosis patients, simultaneously with decreased levels of pro-calpain-1, coinciding with a delay in neutrophil cell death²⁰.

Increased levels of mRNA for calpastatin were detected in mobilized neutrophils, isolated from granulocyte donors pre-treated for 18-20 hours with G-CSF/dexamethasone, as well as in neutrophils cultured *in vitro* in the presence of G-CSF/dexamethasone. A similar increase in calpastatin levels was observed at the protein level, when mobilized neutrophils were compared to the control cells.

Immunoblot analysis of neutrophil lysates showed loss of calpastatin expression upon *in vitro* culture. Addition of G-CSF and dexamethasone to the culture media increased the level of calpastatin after 8 hours of incubation, which was prevented by cycloheximide. However, after 24 hours in culture the cells treated with pro-survival factors such as G-CSF and dexamethasone also displayed strongly reduced levels of calpastatin, albeit still significantly higher than cultured control cells. It appears that the stronger and prolonged activation of calpains allows them to overcome the inhibitory effect of cellular calpastatin, which eventually results in its cleavage. Pharmacological inhibition of calpains partially preserved calpastatin expression during 24 hours in culture, albeit to a

lesser extent than with G-CSF and dexamethasone. Most likely, the activated caspases are responsible for this effect, since neutrophils incubated in the presence of a pan-caspase inhibitor also sustained their calpastatin levels.

Taken together, our data suggest that the calpain/calpastatin system plays an important role in neutrophil apoptosis. Inhibition of calpain activation by increasing the levels of its endogenous inhibitor may be one of the pro-survival mechanisms by which G-CSF and dexamethasone delay the apoptosis in neutrophils used for transfusion purposes. Finally, we showed that intracellular levels of calpastatin correlate with the viability of neutrophils *in vitro* and that pharmacological inhibition of calpains decreases the rate of apoptosis during 24 hours of incubation. A role for calpastatin in apoptosis in neutrophils is supported by data demonstrating that a reduction in its expression levels by anti-sense nucleotides accelerates apoptosis⁵¹.

In sum, we have shown that mobilization of granulocytes for transfusion by the combination of G-CSF and dexamethasone strongly alters the gene expression pattern in circulating neutrophils compared to untreated donor neutrophils. The characteristics of cells used for transfusion can now be studied based on the changed transcriptional program. By means of the changed transcript levels we can now recognize that mobilized neutrophils display changes in subtle functional aspects that previously remained unidentified. These changes include genes involved in immune reactivity, motility, signal transduction and gene transcription, as well as cell viability.

Material and methods

Experimental design

Granulocytes from three different healthy donors (2 males and 1 female) were studied before and after treatment with G-CSF and dexamethasone. Donors received G-CSF (5 µg/kg, subcutaneously), and dexamethasone (8 mg, orally). The study was approved by the local ethical medical committee and in accordance with the Declaration of Helsinki. Blood samples were taken just before the donor treatment (control sample, fresh) and 16-20 hrs after G-CSF and dexamethasone administration (*in vivo* treatment).

Neutrophils from the control sample were isolated and directly prepared for RNA isolation (see below) or cultured overnight in the absence (control sample, apoptosis) or presence of G-CSF (Amgen Europe B.V., Breda, The Netherlands) and dexamethasone (Sigma Aldrich, St. Louis, MO, USA) (*in vitro* treatment). Thus, four pools of RNA were obtained for comparison (see Supplementary Figure S1).

Granulocyte isolation and culture

Heparinized venous blood was collected from the donors, and the granulocytes were isolated as described^{21,22}. In short, the granulocytes and mononuclear cells were separated over isotonic Percoll with a specific density of 1.076 g/ml. Erythrocytes in the pellet were lysed in ice-cold medium containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA, pH 7.4. Granulocytes were washed and resuspended in Hepes-buffered saline solution (HBSS containing 132 mM NaCl, 6.0 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgSO₄, 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) human serum albumin, pH 7.4). Purity of granulocytes isolated with this method was more than 95%. Overnight culture was performed in HBSS with or without addition of 100 ng/mL G-CSF and 1 µM dexamethasone.

RNA isolation, amplification, labeling, and hybridization

Total cellular RNA was extracted from a minimum of 20 x 10⁶ cells with TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the protocol provided by the manufacturer, with the following minor modifications. An additional phenol-chloroform extraction was performed and the isopropanol precipitation at -20°C was facilitated by the addition of 20 µg/mL glycogen (Roche, Almere, The Netherlands). Purity and integrity of the RNA samples were confirmed on the Agilent 2100 bioanalyzer (Agilent Technologies Netherlands B.V., Amstelveen, The Netherlands) by means of the RNA 6000 Nano LabChip kit. Finally, mRNA was amplified with the MessageAmp II Kit (Ambion). Labeling, hybridization, and data extraction were performed at ServiceXS (Leiden, The Netherlands), as has been described elsewhere²³.

Microarray imaging and data analysis

The microarray slides were scanned with the Agilent dual-laser DNA microarray scanner. Default settings of Agilent Feature Extraction preprocessing protocols were used to obtain normalized expression values from the raw scans. Exact protocol and parameter settings are described in the Agilent Feature Extraction Software User Manual 8.5 (<http://chem.agilent.com/scripts/LiteraturePDF.asp?iWHID=37629>). The default Agilent normalization procedure, called Linear & Lowess, was applied. Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) was used for analysis of the data. Genes were defined as differentially transcribed if the average expression level changed at least three-fold compared with unstimulated cells (0 h, control sample) in all three donors.

Quantitative RT-PCR validation of gene expression

PCR amplification was performed on a LightCycler instrument (Roche, Almere, The Netherlands), and analyzed with software version 3.5. The reaction was performed with Lightcycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics, Indianapolis, IN, USA). The annealing temperature used for all primers was 65°C. The reaction mixture consisted of 4 µL of cDNA, 1 µL of relevant primer combination, and 4 µL of SYBR Green I mix in a total volume of 20 µL. All amplified cDNA was compared with the standard within the same run, and in every run the same standard was used, although there was very little variation in the standard between runs.

For amplification, the following LightCycler protocol was used. The chemical cleft of the Taq polymerase was removed by preincubation for 10 minutes at 95°C; the template was amplified for 40 cycles, with annealing of the primers at 65°C. The fluorescence was measured at the end of each cycle at 72°C. At the end of 40 cycles, a melting curve was generated to determine the unique features of the DNA amplified. The specific size of the product was determined on a 1% (w/v) agarose gel. Subsequently, the obtained band was purified by means of the GFX PCR DNA and Gel Band purification kit (Amersham Biosciences) according to the manufacturer's instructions to remove excess dNTPs and primers. The product was sequenced by Big-dye Terminator Sequencing and ABI Prism software (Applied Biosystems, Foster City, CA). The sequence was verified with BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine specificity. All products obtained were unique and had no overlap with other isoforms.

Standard curves and relative quantitation

As a source of cDNA for standard curves to which all samples were normalized, neutrophils were isolated from an apheresis buffy coat obtained from the blood bank North-West (Sanquin). Serial 10-fold dilutions from the cDNA obtained were made to which each sample was quantified with the method described in Technical Note No. LC 13/2001 (Roche Applied Science, Almere, The Netherlands), as has been described elsewhere²⁴.

Immunostaining and FACS analysis

Cell surface expression of various receptors on granulocytes was assayed in total leukocyte samples by flow cytometry (FACS), with saturating concentrations of commercially available monoclonal antibodies (MoAbs), either directly labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or indirectly labeled with allophycocyanin (APC). CD52-FITC, CD55-FITC, CD59-FITC and CD69-PE were from Sanquin Reagents, Amsterdam, The Netherlands; EMR3-FITC and CD177-unconjugated were from AbD Serotec, Oxford, UK; goat F(ab')₂ anti-mouse-IgG-APC was from Southern Biotech, Birmingham, AL, USA).

Samples were analyzed on an LSRII flow cytometer equipped with FACSDiva software (BD). Cells were gated based on their forward and side scatter, and 10,000 gated events were collected per sample (100% positive staining for CD16 and negative for CD36 [monocytes] or CD56 [NK cells] confirmed purity of the analyzed population).

Annexin-V binding

To detect apoptosis, cells were labeled for 10 min on ice with FITC-labeled Annexin V (Bender Med Systems, Vienna, Austria), diluted 1:500 in HBSS, supplemented with 2.5 mM CaCl₂. Annexin-V labeling was followed by a single wash step with the same medium, whereupon the cells were resuspended in HBSS 2.5 mM CaCl₂ containing 1 µg/mL propidium iodide (PI; Sigma). After an additional 5 min on ice, the samples were analyzed on a FACScan flow cytometer (BD). Surviving cells were defined as the cells in the lower left quadrant that stained negative for both Annexin V and PI. 10,000 events were collected for each sample, and data were analyzed with CellQuest Pro software (BD Bioscience).

Western blot analysis

Total cell lysates were prepared by treating the cells with a lysis buffer (250 mM sucrose, 70 mM KCl, 0.5% Triton X-100 (v/v),

0.5% β -octylglucoside (v/v), 2 mM NaVO₄, 1 mM NaF, 1 mM EDTA, supplemented with a complete protease inhibitor cocktail mix (PIM; Roche Diagnostic, Almere, The Netherlands) and 2 mM diisopropylfluorophosphate (DFP; Fluka Chemica, Steinheim, Switzerland) in phosphate-buffered saline [PBS]) for 30 minutes, on ice. Afterwards, samples were mixed with 4x Laemli sample buffer (LSB; 50 mM TrisHCl, pH 6.8, 10% glycerol (v/v), 5 mM DTT [DL-dithiothreitol, Sigma] 1% β -mercaptoethanol, 1% sodiumdodecylsulfate [SDS; *m/v*], 10 μ g/mL bromophenol blue) and boiled for 15 minutes at 95°C. All samples were stored at -20°C before subjection to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Samples were run on 10%, 1.5 mm polyacrylamide gels in a protean-3 mini system (Bio-Rad Laboratories Inc., Veenendaal, The Netherlands). The equivalent of 1.5×10^6 cells was loaded in each lane. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad), which were subsequently blocked for 60 minutes with blocking buffer (5% non-fat dry milk (*m/v*) [Elk; Campina, Zaltbommel, The Netherlands] in Tris-buffered saline, 0.1% Tween-20 (v/v) [TBST]). The membranes were immune-labeled with specific antibodies in blocking buffer containing 2 mM NaN₃, overnight at 4°C. After washing, the membranes were labeled with fluorescently labeled secondary antibodies (either IRDye 800CW or IRDye 700 [Li-COR Bioscience, Lincoln, NE, USA]) and the protein bands were visualized with the Odyssey Infrared Imaging System (Li-COR), and analyzed with the accompanying software (v2.1).

Statistics

Significantly regulated genes were selected with Rosetta Resolver. Genes with a fold change of ≥ 3 together with a *p* value cut-off of ≤ 0.01 (one-way ANOVA test with the Benjamini-Hochberg false-discovery rate correction) were considered significantly different across the different cell populations. Genes differentially expressed in mobilized granulocytes were categorized by reported or putative functions by the use of the OntoExpress program (<http://vortex.cs.wayne.edu/projects.htm#Onto-Express>). Graphs were drawn and statistical analysis was performed with GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA). Data were evaluated by paired, one-tailed student's *t*-test where indicated. The results are presented as the mean $\pm$ SEM, as indicated.

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Temporal and spatial regulation of Bfl-1 expression during the later stages of neutrophil apoptosis

Submitted for Publication

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Abstract

Neutrophils, the most abundant phagocytes of the human immune system, have a very short life span, after which they are rapidly cleared by constitutive, spontaneous, apoptosis. The Bcl-2 family of proteins plays an important role in the regulation of neutrophil apoptosis. Thus far, the anti-apoptotic family member Mcl-1 has received most attention in the context of neutrophil apoptosis. Another anti-apoptotic member, Bfl-1, has only been studied at the mRNA level in neutrophils. In this study, we found that the mRNA level of Bfl-1 is constitutively high in neutrophils and can be further elevated by the anti-apoptotic growth factor G-CSF. At the protein level, Bfl-1 expression was restricted to the later stages of neutrophil apoptosis and was exclusively found in a fraction that contained both the endoplasmic reticulum (ER) and the mitochondria. Bfl-1 protein expression was triggered during early apoptosis in neutrophils and was maintained by calpain inhibition. Although Bfl-1 expression neither prevented the translocation of pro-apoptotic Bax to the mitochondria nor the release of pro-apoptotic factors from the mitochondria, Bfl-1 stabilization was associated with prolonged survival. In sum, the roles that various Bcl-2 family members play during neutrophil apoptosis are determined by their temporal and spatial localization. Bfl-1 is only present during the later stages of this process and, therefore, is not expected to prevent the initiation of the process.

Introduction

Neutrophils, the most abundant phagocytes of the human immune system, have a very short life span, after which they undergo spontaneous apoptosis. The B-cell leukemia (Bcl)-2 family of proteins plays an important role in the regulation of neutrophil apoptosis¹⁻⁴. This family can be divided into three groups, based on their structural and functional similarities. The anti-apoptotic subgroup (including Bcl-2, myeloid cell leukemia (Mcl)-1, Bfl-1/A1, Bcl-xL and Bcl-w) counteracts mitochondrial outer-membrane permeabilization (MOMP) by their pro-apoptotic counterparts. The pro-apoptotic members comprise two subgroups: Bcl-2 effector proteins (including Bax, Bak and Bok) and Bcl-2 homology domain 3 (BH3)-only proteins (such as Bad, Bid, Bim, Noxa and Puma)⁵. After activation or release from their anti-apoptotic counterparts, the BH3-only proteins interact with the Bcl-2 effector proteins⁶. The resulting complex can form supramolecular pores in the mitochondrial outer membrane⁷⁻⁹. This results in the release of pro-apoptotic proteins from the mitochondrial inter-membrane space, such as cytochrome *c*, Smac and Omi^{10,11}. As a consequence, the cell undergoes apoptosis.

Interestingly, Bcl-2 family members do not only localize to the mitochondrial outer membrane, but also in the endoplasmic reticulum (ER) membrane, in fresh as well as in apoptotic cells. In particular, the pro-apoptotic family members Bax and Bak as well as the anti-apoptotic family members Bcl-2 and Bcl-X_L have been shown to influence the ER-Ca²⁺ stores¹²⁻¹⁶. Bcl-2 family members have been shown to associate with inositol triphosphate receptors (IP3Rs) on the ER membrane to regulate their ability to release Ca²⁺ from the ER into the cytosol, as well as with the ER Ca²⁺-ATPase pump (SERCA) to regulate its ability to transfer cytosolic Ca²⁺ to the ER lumen^{13,16,17}.

An important aspect of apoptosis that has recently become apparent is the interaction between the ER and mitochondria^{18,19}. In resting, healthy cells, mitochondria form a dynamic network that undergoes constant fission and fusion and is tightly associated with the ER²⁰. In essence, the mitochondrial network, with its negatively charged matrix, forms a deposit for Ca²⁺, which constantly leaks from the ER. As long as the network is intact, mitochondrial membrane potential ($\Delta\Psi_m$) is maintained, and ER and mitochondria can interact; the Ca²⁺ stress on the cell is kept at a minimum. However, when Bax translocation to the mitochondria promotes mitochondrial fission during the early

stages of apoptosis, and initial caspase-3 activation leads to a loss of $\Delta\psi_m$, Ca^{2+} stress increases exponentially, and amplification of the process of apoptosis finally leads to the execution of cell death^{15;21-25}.

A vitally important factor in the recruitment of fresh neutrophils from the bone marrow is granulocyte-colony stimulating factor (G-CSF). G-CSF has also been shown to delay apoptosis of mature neutrophils, both *in vivo* and *in vitro*²⁶⁻³⁰. Previously, we have demonstrated that the pro-survival effect of G-CSF is mainly due to inhibition of calpain activation³⁰. During neutrophil apoptosis, intracellular Ca^{2+} levels rise gradually, leading to the activation of calpains, a family of Ca^{2+} -dependent cysteine proteases³⁰⁻³³. Calpains degrade the X-linked inhibitor of apoptosis (XIAP), leading to enhanced caspase-3 activation. G-CSF inhibits this rise in intracellular Ca^{2+} , XIAP degradation and caspase-3 activation, and thus inhibits neutrophil apoptosis³⁰.

Previously, the anti-apoptotic Bcl-2 family member Mcl-1 has been implicated as a major player in granulocyte macrophage-colony stimulating factor (GM-CSF)-inhibited neutrophil apoptosis^{2;4;34}. During spontaneous neutrophil apoptosis Mcl-1 is constantly and highly expressed at the mRNA level. However, at the initial stages of neutrophil apoptosis, the protein appears to be degraded by the proteasome at an even faster rate than the mRNA is translated, a process that is delayed by GM-CSF⁴. Thus, it appears that when the intracellular balance is tipped from pro-survival factors (such as Mcl-1) to pro-apoptotic factors (such as Bax), the cells become apoptotic.

In this study, the dynamics of the expression of several Bcl-2 family members in neutrophils during 18 hours in the absence or presence of G-CSF were investigated. We show that G-CSF mainly promotes the mRNA expression and protein stability of the anti-apoptotic Bcl-2 family member Bfl-1. However, the Bfl-1 protein is only temporarily expressed in mitochondria-ER double-positive fractions where it co-localizes with Bax as it translocates to this same fraction during apoptosis. In G-CSF-treated cells, Bfl-1 persists in this fraction and apoptosis is delayed, while in control cells, undergoing spontaneous cell death, Bfl-1 is degraded in a calpain-dependent manner and the cells become apoptotic.

Results

G-CSF induces Bfl-1 expression

To assess gene expression changes in neutrophils during apoptosis, mRNA samples were collected from unstimulated and *in vitro* G-CSF-stimulated neutrophils at 0, 6, 12 and 18 hours and analyzed by RT-MLPA^{35;36}. At 24 hours, the quality of the mRNA samples obtained was insufficient for reliable quantification (not shown). No detectable expression was found of the transcripts for the anti-apoptotic Bcl-2 family members Bcl-2, Bcl-w and Bcl-G or the BH3-only protein Harakiri (not shown). The absence of mRNA from these genes in our samples indicates that our cell preparations were not contaminated with significant numbers of monocytes or lymphocytes, since these cells do express the afore mentioned genes^{2;36}.

Both the anti-apoptotic genes Mcl-1 and Bfl-1 were found to be highly expressed at the mRNA level in neutrophils (Figure 1). Of these transcripts, only Bfl-1 expression was significantly induced by G-CSF at all investigated time points, relative to the control. Of the pro-apoptotic Bcl-2 family members, G-CSF only reduced the expression of Noxa and Bax. Expression of the other pro-apoptotic Bcl-2 family members, such as Bid, was mostly unaffected by G-CSF (Figure 1). Expression of all genes was compared to β 2-microglobulin (B2M), which was stably expressed during incubation in all samples.

Figure 1. Alterations in gene expression of Bcl-2 family members during spontaneous and G-CSF-delayed neutrophil apoptosis. Neutrophils were incubated in the presence (grey lines, triangles) or absence (black lines, squares) of G-CSF (10 ng/mL), and mRNA was isolated every 6 hours for up to 18 hours. Expression of Bcl-2 family members and control genes was determined by MLPA analysis. Values were normalized for β 2-microglobulin (B2M) expression, which was found to be high and stable in both control and G-CSF-treated cells throughout the experiment. Values represent the average ($\pm$ SEM) of five independent experiments performed in duplicate. Statistical significance between control and G-CSF-treated samples was determined by Wilcoxon signed rank test. * $p < 0.05$, ** $p < 0.01$. If no significance is indicated, no significant differences were observed.

Expression of the inhibitor of apoptosis proteins (IAPs, including XIAP, IAP1 [cIAP2] and Apollon [Bruce]) was unaffected by G-CSF (Supplementary Figure S1), while no mRNA expression of IAP2 (cIAP1) was detected (not shown). In addition, G-CSF transiently induced mRNA expression of the endogenous caspase-8 inhibitor, cellular FLICE-like inhibitory protein (c-FLIP). However, in a previous study we detected no delay in caspase-8 activation in G-CSF-treated cells³⁰.

Bfl-1 protein expression is transiently induced in neutrophils and stabilized by G-CSF

Since G-CSF delays neutrophil apoptosis and, of the anti-apoptotic genes, only increases the mRNA expression of Bfl-1, we decided to focus our study on this protein. To assess Bfl-1 protein expression as well as spatial and temporal localization of this protein in neutrophils, sub-cellular fractions were prepared at several time points and were analyzed on Western blot (Figure 2A and B). Apoptosis was monitored in parallel by Annexin-V staining on the flow cytometer (Figure 2C). Bfl-1 expression was exclusively found in the membrane fraction, containing both mitochondria and ER, as indicated by the presence of the mitochondrial marker MnSOD and the ER marker Calnexin. In fresh cells (0h), Bfl-1 expression was hardly detectable in any fraction or in total cell lysates (not shown), but the protein was readily detected after 8 hours of incubation and remained present at 12 hours. However, in the control cells, the protein had completely disappeared at 24 hours, while it remained present in the G-CSF-treated cells. Bax translocation to the membranes and Smac release from the mitochondria were not significantly affected by G-CSF, both peaking after 12 hours (Figure 2B). Interestingly, when the ratio of Calnexin (ER) over MnSOD (mitochondria) was analyzed in the membrane fraction, it became clear that the amount of ER that was co-purified with mitochondria gradually reduced over time in the control cells. In G-CSF-treated cells the ratio slightly increased at the early time points, while it remained relatively high after 24 hours of incubation (Figure 2B). This change in ratio was also found when Calreticulin, another well known ER-marker, was evaluated (not shown). In addition, the Calnexin/MnSOD-ratio did not change in total lysates of the cells (not shown), indicating that Calnexin was not simply degraded during apoptosis.

Figure 2. Bfl-1 is temporarily expressed in control cells during apoptosis, but its expression is maintained in G-CSF-treated cells. Neutrophils were incubated in the presence (grey lines, triangles) or absence (black lines, squares) of G-CSF (10 ng/mL), and sub-cellular fractions were prepared at the indicated times. **(A)** Representative Western blots of the cytosolic and membrane (both ER and mitochondria) fractions of control and G-CSF-treated cells. **(B)** Analyses of the Western blots, as performed on the Odyssey infrared scanner. All values represent the average ($\pm$ SEM) of at least three independent experiments. Values for Bfl-1 represent the relative signal in the membrane fraction normalized for MnSOD, since no Bfl-1 signal was detected in the cytosol. Values for Bax and Smac were calculated by dividing the signal in the membrane and cytosol fractions, respectively, by the total signal of both fractions. The ER/mitochondria ratio was calculated by dividing the Calnexin (CNX) signal by the MnSOD signal. All values were normalized for the control at 0 hours. **(C)** Survival curves of the cells during culture as determined by Annexin-V staining on a flow cytometer. Statistical significance between control and G-CSF treated samples was determined by paired, one tailed student's t-test. * $p < 0.05$.

Inhibition of both calpains and caspases prevents the loss of Bfl-1 expression

Previously, calpain-dependent degradation of Bfl-1 has been described to turn this anti-apoptotic protein into a pro-apoptotic BH3-only protein by cleaving off the BH1 and BH2 domain³⁷. Since we previously found that G-CSF inhibits constitutive calpain activation during neutrophil apoptosis³⁰, we wondered whether the loss of endogenous Bfl-1 expression in the membranes during neutrophil apoptosis was calpain dependent.

Neutrophils were incubated for 8 or 24 hours in the presence of G-CSF, the specific Calpain Inhibitor III (CI3), the proteasome inhibitor MG132, the pan-caspase inhibitor z-VAD-fmk (zVAD) or the calcium chelator BAPTA. MG132 has been shown to stabilize the anti-apoptotic factor Mcl-1⁴, while BAPTA prevents neutrophil apoptosis by chelating intracellular free Ca^{2+} ³⁰. Sub-cellular fractions were prepared after 24 hours and analyzed on Western blot (Figure 3A). Only G-CSF, CI3 and zVAD prevented loss of Bfl-1 expression, while MG132 and BAPTA had no effect (Figure 3B). Bax translocation was comparably increased in all samples, while the amount of Smac in

the cytosol was significantly increased in the MG132-treated cells and decreased in the zVAD-treated cells. Since Smac is degraded by the proteasome after removing XIAP inhibition from caspase-9 and -3, the effect of the proteasome inhibitor is not surprising³⁸. In fresh cells, the ratio of ER/mitochondria in the membrane fractions was high, as indicated by the Calnexin/MnSOD ratio (Figure 3B). In all samples in which Bfl-1 expression was maintained, this ratio remained high during overnight culture, while in the other samples the ratio was severely reduced due to a loss of the Calnexin signal. All of the inhibitors significantly delayed neutrophil apoptosis, as indicated by Annexin-V staining (Figure 3C) and nuclear morphology (not shown).

Figure 3. Both calpain and caspase activities promote the loss of Bfl-1. Neutrophils were incubated for 24 hours in the absence or presence of G-CSF (10 ng/mL), calpain inhibitor 3 (CI3, 20 μ M), the proteasome inhibitor MG132 (50 μ M), the pan-caspase inhibitor zVAD-fmk (zVAD, 20 μ M) or the Ca^{2+} chelator BAPTA-AM (10 μ M). Afterwards, sub-cellular fractions were prepared and analyzed on Western blot. **(A)** Representative Western blot of both fractions, showing Bfl-1 expression in the membrane fraction that contains both mitochondria (MnSOD) and ER (Calnexin). Smac is released from the mitochondria into the cytosol, whereas Bax translocates from the cytosol to the mitochondria/ER. **(B)** Analyses of the Western blots, as performed on the Odyssey infrared scanner. All values represent the average ($\pm$ SEM) of at least three independent experiments. Values for Bfl-1 represent the relative signal in the membranes fraction normalized for MnSOD, since no Bfl-1 signal was detected in the cytosol. Values for Bax and Smac were calculated by dividing the signal in the membrane and cytosol fractions, respectively, by the total signal of both fractions. The ER/mitochondria ratio was calculated by dividing the Calnexin (CNX) signal by the MnSOD signal. All values were normalized for the control at 0 hours. **(C)** Neutrophil survival as determined by Annexin-V staining on the flow cytometer. Cells were analyzed after 8 (black bars) and 24 (white bars) hours. All inhibitors significantly delay neutrophil apoptosis, as compared to the control. Values represent the average ($\pm$ SEM) of at least three independent experiments, performed in duplicate. Statistical significance between control and inhibitor-treated samples was determined by paired, one-tailed student's t-test. * $p<0.05$, ** $p<0.01$.

Caspase inhibition prevents the loss of mitochondrial membrane potential during neutrophil apoptosis

Even though neutrophils hardly utilize their mitochondria for ATP production, they do maintain mitochondrial membrane potential ($\Delta\psi_m$), which is lost during apoptosis^{11;30;39}. $\Delta\psi_m$ was assessed in neutrophils by flow cytometry with the $\Delta\psi_m$ indicator JC-1. This dye forms red fluorescent aggregates in fully charged mitochondria but becomes green fluorescent when $\Delta\psi_m$ is lost and the dye is dispersed (Figure 4A). The fluorescent ratio PE-Texas Red/FITC thus gives an indication of the $\Delta\psi_m$. In control experiments, in which the cells were double labeled with TMRM, an alternative $\Delta\psi_m$ indicator, and FITC-labeled Annexin V, all cells in the population with low $\Delta\psi_m$ were found to be Annexin-V positive, whereas cells in the populations with high $\Delta\psi_m$ were found to be Annexin-V negative (not shown). Even though CI3 and MG132, as well as G-CSF, prevented the early reduction in $\Delta\psi_m$ at 8 hours, only G-CSF, zVAD and BAPTA-treated cells maintained a higher $\Delta\psi_m$ than the control cells after 24 hours (Figure 4B). This indicates that caspase activity as well as elevated intracellular free Ca^{2+} is required for the observed loss of $\Delta\psi_m$, and that Bfl-1 expression does not affect this aspect of neutrophil apoptosis.

Figure 4. Caspase inhibition prevents loss of $\Delta\psi_m$ in the later stages of apoptosis, calpain inhibition only in the early stages. Neutrophils were incubated for up to 24 hours in the absence or presence of G-CSF (10 ng/mL), calpain inhibitor 3 (CI3, 20 μM), the proteasome inhibitor MG312 (50 μM), the pan-caspase inhibitor zVAD-fmk (zVAD, 20 μM) or the Ca^{2+} chelator BAPTA-AM (10 μM). $\Delta\psi_m$ was determined by JC-1 staining on the flow cytometer after 8 (black bars) and 24 (white bars) hours. (A) $\Delta\psi_m$, as determined in the neutrophil population, is lost during apoptosis. The relative ratio of the PE-Texas Red signal over the FITC signal gives a reliable indication of $\Delta\psi_m$. (B) Bar graph of the relative $\Delta\psi_m$, as compared to fresh control cells (C- 0h). G-CSF, CI3 and MG132 significantly delay the loss of $\Delta\psi_m$ during the early stages of apoptosis, but only G-CSF zVAD and BAPTA prevent the loss of $\Delta\psi_m$ in the later stages. All values represent the average ($\pm$ SEM) of at least three independent experiments. Statistical significance between control and inhibitor-treated samples was determined by paired, one-tailed student's t-test. * $p < 0.05$, ** $p < 0.01$.

Bfl-1 expression on ER/mitochondria is induced by caspase activity and reduced by calpain activity

We previously developed a method to analyze neutrophil-derived intact organelles by flow cytometry³⁹. Here, we applied the same method to localize Bfl-1 expression on neutrophil-derived organelles.

Fresh neutrophils were fractionated and, after incubation, the purified organelles were analyzed by flow cytometry after staining with Mitotracker Green, ER-tracker Red and immuno-labeling for the indicated proteins. A substantial portion of the organelles was found to be double-positive for ER-tracker Red and Mitotracker Green (Figure 5A). The same fraction was also found to be positive for Calnexin, which confirms the presence of ER in this fraction. Bfl-1 expression was exclusively found in the ER/Mitochondria double-positive fraction. Although the protein expression of Bfl-1 was very low in fresh cells, and well neigh undetectable on Western blot, the expression

level was sufficiently high to allow analysis by flow cytometry. By incubating the organelles in cytosol in the presence of cytochrome *c*-activated caspases (CC) ⁴⁰ for 1 hour, expression of both Bax and Bfl-1 on the ER/mitochondria containing particles was induced (Figures 5A and B). Addition of caspase-8-truncated Bid (tBid), to mimic early apoptosis, also induced Bax and Bfl-1 expression on these particles, but not significantly (Figure 5B). Although the addition of 1 μ M free Ca^{2+} , similar to the Ca^{2+} levels found in apoptotic neutrophils and sufficient to induce calpain activation (Figure 5C), did induce a certain amount of Bax expression, Bfl-1 expression was severely reduced on the ER/mitochondria-containing particles under these conditions (Figure 5B). The addition of Ca^{2+} also enhanced the activation of caspase-3 (Figure 5C), as expected when calpains degrade the caspase-3-inhibitor XIAP. The combination of activated caspases and Ca^{2+} , as normally found in apoptotic neutrophils, slightly reduced the Bfl-1 expression levels, but not significantly below the levels found on untreated particles. Thus, it appears that early apoptotic stress induces Bfl-1 expression on the ER/mitochondria, while this expression is lost again when Ca^{2+} levels in the cell rise and calpains are activated.

Figure 5. Calpains, but not caspases, are responsible for the loss of Bfl-1 on ER/mitochondria. Neutrophils were fractionated, the organelles incubated in the presence of cytosol with the indicated stimuli, stained with mitotracker green and ER-tracker red, immuno-labeled for Bax and Bfl-1 and analyzed by flow cytometry. **(A)** A significant proportion of the neutrophil-derived particles stains double-positive for both mitotracker green and ER-tracker red. This double-positive fraction also stains positive for Calnexin (blue), further proving the presence of ER. Bfl-1 staining is exclusively found in this population (yellow). Bax expression on these particles, as well as Bfl-1 expression, increases after activating caspases in the cytosol by the addition of cytochrome *c* and dATP. All plots are representative of at least three independent experiments. **(B)** Bar graphs of the relative expression of Bfl-1 and Bax on the ER/mitochondria double-positive particles, after treating the particles in cytosol with either truncated Bid (tBid), cytochrome *c* and dATP (CC), 1 μM Ca^{2+} (High Ca^{2+}) or a combination of the latter two stimuli for 1h. All values represent the average ($\pm$ SEM) of at least three independent experiments. Statistical significance between control and inhibitor-treated samples was determined by paired, one-tailed student's t-test. * $p < 0.05$. **(C)** Control plots showing the activation of calpains after addition of Ca^{2+} and the activation of caspase-3 after addition of CC, as determined in the high-speed supernatants after the experiment.

Both G-CSF and zVAD prevent the rise in intracellular free Ca^{2+} during overnight culture

Since calpain activity appears to be responsible for the degradation of Bfl-1 during neutrophil apoptosis, while the caspase inhibitor zVAD nevertheless prevents the loss of Bfl-1 as demonstrated in Figure 3, we tested the theory that caspases may be responsible for the rise in intracellular free Ca^{2+} as previously observed during neutrophil apoptosis

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Neutrophils were incubated for 24 hours in the presence of the indicated inhibitors, and intracellular free Ca^{2+} levels were determined by loading the cells with a combination of the Ca^{2+} -indicators Fluo 4 and Fura Red and analyzing the signal by flow cytometry after overnight incubation. The ratiometric method of using two Ca^{2+} -indicators simultaneously has been shown to yield a more accurate result than single fluorescence detection⁴¹.

During overnight incubation in a Ca^{2+} containing medium, the fluorescent ratio of Fluo 4 over Fura Red increased significantly (Figure 6), indicating a strong increase in intracellular free Ca^{2+} . G-CSF dramatically reduced this increase, and a similar effect was seen in cells treated with zVAD. Cells treated with the calpain inhibitor Cl3 displayed no significant reduction in intracellular free Ca^{2+} , consistent with the idea that the influx of extracellular Ca^{2+} occurs upstream of calpain activation. The results from previous experiments suggested that the effect of G-CSF depended on *de novo* protein synthesis³⁰. Addition of the protein synthesis inhibitor cycloheximide (CHX) significantly increased the overnight Ca^{2+} influx, while G-CSF had no effect on the Ca^{2+} levels when added in

Figure 6. Caspase activity triggers the increase in intracellular free Ca^{2+} , as observed during neutrophil apoptosis. Neutrophils were incubated for 24 hours in the absence or presence of G-CSF (10 ng/mL), cycloheximide (CHX, 10 $\mu\text{g/mL}$), the combination of G-CSF and CHX, calpain inhibitor 3 (Cl3, 20 μM) or the pan-caspase inhibitor zVAD-fmk (zVAD, 20 μM). Free cytosolic Ca^{2+} was determined by double labeling the cells with Fluo 4 and Fura Red. The relative ratio of the Fluo 4 signal over the Fura Red signal, as determined by flow cytometry, yields a reliable measure for the intracellular free Ca^{2+} concentration. Only G-CSF and zVAD prevented the rise in free Ca^{2+} during overnight incubation, whereas the protein synthesis inhibitor CHX seemed to stimulate the influx of Ca^{2+} . All values represent the average ($\pm$ SEM) of three independent experiments. Statistical significance between control and inhibitor-treated samples was determined by paired, one-tailed student's t-test. ** $p < 0.01$, n.s.: not significant.

Discussion

In the murine myeloblast cell line 32D clone 3 (32Dcl3), G-CSF has been shown to induce granulocytic differentiation by increasing the mRNA expression of the anti-apoptotic Bcl-2 family member Bfl-1/A1 via activation of the transcription factor Wilm's tumour gene 1 (WT1)^{42;43}. In this cell line, signal transduction of the G-CSF receptor occurred via the Janus kinase (Jak)/signal transducers and activators of transcription 3 (Stat3) pathway⁴⁴⁻⁴⁶. In mature human neutrophils, an increase in the mRNA expression of Bfl-1 was also noted after treatment with G-CSF⁴⁷. In addition, the A1-a knockout mice display accelerated spontaneous neutrophil apoptosis³, further stressing the importance of A1/Bfl-1 for neutrophil survival. However, mice express three different A1 genes, whereas humans have only one. Human Bfl-1 has been described to sequester the pro-apoptotic truncated form of the Bcl-2 family member Bid, tBid⁴⁸, as well as to antagonize the Bax-like protein Bak⁴⁹. Although Bfl-1 clearly promotes survival, this ability has been shown to correlate only partially with its ability to bind to the pro-apoptotic Bcl-2 family members⁵⁰. In this study, we demonstrate for the first time the changes in both protein and mRNA expression of endogenous Bfl-1 in primary human neutrophils.

Since we found that the mRNA expression of Bfl-1 is high and rather constant and high in neutrophils, while the protein expression is critically regulated in both space and time, we conclude that overexpression systems can not give accurate information about the endogenous function of Bfl-1. After all, in an overexpressing cell line, Bfl-1 will be expressed continuously throughout the cell, while the endogenous protein is only expressed during the later stages of apoptosis in the mitochondria/ER compartment. As a consequence, it is unlikely that the endogenous protein acts to inhibit Bax translocation or scavenge truncated Bid in neutrophils, as has been suggested previously for other cell types^{49;51}. These results suggest that Bfl-1 expression is translationally controlled. Thus, the high levels of mRNA in neutrophils allow a rapid increase in Bfl-1 protein expression during neutrophil apoptosis. However, the Bfl-1 mRNA 3'-untranslated region does not contain any known binding sites for mRNA-binding proteins.

In healthy, resting, cells mitochondria form a dynamic network that undergoes constant fission and fusion and associates tightly with the ER. Bax translocation to this network triggers fission²⁰. In our current study, we show that Bfl-1 stabilization does not necessarily prevent Bax clustering at the mitochondria and Smac release. However, Bfl-1 expression at the ER/mitochondria does seem to delay the later stages of apoptosis and also correlates with a delay in the dissociation between mitochondria and ER. As long as the mitochondria maintain $\Delta\psi_m$, they can be considered as buffer organelles that take up Ca^{2+} when it leaks from the ER⁵². When mitochondria lose $\Delta\psi_m$, they lose their buffering capacity as well, and Ca^{2+} -stress within the cells increases exponentially, as illustrated in Figure 7. For neutrophils, it appears that this ER/mitochondria association may be an essential element in the process of apoptosis. Even after Bax translocation has occurred and Smac has been released from the mitochondria, as long as the ER and mitochondria associate, the cells do not seem to progress towards full-blown apoptosis. Although the dissociation of mitochondria and ER increases the Ca^{2+} stress on the cell, a sudden rise in intracellular Ca^{2+} can also cause this dissociation²². However, as long as the mitochondrial outer membrane remains intact and Smac is not released, this does not have to result in apoptosis, since caspase activation does not occur. In fact, temporal dissociation of the mitochondria from the ER may protect the mitochondria from Ca^{2+} overload and rupture during, for example, cell activation.

Neutrophil apoptosis appears to proceed in several stages. Initial activation of caspases or cathepsins⁵³ trig-

gers Bax translocation to the mitochondria and ER. As a consequence of Bax translocation, Smac is released from the mitochondria. In addition, Bfl-1 expression is induced on the mitochondria/ER compartment by a pro-apoptotic stimulus, where it apparently remains after translation from mRNA *in situ*, since Bfl-1 protein was never detected in any other compartment of the cell. In BAPTA-treated cells, it appears that the process is inhibited at this stage, before the induction of Bfl-1 expression. As a consequence, Bfl-1 levels in BAPTA-treated cells stay low during overnight incubation. The localization of Bfl-1 in the membrane fraction of neutrophils is remarkable, since Bfl-1 is the only Bcl-2 family member that does not have a transmembrane domain. This suggests that the protein is associated with another protein on the ER/mitochondria. Early caspase activation also results in the influx of extracellular Ca^{2+} , probably preceded by the release of Ca^{2+} from the ER. At this stage of apoptosis, as long as the intracellular free Ca^{2+} concentration is controlled or calpain activation is prevented, the cell does not proceed towards apoptosis. Since caspases are also responsible for the loss of $\Delta\psi_m$ during apoptosis²⁵, they essentially control the intracellular free Ca^{2+} levels by disabling the buffering capacity of the mitochondria. When the intracellular free Ca^{2+} concentration rises above a certain threshold level, calpains become activated. This will then result in the degradation of XIAP³⁹ as well as the loss of Bfl-1. As a consequence, caspase activation is accelerated and the pro-apoptotic signal is greatly amplified, as illustrated in Figure 7.

Figure 7. Three stages of neutrophil apoptosis.

Stage 1: Fully charged mitochondria form a continuous, dynamic, network that associates with the ER. Limited Ca^{2+} leakage from the ER is dispersed in this network. Calpain activity is limited, caspases are not yet activated.

Stage 2: Activation of initiator caspases leads to Bax activation and its translocation to the ER/mitochondria. As a result, the mitochondrial network undergoes fission and Smac is released. Local expression of Bfl-1 prevents further damage. Mitochondrial $\Delta\psi_m$ is maintained and Ca^{2+} -stress on the cell is low. Calpain activity increases, executioner caspases are not yet active. Stimulation of the cells with G-CSF delays the progression from this stage to the final stage of apoptosis.

Stage 3: Due to increased calpain activity, Bfl-1 is proteolytically degraded. The separated/fractionated mitochondria lose $\Delta\psi_m$ and start to dissociate from the ER. As a consequence, the ER- Ca^{2+} -storage becomes depleted (high efflux through calcium channels), which leads to an influx of extracellular Ca^{2+} , increasing Ca^{2+} -stress on the cell and increased calpain activity. Finally, caspases-9 and -3 are also activated and the cell becomes apoptotic.

Grey scale indicates increasing Ca^{2+} -levels, dotted arrows indicate Ca^{2+} -efflux from the ER.

Although the role of Bfl-1 in apoptosis is not entirely clear as yet, it seems to be important in the later stages of apoptosis, just before the final execution of the process. By increasing the protein expression of Bfl-1 after an initial pro-apoptotic trigger, the cell seems to delay the final stage of apoptosis in response to an increase in pro-apoptotic stress. G-CSF-stimulated cells maintain Bfl-1 expression by inhibition of calpain activation, and thus successfully delay their execution. Further research is required into the regulation of Bfl-1 translation during the early stages of neutrophil apoptosis.

Materials and methods

Antibodies and reagents

The rabbit polyclonal antibody against human Bfl-1 was a kind gift from Prof. J. Borst of the Netherlands Cancer Institute, Amsterdam, the Netherlands^{51,54,55}. Mouse anti-Smac/Diablo (#2954) was obtained from Cell Signaling Technology (Boston, MA, USA). Anti-MnSOD was obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Rabbit and mouse (clone 6A7) anti-human-Bax were obtained from BD Biosciences (Franklin Lakes, NJ, USA) and mouse anti-Calnexin (clone TO-5) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

All chemical reagents were obtained from Merck Biosciences (Darmstadt, Germany), unless otherwise indicated.

Cell preparation and culture

Neutrophils were isolated from the heparinized blood of healthy individuals after informed consent had been acquired according to the Helsinki declaration, by centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) and subsequent lysis of erythrocytes as described⁵⁶. Neutrophil preparations were typically >97% pure, with the contaminating cells being mostly eosinophils. Cells were cultured in Hepes-buffered saline solution (HBSS; 132 mM NaCl, 20 mM Hepes, 6 mM KCl, 1 mM MgSO₄, 1.2 mM K₂HPO₄, 1 mM CaCl₂, pH 7.4) supplemented with 1% (v/v) human serum albumin (Cealb; Sanquin, Amsterdam, The Netherlands) and 5 mM glucose at a concentration of 5x10⁶ cells/mL in polypropylene round-bottom tubes of 14 mL (BD Biosciences, Franklin Lakes, NJ, USA). Incubations were performed in a shaking water bath at 37°C. Cells were incubated in the presence of 10 ng/mL clinical grade G-CSF (Neupogen; Amgen, Breda, The Netherlands), where indicated.

RNA preparation and MLPA

Total cellular RNA was extracted from a minimum of 20 x 10⁶ cells with TRIzol reagent (Invitrogen, Breda, The Netherlands) according to the protocol provided by manufacturer, with the following minor modifications. An additional phenol-chloroform extraction was performed and the isopropanol precipitation at -20°C was facilitated by the addition of 20 µg/mL glycogen (Roche, Almere, The Netherlands).

MLPA (multiplex ligation-dependent probe amplification) was essentially performed as described³⁵, with the R011 probeset (MRC-Holland, Amsterdam, The Netherlands). In brief, RNA samples (40–60 ng of total RNA) were first reverse transcribed by means of a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probes. Annealed oligonucleotides were covalently linked by Ligase-65 at 54°C (MRC-Holland, Amsterdam, The Netherlands). Ligation products were amplified by PCR (33 cycles, 30 s at 95°C; 30 s at 60°C; and 1 min at 72°C) via one unlabeled and one FAM-labeled primer (10 pMol). After the PCR stage, aliquots of samples were mixed with Genescan-500 ROX size standards and run on an ABI 3100 capillary sequencer (Applied Biosystems, Warrington, UK) in Genescan mode.

Data were analyzed with Genemapper v4.0 (ABI) and exported for further analysis with Microsoft Excel spreadsheet software. The sum of all peak data was set at 100% to normalize for fluctuations in total signal between samples. Gene expression levels were calculated relative to β2-microglobulin expression in all samples, which was set to 1.

Cell fractionation and Western blot

Sub-cellular fractions were prepared from 5x10⁶ cells by washing the cells once in ice-cold phosphate-buffered saline (PBS) before resuspension in cytosol extraction buffer (250 mM sucrose, 70 mM KCl, 250 µg/mL digitonin, complete protease inhibitor cocktail mix [PIM; Roche Diagnostics, Almere, The Netherlands] and 2 mM diisopropylfluorophosphate [DFP; Fluka Chemica, Steinheim, Switzerland] in PBS) at a concentration of 10⁸ cells/mL. Cells were incubated for 15 min on ice, after which they were centrifuged for 5 min at 1000xg, 4°C. The supernatant represented the cytosolic fraction; the pellet, containing both the mitochondria and most of the ER, was dissolved in mitochondrial lysis buffer (100 mM NaCl, 10 mM MgCl₂, 2 mM EGTA, 2 mM EDTA, 1% (v/v) NP-40, 10% (v/v) glycerol [Sigma], PIM, 2 mM DFP in 50 mM TrisHCl buffer at pH 7.5) and was incubated for another 15 min on ice. After incubation, the samples were centrifuged at 10,000xg for 10 min at 4°C. The supernatant, containing the mitochondrial content and associated ER, represented the membrane fraction. The insoluble pellet was discarded. Both fractions were dissolved in Laemmli sample buffer (LSB; 50 mM TrisHCl, pH 6.8, 10% glycerol (v/v), 5 mM DTT [DL-dithiothreitol, Sigma-Aldrich], 1% 2-mercaptoethanol, 1% sodium dodecylsulphate (SDS) (w/v), 100 µg/mL bromophenol blue) and were boiled for 10 min at 95°C. All samples were stored at -20°C before subjection to SDS-polyacrylamide gelelectrophoresis (SDS-PAGE).

Samples were run on 12%, 1.5 mm, polyacrylamide gels in a protean-3 mini system (Bio-Rad Laboratories, Veenendaal, The Netherlands). The equivalent of 1.5×10^6 cells was loaded in each lane. After electrophoresis, the proteins were transferred to polyvinyl difluoride membranes (Immobilon-FL; Millipore, Billerica, MA, USA), which were subsequently blocked for 30 min in blocking buffer (5% non-fat dry milk (w/v) [Elk; Campina, Zaltbommel, The Netherlands] in Tris-buffered saline, 0.1% Tween-20 (v/v) (TBST)). Blots were immuno-labeled with specific antibodies against the indicated proteins in blocking buffer containing 2 mM NaN_3 , overnight at 4°C. After washing the blots in TBST, they were treated with IRDye-labeled secondary antibodies directed against the primary antibodies (goat anti-rabbit-IgG IRDye 680 or goat anti-mouse-IgG IRDye 800CW; LI-COR Biosciences, Lincoln, NE, USA). Labeling was followed by another round of washing in TBST before detection of the specific signals on an Odyssey Infrared Imager (LI-COR).

Flow cytometry

To detect apoptosis, cells were labeled for 10 min on ice with fluorescein-isothiocyanate (FITC)-labeled Annexin V (Bender Med Systems, Vienna, Austria), diluted 1:500 in HBSS, supplemented with 2.5 mM CaCl_2 . Annexin-V labeling was followed by a single wash step with the same medium, whereupon the cells were resuspended in HBSS 2.5 mM CaCl_2 and analyzed by flow cytometry on an LSRII flow cytometer (BD).

To detect changes in $\Delta\psi_m$, the cells were loaded for 15 min at 37°C with 0.5 μM JC-1 (Molecular Probes, Eugene, OR, USA) in HBSS, containing 1 μM tetraphenyl boron to facilitate entry of the dye into the cells, and were analyzed immediately by flow cytometry. To determine a background value for $\Delta\psi_m$, cells were incubated with 1 μM CCCP (Calbiochem, La Jolla, CA, USA), which is sufficient to completely abrogate $\Delta\psi_m$. Relative $\Delta\psi_m$ was calculated as a ratio of the fluorescent signal in the red (PE-Texas Red) channel over the green (FITC) channel.

Preparation and analysis of neutrophil organelles

Cells were resuspended at a concentration of $200 \times 10^6/\text{mL}$ in mito buffer (0.2 mM EDTA, 0.25 mM sucrose, 10 mM Tris-HCl, pH 7.8) supplemented with a protease inhibitor mixture (PIM) of two Complete tablets per 50 mL (Roche Diagnostics, Almere, The Netherlands), 1 mM 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride (Pefabloc SC, Roche) and 2 mM DFP. After the addition of 10 mM triethanolamine and 0.1 mg/mL digitonin the cells were incubated for 10 min on ice, homogenized by 50 strokes in a pre-cooled glass-glass Dounce tissue homogenizer with the tight piston, and centrifuged at 700xg for 10 min at 4°C. The supernatant was saved, the pellet resuspended in the same volume of mito buffer supplemented with 0.1 mg/mL digitonin, homogenized and centrifuged once again.

The combined supernatants, containing all organelles and the cytosol but not the nuclei and intact cells, were incubated in the presence of either 100 nM tBid (R&D Systems, Minneapolis, MN, USA), 25 $\mu\text{g}/\text{mL}$ cytochrome *c* and 500 μM dATP (Sigma-Aldrich), 1 μM free Ca^{2+} as determined with the Bound and Determined program (v4.0)⁵⁷, or a combination of stimuli for 1 hour at 37°C.

After 1 hour of incubation at 37°C, the organelles were separated from the cytosol by high speed centrifugation (HSC) at 15,000xg and resuspended in mitochondrial assay buffer (MAB; 120 mM KCl, 5 mM KH_2PO_4 , 1 mM EDTA, 1 mM MgCl_2 , 3 mM Hepes, pH 7.8, 1% (v/v) human serum albumin and 2 mM glycerolphosphate) containing 0.1 μM Mitotracker Green and 0.5 μM ER-Tracker Red (BODIPY TR glibenclamide; Molecular Probes, Invitrogen, Carlsbad, CA, USA). After 30 min of incubation at 37°C, the samples were again subjected to HSC, resuspended in MAB containing the primary antibodies (all diluted 1:200), left on ice for 30 min, subjected to HSC, resuspended in MAB containing the secondary antibodies (goat-anti-mouse Alexa Fluor 405 and goat-anti-rabbit Alexa fluor 750; Molecular Probes), diluted 1:500 and incubated on ice for another 30 min. Finally, the samples were washed once in MAB and analyzed on an LSRII flow cytometer (BD Biosciences), equipped with FACSDiva software. To detect small particles, forward scatter (FSC) was set to 600 and side scatter (SSC) to 550. All gates and marker settings were based on unstained or single stained samples. For each sample 500,000 events were collected.

Calpain and caspase assay

Calpain and caspase-3 activities were determined in high speed supernatants, derived from the mitochondria and ER purifications. Samples were stored at 4°C until use. Protease activity was determined by the addition of 100 μL of reaction buffer (10 mM Hepes, pH 7.4, 20% glycerol, 5 mM DTT, and 50 μM fluorescent substrate for caspase-3 (Ac-IETD-AMC; Alexis Biochemicals, Lausen, Switzerland) or Calpain Substrate II (Suc-LY-AMC; Calbiochem, Darmstadt, Germany) to detect calpain activity) to 20 μL of the sample. The fluorescence increase was measured in a SpectraFluo Plus spectrophotometer (Tecan,

Zürich, Switzerland) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm during 1 hour of incubation at 37° at a kinetic interval of 30 seconds.

Determination of cytosolic free Ca^{2+}

Cytosolic free Ca^{2+} was determined by loading the cells with 1 μ M of the Ca^{2+} -indicator fluo-4 acetoxymethyl (AM) ester (Molecular Probes), which becomes fluorescent after binding free Ca^{2+} , in combination with Fura Red AM (Molecular Probes), which loses fluorescence after binding free Ca^{2+} , for 45 min at 37°C. After loading, the cells were washed once in HBSS, resuspended in HBSS at a concentration of 5×10^6 cells/mL and incubated overnight in the presence of the indicated stimuli and inhibitors. Afterwards, fluorescence was determined on an LSRII flow cytometer. The Fluo4 signal was collected in the Pacific Blue channel and the Fura Red signal in the Texas Red channel. The calculated ratio of the Fluo 4 signal over the Fura Red signal is indicative of the increase in free cytosolic Ca^{2+} . All values were normalized for the fluorescent ratio found in a fresh control sample of the same donor.

Statistical analysis and image processing

Graphs were drawn and statistical analysis was performed with GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego, CA, USA). The results are presented as the mean $\pm$ s.e.m., as indicated. Data were evaluated by paired, one-tailed student's *t*-test or Wilcoxon signed rank test, where indicated. The criterion for significance was $P < 0.05$ for all comparisons. Images were processed in Adobe Photoshop CS (Adobe Systems Inc., San Jose, CA, USA) and CorelDRAW 11 (Corel Corporation, Ottawa, Ontario, Canada).

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Supplemental Figure S1. Alterations in gene expression of apoptosis-related proteins during spontaneous and G-CSF-delayed neutrophil apoptosis. Neutrophils were incubated in the presence (grey lines, triangles) or absence (black lines, squares) of G-CSF (10 ng/mL), and mRNA was isolated every 6 hours for up to 18 hours. Gene expression was determined by MLPA analysis. Values were normalized for β 2-microglobulin (B2M) expression, which was found to be high and stable in both control and G-CSF-treated cells throughout the experiment. Values represent the average ($\pm$ SEM) of five independent experiments performed in duplicate. Statistical significance between control and G-CSF-treated samples was determined by Wilcoxon signed rank test. * $p < 0.05$, ** $p < 0.01$. If no significance is indicated, no significant differences were observed.

Summary, Discussion and Prospective

Summary and discussion

In this thesis, the various roles of mitochondria in neutrophil apoptosis are discussed. Neutrophil mitochondria maintain $\Delta\psi_m$, but do not contribute significantly to the cellular energy homeostasis. As demonstrated in **chapter 2**, only complex III of the respiratory chain complexes contributed significantly to $\Delta\psi_m$ in neutrophils. However, complex III displayed a severely reduced cytochrome *c* reductase activity. It appeared that complex III mainly received its electrons from the glycerol-3-phosphate shuttle via the glycolysis, instead of receiving them from complexes I and II. Thus, complex III functions to maintain the high rate of glycolysis required for neutrophil functions. Respiratory supercomplexes, required for efficient electron transfer, were almost absent in neutrophils. As a consequence, complex III in neutrophil mitochondria does not transfer electrons to complex IV via the reduction of cytochrome *c*, but rather transfers these electrons directly to molecular oxygen, producing mROS (**chapter 3**).

The consequences of mROS production in neutrophil mitochondria are further discussed in **chapter 3**. From studies with the mitochondria-targeted ROS scavenger MitoQ, it became apparent that mROS do not contribute to neutrophil apoptosis as such, but are essential signaling intermediates in neutrophil survival. TNF- α , a potent survival factor for neutrophils, induced a strong production of mROS. The pro-survival effect of TNF- α depends on mROS production, as indicated by the fact that MitoQ almost completely cancelled this effect. These findings may have important implications for inflammatory pathologies, wherein neutrophils, with a TNF- α -induced extended lifespan, damage healthy tissue. Treating patients with mitochondria targeted-ROS scavengers may alleviate the inflammatory conditions by re-inducing neutrophil apoptosis. Thus, neutrophil mitochondria may represent attractive therapeutic targets.

Patients with the mitochondrial disorder BTHS often suffer from neutropenia. In **chapter 4** we demonstrate an inverse linear correlation between annexin-V binding to the neutrophils in BTHS patients and the number of circulating neutrophils. This indicates that BTHS neutrophils might display PS in the absence of apoptosis and hence are prone for premature clearance from the circulation. Mitochondria in BTHS neutrophils display a reduced $\Delta\psi_m$, irrespective of the cell count. As a consequence, the cells produce more lactate and more mROS. We hypothesize that the reduction in $\Delta\psi_m$ reduces the ability of the mitochondria to function as buffers for Ca^{2+} when it leaks from the ER. Increased cytosolic Ca^{2+} levels in combination with increased mROS in BTHS neutrophils may inhibit the activity of the phospholipid flippase, which transports PS from the outer leaflet of the plasma membrane to the inner leaflet, while activating the scramblase, which randomly distributes the phospholipids. The ratio of scramblase activity over flippase activity is higher in neutrophils than in PBMC (**chapter 4**). Therefore, this hypothesis might explain why only the neutrophils are affected in BTHS. In addition, this hypothesis suggests another important function for mitochondria in neutrophils, *i.e.* as Ca^{2+} buffers.

During apoptosis, $\Delta\psi_m$ is lost (**chapter 5**). However, loss of $\Delta\psi_m$ alone did not trigger or accelerate apoptosis (**chapter 3**). G-CSF delayed neutrophil apoptosis but only slightly delayed the loss of $\Delta\psi_m$. In **chapters 5 and 6** we investigated the mechanisms by which G-CSF delays neutrophil apoptosis at several levels. G-CSF did not affect any of the apoptotic events upstream of mitochondrial acceleration, such as caspase-8 activation, Bid cleavage and Bax translocation. Even the release of pro-apoptotic proteins (*i.e.* Smac) was not prevented by G-CSF. However, G-CSF did prevent the activation of caspases-9 and -3, downstream from the mitochondria. We discovered that G-CSF pre-

vents the influx of extracellular Ca^{2+} during neutrophil apoptosis and hence the activation of calpains, Ca^{2+} -activated cysteine proteases. Calpains are responsible for the degradation of XIAP, and hence for the activation of caspases-9 and -3 (**chapter 5**). In addition, G-CSF increased the expression of calpastatin, the endogenous inhibitor of calpains, both at the mRNA as well as at the protein level (**chapter 6**). Thus, the regulation of Ca^{2+} homeostasis represents an important checkpoint in neutrophil apoptosis.

This function was further investigated in **chapter 7**. The focus of this chapter is on the anti-apoptotic Bcl-2 family member Bfl-1. Expression of mRNA for this protein was enhanced by G-CSF and very high in resting neutrophils, while the expression remained stable during apoptosis. However, at the protein level, Bfl-1 was only expressed during the later stages of spontaneous apoptosis in a fraction that contained both the ER and the mitochondria. G-CSF did not induce the protein expression of Bfl-1, but only delayed its degradation by calpains. It appeared that Bfl-1 expression was translationally controlled and that a pro-apoptotic stimulus was required to trigger Bfl-1 translation. On isolated organelles, Bfl-1 expression on an ER/mitochondria double-positive fraction could only be induced by activating caspases. Thus, Bfl-1 seems to act as a stress-response protein in neutrophils and, because of its spatial and temporal localization, does not prevent the activation and translocation of Bax or the formation of the permeability transition pore (PTP). Interestingly, separation of the mitochondria and the ER only seemed to occur after Bfl-1 had been degraded, while stabilization of this protein delayed this separation. These results suggest that the separation of these organelles may also represent an important checkpoint in neutrophil apoptosis, and that Bfl-1 may be involved in the inhibition of this process. In theory, mitochondria lose part of their Ca^{2+} -buffering capacity after separation from the ER, since Ca^{2+} is thought to be transferred between these organelles through direct interactions, thus Ca^{2+} -induced stress on the cell increases after separation of the mitochondria from the ER. In addition, Ca^{2+} appeared to play an important role in the loss of $\Delta\psi_m$ during apoptosis, while early caspase activation appeared to be essential for the rise in intracellular Ca^{2+} during neutrophil apoptosis. However, the exact mechanism by which Ca^{2+} influx in neutrophils is triggered during apoptosis remains to be determined.

In conclusion, neutrophil mitochondria control the life-span of neutrophils at several levels. Not mere bags of toxic proteins, these organelles have a dynamic role in neutrophil survival and death. A particularly important aspect of neutrophil mitochondria seems to be their function as Ca^{2+} buffers. Although reducing $\Delta\psi_m$ with inhibitors or uncouplers does not accelerate normal neutrophil apoptosis, because caspase activation or formation of the PTP is not accelerated either, loss of $\Delta\psi_m$ in the presence of active initiator caspases would probably be detrimental for the cell. Also, in the absence of $\Delta\psi_m$ neutrophils would be less likely to survive in a pro-inflammatory environment, when their services are needed most.

Prospective

Many questions, concerning the process of apoptosis in general and apoptosis in neutrophils in particular, remain unanswered. For example, the functional role of the pro-apoptotic proteins concealed within the mitochondria, such as Smac, Omi and AIF, has hardly been investigated. It is becoming increasingly clear that such proteins have a role outside apoptosis, but the nature of this role is still elusive¹. A similar problem exists with the Bcl-2 family of proteins. Over-expression of these proteins either inhibits or enhances apoptosis, but under physiological conditions these proteins may play completely different roles, for example in lipid transport, membrane reorganization or Ca^{2+}

homeostasis²⁻⁴. Neutrophils do not express Bcl-2, but several members of the Bcl-2 family certainly play a role in their apoptosis. Most prominent amongst these proteins in neutrophils are Mcl-1 and Bfl-1. The main function of Mcl-1 in neutrophils seems to be inhibition of Bax translocation⁵, but this thesis has demonstrated that the role of Bfl-1 in the apoptotic process is far from obvious. The function of Bax itself is also not directly clear. This protein is a main contributor to the formation of the PTP. However, it may also be involved in the normal dynamics of mitochondrial fusion and fission^{6,7}. It has been proposed that fission of the mitochondrial network protects the organelle from Ca²⁺-induced damage⁸. Such a hypothesis sheds a completely different light on the translocation of Bax to the mitochondria during increased Ca²⁺-induced stress. From the perspective of the mitochondria, a fission event mediated by Bax may be protective, rather than damaging. Only continued translocation of Bax during full-blown apoptosis may be enough for the formation of the PTP, while initial Bax translocation could simply be considered a cellular stress response.

Similar issues can be raised about the proteases involved in apoptosis. Both calpains and caspases have roles outside apoptosis. In fact, calpains are constitutively active in neutrophils and their activity is merely enhanced during apoptosis (**chapters 5 and 7** of this thesis). The targets for calpains outside apoptosis have hardly been studied, and little is known about their physiological role, but some studies suggest they may be involved in neutrophil migration^{9,10}. Diverse roles for caspases that were formerly considered apoptotic have recently been unveiled. Caspase-8, for example, seems to have a signaling function in the activation of NF- κ B after it has been activated¹¹. In this thesis, it is also shown that the activation of caspase-8 does not have to lead to apoptosis (**chapter 5**). Currently, one can only speculate about the roles of the other apoptotic caspases, such as caspase-10, outside apoptosis.

Finally, the role of the IAP family in apoptosis is also far from clear. All members of the IAP family seem to inhibit apoptosis when over-expressed. However, at physiological levels, only XIAP is a true inhibitor of apoptotic caspases¹². IAP-1 and -2 are currently thought to be involved in TNF- α receptor signaling¹³. Neuronal apoptosis inhibitory protein (NAIP), the finding member of this family, is now thought to function as an intracellular immune receptor for flagellated bacteria¹⁴. In this thesis, it was observed that XIAP is degraded in a calpain-dependent manner (**chapter 5**). It has been suggested that the fragments are degraded by the proteasome¹⁵, but it could equally be true that these fractions acquire a new, as yet unidentified, role.

Interestingly, most of the proteins involved in apoptosis have close counterparts involved in innate immunity. Apaf-1, activator of caspase-9, is a member of the Nod-like receptor (NLR) family of proteins, as is NAIP¹⁶. XIAP is a closely related protein. Most of the non-apoptotic caspases play a role in processing regulatory cytokines of the immune system or are involved in immune signaling¹⁶. It is, therefore, not surprising that immune signaling and apoptosis are such closely related processes. The TNF- α receptor family of proteins is either involved in immune activation or in conveying cell death¹⁷, all of the cytokines discussed in this thesis prepare neutrophils for immune surveillance as well as protecting the cells from apoptosis (**chapters 3, 5-7**). In addition, cellular metabolism is also closely related to innate immunity and cell death¹⁸. Most of these functions converge on the mitochondria. This makes neutrophils the ideal cells for studying these relationships. After all, the neutrophil is a central player in innate immunity, has an active apoptotic program and a peculiar metabolism with a proven role in both cell death and immune functions. For the future of research in either the field of apoptosis, innate immunity or metabolism, these relationships can not be ignored and deserve further clarification.

Figure 1. Side by side comparison of the initiation of inflammation and apoptosis. Bacterial flagelling is recognized by the leucine rich repeats (LRR) of Ipaf, which consequently unfolds. This leads to the formation of an inflammasome, activation of caspase-1 and subsequent inflammation (left panel). Cytochrome *c* (Cyt. *c*) is recognized by the WD40 repeats of Apaf, which consequently unfolds. This leads to the formation of an apoptosome, activation of caspase-9 and subsequent apoptosis (right panel).

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List of common abbreviations

List of common abbreviations

3NP	3-nitropropionate
AIF	Apoptosis inducing factor
Bcl-2	B-cell leukemia-2
BIR	Baculovirus IAP repeat
BTHS	Barth syndrome
CHX	Cycloheximide
CI3	Calpain-inhibitor 3
CNX	Calnexin
$\Delta\psi_m$	Mitochondrial membrane potential
ER	Endoplasmic reticulum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIF-1	Hypoxia inducible factor-1
IAA	Mono-iodo-acetic acid
IAP	Inhibitor of apoptosis
Mcl-1	Myeloid cell leukemia-1
MnSOD	Mn-Super-oxide dismutase
mROS	Mitochondria-derived reactive oxygen species
NF- κ B	Nuclear factor- κ B
OXPHOS	Oxidative phosphorylation
PBMC	Peripheral blood mononuclear cells
PMN	Polymorphonuclear cells
PKC- δ	Protein kinase C- δ
PTP	Premeability transition pore
ROS	Reactive oxygen species
Smac	Second mitochondrial activator of apoptosis
TNF- α	Tumor necrosis factor- α
XIAP	X-linked inhibitor of apoptosis

Samenvatting in het Nederlands

Neutrofiele granulocyten, of kortweg neutrofielen, zijn de meest voorkomende cellen van ons immuunsysteem. In het bloed van een gezond, volwassen mens treft men ongeveer 2-3 miljard van deze cellen per liter aan. Hun voornaamste taak is het opeten (fagocyteren) en doden van schadelijke micro-organismen, met name bacteriën en schimmels. Zij doen dit met behulp van een arsenaal aan schadelijke stoffen, zoals eiwitplitsende enzymen en zuurstofradicalen. Om te voorkomen dat verouderde neutrofielen schade aan de gezonde weefsels aanbrengen, leven ze maar heel kort: Ongeveer 24 uur in de circulatie en daarna nog 2-3 dagen in de weefsels. Neutrofielen zijn als het ware geprogrammeerd om aan het einde van die tijd zelfmoord te plegen, een proces dat apoptose wordt genoemd. Een cel die in apoptose gaat, breekt zijn eigen DNA af en verpakt zijn inhoud in kleine pakketjes die eenvoudig door andere cellen kunnen worden opgenomen voor hergebruik, zonder dat de inhoud van de cel vrijkomt. Dit proefschrift draait om de rol die de mitochondria spelen in het proces van neutrofiel apoptose. Mitochondria zijn kleine organen, zogenaamde ‘organellen’, die verschillende functies hebben in de cel. In **hoofdstuk 1** wordt een overzicht gegeven van de literatuur over de rol van mitochondria in apoptose, zoals die was verschenen vóór aanvang van het onderzoek dat in de rest van dit proefschrift wordt beschreven.

In de meeste cellen spelen de mitochondria een belangrijke rol bij de energievoorziening. Als cellen glucose opnemen wordt dit in het proces van de glycolyse omgezet in pyruvaat. Hierbij wordt ook een kleine hoeveelheid energie in de vorm van ATP vastgelegd. Als dit pyruvaat echter door de mitochondria verder kan worden verwerkt in de zogenaamde ademhalingsketen, waarin het volledig wordt omgezet in CO₂ en water in een zuurstofafhankelijk proces, kan er veel meer energie uit worden gewonnen. Als cellen geen beschikking hebben over zuurstof, wordt pyruvaat omgezet in melkzuur (lactaat). Op deze manier kan de glycolyse gewoon door blijven gaan en ATP produceren. Het nadeel is wel dat de afbraak van glucose zonder zuurstof relatief inefficiënt verloopt en bovendien leidt tot verzuring van het weefsel. Het opmerkelijke aan neutrofielen is dat ze wel mitochondria hebben, maar deze niet lijken te gebruiken om ATP te produceren. In plaats daarvan verwerven neutrofielen alleen energie uit de glycolyse en produceren dus relatief veel lactaat. Neutrofiel mitochondria hebben bovendien een membraanpotentiaal, aangeduid als $\Delta\psi_m$ (delta-psi-m). Dat wil zeggen dat de mitochondria binnenin, in hun matrix, negatief geladen zijn ten opzichte van de buitenkant. Normaal gesproken wordt dit effect veroorzaakt door activiteit van de ademhalingsketen, die negatief geladen elektronen, afkomstig van de afbraak van pyruvaat, transporteert en positief geladen protonen over de membraan naar buiten pompt. Drie van de vier eiwitcomplexen waaruit de ademhalingsketen bestaat (complex I, III en IV), zijn verantwoordelijk voor het pompen van protonen. Een vijfde complex, aangeduid als complex V of het F₁/F₀-ATPase, gebruikt normaal gesproken de zo ontstane protonengradiënt om ATP te produceren. In **hoofdstuk 2** van dit proefschrift wordt de vraag behandeld hoe neutrofiel mitochondria wel een $\Delta\psi_m$ in stand kunnen houden terwijl ze geen ATP produceren.

Het bleek dat in neutrofielen van de ademhalingscomplexen alleen complex II en V een relatief normale activiteit vertoonden, terwijl de activiteit van de andere complexen significant lager was dan in controlecellen. Complex II is geen protonenpomp, terwijl complex V wel protonen kan pompen maar alleen als het ATP verbruikt. Echter, bij gebruik van chemische remmers van de verschillende ademhalingscomplexen bleek dat alleen remming van complex III in neutrofielen tot een verlaagde $\Delta\psi_m$ leidde. Remming van de andere complexen had geen of weinig effect op de $\Delta\psi_m$ en geen effect op cellulaire ATP niveaus. Vooral complex III remming leidde tot verhoogde lactaatproductie door neutrofielen. Dit kon worden verklaard doordat de ademhalingscomplexen in neutrofielen niet in grotere supercomplexen waren georganiseerd. Eerder was al aangetoond dat in bijvoorbeeld hartspiercellen de

organisatie van ademhalingscomplexen in supercomplexen, waarbij de verschillende ademhalingscomplexen direct met elkaar in contact staan, leidt tot een meer efficiënte overdracht van electronen tussen de complexen. Complex III kan zijn ontvangen electronen dus minder efficiënt afstaan aan zijn substraat, cytochroom *c*, als het niet in direct contact staat met complex IV, zelfs als dat substraat in voldoende mate aanwezig is. Bovendien zijn de concentraties van zowel cytochrome *c* als complex IV in neutrofielen erg laag. Dit verklaart waarom complex III in neutrofielen minder actief lijkt (dat wil zeggen; verlaagde cytochroom *c* reductase activiteit heeft), terwijl het toch als protonenpomp kan blijven functioneren. Bovendien bleek complex III in neutrofielen zijn electronen niet van complex I en II te ontvangen, zoals in normale cellen, maar vooral vanuit de zogenaamde glycerol-3-fosfaat shuttle. Door electronen via glycerol-3-fosfaat af te geven aan complex III kan de glycolyse zichzelf aan de gang houden zonder al te veel lactaat te produceren. Dat complex III in neutrofielen vooral afhankelijk is van glycerol-3-fosfaat betekent dat de ademhalingsketen in neutrofielen vooral een rol heeft in de regulatie van de glycolyse, zonder zelf veel aan de ATP productie bij te dragen.

In **hoofdstuk 3** worden de consequenties beschreven van deze onderbroken ademhalingsketen in neutrofielen. Normaal gesproken levert complex III zijn electronen af aan complex IV, dat deze aan zuurstof koppelt en water produceert. In neutrofielen is complex IV echter vrijwel niet aanwezig en blijven de electronen dus achter in complex III. Complex III kan zijn electronen wel kwijt aan zuurstof, maar produceert daarbij geen water maar het zeer schadelijke superoxide. Dit blijkt in neutrofielen voortdurend te gebeuren. Normaal gesproken wordt verondersteld dat de productie van superoxide in de mitochondria bijdraagt aan de veroudering van de cel. Verrassend genoeg bleek dit in neutrofielen niet zo te zijn. Integendeel; de continue productie van superoxide bleek zelfs nodig voor de verlengde overleving van de cellen onder invloed van pro-inflammatoire factoren, zoals TNF- α . Deze factoren worden normaal gesproken gemaakt op de plaats van een ontsteking en dienen om afweercellen, zoals neutrofielen, aan te trekken en te activeren zodat ze hun werk kunnen doen. TNF- α bleek de productie van superoxide in neutrofiel mitochondria sterk te verhogen, terwijl MitoQ, een antioxidant dat speciaal ontwikkeld is om alleen in de mitochondria superoxide weg te vangen, niet alleen het niveau van de superoxide sterk verlaagde, maar ook het overlevingseffect dat TNF- α op neutrofielen had volledig teniet deed. De productie van superoxide in de mitochondria van neutrofielen speelt dus een belangrijke rol in het ontstekingsproces door de levensduur van deze cellen te verhogen onder invloed van pro-inflammatoire factoren.

In het volgende hoofdstuk, **hoofdstuk 4**, worden de eigenschappen van de mitochondria in neutrofielen van patiënten met Barth syndroom (BTHS) beschreven. BTHS is een zeer zeldzame mitochondriale aandoening, die zich bij sommige patiënten onder meer uit in een verlaagd aantal neutrofielen in de circulatie (neutropenie), wat tot een verhoogd infectierisico leidt. Hoewel de neutrofielen van BTHS patiënten niet sneller of eerder in apoptose gaan, vertonen ze wel een verhoogde expressie van het vetzuur phosphatidylserine (PS) in hun buitenmembraan. Normaal gesproken wordt PS actief binnenin de cel gehouden en is de expressie daarvan op de buitenmembraan een teken van apoptose, en bovendien een signaal voor andere cellen om de cel die PS laat zien op te ruimen. Het lijkt er dus op dat in patiënten met BTHS de gezonde neutrofielen eerder worden opgeruimd. Hoewel de mitochondria in BTHS neutrofielen altijd defect waren, hadden niet alle patiënten last van neutropenie. Daarbij vertoonden de andere cellen van het immuunsysteem, die dezelfde mitochondriale gebreken hadden, geen verdere defecten. Een verklaring voor dit fenomeen moet waarschijnlijk worden gezocht in de rol van mitochondria in de calciumhuishouding van de cel.

Positief geladen calciumionen (Ca^{2+}) worden in de cel opgeslagen in het endoplasmatisch reticulum (ER).

Ca^{2+} speelt een belangrijke rol in de activatie van veel processen, onder meer in het transport van PS van binnen in de cel naar buiten. Ca^{2+} lekt voortdurend uit het ER, maar wordt daarin ook weer vlug opgenomen. Het kan echter ook door de mitochondria worden opgenomen, die een groot negatief geladen compartiment in de cel vormen en daardoor als een soort buffers fungeren voor Ca^{2+} . In BTHS patiënten is echter de $\Delta\psi_m$ verlaagd en dus de bufferende werking van de mitochondria aangetast, omdat ze minder negatief geladen zijn. Het gevolg is een verhoogde Ca^{2+} concentratie in de cel. Er zijn twee enzymen verantwoordelijk voor de verdeling van PS over het celmembraan. Het ene, scramblase, verdeelt PS willekeurig over het binnen- en buitenmembraan, is traag en wordt door Ca^{2+} gestimuleerd. Het andere enzym, flippase, brengt PS alleen naar binnen, is snel, ATP afhankelijk en wordt door Ca^{2+} geremd. Als Ca^{2+} in een cel verhoogd is, kan dit dus netto tot verhoogde PS expressie in het buitenmembraan leiden, mits de scramblase-activiteit groter wordt dan de flippase-activiteit. Omdat in gezonde neutrofielen de scramblase-activiteit ten opzichte van de flippase-activiteit al hoger bleek te zijn dan in andere afweercellen, maar nog niet hoog genoeg om onder normale omstandigheden tot PS vertoning aan de buitenzijde te leiden, zal een kleine remming van flippase en activatie van scramblase in neutrofielen eerder tot PS expressie leiden dan in de andere afweercellen. Dit verklaart waarschijnlijk waarom in patiënten met BTHS alleen de neutrofielen een verhoogde PS expressie vertonen, en dus vervroegd worden geklaard.

Uit hoofdstuk 4 is de rol van mitochondria als Ca^{2+} buffers naar voren gekomen; deze rol wordt in de laatste drie hoofdstukken verder uitgediept. In **hoofdstuk 5** hebben we de neutrofielen gedurende 24 uur gevolgd terwijl ze in apoptose gingen. Bovendien hebben we het effect van de klinisch relevante groeifactor G-CSF op neutrofiel apoptose bestudeerd. Apoptose is een proces dat in een aantal duidelijk gedefinieerde stappen en in een bepaalde volgorde verloopt. Door op bepaalde tijdstippen de verschillende stappen van het proces in aan- of afwezigheid van G-CSF te onderzoeken hoopten wij een duidelijker beeld van neutrofiel apoptose te krijgen en bovendien uit te vinden in welk stadium van het proces G-CSF precies ingrijpt. Een belangrijke stap in apoptose is het vrijkomen van bepaalde eiwitten uit de mitochondria, zoals cytochroom *c* en Smac, die het proces aanzienlijk versnellen. Cytochroom *c*, een onderdeel van complex IV van de ademhalingsketen, komt nauwelijks voor in neutrofielen, maar Smac wel. Nu bleek dat G-CSF helemaal geen invloed had op de translocatie van het eiwit Bax naar de mitochondria, dat de gaten maakt waardoor Smac wordt losgelaten, of op het vrijkomen van Smac zelf. De stappen die benedenstrooms van deze evenementen plaatsvonden, met name de activatie van caspase-3, het enzym dat de laatste fase van apoptose uitvoert, werden echter wel geremd door G-CSF. Bovendien bleek G-CSF de instroom van extracellulaire Ca^{2+} tijdens neutrofiel apoptose te remmen. Ca^{2+} regelt niet alleen de PS expressie in de celmembraan, maar activeert ook de zogenaamde calpainen. Dit zijn zogenaamde proteolytische enzymen, dat wil zeggen: eiwitten die andere eiwitten specifiek in stukken knippen. Een belangrijk doelwit voor deze calpainen in neutrofielen is XIAP. Dit is een eiwit dat aan caspase-3 bindt en voorkomt dat caspase-3 geactiveerd kan worden. Normaal gesproken wordt de binding van XIAP aan caspase-3 opgeheven door Smac, dat uit de mitochondria afkomstig is, maar in neutrofielen bleek de degradatie van XIAP door calpainen ook noodzakelijk. Door de instroom van extracellulair Ca^{2+} te remmen, voorkomt G-CSF de activatie van de calpainen en dus de activatie van caspase-3 en de executie van apoptose.

In **hoofdstuk 6** hebben we een studie gedaan naar de genexpressie in neutrofielen geïsoleerd uit donoren die behandeld waren met G-CSF en dexamethasone. Deze behandeling wordt regelmatig toegepast om neutrofielen te verwerven voor transfusie. Als een patiënt een zware chemotherapie ondergaat kan het gebeuren dat de patiënt

tijdelijk neutropeen wordt. Er dreigt dan ernstig infectiegevaar. In die situatie kan een familielid van de patiënt gevraagd worden als neutrofielendonor op te treden. Om het aantal circulerende neutrofielen in de donor tijdelijk te verhogen wordt deze donor met G-CSF/dexamethasone behandeld. Daarna kan bloed afgenomen worden, waaruit dan een zogenaamd granulocytenconcentraat wordt bereid waarmee de patiënt kan worden behandeld. Uit een eerdere studie bleek dat de zo verworven neutrofielen in functie niet wezenlijk verschilden van normale neutrofielen, maar dat ze wel langer bleven leven. Uit de genexpressiestudie in hoofdstuk 6 bleek dit voornamelijk te komen doordat in de neutrofielen van behandelde donoren het gen voor calpastatin, de natuurlijke remmer van calpainen, verhoogd tot expressie kwam, dat wil zeggen dat er meer calpastatin werd aangemaakt in de cellen. Het netto effect van de behandeling met G-CSF/dexamethasone was dus wederom remming van de calpainen.

In het laatste hoofdstuk wordt een meer beperkte genexpressiestudie beschreven die we in een tijdreeks hebben uitgevoerd. Hieruit kwam het gen Bfl-1 naar voren als een ander gen waarvan de expressie door G-CSF werd verhoogd. Bfl-1 is een anti-apoptotisch lid van de Bcl-2 familie van eiwitten, waarvan Bax een pro-apoptotisch lid is. Hoewel het gen van Bfl-1 constant in hoge mate tot expressie komt in neutrofielen, bleek het eiwit alleen tijdens apoptose aanwezig te zijn. Bovendien was het niet in de hele cel aanwezig, maar exclusief in een compartiment waarin ook de mitochondria en het ER zaten. Een eerdere studie had Bfl-1 aangewezen als een mogelijke remmer van Bax, maar omdat het in neutrofielen pas aanwezig is als Bax al naar de mitochondria is getransloceerd, lijkt het die rol in neutrofielen niet te vervullen. Zeker is dat Bfl-1 in de latere stadia van apoptose door calpainen wordt afgebroken. Doordat G-CSF calpainen remt en bovendien de expressie van Bfl-1 verhoogt, werd Bfl-1 niet afgebroken in cellen die met G-CSF waren behandeld. De functie van Bfl-1 tijdens neutrofiel apoptose, is echter nog onduidelijk. Mogelijk heeft Bfl-1 iets te maken met de dissociatie van de mitochondria en het ER die tijdens neutrofiel apoptose optreedt. Deze dissociatie is belangrijk, omdat het de werking van de mitochondria als Ca^{2+} buffers vermindert. Er zal echter meer fundamentele studie naar Bfl-1 moeten worden gedaan voordat we conclusies kunnen trekken over de rol van dit eiwit tijdens neutrofiel apoptose.

Kort samengevat komt het er op neer dat mitochondria verscheidene essentiële functies vervullen tijdens neutrofiel apoptose. Hun functie is niet beperkt tot het loslaten van pro-apoptotische eiwitten, zoals oorspronkelijk werd gedacht, maar bestrijkt ook de Ca^{2+} -huishouding en superoxide productie. Bovendien spelen neutrofiel mitochondria een belangrijke rol in de regulatie van de glycolyse. Deze resultaten geven ons meer inzicht in het proces van neutrofiel apoptose en benadrukken bovendien het belang van de mitochondria in dat proces.

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