CELL DEATH AND CLEARANCE – STUDIES OF HUMAN NEUTROPHILS FROM BLOOD AND TISSUE

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Cover illustration photo: 3D rendition from confocal sections showing an apoptotic human neutrophil stained with APC-conjugated Annexin V

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Abstract:

Neutrophils are phagocytic cells that typically migrate from circulation to tissues in order to combat microbial invasion. The journey from blood to tissue involves mobilization of intracellular organelles which results in modifications of surface markers (e.g., exposure of receptors involved in adhesion, chemotaxis and phagocytosis) that render neutrophils a primed/activated phenotype distinct from that of resting blood neutrophils. Neutrophils contain a substantial arsenal of tissue destructive factors, which could be hazardous for the environment if released in an uncontrolled fashion. Therefore, neutrophil apoptosis and clearance of the dead bodies is of outmost importance and a necessity for resolution of the inflammation.

Apoptosis of neutrophils can be modulated *in vitro;* typically pro-inflammatory danger signals delay apoptosis. The acute phase protein serum amyloid A (SAA) delayed neutrophil apoptosis *in vitro*, an effect that was blocked by inhibition of the receptor P2X7. Blocking of P2X7 also inhibited prolonged survival mediated by other stimuli indicating that P2X7 is not an actual SAA receptor, but instead involved in anti-apoptotic signaling in general. Clearance of apoptotic cells can also be modulated *in vitro*, e.g., by opsonization. This was shown for Galectin-3 that increased the clearance of apoptotic neutrophils by monocyte-derived macrophages. Galectin-3 enhanced the proportion of macrophages that engulfed apoptotic cells but also the number of ingested neutrophils in each macrophage.

Apoptosis is well studied in resting neutrophils purified from peripheral blood, but how the process is modulated in tissue neutrophils is relatively unknown. We investigated the apoptotic process in tissue neutrophils from two different inflammatory settings, skin chambers on healthy subjects and synovial fluid from patients with inflammatory arthritis. Skin chamber neutrophils were totally resistant to anti-apoptotic stimulation, which was in stark contrast to neutrophils from synovial fluid that responded well to anti-apoptotic stimulation. Also, neutrophils from skin chambers showed an activated phenotype, while neutrophils from synovial fluid surprisingly displayed a phenotype similar to that of resting blood neutrophils. Thus, the tissue neutrophils in our studies behaved fundamentally different. If this means that every inflammatory setting is unique remains to be evaluated in future studies.

Key words: neutrophils, apoptosis, transmigration, phagocytes

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

I K Christenson, L Björkman, C Tängemo, and J Bylund
Serum Amyloid A inhibits apoptosis of human neutrophils via a P2X7-sensitive pathway independent of formyl peptide receptor-like 1
Journal of Leukocyte Biology (2008) 83(1):139-48

II A Karlsson*, K Christenson*, M Matlak, Å Björstad, KL Brown, E Telemo, E Salomonsson, H Leffler, and J Bylund
Galectin-3 functions as an opsonin and enhances macrophage clearance of apoptotic neutrophils
*Glycobiology (2009) 19(1):16-20. *=joint first authors*

III K Christenson, L Björkman, J Karlsson, M Sundqvist, C Movitz, DP Speert, C Dahlgren, and J Bylund
In vivo transmigrated neutrophils are resistant to anti-apoptotic stimulation
Journal of Leukocyte Biology (2011) epub ahead of print

IV K Christenson, L Björkman, C Movitz, and J Bylund
Cell death processes in neutrophils from synovial fluid
In manuscript

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ABBREVIATIONS

AIF	Apoptosis-inducing factor
Bcl-2	B-cell lymphoma-2
Caspases	Cysteine-dependent aspartate-directed proteases
CGD	Chronic granulomatous disease
CR	Complement receptor
CRP	C-reactive protein
DAMPs	Damage-associated molecular patterns
DISC	Death-inducing signaling complex
DMARD	Disease-modifying anti-rheumatic drug
GMCSF	Granulocyte macrophage colony-stimulating factor
HMGB1	High mobility group box 1
IL	Interleukin
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MOMP	Mitochondrial outer membrane permeabilization
MPO	Myeloperoxidase
MRSA	Methicillin-resistant *Staphylococcus aureus*
NSAID	Non-steroidal anti-inflammatory drug
PAMPs	Pathogen-associated molecular patterns
PGN	Peptidoglycan
PS	Phosphatidylserine
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SAA	Serum amyloid A
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand

INTRODUCTION

Apoptosis, or programmed cell death, is a fundamental cellular process necessary for fetal development and the subsequent function of all multicellular life. As a controlled "quiet" type of death, apoptosis does not disturb the surrounding and is thereby a physiological way for the body to dispose of no longer needed cells. Development of new life involves repeated removal and replacement of cells that already have out-played their role and is therefore totally dependent of functional apoptosis and subsequent clearance of the apoptotic cells. A model system in which apoptosis during development has been completely mapped is the maturation of *Caenorhabditis elegans*. In all, 131 out of 1090 cells need to undergo apoptosis before this small nematode is fully developed [1, 2]. Apoptosis is not only important during development, but also to maintain cellular homeostasis in various settings in adult organisms. Tissues are in constant need of renewal due to old age, growth or damage and most cell types can be re-produced to substitute others. Hence, a controlled death of cells that need to be replaced is of outmost importance to prevent massive cell accumulation. Insufficient apoptosis can result in a variety of human disorders, e.g., cancer, auto-immunity, and neurodegenerative diseases. Apoptosis is especially important in cells participating in the immune system of most organisms, a supremely complex system that defends our bodies from the constant threat of surrounding microorganisms. Among immune cells, the ability to enter programmed cell death is central for fine tuning of immune responses and to ascertain a well-balanced state that keep us healthy.

This thesis deals with cell death of human neutrophils, professional phagocytes belonging to the innate immune system. Neutrophils are key entities for eliminating invading microbes, but may also cause profound damage to other host cells in tissues to which they transmigrate upon local irritation. Thus, the activity and longevity of neutrophils need to be appropriately balanced. After describing cell death in general an introduction to human neutrophils and their actions will follow. Finally, the thesis will commence with specific aspects on the life and death of neutrophils in blood as opposed to tissues and how these processes affect the overall immune responses.

GENERAL ASPECTS OF APOPTOSIS

Many features of apoptotic cell death are shared by a wide variety of cells. Most important among these general attributes is that apoptosis in a controlled way enables a cell to die without releasing its intracellular constituents. This is accomplished by keeping the plasma membrane intact and impermeable to macromolecules at the same time as the cellular innards are degraded [3]. The integrity of the plasma membrane is, as will be described in detail below, supremely important with regards to immunity and immune cells, but common for most cell types is that apoptosis results in non-functional and inert cell corpses that can be removed from the system by neighboring phagocytes, a process known as clearance [4, 5]. Apoptotic cell death is also important from a recycling perspective, as useful contents, e.g., iron in red blood cells, can be reused by the phagocytes that clears the dead corpses [6]. The apoptotic process involves a number of characteristic morphological changes like nuclear condensation, cleavage and fragmentation of DNA and decomposition of the cytoskeleton [7, 8]. Even if the surface membrane remains impermeable, the apoptotic process also involves loss of membrane potential, both of mitochondrial and surface membranes [9]. Mitochondrial membrane permeabilization plays a major role in apoptotic signaling which will be described more thoroughly below.

The plasma membrane consists of a variety of lipids and alteration in surface potential will result in redistribution of the surface components. One example is phosphatidylserine (PS) which is a phospholipid normally localized on the inner leaflet of surface membranes, but during apoptosis PS becomes exposed on the outer leaflet of the membrane [10]. The exposure of PS, as well as other surface markers, functions as recognition signals to adjacent phagocytes and thus facilitates the clearance of the apoptotic cells [10, 11] (further described in a section below). Exposure of PS and internal morphological as well as biochemical changes also provides opportunities to monitor the apoptotic process in experimental settings (as described in Appendix I).

Caspases

One defining feature of the apoptotic process is an intracellular proteolytic cascade involving caspases, i.e., cysteine-dependent aspartate-directed proteases. Fourteen caspases have been identified in humans so far and at least seven of them are involved in cell death [12]. These caspases are divided into initiator caspases (caspase-2, -8, -9 and -10) and effector caspases (caspase-3, -6 and -7) [13, 14]. Caspases exist within the cells as inactive pro-enzymes (zymogens) which are either activated by proteolytic cleavage or by interaction with activating proteins and are then converted to functional proteolytic enzymes. Introduction of apoptotic signaling results in a

hierarchical cascade of activated caspases where initiator caspases cleaves and activates the downstream effector caspases which in turn initiates degradation of internal cellular parts [15]. Caspase-3 for example, has been shown to be involved in both DNA fragmentation [16, 17] and reorganization of the cytoskeleton [18]. However, even if caspases plays a major part in the apoptotic process, apoptosis may also be executed by caspase-independent proteins [19], which will be further discussed in a later section.

Initiation of apoptosis

The apoptotic process can be induced by stimulation of the intrinsic or the extrinsic pathway, i.e., either by internal factors (such as starvation or internal damage) or by external signals from other cells.

The intrinsic pathway – death from within

The intrinsic pathway is also called the Bcl-2 regulated or mitochondrial pathway because of the involvement of the B-cell lymphoma-2 (Bcl-2) family of proteins and the release of pro-apoptotic mediators from the inter-membrane space of mitochondria. The common feature for the Bcl-2 proteins is that they contain one or more Bcl-2 homology domains (BH-domains) which are needed for heterodimeric interaction between the family members. The Bcl-2 family members are divided into anti-apoptotic proteins, containing four BH-domains (e.g., Mcl-1, Bcl-x) and pro-apoptotic proteins with three or less BH domains. The latter are subdivided in two groups, Bax family members (e.g., Bax, Bak) and the so-called BH3-only proteins (e.g., Bid, Bad) [20, 21]. In viable cells, anti-apoptotic proteins inhibit pro-apoptotic Bax family members. Upon intrinsic apoptotic stimulation, BH3-only proteins liberate the Bax and Bak by binding to anti-apoptotic proteins. Some BH3-only proteins are also suggested to activate the Bax family members directly [21, 22]. Activated Bax and Bak proteins are incorporated in the mitochondrial membrane and induce mitochondrial outer membrane permeabilization (MOMP), which results in release of death-inducing proteins like cytochrome c, apoptosis-inducing factor (AIF) and Smac/DIABLO from the inter-membrane space [23]. In addition, MOMP results in loss of mitochondrial functions. Cytochrome c initiates activation of the caspase cascade via caspase-9 and to ensure caspase activation, Smac/DIABLO also neutralizes proteins with inhibitory effect on caspases [23]. MOMP does not only result in the release of caspase-activating proteins, but some of them are caspase-independent, e.g., AIF and endonuclease G [24, 25]. The AIF translocates directly to the nucleus and causes DNA fragmentation and chromatin condensation without activation of the caspase cascade [26, 27]. This means that AIF can execute its functions even if the caspase cascade is inhibited [19].

The extrinsic pathway – death from beyond

The extrinsic pathway is triggered by interaction between specific death ligands and death receptors located on the surface membranes of many cell types. Several death receptors have been characterized, e.g., TNFR1, Fas/CD95 and the receptor for TNF-related apoptosis-inducing ligand (TRAIL). These are activated by their respective ligands belonging to the TNF superfamily; TNF, FasL, and TRAIL [28]. Among these, Fas/CD95 is the best characterized and is involved in cell death of several cell types, e.g., neurons, hepatocytes and lymphocytes [29-32]. FasL can be expressed as a membrane protein on various cells but may also exist in a soluble form after cleavage by metalloproteinases [33, 34]. Death receptors consist of an extracellular part combined with a death domain in the cytoplasmic region. Death receptors ligands are often homotrimeric structures and their ligation to death receptors initiates cross-linking to other death receptors and clustering of death domains. This is followed by recruitment of adaptor proteins, e.g., FADD or TRADD, and interaction with procaspase-8 to form a death-inducing signaling complex (DISC) [35, 36]. Procaspase-8 is cleaved to active caspase-8 in this complex and functions as a central mediator of apoptosis, promoting cell death by cleavage of the downstream effector caspases, e.g., caspase-3 which in turn results in degradation of intracellular macromolecules [37-39]. Active caspase-8 also functions as a link to intrinsic signaling as it activates the BH3-only protein Bid, which triggers MOMP, cytochrome c release and the effector caspases as described above [28, 40].

THE IMMUNE RESPONSE TO DANGER

Throughout life, we are all living under constant threats from a multitude of pathogens but only in exceptional cases does this affect our health. The reason to why we are not afflicted by this diversity of possible diseases is an incredibly well-functioning immune system.

Innate and adaptive immunity

Simplified, the immune system can be divided into two over-lapping parts, the innate and the adaptive immunity. The adaptive immunity relies on gradual maturation of recognition structures (antibodies as well as receptors) that enables highly specific recognition of virtually any possible threat. The downside of this set-up is that it takes time for the adaptive immune system to learn what structures that should be recognized and to develop the specific recognition. The adaptive immunity is an incredibly complex system that evolved relatively recently. In contrast, innate immunity is a very old and evolutionarily conserved system that relies on inherited recognition structures. The innate immune system constitutes the major defense against microbes in most organisms ranging from very primitive species to highly developed mammals [41]. As our first line of defense, the innate immunity is activated by conserved danger signals stemming from microbial invasion and tissue damage. A rapid initial defense mediated through the innate immunity either clears or restricts the damage until the slower, but more specific adaptive immune system is activated.

As the cells of adaptive immunity come in touch with unknown danger molecules it takes days to weeks to produce specific antibodies directed to the foreign structures, a time-period covered by the rapidly alerted innate immune cells [42]. Cells of the adaptive immunity are generally the main driving force behind chronic inflammation in auto-immune diseases, but activated adaptive immune cells also alert the innate immune system with additional acute inflammation as a consequence. Hence, the separation of the immune system into innate and adaptive immunity is in many ways a theoretical construction; in real life the two systems overlap and presumably they partly function side by side in the bodily defense.

Initiation of innate immunity – danger signals

The keystone in the innate immunity is recognition of danger signals such as structural molecular patterns. Danger signals can be derived either from microbes, so-called pathogen-associated molecular patterns (PAMPs), or be of endogenous origin, designated damage-associated molecular patterns (DAMPs). The PAMPs consist of a diversity of conserved microbial structures with molecular patterns totally foreign for the human body (non-self). One

example of PAMPs is formylated peptides expressed by bacteria. Human peptides are not formylated and are thereby easily distinguished from the bacterial counterparts [43]. In contrast to eukaryotic cells, bacteria are also surrounded by a cell wall consisting of a variety of structures recognized as danger signals, typically by toll-like receptors (TLRs) on innate immune cells [44]. The DAMPs are self-molecules normally not exposed to innate immune cells unless as a consequence of damage and destruction; DAMPs can be nuclear or cytosolic proteins suddenly exposed to the environment by accident. One prototypic example of a DAMP is the DNA-binding protein High mobility group box 1 (HMGB1) that binds to a pattern recognition receptor known as RAGE. Several other RAGE agonists like AGE, members of the S100 family and amyloid-β also function as DAMPs [45].

Recognition of danger signals and inflammatory responses

Macrophages and dendritic cells are the sentinels of the innate immunity, being resident in tissues all over the body and well equipped to recognize both PAMPs and DAMPs. The spreading of sentinels is beneficial, since regardless of where intruding microbes enter the body, or tissue damage occurs, it will be detected by sentinels in the vicinity. Recognition of danger signals results in release of signaling molecules which initiate an acute inflammatory event at the damaged site including accumulation of innate effector cells.

Inflammation is characterized by five cardinal symptoms; heat, redness, swelling, pain and loss of function. Inflammatory mediators such as prostaglandins, leukotrienes and thromboxanes, transformed from arachidonic acid, are partly responsible for these symptoms, inducing a relaxing effect on the blood vessels which causes increased blood flow and facilitates the permeabilization of micro-vessels that are branched at the affected site [46]. Prostaglandins are also involved in causing pain, together with released cytokines and cell accumulation, and a cause of fever [42]. The acute inflammatory process not only takes place locally but may also result in systemic effects, most notably the acute phase reaction, i.e., *de novo* synthesis of acute phase proteins in the liver. A typical sign of ongoing inflammation is enhanced concentrations of acute phase proteins, e.g., complement factors, C-reactive protein (CRP) and serum amyloid A (SAA) in circulation. The complement system is important for the innate immune defense through a variety of mechanisms, both in circulation and in tissues. It has antimicrobial action, but also provides complement factors that function as chemoattractants or opsonins. Measurement of CRP concentration in blood is frequently used in hospitals as a clinical marker, since increased levels of this protein implicate bacterial infection or ongoing inflammation. Also increased levels of SAA are used in diagnostics of inflammatory diseases; suggested roles of SAA in inflammation will be described in a later section.

LIFE OF NEUTROPHILS

As stated above, acute inflammation involves local accumulation of innate immune effector cells, most notably neutrophils around which this thesis is focused. Neutrophils are professional phagocytes that engulf both microbes and damaged cells and destroy them using a rich weaponry of intracellulary stored toxins and enzymes (described in more detail below). Neutrophils are the most abundant of leukocytes in circulation where they directly clear microbes that may have entered the blood stream. Therefore, minor cuts and wounds very seldom result in sepsis (infection of the blood) and systemic inflammation. However, microbial invasion typically takes place, not in blood, but in other tissues where a local acute inflammatory response is triggered. Circulating blood neutrophils are very swiftly alerted by the local inflammation and can infiltrate an inflammatory focus within hours to clear the site from unwelcome objects [42]. Compared to other cell types, neutrophils are very short-lived and they are pre-programmed to die by apoptosis after fulfillment of their functions as phagocytes. The life and functions (Fig. 1), but above all the death of neutrophils will be thoroughly described in the following sections.

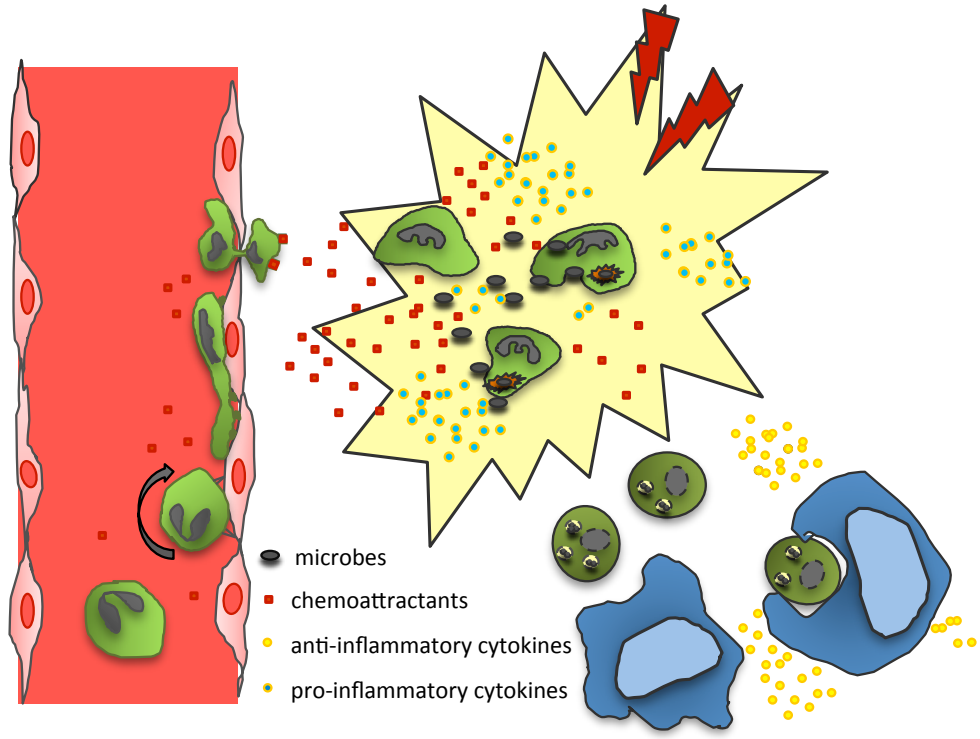

Figure 1: Life and death of neutrophils. Neutrophils circulate in the blood vessels in a resting state. Pro-inflammatory cytokines and chemoattractants activate both endothelium and neutrophils to upregulate surface molecules needed for attachment to and rolling along the endothelial lining. More firm adhesion is followed by transmigration towards a chemotactic gradient. In the tissue, neutrophils ingest and destroy microbes and cell debris after which they die by apoptosis and are rapidly cleared by macrophages. Clearance is accompanied by release of anti-inflammatory cytokines that participate in resolution of inflammation.

Neutrophil physiology and degranulation

Neutrophils differentiate from myeloid stem cells in the bone marrow and after complete maturation they are released to the circulation [47]. In contrast to most other cell types, *de novo* synthesis of proteins occurs rather sparsely in neutrophils [48, 49]. Instead the neutrophil cytoplasm is filled with different granules and vesicles, i.e., intracellular storage organelles containing a variety of molecules needed at different time points and stages of the neutrophil life. These different intracellular organelles are formed in a very specific order during cellular maturation in the bone marrow; the azurophil granules are formed first followed by the specific granules, the gelatinase granules and finally the secretory vesicles, the latter are formed through endocytosis of the plasma membrane. During formation the granules are packed with a wide range of newly synthesized proteins, e.g., components required for microbial killing or surface structures such as adhesion molecules like complement receptors (CR)-1 and -3, chemotactic and phagocytic receptors.

Upon cell activation, granules are mobilized to the cell surface, or to intracellular compartments, in opposite order of formation, i.e., the last formed granules are the first and most readily mobilized [47, 50]. The secretory vesicles and the gelatinase granules are primarily mobilized to the plasma membrane; upregulation of phagocytic receptors in this way renders neutrophils optimally prepared, or primed, for subsequent antimicrobial action. The specific- and particularly the azurophil granules instead fuse with internal organelles (typically the phagosome) and only rarely with the cell surface.

Once the granules have been mobilized they cannot be re-generated [50], hence degranulation is a one way road to cellular alteration. The separation of proteins into different compartments is beneficial as molecules of need can be rapidly mobilized without time-consuming regulation at the gene level. It also serves a protective function as granular proteolytic proteins can be of potential danger to adjacent cells if exposed at an improper occasion. After maturation in the bone marrow, the non-activated neutrophils are released to the blood system and circulate there in a resting state, until they are reached by activating alarm signals from tissue.

The journey from blood to tissue

Danger signals from invading pathogens or damaged cells are very quickly detected by sentinels in tissue that release cytokines and chemokines to alert effector cells in circulation. These alarm signals primarily activate endothelial cells to upregulate new surface markers, e.g., E- and P-selectins on the apical side of their surface [51, 52]. The endothelium also secretes chemokines to

activate leukocytes passing in the blood and the neutrophils will respond rapidly to this invite.

Shedding of L-selectin and upregulation of complement receptors

The L-selectin expressed on the neutrophil surface mediates a loose tethering to selectins and glycoreceptors on the endothelium, making it possible for the neutrophils to start rolling along the vessel lining [52]. Interaction with the endothelium also results in fusion of the easily mobilized secretory vesicles to the neutrophil surface, thereby exposing new surface receptors, e.g., CR1 and CR3. Such changes in surface composition concomitant with the shedding of L-selectin from the cell surface facilitate firm adhesion to endothelial cells and typically precede extravasation [53, 54]. The shedding of L-selectin is not directly dependent on degranulation, but rather executed by activation of proteinases such as the metalloproteinase ADAM-17 [55].

Chemotaxis

During the subsequent migration through the vessel lining, neutrophils are further degranulated and some proteolytic proteins (e.g., gelatinase) are released that facilitate the movement in tissue by degradation of the matrix [50]. Migration from blood to tissue is orchestrated by chemoattractants which interact with mobilized receptors on the neutrophil surface [56]. The chemoattractants form gradients with increasing concentrations in the vicinity of the inflammatory focus, directing neutrophils towards this site. One group of powerful chemoattractants are the PAMPs formylated peptides (described above) which interacts with mobilized chemotactic receptors on the activated neutrophils [57].

Hence, the transmigration from blood to tissue typically requires activation and priming of the neutrophils by granule mobilization and subsequent release of a variety of granule proteins and altered surface composition. This means that the transmigrated neutrophils in many ways are distinct, phenotypically as well as functionally, from neutrophils left in circulation.

Microbial killing and collateral tissue damage

Neutrophils have several functions and all of them lead in the same direction, to protect the body from dangerous objects of either microbial or endogenous origin. As described above, neutrophils are primed when reaching the inflammatory site, all ready to deal with invading threats.

Phagocytic uptake

Neutrophils are phagocytic cells and interact with their prey using a variety of phagocytic receptors. The binding can be direct or facilitated by opsonization, i.e., coating of the prey by, e.g., complement factors or antibodies [42]. After

physical connection, neutrophils engulf their prey by protruding their surface membrane around the prey which finally is enclosed in a plasma membrane-derived phagosome [58]. The phagosome will then mature by fusing with specific and azurophil granules forming a phagolysosome.

Reactive oxygen species – potent antimicrobial molecules

An important neutrophil weapon against microbes is the production of toxic reactive oxygen species (ROS). The ROS production is carried out by an enzyme complex, the NADPH-oxidase, which consists of membrane bound proteins as well as cytoplasmic components [59]. The NADPH-oxidase transports electrons over the membranes to reduce molecular oxygen (O_2), to superoxide anion (O_2^-) either in the extracellular milieu or in intracellular compartments, e.g., phagosomes. The O_2^- can dismutate spontaneously to form hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO), stored in azurophil granules, will after mobilization transform H_2O_2 to other reactive oxygen metabolites, e.g., hypochlorus acid (HOCl) [41, 60, 61].

The ROS are typically produced in the phagosomal membrane, directing their toxic effects (e.g., lipid peroxidation, oxidation of tyrosine residues and destruction of heme-containing molecules) towards the engulfed prey. The importance of ROS production as a part of antimicrobial defense is seen in patients suffering from chronic granulomatous disease (CGD). Patients with CGD lack a functional NADPH-oxidase which results in incapacity to produce ROS (Paper III) and recurrent severe infections [62].

Oxygen-independent microbial killing

Even if ROS production is an efficient way to kill microbes, neutrophils are also armed with other harmful substances like proteolytic enzymes and anti-microbial peptides. Examples of the latter are defensins and cathelicidin stored in azurophil and/or specific granules. These anti-microbial peptides are effective against many pathogens including bacteria, viruses and fungi. Mechanistically, anti-microbial peptides act primarily by lysing membranes, but some have also been ascribed immunomodulatory effects, e.g., functioning as chemoattractants, mediators of cytokine production and modulators of apoptosis [63]. Proteases also play an important part in neutrophil function, both by degrading the ingested prey and as proteolytic activators of inflammatory mediators, e.g., cytokines and chemokines [64].

Neutrophil accumulation is a risk for the tissue

In conclusion, neutrophils are potent phagocytic cells that destroy ingested prey with an arsenal of toxic substances and proteolytic enzymes most of which are preformed and stored in granules that fuse with the phagosome. In addition to this arsenal of stored toxic substances, neutrophils can also

produce significant amounts of IL-8 (Paper I, III and IV), a potent chemoattractant that attracts more neutrophils to the inflammatory focus. Hence, an abundance of potentially harmful and/or pro-inflammatory molecules will be at risk to be released in the tissue surrounding an inflammatory site. Therefore it is of outmost importance that neutrophils never start leaking out their innards and to avoid this, neutrophils are destined to undergo apoptosis after which they are removed from the site by other phagocytes.

DEATH OF NEUTROPHILS

Neutrophils are in general regarded as very short-lived cells with a proposed lifespan of between 10 hours [65] and 5 days [66] in circulation, i.e., there are very diverse opinions. In our lab, we often use human neutrophils separated from one day old human buffy coats. Many of our functional assays show that neutrophils can be at least 20-24 hours old and still be viable and functional in the same way as freshly prepared blood neutrophils.

As discussed above, neutrophils spend a major part of their relatively short life circulating in the blood stream, waiting to be summoned to the tissue. If no signals alert them to leave circulation, aged neutrophils will be removed from the blood vessels, most likely via uptake by the liver or spleen [65]. This probably occurs as soon as they demonstrate apoptotic features; we never find apoptotic neutrophils in freshly drawn blood. Removed neutrophils are continuously replaced by cells released from bone marrow in order to keep homeostasis [67]. However, since the most important part of neutrophil life takes place in tissue during an inflammatory event, cell death in inflamed tissues is an important and exciting topic to study, and has thus been the focus of this thesis.

Acute inflammation is accompanied by a massive mobilization and activation of neutrophils and other immune cells, and a variety of released inflammatory mediators keep the inflammation going. As described above, neutrophils store their powerful arsenal of hazardous factors, e.g., proteolytic enzymes and a variety of toxic molecules, within intracellular compartments. As phagocytes, neutrophils may also contain ingested prey that is degraded within the cell. This means that neutrophils are of potential danger not only to microbes, but also to the environment, if the cell integrity is disturbed and intracellular content are free to leak out [68], a process referred to as necrosis.

Neutrophil necrosis is thus a pro-inflammatory event (Fig. 2) that can lead to prolonged inflammation (described below), and to avoid this, the neutrophils are instead destined to die by apoptosis. As previously described, apoptosis is a type of cell death that maintains membrane integrity and thus spares the surrounding tissue from additional damage and avoids further activation of innate immunity that would otherwise be caused by leaking DAMPs. Apoptotic neutrophils are swiftly engulfed by adjacent phagocytes, typically macrophages, which release anti-inflammatory cytokines upon ingestion of the dead cells (Fig. 2), dampening inflammation and facilitating its eventual resolution [69]. As will be described in more detail below, it is important that clearance of apoptotic neutrophils is rapid, as apoptotic cells will lose membrane integrity over time and become (secondary) necrotic [70].

In conclusion, neutrophil apoptosis and clearance are central processes aimed at tipping the inflammatory balance in the direction of resolution (Fig. 2). The balance between resolution and enhancement of an inflammatory event thus depends on how neutrophils die and for how long they remain at the inflammatory site. However, neutrophil cell death, both apoptosis and necrosis, as well as clearance, are affected by the inflammatory milieu and the processes can be either enhanced or decreased depending on factors at the inflammatory site which will be further described below.

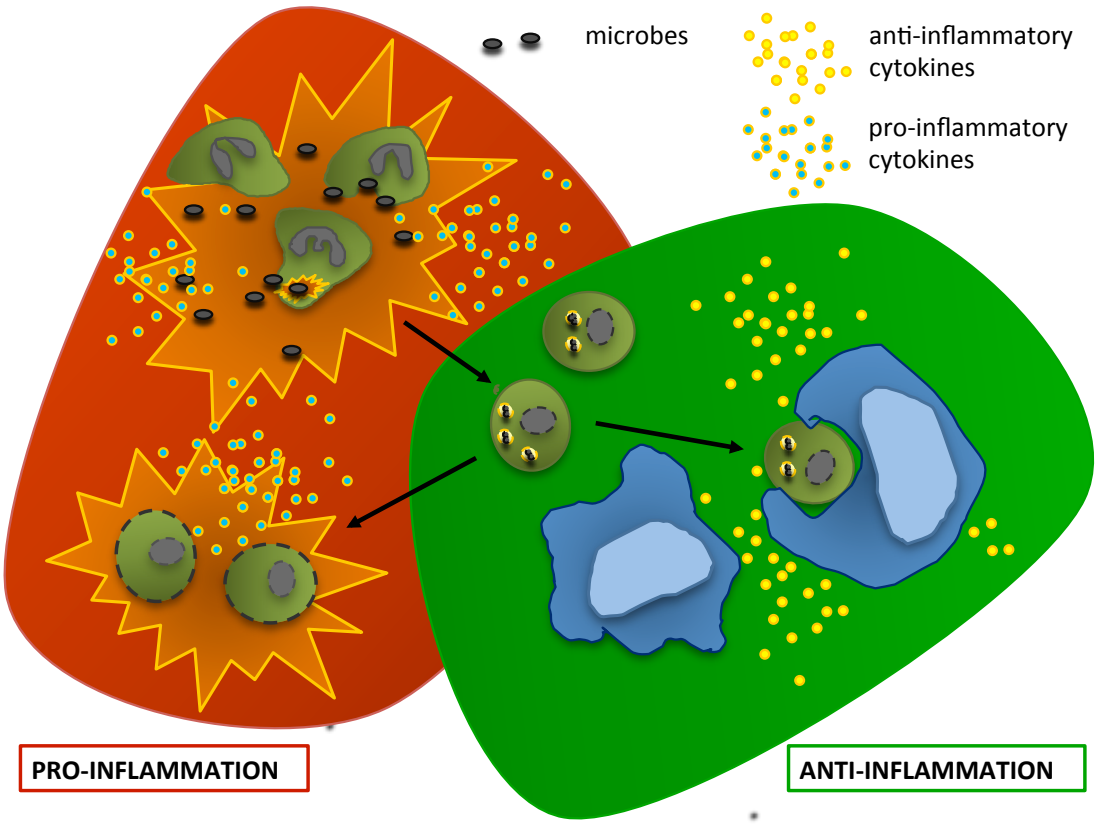

Figure 2: Cell death and clearance – denominators of the inflammatory outcome. Neutrophils exist in three major states in inflammation, viable, exerting their functions as phagocytes, or dead - either by apoptosis or necrosis. Whereas the viable and necrotic states are regarded as pro-inflammatory, apoptosis and subsequent clearance of dead corpses are anti-inflammatory events which dampen the inflammation.

Modulation of neutrophil apoptosis

Apoptosis can be induced in practically all cell types, but in contrast to most other cells, neutrophils are short-lived and destined to die by spontaneous apoptosis. Spontaneous neutrophil apoptosis occurs much like classic apoptosis (described above) with degradation of internal structures within an intact surface membrane. Apoptotic neutrophils are non-functional and no longer capable of, e.g., degranulation, ROS-production or phagocytosis, and the dead bodies are swiftly cleared by other phagocytes, e.g., macrophages in the vicinity.

Spontaneous apoptosis in neutrophils

Spontaneous apoptosis is initiated by activation of the intrinsic pathway, i.e., within the cell. Instead of Bcl-2, neutrophils express other anti-apoptotic proteins such as Mcl-1. The Mcl-1 is expressed in viable cells, but is gradually reduced during the apoptotic process [71]. Bax proteins are constitutively expressed in neutrophils, and as Mcl-1 decreases, Bax are released from Mcl-1:Bax heterodimers and become free to translocate to the mitochondrial membrane and induce MOMP [72, 73] with subsequent activation of death processes.

Neutrophil apoptosis is mainly caspase-dependent but Liu *et al* have suggested also a caspase-independent cell death for TNF-α treated neutrophils if the caspase-dependent pathway is inactivated [74]. This is consistent with the fact that a general caspase inhibitor cannot totally block spontaneous apoptosis of neutrophils (Paper III). As described above, apoptotic signaling involves release of death-inducing proteins from permeabilized mitochondrial membranes also without involvement of caspases [24], which could be a possible mechanism also in neutrophils [75, 76].

Danger signals mediate delayed apoptosis

Even if neutrophils are programmed to undergo spontaneous apoptosis, the process can be modulated, either accelerated or delayed, by a variety of factors. A diversity of PAMPs mediate prolonged neutrophil survival, i.e., act as anti-apoptotic stimuli. Some examples of anti-apoptotic PAMPs are components of bacterial cell walls and membranes, e.g., lipopolysaccharide (LPS), peptidoglycan (PGN) and lipoteichoic acid (LTA) which all mediates signaling via pattern recognizing Toll-like receptors (TLRs). *In vitro* studies have demonstrated that these microbial-derived factors have very potent anti-apoptotic effects on human blood neutrophils (Paper I, III and IV). Phagocytosis of certain bacteria has been shown to increase the life span of neutrophils *in vitro* [77], although these are exceptions since phagocytosis of microbes most often leads to enhanced cell death, as will be discussed below.

In addition to PAMPs, several factors of endogenous origin such as cytokines and chemokines have been shown to postpone neutrophil apoptosis. The cytokine GMCSF stimulates maturation and release of new granulocytes and monocytes from bone marrow, but is also present at inflammatory sites (Paper III and IV). *In vitro*, GMCSF is a very efficient anti-apoptotic stimulus for blood neutrophils [78], just as potent as bacterial-derived LPS (Paper I, III and IV). Anti-apoptotic stimulation with LPS or GMCSF results in decreased activity of caspase-3 and -7, indicating a block of caspase-activating enzymes during anti-apoptotic signaling (Paper III). Other pro-inflammatory cytokines,

e.g., IL-1β, IL-6 and INF-γ [79], and chemoattractants like IL-8 [80] and C5a [81], have also been suggested to prolong neutrophil survival *in vitro*, although the opinions are divided regarding some of these factors [78]. In our hands, neither IL-8 nor C5a show any effect on neutrophil apoptosis, and a question is if chemoattractant receptors are involved in anti-apoptotic signaling at all (Paper I).

In Paper I, we show that the acute phase protein serum amyloid A (SAA) has an anti-apoptotic effect on human neutrophils *in vitro* (Paper I). SAA has previously been shown to have a chemotactic effect, and to mediate ROS-production via a chemoattractant receptor [82-85], but in our study we ruled out the possibility for this receptor to be involved in the SAA-mediated enhancement of neutrophil survival. Instead, we found that inhibition of the surface receptor P2X7 [86] totally blocked the anti-apoptotic effect mediated by SAA suggesting that P2X7 could be an SAA receptor. However, inhibition of P2X7 also blocked prolonged survival mediated by LPS and GMCSF, which indicates that P2X7 instead is vital for anti-apoptotic signaling in general (Paper I). A saving clause regarding SAA is that the main part of all SAA studies showing biological effects (including Paper I) is performed with a recombinant hybrid form of human SAA (a combination of the isoforms SAA1 and SAA2) that is not found *in vivo*. In contrast to the recombinant hybrid molecule, endogenous SAA has been shown to be remarkably inert [87].

Accelerated neutrophil apoptosis

Acceleration of spontaneous apoptosis can be achieved by a variety of factors that activate the intrinsic pathway, e.g., internal cellular stress by serum starvation or DNA damage due to UV-radiation. The anti-Fas/CD95 antibody is also frequently used to enhance neutrophil apoptosis *in vitro* by mimicking the binding of Fas ligand to its receptor (Paper I, II, III, IV and Appendix I). As described above, activation of the Fas receptor involves cross-linking of death receptors and the formation of DISC that includes procaspase-8. Subsequently, caspase-8 mediates cleavage of downstream effector caspases [37-39]. This is consistent with our data, showing that stimulation with anti-Fas/CD95 antibody results in increased activation of caspase-3 and -7 (Paper III). Another death receptor ligand, TNF-α, has been shown to have divergent effects on neutrophil life-span; while low concentrations delay apoptosis, higher concentrations instead induce apoptosis [88].

Phagocytosis of a variety of bacteria enhances neutrophil apoptosis [89]. The mechanisms found responsible for accelerated apoptosis after phagocytosis are several. However, worthy of specific attention is the finding that elevated neutrophil apoptosis after phagocytosis is dependent on ROS production, conclusions mainly drawn from experiments performed in the presence of

NADPH-oxidase inhibitors that do not affect spontaneous apoptosis [90-92]. As mentioned, ROS-production is one of the most powerful weapons in the neutrophil defense against engulfed microbes, and known to induce apoptosis in other cell types [93]. ROS have also been suggested to be direct mediators of spontaneous apoptosis in neutrophils, a conclusion mainly stemming from studies showing decreased cell death of neutrophils from patients with CGD, which lacks the capacity to form ROS (Paper III; [94, 95]. However, experiments with CGD neutrophils that readily undergo apoptosis after phagocytosis of certain microbes contradict the notion that phagocytosis-induced apoptosis needs to be ROS-mediated [95-97].

Clearance of apoptotic neutrophils

Since apoptotic neutrophils will eventually become leaky and disintegrate, apoptosis of these cells would be totally pointless if they were not removed from the inflammatory site before entering secondary necrosis. As mentioned above, clearance is accompanied by release of anti-inflammatory cytokines, e.g., TGF-β, that suppress the action of pro-inflammatory cytokines in an autocrine/paracrine fashion [69]. Rapid and efficient clearance of apoptotic neutrophils by macrophages (Fig. 3) is therefore a vital step towards termination of inflammatory events (Fig. 1).

Recognition of apoptotic cells

It is of course important that phagocytes are able to distinguish between apoptotic and viable cells. Mix-up is avoided as the apoptotic process includes exposure of surface molecules recognized as “eat-me” signals by neighboring phagocytes. Exposure of PS on the external side of apoptotic cells [106] is such an “eat-me” signal and has been shown to function through PS binding to, and activation of, a presumptive PS-receptor on the macrophages [5]. Also a diversity of other macrophage receptors, like CD36 [107], RAGE [108] and possibly also CD14 [109], have been reported to bind PS on apoptotic neutrophils. Also scavenger receptors can be involved in the recognition and uptake of apoptotic neutrophils [110-112]. Phagocytosis of viable cells is avoided by recognizable structures on the surface of living cells. Negative regulation of phagocytosis by interaction of Sirp1α and CD47 expressed on macrophages and red blood cells, respectively, has been shown [113]. On viable neutrophils, presence of CD47 functions as a “do not eat me signal” to avoid phagocytosis by macrophages [114].

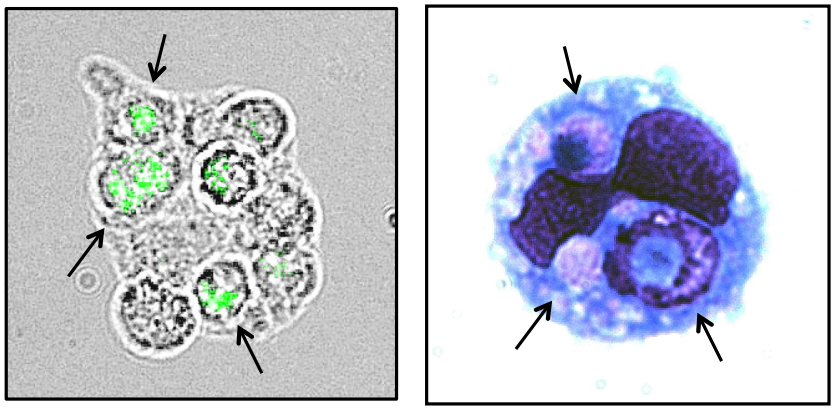

Figure 3: Macrophage clearance of apoptotic neutrophils – *in vitro* and *in vivo*. Microscopic evaluation of clearance indicates that the process appears remarkably similar *in vitro* (left) and *in vivo* (right). The *in vitro* image show clearance of galectin-3 opsonized CFDA-stained neutrophils by human monocyte-derived macrophages and the *in vivo* image is from bronchoalveolar lavage fluid (courtesy of Margaretha Smith and the Lung Immunology Group, GU). Arrows indicate engulfed neutrophils.

Modulation of clearance

Phagocytosis of apoptotic neutrophils can be facilitated by macrophage-derived ROS and we and others have shown that CGD macrophages display decreased clearance [119-122]. A variety of mechanisms by which ROS increase clearance have been suggested, but our data indicates that oxidation of macrophage receptors could at least be partially responsible [122].

An inflammatory site also contains soluble proteins that may influence apoptotic clearance. Such proteins are the complement factors, which through opsonization, i.e., coating, of apoptotic cells facilitate engulfment [123]. Even if macrophages recognize apoptotic cells by defined surface structures, opsonins facilitate the up-take of dead cells by acting as bridging molecules between the cells [124]. A novel protein with inflammatory potential, galectin-3, has been shown to be involved in several stages of inflammation, [125] e.g., neutrophil activation [126], microbial recognition and phagocytosis [127]. Galectin-3 *per se* has the potential to form pentamers and even larger protein lattices [125], and it is possible that galectin-3 can have cross-linking (opsonizing) and aggregating functions also *in vi*vo. We have shown that galectin-3 functions as an opsonin for apoptotic neutrophils, enhancing the clearance by conveying a link to monocyte-derived macrophages (Paper II). An interesting notion was that galectin-3 bound equally well to viable and apoptotic neutrophils. However, opsonisation of viable neutrophils did not result in clearance (Paper II), confirming that the exposure of apoptotic markers is still necessary for proper clearance to occur.

Neutrophil necrosis

If neutrophil apoptosis and phagocytic clearance of apoptotic cells are viewed as a quiescent, physiological way of getting rid of neutrophils and to terminate the inflammatory process (Fig. 2), there is also a pathological type of neutrophil death, necrosis. As described above, necrosis is a violent type of cell death with a pro-inflammatory outcome mediated by release of harmful intracellular content that can pass over the membranes unhampered [70]. Apart from proteolytic enzymes capable of causing direct tissue damage, there is also leakage of DAMPs and some preformed cytokines from necrotic neutrophils that counteracts resolution of inflammation. Secondary necrosis, when apoptotic cells gradually lose membrane integrity over time, is often seen with neutrophils, not least after *in vitro* culture. The transition from an apoptotic to a necrotic state can be accelerated by ingested microbes, e.g., methicillin resistant *Staphylococcus aureus* (MRSA) [98], *Burkholderia cenocepacia* [96], or *Streptococcus pneumoniae* [99]. Apoptotic cells are also susceptible to soluble factors; *in vitro* studies show that membranes of apoptotic neutrophils are swiftly permeabilized by the human cathelicidin LL-37, whereas viable cells are not affected [68, 100, 101]. The LL-37 effects on apoptotic membranes are independent of receptor-ligand interactions, but the exact mechanism remains elusive [68]. We have recently found that PSMα2, a peptide derived from community-associated MRSA [102] also permeabilizes membranes of apoptotic neutrophils selectively (Forsman H. *et al*. submitted), indicating that the ability is not unique for LL-37.

In addition to the secondary necrotic process affecting apoptotic cells, viable neutrophils may also become necrotic directly due to physical damage [103], and a variety of microbial toxins [104, 105].

Failure to recognize PS on apoptotic cells, can lead to accumulation of cell corpses and secondary necrosis followed by augmented inflammation and ultimately development of auto-immunity [115]. Along these lines, the auto-immune disorder Systemic Lupus Erythematosus (SLE) is accompanied by impaired clearance of apoptotic and necrotic cells that triggers the adaptive immune defense to react against these dead neutrophils [116]. SLE is a multi-systemic disorder characterized by hyper-activated B-cells producing anti-bodies against auto-antigens presented by T-cells. Many of these auto-antigens are suggested to stem from lingering necrotic neutrophils [116-118].

THE STUDY OF HUMAN TISSUE NEUTROPHILS

Human neutrophils are fairly well studied, both regarding functions and cell death, but a major part of all *in vitro* studies is performed on neutrophils isolated from peripheral blood. This is of course due to the fact that standardized protocols are in use that enables purification of high numbers of neutrophils from limited volumes of peripheral blood. However, since many crucial neutrophil processes take place after cells have left the blood stream there is an unfortunate lack of data on neutrophils that have been collected from tissues.

As described above, the residing dogma states that neutrophils need to be primed and activated in order to leave the blood vessels and migrate to the inflammatory site [53, 54]. Such changes, e.g., exposure of necessary receptors for attachment and chemotaxis, are partly due to mobilization of new granule proteins to the cell surface. Activation/degranulation is a one-way process with no possibility to re-generate the granules that have been mobilized or to replace the L-selectin that has been shed off. This leaves the activated neutrophils with a phenotypically different appearance compared to the resting cells. Priming can be partly mimicked *in vitro* by addition of priming factors like TNF-α or low concentrations of chemoattractants to blood neutrophils which induce an activated phenotype that lacks of L-selectin and shows enhanced expression of CR1 and CR3 on the surface (Paper IV). The transmigration process can be studied *in vitro*, where blood neutrophils are allowed to migrate over endothelial or epithelial cell layers or artificial membranes. Such studies demonstrate that transmigration indeed results in a primed/activated state as the cells lack L-selectin (unpublished data), has increased phagocytic capacity [128] and show prolonged survival [129, 130]. Although it needs to be pointed out that even if *in vitro* priming by TNF-α or models of transmigration are useful tools, the results are far from identical to those obtained with *in vivo* transmigrated neutrophils [131]. So, to fully evaluate the capacity, functions and death of neutrophils, it is important to also direct interest to neutrophils isolated from tissues.

Isolation of tissue neutrophils is associated with many experimental considerations and technical limitations. Microbial infections in tissue is naturally an important cause for neutrophil assembly and limited numbers of cells have been collected from a number of human sources in association with infection, e.g., saliva [132], cerebrospinal fluid [133], urine (our unpublished data) or bronchoalveolar lavage (BAL) fluid [134, 135]. However, these approaches are not only limited by inadequate cell yields, but also by a variety of confounding factors such as the presence of microbes which makes the situation very complex. To avoid involvement of microbes, we have chosen to

employ two different aseptic methods for the study of tissue neutrophils, a skin chamber model (Paper III) and aspiration of synovial fluid from patients with inflammatory arthritis (Paper IV). An advantage with these methods, in addition to the aseptic environment, is the possibility to isolate neutrophils from peripheral blood as well as tissue, from the same subjects. As will be described below, these methods often gives abundant neutrophil populations of relatively high purity. Studying these two types of transmigrated neutrophils have shown that tissue neutrophils may be phenotypically very different, depending on the model used.

The skin chamber model

An aseptic way to receive *in vivo* transmigrated human neutrophils is to generate a local skin inflammation, e.g., on the forearm, from where cells can later be collected. This can be achieved by a previously described skin chamber model [136]. Using this technique, skin blisters are created by application of negative pressure, lifting the epidermis from the underlying tissue. After removal of the blister roofs, chambers are adjusted over the non-bleeding lesions and autologous serum is added to the chamber wells (Fig. 4). Serum proteins and factors released from cells that are in contact with the skin lesion will create a chemotactic gradient which induces cells to leave the circulation and transmigrate into the skin chamber fluid. This method attracts different types of leukocytes; Kuhns *et al.* show that mononuclear cells predominate during the first eight hours, after which they are outnumbered by increasing amounts of neutrophils up to twenty-four hours [137]. When collecting the cells from the skin chambers after 20 hours, yields are between 5-30 million cells with a purity of >85% neutrophils (Paper III). Thus, the skin chamber model offers a controlled acute inflammatory event where cells can be collected at a fixed time-point.

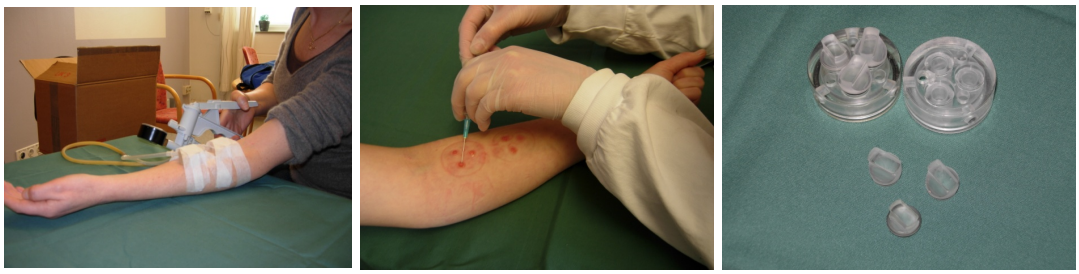

Figure 4: Skin chamber model. Skin blisters are generated on the volar part of the forearm by negative pressure (left). The blister roofs are removed (middle) and the non-bleeding skin lesions are covered by new chambers (right) that are filled with autologous serum. Chambers are kept for 20 hours after which neutrophils are collected.

Skin chamber neutrophils have a primed/activated phenotype

Transmigrated neutrophils acquired from skin chambers have been well studied regarding activation and functional status. In comparison to neutrophils from blood, skin chamber neutrophils have a primed phenotype as discussed above, lacking L-selectin and expressing enhanced levels of CR1 and CR3, indicating complete mobilization of secretory vesicles (Paper III and [138]). Presence of the granule proteins gelatinase, lactoferrin and MPO in chamber fluid has been observed, showing also partial mobilization of gelatinase, specific and to a minor extent, azurophil granules [138]. These studies thus confirm that neutrophils degranulate as they migrate to tissue. In Paper III, we show that freshly isolated skin chamber neutrophils contained increased levels of IL-8 compared to neutrophils isolated from blood. The level of this pro-inflammatory cytokine was further enhanced in response to TLR agonists (Paper III). The cell-free skin chamber fluid has been investigated and compared to sera regarding cytokine content and are shown to contain elevated levels of pro-inflammatory cytokines, e.g., IL-8, IL-6, TNF-α (Paper III and [137]).

Skin chamber neutrophils have also been shown to be primed with respect to the function of the NADPH-oxidase and respond with enhanced ROS production to various stimuli, e.g., a formylated peptide [139, 140], and galectin-3 [126]. Both C5a and IL-8 mediate ROS-production in *in vitro*-primed blood neutrophils. However, this does not hold true for skin chamber neutrophils, probably due to the abundance of these factors in the chamber fluid [139]. Hence, previous interaction with these chemoattractants during transmigration to the skin chambers may desensitize the cells to these agonists due to temporary occlusion of signaling pathways or internalization of the occupied receptors [139, 140]. These findings indicate that C5a and IL-8 are, at least in part, responsible for attracting neutrophils to the skin chambers.

Apoptosis of skin chamber neutrophils differs from that of blood cells

Thus, the functions of skin chamber neutrophils have been rather well investigated, but how these cells behave regarding cell death processes have been over-looked. We therefore set out to evaluate the apoptotic process in transmigrated neutrophils obtained by this method (Paper III).

Although the skin chamber fluid contained a variety of pro-inflammatory cytokines, spontaneous apoptosis after overnight incubation was similar in neutrophils from skin chambers and peripheral blood, i.e., the populations of viable and apoptotic cells were approximately equal (Paper III). Neutrophils from blood are responsive to a wide range of anti-apoptotic factors as described earlier. However, the skin chamber neutrophils were totally non-

responsive to anti-apoptotic stimulation as none of the factors used, including TLR agonists and GMCSF, could enhance survival of the cells. This indicates that the transmigrated neutrophils, although not more apoptotic *per se*, were dedicated to an apoptotic pathway and that this dedication could not be reversed by anti-apoptotic stimulus. The findings could not be explained by absence of functional receptors as skin chamber neutrophils produced increased levels of IL-8 after stimulation with TLR agonists.

We also allowed neutrophils from blood to transmigrate through artificial membranes into skin chamber fluid to see if we could mimic the *in vivo* situation. However, the blood neutrophils were still able to respond to anti-apoptotic stimulation after transmigration. Thus, our study shows that tissue neutrophils are significantly different from blood neutrophils regarding regulation of cell death and that a combination of factors is needed to accomplish these changes (Paper III).

Inflamed synovial fluid

Synovial fluid from inflamed knee joints is often a very leukocyte-rich milieu. In our study synovial fluids were aspirated from patients with inflammatory arthritis, i.e., auto-immune or auto-inflammatory disorders, where the duration of the acute inflammatory event was in the range of 1-3 weeks (Paper IV). In auto-immune diseases, the adaptive immune-system is triggered and production of auto-antibodies against endogenous molecules activates immune cells to defend the body against these “danger labeled” molecules. One example of a chronic inflammatory disease where antibodies may be produced against endogenous peptides is rheumatoid arthritis (RA). RA is characterized by recurrent episodes of acute inflammation in the synovial joints that eventually leads to bone erosion [141]. On these occasions the innate immunity is triggered, seen as a major influx of neutrophils to the afflicted joints (Paper IV). Neutrophils have been ascribed a destructive role in inflammatory joint diseases, mediated by, e.g., excessive production of ROS and release of proteolytic enzymes that contribute to bone destruction [142, 143]. Thus, beside the acute inflammatory situation, inflammatory arthritis also involves an underlying chronic inflammation resulting in a complex setting with, e.g., involvement of lymphocytes. Hence, the cell types in the aspirated synovial fluid varied due to cause of inflammation and duration of the inflammatory event (Paper IV), i.e., the inflammatory settings in inflamed joints are quite diverse and complex compared to the rather controlled situation in the skin chambers.

The phenotype of synovial fluid neutrophils resembles that of blood neutrophils

In several studies investigating neutrophils collected from synovial fluid the cells are subjected to a density gradient centrifugation step after aspiration in order to exclude other leukocytes [71, 144]. In our study, this step was avoided since centrifugation through the density-regulator Ficoll can have a priming effect on neutrophils from synovial fluid (Paper IV and unpublished data). In contrast to other studies and to our surprise, we found neutrophils from synovial fluid in a rather resting state, very similar to the phenotype of blood neutrophils (Paper IV). The newly aspirated neutrophils still presented L-selectin on the cell surface and even if the cells showed increased surface expression of CR3 compared to blood neutrophils, the expression could be further enhanced after TNF-α priming, i.e., the cells were primable.

Apoptosis in synovial fluid neutrophils resembles that of blood cells

Synovial fluid has been described to be a relatively rich source of inflammatory mediators (Paper IV and [145]) and several studies have evaluated what impact cell-free synovial fluid has on neutrophil survival. Most of these studies have been performed with blood neutrophils and conclusions are in conflict; some researchers claim that synovial fluid increases neutrophils apoptosis [146], while other studies suggest the opposite [144]. The discrepancies could possibly stem from the fact that the inflamed synovia is hypoxic [147, 148], and since pro-apoptotic effects predominate under normoxia, while anti-apoptotic pathways are triggered during hypoxia [71], oxygenation of the tissue may be a factor that influences the findings on cell survival. The issue of apoptosis in synovial cells thus merits further investigation.

In Paper IV, we investigated neutrophils from synovial fluid regarding cell death processes and discussed them in comparison to the skin chamber neutrophils in Paper III. Neutrophils from synovial fluid showed increased levels of spontaneous apoptosis compared to blood neutrophils after overnight incubation, which is in contrast to the skin chamber neutrophils (Paper III). Neutrophils from synovial fluid were also fully responsive to anti-apoptotic stimulation, which was in stark contrast to the skin chamber neutrophils. The ability of synovial fluid neutrophils to decrease the apoptotic rate upon stimulation is in agreement with the only other study that has tested apoptosis in these tissue cells [149]. As described above, neutrophils from synovial fluid were surprisingly unprimed regarding surface markers and IL-8 content. This again is very different from the IL-8 rich skin chamber neutrophils (Paper IV). Hence, the two studies of tissue neutrophils (Paper III and IV) indicate that both life and death are highly dependent on the inflammatory setting at the affected site.

CONCLUDING REMARKS

As described above, apoptosis and subsequent clearance of inflammatory cells are necessities for proper resolution of the inflammatory process and any disturbances in these events may have dire consequences for the inflammatory outcome. Hence, neutrophil cell death and clearance have been thoroughly investigated in cells isolated from peripheral blood, but there is a surprising lack of published data on how these processes are regulated in tissue-derived cells. As the transmigration process involves an alteration of the cells from a resting to a primed and even activated state, it is reasonable to believe that these changes also affect the apoptotic signaling pathways. *In vitro* transmigration over an epithelial cell line has been shown to result in prolonged neutrophil survival [130]. This is consistent with a study by Theilgaard-Monch *et al.* [150] where up-regulation of anti-apoptotic genes in skin chamber neutrophils is shown, concomitant with down-regulation of genes encoding pro-apoptotic molecules. However, the transmigrated neutrophils in our studies did not follow this pattern but instead showed spontaneous apoptosis equal (skin chamber neutrophils; Paper III) or enhanced (synovial fluid neutrophils; Paper IV) compared to blood neutrophils from the same donors. This shows that neutrophil viability is not regulated only at transcriptional level but that primarily post-translational mechanisms decide over neutrophil life and death.

Neutrophils from skin chambers and synovial fluid neutrophils were markedly different in terms of how they responded to modulation of apoptosis. While neutrophils from skin chambers were completely non-responsive to anti-apoptotic stimulation, neutrophils from synovial fluid responded similar to resting blood neutrophils, i.e., with delayed apoptosis to a wide variety of stimuli (Paper III and IV). The main question is of course why these two types of transmigrated neutrophils responded so differently? In the section below, I will highlight and discuss some possible explanations to these disparities.

The background to the two inflammatory events is of course very different; in one case aseptic skin lesions on healthy individuals and in the other case erosive knee joints of patients with chronic auto-immune or auto-inflammatory disease. Accumulation of neutrophils is in both situations a result of acute inflammation, but the patients with inflammatory arthritis also have an underlying chronic inflammation. The settings of these two inflammatory events are thus quite dissimilar with different milieus containing diverse mixtures of soluble factors and cells. Many immune cells are excellent producers of both pro- and anti-inflammatory cytokines, especially lymphocytes that are central cells in chronic inflammation. The

lymphocyte count in synovial fluid varied substantially between patients, possibly due to the duration of the inflammatory event. Lymphocytes were present also in skin chambers but to a lesser degree compared to the synovial fluid. A multiplex cytokine assay performed with cell-free fluids from skin chambers or inflamed joints also indicated different composition of cytokines in the two models (Paper III and IV). The respective inflammatory milieus may of course have different effects on neutrophils regarding apoptotic signaling. We have seen that some skin chamber fluids enhance survival of blood neutrophils, while others do not affect the spontaneous apoptosis at all (unpublished data). There are also contradictory opinions about how inflamed synovial fluid affects the survival of blood neutrophils [146, 151]. In Paper I we show that the apoptosis of blood neutrophils was easily delayed with anti-apoptotic factors of different origin. However, this prolonged survival was overall blocked by inhibition of the surface receptor P2X7 (Paper I). This pattern is remarkably similar to that seen in skin chamber neutrophils, as they too were totally non-responsive to anti-apoptotic stimulation (Paper III). It may be that the inflammatory milieu in the skin chambers contains a blocking factor for P2X7, and that this factor may be lacking (or be inactive) in the synovial cavity. To evaluate the impact of the inflammatory milieu, blood neutrophils were incubated in fluid from skin chambers. Since the neutrophils collected from chambers were totally unresponsive to anti-apoptotic stimulation this could mean that also blood neutrophils might be just as unresponsive after exposure to the same milieu. However, blood neutrophils were still responsive to anti-apoptotic stimulation after this treatment (Paper III), indicating that soluble mediators at the inflamed site are not the solely determinant factors. It is still possible that P2X7 is inactivated during the actual transmigration process, as opposed to during the cell lingering in the inflamed target fluid, and that such inactivation for some reason occurs during the journey to skin chambers, but not to synovial fluid.

Another aspect to consider is that the synovial fluid is aspirated from patients with active inflammatory arthritis, while the skin chambers are used on healthy individuals. All the patients were under treatment with orally administrated anti-inflammatory drugs, and this may have impact on neutrophil signaling and function, both in blood and synovial fluid. It has previously been suggested that one of the anti-inflammatory properties of NSAIDs and DMARDs is that they enhance neutrophil apoptosis [152, 153]. However, overnight incubated blood neutrophils from patients with inflammatory arthritis and neutrophils from healthy individuals showed very similar amounts of spontaneous apoptosis, indicating that the anti-inflammatory therapy did not influence our results.

An additional difference between neutrophils from skin chambers and synovial fluid neutrophils is the duration of the inflammatory event that initially triggered the neutrophil accumulation. The skin chamber model is a controlled inflammatory situation where the cells are collected after 24 hours. Neutrophils have been shown to arrive primarily during the last twelve hours of this period [154, 155]. The duration of the inflammatory events in the knee joints can vary from one to three weeks, which makes the age of the collected neutrophils impossible to determine. Similarities to blood neutrophils might implicate that neutrophils from synovial fluid are rather newly recruited at the aspiration time. Recurrent inflammatory episodes in rheumatic disease are generally accompanied with enhanced level of leukocytes in circulation. This implicates that neutrophils in the joint can be replaced by a continual migration of new neutrophils if the turn-over rate is increased at the afflicted site.

The inflamed joint is believed to be a rather oxygen-free milieu [148] and several studies have suggested that neutrophil survival is increased under hypoxic conditions [71, 151, 156]. Increased survival while in the joint may mean that neutrophils in synovial fluid instead are generally older than the neutrophils in the skin chambers. Perhaps this could be an explanation to why the neutrophils from synovial fluid show increased spontaneous apoptosis after overnight incubation compared to their blood counterparts, while skin chamber neutrophils did not. Hypoxia, as a consequence of increased cellular consumption of existing oxygen is plausible to occur at inflammatory sites *in vivo* given the increase in cell number and activity. Possibly, the artificial inflammatory setting in skin chambers provides a less hypoxic milieu. This could contribute to the disparity in responses to anti-apoptotic stimulation between the two types of transmigrated neutrophils.

I have previously discussed the transmigration process and how it results in priming and activation of the resting blood neutrophils. A major difference between neutrophils from skin chambers and from synovial fluid is that the latter are, in every investigated aspect except spontaneous apoptosis, much more similar to resting neutrophils from blood than primed neutrophils from skin chambers (Paper III and IV). Thus, the synovial cells seem not to have undergone the same degranulation processes as the skin chamber cells, processes that are thought to be necessary for proper migration from the blood stream to the inflammatory focus. One possible explanation to this would be if the neutrophils in the synovial cavity arrived there directly from the adjacent bone marrow, and not by classic transmigration from the blood vessels. Tissue damage and erosion of cartilage and bone are connected to recurrent inflammation in the synovial joints of patients with inflammatory arthritis. Such bone destruction can result in breakage of the cortical barrier

and thereby provide a physical connection to the bone marrow [157]. In this scenario, naïve neutrophils would be free to reach the inflamed joint directly, without passing the blood vessels, explaining their rather resting phenotype.

Cells from the two types of inflamed tissues investigated in this thesis behave fundamentally different and it is possible that each and every type of inflamed tissue has its unique properties. At present, we can only state that transmigrated neutrophils from inflamed synovial fluid and skin chambers are different from one another and that modulation of the apoptotic process in transmigrated neutrophils collected from other types of tissues needs to be studied further to get a more complete picture of how aseptic inflammation is related to cell death processes. Thus, the studies presented in this thesis mirror the complexity of inflammatory milieus and how this may have an impact on the cells herein as well as, perhaps, the outcome of the inflammatory event. But is the milieu a determining factor and could conclusions regarding this milieu be drawn without taking the journey there into account? Simplified *in vitro* investigations of blood neutrophils is naturally of great value, since they provide a basic knowledge about how resting blood neutrophils responds to various inflammatory mediators, and such knowledge is needed to get a basic comprehension of the inflammatory milieu. However, is it reasonable to evaluate one inflammatory factor without the whole inflammatory context, and are responses in blood neutrophils translatable to how neutrophils from inflammatory tissues would respond to a similar treatment? These are very difficult questions to answer, but nonetheless important to ask.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Det medfödda immunförvaret består till stor del av neutrofiler som skyddar oss mot främmande mikroorganismer, t ex bakterier, virus och svampar. Neutrofilerna är de vanligaste vita blodkropparna och de cirkulerar i blodet i ett vilande tillstånd i väntan på att bli kallade till kroppens olika vävnader på grund av skada och/eller bakterieinvasion. När bakterier tar sig förbi huden eller slemhinnorna och in till kroppens vävnader, kommer de celler som finns intill att känna av dem och skicka ut alarmsignaler. När neutrofilerna i blodet nås av dessa signaler kommer de att aktiveras och mobilisera granule (intracellulära förråd av proteiner) till ytan, vilket gör dem redo att förflytta sig från blodet och ut till det skadade området för att avdöda mikroorganismer. Neutrofiler som förflyttat sig till ett skadat område är alltså aktiverade och har fått en annan fenotyp än de neutrofiler som finns kvar i blodet.

Neutrofilernas viktigaste uppgift är att fagocytera (”äta upp”) och avdöda mikroorganismer med hjälp av ett stort antal olika intracellulärt lagrade bakteriedödande substanser t ex reaktiva syreradikaler (ROS) och antimikrobiella peptider, men de fagocyterar även delar av skadade celler. Efter avslutat uppdrag är neutrofilerna programmerade att dö genom att gå i apoptos. Apoptotisk cellöd innebär att de inre delarna i cellen bryts ner, men att cellmembranet runtomkring behålls intakt. Andra fagocyterande celler som t ex makrofager som finns i närheten ”städar bort” de apoptotiska neutrofilerna genom att omsluta dem med sitt cellmembran och samtidigt frisätter de antiinflammatoriska cytokiner som bidrar till att inflammationen avslutas. De här processerna är viktiga av flera skäl eftersom de förhindrar toxiska substanser som finns i neutrofilerna från att läcka ut och skada den omgivande vävnaden, men också att stora mängder döda neutrofiler ansamlas på ett ställe vilket skulle kunna resultera i en förlängd inflammation. Neutrofil apoptos och avlägsnandet av de döda neutrofilerna är alltså en viktig del av kroppens immunförsvar mot främmande mikroorganismer.

I mitt doktorandprojekt har jag studerat hur apoptosprocessen hos neutrofiler påverkas av olika faktorer som tros vara av betydelse vid inflammatoriska händelser, men också hur makrofagers upptag av apoptotiska neutrofiler påverkas av omgivande faktorer. I de två första arbetena har jag studerat neutrofiler som separerats från blod. Apoptosen hos blodneutrofiler är väldigt lätt att modulera genom tillsats av olika faktorer som antingen skyndar på eller fördröjer apoptosen. Anti-apoptotiska faktorer kan vara proteiner som härstammar från cellväggen eller cellmembranet hos bakterier men kan också komma från den egna vävnaden t ex pro-inflammatoriska cytokiner. I de två sista arbetena har jag undersökt apoptosprocessen hos neurofiler som lämnat blodbanan på grund av lokal inflammation i två olika

typer av vävnad. I det ena fallet har vi använt en hudkammarmodell på friska försökspersoner där små hudskador i det yttre hudlagret, skapade med hjälp av en vaccumpump, resulterar i att neutrofiler vandrar ut från blodet och in i serumfyllda hudkammare som placerats på underarmens insida. I det andra fallet har vi studerat neutrofiler från syovialvätska som tappats ur inflammerade knäleder hos patienter med olika typer av inflammatoriska artriter.

Arbete 1: I samband med akut inflammation så sker också systemiska effekter t ex ökad koncentration av akutfaspoteiner i blodet. Ett exempel är serum amyloid A (SAA) som ofta ökar kraftigt i blodet vid vissa inflammatoriska tillstånd, t. ex reumatoid artrit (RA). Vi undersökte hur SAA påverkade apoptosen av humana blodneutrofiler. Förutom en rekombinant form av SAA (rSAA) använde vi också SAA framrenat ur plasma från patienter med RA och vi kunde se att båda SAA formerna hade en anti-apoptotisk effekt på neutrofiler från blod. Flera olika receptorer har visats vara inblandade i SAA-signalering, bl a FPRL1 som är en receptor som associerats med neutrofil förflyttning och frisättning av ROS. Våra resultat tydde dock på att denna receptor inte är inblandad i SAA´s påverkan på neutrofil celldöd. En annan receptor som har kopplats samman med SAA signalering är P2X7. Inhibering av P2X7 blockerade SAA´s anti-apoptotiska effekt, men den blockerade också den anti-apoptotiska effekten från andra stimuli. Detta tyder på att P2X7 inte är en SAA receptor, utan att den istället verkar ha en generell roll vid anti-apoptotisk signalering.

Arbete 2: Galektin-3 är ett sockerbindande protein vars koncentration ökar i blodet vid vissa inflammatoriska tillstånd. Proteinet består av en sockerbindande del, CRD regionen och en N-terminal del som kan binda andra galektin-3 molekyler och bilda komplex. Här undersökte vi hur galektin-3 påverkar makrofagers upptag av apoptotiska neutrofiler. Monocyter från blod odlades i närvaro av en tillväxtfaktor under en vecka för att utvecklas till makrofager och fick sedan fagocytera apoptotiska neutrofiler i närvaro eller frånvaro av galektin-3. Vi kunde se att närvaro av galektin-3 ökade både antalet fagocyterande makrofager och mängden apoptotiska neutrofiler varje makrofag tog upp. En tänkbar förklaring till den ökade fagocytosen i närvaro av galektin-3 kan vara att galektin-3 binder till cellytan hos både apoptotiska neutrofiler och makrofager och fungerar som en brygga mellan cellerna vilket underlättar upptaget.

Arbete 3: Apoptos och hur den apoptotiska processen påverkas av olika faktorer är grundligt studerad i neutrofiler separerade från humant blod. En stor del av neutrofilerna kommer dock inte att dö i blodet utan i olika typer av vävnader efter att ha transmigrerat dit för att försvara kroppen mot

mikroorganismer som tagit sig förbi hudbarriären eller slemhinnorna. Här undersökte vi därför hur apoptosprocessen kunde moduleras i neutrofiler som förflyttat sig ut från blodbanan till hudkammare som placerats på underarmens insida hos friska försökspersoner. Hudkammarneutrofilerna visade sig stämma med den gängse bilden av transmigrerade neutrofiler så till vida att de var aktiverade och uttryckte receptorer som är associerade med förflyttning från blodbanan. Dock visade det sig att de stimuli som vanligtvis används för att fördröja apoptosen hos blodneutrofiler inte alls påverkade apoptosen hos hudkammarneutrofilerna trots att de uttryckte receptorer för anti-apoptotiska faktorer. Receptorerna visade sig vara funktionella, eftersom de kunde förmedla produktion av den pro-inflammatoriska cytokinen IL-8. Den här studien visar att transmigreringsprocessen från blod till vävnad påverkar neutrofilerna på flera sätt. Resultaten tyder på att neutrofil apoptos regleras olika i blod och efter transmigrering till vävnad vilket kan vara värt att ta i beaktande vid apoptosstudier.

Arbete 4: Inflammatoriska artriter innebär perioder med ökad inflammation i olika leder och detta resulterar i massiv utvandring av neutrofiler och vätska till det skadade området. Här har vi studerat neutrofiler från ledvätska som tappats från knäleder hos patienter med olika inflammatoriska sjukdomar och jämfört dessa med blodneutrofiler från samma person. Vi jämförde också med resultaten från hudkammarstudien (arbete 3). Som jag tidigare har nämnt, så behöver neutrofiler förändras och aktiveras för att kunna förflytta sig från blodet till vävnaden och för att känna igen och avdöda bakterier. Till vår stora överraskning verkade neutrofilerna från ledvätska vara vilande, dvs de uppvisade inte de förändringar som vanligtvis är förknippade med transmigration och de verkade inte heller innehålla IL-8 som hudkammarneutrofilerna gjorde. Precis som hos neutrofiler från blod kunde apoptosen hos neutrofilerna från ledvätskan fördröjas med anti-apoptotiska faktorer. Detta är en tydlig skillnad mot de neutrofiler som transmigrerat till hudkammare, eftersom de inte alls svarade på anti-apoptotisk stimulering. De två typerna av vävnadsneutrofiler, från hudkammare och ledvätska, liknade alltså inte alls varandra, vare sig gällande aktiveringsgrad eller apoptos och dessa skillnader gör att det är svårt att dra några generella slutsatser om vävnadsneutrofiler. Kanske är det helt enkelt är så att varje inflammatorisk vävnad är unik och därmed påverkar de celler som befinner sig där på olika sätt. För att avgöra detta krävs det flera studier där man undersöker utvandrade neutrofiler i andra typer av inflammatorisk vävnad.

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