Chapter 1

Introduction
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1 Granulocytes, mediators of inflammation

The human body contains about five liters of blood, accounting for 7% of body weight. Red blood cells constitute about 45% of this volume and white blood cells around 1%, the rest being the liquid blood plasma. The white blood cells, or leukocytes, can be sub-divided into: granulocytes, monocytes and lymphocytes. The granulocytes, as their name suggests, contain many granules in their cytoplasm. They represent the largest group of leukocytes and can themselves be subdivided in neutrophils, eosinophils and basophils. Neutrophils are the most common type of granulocytes and are also called polymorph nuclear cells, due on their multi-lobed nucleus. Their main task in the human body is to phagocytose and destroy invading microorganisms especially bacteria. Eosinophils, which form the second group help to destroy invading parasites and are involved in allergic inflammatory responses. Most leukocytes function in tissues while blood acts to transport cells to where they are required. Normally, granulocytes have a short life span and undergo spontaneous apoptosis. However, upon interaction with inflammatory cytokines, their life span can be increased.

A local infection or injury in tissue rapidly attracts leukocytes to the effected region as part of the inflammatory response, helping to fight the infection or heal the wound. This complex inflammatory response is mediated by multiple inflammatory proteins, which are produced locally by a variety of cells. Once, the granulocytes have arrived at the inflammatory site, they recognize opsonized microorganism’s. They express surface receptors, allowing them to attach to a variety of microorganisms. Granulocytes ingest the infecting microorganisms by a process known as phagocytosis. The ingested bacteria are then killed by reactive oxygen species (ROS) derived from superoxide produced by an activated, phagosome bound, NADPH-dependent oxidase and proteolytic enzymes released from intracellular granules. Additionally, other proteins produced at the inflammatory site by, for example, by the granulocytes themselves, are released into the blood and stimulate the bone marrow to produce more granulocytes. This result in a positive feedback loop recruiting more immune cells to the site of inflammation. Finally, for rapid resolution of inflammation, granulocyte apoptosis and the subsequent recognition and removal of these apoptotic cells by macrophages is important. The molecular mechanisms underlying regulation of these neutrophil effector functions (described later in more detail) are complex and still not completely understood.

This efficient and aggressive response makes the granulocyte an efficient killer of, and protector against, invading pathogens. Consequently, a defect in their functionality can lead to dramatic immune failure, such as that observed in chronic granulomatous disease (CGD), a disorder of the NADPH dependent oxidase, Chediak-Higashi syndrome (caused by a granule defect) or neutrophil adhesion deficiencies. Additionally, dysregulation of one of the effector functions can result in chronic inflammation, which can cause aspecific tissue damage and improper tissue functioning. Inflammatory disorders such as Chronic Obstructive Pulmonary Disease (COPD) or Allergic Asthma are both characterized by chronic lung inflammation. COPD is correlated with neutrophils infiltrated into the lung and chronic asthma with infiltrated eosinophils, contributing to the pathogenesis of these inflammatory disorders. Correct differentiation towards mature and functional granulocytes, as well as proper and tight regulation of granulocyte effector functions, are therefore critical to eliminate invading pathogens in our body, without inducing chronic inflammation.

2 Maturation of human granulocytes

Hematopoietic progenitor cells present in the bone marrow can differentiate into lymphoid and myeloid lineages. Cells of the lymphoid lineage consist of T- and B-cells and are involved in humoral- and cellular defense. The innate immune system is composed of cells of the myeloid lineage and includes the monocytes, macrophages and granulocytes. The maintenance of normal numbers of these terminally differentiated cells is regulated by continuous self-renewal, control of proliferation and differentiation of CD34+ hematopoietic stem cells. This process is closely coordinated by expression of, cytokines and colony stimulating factors, their receptors, and transcription factors. General myelopoiesis is driven by cytokines, such as GM-CSF and IL-3. However, terminal differentiation towards the neutrophilic and eosinophilic lineage can be directed by G-CSF and IL-5 respectively. Based on
nuclear shape and formation of granules, neutrophil differentiation can be divided into several stages: myeloblast, promyelocyte, myelocyte, banded cells, which contain a banded-shaped nucleus, and segmented cells, which contain a segmented-shaped nucleus (Fig. 1). During maturation, proteolytic enzymes and membrane-bound proteins are synthesized and stored in granules. At the stage of the myeloblast or pro-myelocyte, formation of azurophilic granules occurs, which are characterized by their content of hydrolytic and anti-bacterial proteins such as elastase, defensins and myeloperoxidase (MPO) (Fig. 1). At the myelocytic stage, specific granules arise which predominantly express lactoferrin and are characterized as MPO negative. At this stage, synthesis of the fMLP receptor and CD11b (β2 integrin) is initiated and these stay present in the membranes of the granules formed at this stage of differentiation. Gelatinase positive granules belonging to the specific granules are formed around the stage of banded cells. Finally, at the segmented stage of the neutrophil the secretory vesicles are formed, of which the membranes are rich with receptors. Mature granulocytes express a large number of receptors not only on the membrane of the granules but also on the cell surface. Under normal conditions, granulocytes, especially neutrophils, have a short life span and spontaneously undergo apoptosis. However, when infection or inflammation is ongoing, the receptors expressed on the cell surface are stimulated by inflammatory mediators. This results in extension of their life span, and activation of granulocyte effector functions, which finally leads to elimination of pathogens (described in more detail in paragraph 1.5).

**Figure 1: Maturation of human granulocytes.**

Differentiation of hematopoietic stem cells towards neutrophils involves several stages. Myeloblast, myelocyte, metamyelocyte, banded cells and segmented cells. At the stage of the myeloblast the formation of azurophilic granules occurs containing the component synthesized at that stage as indicated. At the myelocyte stage specific granules are formed with lactoferrin as a component. At the metamyeloid/banded stage gelatinase positive granules are formed and finally at the last stage the segmented granules are formed. At which stage differentiation of eosinophils diverge from the neutrophilic lineage is unclear.
3 Surface receptors involved in granulocyte activation

Several classes of receptors, expressed on the surface of human granulocytes have been shown to be important for granulocyte differentiation, priming or activation. Important groups of cell surface receptors expressed by granulocytes are the cytokine receptors, the tumor necrosis factor (TNF) receptors, the G-protein coupled receptor (GPCR) family, the integrins and Fc receptors. Upon ligand binding, cytokine receptors undergo homodimerization, and TNF receptors undergo oligomerization to form trimers \(^{29}\). The GPCRs include those for formyl-peptides (fMLP), Platelet Activating Factor (PAF) and Interleukin-8 (IL-8) \(^{30};\ 31\). These receptors transduce signals through associated G-protein subunits that interact with the intracellular tail of the receptor. Integrins and Fc-receptors also represent an important group, and these single-transmembrane-domain receptors require cross-linking for activation \(^{32}\). In this thesis work is mainly focussed on the cytokine receptors and the G-protein coupled receptors.

3.1 Signaling through Cytokine receptors

Cytokines can induce proliferation, differentiation, priming and survival signals in human granulocytes by binding to their cognate receptors. The cytokine receptor family consists of (i) Growth Hormone Receptor subfamily, which form homodimers, such as the granulocyte colony stimulating factor receptor (G-CSFR), (ii) the interleukin-3 (IL-3) receptor subfamily, which form heterodimers with a common chain (\(\beta_c\)), e.g. IL-3R, IL-5R and GM-CSFR, (iii) the IL-6 receptor subfamily, which form complexes with gp130, (iv) the IL-2 receptor subfamily which form complexes with IL2R\(\gamma\), and (v) the class II cytokine receptor family which includes the IL-10 and interferon receptors \(^{29}\). Dimerization of cytokine receptors results in a conformational change, leading to receptor activation. These receptors do not contain an intrinsic kinase domain, but their activation is mediated by binding of cytoplasmic tyrosine kinases such as Src kinases (Lyn, Hck and Fes) and the Janus Kinase family (JAK) \(^{33};\ 35\). Upon cytokine binding to the receptor, JAKs can directly phosphorylate tyrosine residues in the cytoplasmic tail of the receptor \(^{36};\ 37\). These phospho-tyrosines can then recruit cytoplasmic signaling molecules containing SH2-domains to the receptor complex, such as members of the signal transducer and activator of transcription (STAT) family \(^{38}\). Other signaling molecules are activated by receptor phosphorylation, which include small GTPases such as p21Ras, and phosphatidylinositol-3-kinase (PI3K) \(^{39};\ 41\). The adaptor protein Shc docks to the phosphorylated receptor and in turn interacts with mSos, an exchange factor for p21Ras. Activated p21Ras can subsequently activate the Raf-MEK-MAPK pathway \(^{42};\ 43\). Initiation of the PI3K pathway at receptor level is not completely clear. However, it has been suggested to be dependent on the activity of Src kinases \(^{44};\ 45\). An alternative mechanism has been demonstrated for the \(\beta_c\). In this model, serine phosphorylation of the \(\beta_c\), mediated by protein kinase A, forms a docking site for the 14-3-3 adaptor protein. This association is needed for PI3K recruitment to the receptor and activation of downstream targets for PI3K \(^{46}\).

3.2 Signaling through G-protein coupled receptors

Chemoattractants and chemokines are important inflammatory mediators for granulocytes, and they activate G-protein coupled receptors (GPCRs) expressed on the cell surface \(^{47}\). Chemokines are small proteins with four conserved cysteine residues forming two essential disulphide bonds and can be divided into CXC and CC, C and CX3C chemokines. To date a large number of chemoattractant receptors have been cloned \(^{48};\ 49\). These include the fMLP and C5a receptor, sixteen CXC chemokine receptors (CXCL1-CXCL16), twenty eight CC chemokine receptors (CCL1-CCL28), two C chemokine receptor (XCL1,2) and one CX3C chemokine receptor (CX3CL) \(^{50}\). All these receptors possess seven transmembrane domains, which are characteristic of G-protein-coupled receptors.
Seven-membrane spanning or “serpentine” receptors are characterized by the association of heterotrimeric G-proteins with the cytoplasmic tail of the receptor and therefore also termed G-protein coupled receptors. These heterotrimeric G-proteins consist of an $\alpha$- and a $\beta\gamma$-subunit. Agonist binding to the receptor results in the exchange of GTP for GDP on the $G\alpha$ subunit, resulting in dissociation of $G\beta\gamma$. Twenty seven different $G\alpha$ subunits have been described, and these can be subdivided into four groups based on sequence homology and interaction with effector molecules: $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12}$. Furthermore, there are five different $G\beta$ subunits and 10 different $G\gamma$ subunits.

Chemoattractant receptors, such as the IL-8 receptor or tMLP receptor, are coupled to $G\alpha_i$ proteins, a class of G-protein that is sensitive to pertussis toxin (PTX). PTX is a bacterial toxin that specifically modifies a subset of $G\alpha$ subunits including $G\alpha_i$, but not the $G\alpha$ subunits of the $G_s$, $G_q$ or $G_{12}$ classes. Evidence exist that chemoattractant induced signaling is mediated by the $G\beta\gamma$ subunits. It has been demonstrated that the $G\beta\gamma$ subunit released from $G\alpha_i$ proteins can activate phospholipase (PLC-$\beta_2$, PLC-$\beta_3$). Indeed, purified G$\beta\gamma$ proteins can directly activate recombinant PLC-$\beta_2$ as well as PLC-$\beta_3$. In addition, PI3K activation, which is also stimulated by chemoattractants, has also been described to be activated directly by $G\beta\gamma$ subunits. Based on this, together with the finding that many responses to chemoattractants are PTX sensitive, a role for the $G\alpha_i$ and $G\beta\gamma$ has been suggested to mediate chemoattractant induced responses in human granulocytes.

4 Signaling pathways regulating granulocyte activation

The final activation state of human granulocytes is dependent on the stimulus, the receptor-type and the intracellular signaling pathways which are activated. Although a plethora of intracellular signaling molecules are activated upon stimulation of human neutrophils, it is still unclear which signal transduction pathways specifically lead to priming versus activation of these phagocytes. This section focuses on signaling “modules” activated by a wide variety of stimuli. These pathways, which include the small GTPases, MAPK, PI3K and Ca$^{2+}$/calmodulin have been suggested to play critical roles in regulating neutrophil effector functions.

4.1 Small GTPases

Small GTPases play a crucial role in transducing signals from receptors to downstream effector molecules. They can be divided into the Ras superfamily, which includes Ras, Rap1 and Ral and the Rho family, which includes RhoA, -B and -C, Rac1 and -2 and Cdc42. All GTPases cycle between inactive GDP-bound forms and active GTP-bound forms. GDP-GTP exchange and hydrolysis of GTP to GDP are catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively. Binding of GTP occurs due to the fact that GTPases have a high affinity for GTP and because GTP is in a ten times molar excess over GDP in the cytosol. GEFs enhance GTP-binding by stabilizing the nucleotide free transition state during the exchange of GDP for GTP. As suggested by their name, GTPases can themselves hydrolize the bound GTP to GDP. GAPs enhance this intrinsic GTPase activity. Several small GTPases have been implicated in regulating neutrophil function. Activation of Ras and Rap1 by both cytokines and chemoattractants has been reported in human neutrophils, while Ral is only activated upon chemoattractant stimulation via GPCRs. Ras has been shown to activate MAPK and PI3K, both of which are thought to be important in granulocyte function. Additionally, a role for Rap1 in activating the respiratory burst has been postulated, possibly by playing a role in the correct targeting or assembly of the NADPH oxidase complex. Within the Rho family, Rac2 is highly expressed in human neutrophils and is activated upon stimulation of serpentine receptors but not by stimulation with GM-CSF or TNF. Rac2 has also been shown to be present in the NADPH oxidase complex of human neutrophils where it is critical in the production of reactive oxygen species (ROS). Additionally, a role for the Rho family of GTPases in actin polymerization and migration has been demonstrated. Rac is known to regulate actin assembly in a variety of cells, resulting in cell spreading and the formation of lamellipodia. RhoA activity has been suggested to play a role in the generation of stress fibers or focal adhesions. In leukocytes however, focal adhesions and stress fibers are not formed. Additionally, RhoA has been implicated in generation of actomyosin-based contractility, via the activation of Rho kinase (ROCK) and in
human granulocytes, a role for RhoA and ROCK in the detachment of migrating granulocytes has recently been demonstrated 77.

### 4.2 Mitogen Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) form a three-component kinase module, consisting of a MAPK, an upstream MAPK kinase and a MAPK kinase kinase (Fig. 2). MAPKs can be subdivided into three families: the Extracellular Regulatory Kinases (ERKs), p38 kinases and c-Jun amino-terminal Kinases (JNKs) 78. For activation, MAPKs require phosphorylation on a tyrosine and threonine residue by MAPKKs, which are characteristically dual specificity kinases catalyzing the phosphorylation of both sites 70.

The best characterized MAPK cascade consists of Raf isoforms (MAPKKK), MEK1/2 (MAPKK) and ERK1/2 (MAPK) and is regulated by the Ras small GTPase 79. This pathway is thought to be important for growth and differentiation of various cell types. However, in terminally differentiated, non-dividing cells, such as neutrophils, this pathway may play an additional role. p38 MAPK and JNK tend to be activated by cellular stress including heat shock, osmotic shock, cytokines, anti-oxidants, UV and DNA damage. JNK is thought to be involved in mitogenic or anti-apoptotic signals, while p38 has been found to play a role in stress induced apoptosis 78. In neutrophils, LPS, fMLP, PAF and GM-CSF can all activate ERK1/2 80; 81. In general, TNFα fails to activate ERK1/2, although contradictory findings have been reported 66; 82; 83. p38 activation is also widely induced by agonist stimulation of the various receptor classes, although GM-CSF induced p38 activity is minimal 82. JNK has been demonstrated to be activated by GM-CSF, IL-3, IL-5 and TNFα in human and mouse hematopoietic cell lines 80. However, stimulation of JNK activity in human granulocytes has so far not been demonstrated 81; 82.

### 4.3 Phosphatidylinositol 3-kinase

Another signal transduction pathway playing a critical role in both the priming and activation of human neutrophils involves a lipid kinase, phosphatidylinositol 3-kinase (PI3K). This enzyme phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (P1), P(4)phosphate and P(4,5)diphosphate. PI3K consist of two subunits, a catalytic subunit and an adaptor subunit and can be divided into several classes. Class I PI3K can be subdivided into two groups according to the associated subunits 84. Class 1A PI3Ks (p110α/β) associate with adaptor subunits containing Src homology-2 (SH2) domains and are activated by binding to phosphorylated tyrosine residues. Class 1B PI3Ks (p110y) however, are stimulated by G-protein βγ subunits, and do not interact with the SH2-domain-containing adaptors, but associate with the adaptor molecule p101 85 (Fig. 3A). It has been demonstrated that GM-CSF, PAF and fMLP all stimulate PI3K activity 66; 86. Interestingly, in neutrophils, GPCRs are capable of activating both, Class 1A and 1B 56; 87; 88. PI3Ks themselves can regulate a diverse group of downstream serine/threonine kinases including PKC isoforms, p70 S6 kinase and Protein Kinase B (PKB) 89. Phosphorylation of P(4)P or P(4,5)P2 by PI3K leads to formation of P(3,4)P2 and P(3,4,5)P3. These lipid products form docking sites for

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Figure 2: MAPK regulated pathway

<table>
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<tr>
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<th>Stress</th>
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<td>ERK1/2</td>
<td>p38</td>
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proteins containing a PH domain. PKB was one of first the proteins identified as containing a PH domain, and a firm link between PI3K and PKB activation has been established. Activation of PI3K results in PKB translocation to the membrane (Fig. 3A). However for full activation, PKB needs additional phosphorylation on threonine 308, localized in the kinase domain and on serine 473 in the C-terminal regulatory region of PKB (Fig. 3B). Phosphoinositide-dependent kinase-1 (PDK1) was identified by its ability to phosphorylate PKB at threonine 308 in vitro in the presence of Pi(3,4)P2 or Pi(3,4,5)P3. Integrin linked kinase (ILK) has been shown to phosphorylate PKB on Ser 473 in vitro and when over expressed in cells. However, other studies have indicated that ILK may not directly phosphorylate PKB at Ser473. Other findings have shown that PDK1 can interact with a region of the C-terminus of the protein kinase C-related kinase-2 (PRK2), termed the PDK1-interacting fragment (PIF). This interaction converts PDK1 from an enzyme that phosphorylates PKB on only Thr308, to a kinase that phosphorylates both Thr308 and Ser473 of PKB. However, a more recent report has suggested that the PIF-binding pocket in PDK1 is not actually required for PKB phosphorylation, thus it remains unclear which kinase is responsible for Ser473 phosphorylation in vivo. Additionally, agonists that increase intracellular Ca2+ levels have been reported to activate PKB in a PI3K-dependent manner. PKB phosphorylation was reported to be mediated by Ca2+/calmodulin dependent kinase (CaMKK) on threonine 308. PKB activity correlates with survival signals in many cell types, and it has been shown that it can inactivate pro-apoptotic molecules, such as glycogen synthase kinase 3 (GSK-3) and forkhead transcription factors. In human neutrophils, activation of PI3K and PKB can occur upon stimulation of almost all cytokines, chemoattractants and chemokines and a role for PI3K in the neutrophil effector functions has been proposed in many studies.

Figure 3: PI3K regulated pathway

Activation of PI3K can occur via activation of serpentine receptors or cytokine receptors. In general PI3Kγ can be activated by GPCRs via the βγ subunit of G-protein. PI3Kα can be activated by upon activation of cytokine receptors. Activated PI3K phosphorylates lipids in the membrane thereby creating a docking site for PH domain containing proteins such as PKB (A). For full activation PKB needs to be phosphorylated on Threonine 308 and Serine 473. Ser 473 is phosphorylated by PDK1 which is similarly recruited to the membrane. Phosphorylation of Thr 308 possibly occurs via the PIF domain of PRK2 generating a PDK1 which can phosphorylate both sites or directly via CaMKK or ILK (B).
**4.4 Ca\(^{2+}\), calmodulin and Ca\(^{2+}\)-Calmodulin dependent kinases**

In resting cells, the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{i}\)]) is kept low (approximately 10-100 nM). It is transported out of the cytosol by Ca\(^{2+}\)-ATPases in the plasma membrane or in the intracellular stores such as the endoplasmatic reticulum. Upon stimulation of G-protein-coupled receptors by agonists such as fMLP, IL-8, C5a and PAF, or engagement of integrins and Fc-receptors, activation of phospholipase-C (PLC)β or PLCγ occurs. PLCβ and PLCγ catalyze the conversion of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)). DAG activates protein kinase C, and IP\(_3\) induces Ca\(^{2+}\) release from stores by binding to IP\(_3\) receptors. The fast release from the internal stores is followed by a later entry of extracellular Ca\(^{2+}\) across the cell plasma-membrane. Both, duration and localization of changes in [Ca\(^{2+}\)], and the intracellular distribution of Ca\(^{2+}\) effector proteins can determine the effect of Ca\(^{2+}\) on cellular functions.

The effects induced by changes in [Ca\(^{2+}\)] are mediated by EF-hand motif containing proteins, such as calmodulin. Calmodulin is a 17 kDa, ubiquitously expressed molecule which undergoes a conformational change upon binding of four Ca\(^{2+}\) ions. Ca\(^{2+}\)-bound calmodulin targets downstream effector molecules by binding to their calmodulin binding domain (CBD) and inducing activation of these targets (Fig. 4A). An important group of Ca\(^{2+}\)/Calmodulin-dependent proteins are the CaM kinases, which include CaMKI, II, IV and CaMKK. As well as the CBD, CaMKs contain an autoinhibitory domain (AID). In an inactive state, the autoinhibitory domain functions as a pseudo-substrate, interacting with the more N-terminal kinase domain. Upon calmodulin binding, the kinase undergoes a conformational change, whereby the interaction of the AID with the kinase domain is lost (Fig. 4B). CaMKII is the best studied member of the CaMK family. It is ubiquitously expressed, and has been reported to phosphorylate a variety of substrates. Four isoforms of CaMKII exist, as well as splice variants of CaMKII α, β, γ and δ. The γ and δ isoforms are expressed in most cells whereas the α and β isoforms are predominantly expressed in neural tissue, where they regulate long term potentiation and neurotransmitter release. CaMKII is an oligomeric protein consisting of twelve subunits. Its activation occurs by Ca\(^{2+}\)/calmodulin binding, and is followed by immediate autophosphorylation on threonine 286 and 306 by intra- and inter-subunit catalysis. This autophosphorylation decreases the dissociation rate for Ca\(^{2+}\)/calmodulin when the intracellular Ca\(^{2+}\) concentration diminishes. Furthermore, even after dissociation of Ca\(^{2+}\)/calmodulin, CaMKII stays partially active, resulting in a prolonged response to transient elevation of intracellular Ca\(^{2+}\) levels. CaMKII is localized in both the nucleus and the cytosol. In the nucleus, it has been suggested to play a role in regulation of gene transcription by phosphorylation the transcription factor CREB.

CaMKI and CaMKIV are closely related monomeric protein kinases, but their distribution in tissues and cells is different. CaMKI is cytosolic and is ubiquitously expressed. CaMKIV is predominantly present in the nucleus and is expressed in neural tissue, T-cells and testis. Both are activated by binding of Ca\(^{2+}\)/calmodulin and their activity is enhanced after phosphorylation by CaMKK (Fig. 5). CaMKIV becomes additionally autophosphorylated on Ser-12 and Ser-13. CaMKIV has been suggested to stimulate gene transcription by direct phosphorylation of CREB, activating transcription factor 1 (ATF-1) and serum response factor (SRF), possibly via Ca\(^{2+}\)-dependent activation of the MAPK pathway. The role of CaMKI is less clear, but in vitro it is able to stimulate the phosphorylation of CREB and ATF-1, and of synapsin-1 and synapsin-2. The mechanism of CaMKK activation is comparable to that of other CaMK family members. It has a Ca\(^{2+}\)/calmodulin binding domain and is autophosphorylated. However, CaMKK activity is not completely dependent on Ca\(^{2+}\)/calmodulin binding, but rather it enhances its activity. The two known isoforms, CaMKKα and β, do not seem to differ in function. However, based on similar tissue distribution, it has been suggested that CaMKKβ is more specifically involved in regulation of CaMKIV activity.
Figure 4: Activation model of calmodulin and Ca$^{2+}$/calmodulin dependent kinases

(A) When Ca$^{2+}$ levels rise in the cytoplasm, calmodulin undergoes a conformational change by binding four Ca$^{2+}$ ions. Activated calmodulin is then able to bind target molecules that contain a calmodulin Binding Domain (CBD), such as the family of Ca$^{2+}$/calmodulin dependent kinases. (B) CaMKs contain a catalytic domain, an autoinhibitory domain (AID) and a calmodulin binding domain (CBD). In an inactive state, the AID acts as a pseudo-substrate by interacting with the catalytic kinase domain. Upon calmodulin binding the kinase undergoes a conformational change and the interaction of the AID with the catalytic domain is lost. CaMKI and IV are known to be further activated by CaMK kinase (CaMKK).

5 Granulocyte effector functions

The activation of specific intracellular pathways by the various receptor classes leads to regulation of specific granulocyte effector functions. The molecular mechanisms involved in priming, migration, ROS formation and apoptosis are discussed in this section.

5.1 Priming

Granulocyte functioning can be rapidly amplified by environmental factors through a mechanism termed 'priming', which is independent of protein synthesis. For example, traces of bacterial lipopolysaccharide (LPS), which by itself does not cause activation of the respiratory burst, induces an enhanced formation of reactive oxygen species (ROS) upon subsequent stimulation with chemotactic factors such as fMLP. Activation refers to processes that lead to a measurable alteration in cells, for example degranulation. Priming, however, refers to a process whereby the response of cells to a subsequent (activating) stimulus is amplified if these cells were pre-exposed to a (priming) stimulus (Fig. 5). Neutrophils are normally not activated by formyl peptides in the context of respiratory burst activation. However, addition of platelet activating factor (PAF), GM-CSF and TNFα to human granulocytes primes the generation of ROS-release upon a subsequent activation with chemoattractant. Priming effects can have distinct activation profiles depending on the agonist employed. For example, priming by LPS or GM-CSF requires several minutes and is maximal for 30-60 minutes, whereas priming by PAF occurs very rapidly and is maximal within a few minutes.

A role for Ca$^{2+}$ has been suggested in certain aspects of the priming process. Indeed a transient increase in [Ca$^{2+}$] is itself sufficient to prime human granulocytes as demonstrated by the addition of ionomycin, a calcium ionophore. However, cytokines such as GM-CSF and IL-5 can induce priming without inducing a rise in [Ca$^{2+}$]. This priming activity is accompanied by induction of protein tyrosine phosphorylation. Priming has indeed been shown to be inhibited by protein tyrosine kinase inhibitors. MAPKs appear to be activated by almost all granulocyte priming agents, and a role for ERKs in priming the respiratory burst has been suggested although this has...
not been confirmed by all studies\textsuperscript{66, 131-134}. PI3K is also thought to play a role in priming of granulocyte effector functions. Several studies have demonstrated that addition of the PI3K inhibitors wortmannin or LY294002 to neutrophils results in an inhibition of fMLP-stimulated respiratory burst\textsuperscript{66; 80; 131-137}. Recently it has been proposed that the priming action of GM-CSF on neutrophil respiratory burst may involve phosphorylation of p47\textsuperscript{phox}, a component of the cytosolic NADPH oxidase complex. This phosphorylation was inhibited by wortmannin suggesting a role for PI3K in this process\textsuperscript{138}.

**Figure 5: Priming**

Inflammatory factors such as PAF (released by phagocytes), GM-CSF or TNF\textsuperscript{α} (released by mononuclear cells) or LPS (bacterial component) can bring granulocytes in a pre-activated or primed state. Unprimed cells are not able to respond on for example fMLP treatment, whereas primed cells can perform granulocyte effector functions upon fMLP treatment, such as degranulation and respiratory burst.

### 5.2 Migration

Chemoattractant-induced activation leads to firm adhesion and spreading of granulocytes on the endothelium. This is mediated by integrins expressed on their surface which interact with ligands on endothelial cells\textsuperscript{139; 140}. Subsequently, cells migrate through the endothelial layer, towards the inflammatory site, in a process termed diapedesis (Fig. 6). It has been suggested that neutrophils may not migrate “between” endothelial cells but actually pass “through” cells, although this is still controversial\textsuperscript{141}. After diapedesis through the endothelium, cells migrate towards the site of inflammation by sensing a chemotactic gradient and interacting with proteins (collagens and fibronectin) that form the extracellular matrix\textsuperscript{142}. Signals generated by chemoattractants and cross-linking of integrins, leads to extension of the leading front of the cells called the lamellipodium, and contraction of the rear of the cell, the uropod. fMLP, C5a and IL-8 are potent chemotactic factors for neutrophils.
Figure 6: Migration

During inflammation granulocytes adhere and spread to the endothelium, followed by migration through the endothelium layer termed “diapedesis”. Sensing a chemotactic gradient, granulocytes migrate to the site of inflammation by extension of the leading front, the lamellipodium and contraction of the rear of the cell, the uropod.

Chemoattractants can induce two types of movement; chemotaxis, which is “directed” movement, and chemokinesis which is “non-directional” or “random” movement. Chemotaxis is predominantly induced by chemoattractants such as fMLP and IL-8, whereas cytokines such as GM-CSF induce chemokinesis. The signaling pathways regulating migration have been characterized to a preliminary degree. For example, the role of ERK and PI3K has been studied by the use of pharmacological inhibitors. Utilising boyden chemotaxis chambers, the MAPK kinase (MEK) inhibitor PD98059 was found to have no effect on movement by cytokines or chemoattractants. However, when studied in agarose migration assays, PD98059 was found to inhibit migration. p38 has also been implicated indirectly in fMLP induced chemotaxis. However, p38 activation was not required for IL-8 induced migration.

In terms of a role for PI3K in migration, many contradictory findings have been reported. Inhibitors of PI3K prevent neutrophil migration induced by GM-CSF, however no inhibition of migration is observed after stimulation with PAF or fMLP. This suggests that PI3K is involved in regulating chemokinesis rather than chemotaxis. However, another study demonstrated that IL-8 induced chemotaxis was inhibited by both wortmannin and LY294002. Furthermore, knockout mice lacking PI3Kγ demonstrated an inhibited response to chemoattractant induced migration. This apparent discrepancy between human, and mouse neutrophils could be due to the involvement of other PI3K isoforms in human neutrophils, or possibly to the use of different assay systems/conditions. Recently, it has been suggested that PI3K effects directionality rather than motility. Several reports have suggested a role of [Ca2+] in neutrophil migration. Integrins are translocated from the uropod to the leading edge via endosomes and this appears to be a Ca2+ dependent process, since buffering of [Ca2+] has been shown to inhibit the recycling of the integrins. Moreover, it has been shown that eosinophils have low [Ca2+] concentrations in the front of the cell and high [Ca2+] in the uropod. Although this has not be demonstrated in neutrophils, such Ca2+ distribution might enable release of cell attachments in the uropod, since it has been proposed that uropod retraction of migrating neutrophils requires myosin II, which is activated by a Ca2+ dependent mechanism.

5.3 Respiratory burst

When neutrophils have been mobilized to the site of inflammation, pathogens are removed through phagocytosis and generation of anti-microbial oxidants by the NADPH oxidase complex in the so called “respiratory burst”. The NADPH oxidase is a multicomponent enzyme complex that is dormant in resting cells but becomes highly active during the phagocytosis of invading pathogens. The activated NADPH oxidase is composed of five components: flavocytochrome b558, p47phox, p67phox, p40phox and the small GTPase Rac2. Cytochrome b558 itself is composed of two subunits, gp91phox and p22phox. In the resting state, the subunits of NADPH oxidase are localized on...
the membrane of specific granules (gp91\textsubscript{phox} and p22\textsubscript{phox}) and the cytoplasm (p47\textsubscript{phox} and p67\textsubscript{phox} and p40\textsubscript{phox}). In response to stimulation with inflammatory mediators, the cytosolic subunits translocate to the membrane and associate with the membrane-bound subunits to form an activated enzyme complex (Fig. 7). The core part of the active enzyme is formed by p67\textsubscript{phox}, Rac2 and cytochrome b558. p47\textsubscript{phox} is required for the assembly of the active enzyme complex acting as an adaptor for p67\textsubscript{phox} translocation \textsuperscript{159-161}. However p40\textsubscript{phox} has also been shown to be critical for the function of the NADPH oxidase \textsuperscript{162}.

fMLP can effectively induce activation of the respiratory burst, however it requires prior priming of the cells with cytokines, chemotaxtants or lipopolysaccharides \textsuperscript{4}. It has been shown that fMLP induces translocation of p47\textsubscript{phox} to the plasma membrane, possibly by enhancing its phosphorylation state \textsuperscript{138}. A role for the MAPKs in this phosphorylation induced translocation has been proposed, since p47\textsubscript{phox} contains a consensus phosphorylation site for MAPKs \textsuperscript{138}. However, most studies utilizing PD98059 have demonstrated that ERKs do not appear to play a crucial role, since inhibition of MEK did not effect fMLP induced respiratory burst in cells primed with GM-CSF, TNF\textalpha or PAF \textsuperscript{66}. On the other hand, inhibitors of PI3K can potentely inhibit fMLP-induced respiratory burst in GM-CSF, PAF and TNF\textalpha primed cells \textsuperscript{66}. Recently, a direct role for PI3K in the respiratory burst has been demonstrated. The Phox homology (PX) domains of p47\textsubscript{phox} and p40\textsubscript{phox} have been found to bind to phosphorylated phosphatidylinositol-lipids which are products of PI3K activity \textsuperscript{162}; \textsuperscript{163}. A critical role for changes in [Ca\textsuperscript{2+}]i in the activation of the respiratory burst has also been described \textsuperscript{4}; \textsuperscript{155}. Depletion of [Ca\textsuperscript{2+}]i completely abolishes fMLP induced superoxide production, while addition of Ca\textsuperscript{2+}-ionophore primes fMLP induced NADPH oxidase activation \textsuperscript{124}. The mechanism by which [Ca\textsuperscript{2+}]i, regulates oxidase activity still remains unclear.

\textbf{Figure 7: Respiratory burst}

In a resting state, the subunits of NADPH oxidase are localized to the membrane of specific granules (gp91\textsubscript{phox} and p22\textsubscript{phox}) and the cytoplasm (p47\textsubscript{phox}, p67\textsubscript{phox}, p40\textsubscript{phox} and Rac2). In response to inflammatory mediators, cytosolic subunits translocate to the membrane and fuse with the membrane-bound subunits to form an activated enzyme complex, which then can lead to the formation of reactive oxygen species.

\textbf{5.4 Apoptosis}

Granulocyte apoptosis and the subsequent recognition and removal of apoptotic cells by phagocytes is critical in the rapid resolution of inflammation \textsuperscript{6}. This is necessary to avoid unwanted tissue damage through the release of granule proteases from necrotic granulocytes. \textit{In vivo} and \textit{in vitro} granulocytes undergo apoptosis spontaneously, however granulocytes present at inflammatory sites have increased life span \textsuperscript{3}. The molecular mechanisms underlying the control of granulocyte apoptosis remain largely unknown. In culture 50-70\% of neutrophils undergo constitutive apoptosis within 20 hours; with eosinophils it can take up two days to achieve equivalent rates of apoptosis \textsuperscript{164}; \textsuperscript{165}. Priming agents such as GM-CSF and bacterial products such as LPS can enhance the life-span of human neutrophils. GM-CSF induces a rapid activation of Lyn, a Src family tyrosine kinase, and Lyn-antisense treatment of neutrophils reverses the survival-promoting effect of GM-CSF \textsuperscript{166}; \textsuperscript{167}.

Control of apoptosis in various cell types correlates with the expression of BCL-2 family proteins. The BCL-2 family includes both pro- and anti-apoptotic molecules. The ratio between these two subsets helps to determine, in part, the susceptibility of cells to a death signal \textsuperscript{168}. Pro-apoptotic members of the BCL-2 family are thought to control mitochondrial permeability transitions allowing the release of cytochrome c and apoptosis inducing factor (AIF), which are both involved in the execution phase of apoptosis. Anti-apoptotic members are localized in the membrane of the mitochondria and are thought to act as “guardians of the mitochondria” \textsuperscript{169}. Mature human
neutrophils do not express the anti-apoptotic protein Bcl-2, but do constitutively express the pro-apoptotic proteins Bax, Bid, Bak and Bad. Eosinophils have been found to contain the pro-apoptotic Bax, but also express the anti-apoptotic proteins Bcl-xL and low levels of Bcl-2. This might explain differences in the rate of spontaneous apoptosis between neutrophils and eosinophils. Expression of several anti-apoptotic family members in neutrophils has been shown, although contradictory results have been reported. The expression of the anti-apoptotic Mcl-1 protein has been shown to be decreased upon the onset of apoptosis. Treatment of neutrophils with agents that delay apoptosis increase or maintain the Mcl-1 levels, providing a mechanism to explain cytokine-mediated increased survival.

Thus far, the intracellular signaling pathways regulating neutrophil apoptosis, and the expression of A1 and Mcl-1 are undefined. Within the MAPK family, a role for ERK in regulating apoptosis has been suggested, since PD98059 can inhibit GM-CSF induced survival. p38-dependent signaling pathways have also been implicated in regulating neutrophil apoptosis. Apoptosis induced by stress stimuli such as UV, hyperosmolarity or sphingosine are thought to require p38 activity. In contrast, Fas-induced apoptosis or spontaneous apoptosis can occur independently of p38 activation. Both pathways have been shown to utilize members of the caspase family. Few studies so far have analyzed the role of PI3K in the regulation of neutrophil apoptosis. However, initial data suggests that in neutrophils, like many other cell-types, PI3K activity is critical for cytokine-mediated rescue from spontaneous apoptosis. PI3K can activate PKB which in turn may phosphorylate Bad, thereby decreasing its pro-apoptotic effect. Ca^{2+} may also play a role in neutrophil survival, since transient elevations in cytosolic free Ca^{2+} have been shown to retard subsequent apoptosis. A potential mechanism for this has been suggested by the recent observation that Ca^{2+} can promote cell-survival by directly activating PKB through CaMK kinase, although thus far this has not been confirmed in human neutrophils.

6 Specificity in granulocyte signal transduction

As described above, granulocytes exhibit specific effector functions. However, extracellular factors, which can lead to priming or activation of human neutrophils by binding to specific receptors activate a plethora of overlapping intracellular signaling pathways in these cells. How then is specificity obtained in intracellular signal transduction, resulting in regulation of granulocyte effector functions? First of all, specificity in intracellular signaling can be achieved by the restricted expression patterns of receptors, molecular signaling molecules and/or transcription factors. For example, expression of the IL-8 Receptor (CXCL8) is restricted to neutrophils. Similarly, the components of the NADPH oxidase, regulating the respiratory burst, are highly expressed in human granulocytes. Secondly, strength of activation signals can create specificity. Whereas low concentration of IL-3 in combination with GM-CSF or EPO results in differentiation of progenitor cells towards granulocytes or erythrocytes, high concentrations of IL-3 in the presence of the other growth factors does not lead to differentiation, but rather promotes self renewal. Additionally, the strength and kinetics of intracellular signaling molecules can be critical. For example, in the PC12 cell line, transient ERK activation by EGF results in proliferation, whereas sustained ERK activation induced by NGF results in differentiation. Thirdly, localization of the signaling molecules are of obvious importance in mediating signaling specificity. Molecules which are needed for cell migration are recruited actively to the lamellipodium and activation of the respiratory burst needs translocation of several subunits to the membrane of the phagosome. Changes in localization can therefore dramatically effect granulocyte effector functions. Thus specificity can be reached by regulation of expression, duration of activation, activation-strength and localization of specific signaling molecules. Figure 8 shows an overview of the signaling molecules which are thought to be involved in the regulation of the specific granulocyte effector functions.
**Figure 8**: Signaling molecules involved in granulocyte effector functions.
7 Scope of this thesis

Human granulocytes play an important role in host defense by mediating inflammatory responses to invading microorganisms. Defects in granulocyte differentiation or improper functioning of these cells can lead to disease pathogenesis. Granulocyte maturation, priming and activation are under the control of cytokines and chemoattractants. This study focuses on the intracellular signaling mechanism regulating granulocyte differentiation and functioning. To address this, several points of research or questions were investigated.

1) What are the roles of intracellular pathways in the cytokine induced maturation of hematopoietic progenitors towards granulocytes?

To address this question, an in ex-vivo differentiation model was developed, in which isolated umbilical cord blood CD34+ progenitors were differentiated towards neutrophils. In Chapter 2 we have investigated the differences in the maturation program induced by G-CSF or a combination of IL-3 and G-CSF. By using pharmacological inhibitors we demonstrate the importance of PI3K and MEK in the maturation process of hematopoietic progenitors towards neutrophils.

2) Identification of novel kinases present in human granulocytes.

Since granulocytes have specific effector functions we hypothesized that specific signaling molecules could be present in these cell. To this purpose we describe in Chapter 3 polymerase chain reaction analysis using degenerate primers against conserved regions of kinases, leading to the identification of CKLiK. We characterize CKLiK as a Ca2+ and calmodulin dependent kinase, whose expression is restricted to granulocytes. For analysis of the role of CKLiK in granulocyte functioning, we describe in Chapter 4 the role of Ca2+ and calmodulin regulated pathways by using a Ca2+ ionophore and a calmodulin antagonist. In Chapter 5 we describe the regulation and functional characterization of CKLiK in human granulocytes, by using an antibody against CKLiK and an cell permeable inhibitory peptide.

3) What genes are regulated by cytokines in human granulocytes?

Exposure of inflammatory mediators, such as GM-CSF, to mature granulocytes leads to a pre-activated or primed phenotype. In Chapter 6 we identified GM-CSF regulated genes, by a differential display technology, in which the gene encoding 5-lipoxygenase activating protein was identified as a cytokine regulated gene in vitro as well as in vivo (Chapter 6).

Taken together this thesis provides novel insights into granulocyte signal transduction pathways and identifies new signals that can control granulocyte maturation, priming, activation and functioning.
References


Introduction


Introduction
