Signal transduction pathways regulating granulocyte differentiation and function

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Omslag: Wilma Vereecken
Illustraties: Richard Stark

Print: FEBODRUK BV, Enschede

De uitgave van dit proefschrift werd mede mogelijk gemaakt door financiële steun van

Afdeling Longziekten
Dr. Ir. Van de Laar Stichting
J. E. Jurriaanse Stichting
GlaxoSmithKline
Novartis

ISBN: 90-393-3090-5

Signal transduction pathways regulating granulocyte differentiation and function

Signaal transductie routes betrokken bij de
differentiatie en activatie van humane granulocyten

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit van Utrecht
op gezag van de rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
dinsdag 3 september 2002 des middags te 14.30 uur

door

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Geboren op 22 augustus 1971 te Wijchen

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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 [1]. Eosinophils, which form the second group help to destroy invading parasites and are involved in allergic inflammatory responses [2]. 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 [3].

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 [4] and proteolytic enzymes released from intracellular granules [5]. 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 [6]. 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 [1]. 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 [7], Chediak-Higashi syndrome (caused by a granule defect) [8] or neutrophil adhesion deficiencies [9]. Additionally, dysregulation of one of the effector functions can result in chronic inflammation, which can cause aspecific tissue damage and improper tissue functioning [10]. 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 [11-14]. 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 [15-19]. General myelopoiesis is driven by cytokines, such as GM-CSF and IL-3 [20; 21]. However, terminal differentiation towards the neutrophilic and eosinophilic lineage can be directed by G-CSF and IL-5 respectively [15]. Based on

nuclear shape and formation of granules, neutrophil differentiation can be divided into several stages [22]: 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) [22] (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 [23; 24]. 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 [19; 25-27]. 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 [28]. 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).

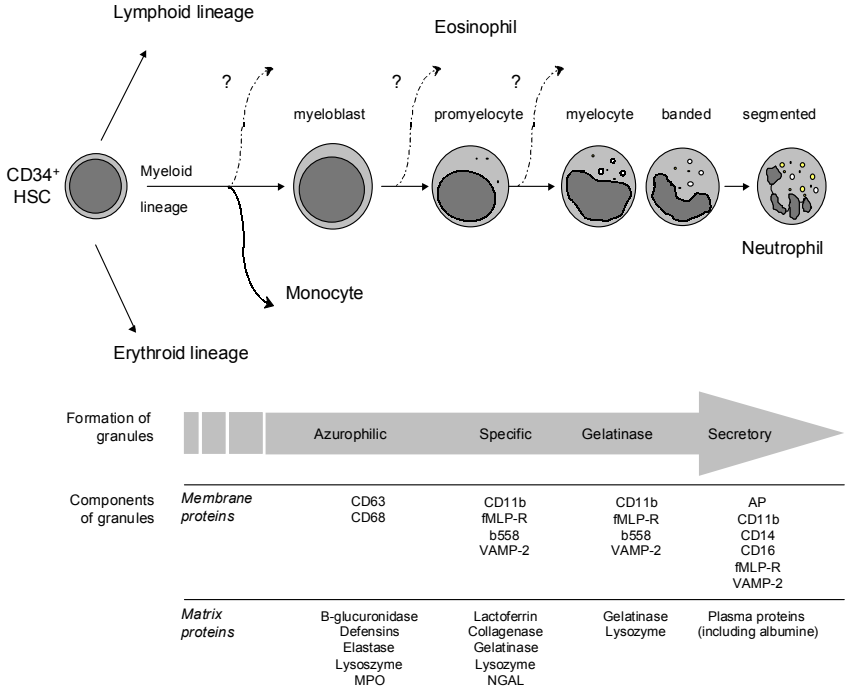

Figure 1: *Maturation of human granulocytes.*
Differentiation of hematopoietic stem cells towards neutrophils involves several stages. Myoblast, myelocyte, metamyelocyte, banded cells and segmented cells. At the stage of the myeloblast the formation of azurophilic granules occurs containing the component 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 (βc), 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 IL2Rγ, 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 βc. In this model, serine phosphorylation of the βc, 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 CX_3C 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 [51]. These heterotrimeric G-proteins consist of an α- and a βγ-subunit. Agonist binding to the receptor results in the exchange of GTP for GDP on the Gα subunit, resulting in dissociation of Gβγ. Twenty seven different α 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 $G\alpha_{12}$ [52]. Furthermore, there are five different β subunits and 10 different γ subunits. Chemoattractant receptors, such as the IL-8 receptor or fMLP receptor, are coupled to $G\alpha_i$ proteins, a class of G-protein that is sensitive to pertussis toxin (PTX) [53]. PTX is a bacterial toxin that specifically modifies a subset of Gα subunits including $G\alpha_i$, but not the α subunits of the Gs, Gq or G12 classes. Evidence exist that chemoattractant induced signaling is mediated by the Gβγ subunits. It has been demonstrated that the βγ subunit released from $G\alpha_i$ proteins can activate phospholipase (PLC) β2 [54-57]. Indeed, purified Gβγ proteins can directly activate recombinant PLC-β2 as well as PLC-β3 [58-60]. In addition, PI3K activation, which is also stimulated by chemoattractants, has also been described to be activated directly by βγ subunits [61]. Based on this, together with the finding that many responses to chemoattractants are PTX sensitive, a role for the $G\alpha_i$ and βγ 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 [62; 63] and the Rho family, which includes RhoA, -B and -C, Rac1 and -2 and Cdc42 [64]. 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 [65]. 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 hydrolyze the bound GTP to GDP. GAPs enhance this intrinsic GTPases activity. Several small GTPases have been implicated in regulating neutrophil function. Activation of Ras [66] and Rap1 [67] by both cytokines and chemoattractants has been reported in human neutrophils, while Ral is only activated upon chemoattractant stimulation via GPCRs [68]. Ras has been shown to activate MAPK and PI3K, both of which are thought to be important in granulocyte function [66; 69; 70]. 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 [67]. 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 [71]. 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) [72-75]. Additionally, a role for the Rho family of GTPases in actin polymerization and migration has been demonstrated [64]. Rac is known to regulate actin assembly in a variety of cells, resulting in cell spreading and the formation of lamellipodia [76]. 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) [64] 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].

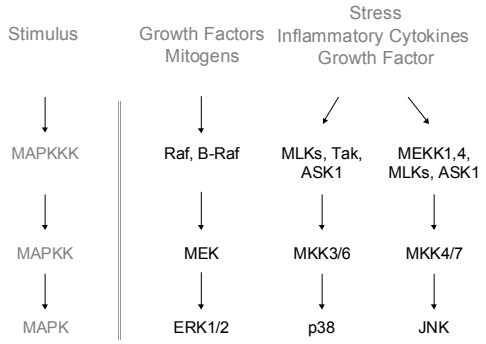

Figure 2: *MAPK regulated pathway*

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 (PI), PI(4)phosphate and PI(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 (p110γ) 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 [66; 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 PI(4)P or PI(4,5)P_2 by PI3K leads to formation of PI(3,4)P_2 and PI(3,4,5)P_3. These lipid products form docking sites for

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 [90]. 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 $PI(3,4)P_2$ or $PI(3,4,5)P_3$ [91]. Integrin linked kinase (ILK) has been shown to phosphorylate PKB on Ser 473 *in vitro* and when over expressed in cells [92]. However, other studies have indicated that ILK may not directly phosphorylate PKB at Ser473 [93]. 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) [94]. 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 [95], thus it remains unclear which kinase is responsible for Ser473 phosphorylation *in vivo*. Additionally, agonists that increase intracellular Ca^{2+} levels have been reported to activate PKB in a PI3K-dependent manner. PKB phosphorylation was reported to be mediated by Ca^{2+}/calmodulin dependent kinase kinase (CaMKK) on threonine 308 [96] (Fig. 3B). 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 [97]. In human neutrophils, activation of PI3K and PKB can occur upon stimulation of almost all cytokines, chemoattractants and chemokines [98] and a role for PI3K in the neutrophil effector functions has been proposed in many studies [66; 87; 99].

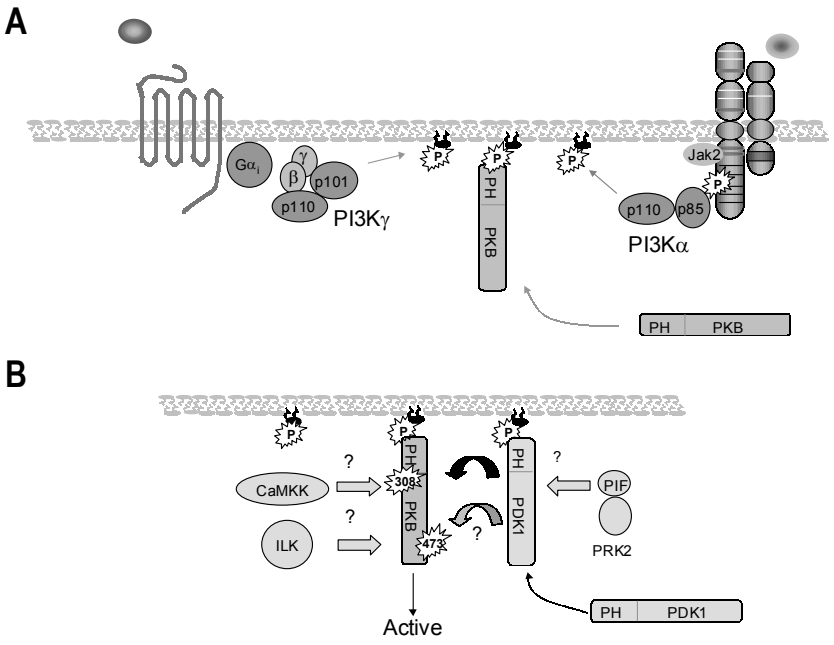

Figure 3: *PI3K regulated pathway*

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 [100]. PLCβ and PLCγ catalyze the conversion of phosphatidylinositol 4,5,-biphosphate (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 [101]. 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 $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ are mediated by EF-hand motif containing proteins, such as calmodulin [102]. Calmodulin is a 17 kDa, ubiquitously expressed molecule which undergoes a conformational change upon binding of four Ca^{2+} ions [103]. 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 [104]. 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 [105]. Four isoforms of CaMKII exist, as well as splice variants of CaMKII α, β, γ and δ [106]. 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 [105]. CaMKII is an oligomeric protein consisting of twelve subunits [107]. 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 [108; 109]. 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 [110].

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 [104]. 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 [111]. CaMKIV has been suggested to stimulate gene transcription by direct phosphorylation of CREB, activating transcription factor 1 (ATF-1) and serum response factor (SRF) [112], possibly via Ca^{2+}-dependent activation of the MAPK pathway [113]. The role of CaMKI is less clear, but *in vitro* it is able to stimulate the phosphorylation of CREB and ATF-1 [114], and of synapsin-1 and synapsin-2 [115]. 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 [116]. 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 [117].

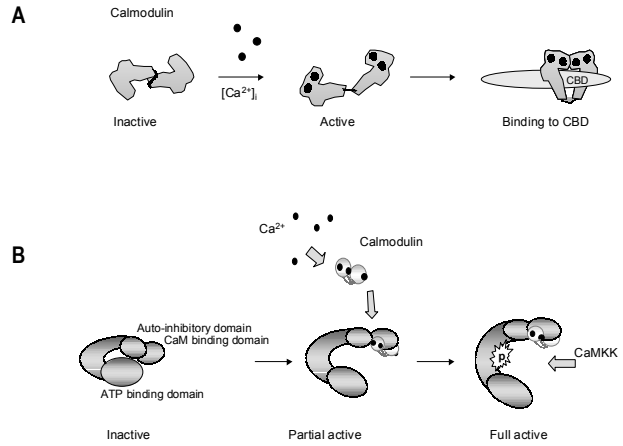

Figure 4: *Activation model of calmodulin and Ca^{2+}/calmodulin dependent kinases*
(A) When Ca^{2+} levels rise in the cytoplasm, calmodulin undergoes a conformational change by binding four Ca^{2+} ions. Activated calmodulin is then able to bind target molecules that contain a calmodulin Binding Domain (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 [118]. 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 [119]. 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 [80; 120-122]. 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 [123].

A role for Ca^{2+} has been suggested in certain aspects of the priming process. Indeed a transient increase in $[Ca^{2+}]_i$ is itself sufficient to prime human granulocytes as demonstrated by the addition of ionomycin, a calcium ionophore [124]. However, cytokines such as GM-CSF and IL-5 can induce priming without inducing a rise in $[Ca^{2+}]_i$. This priming activity is accompanied by induction of protein tyrosine phosphorylation. Priming has indeed been shown to be inhibited by protein tyrosine kinase inhibitors [125-127]. MAPKs appear to be activated by almost all granulocyte priming agents [66; 128-130], and a role for ERKs in priming the respiratory burst has been suggested [128] although this has

not been confirmed by all studies [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 [66; 80; 131-137]. Recently it has been proposed that the priming action of GM-CSF on neutrophil respiratory burst may involve phosphorylation of $p47^{phox}$, a component of the cytosolic NADPH oxidase complex. This phosphorylation was inhibited by wortmannin suggesting a role for PI3K in this process [138].

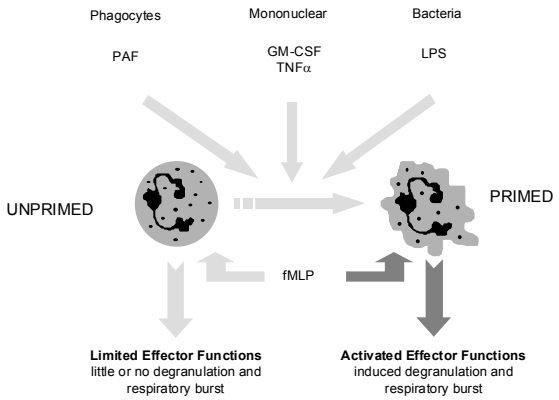

Figure 5: *Priming*
Inflammatory factors such as PAF (released by phagocytes), GM-CSF or TNFα (released by mononuclear cells) or LPS (bacterial component) can bring granulocytes in a pre-activated or primed state. Unprimed cells are not able to respond on for example fMLP treatment, whereas primed cells 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 [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 [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 [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.

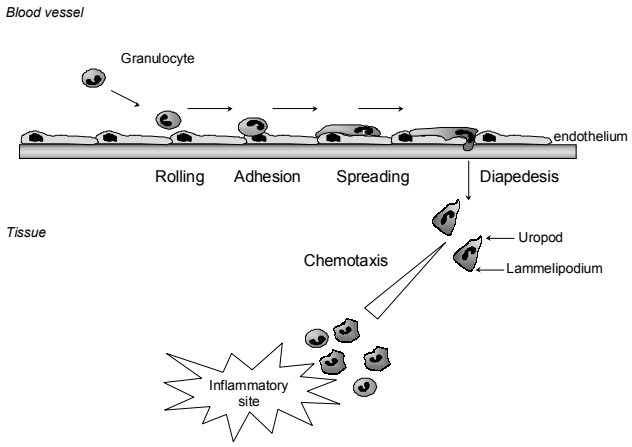

Figure 6: *Migration*
During inflammation granulocytes adhere and spread to the endothelium, followed by migration through the endothelium layer termed "diapedesis". Sensing a chemotactic gradient, granulocytes migrate to the site of inflammation by extension of the leading front, the lamellipodium and 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 [66; 143]. 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 [66]. However, when studied in agarose migration assays, PD98059 was found to inhibit migration [144]. p38 has also been implicated indirectly in fMLP induced chemotaxis [145]. However, p38 activation was not required for IL-8 induced migration [146]. 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 [66]. 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 [146]. Furthermore, knockout mice lacking PI3Kγ demonstrated an inhibited response to chemoattractant induced migration [147-149]. 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 [150]. Several reports have suggested a role of $[Ca^{2+}]_i$ in neutrophil migration. Integrins are translocated from the uropod to the leading edge via endosomes [151] and this appears to be a Ca^{2+} dependent process, since buffering of $[Ca^{2+}]_i$ has been shown to inhibit the recycling of the integrins. Moreover, it has been shown that eosinophils have low $[Ca^{2+}]_i$ concentrations in the front of the cell and high $[Ca^{2+}]_i$ in the uropod [152]. Although this has not be demonstrated in neutrophils [153], such Ca^{2+} 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 Ca^{2+} dependent mechanism [154].

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 b_{558}, $p47^{phox}$, and $p67^{phox}$, $p40^{phox}$ and the small GTPase Rac2 [155]. Cytochrome b_{558} itself is composed of two subunits, $gp91^{phox}$ and $p22^{phox}$. In the resting state, the subunits of NADPH oxidase are localized on

the membrane of specific granules (gp91^{phox} and p22^{phox}) and the cytoplasm (p47^{phox} and p67^{phox} and p40^{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^{phox}, Rac2 and cytochrome *b*558. p47^{phox} is required for the assembly of the active enzyme complex acting as an adaptor for p67^{phox} [156-158]. The role of p40^{phox} is thought to be a regulator of p67^{phox} translocation [159-161]. However p40^{phox} has also been shown to be critical for the function of the NADPH oxidase [162].

fMLP can effectively induce activation of the respiratory burst, however it requires prior priming of the cells with cytokines, chemoattractants or lipopolysaccharides [4]. It has been shown that fMLP induces translocation of p47^{phox} to the plasma membrane, possibly by enhancing its phosphorylation state [138]. A role for the MAPKs in this phosphorylation induced translocation has been proposed, since p47^{phox} contains a consensus phosphorylation site for MAPKs [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, TNFα or PAF [66]. On the other hand, inhibitors of PI3K can potently inhibit fMLP-induced respiratory burst in GM-CSF, PAF and TNFα primed cells [66]. Recently, a direct role for PI3K in the respiratory burst has been demonstrated. The Phox homology (PX) domains of p47^{phox} and p40^{phox} have been found to bind to phosphorylated phosphatidylinositol-lipids which are products of PI3K activity [162; 163]. A critical role for changes in $[Ca^{2+}]_i$ in the activation of the respiratory burst has also been described [4; 155]. Depletion of $[Ca^{2+}]_i$ completely abolishes fMLP induced superoxide production, while addition of Ca^{2+}-ionophore primes fMLP induced NADPH oxidase activation [124]. The mechanism by which $[Ca^{2+}]_i$ regulates oxidase activity still remains unclear.

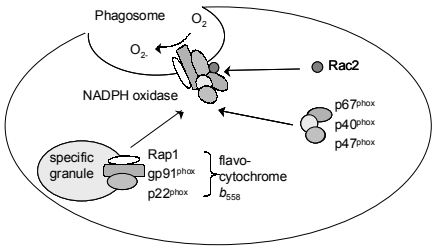

Figure 7: *Respiratory burst*
In a resting state, the subunits of NADPH oxidase are localized to the membrane of specific granules (gp91^{phox} and p22^{phox}) and the cytoplasm (p47^{phox}, p67^{phox}, p40^{phox} and Rac2). In response to inflammatory mediators, cytosolic subunits translocate to the 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.

5.4 Apoptosis

Granulocyte apoptosis and the subsequent recognition and removal of apoptotic cells by phagocytes is critical in the rapid resolution of inflammation [6]. This is necessary to avoid unwanted tissue damage through the release of granule proteases from necrotic granulocytes. *In vivo* and *in vitro* granulocytes undergo apoptosis spontaneously, however granulocytes present at inflammatory sites have an increased life span [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 to two days to achieve equivalent rates of apoptosis [164; 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 [166; 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 [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 mitochiondria and are thought to act as “guardians of the mitochondria” [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 [170]. Eosinophils have been found to contain the pro-apoptotic Bax, but also express the anti-apoptotic protein Bcl-x_L and low levels of Bcl-2 [171]. 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 [170]. 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 [172].

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 [173]. 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 [174]. 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 [174]. 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 [167; 173]. PI3K can activate PKB which in turn may phosphorylate Bad, thereby decreasing its pro-apoptotic effect [175; 176]. 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 [177]. 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 [96], 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 [178; 179]. 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 [180]. 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.

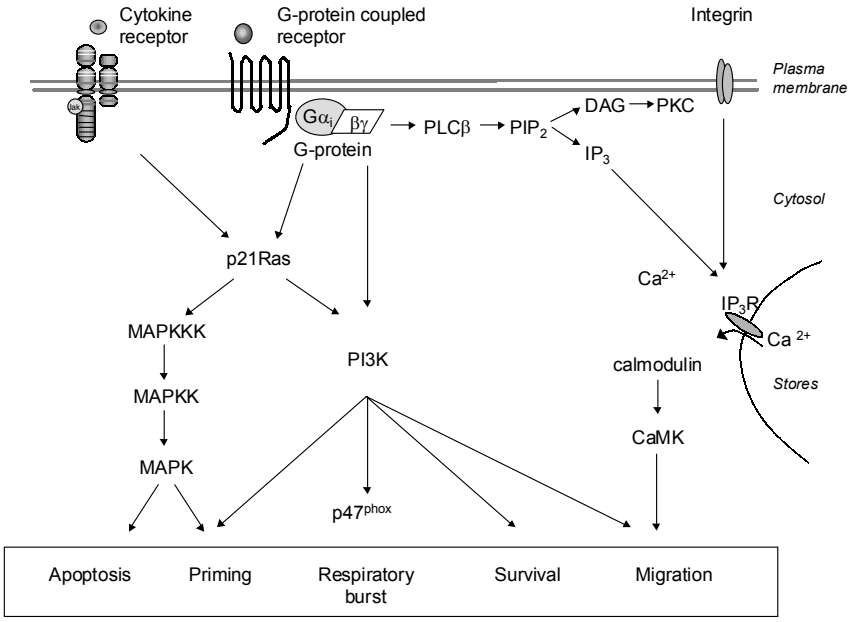

Figure 8: *Signaling molecules involved in granulocyte effector functions*

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 Ca^{2+} 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 Ca^{2+} and calmodulin regulated pathways by using a Ca^{2+} 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.

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Chapter 2

Critical roles for phosphatidylinositol-3 kinase and mitogen activated kinase kinase in cytokine-induced neutrophil differentiation

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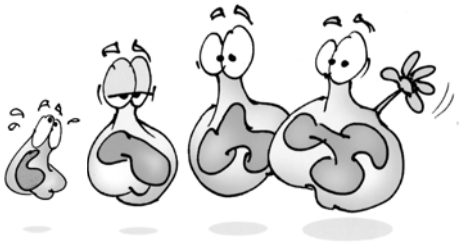
In preparation



Abstract

The production of mature blood cells, termed hematopoiesis, is a carefully orchestrated series of events involving self-renewal and differentiation of primitive pluripotent stem cells. Dysregulation of this complex process can result in severe clinical conditions ranging from aplasia of the bone marrow to leukemia. Differentiation is mediated by processes that are initiated by specific cytokine receptors, as well as processes that are intrinsically present in stem cells. To investigate the role of cytokine-activated signaling pathways in myeloid differentiation, we developed an ex-*vivo* maturation model. In this system, human umbilical cord blood $CD34^+$ progenitor cells can be differentiated to mature neutrophils by addition of granulocyte-colony stimulating factor (G-CSF). First, we compared the effects of a combined cytokine-treatment utilizing interleukin (IL)-3/G-CSF, versus only G-CSF, on proliferation and differentiation. Maturation in the presence of IL-3 resulted in enhanced proliferation compared to G-CSF treatment alone. However, G-CSF induced differentiation in the absence of IL-3 resulted in a greater number of terminally differentiated neutrophils, as measured by lactoferrin- and CD11b expression, morphology, and capacity to produce reactive oxygen species (ROS). We then investigated the role of phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase kinase (MEK) in G-CSF induced $CD34^+$ cell maturation using the specific pharmacological inhibitors LY294002 and PD98059 respectively. Inhibition of PI3K induced cell cycle arrest, but interestingly also resulted in impaired differentiation as was apparent by a reduction of lactoferrin positive cells and histological staining. In contrast, inhibition of MEK resulted in reduced proliferation, without significantly effecting G-CSF induced differentiation. These results demonstrate critical roles for both PI3K and MEK in the cytokine induced maturation of human $CD34^+$ progenitor cells towards neutrophils. Furthermore, our data suggest that cell cycle arrest *per se* does not result in induction of an intrinsic neutrophil differentiation program.

Introduction

Hematopoiesis is a complex and carefully orchestrated series of events involving self-renewal and differentiation of primitive pluripotent stem cells [1]. Dysregulation of hematopoietic differentiation can result in the development of a variety of pathological conditions ranging from aplasia of the bone marrow to aberrant differentiation of myeloid progenitors in diseases such as myelodysplastic syndromes and leukemia. The molecular mechanisms underlying how hematopoietic cytokines exert lineage-dominant effects on the differentiation process *in vivo* are not completely understood. The "inductive model" postulates that specific extracellular signals force stem cells to commit to a particular hematopoietic lineage. This model requires that the intracellular signals generated by cytokine-stimulation are specific and non-interchangeable [2]. However, other studies have reached alternative conclusions favoring a "stochastic model" [3]. This predicts that hematopoietic stem cell fate decisions are independent of extracellular signals and the role of cytokines is to provide non-specific survival and proliferation signals. That is, cytokines simply permit the survival, proliferation and development of intrinsically committed cells.

The effects of cytokine-stimulation are mediated by cell surface receptors belonging to the cytokine receptor superfamily, which signal by activating JAK tyrosine kinases [4]. Interaction of cytokines with their cognate receptors expressed on hematopoietic progenitor cells, results in activation of a plethora of intracellular signaling pathways that are thought to control hematopoiesis by regulation of transcription [5-7]. Activation of members of the STAT transcription factor family by multiple cytokines is thought to play a critical role in the control of hematopoietic cell growth and differentiation (reviewed in [8]). For example, using a mouse myeloid cell line, LGM-1, exogenously expressing the G-CSFR, STAT3 has been shown critical for neutrophil differentiation [9]. Addition of G-CSF to these cells resulted in a block in proliferation, down-regulation of c-*myc,* and differentiation into cells with lobed nuclei. These changes were blocked by introduction of a dominant-negative STAT3 mutant. Several additional intracellular signaling molecules are activated by multiple cytokines, including phosphatidylinositol-3 kinase (PI3K) and Mitogen Activated Protein Kinase Kinase (MAPKK or MEK). Both signaling molecules pathways have been shown to be induced by a variety of hematopoietic cytokines including IL-3, GM-CSF and G-CSF [10 11 12].

PI3K has been shown to be involved in growth factor dependent survival and proliferation of a variety of cell lines [13]. PI3K knockout mice lacking the p110α catalytic subunit die around embryonic day 9.5, which corresponds with a failure of the embryonic cells to proliferate [14]. One mechanism for this is suggested by studies demonstrating that PI3K is involved in the regulation of the cell cycle through transcriptional induction of cyclin D [15-17]. Additionally, the stabilization of cyclin D protein has been suggested to be PI3K-dependent [18; 19]. Furthermore, PI3K appears to play a role in down-regulation of the cyclin-dependent kinase inhibitors (CDKIs), such as $p27^{KIP1}$. This is thought to be mediated, at least in part, via inactivation of the members of the Forkhead family of transcription factors [20; 21 22]. PI3K has also been linked with survival signals [23]. Transgenic mice expressing an active form of PI3K have increased numbers of T lymphocytes exhibiting reduced apoptosis, and develop lymphoproliferative disorders [24].

Cytokine receptor activation also results in activation of the small GTPase Ras, which in turn can activate the protein kinase Raf. Raf activates the dual-specific MAPK Kinase (MEK), which then can phosphorylate Extracellular Regulated Kinase (ERK). Ras plays a critical role in regulating cell cycle control, possibly through down regulation of $p27^{KIP1}$ and the up regulation of cyclin D1 expression [25-29]. A direct role for MEK-ERK in cell cycle control has been proposed since treatment of cells with a pharmacological inhibitor of MEK (PD98059) or transfection of dominant-negative ERK correlates with decreased cyclin D1 expression [30; 31].

The function of PI3K- and MEK-mediated signaling in the processes driving hematopoiesis remains unclear. In this study we developed an ex-*vivo* model system based on the cytokine induced differentiation of human umbilical cord blood stem cells towards neutrophils. Cytokines and colony stimulating factors including FLT-3 ligand, Stem Cell Factor (SCF), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and Granulocyte-Colony Stimulating Factor (G-CSF) have been demonstrated to be important for granulocyte maturation [32-34]. G-CSF has been demonstrated to play a critical role, as is exemplified by the neutropenia found in G-CSF deficient mice [35]. Here we investigate a role for PI3K and MEK in the cytokine-induced proliferation and differentiation of primary human $CD34^+$ progenitor cells towards neutrophils. We have compared maturation of isolated primary umbilical cord blood $CD34^+$ stem cells in the presence of either a combination of IL-3 and G-CSF, or G-CSF alone. Differentiation in the presence of IL-3 increased the expansion of cells but reduced the number of

mature, functional neutrophils compared to G-CSF alone. Inhibition of PI3K during G-CSF induced maturation of $CD34^+$ stem cells resulted in a block in proliferation and furthermore the capacity of these progenitor cells to differentiate was also abrogated. In contrast, addition of the MEK inhibitor PD98059 during the maturation process resulted in decreased proliferation, but no effect on differentiation. These data delineate critical roles for PI3K and MEK in cytokine driven differentiation and proliferation of primary hematopoietic cells towards neutrophils. Furthermore, our data suggest that cell cycle arrest *per se* does not result in induction of an intrinsic neutrophil differentiation program.

Materials and Methods

Reagents

Human growth factors and cytokines were used to induce differentiation. Stem Cell Factor (SCF) and FLT-3 ligand were purchased from Peprotech (Rocky Hill, NJ), IL-3 and G-CSF were obtained from Strathmann (Hamburg, Germany) and GM-CSF was purchased from Endogen (Woburn, MA). The pharmacological inhibitors PD98059 and LY294002 were purchased from Biomol (Plymouth Meeting, PA). The PE-conjugated antibody against lactoferrin was obtained from Beckman Coulter (Mijdrecht, The Netherlands) and Monoclonal antibody against CD11b (44A) was obtained from American Type Culture Collection (Rockville, MD).

Isolation of $CD34^+$ stem cells and culturing towards neutrophils

$CD34^+$ cells were isolated as previously described [36]. In brief, mononuclear cells were isolated from umbilical cord blood by density centrifugation over isotonic ficoll solution (Pharmacia, Uppsula, Sweden). Immunomagnetic selection with hapten conjugated antibody against CD34 was used to isolate $CD34^+$ cells (Miltenyi Biotech, Auburn, USA). $CD34^+$ cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Paisley, UK) supplemented with 10% FCS, 50 μM β-mercaptoethanol, 10 U/ml penicillin, 10 μg/ml streptamycin, and 2mM glutamine at a density of $2\text{-}3x10^5$ cells/ml. Cells were differentiated towards neutrophils upon addition of SCF (50 ng/ml), FLT-3 (50 ng/ml), GM-CSF (0.1 nmol/l), IL-3 (0.1 nmol/l), and G-CSF (30 ng/ml). Every 3-4 days, cells were counted and fresh medium was added to a density of $5x10^5$ cells/ml. After 3 days of differentiation, cells were cultured further with IL-3 and G-CSF and from day 7 cells were cultured in the presence of G-CSF or with G-CSF and IL-3 as indicated. In some experiments, inhibitors were added upon isolation, and similar to the cytokines, refreshed every 3-4 days.

Morphological analysis of differentiating stem cells

Morphology of differentiating stem cells was analyzed by May-Grünwald Giemsa staining. During the maturation process cytospins were prepared from 10^5 cells. Slides were dried and fixed for 10 minutes in 100% methanol and May-Grünwald Giemsa staining was performed. In short, cytospins were stained in a 50% Eosin Methylene Blue solution for 20 minutes according to May-Grünwald (Sigma-Aldrich GmbH, Seelze, Germany). These were subsequently rinsed in water for 5 seconds, and the nuclei were counter-stained with 10% Giemsa solution (Merck, Darmstadt, Germany) for 15 minutes. Cells were imbedded in entellan (Merck, Darmstadt, Germany) and covered by a glass coverslip for preservation. Based on cytoplasmic staining and morphology of the nuclei, cells were counted as belonging to: 1) premature cells (which include blast cell and promyelocytes); 2) myelocytes (which include myelocytes and banded cells); 3) segmented cells, and 4) highly segmented cells.

Analysis of neutrophil differentiation markers

Lactoferrin positive cells were detected using a PE-conjugated antibody against lactoferrin (Beckman Coulter, Mijdrecht, The Netherlands). Staining was performed as described in the manufacture's protocol, although adapted for stem cells. In brief, $3x10^5$ cells were first washed in PBS and resuspended in 100 μl IntraPrep fixation reagent. After 15 minutes of incubation at room temperature, cells were washed and gently resuspended in 100 μl IntraPrep permeabilisation reagent. After 5 minutes of incubation, anti-lactoferrin-PE was added and incubated for another 15

minutes. Cells were washed and resuspended in PBS containing 0.5% formaldehyde. Number of lactoferrin positive cells were detected by FACS analysis (FACSvantage, Becton Dickinson).
CD11b positive cells were analyzed using the mouse monoclonal antibody 44A against CD11b. 10^5 cells were collected and washed in cold PBS2+ (PBS, 10% GPO, 10% sodium citrate). Cells were incubated for 30 minutes on ice with anti-CD11b. Subsequently, cells were washed, and incubation was performed with a secondary goat anti mouse FITC labeled antibody (Becton Dickinson, San Jose, CA) for 30 minutes on ice. Cells were washed and CD11b positive cells were detected by FACS analysis (FACSvantage, Becton Dickinson).

Analysis of respiratory burst

The production of reactive oxygen species was measured by dihydrorhodamine staining. 400 µl of $1.25x10^6$ cells/ml were as indicated first primed with GM-CSF (10^{-10}M) for 20 minutes at 37^0C. Dihydrorhodamine 123 (DHR123) (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 100 ng/ml and incubated for 10 minutes at 37^0C. Respiratory burst was initiated by fMLP (10^{-6}M) or PMA (100ng/ml). Stimulation was stopped by washing with cold PBS2+. Cells were resuspended in PBS2+ and analyzed by FACS.

Measurement of apoptosis and proliferation.

Apoptosis of differentiating stem cells was measured by analyzing Annexin V-fluorescein isothiocyanate (FITC) binding (Bender Medsystems; Vienna, Austria). 10^5 cells were washed with PBS and resuspended in binding buffer (10 mM HEPES NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Subsequently cells were incubated with Annexin V-FITC for 15 minutes at room temperature in the dark, washed with PBS and resuspended in binding buffer. Propidium iodide was added (1µg/ml) and the percentage of apoptotic cells were detected by FACS analysis (FACSvantage, Becton Dickinson). For proliferation assays, the number of viable cells were counted by trypan blue exclusion. Cell cycle analysis was determined as previously described [22]. $3x10^5$ cells were fixed in 70% ethanol at -20^0C for 2 hours. Cells were gently spun down and permeabilized in 200µl 0.1% Triton X-100, 45mM Na_2HPO_4 and 25mM sodium citrate at 37^0C for 20 minutes. Next, 750µl of 0.1% Triton X-100, 10mM PIPES, 2mM $MgCl_2$, 40µg/ml RNase and 20µg/ml propidium iodide, was added and incubated for 30 minutes in the dark. DNA content was analyzed by FACS.

Results

G-CSF delays proliferation and enhances neutrophil differentiation

To investigate the role of different cytokine-activated signaling pathways in human neutrophil maturation, we developed a system in which myeloid progenitor cells can be differentiated to mature neutrophils ex-*vivo*. $CD34^+$ hematopoietic stem cells (HSCs), enriched from human umbilical cord blood (UCB), were cultured as described in Material and Methods. Cells were passaged at day six and differentiated towards neutrophils in the presence of IL-3/G-CSF, or G-CSF alone and the effect on both proliferation and differentiation was analyzed. Culturing HSCs with IL-3/G-CSF resulted in a 565.2 ± 79.7 fold increase in cell number at day 16. However, treatment with G-CSF alone, resulted in greatly reduced proliferation of CD34+ stem cells (348.1 ± 29.0) (Fig. 1A).

To quantify the neutrophilic differentiation of stem cells treated with IL-3/G-CSF, or G-CSF alone, we analyzed the percentage of lactoferrin positive cells, a neutrophil specific granule protein, and CD11b expression on the cell surface of neutrophils. Treatment of G-CSF compared to the combined treatment of IL-3/G-CSF resulted in a dramatic increase in lactoferrin positive cells to 36.7%, compared with 18.6% at day 13 (Fig. 1B). The amount of lactoferrin positive cells in the G-CSF treated population further increased to 46.7% at day 16, whereas in the combined treatment the number of lactoferrin positive cells did notincrease further. Analysis of CD11b expression demonstrated greatly enhanced levels at day 16 when cells were treated with G-CSF alone, compared to the population treated with IL-3/G-CSF (Fig. 1C).

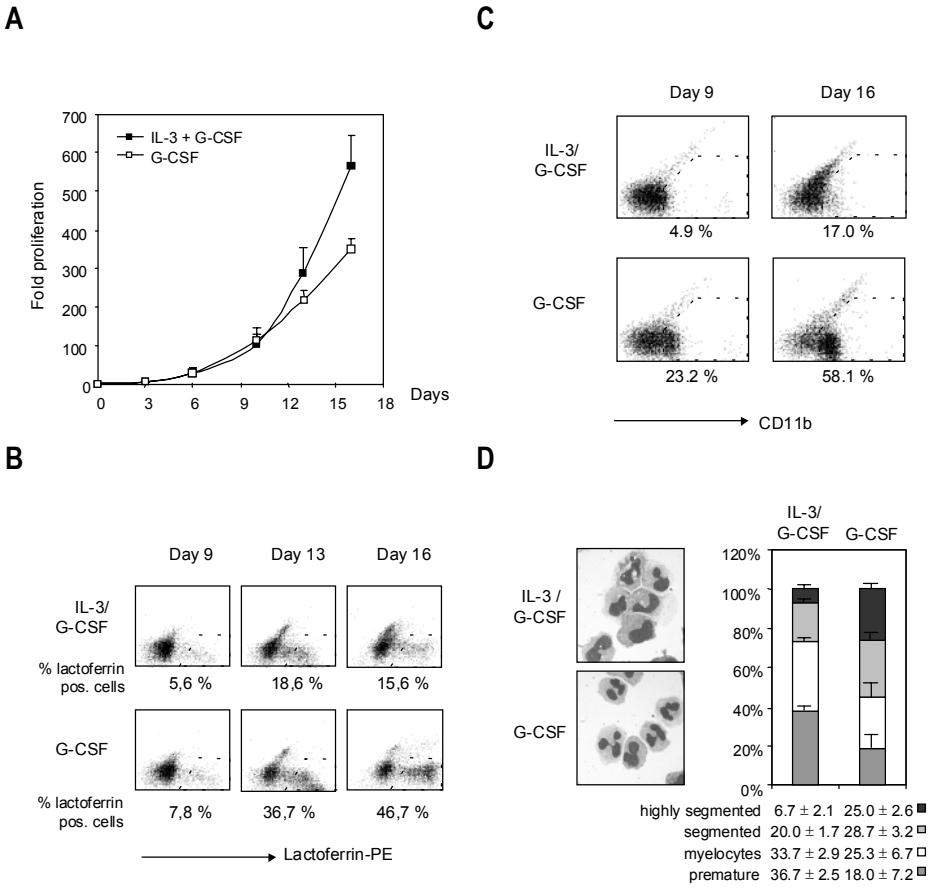


Figure 1: *Effect of cytokine treatment on the maturation of HSCs towards neutrophils.*
$CD34^+$ cells isolated from cord blood were cultured for the first three days with a cocktail of cytokines as described in Materials and Methods. At day three only IL-3 and G-CSF were added, at day six cells were divided and cultured further with IL-3 and G-CSF or with G-CSF alone. **(A)** Proliferation was analyzed by counting number of viable cells every 3-4 days and fold proliferation was calculated. **(B)** Differentiation was analyzed by detection of lactoferrin positive cells at day 9, 13, and 16 as described in Materials and Methods. The percentage of highly lactoferrin positive cells, gated as shown, was determined by FACS analysis at 9, 13 and 16 days as described in Materials and Methods. **(C)** Expression of CD11b on the cell surface of differentiating cells was detected by FACS analysis. The percentage of CD11b positive cells, gated as shown in the first figure, are indicated **(D)** Determination of differentiation stage was analyzed by May-Grunwald Giemsa staining and cells were counted as described in Materials and Methods. Representative pictures of differentiated cells at day 16 are also depicted. Graphs represent mean percentage cells of different stages. All data represent the mean of four independent experiments.

Histological analysis of neutrophil differentiation was determined by May-Grunwald Giemsa staining as described in Materials and Methods. Stem cells treated with G-CSF alone exhibited a maturer neutrophil phenotype, including segmented and highly segmented cells, which represented almost 53.7% of the total population (Fig. 1D). In contrast, cells treated with IL-3/G-CSF contained only 26.7% of mature neutrophilic cells (Fig. 1D). In Figure 1D representative pictures of cells after 16 days of differentiation are depicted for both conditions. These results demonstrate that the combined IL-3 and G-CSF treatment drives the expansion of the HCS more efficiently than G-CSF, whereas G-CSF treatment gives rise to more terminally differentiated neutrophils.

Production of reactive oxygen species correlates with enhanced differentiation

Although we have demonstrated that treatment with G-CSF alone enhances neutrophil differentiation of HSC, as determined by the expression of granulocytic markers, and histological staining, this doesn't measure functional maturity of these cells. Thus, we further analyzed the ability of differentiated neutrophils to produce reactive oxygen species (ROS). ROS production by the so-called "respiratory burst" is a complex process that requires correct

assembly of the NADPH oxidase enzyme complex. The NADPH oxidase is a multi-subunit complex composed of five components: flavocytochrome b_{558}, $p47^{phox}$ and $p67^{phox}$ and $p40^{phox}$ and the small GTPase Rac2, which all have to be present for ROS production [37]. We first tested Phorbol Myristate Acetate (PMA) induced ROS production, as an indication of the functionality of the NADPH oxidase complex. After six days of differentiation, no ROS production was observed in response to addition of PMA (Fig. 2A). However by day nine, the required components of the NADPH oxidase complex were apparently present since PMA-treatment resulted in ROS production (Fig. 2A). Comparison of cells differentiated with the different cytokine combinations demonstrated greatly enhanced ROS production with G-CSF treatment alone compared to combined IL-3/G-CSF treatment, suggesting terminal neutrophilic differentiation. A more physiological method of inducing the respiratory burst was tested utilizing the chemoattractant fMLP, a potent activator of ROS formation in pre-activated or "primed" neutrophils [38]. Differentiated cells were first primed with GM-CSF for 20 minutes prior to stimulation with fMLP. HSCs differentiated towards neutrophils in the presence of G-CSF alone showed a greatly increased response to fMLP treatment compared to treatment with both IL-3/G-CSF (Fig. 2B). In conclusion, G-CSF regulates the differentiation of HCS towards functionally mature neutrophils more efficiently when IL-3 induced signaling pathways are not concomitantly activated.

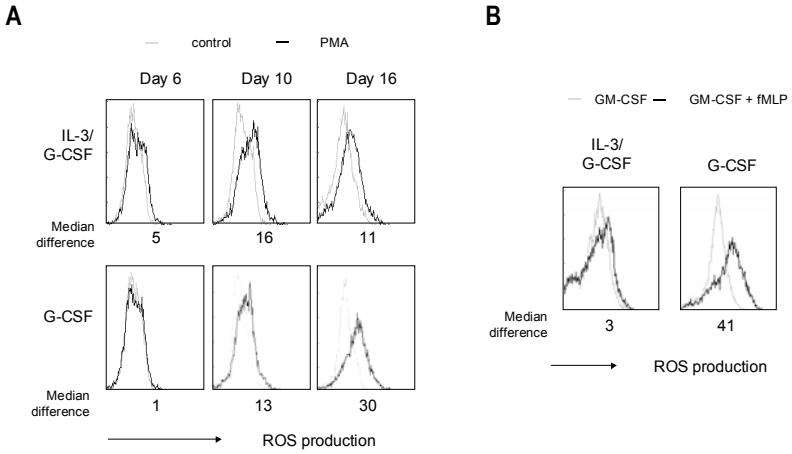


Figure 2: *Analysis of the production of reactive oxygen species.*
The ability of differentiated cells to produce reactive oxygen species was analyzed by DHR123 staining as described in Materials and Methods. Cells were differentiated for 16 days with IL-3/G-CSF or G-CSF. **(A)** PMA induced formation of reactive oxygen species during differentiation. Unstimulated cells of the same day were used as a control. **(B)** Respiratory burst induced by fMLP at day 16. Cells were pre-activated with GM-CSF for 20 minutes prior to addition of fMLP. Values below graphs indicate the median difference of PMA or fMLP treated cells versus untreated cells of the same population. Data are representative of three independent experiments.

Inhibition of PI3K induces cell cycle arrest and blocks differentiation to neutrophils.
PI3K has been linked to regulation of proliferation and survival in many cell systems [23; 39; 40; 41]. However, little is known concerning the role of PI3K in the differentiation of human hematopoietic cells. We investigated the role of PI3K on G-CSF induced maturation of $CD34^+$ stem cells utilizing the specific pharmacological inhibitor LY294002 [42]. Differentiation towards the myeloid lineage was initiated by a cytokine cocktail as described in Materials and Methods. Treatment with LY294002, totally abrogated proliferation (Fig. 3A) and this was not due to enhanced cell death since we observed only a very modest elevation in the amount of apoptotic cells as analyzed by annexin-V binding (Fig. 3B). Analysis of the DNA content of cells demonstrated a decrease in the number of dividing cells in the presence of LY294002 within 24 hours of treatment (Fig. 3C). Additionally we analyzed whether the arrested cells were still able to undergo differentiation induced by G-CSF. Cells were cultured for 13 days and lactoferrin expression and histological staining were analyzed. No lactoferrin positive cells were observed in the LY294002 treated cells (Fig. 3C). Histological staining demonstrated a lack of mature neutrophils (Fig. 3D). Taken together these results indicate that PI3K activity is critical for regulating proliferation as opposed to survival of CD34+ stem cells, as well as playing an important role in the maturation towards neutrophils.

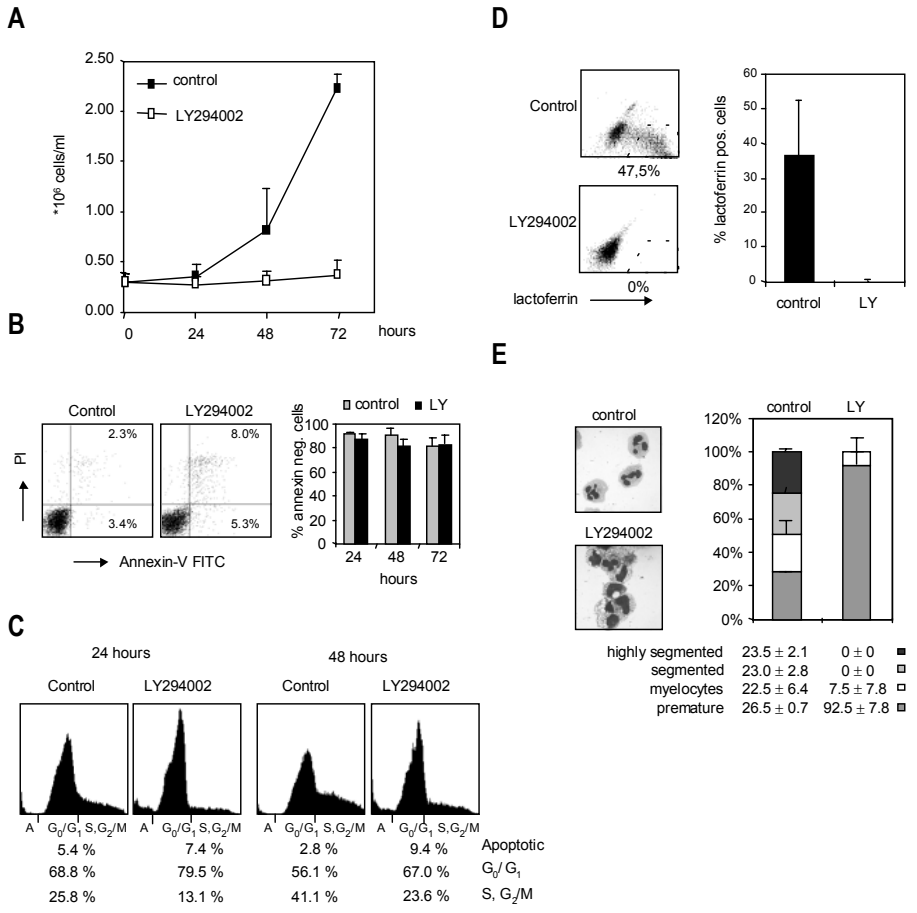


Figure 3: *The effect of LY294002 on the proliferation and differentiation of hematopoietic progenitor cells.*

$CD34^+$ stem cells were cultured in the absence or presence of 20 μM LY294002. **(A)** Proliferation of $CD34^+$ stem cells was analyzed by counting viable cells after 24, 48 and 72 hours. **(B)** The percentage of apoptotic progenitor cells was determined after 24, 48 and 72 hours. Percentage of early apoptotic cells (annexin-V positive, PI negative) and percentage of late apoptotic cells (annexin-V and PI positive) are indicated. Numbers of viable cells are indicated as annexin-negative cells (right panel). **(C)** Cell cycle profiles are depicted of cells cultured for 24 and 48 hours with or without LY294002. **(D)** The effect of LY294002 on the differentiation of HCS towards neutrophils. Hematopoietic progenitor cells were cultured for 15 days without (control) or with LY294002. Representative dotplots of lactoferrin staining, as gated in Figure 1B, are indicated. Graphs represent the mean percentage of lactoferrin positive cells of three independent experiments **(E)** Determination of differentiation stage was analyzed by May-Grunwald Giemsa staining as described in Figure 1D. Graphs represent the mean percentage cells of the different stages. All data are representing the mean of three independent experiments.

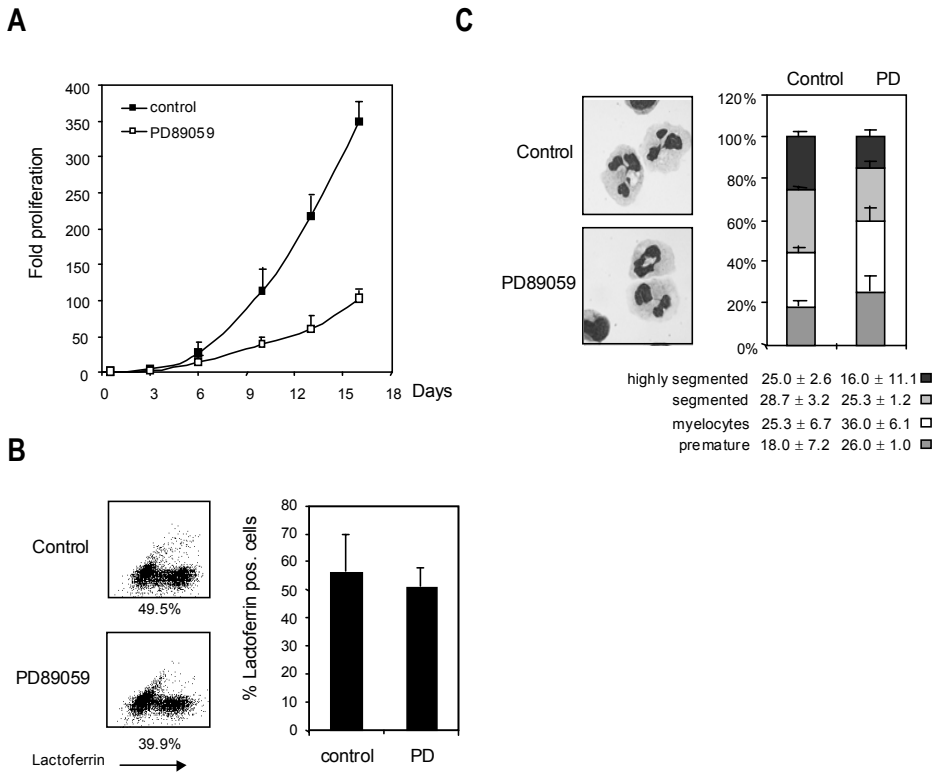


Figure 4: *Effect of PD89059 on the maturation of HCS towards neutrophils.*

$CD34^+$ stem cells were differentiated with G-CSF and cultured in the absence or presence of 20μM PD89059. **(A)** Proliferation of $CD34^+$ stem cells was analyzed by counting viable cell numbers **(B)** Differentiation was analyzed by lactoferrin staining. Representative dotplots showing he percentage of lactoferrin positive cells, as gated in Figure 1B, are shown. Graphs represent the mean percentage of lactoferrin positive cells of three independent experiments **(E)** Determination of differentiation stage was analyzed by May-Grunwald Giemsa staining as described in Figure 1D. Graphs represent the percentage cells of the different stages. All data represent at least three independent experiments.

Inhibition of MEK reduces proliferation without effecting neutrophil differentiation

Another well-characterized signaling pathway downstream of multiple cytokine receptors is the Ras-Raf-MEK-MAPK pathway [43]. It has been suggested that this pathway is involved in the G-CSF induced regulation of the cell cycle in myeloid cell lines [44-46]. However, little is known concerning the role of this pathway in the differentiation of human hematopoietic progenitors towards neutrophils. For this purpose, we differentiated $CD34^+$ stem cells with G-CSF in the presence or absence of the specific MEK inhibitor PD98059. We observed a growth inhibitory effect of PD89059 on treated cells, with a reduction to about 30% of the number of cells compared to the control at day 16 (Fig. 4A). Analysis of the role of MEK inhibition on differentiation demonstrated no significant differences in the percentage of lactoferrin positive cells (Fig. 4B) or the number of CD11b positive cells (data not shown). Morphologic analysis by May-Grunwald Giemsa staining in the PD89059 treated cells compared to the control cells also demonstrated no reduction in the number of mature, differentiated cells (Fig. 4C). Taken together these data suggest a role for MEK in regulating proliferation, but no significant role in the differentiation of hematopoietic progenitors towards neutrophils.

Discussion

The production of mature blood cells, hematopoiesis, involves a carefully orchestrated series of events involving self-renewal and differentiation of primitive pluripotent stem cells. There is a growing understanding of the complexity of the intrinsic and extrinsic controls built into this system to maintain its integrity. The external influences are many and diverse, encompassing interactions of developing hematopoietic cells with extracellular matrix molecules, a variety of stromal cell types and with growth factors. A large family of hematopoietic cytokines is able to control the proliferation, differentiation and survival of progenitor cells. Interleukin (IL)-3, for example, stimulates the growth of multipotential progenitor cells that will eventually differentiate into granulocytes, macrophages, megakaryocytes and erythrocytes. Their ability to stimulate proliferation works primarily during early hematopoiesis without specifying the final differentiation fate of the progenitor. Other cytokines act at a later stage to direct the final fate of a cell. G-CSF is, for example, critical for the terminal differentiation of myeloid progenitors to neutrophils, and granulocyte-macrophage colony-stimulating factor (GM-CSF) has its actions restricted primarily to neutrophil and monocyte lineages. These late acting cytokines are thought to initially stimulate cellular proliferation followed by differentiation to a non-proliferating mature cell. It is the combinations of cytokines acting on a particular hematopoietic progenitor that will initiate a specific developmental program determining the final differentiation fate of the target cell. These signals result in the activation of distinct downstream signal transduction pathways resulting in changes in the regulation of expression, or activation of several families of transcription factors.

In this study, we developed an ex-*vivo* model system in which cytokine induced differentiation of human hematopoietic progenitors towards neutrophils could be investigated. Maturation in the presence of IL-3 together with G-CSF resulted in increased cell expansion, however under these conditions the number of mature neutrophils was considerable lower, compared to cells treated with G-CSF alone (Fig. 1). This suggests that these two cytokines induce distinct signaling pathways regulating proliferation and/or differentiation. However, data from various cell lines have shown that the intracellular pathways activated by the G-CSF receptor and the IL-3 receptor are highly overlapping [8; 47-50]. The differences observed between these two cytokines could be explained due to differences in signal strength, or possibly the duration of activation of various intracellular signaling pathways. These differences have been observed in the rat pheocchromocytoma PC12 cell line stimulated with either epidermal growth factor (EGF) or nerve growth factor (NGF) (reviewed in [51] and [52]). Both factors induce similar intracellular signal transduction pathways, however EGF induces proliferation, while NGF induces differentiation. However, in this system NGF induces a sustained, whereas EGF induces transient ERK activation. This has led to the proposal that the duration of ERK activation is important for determining whether a particular stimulus may result in a differentiation or proliferation response. Since both IL-3 and G-CSF induce PI3K activation but only G-CSF triggers neutrophil differentiation, a similar mechanism might be possible.

The results obtained from the comparison of G-CSF treatment versus a combined IL-3/G-CSF treatment suggests that decreased proliferation is linked to increased differentiation and *vice versa*. Indeed it has been reported that over-expression of CDK inhibitors $p27^{kip1}$ and $p21^{Cip1}$ in the absence of differentiation agents can lead to terminal differentiation of promonocytic cells (U937) [53]. Furthermore, myeloid progenitor cells isolated from $p27^{kip1}$-deficient mice show significantly increased proliferation and reduced differentiation in response to G-CSF [54]. Additionally, it has been described that retinoic acid induced differentiation of human myeloid cell lines correlates with cell cycle arrest, associated with sequential down regulation of cyclin E, c-myc and upregulation of $p27^{kip1}$ [55]. However our data, utilizing the PI3K inhibitor LY294002, demonstrate that a cell cycle arrest *per se* is not in itself sufficient to induce differentiation (Fig. 3). In the maturation of megakaryocytes, thrombopoietin or c-kit ligand can promote proliferation and differentiation at the same time [56; 57]. An additional study demonstrated that Insulin Growth factor 1 (IGF-1) increased the early development of vitamin D3 induced macrophage differentiation, which was associated with elevated cyclin E levels, hyperphosphorylated Rb protein, and suppression of $p27^{Kip1}$ levels [58]. These data,

together with our findings suggest that the initial steps of differentiation can occur concomitantly with the progression through the cell cycle.

Although a role for PI3K in cellular proliferation has been well established (reviewed in [40]), little is known concerning the potential role of PI3K in differentiation. Mice lacking the regulatory p85α subunit of PI3K have reduced numbers of mature B-cells [59 60]. One mechanism by which PI3K might regulate differentiation is through regulation of the Retinoblastoma protein (Rb) during the maturation process. Rb mediates a block in the G_1-phase of the cell cycle by inhibiting the E2F family of transcription factors, thereby preventing transcription of genes necessary for further progression in the cell cycle [61]. Phosphorylation of Rb results in release of Rb from E2F and transcription can proceed. While having a clearly defined role in transcriptional regulation of the cell cycle, Rb proteins have also been shown to direct the differentiation program in myoblasts, adipocytes and hematopoietic cells, since these cells fail to differentiate in the absence of Rb [62]. Furthermore, high levels of Rb are induced and sustained during erythroid differentiation while Rb is down regulated during granulocytic maturation [63]. PI3K has been previously implicated in regulating E2F-mediated transcription through Rb [64-66]. Additionally, a link between PI3K and p130 Rb-like protein levels has been described [67]. In this manner PI3K might be able to regulated differentiation through regulation of Rb phosphorylation and protein levels. Figure 5, schematically represents the role for PI3K in both proliferation and differentiation during the maturation process. While multiple cytokines and growth factors, such as IL-3, are unable to induce differentiation, they are potent in activating PI3K. Therefore PI3K activity alone is unlikely to be sufficient to induce differentiation.

We have also demonstrated a role for MEK in regulating the proliferation of human $CD34^+$ progenitors towards neutrophils. A potential mechanism underlying this observation for MEK is through the upregulation of cyclin D1 expression [30]. Sustained activation of ERK1 by MEK was demonstrated to be required for the continued expression of cyclin D1 in the G_1 phase of the cell cycle. Another possible target for MEK is the transcription factor c-myc, which is a critical regulator of cellular proliferation [68]. Indeed, it has previously been demonstrated that MEK is required for CSF-1 mediated c-*myc* transcription and cytokine induced proliferation [69]. Activation of ERK by MEK also results in the direct phosphorylation of c-myc, which acts to stabilize the protein [70]. c-myc stability can also be increased through PI3K by inhibiting GSK-3, a kinase which itself can phosphorylates c-myc, targeting it for ubiquitination [70]. A recent study has analyzed the effect of mutation of tyrosine 764 in the intracellular region of the G-CSF in primary mouse bone marrow cells. Cells expressing this G-CSFR mutant responded with reduced proliferation in response to G-CSF, without having an effect upon differentiation [71]. This mutation was also associated with a reduced ERK activity, further supporting a critical role for the MEK-ERK pathway in proliferation (Fig. 5).

In this study, we have analyzed the role of various cytokine-induced intracellular signaling pathways in an ex-*vivo* differentiation model utilizing primary human hematopoietic stem cells. We have demonstrated critical roles for PI3K and MEK in neutrophil maturation, a process involving control of both proliferation and differentiation. Importantly, we have identified PI3K as a novel player in the process of myeloid differentiation. Our data also suggest that cell cycle arrest *per se* does not result in induction of an intrinsic neutrophil differentiation program. Further work will be required to understand the molecular mechanisms underlying these observations.

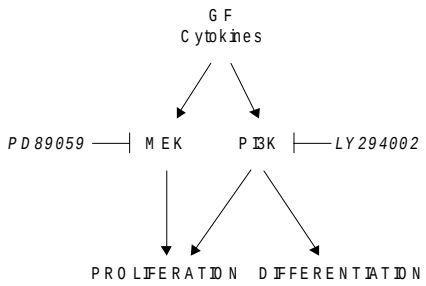


Figure 5. *Schematic representation of the role of PI3K and MEK in maturation of hematopoietic stem cells*. PI3K has a critical role in both proliferation and differentiation. Although PI3K is necessary in these processes, it is probably not sufficient to induce differentiation. MEK has a significant role only in proliferation.

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Chapter 3

Identification and characterization of CKLiK a novel granulocyte Ca^{2+}/calmodulin-dependent kinase

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Blood, 2000, 96, 3215-3223

Abstract

Human granulocytes are characterized by a variety of specific effector functions involved in host defense. Several widely expressed protein kinases have been implicated in the regulation of these effector functions. We employed a PCR-based strategy to identify novel granulocyte specific kinases. A novel protein kinase cDNA with an open reading frame of 357 amino acids was identified with homology to calcium-calmodulin-dependent kinase I (CaMKI). We have termed it CaMKI-like kinase (CKLiK). Analysis of *CKLiK* mRNA expression in hematopoietic cells demonstrated an almost exclusive expression in human polymorphonuclear leukocytes (PMN). Upregulation of CKLiK mRNA occurs during neutrophilic differentiation of CD34+ stem cells. CKLiK kinase activity was dependent on Ca^{2+} and calmodulin as analysed by *in vitro* phosphorylation of cAMP responsive element modulator (CREM). Furthermore, CKLiK transfected cells treated with ionomycin demonstrated an induction of CRE binding protein (CREB) transcriptional activity compared to control cells. Additionally, CaMK-kinaseα enhanced CKLiK activity. *In vivo* activation of CKLiK was shown by addition of IL-8 to a myeloid cell line stably expressing CKLiK. Furthermore inducible activation of CKLiK was sufficient to induce ERK MAP kinase activity. These data identify a novel Ca^{2+}/calmodulin-dependent PMN specific kinase that may play a role in Ca^{2+}-mediated regulation of human granulocyte functions.

Introduction

Human polymorphonuclear leukocytes (PMN), which include neutrophilic and eosinophilic granulocytes, play an important role in host defense against invading microorganisms[1]. Recruitment and activation of these cells *in vivo* occurs in a multistep process that involves many different membrane bound receptors activating an array of diverse intracellular signaling molecules. In short, PMNs in the peripheral blood enter a pre-activated state by interacting with cytokines liberated from the inflammatory locus. This is followed by attachment to the endothelium, which is mediated by the interaction with adhesion molecules expressed on the surface of activated endothelial cells. Release of chemokines at the site of inflammation is responsible for the migration of PMNs to this locus. Finally upon recognition of the inciting agent by PMNs, phagocytosis, secretion of toxic proteins and activation of membrane bound NADPH-oxidase generating reactive oxygen intermediates ensues [2;3]. Furthermore, rapid induction of apoptosis in PMNs and subsequent removal of apoptotic cells are important in the rapid resolution of inflammation [4; 5]. An unfortunate consequence of activation *in vivo* is tissue damage during acute inflammation and, therefore, the activity of granulocytes is under tight control.

PMNs express a wide variety of receptors on their plasma membrane steering the process of priming and activation. Upon binding of inflammatory mediators, such as formyl peptides, lipopolysaccharides, chemokines or cytokines, the receptor transmits a signal to the cell interior resulting in the initiation of a cascade of intracellular events. Phosphorylation of effector molecules by kinases is critical for transducing intracellular signals. Thus far several classes of kinases including (i) serine kinases, such as mitogen activated (MAP) kinases; (ii) lipid kinases such as phosphatidylinositol-3 kinase (PI-3K); (iii) tyrosine kinases including the src kinases; (iv) cAMP-dependent kinases, and (v) Ca^{2+}-dependent kinases, are activated in response to inflammatory mediators in human granulocytes.

A role for these widely expressed kinases in neutrophil functions has been intensively studied utilising pharmacological inhibitors. A role for MAP kinases, $p42^{ERK1}$ and $p42^{ERK2}$ in chemotaxis, respiratory burst and PAF release is suggested, although the use of a pharmacological inhibitor for MEK has resulted in contradictory findings[6; 7]. A clearer role for PI-3K in neutrophil migration and respiratory burst has been demonstrated by use of PI-3K inhibitors wortmannin and LY294002[7-11]. A role for PKC has also been postulated in a variety of granulocyte effector functions and PKA is suggested to be involved in down-regulating the respiratory burst[12; 13]. Recently a role for src kinases in adhesion-dependent degranulation has also been described[14]. Although inhibitory studies support a role for these kinases in regulating neutrophil functions, their activation is not specific for these granulocyte effector functions since they are widely expressed. Furthermore pharmacological inhibitors are often limited in their specificity, making the interpretation of data more complex[15].

Changes in cytosolic free Ca^{2+} are described during activation of several neutrophil responses, such as degranulation, respiratory burst and adhesion. An important role for Ca^{2+} in these processes has been suggested[16; 17] and, therefore, Ca^{2+}-dependent kinases may well be involved. In addition a role for the Ca^{2+}-dependent phosphatase, calcineurin has been shown in the Ca^{2+}-dependent recycling of integrins to the front of migrating neutrophils[18], while a role for calmodulin and CaMKII is suggested in O_2^- production[19].

In this report we describe the identification of a novel protein kinase, which we have termed CKLiK (CaMKI-like kinase). This kinase is predominantly expressed in human PMNs and is regulated by Ca^{2+} and calmodulin. Interleukin-8 (IL-8), which is a potent activator of neutrophil effector functions, induces activation of CKLiK and we show that an inducible active mutant of CKLiK induces ERK MAP kinase activation. These data identify a novel Ca^{2+}/calmodulin-dependent protein kinase expressed in PMNs that may play a role in transducing chemokine-induced signals regulating human granulocyte functions.

Materials and Methods

Cells, reagents and antibodies

COS cells were cultured in Dulbecco's Modified Eagle Medium (Life Technologies, Breda, The Netherlands) supplemented with 8% heat-inactivated FCS. $CD34^+$ stem cells were cultured in Iscoves Modified Dulbecco medium (IMDM) supplemented with 2 mM glutamine, 8% Hyclone and cytokines. Murine myeloid 32D cells were cultured

in RPMI 1640-glutamate supplemented with 8% Hyclone serum (Life Technologies, Breda, The Netherlands) and mouse IL-3[20]. 32D cells were stably transfected with pBabe-HA_CKLiK by electroporation (0.26 V, 1000 msec, 100μFD) and selected with puromycin (1μg/ml; Sigma, Zwijndrecht, The Netherlands) at concentration of 1μg/ml. Ionomycin was purchased from Calbiochem (La Lolla, CA, USA). Calmodulin was a kind gift of Dr. R. de Groot. Monoclonal antibodies, 12CA5 against HA epitope were purchased from Boehringer Mannheim Biochemicals (Almere, The Netherlands). Polyclonal anti-VSV tag antibodies were obtained from Medical & Biological Laboratories (MBL, Japan). Human interleukin-8 (IL-8), Trombopoetin (TPO), fms like tyrosine kinase-3 (FLT-3) ligand and stem cell factor were purchased from PeproTech (Rocky Hill, NJ, USA). Human interleukin-3 (IL-3) and granulocyte colony stimulating factor (G-CSF) were purchased from Strathmann Biotech (Hamburg, Germany) and IL-6 was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Phospho-CREB-Ser133 antibodies were obtained from New England Biolabs Inc. (Beverly, MA, USA). 4-hydroxy-tamoxifen and W7 were purchased from Sigma (Zwijndrecht, The Netherlands) while PD98059 was purchased from Biomol (Plymouth Meeting, PA).

Identification and cloning of granulocyte kinases

For the identification of novel kinases in human granulocytes PCR was performed using degenerate primers as previously described [21]. Forward primers were based around the consensus amino acid sequence HRDLKPEN, which corresponded to the conserved regions in the serine/threonine protein kinase catalytic domain. Oligonucleotides were also designed against the DXWXXG amino acid motif approximately 65 amino acids downstream and used as reverse primers. PCR was performed on granulocyte cDNA with Taq polymerase (Perkin Elmer, Roche Molecular Systems Inc, Branchburg, New Jersey, USA) using excess of degenerate primers (1μg) under conditions of 10mM TRIS-Cl pH 8.3, 50mM KCl, 0.2mM dNTPs and 0.8mM $MgCl_2$. Denaturation, annealing and extension temperatures of 94^0C, 52^0C and 72^0C respectively were used. PCR products of approximately 200 bp were cloned into pGEM-T vector (Promega Corporation, Madison, WI, USA) and sequenced. The identified sequences were screened against the BLAST database (http://www.ncbi.nlm. nih.gov /BLAST).

Full-length cDNA was obtained by screening a λZAPII eosinophilic library [22] with the amplified PCR fragment as a probe. After plaque purification, the pBluescript SK- phagemids containing positive inserts were isolated by *in vivo* excision using M13K07 helper phages (Promega Corporation, Madison, WI, USA), and subsequently characterized by restriction mapping and sequencing.

Isolation human leukocytes

Blood was obtained from healthy volunteers at the donor service of the University Medical Center (Utrecht the Netherlands). Granulocytes were isolated from 0.4% (wt/vol) trisodium citrate (pH 7.4) treated blood as previously described[23]. In short, mononuclear cells were removed from granulocytes by centrifugation over isotonic Ficoll from Pharmacia (Uppsula, Sweden). After lysis of the erythrocytes in an isotonic NH_4Cl solution, neutrophils were washed and resuspended in incubation buffer (20mM HEPES, 132mM NaCl, 6mM KCl, 1mM $MgSO_4$, 1.2mM KH_2PO_4, 5mM glucose, 1mM $CaCl_2$). Granulocytes were incubated for 30 minutes at 37^0C before experiments were performed. Monocytes were further separated from mononuclear lymphocytes by elutriation as described previously[24].

Isolation and differentiation of $CD34^+$ stem cells

$CD34^+$ cells were isolated using the MACS $CD34^+$ progenitor cells isolation kit from Miltenyi Biotech (Auburn, CA). First mononuclear cells were isolated from human umbilical cord blood cells by density centrifugation over a Ficoll solution. Cells were blocked and additionally incubated with a CD34 antibody in a phosphate buffer containing 2mM EDTA and 0,5% BSA (Buffer M). Magnetic beads recognizing the CD34 antibody were added and the suspension was applied to an MS^+ separation column placed in the magnetic cell separator MiniMACS. $CD34^-$ cells were allowed to pass through the column by three wash steps with buffer M. $CD34^+$ cells were eluted with buffer M by removal of the column from the separator. To obtain a pure $CD34^+$ cell population the magnetic separation step was repeated on a second column. After final elution stem cells were counted and cultured. Before differentiation, $CD34^+$ stem cells were proliferated for one week in the presence of TPO (10ng/ml), SCF (50ng/ml), FLT-3 ligand (50ng/ml) and IL-6 (2ng/ml). At day 0 differentiation was started by addition of SCF, FLT-3 ligand,

IL-3 (10ng/ml) and G-CSF (30ng/ml). At day 3, 7, 10, 14 and 17 IL-3 and G-CSF were added and at day 21 only G-CSF was added to the cells. At each point cells were counted, diluted to $0.5*10^6$ cells/ml and if possible samples were taken for mRNA isolation.

RNaseProtection

Total mRNA from human monocytes, lymphocytes, granulocytes and hematopoietic cell lines HL60, U937 and THP1 were isolated. In short, 10^8 cells were lysed in 2ml GIT-C solution (6M guanidine thiocyanate, 25mM sodiumcitrate, 0.5%N'Lauroyl-sacosine, 100mM β-mercapto-ethanol) and RNAs were further isolated by phenol extraction and ethanol precipitation. ^{32}P-UTP labelled antisense RNA transcript, corresponding to the original PCR fragment coding the catalytic domain of CKLiK, was generated using the Riboprobe in vitro Transcription system (Promega Corporation, Madison, WI, USA) and used as a RNA-probe. As internal control a 90 bp antisense RNA probe of GAPDH was used. Total RNAs samples (10μg) were lyophilised and resuspended in 2μl DEPC water. Hybridisation was performed with 10^5 cpm of each antisense RNA probe in 25μl 80% formamide, 40mM PIPES pH 6.4, 400mM NaCl and 1mM EDTA overnight at 45^0C. Subsequently probes were incubated for 1 hour in RNAse buffer (10mM Tris-Cl pH 7.5, 5mM EDTA, 300mM NaCl supplemented with 0.15μl/ml T1 RNase) at 37^0C to degrade unhybridised RNA. The reaction was stopped by addition of 10μl proteinase K (5mg/ml) and 10% SDS. Hybridised (double stranded) RNA was purified by phenol-extraction and ethanol precipitation. The remaining pellet was resuspended in 2μl DEPC water and 2μl RNA loading buffer (80% formamide, 10mM EDTA, 1 mg/ml xylene cyanol, 1 mg/ml bromphenol blue). Samples were heated for 5 minutes at 95^0C and analyzed by polyacrylamide gel electrophoresis.

DNA constructs

Epitope-tagged CKLiK was generated by PCR using the oligonucleotides forward *Xho*I; 5'-CCGCTCGAGTATGGCCCGGGAGAACGGC-3' and reverse *Kpn*I; 5'-CCGGTACCCAAGTAG-CTGACATTACAGG-5') and ligated by *Xho*I/*Kpn*I digest into pMT-HA. HA_CKLiK-309 and HA_CKLiK-296 were also generated by PCR using reverse primers introducing a stopcodon at amino acid 310 or 297 (309:5'-GCATTTCATGCTTGGCACCATT-3',296; 5'-GCTCTAGATCACTGGGCGCTGACG GACTC-3 '). PCR products were cloned into pGEM-T vector and subcloned into pMT2-HA vector. GFP-tagged CKLiK was generated by PCR and recloned in frame into pEGFP-C2 vector (Clontech Laboratories Inc., California, USA). HA_CKLiK was cloned from pMT-HA into pBabe[25] by *BamH*I/ *EcoR*I digest. Untagged CKLiK-296 was obtained by PCR cloned and into pSG5[26]. VSV tagged CaMKKα was generated by PCR on rat brain tissue cDNA using the oligonucleotides 5'-CAGTCGACCAGGAATATCC ACGGACTGA-3' and 5'-ATAGCGGCCGCCGGATGCAGCCTCATCTTC-3' and cloned into the pMT2-VSV vector. Tamoxifen inducible CKLiK construct, ER_CKLiK-296 was generated by PCR and cloned into the pCDNA3-ER-N vector. The constructs for HA_PKB and HA_ERK have been previously described [27]. CREB_GAL4, CREBS133A_GAL4, GAL4CAT constructs were previously described in [28].

In vitro kinase assay

COS cells were transiently transfected with 10μg of HA-tagged CKLiK-WT, CKLiK-309 or CKLiK-296 using calcium phosphate precipitation and the medium refreshed 16 hours later. For the CKLiK kinase assay, 24 hours later cells were stimulated with or without ionomycin for 5 minutes, washed twice with cold PBS and lysed in a buffer containing, 1% NP-40, 20mM Tris-Cl pH 7.5, 150mM NaCl, 10% glycerol and 10mM $MgCl_2$ supplemented with 10μg/ml aprotinin, 1mM leupeptin, 1mM PMSF, 1mM benzamidine and 1mM Na_3VO_4. 32D cells stably expressing CKLiK-WT, were mIL-3 and serum starved for 4 hours and stimulated with hIL-8 (10^{-7}M). Cells were washed and lysed as buffer described above. Lysates were pre-cleared for 20 min with protein-A beads and immunoprecipitated with 12CA5 antibody. Immunoprecipitates were washed twice in lysis buffer and twice in dilution buffer containing 10mM Tris-Cl pH 7.4 and 20mM $MgCl_2$. Kinase assay was performed in the presence 10mM Tris-Cl pH7.4, 20mM $MgCl_2$, 1mM DTT, 50μM ATP, 0.1μl γ-^{32}P-dATP in the presence or absence of 1mM

$CaCl_2$ and 0.5μg calmodulin, 5μg CREMβ (33kDa) or mutated CREMτ-S117A (42kDa) as substrate [29]. PKB and ERK kinase assay were performed as described previously[27].

CAT assay

COS cells were transiently transfected with 4μg CKLiK-WT, -309 or -296, together with 2μg CREB_GAL4 or CREB-S133A_GAL4 fusion expression plasmids and 2μg GAL4CAT reporter construct using calcium phosphate precipitation. After 16 hours cells were washed twice and medium refreshed. Eight hours later cells were incubated overnight with 1μM ionomycin. Cells were lysed by repeated freeze-thawing in 100 μl 250mM Tris-Cl pH7.4 and 25mM EDTA. 50μl of cellular extract was incubated in a total volume of 100μl containing 250mM Tris-Cl 7.4, 2% glycerol, 0.3mM Butyryl Coenzyme A and 0.05μCi ^{14}C-Chloramphenicol for 2 hours at 37^{0}C. Reaction products were extracted using 400μl xylene/pristane (1:2) and the percentage of acetylated products was determined using liquid scintillation counting. A lacZ reporter was used to correct for transfection efficiency. Data represent at least 3 independent experiments ± SEM.

Detection of GFP fusion-protein localization

Plasmids encoding CKLiK and active CKLiK-296 containing N-terminal enhanced Green Fluorescent were transiently transfected in COS cells which were grown on coverslips. 36 hours after transfection COS cells were washed with PBS, fixed with 70 % ice-cold methanol, and examined by fluorescence microscopy.

Western blotting

After stimulation 10^6 neutrophils/point were lysed in 1x Laemmli sample buffer. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were probed with anti-phospho-CREB-Ser133 antibodies (New England Biolabs Inc., Beverly, MA, USA) and swine anti-rabbit peroxidase-conjugated antibodies (DAKO, Denmark), following detection by enhanced chemiluminescence (ECL, Amersham). Protein kinase expression controls were performed by utilizing 12CA5 and rabbit anti-mouse peroxidase conjugated antibodies (DAKO, Denmark) to detect HA_CKLiK, HA_ERK and HA_PKB. Detection of CaMKK-VSV was performed with anti-VSV antibodies and swine anti-rabbit peroxidase-conjugated antibodies. Hybridization's were followed by detection with enhanced chemiluminescence (ECL, Amersham).

Results

Cloning and expression of CKLiK

While several kinases have been implicated in the control of granulocyte effector functions[12; 30], most of these proteins are widely expressed. To identify novel granulocyte specific kinases, degenerate primers against conserved kinase catalytic domains were utilized as previously described[21]. The central core of the catalytic domain consists of subdomains VI and IX and contains two well-conserved amino acid triplets APE and DGF[31]. Degenerate oligonucleotides were designed against subdomains VI and IX and used in a PCR on cDNA of human PMNs. Under stringent conditions PCR products of approximately 200bp were amplified, cloned and sequenced. All cloned PCR products contained the two conserved amino acid triplets and many were identical to previously identified kinases (data not shown). However one clone exhibited homology with Calcium/Calmodulin-dependent kinase I (CaMKI) and subsequently termed CKLiK (CaMKI Like Kinase).

To isolate full-length *CKLiK* cDNA, a human eosinophil library was screened with the amplified PMN cDNA PCR fragment. Several positive clones were isolated and sequenced, and one cDNA of 1.7 kb contained a 357 amino acid open reading frame encoding a protein of 40 kDa as determined by *in vitro* transcription/translation (data not shown). In Figure 1 the complete cDNA and the translated amino acid sequence is depicted. The context of the ATG codon was in good agreement with eukaryotic translation start-sites[32]. As shown in Figure 2a comparison of the predicted CKLiK protein sequence in BLAST database revealed a 77% homology of CKLiK with CaMKI on amino acid level. The greatest divergence between the two sequences occurs at the N- and C-terminus.

To determine the distribution of CKLiK expression we analyzed mRNA levels in primary human leukocytes and hematopoietic cell lines by RNase protection (Fig. 2b). mRNA from lymphocytes, which includes T and B-cells, monocytes and PMNs were analyzed. Additionally three myeloid cell lines HL60 (promyelocytic leukemia), U937 (histiocytic lymphoma) and THP1 (acute monocytic leukemia) were analysed for *CKLiK* expression. As an internal control we utilized a GAPDH probe (Fig 2b; lower panel). Human PMNs contain high levels of *CKLiK* (Fig.2b; lane 3), while little to no expression was observed in monocytes and lymphocytes (Fig. 2b; lane 1-2). In the different lymphoid and myeloid cell lines we couldn't detect *CKLiK* (Fig. 2b; lane 4-6). Since CKLiK expression is apparently only detected in mature myeloid cells, we analyzed the expression of CKLiK during differentiation of $CD34^+$ cord blood stem cells towards the neutrophilic lineage. Although after ten days of differentiation CKLiK mRNA was present, increased mRNA levels are indeed detected during terminal differentiation at day 28 (Fig 2c). Similar results were obtained during the differentiation towards eosinophils in the presence of interleukin-5 (data not shown).

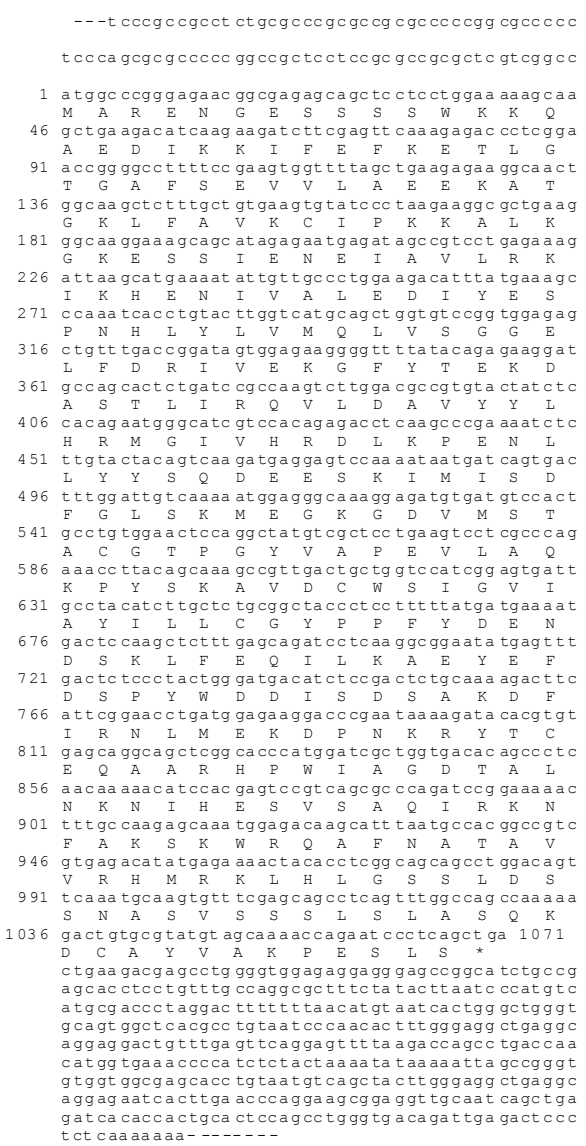

Figure 1: *CaMKI-like kinase, CKLiK, nucleotide sequence and open reading frame.*

Full-length sequence of the novel kinase was obtained by screening an eosinophil cDNA λZAPII phage library using the initial degenerate PCR product as a probe. Sequencing was performed and the open reading frame was analyzed.

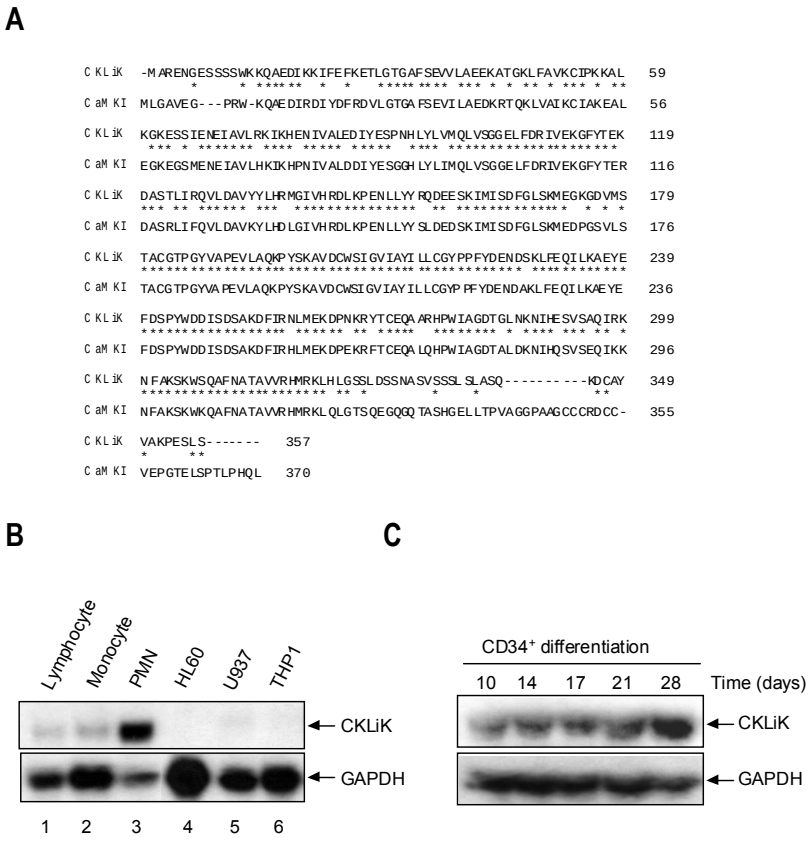

Figure 2: *CKLiK is homologous to CaMKI and highly expressed in human granulocytes.*
(A) Alignment of CKLiK with CaMKI. Residues identical to CaMKI are indicated by a " * " and gaps in the alignment are indicated with a dash (-).The amino acid sequence is indicated on the right. **(B)** Distribution of CKLiK was determined by RNase Protection. The PCR product encoding the catalytic domain of CKLiK was used to generate a radiolabeled RNA probe. RNA isolated from primary hematopoietic cells and cell-lines were hybridized for CKLiK expression. A GAPDH probe was used as a control. Lanes 1-3 represent primary leukocytes (1; lymphocytes, 2; monocytes, 3; PMNs) and lane 4-6 represent lymphoid and myeloid cell lines as indicated. **(C)** Expression of CKLiK in cord blood derived $CD34^+$ stem cells during differentiation towards neutrophils was analyzed by RNase Protection as in (B). Timepoints indicate the number of days differentiated towards neutrophilic lineage in the presence of G-CSF.

CKLiK kinase activity is dependent on Ca^{2+}/calmodulin and can regulate CREM and CREB

As mentioned, CKLiK has homology with the Ca^{2+}/calmodulin-dependent kinase family. The CaM kinases belong to the serine/threonine class of kinases and include CaMKI, CaMKIV and the upstream CaMKK[33]. To investigate whether Ca^{2+} and calmodulin regulated CKLiK we utilized CRE binding protein (CREB) or CRE modulator (CREM) as downstream targets. CREB and CREM belong to the basic-leucine-zipper (bZip) class of transcription factors. Serine 133 for CREB and serine 117 for CREM have been shown to be phosphorylated by several kinases including CaMKI *in vitro*[2 8; 34], resulting in interaction with transcriptional co-activators and induction of transcription [35].

CKLiK kinase activity was analyzed by *in vitro* kinase assay, utilizing CREM as a substrate and by transcriptional reporter assays utilizing CREB_GAL4 fusion constructs [28]. CKLiK was immunoprecipitated from transfected COS cell lysates and immunecomplex kinase assays performed (Fig 3a). CREMβ (33kDa) or a mutant CREMτ (42kDa) which has a serine to an alanine substitution on position 117 (CREMτ-S117A), were utilized as substrates (Fig. 3a). Due to the different size of the two isoforms, this allows the specific phosphorylation of Ser-117 to be analyzed within a single assay. The assay was performed in the absence or presence of Ca^{2+} and calmodulin. As a positive control for this assay we utilized recombinant CaMKI, which potently phopshorylated CREMβ. Due to the high level of recombinant CaMKI, some background phosphorylation of the mutated form of CREM (CREMτ-S117A)

was detected. A clear phosphorylation of CREMβ but not CREMτ-S117A by CKLiK was observed in the presence of Ca^{2+}/Calmodulin and this was enhanced by prior ionomycin treatment (Fig. 3a). A small activation of CKLiK was also observed by ionomycin treatment in the absence of Ca^{2+}/Calmodulin during the kinase assay (data not shown). For transcriptional reporter assays, cells were transfected with HA_CKLiK, a GAL4-CAT reporter construct in combination with a CREB_GAL4 fusion construct or a mutant thereof, in which the phosphorylation site (serine-133) is substituted by an alanine (CREB-S133A_GAL4) (Fig. 3b). Ionomycin treatment resulted in a 5-fold induction of reporter-activity in cells expressing CKLiK with only a modest increase in control cells (Fig. 3b; left panel). CKLiK had no effect when using the mutant form of CREB (Fig 3b; right panel). These results demonstrate that CKLiK is regulated by Ca^{2+}/calmodulin and that active CKLiK can directly phosphorylate CREM, and activate CREB enhancing it's transactivation capacity.

C-terminal truncation of CKLiK results in a constitutively active mutant

Sequence alignment with CaMKI and the results described above suggests that CKLiK contains a calmodulin binding domain regulating its activity. To investigate this hypothesis we generated two truncation mutants (Fig. 4a). Truncation of the predicted calmodulin-binding domain (CKLiK-309) should result in an inactive kinase. Indeed mutant CKLiK-309, lacking residues 310-357, remains inactive even in the presence of Ca^{2+}/calmodulin, unable to phosphorylate CREM (Fig. 4b) or to activate CREB mediated transcription after ionomycin stimulation (Fig. 4c). However, if CKLiK also contains an auto-inhibitory domain similar to CaMKI then, removal of this domain (CKLiK-296) should generate a constitutively active kinase. As was predicted truncation of residues 297-357 generated a constitutively active CKLiK. CREM was phosphorylated by CKLiK-296 in the absence of Ca^{2+}/calmodulin (Fig. 4b) and CKLiK-296 greatly enhanced CREB mediated transcription, which was not further enhanced by ionomycin addition (Fig 4c). Since we demonstrated that activated CKLiK could phosphorylate CREM and activate CREB mediated transcription we were interested to determine whether CKLiK was located in the cytoplasm or nucleus. We generated Green

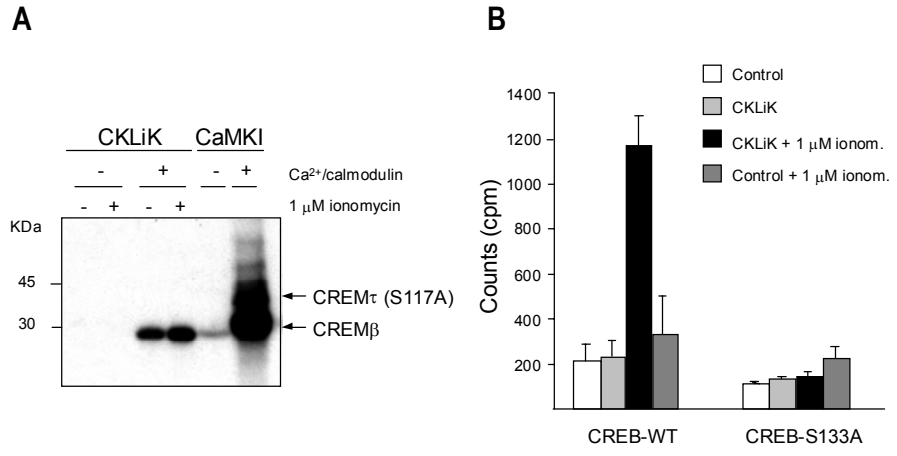

Figure 3: *CKLiK activity requires Ca^{2+}/Calmodulin and regulates the transcription factors CREM and CREB.*

(A) COS cells were transfected with 10μg HA_CKLiK. Recombinant CaMKI was used as a positive control. Cells were stimulated with or without 1μM ionomycin as indicated. HA_CKLiK was immunoprecipitated from whole cell lysates and kinase assays were performed in the presence or absence of Ca^{2+}/calmodulin as indicated. CREMβ-WT (33kDa) and mutated CREMτ-S117A (42kDa) were used as substrate. Data represent one of four independent experiments. **(B)** Cells transfected with GAL4-CAT reporter construct (2μg) and fusion construct of CREB_GAL4 or CREB-S133A_GAL4 (2μg) (indicated as CREB-WT and CREB-S133A respectively), were co-transfected, with CKLiK (4μg) or with control vector as indicated. Stimulation with or without 1μM ionomycin and CAT reporter assays were performed as described in Materials and Methods. Data are indicated as counts per minute (cpm) and represent at least three independent experiments ± S.E.M.

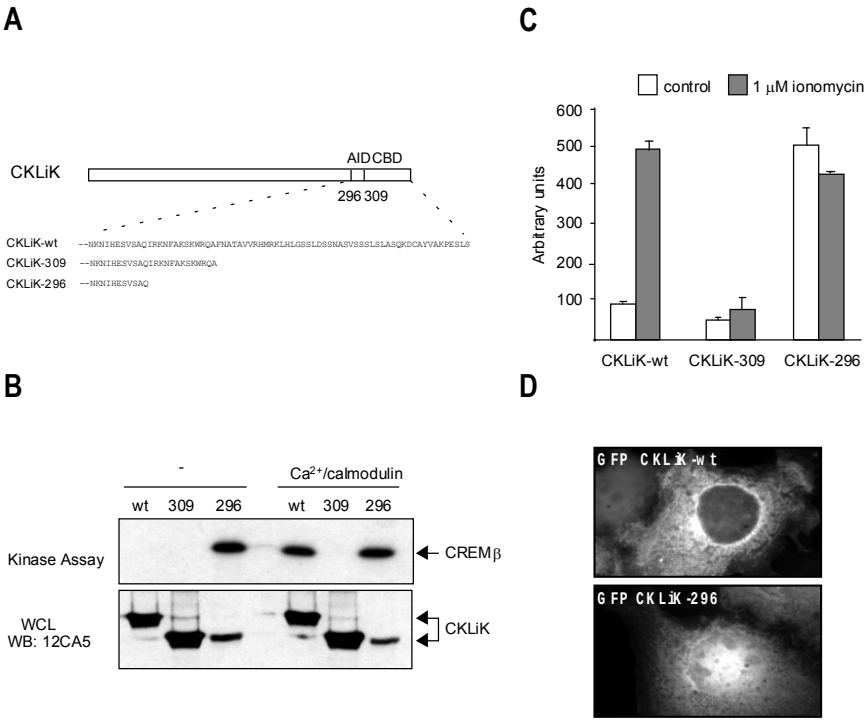

Figure 4: *A constitutively active CKLiK mutant activates CREB and CREM independently of Ca^{2+} /Calmodulin.*
(A) Schematic representation of the predicted calmodulin binding domain (CBD) and the autoinhibitory domain (AID) of CKLiK. Truncation mutants of these two domains are indicated. In CKLiK-309 the predicted CBD and in CKLiK-296 the predicted CBD and AID were deleted. **(B)** *In vitro* kinase assays. COS cells were transfected with 10μg HA_CKLiK-WT (wt), HA_CKLiK-309 (309) or HA_CKLiK-296 (296) as indicated. Immunoprecipitations were performed and CREM phosphorylation was detected in the presence or absence of Ca^{2+}/calmodulin as indicated. Expression of the different forms of CKLiK was detected by western blotting of cell lysates with 12CA5 antibody. **(C)** Reporter assays. Cells were transfected with GAL4-CAT reporter construct, CREB-GAL4 (2μg) and co-transfected, with CKLiK-WT or mutated CKLiK-309 or CKLiK-296 (4μg) as indicated. 18 hours prior to harvesting cells were stimulated with (grey bars) or without (white bars) 1μM ionomycin and reporter-assays were performed. Data represent at least three independent experiments ± S.E.M, and was corrected for transfection efficiency. **(D)** Active CKLiK is localized in the nucleus. Cells were grown on coverslips and transfected with CKLiK and active CKLiK-296 containing N-terminal enhanced Green Fluorescent Protein (eGFP). 36 hours after transfection cells were examined by fluorescence microscopy for CKLiK localization.

Fluorescent Protein (GFP) containing fusion constructs of CKLiK-wt and the active CKLiK-296. COS cells were grown on coverslips and transfected with CKLiK and CKLiK-296 containing N-terminal GFP fusion. Localization was examined by fluorescence microscopy (Fig. 4d). GFP-CKLiK was clearly located in the cytoplasm, whereas the active form of CKLiK exhibited a strong nuclear fluorescence. Since the active form of CKLiK is detected in the nucleus it suggests that CKLiK may regulate transcription through nuclear localization.

Ca^{2+}/calmodulin kinase kinaseα enhances CKLiK activity

Although the binding of Ca^{2+}/calmodulin is known to activate CaM kinases, an additional activation of CaMKI and CAMKIV by CAMK kinase (CAMKK) has previously been described[36-38]. CaMKK phosphorylates CAMKI and CaMKIV in the activation-loop at position Thr-177 and Thr-196/Thr-200 respectively, stabilizing and thereby enhancing kinase activity. CKLiK contains a threonine at position 180 in alignment with Thr-177 of CaMKI and Thr-200 of CAMKIV. To investigate the effect of CAMKKα on the CKLiK activity we performed kinase and reporter assays. Co-transfection of CaMKKα with CKLiK resulted in an enhanced CREM phosphorylation (Fig. 5a; right panel). In the absence of Ca^{2+}/calmodulin in the kinase assay, CaMKKα enhanced CKLiK induced CREM phosphorylation (Fig. 5a; left panel). Similar results were found utilizing reporter assays (Fig. 5b). Co-transfection

of CKLiK with CaMKKα resulted in an elevated reporter activity both in untreated (Fig. 5b; left panel) and ionomycin treated cells (Fig. 5b; right panel). CaMKKα alone had no effect on CAT activity, suggesting that CaMKKα cannot itself activate CREB directly. Interestingly CaMKKα can enhance CKLiK activity in the absence of Ca^{2+} in the *in vitro* kinase assay. Indeed there are indications for CaMKKα induced CaMK activity in the absence of Ca^{2+} and calmodulin [33; 39]. However, since there is Ca^{2+}/calmodulin present in the transfected cells, phosphorylation of CKLiK by CaMKKα may lead to stabilization of the CKLiK-Ca^{2+}-calmodulin complex resulting in enhanced kinase activity.

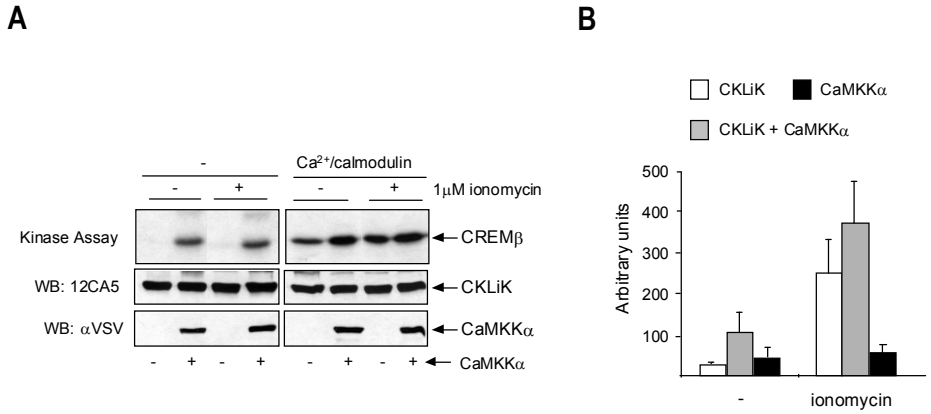

Figure 5: *CaMKKα enhances CKLiK activity.*

(A) HA_CKLiK (8μg) was transfected in COS cells with or without CaMKKα_VSV (2μg). Prior to harvesting, cells were stimulated with or without 1μM ionomycin as indicated. HA_CKLiK was immunoprecipitated and kinase assays were performed using CREM as substrate in the presence or absence of Ca^{2+}/calmodulin. Equal protein expression was demonstrated by western blotting. For CKLiK antibody 12CA5 (middle panel) and for CaMKKα antibody anti-VSV (bottom panel) is used. **(B)** CREB_GAL4 and CKLiK (4μg) were co-transfected without or with CaMKKα (2μg) as indicated. CaMKKα was transfected without CKLiK as a negative control. 18 hours prior to harvesting, cells were stimulated with or without 1μM ionomycin and reporter-assays were performed. Data represent at least three independent experiments ± S.E.M and are corrected for transfection efficiency.

Activation of CKLiK by IL-8 in bone marrow derived myeloid precursor cells

Since agonist stimulation of G-protein coupled receptors results in changes of $[Ca^{2+}]_i$, these receptors are potential activators of CKLiK. The IL-8 receptor is highly expressed on human neutrophils and IL-8 can activate neutrophil effector functions, such as chemotaxis. In order to investigate whether IL-8 can activate CKLiK, we utilized a 32D model system. 32D cells are IL-3-dependent bone marrow derived myeloid precursor cells [40]. Since these cells express endogenous IL-8R [41] we generated stable 32D cell lines expressing HA_CKLiK and used these cells to measure IL-8 induced CKLiK activity. IL-8 was indeed able to induce an increase in intracellular Ca^{2+} in these cells (Fig. 6a). IL-8 stimulation of HA_CKLiK expressing 32D cells, resulted in a rapid transient increased CKLiK activation (Fig. 6b), whereas in Ca^{2+}-depleted cells, IL-8 induced CKLiK activity was completely abolished (data not shown). CKLiK activation parallels the rise in intracellular Ca^{2+} induced by IL-8 (Fig. 6a). Additionally, W7, an antagonist of calmodulin completely inhibited the IL-8 induced CKLiK activity (Fig 6c). CKLiK activity was also inhibited by W7 in the ionomycin treated cells, indicating that calmodulin is indeed critical for the CKLiK kinase activity stimulated by agents, which induce a Ca^{2+} influx.

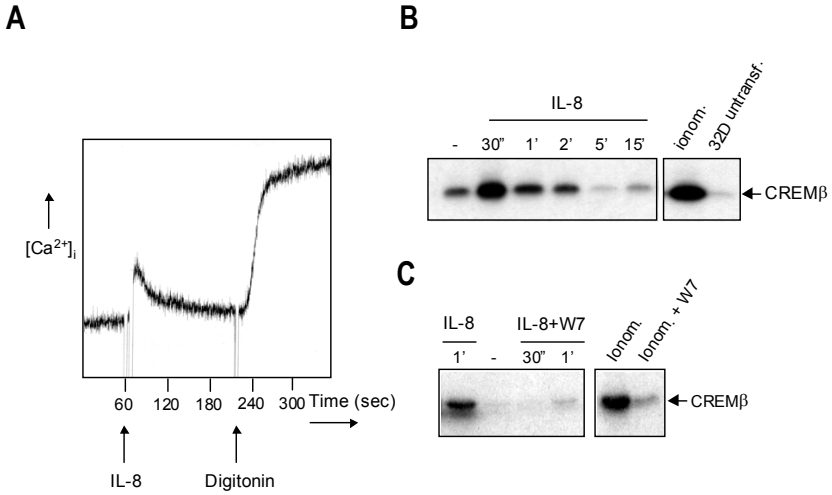

Figure 6: *IL-8 stimulation of bone marrow derived myeloid precursor cells activates CKLiK*
(A) Ca^{2+} response of 32D cells to hIL-8 (10^{-7}M). Cell were incubated with INDO-AM for 45 minutes and washed twice. $[Ca^{2+}]_i$ concentrations were measured by dual excitation at a wavelength of 340nm and detected at 390nm using a Hitachi F4500 fluorescence spectrophotometer. Digitonin was added for 100% $[Ca^{2+}]_i$ as indicated. **(B)** IL-8 induces CREB phosphorylation in 32D cells stably expressing wild type HA_CKLiK. Cells were starved for four hours and stimulated with IL-8 (10^{-7}M) for indicated time periods. HA-tagged CKLiK was immunoprecipitated and kinase assays performed in the absence of Ca^{2+}/calmodulin utilizing CREM as a substrate. Ionomycin treated and untransfected 32D cells were used as positive and negative controls respectively. Data represent one of at least 3 independent experiments. **(C)** W7 inhibits CKLiK kinase activity. HA_CKLiK stable 32D cells were starved and treated for 15 minutes with or without the calmodulin inhibitor W7 prior to IL-8 (10^{-7}M) stimulation. Immunecomplex kinase assays were performed as in (B).

Activation of downstream signaling pathways by CKLiK

To identify potential downstream targets of CKLiK, we analyzed the effect of CKLiK on the activation of the ERK1 MAP kinase and Protein kinase B (PKB), an effector of PI-3K. The regulation of ERKs and PKB by $[Ca^{2+}]_i$ has been demonstrated in several systems[42; 43]. Cells were transfected with HA-tagged-ERK or -PKB and co-transfected with constitutively active CKLiK (CKLiK-296) or CaMKKα and immunecomplex kinase assays were performed. Stimulation with 20% FCS for 10 minutes was used as a positive control. Co-transfection of CKLiK-296 had no effect on PKB activity, while co-transfection of CaMKKα potently activated PKB similar to levels induced by 20% FCS (Fig. 7a; upper panel). This is in agreement with the direct PKB phosphorylation by CaMKK described by Yano et al.[42]. Interestingly however, co-transfection of

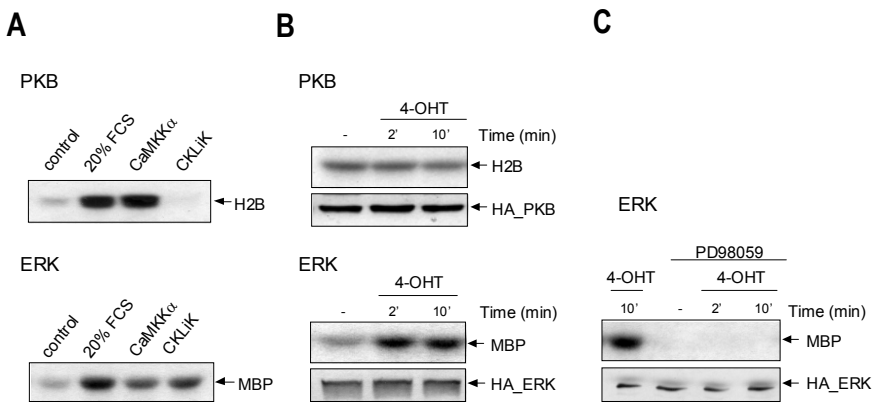

Figure 7: *CKLiK activates ERK1 MAP kinase but not PKB.*

(A) HA_PKB or HA_ERK1 (2μg) were transfected in COS cells with or without CaMKKα (8μg) or CKLiK-296 (8μg) as indicated. 20 hours prior to harvesting cells were serum starved. Unstimulated and 20%FCS treated cells were used as controls. HA_PKB or HA_ERK were immune precipitated and kinase activity was measured using Histone 2B (H2B) or Myelin Basic Protein (MBP) as a substrates. Data represent one of at least four independent experiments. **(B)** HA_ERK1 or HA_PKB (2μg) were co-transfected with ER_CKLiK-296 (8μg) in COS cells. Prior to harvesting cells were stimulated with 4-OH tamoxifen (4-OHT) for 2 or 10 minutes. Immunoprecipitations and kinase assays were performed as in (A) using H2B or MBP as a substrate. Equal expression of HA_PKB and HA_ERK were analyzed by western blotting of whole cell lysates with 12CA5 antibody. **(C)** PD098059 inhibits the CKLiK induced ERK activity. COS cells were transfected with HA_ERK1 (2μg) and ER_CKLiK-296 (8μg). Cells were treated with or without the MEK inhibitor PD098059 (50μM) for 15 minutes prior to stimulation with 4-OHT for indicated time-periods. Cells were lysed and immunecomplex kinase assays were performed as previously described. Equal expression of HA_ERK1 was analyzed by western blotting.

constitutively active CKLiK-296 resulted in an increase in ERK1 activation (Fig. 7a; lower panel). To avoid the possibility of autocrine effects due to over-expression of active CKLiK, we constructed a tamoxifen-inducible active CKLiK (ER_CKLiK-296).

When transfected into cells, ER_CKLiK-296 is actively repressed by binding of heat-shock proteins. Upon addition of the oestrogen derivate, 4-hydroxy-tamoxifen (4-OHT), heat-shock proteins dissociate and CKLiK-296 is directly activated [44]. This system has been successfully utilized for other protein kinases [45]. Cells were transfected with ER_CKLiK-296 and HA epitope tagged ERK1 or PKB. Prior to harvesting cells were stimulated with 4-OHT and ERK/PKB kinase activity was measured. After two minutes of treatment an increased ERK activation was detected, whereas no PKB activation occurred (Fig. 7b). The CKLiK induced ERK activity could be blocked by the MEK inhibitor PD98059 (Fig. 7c), suggesting that CKLiK activates signaling molecules upstream in the MAPK pathway. Thus, these results suggest a role for CKLiK in regulation of ERK MAPK kinases at the level of MEK or higher.

Discussion

In this paper we describe the identification and characterization of a novel protein serine kinase which is highly expressed in human PMNs. These cells, which include neutrophils and eosinophils, are characterized by a variety of specific effector functions that regulate host defense. While several protein kinases, have been previously postulated to regulate some of these effector functions through pharmacological inhibition studies, these proteins are ubiquitously expressed and thus more likely to be involved in general cellular processes. Furthermore the use of pharmacological inhibitors is often complicated by aspecific effects [15; 46].

By utilizing degenerate PCR primers against conserved catalytic kinase domains in a polymerase chain reaction on cDNA templates from human granulocytes, we have identified a novel protein kinase with an open reading frame of

357 amino acids (Fig.1). This novel kinase shows homology with Calcium/calmodulin-dependent Kinase I (CaMKI) and therefore we have termed this kinase CaMKI-like kinase (CKLiK). CaMKI belongs to the family of Ca^{2+} and calmodulin-dependent kinases, which includes CaMK I, II and IV as well as myosin light chain kinase, phosphorylase kinase and elongation factor 2 kinase [47; 48]. CaMKI itself has a wide tissue distribution and can phosphorylate a number of substrates *in vitro*, including the synaptic vesicle-associated proteins synapsin 1 and synapsin 2 [49] and cAMP response element binding protein (CREB)[28]. Interestingly, analysis of CKLiK mRNA in hematopoietic cells, revealed high and almost exclusive expression levels in PMNs, suggesting a role for CKLiK in human granulocytes (Fig. 2). Upregulation of CKLiK mRNA was also observed in cord blood $CD34^+$ stem cells differentiated towards neutrophils, which may indicate the importance of this kinase in terminally differentiated myeloid cells. In granulocytes, changes in $[Ca^{2+}]_i$ have been associated with multiple functions, including degranulation, phagosome-lysosome fusion, regulation of cytoskeletal binding proteins and transcriptional control [16; 17]. These are thus potential processes whereby CKLiK may play a role.

Based on crystal structure and mutational-analysis of CamKI a model has been proposed for CaM Kinases regulation [50; 51]. When Ca^{2+} levels rice within the cytosol, calmodulin binds Ca^{2+} and is capable of interacting with calmodulin binding proteins such as CaMK. Inactive CaMKs are in a folded configuration whereby the N-terminus acts as a pseudo-substrate by interacting with an auto-inhibitory domain. Upon Ca^{2+} recruitment, calmodulin binds to the calmodulin-binding-domain located on the C-terminal of CaMK, which then results in unfolding and auto-phosphorylation of CaMK. It appears that CKLiK is also similarly regulated, since truncation of the predicted calmodulin binding domain or the auto-inhibitory domain resulted in an inactive and constitutively active CKLiK respectively (Figure 4).

Additionally we show that CKLiK can directly activate two members of the CREB/ATF family of transcription factors, CREM and CREB by phosphorylation of serine residues 117 and 133 respectively (Fig. 3). Although CKLiK is located in the cytoplasm, the constitutively active mutant was clearly translocated to the nucleus (Fig. 4d). Possibly this enhanced nuclear translocation uncouples CKLiK from normal regulation resulting in enhanced transcriptional activation. For CaMKIV it has been suggested that it can phosphorylate CREB *in vivo* since it shows nuclear localisation[52]. Although a role for CREB in myeloid cells has been proposed in mediating cytokine or chemokine effector functions [53-56], it remains to be determined if CREB and CREM are physiological substrates for CKLiK in human granulocytes. Indeed we were able to demonstrate CREB phosphorylation by IL-8 in human PMNs (data not shown).

Co-transfection of CaMK kinase (CaMKKα) resulted in an enhanced CKLiK activity measured by *in vitro* phosphorylation of CREM and *in vivo* CREB mediated transcription activation. Enhancement of CKLiK activity by CaMKK is possible via phosphorylation of the threonine residue on position 180, since this is in consensus with threonine residues 177 and 196/200 of CaMKI and CaMKIV respectively. Interestingly CaMKKα induced CKLiK activity in the absence of Ca^{2+} in the *in vitro* kinase assay (Fig. 5). There is indication of the generation of Ca^{2+}-independent CaMKIV activity by CaMKKα, however this is not the case for CaMKI [33; 39]. What the mechanism is for generating this enhanced CKLiK activity is unclear, although this may be a relevant *in vivo*. As previously mentioned it may be due to stabilization of the CKLiK-Ca^{2+}- calmodulin complex allowing for example a prolonged kinase activity similar to the regulation of CaMKII [57], which might be needed for regulation of transcription.

IL-8 induced CKLiK activation is very rapid and corresponded to the IL-8 induced rise in $[Ca^{2+}]_i$ (Fig. 6). The IL-8 receptor is highly expressed on human neutrophils and could be a possible upstream physiological receptor for CKLiK. IL-8 is known to induce chemotaxis, and recently a role for intracellular Ca^{2+} in IL-8 induced migration has been described indicating a possible role of CKLiK in this process [58; 59]. A role for Ca^{2+} and calmodulin-dependent kinases in apoptosis has also been recently demonstrated, since CaMKK was found to directly activate PKB and thereby rescue apoptosis[42]. While we found no activation of PKB by CKLiK, we demonstrated an elevated ERK activity by inducing CKLiK activation, which could be blocked by the MEK inhibitor PD098059 (Fig. 7). As previously eluded to, pharmacological inhibitory studies have suggested that ERK MAP kinases possibly play a role in chemotaxis, or respiratory burst and PAF release [6; 7; 60]. The mechanism by which CKLiK can activate ERKs however remains to be resolved, although it presumably occurs upstream of ERK.

In conclusion the approach of degenerate PCR resulted in a successful isolation of a novel PMN protein kinase. We have demonstrated that this kinase is highly and predominantly expressed in human PMNs and terminally

differentiated myeloid stem cells and that Ca^{2+}/calmodulin regulates its activity. IL-8, which is a potent activator of neutrophil effector functions, is capable of activating CKLiK in bone marrow derived myeloid cells. Furthermore a role for CKLiK is suggested in regulation ERK MAP kinases, since induced CKLiK activity was sufficient to activate ERK1. Therefore the restricted expression of CKLiK in granulocytes can allow the transduction of granulocyte specific signals induced by a general signal transduction event: the rise in $[Ca^{2+}]_i$, initiated by inflammatory mediators.

Acknowledgements

We would like to thank Rolf de Groot for supplying the CREMτ, CREMβ-S117A and CaMKI proteins and the GAL4 reporter constructs, CREAL4 and CREB-S133A_GAL4 fusion constructs. We would also like to thank Sigrid Oomen and Ivo Touw (Dept. Hematology, Erasmus University Rotterdam) for technical assistance and advice in the generation of 32D stable cell lines.

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Chapter 4

Role of Ca^{2+}/calmodulin-regulated signaling pathways in chemoattractant induced neutrophil effector functions

Comparison with the role of phosphatidylinosiyol-3 kinase

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Submitted

Abstract

In human neutrophils, both changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and activation of phosphatidylinositol-3 kinase (PI3K) have been proposed to play a role in regulating cellular function induced by chemoattractants. In this study we have investigated the role of $[Ca^{2+}]_i$ and its effector molecule calmodulin in human neutrophils. Increased $[Ca^{2+}]_i$ alone was sufficient to induce phosphorylation of extracellular signal-regulated protein kinase 2 (ERK2), p38 mitogen activated kinase (p38 MAPK), Protein Kinase B (PKB) and Glycogen Synthase Kinase-3 α (GSK-3α). Inhibition of calmodulin using a calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), did not effect fMLP induced ERK, p38 or GSK-3 phosphorylation, but attenuated fMLP induced PKB phosphorylation. PCR analysis of human neutrophil cDNA, demonstrated variable expression of members of the Ca^{2+}/calmodulin-dependent kinase family. The roles of calmodulin and PI3K in regulating neutrophil effector functions were further compared. Neutrophil migration was abrogated by inhibition of calmodulin, while no effect was observed when PI3K was inhibited. In contrast, production of reactive oxygen species was sensitive to inhibition of both calmodulin and PI3K. Finally, we demonstrate that chemoattractants are unable to modulate neutrophil survival, despite activation of PI3K and elevation $[Ca^{2+}]_i$. Taken together, our data indicate critical roles for changes in $[Ca^{2+}]_i$ and calmodulin in regulating neutrophil migration and respiratory burst and suggest that chemoattractant induced PKB phosphorylation may be mediated by a Ca^{2+}/calmodulin sensitive pathway in human neutrophils.

Introduction

Neutrophils form a first line of host defense in the human immune system being recruited to inflammatory sites in response to infection or tissue injury. Here they phagocytose and kill invading pathogens [1-3]. One of the responses of neutrophils to inflammatory mediators such as chemoattractants, is the migration towards the site of infection. This migration involves firm adhesion and attachment to the endothelium, diapedesis and interaction with extracellular matrix proteins [4]. Secondly, activated neutrophils initiate the NADPH oxidase system, which causes reactive oxygen species (ROS) formation, resulting in efficient killing of pathogens [5]. For resolution of inflammation, removal of neutrophils by programmed cell death is essential to avoid tissue damage, which can be caused by excessive release of granule proteases or inappropriate production of ROS [6].

Chemoattractants are potent activators of neutrophil effector functions. In neutrophils they stimulate G-protein coupled receptors (GPCRs) which in turn activate the trimeric G-proteins. Exchange of guanosine diphosphate (GDP) with guanosine triphosphate (GTP) bound to the Gα subunit, results in dissociation of the Gβγ heterodimer, which subsquently leads to the activation of phospholipase Cβ (PLCβ)2 and PLCβ3, resulting in hydrolysis of $PI(4,5)P_2$ (PIP_2) into diacylglycerol (DAG) and inositol-3 phosphate (IP_3) [7;8]. The dissociation of the Gβγ subunit can also result in activation of phosphatidylinosytol-3-OH kinase gamma (PI3Kγ) [9;10]. However it has also been described that chemoattractant induced GPCR stimulation can also activate the p85-associated Class 1A PI3K, through an as yet undefined mechanism [11;12].

Both PLCβ and PI3Ks have been described to be important in mediating chemoattractant induced activation of neutrophil effector functions [7;8;10;11;13;14]. Upon neutrophil activation, PI3K is recruited to the membrane where it can phosphorylate phosphoinositides (PI) at the D-3 position of the inositol ring. These phosphorylated lipids, preferentially $PI(3,4)P_2$ and $PI(3,4,5)P_3$, act as second messengers forming docking sites for molecules that posses a plextrin homology (PH) domain such as Protein Kinase B (PKB) [15-17]. As previously mentioned PLCβ hydrolyses $PI(4,5)P_2$ into DAG and IP_3. IP_3 initiates the release of Ca^{2+} from the endoplasmatic reticulum, resulting in a rise in cytoplasmic Ca^{2+}. Although their have been several suggested functions for this rise of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), a clear role for elevated $[Ca^{2+}]_i$ in neutrophil function has not been resolved [18;19]. An important downstream regulator of Ca^{2+} is the ubiquitously expressed protein calmodulin [20]. Calmodulin is activated by binding of four Ca^{2+} ions, which results in a conformational change. Once calmodulin is activated it can bind to downstream targets, such as the Ca^{2+}/calmodulin-dependent kinases (CaMK) [21;22]. CaMKs form a family of serine/threonine kinases which can be activated by binding of Ca^{2+}/calmodulin and includes, CaMKI, CaMKII, CaMKIV, CKLiK and CaMKK. Until now there is no evidence for a specific role of these kinases in human granulocytes. However, recently we have demonstrated that CKLiK mRNA is highly specifically expressed in human neutrophils [23].

Stimulation of human neutrophils with chemoattractant also regulates the activation of various intracellular protein kinases. Downstream signal molecules such as extracellular regulated kinase (ERK), Protein Kinase B (PKB) and the 38 kD mitogen activated protein kinase (p38) have also been implicated in the regulation of neutrophil effector functions [11;24;25]. However it has not been investigated whether the activation of these signaling molecules might be mediated by either activation of PI3K or by a rise in $[Ca^{2+}]_i$ or by both.

Here we show that that elevated $[Ca^{2+}]_i$ induces phosphorylation of downstream signaling molecules similarly to chemoattractants. Inhibition of calmodulin resulted in a diminished PKB phosphorylation, but had no effect on ERK, p38 and GSK-3 phosphorylation. GPCR mediated migration of human neutrophils was found to be dependent on calmodulin activity, but not on PI3K. However, fMLP induced respiratory burst demonstrated dependency on both calmodulin and PI3K activity. These data demonstrate critical roles for changes in $[Ca^{2+}]_i$ and calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions such as migration and formation of reactive oxygen species.

Materials and Methods

Reagents and antibodies

PAF (platelet-activating factor), fMLP (N-formyl-methionyl-leucyl-phenylalanine) and cytochrome c were purchased from Sigma (St. Louis, MO). Recombinant human GM-CSF was obtained from Genzyme (Boston, MA). Ionomycin was puchased from Calbiochem (La Lolla, CA) and human IL-8 from PrepoTech (Rocky Hill, NJ). *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W7) was obtained from Sigma (Zwijndrecht, The Netherlands). LY294002 was purchased from Biomol (Plymouth Meeting, PA). Polyclonal phospho-p42/44 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Thr182), phosphoPKB (Ser473) and phospho-GSK-3α/β (Ser21/9) antibodies were obtained from Cell Signaling (Beverly, MA). ERK-2 (C-14), actin (I-19) and p38 (C20) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Isolation of human neutrophils

Blood was obtained from healthy volunteers at the donor service of the University Medical Center (Utrecht, The Netherlands) anti-coagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4). Neutrophils were isolated as previously described [26]. In short, mononuclear cells were depleted from neutrophils by centrifugation over isotonic Ficoll from Pharmacia (Uppsula, Sweden). After lysis of the erythrocytes in an isotonic NH_4Cl solution, neutrophils were washed and resuspended in incubation buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$ and 0.5% HSA). Neutrophils were incubated for 30 minutes at 37^0C before experiments were performed.

RNA isolation, cDNA synthesis and PCR

mRNA was isolated from neutrophils by lysing cells ($25x10^6$) in 400 µl GITC solution (4 M guanidine-isothiocyanate, 25 mM sodium citrate, 1 mM β-mercaptoethanol, 0.5% sarkosyl and 0.1% antifoam). RNAs were further isolated by phenol extraction and ethanol precipitation. To remove possible DNA contamination, the RNA solution was treated with DNAse I (Clontech Laboratories, Palo Alto, CA) for 30 minutes at 37ºC and RT PCR was performed with of purified RNA (1 µg). PCR was performed with primers (100 ng) of human CaM kinase family members (CaM kinase I, II, IV, CKLiK and CaMK kinase). The following oligonucleotides were used: CaMKI forward: 5'-CGGAGGACATTAGAGACA-3', reverse: 5'-CTCGTCATAGAAGGGAGG-3'; CaMKII forward: 5'-GGTTCACGGA-CGAGTATC-3', reverse: 5'-TGGCATCAGCTTCACTGTA-3'; CaMKIV forward: 5'-GATGAAAGAGGCGATCAG-3', reverse: 5'-TAGGCCCTCCTCTAGTTC-3'; CKLiK forward: 5'-GGCAAAGGAGATGTGATG-3', reverse: 5'-CTGCTCGAAACACTTGC-3' and CaMKK forward: 5'-TCTCCATCACGGGTATGC-3' and reverse: 5'-GCGTCACT-GCCCTTGAAT-3'. As a control β-actin primers were used as described [27]. Taq polymerase was used under conditions of 10 mM Tris-Cl pH 8.3, 50 mM KCl, 0.2 mM dNTPs and 0.8 mM $MgCl_2$ (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ). PCR products were visualized on a 1% agarose gel.

Cell lysates and western blotting

Neutrophils were stimulated with fMLP (1 µM), IL-8 (10^{-8} M) or ionomycin (1 µM) for several time points and pre-treated if necessary with 5 to 50 µM W7 for 20 minutes at 37ºC. For Western blotting with phospho-ERK or phospho-p38 antibodies, cells (10^6) were lysed in 40 µl sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 2% β-mercaptoethanol) and boiled for 5 minutes at 95ºC. For western blotting with phospho-PKB or phospho-GSK-3 antibodies, cells ($4x10^6$) were lysed in 40 µl lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM EDTA, 1% Triton X100, 1 mM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM benzamidin, 1 mM PMSF and 1 mM DFP). Lysates were spun down at 4ºC for 5 minutes and 5x sample buffer was added to the supernatant before boiling the samples.

Analysis of neutrophil migration

Migration experiments were performed as described previously [28]. In short, glass coverslips were coated with a Hepes buffer containing 0,5% human serum albumine (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2

mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$). Purified neutrophils (10^6/ml Hepes buffer) were first incubated at 37ºC for 20 minutes and pre-treated with W7 (50 μM) or LY294002 (20 μM) for 10 minutes. Neutrophils were allowed to attach to the coverslip for 10-15 minutes at 37ºC. Medium was removed and the cells were washed two times with Hepes buffer. The coverslip was then inverted in a droplet of medium containing 10^{-8}M IL-8 and sealed with a mixture of beeswax, paraffin, and petroleum jelly (1:1:1, wt/wt/wt). Cell tracking at 37ºC was monitored by time–lapse microscopy and analyzed by custom-made macro (A.L.I.) in image analysis software (Optimas 6.2; Media Cybernetics, Silver Spring, MD). Cell migration was followed for 10 minutes making a picture every 20 seconds.

Measurement of ROS production

Respiratory burst was measured by ROS induced cytochrome c reduction [29]. Assay was performed as previously described [30]. Neutrophils ($4x10^6$ cells/ml) were pre-incubated with W7 (20 or 50 μM) or LY294002 (20 μM) for 20 minutes and GM-CSF (10^{-10}M) to prime the cells. Cytochrome c (75 μM) was added and transferred to a microtitre plate and placed in a thermostat-controlled plate reader (340 ATTC; SLT Lab Instruments, Salzburg, Austria). ROS production was induced by stimulation with 1 μM fMLP. Cytochrome c reduction was immediately measured every 12 seconds as an increase in absorbance at 550 nm.

Measurement of Apoptosis assay

Apoptosis was measured by analyzing Annexin V-fluorescein isothiocyanate (FITC)- binding (Bender Medsystems; Vienna, Austria). In short neutrophils were resuspended in Hepes buffered RPMI containing 8% serum (Hyclone) at a concentration of 10^6/ml and treated with GM-CSF (10^{-10} M) or IL-8 (10^{-7} M) and incubated at 37ºC for indicated time periods. Cell were stored at 4^0C until last incubation timepoint had been reached. Cells were washed with PBS and resuspended in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Subsequently cells were incubated with Annexin V-FITC for 15 minutes at room temperature in the dark, washed with PBS and resuspended in binding buffer. Propidium iodide was added (1 μg/ml) and the percentage of apoptotic cells was detected by FACS analysis (FACSvantage, Becton Dickinson).

Results

Comparison of intracellular signaling pathways activated by chemoattractants and elevated $[Ca^{2+}]_i$

Stimulation of human neutrophils with chemoattractants has been reported to result in the activation of several intracellular signaling pathways. It is of were interest to determine whether changes in $[Ca^{2+}]_i$ alone could modulate these responses. To this end, we analyzed if activation of several kinases could be induced by addition of the Ca^{2+} ionophore ionomycin, or the chemoattractant fMLP. The phosphorylation state of ERK1/2, p38, PKB and GSK-3 after stimulation of neutrophils with ionomycin or the chemoattractant fMLP was compared. Addition of fMLP resulted in a rapid phosphorylation of ERK2 and p38, being optimal at approximately one minute after stimulation (Fig. 1A, left panel). Elevation of $[Ca^{2+}]_i$, by ionomycin addition, was also sufficient to induce ERK2 phosphorylation (Fig. 1B, upper left panel). This was optimal after 30 seconds and was maintained for at least 15 to 30 minutes. Similarly, ionomycin also induced rapid phosphorylation of p38 (Fig. 1A, lower left panel).

PI3K activation results in the recruitment of PKB to the membrane were kinases, such as PDK1, phosphorylate PKB. Recently it has been demonstrated that CaMKK phosphorylates PKB suggesting a role for Ca^{2+} in the activation of PKB [31]. Treatment with ionomycin was indeed sufficient to induce PKB phosphorylation in human neutrophils (Fig 1A, upper right panel). Additionally, GSK-3 a direct target of PKB mediated phosphorylation, was also phosphorylated upon ionomycin treatment (Fig 1A, lower right panel). Both PKB and GSK-3α phosphorylation after ionomycin treatment were still elevated after 30 minutes. Taken together, increased $[Ca^{2+}]_i$ alone is sufficient to induce phosphorylation of ERK2, p38, PKB and GSK-3α similar to receptor-mediated activation by chemoattractants.

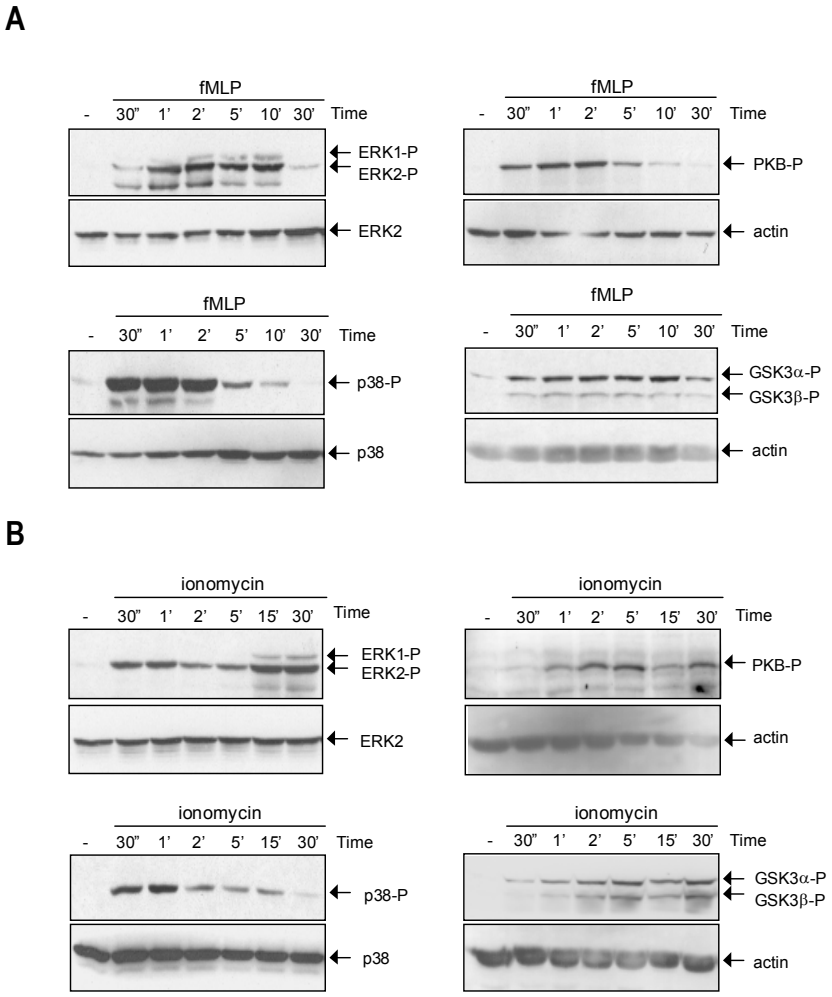

Figure. 1: *Regulation of neutrophil signaling pathways, by fMLP and ionomycin..*
Human neutrophils were isolated and stimulated with **(A)** 1 μM fMLP or **(B)** 1 μM ionomycin for indicated time points. Cells were lysed and western blotting was performed as described in Materials and Methods. Phosphospecific antibodies were used for ERK-P, p38-P, PKB-P and GSK3-P. The levels of ERK2, p38 and actin were determined as a control for equal loading of proteins. Data are representative of three independent experiments.

Calmodulin is critical for chemoattractant mediated PKB phosphorylation in human neutrophils

Increased $[Ca^{2+}]_i$ is sufficient to regulate multiple intracellular signaling pathways. Therefore the role of calmodulin, the major downstream effector of Ca^{2+}, in the regulation of intracellular signaling was analyzed by using the calmodulin inhibitor W7 [32]. ERK2 phosphorylation was only partially inhibited at a concentration of 50 μM W7 (Fig. 2A, upper panel) and no effect was observed on the regulation of p38 when calmodulin was inhibited (Fig. 2A, lower panel). A dramatic inhibition of fMLP induced PKB-phosphorylation was observed, which occurred in a

concentration-dependent manner (Fig. 2B, upper panel). Surprisingly, GSK-3 that has been previously demonstrated to be a downstream target of PKB in several cell types, exhibited no sensitivity to W7 (Fig. 2B, lower panel). In conclusion, these results demonstrate that chemoattractant mediated PKB activation, unlike ERK2 and p38, is dependent on calmodulin activity. Additionally, these data suggest that fMLP induced GSK-3 phosphorylation in human neutrophils can occur in a PKB-independent manner.

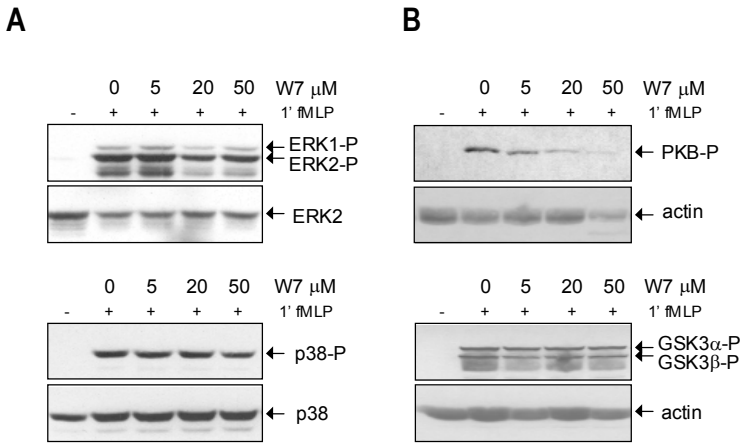

Figure 2: *Effect of calmodulin inhibitor W7 on protein phosphorylation in neutrophils after fMLP stimulation.*
Isolated neutrophils were incubated with DMSO or increasing concentrations of W7 (5-50 μM) for 20 minutes before stimulation with 1 μM fMLP for one minute. Cells were lysed and phosphorylated proteins detected by western blotting as described in Materials and Methods. **(A)** ERK-P and p38-P. **(B)** PKB-P and GSK3-P. The levels of ERK2, p38 and actin were determined as a control of equal loading. Data are representative of at least three independent experiments.

Expression of Ca^{2+}/calmodulin-dependent kinases in human neutrophils

Binding of Ca^{2+} to calmodulin enables it to interact with and activate Ca^{2+}/calmodulin-dependent kinases (CaMKs). Previous reports have demonstrated that CaMKs can phosphorylate PKB, ERK and JNK *in vitro* [23;31;33]. However, little is known about the expression of kinases directly activated by changes in $[Ca^{2+}]_i$ in human neutrophils In order to identify CaMKs expressed in human neutrophils, RT-PCR was performed with neutrophil cDNA and specific CaM kinase primers for CaMKI, CaMKII, CaMKIV, CKLiK and CaMKK. Expression of CKLiK, CaMKK, CaMKII and low amounts of CaMIKI were detected. However, no CaMKIV expression was observed (Fig. 3).

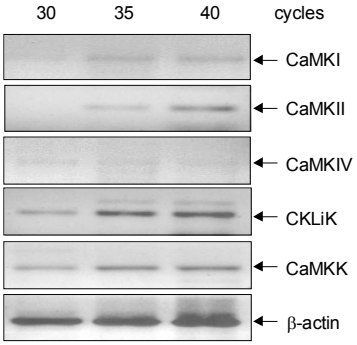

Figure 3: *Expression of Ca^{2+}/calmodulin dependent kinases in neutrophils.*
cDNA was synthesized from neutrophil mRNA as described in Materials and Methods. PCR with specific primers for CaMKI, II, IV, CKLiK, CaMKK was performed with 30, 35 or 40 reaction cycles. β-actin primers were as a control. PCR fragments were visualized on gel. Data are representative of three independent experiments.

Chemoattractant mediated neutrophil migration is abrogated by inhibition of calmodulin but not by inhibition of PI3K

In order to investigate the role of $[Ca^{2+}]_i$ and calmodulin in neutrophil function, two processes involved in host defense, migration and the generation of ROS were investigated. During neutrophil migration, activation of PI3K and changes in $[Ca^{2+}]_i$ occur as a results of chemoattractant induced GPCR activation. Although a specific role for $[Ca^{2+}]_i$ in regulating migration has not been defined, some aspects of the migration process have been shown to be at least partially controlled by changes in $[Ca^{2+}]_i$ [34]. Therefore, analysis of the role of calmodulin and PI3K in IL-8 induced neutrophil migration on albumin coated coverslips was performed. IL-8 is a known potent activator of neutrophil migration and activates the GPCRs, CXCL1 and CXCL2, within the same family of the fMLP receptor [35]. Migration of neutrophils was imaged in the presence of IL-8 for 10 minutes, and the effect of specific inhibitors of calmodulin (W7) or PI3K (LY294002), were analyzed by recording migratory tracks (Fig. 4A, upper panels). To obtain a better comparison, the tracks were centered in Figure 4A (lower panels). Upon IL-8 stimulation, neutrophil migration was markedly increased. Both the migration distance and the migration speed is clearly elevated by IL-8 treatment from 1.68±0.35 to 7.08±1.33 μm per minute (Fig. 4B). Pre-incubation with W7 abrogated the IL-8 induced migration completely, whereas inhibition of PI-3K with LY294002 showed no effect on the IL-8 induced migration on albumin (see Fig. 4A+B). Similar results were also observed for fMLP induced migration (data not shown). These results suggest that PI3K is not necessary for chemokine induced neutrophil migration and demonstrate a critical role for calmodulin, and thus changes in $[Ca^{2+}]_i$, in IL-8 induced neutrophil migration on albumin coated surfaces.

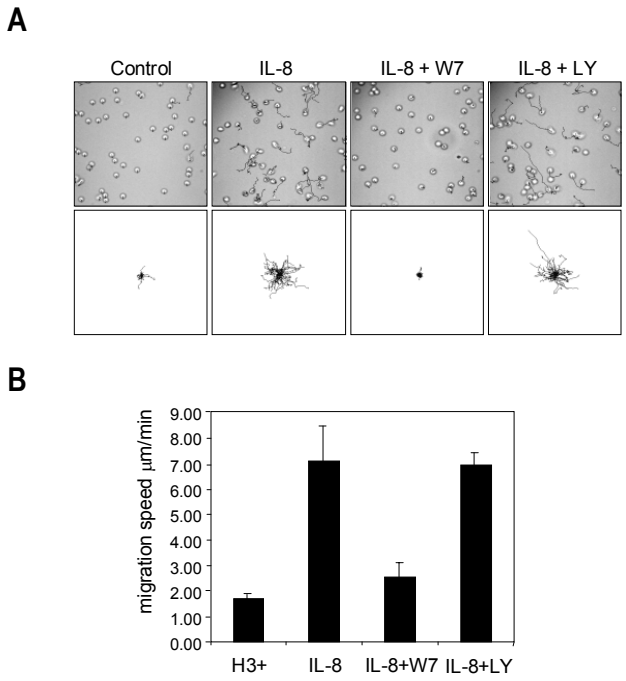

Figure 4: *Effect of W7 and LY294002 on IL-8 induced neutrophil migration.*

Migration of neutrophils was monitored by video microscopy. Cells were pre-incubated as indicated with 50 μM W7 or 20 μM LY294002 for 10 minutes, followed by attachment to albumine coated coverslips for 15 minutes. **(A)** Neutrophil migration was induced by 10^{-8}M IL-8 and imaged every 20 seconds for 10 min. (A, upper panels) Tracks and image of cells are shown as migration tracks of individual cells. (A, lower panels) Centered tracks are depicted. **(B)** Average migration speed of at least three independent experiments are calculated and expressed as μm per minute ± std.

Both calmodulin and PI3K are critical in generation of reactive oxygen species in human neutrophils

Stimulation of neutrophils with the chemoattractant fMLP induces the rapid formation of ROS. This process, termed the respiratory burst, is initiated by the association of the intracellular multi-protein complex NADPH oxidase, which catalyzes the production of ROS and results in efficient killing of invading pathogens [5]. The effect of the calmodulin inhibitor W7 on fMLP induced ROS production was investigated and compared with the effect of the

PI3K inhibitor LY294002. The respiratory burst is dependent on prior priming of cells with cytokines, chemoattractants, or lipopolysaccharides [1;36]. Neutrophils were therefore pre-treated with GM-CSF. W7 or LY294002 were added 20 minutes before stimulation with fMLP. As shown in Figure 5 unprimed cells were unable to activate the respiratory burst upon fMLP stimulation. However cells first primed with GM-CSF induced a rapid production of ROS. Pre-treatment with W7 resulted in a concentration dependent inhibition of ROS production, which was also observed with the PI3K inhibitor LY294002, as we demonstrated before [11;30]. These data indicate that both PI3K and calmodulin are necessary for optimal chemoattractant mediated ROS production in human neutrophils.

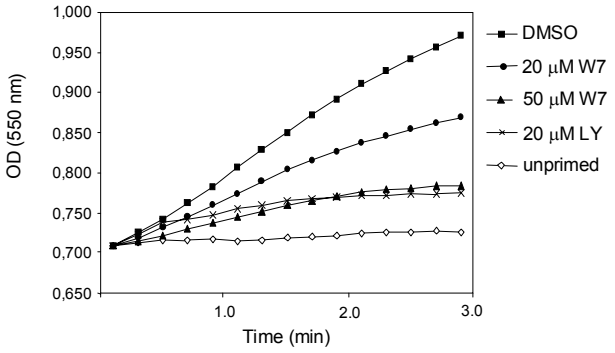

Figure 5: *Comparison of the effect of W7 and LY294002 on the respiratory burst in human neutrophils.*
Cells were isolated and treated without (open mark) and with 10^{-10} M GM-CSF (closed marks) to prime the cells. Before initiation of the respiratory burst with 1 μM fMLP, cells were pre-treated with DMSO, 20 μM W7, 50 μM W7 or 20 μM LY294002 for 20 minutes as indicated. ROS production was measured by cytochrome c reduction resulting in a change in absorption at a wavelength of 550 nm. Average of three independent experiments are depicted.

Chemokines in contrast to cytokines play no role in regulating neutrophil survival

Neutrophil apoptosis, and recognition and removal of cells by macrophages is an essential event in the termination of inflammation and prevention of damage to host tissue. Neutrophils are intrinsically committed to programmed cell death, however inflammatory cytokines such as GM-CSF can delay this process [30;37;38]. Furthermore there are some indications that other inflammatory mediators may be able to inhibit neutrophil apoptosis [39]. In this study, we wished to determine whether indeed chemoattractants could protect human neutrophils from apoptosis. One of the early events during apoptosis is the appearance of phosphatidylserine on the extracellular surface of cells. Phosphatidylserine can bind to (FITC-labeled) annexin-V and in this way functions as a marker of programmed cell death (Fig. 7A). After 16 hours, 78% of all neutrophils were annexin-V positive. Relative to this amount of apoptotic cells (average of individual experiments, considered to be 100% in Fig. 7B), 35% of these cells were rescued from apoptosis after treatment with GM-CSF. IL-8 however, showed no additional survival compared with the untreated neutrophils. We hypothesized that IL-8 might possibly show an additional effect to GM-CSF in the delay of apoptosis. However, also here no additional effect of IL-8 was observed when neutrophils were treated with both GM-CSF and IL-8 (Fig. 7A+B). Therefore chemoattractant induced activation of PI3K and elevation of $[Ca^{2+}]_i$ were insufficient in the protection of neutrophils against apoptosis.

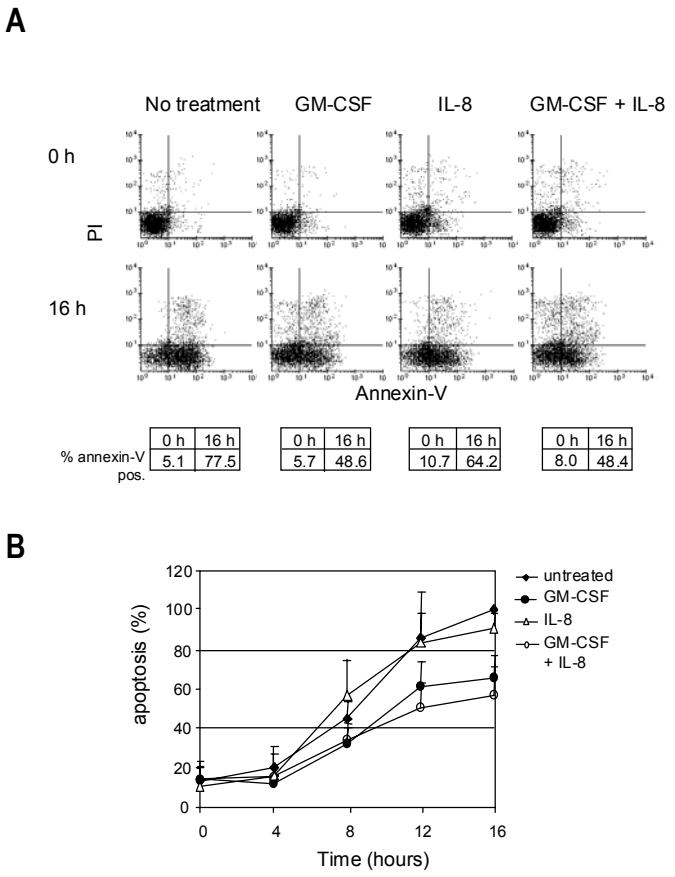

Figure 6: *Comparison of the effects of cytokines and chemokines on neutrophil survival.*
Isolated neutrophils were pre-incubated at 37°C in Hepes buffered RPMI containing 8% serum. Cells were treated for 16 hours with indicated 10^{-10}M GM-CSF or/and 10^{-7}M IL-8 or a combination. **(A)** The amount of apoptotic cells was analyzed by annexin-V FITC and propidium iodide staining. FACS Dotplots of 0 and 16 hours are depicted and % of annexin-V positive cells are displayed in tables. **(B)** Average of % annexin-V positive cells, of at least four independent experiments are depicted and error bars (std.) are indicated. Total amount of apoptotic cells at 16 hours in untreated conditions were corrected to 100%.

Discussion

In this study the role of Ca^{2+} and its downstream effector molecule calmodulin in GPCR regulated signaling pathways in human neutrophils was investigated. Additionally, the role of calmodulin and PI3K, in chemoattractant induced neutrophil effector functions, such as migration and respiratory burst were compared. Treatment of cells with the calcium ionophore ionomycin, resulted in the phosphorylation of ERK2, p38, PKB and GSK-3α (Fig. 1). This demonstrates that increased $[Ca^{2+}]_i$ in itself is sufficient to activate these kinases and suggests that the elevated $[Ca^{2+}]_i$ generated by chemoattractants might be important in activation of downstream signaling events. Compared to fMLP treatment, ionomycin illustrated a prolonged phosphorylation of ERK2, PKB and GSK-3α in these cells. This might be due to the continuously elevated $[Ca^{2+}]_i$ induced by ionomycin, or it may indicate that elevation of $[Ca^{2+}]_i$ alone is not sufficient to activate inhibitory signaling pathways normally responsible for ERK, PKB and GSK-3 dephosphorylation.

Analysis of the effect of calmodulin inhibition on signaling pathways induced by chemoattractants in human neutrophils showed a minor inhibition of ERK- and a dramatic inhibition of PKB phosphorylation (Fig. 2). Although there are some indications that CaMKIV and CKLiK may influence ERK activation *in vivo* [23;33], our data suggests that calmodulin is at least not essential in GPCR regulated ERK activity. Importantly, PKB phosphorylation was completely abolished by inhibition of W7 (Fig. 3b). In general, generation of PIP_3 by PI3K initiates recruitment of PKB though its PH domain to the membrane. However, for activation of PKB, phosphorylation on serine 473 and threonine 308 are necessary and additional kinases are involved. It has been described that CaMKK can phosphorylate PKB directly [31]. These data, together with our RT-PCR expression data, suggest that CaMKK could play an important role in PKB activation in human neutrophils stimulated by chemoattractants. Whether CaMKK is recruited to the membrane or can activate PKB cytosolically in human neutrophils still has to be elucidated. Interestingly, GSK-3 which has previously been shown to be a downstream target of PKB in several cell types, exhibited no sensitivity to calmodulin inhibition. Since we observed complete inhibition of PKB phosphorylation it appears that PKB activation is not necessary for chemoattractant induced GSK-3 phosphorylation in human neutrophils. GSK-3 phosphorylation has also been described to be mediated by protein kinases other then PKB. For example growth factors can inhibit GSK-3 activity by means of the classical MAPK pathway [40]. There are also indications that phosphorylation of GSK-3 can be mediated by Protein Kinase A or by a pathway that involves the mammalian target of rapamycin (mTOR) [41;42]. In guinea pig neutrophils it has been demonstrated that GSK-3 phosphorylation could only be inhibited by dual treatment of the PI3K inhibitor wortmannin and the MEK inhibitor PD98059 [43]. Furthermore, our data illustrate that fMLP induced GSK-3 phosphorylation is more sustained relative to PKB phosphorylation, and the kinetics are rather similar to that fMLP induced ERK phosphorylation (see Fig. 1).

We demonstrate an inhibition of neutrophil migration by W7 (Fig. 4). A calmodulin-dependent kinase which might be involved in this process is myosin light chain kinase (MLCK). This kinase phosphorylates the light chain of myosin II which is thought to be important for contraction at the rear of the cell [44]. Furthermore Ca^{2+} is suggested to play a role in the recycling of integrins, which involves cell adhesion and attachment [45]. Additionally it had been described that CaMKII counteracts the calcineurin induced affinity switch of $\alpha_5\beta_1$ integrin in CHO cells, suggesting also a role for Ca^{2+}/calmodulin-dependent molecules in regulating the affinity state of integrins on neutrophils [46].

Although PI3K has been suggested to play a role in signaling to the actin cytoskeleton [9], no inhibitory effect of LY294002 on chemoattractant induced migration was observed, suggesting a minor role for PI3K in chemoattractant induced migration. Although we have demonstrated this before [11], contradictory results have also been described [47]. Furthermore, it has been shown that neutrophils isolated from knockout mice lacking PI3Kγ demonstrated an inhibited response to chemoattractant induced migration [8;14]. Therefore, a role for PI3K in neutrophils migration cannot be ruled out. Recently, it has been described that PI3K rather affects directionality and not motility [48], which might be in agreement with our findings, since our assays predominantly detects motility.

We also demonstrate that fMLP induced ROS production was dependent on both, calmodulin and PI3K. In guinea pig neutrophils is has been demonstrated that inhibition of calmodulin results in inhibition of Rac and p21 activated kinases (PAKs) [49]. Involvement of Rac in ROS production has been demonstrated [5] and a role for PAK has been suggested in phosphorylation the 67 kDa subunit of the NADPH complex [50]. However in human neutrophils we

have shown that Rac activation was independent of changes in $[Ca^{2+}]_i$ [51]. A role for PKB has been suggested in phosphorylation the 47 kDa subunit of the NADPH complex, $p47^{phox}$, since membrane targeted PI3K leads to PKB phosphorylation and $p47^{phox}$ phosphorylation [52]. Recently a role for PI3K in the respiratory burst has been shown, since it has been demonstrated that the Phox homology (PX) domains in $p47^{phox}$ and $p40^{phox}$ binds to different phosphorylated phosphatidylinositol [53;54]. Although direct activation of components in the multi-protein complex by PKB has not been shown, it is possible that the inhibitory effect of W7 on the generation of ROS may be mediated by PKB.

As demonstrated in Figure 6, neutrophil apoptosis was unaffected by chemokine stimulation. In previous reports we have demonstrated a role for the PI3K-PKB pathway in cytokine-mediated delay of apoptosis in human neutrophils [30]. Although IL-8 induces activation of PI3Kand PKB and increases $[Ca^{2+}]_i$, our results demonstrate that it does not effect neutrophil survival. The divergence effects on apoptosis obtained by cytokines and chemokines might be due to the difference in kinetics. Chemokines transduce rapid signaling events, while cytokines signaling is relatively slow. Furthermore, no additional effect on GM-CSF delayed apoptosis by IL-8 could be observed. It has been suggested that Ca^{2+}-induced PKB phosphorylation might lead to survival [31]. However, here we demonstrate that there is no role for chemoattractants and thus $[Ca^{2+}]_i$ in regulating neutrophil apoptosis.

Taken together, we demonstrated that elevations of $[Ca^{2+}]_i$ and calmodulin play central roles in chemoattractant induced neutrophil effector functions, such as migration and respiratory burst, which partially overlaps with PI3K regulated functions. A possible convergence of PI3K- and Ca^{2+}/calmodulin regulated pathways might be at the level of PKB activation, in which PI3K recruits PKB to the plasma membrane and calmodulin-regulated kinases mediate its phosphorylation. Further work is necessary to determine precisely which CaMKs are important in the Ca^{2+}/calmodulin mediated effector functions of human neutrophils.

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Chapter 5

Functional characterization of CaMKI Like Kinase in human granulocytes: A role in respiratory burst and migration

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Submitted

Abstract

Human granulocytes, which include neutrophils and eosinophils, respond to inflammatory mediators by activating specific effector functions, finally resulting in the elimination of invading microorganisms. Activation of these granulocyte effector functions, such as induction of the respiratory burst, are regulated by a variety of ill-defined signaling pathways. Recently, we identified a novel protein kinase cDNA with high homology to Ca^{2+}-calmodulin-dependent kinase I (CaMKI), termed CaMKI-like kinase (CKLiK), which showed restricted mRNA expression to human granulocytes. In this study the expression, regulation and function of this novel protein kinase has been investigated in human granulocytes. The *CKLiK* gene was found to be localized on chromosome 10. Antibodies against the C-terminus of CKLiK, which demonstrates no homology with CaMKI. CKLiK was detected in $CD34^{+}$ derived neutrophils and eosinophils, as well as in mature peripheral granulocytes. Analysis of CKLiK kinase activity showed dependency on Ca^{2+} and calmodulin. Furthermore, activation of human granulocytes by inflammatory mediators, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) and Platelet Activating Factor (PAF) resulted in induction of CKLiK activity, in parallel with a rise of intracellular Ca^{2+} $[Ca^{2+}]_i$. In order to study the function of CKLiK in human granulocytes, we generated a cell permeable CKLiK peptide inhibitor (CKLiK297-321). This peptide was able to inhibit CKLiK kinase activity in a concentration-dependent manner. Since both production of reactive oxygen species (ROS) in human granulocytes and migration have previous been shown to require Ca^{2+}, we investigated the effect of this peptide on these processes. fMLP-induced ROS production was potently inhibited by CKLiK(297-321). Additionally, migration of neutrophils on albumin coated surfaces was perturbed. These findings suggest a critical role for CKLiK in modulating functional responses in human granulocytes.

Introduction

Human granulocytes play an important role in host defense against invading pathogens[1]. During inflammation, granulocytes in the peripheral blood become primed or pre-activated, leave the bloodstream by diapedesis through the endothelium and migrate towards the site of inflammation [2]. At the inflammatory site granulocytes become highly activated, secrete proteolytic enzymes by degranulation and form cytotoxic reactive oxygen species (ROS) to eliminate invading microorganisms [3]. Inflammatory mediators, such as chemokines and chemoattractants that activate the G-protein coupled receptors (GPCR) initiate these granulocyte effector functions. Binding of ligand to GPCRs results in activation of trimeric G-proteins and subsequent dissociation of the Gα and Gβγ subunits. The dissociation of the Gβγ subunit can result in activation of phosphatidylinosytol-3-OH kinase (PI3K) and PLCβ. PLCβ activation can in turn lead to the hydrolysis of phosphatidylinositol (4,5)phosphate (PIP_2) producing diacylglycerol (DAG) and inositol-3 phosphate (IP_3) [4]. Subsequently, IP_3 binds to its receptor on the Ca^{2+} stores, resulting in Ca^{2+} release into the cytoplasm. Elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in granulocytes has been observed during activation of several granulocyte effector functions, for example in fMLP induced respiratory burst [5; 6]. Additionally, Ca^{2+} has been implicated in the regulation of migration, since Ca^{2+} buffered neutrophils demonstrated an abrogated integrin recycling and rear release [7 8]. However very few specific proteins have been identified that sense changes in $[Ca^{2+}]_i$ and transduce these signals modulating granulocyte functions.

Recently we identified a novel protein kinase CKLiK, which mRNA is highly expressed in human granulocytes [9]. CKLiK is highly homologous with CaMKI, a member of the Ca^{2+}/calmodulin-dependent kinase (CaMK) family [10] . Based on the crystal structure of CaMKI a general model for the activation of CaMKs has been proposed [11]. When Ca^{2+} levels rise in the cytosol, calmodulin, a ubiquitously expressed Ca^{2+}-binding protein, undergoes a conformational change, which results in binding to the calmodulin binding domain present in CaMK family members [12]. Inactive CaMKs are in a "folded" configuration whereby the autoinhibitory domain acts as a pseudosubstrate by interacting with the N-terminal kinase domain. The calmodulin-binding domain is located on the C-terminus of CaMK. Calmodulin binding results in "unfolding" and subsequent activation of CaMK [10]. For CaMKII this activation has been correlated with autophosphorylation [13]. CaMKIV has also been proposed to be autophosphorylated, but not in the activation loop which is the case for CaMKII [14]. For CaMKI this remains to be proven. Full activity of CaMKI and CaMKIV is obtained by phosphorylation of a threonine residue by CaMKK in their activation loop [15; 16; 17]. CKLiK activity is also enhanced by co-expression of CaMKK, and CKLiK indeed has a threonine residue in the activation loop suggesting regulation by CaMKK [9].

In this study we characterized the expression, regulation and function of this novel Ca^{2+}/calmodulin dependent kinase in human neutrophils and eosinophils. We identified *CKLiK* as a gene located on chromosome 10 (10p14). CKLiK expression was detected in human eosinophils and neutrophils by utilizing a novel antibody raised specifically against CKLiK. Furthermore, we demonstrate that CKLiK can be activated by physiological relevant inflammatory mediators which induce elevations of $[Ca^{2+}]_i$. By the generation of a cell permeable inhibitory peptide for CKLiK we were able to investigate a role of CKLiK in granulocyte effector functions. Addition of this peptide led to an inhibited respiratory burst and an abrogated migration, suggesting a role for CKLiK in these effector functions in human granulocytes.

Materials and methods

Cells, reagents and antibodies

Murine myeloid 32D cells were cultured in RPMI 1640-glutamate supplemented with 8% Hyclone serum (Life Technologies, Breda, The Netherlands) and mouse IL-3. Generation of 32D cells stably expressing pBabe-HA_CKLiK has been described previously [9]. Stemcell factor and hFLT-3 ligand were purchased from Peprotech (Rocky Hill, NJ), hIL-3 and hG-CSF were obtained from Strathmann (Hamburg, Germany), GM-CSF was purchased from Endogen (Woburn, MA, USA), hIL-5 was a kind gift of Dr. I. Uings (GlaxoSmithKline, Stevenage, UK). fMLP (N-formyl-methionyl-leucyl-phenylalanine) was purchased from Sigma (St. Louis, MO). Calmodulin was a kind gift

of Dr. R. de Groot. Monoclonal antibodies, 12CA5 against HA epitope were purchased from Boehringer Mannheim Biochemicals (Almere, The Netherlands). Cell permeable CKLiK(297-321) was designed by linking it to the 11 amino acid sequence of the HIV tat protein (YGRKKRRQRRRIRKNFAKSKWRQAFNATAVVRHMRK). An arbitrary tat-control peptide was used as a negative control

Generation of CKLiK antibody

Antibodies against the C-terminal part of CKLiK were generated by immunizing rabbits with an eleven amino acid synthesized peptide specific for CKLiK, CAYVAKPESLS, coupled to Keyhole Limpet Hemocyanin. Antibody purification was performed by affinity chromatography. In short, the immunogenic peptide was coupled to an agarose carrier support utilizing a SulfoLink kit (Pierce, Rockford, IL, USA). Antibodies against were allowed to bind to the peptide coupled beads, washed with 100 mM TRIS pH 8.0 and eluted with Glycine pH 3.0. Purified anti-CKLiK was dialyzed against PBS and stored at 4^0C containing 0.02% sodium azide.

Isolation of human neutrophils and eosinophils.

Blood was obtained from healthy volunteers at the donor service of the University Medical Center (Utrecht, The Netherlands). Granulocytes were isolated from 0.4% (wt/vol) trisodium citrate (pH 7.4) treated blood as previously described [18]. In short, mononuclear cells were depleted by centrifugation over isotonic Ficoll from Pharmacia (Uppsula, Sweden). After lysis of the erythrocytes in an isotonic ice-cold NH_4Cl solution, granulocytes were washed and resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$ and 0.5% HSA). Eosinophils were separated from neutrophils by negative immunomagnetic selection with anti-CD16-conjugated microbeads (MACS Miltenyi Biotech, Auburn, USA) [19]. Purity of eosinophils was > 98%.

Isolation and culture of human $CD34^+$ cells

$CD34^+$ cells were isolated as previously described [20]. In short, mononuclear cells were isolated from umbilical cord blood by density centrifugation over isotonic ficoll solution (pharmacia, Uppsula, Sweden). Immunomagnetic selection with hapten conjugated antibody against CD34 was used to isolate $CD34^+$ cells (Miltenyi Biotech, Auburn, USA). $CD34^+$ cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Paisley, UK) supplemented with 10% FCS, 50 μM β-mercaptoethanol, 10 U/ml penicillin, 10 μg/ml streptamycin, and 2mM glutamine at a density of 0.3 x 10^6 cells/ml. Cells were differentiated towards neutrophils or eosinophils upon addition of SCF (50 ng/ml), FLT-3 (50 ng/ml), GM-CSF (0.1 nmol/l), IL-3 (0.1 nmol/l), and G-CSF (10 ng/ml) for neutrophils differentiation or IL-5 (0.1 nmol/l) for eosinophil differentiation. Every 3 or 4 days, cells were counted and fresh medium was added to a density of $0.5x10^6$ cells/ml. After 3 days of differentiation, only IL-3 and G-SCF or IL-5 were added to the cells as appropriate.

Analysis of CKLiK expression

CKLiK expression was analyzed by immunoprecipitation and western blotting. For this, cells were stimulated as indicated and washed twice with cold PBS and lysed in a buffer containing; 1% NP-40, 20mM Tris-Cl pH 7.5, 150mM NaCl, 10% glycerol and 10mM $MgCl_2$ supplemented with 10μg/ml aprotinin, 1mM leupeptin, 1mM phenyl-methylsulfonylfluoride (PMSF), 1mM benzamidine 1mM Na_3VO_4 and 1mM diisopropyl-phosphorofluoride (DFP). Lysates were pre-cleared for 20 min with protein-A sepharose and CKLiK was immunoprecipitated with anti-CKLiK for 1-2 hours. Immunoprecipitates were washed twice in cold PBS and denaturated in sample buffer by boiling for 5 minutes. Samples were separated by a 12.5% SDS-PAGE and analyzed by western blotting. Membranes were blocked with 5% low-fat milk for 1 hour before hybridizing anti-CKLiK in 0.5% low-fat milk for 2 hours and swine anti-rabbit peroxidase-conjugated antibody (swarpo) or protein-A peroxidase-conjugated ($protA^{hrp}$) in 0,5% milk for 1 hour.

CKLiK Kinase assay

CKLiK kinase assays were performed as previously described [9]. In short, cells were stimulated as indicated, washed with cold PBS and lysed in lysis buffer as described above. Lysates were pre-cleared for 20 min with protein-A sepharose beads and immunoprecipitated with CKLiK polyclonal antibody. Immunoprecipitates were washed twice

in lysis buffer and twice in dilution buffer containing 10mM Tris-Cl pH 7.4 and 20mM $MgCl_2$. Kinase assay was performed in the presence 10mM Tris-Cl pH 7.4, 20mM $MgCl_2$, 1mM DTT, 50μM ATP, 0.1μl γ-^{32}P-dATP in the presence or absence of 1mM $CaCl_2$ and 0.5μg calmodulin, 5μg CREMβ as substrate [21]. After 20 minutes of incubation at room temperature, reactions were stopped by adding Laemli protein sample buffer and then boiled for 3 minutes. Samples were separated on a 12.5% SDS-PAGE gel and analyzed by autoradiography.

Detection of $[Ca^{2+}]_i$

Cells ($4x10^6$ cells) were loaded with 1.25 μM Fura-2AM (Molecular probes, Eugene, OR, USA) for 15 minutes, then washed and kept at room temperature. Cells were stimulated with indicated chemoattractants in a quartz cuvette (400 μl $2x10^6$/ml). The fluorescence was measured under stirring conditions at 37^0C in a Hitachi F-4500 fluorescent spectrophotometer (Hitachi Ltd, Tokyo, Japan), using a multi-wavelength timescan program. Fura-2 fluorescence was measured at 340 nm (F1) and 380 nm (F2) at 510 emission.

Analysis of respiratory burst

Respiratory burst was measured by ROS-induced cytochrome c reduction [22]. Assay was performed as previously described [23]. In short, granulocytes ($4x10^6$ cells/ml) were pre-incubated with CKLiK(297-321) or a tat-control peptide for 20 minutes and with GM-CSF (10^{-10}M) to prime the cells. Cytochrome c (75 μM) was added and samples were transferred to a microtitre plate and placed in a thermostat-controlled plate reader (340 ATTC; SLT Lab Instruments, Salzburg, Austria). ROS production was induced by stimulation with 1 μM fMLP. Cytochrome c reduction was immediately measured every 12 seconds as an increase in absorbance at 550 nm.

Analysis of neutrophil migration

Migration experiments were performed as described previously [24]. In short, glass coverslips were coated with a Hepes buffer containing 0.5% human serum albumine (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$). Purified neutrophils (10^6/ml Hepes buffer) were allowed to attach to the coverslip for 10-15 minutes at 37ºC. Medium was removed and the cells were washed two times with Hepes buffer. The coverslip was then inverted in a droplet of medium containing 10^{-8}M fMLP and sealed with a mixture of beeswax, paraffin and petroleum jelly (1:1:1, wt/wt/wt). Cell tracking at 37ºC was monitored by time–lapse microscopy and analyzed by custom-made macro (A.L.I.) in the image analysis software (Optimas 6.1; Media Cybernetics, Silver Spring, MD). Cell migration was followed for 10 minutes making a picture every 20 seconds.

Results

CKLiK gene is located on chromosome 10

Recently the draft sequence of the human genome has been completed [25]. It is of interested to determine the chromosomal localization of the *CKLiK* gene. A screen of the genomic sequence (ncbi.nlm.nih.gov/BLAST) identified the *CKLiK* gene as being located on chromosome 10 at position 10p14. The *CKLiK* gene consists of 10 exons forming the complete CKLiK mRNA. Exon 1 is localized at position 12950 K, and exon 10 ends at 13490 K, encoding a region of 540,000 bp (Fig. 1A). As expected, CKLiK was found to contain a kinase domain an ATP binding domain, a calmodulin binding domain and an autoinhibitory domain, as is found in all Ca^{2+}/calmodulin-dependent kinases. In order to define other potential domains we scanned the amino-acid sequence of CKLiK against protein databases (scansite.mit.edu). Analysis suggested that the C-terminal sequence AYVAKPESLS of CKLiK may form a recognition site for PDZ domain containing proteins. We know from our previous findings that CKLiK shows high homology with CaMKI. In Figure 1B a phylogenetic tree of Ca^{2+}/CaM dependent kinases is depicted, and CKLiK is indeed most homologous with CaMKI. Also closely related are the kinases DJ27L16.1, also called CaMK1G (ass.nr: NM_020439), and AMPK-Like (ass.nr: AAF74509). Both are recently sequenced kinases whose specific functions are unknown.

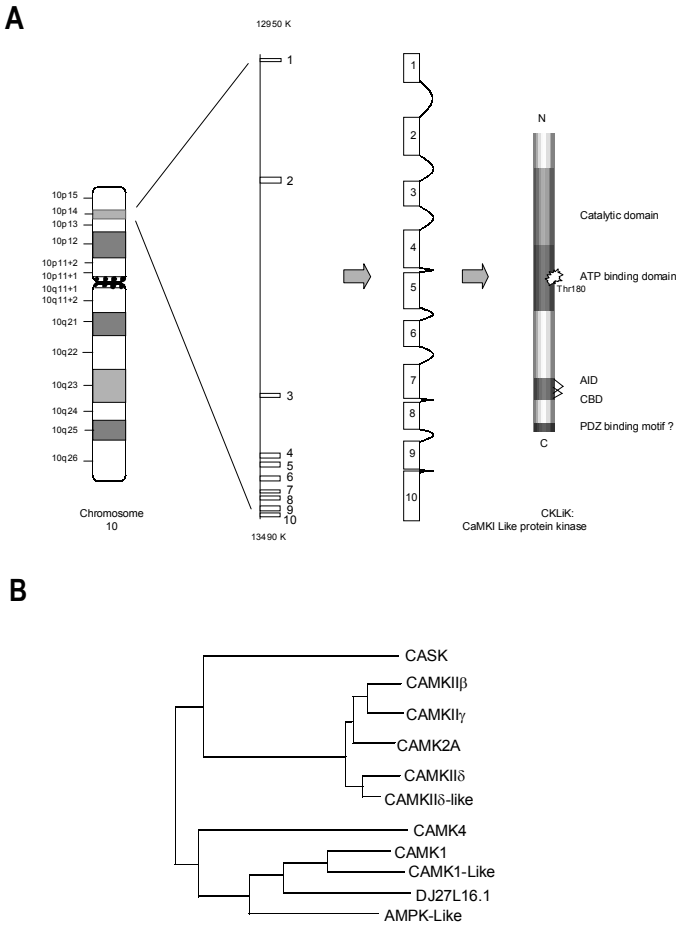

Figure 1: *Chromosomal localization of CKLiK.*
The *CKLiK* gene is located on chromosome 10 at position 10p14 **(A)**. The gene overlaps a region of 530,000 bp and consist of 10 exons, which forms the CKLiK mRNA. CKLiK contains a catalytic domain, an ATP binding domain, an auto inhibitory domain (AID), a calmodulin binding domain (CBD) and a hypothetical PDZ binding motif. **(B)** Localization of CKLiK within the phylogenetic tree of the Ca^{2+}/calmodulin regulated kinase family.

Characterization of CKLiK antibody

To study the expression of CKLiK in human granulocytes we generated a specific polyclonal antibody. A 10 amino acid peptide derived from the C-terminal part of CKLiK was used to immunize rabbits. This sequence was found to be specific for CKLiK and not for CaMKI or other CaMK family members (Fig. 2A). Purification of the antibody was performed by affinity chromatography using the CKLiK-peptide coupled to agarose beads. In order to test the antibody, we utilized 32D cells stably expressing epitope-tagged CKLiK (HA-CKLiK) [9] and checked whether the anti-sera could be used for western blotting (Fig. 2B), immunoprecipitations (Fig. 2C) and immunecomplex kinase assays (Fig 2D). Both, CKLiK in whole cell lysates, and immunoprecipitated CKLiK could be detected, but not in the control 32D cells lacking CKLiK (Fig. 2B, C). Additionally, the functional kinase assays performed with the anti-CKLiK, demonstrated comparable results obtained with anti-HA (Fig. 2D). Thus, the antibody appears to be specific for CKLiK and can be used for analysis of protein levels and kinase activity in primary granulocytes.

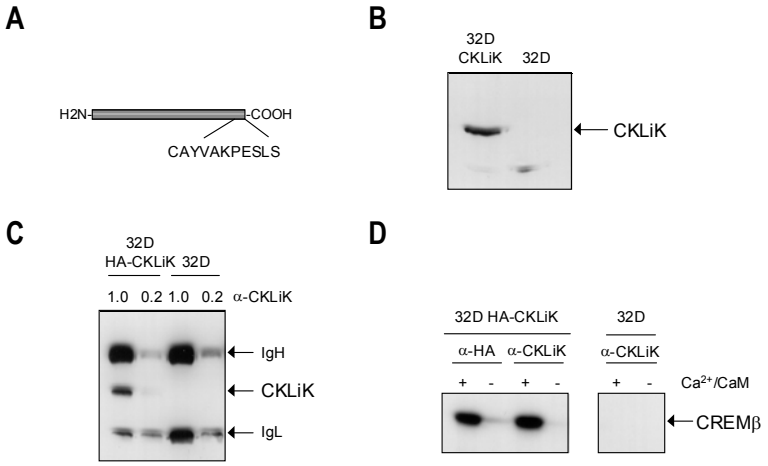

Figure 2: *Characterization of CKLiK antibody.*
(A) An antibody against the C-terminal portion of CKLiK was generated by immunizing rabbits with a KLH-coupled peptide as indicated. **(B)** Western blots with CKLiK antisera were performed. 32D (10^6) cells stably expressing an N-terminal epitope tagged CKLiK (32D HA-CKLiK) or control cells (32D) were lysed, seperated by SDS-PAGE and western blotting was performed as described in Materials and Methods. **(C)** Immunoprecipitations were performed with CKLiK antibody. 32D (10^7) cells were lysed and immuno-precipitations were performed with 1µl and 0.2µl purified CKLiK antibody (α-CKLiK) as indicated. **(D)** Immunecomplex kinase assays were performed with or without Ca^{2+} and calmodulin using the CKLiK antibody and an antibody against the epitope-tag (α-HA) as a control. CREMβ was used as a substrate.

CKLiK activity in human granulocytes is dependent on Ca^{2+}/calmodulin

We previously demonstrated high mRNA expression of CKLiK in human granulocytes [9]. Here we analyzed CKLiK protein expression in neutrophils and eosinophils derived by the differentiation of hematopoietic $CD34^+$ stem cells cultured with G-CSF or IL-5 respectively, and isolated peripheral blood granulocytes. In differentiated cells, CKLiK was found present in both neutrophils and eosinophils (Fig. 3A, left panel). We observed a higher expression of CKLiK in differentiated eosinophils then neutrophils. In peripheral blood neutrophils a low expression of CKLiK was observed, compared to the expression in eosinophils (Fig. 3A, right panel). To demonstrate whether immunoprecipitated CKLiK indeed requires Ca^{2+} and calmodulin for kinase activity we differentiated stem cells towards neutrophils and eosinophils and performed immunoprecipitation, using CKLiK-antibody. CKLiK kinase assays were performed utilizing CREMβ as a substrate. Indeed, only in the presence of Ca^{2+} and calmodulin CKLiK kinase activity could be observed (Fig. 3B, upper panels). Using peripheral blood eosinophils and neutrophils we observed elevated CKLiK kinase activity in the presence of Ca^{2+} and calmodulin (Fig. 3B, lower panels). A low basal kinase activity was observed in the absence of Ca^{2+}/calmodulin in peripheral neutrophils. It is possible that neutrophils display a constitutively low CKLiK kinase activity, however we cannot exclude that CKLiK becomes modestly activated during cell isolation.

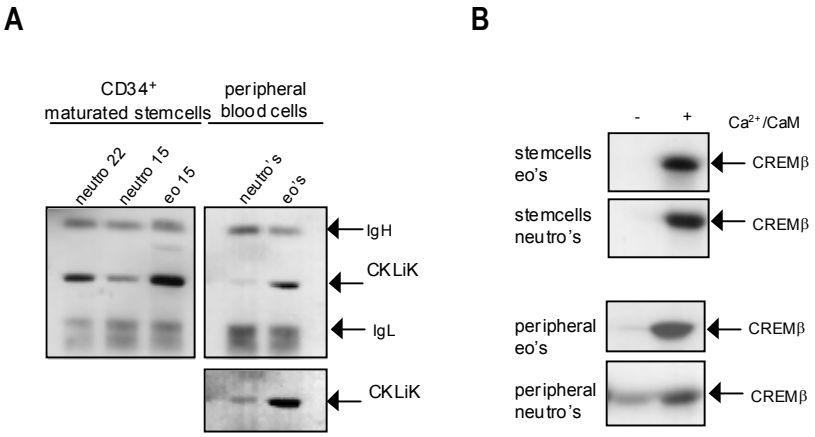

Figure 3: *Expression and activation of CKLiK in human eosinophils and neutrophils.*
(A) CKLiK was immunoprecipitated with CKLiK antibody from stem cells differentiated towards neutrophils ($2.5x10^7$ and $1.5x10^7$ as indicated) and eosinophils ($1.5x10^7$) (left panel) and from peripheral blood neutrophils ($2x10^7$) and eosinophils (10^7) (right panel, with a longer exposure below) and separated by SDS-PAGE,. Western blot analysis were performed with the CKLiK antibody. Heavy and light chain of CKLiK antibody and immunoprecipitated CKLiK are indicated. **(B)** Kinase activity of CKLiK is dependent on Ca^{2+}/calmodulin. Kinase assays were performed in the absence or presence of Ca^{2+} and calmodulin with CKLiK immunoprecipitated from stem cells differentiated towards neutrophils and eosinophils (upper panels) and with CKLiK obtained from peripheral blood eosinophils and neutrophils (lower panels). Data are representative of three independent experiments.

Inflammatory mediators activate CKLiK in human granulocytes.
Granulocytes can be activated by a variety of inflammatory mediators. Since CKLiK is activated by Ca^{2+} and calmodulin the induction of CKLiK activity by physiologically relevant agents that induce a rise in $[Ca^{2+}]_i$ was investigated. Purified neutrophils were stimulated with fMLP, and eosinophils with PAF for the times indicated. Subsequently, cells were lysed and CKLiK kinase assays were performed. Phosphorylation of CREMβ was observed in neutrophils after only 30 seconds of stimulation with fMLP (Fig. 4A), while eosinophils reached a maximum CKLiK activity after 1 minute (Fig. 4B). Activity of CKLiK in both neutrophils and eosinophils decreased after 2 minutes. This transient activation of CKLiK follows the rise of intracellular Ca^{2+} normally observed after activation of G-protein-coupled receptors in these cells as demonstrated in Figure 4C .

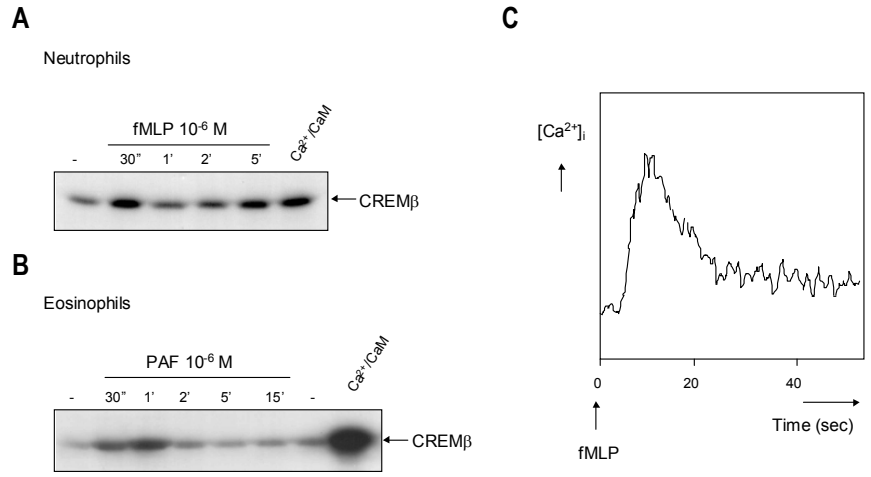

Figure 4: *CKLiK activity is induced by inflammatory mediators.*
Peripheral blood neutrophils **(A)** and eosinophils **(B)** were isolated and stimulated with fMLP (10^{-6}M) or PAF (10^{-6}M) respectively. Cells were lysed ($3x10^7$ cells/point), immunoprecipitated with CKLiK antibody and kinase assays performed without addition of Ca^{2+} and calmodulin, except for positive controls. **(C)** Ca^{2+} response of neutrophils to fMLP. Cell were loaded with Fura-2AM for 15 minutes and washed. Changes in $[Ca^{2+}]_i$ were measured at 340 nm (F1) and 380 nm (F2) at 510 emission using a Hitachi F4500 fluorescence spectrophotometer. Similar Ca^{2+} responses were obtained after PAF stimulation of eosinophils (data not shown).

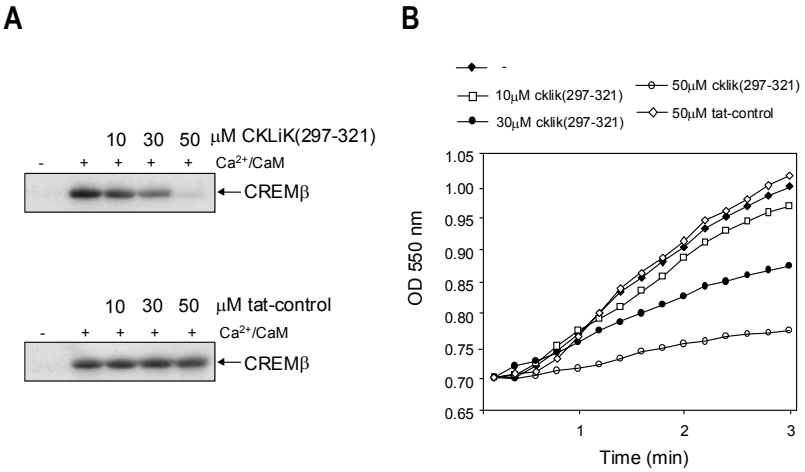

Figure 5: *CKLiK(297-321) inhibits CKLiK kinase activity and respiratory burst.*
A cell permeable CKLiK peptide YGRKKRRQRRRIRKNFAKSKWRQAFNATAVVRHMRK was synthesized. **(A)** 32D cells stably expressing HA-CKLiK were lysed and CKLiK was immunoprecipitated. CKLiK kinase assays was performed with increasing concentrations of CKLiK(297-321) as indicated. CREMβ was used as a substrate. A non-relevant tat-control peptide was used a negative control. **(B)** Effect of CKLiK(297-321) peptide on respiratory burst. Granulocytes were isolated and treated GM-CSF (10^{-10}M) to prime the cells. Cells were then treated without (-) or with increasing concentrations of CKLiK(297-321) or a tat-control peptide for 20 minutes. Respiratory burst was initiated with fMLP (10^{-6}M). ROS production was measured by cytochrome c reduction resulting in a change in absorption at a wavelength of 550nm. Average of three independent experiments are depicted.

CKLiK(297-321) reduces ROS production in human granulocytes.
While we have demonstrated expression and regulation of CKLiK in human granulocytes, a role for this kinase in regulating granulocyte effector functions remains unclear. To investigate this question an inhibitory CKLiK peptide, ($_{297}$IRKNFAKSKWRQAF-NATAVVRHMRK$_{321}$) was generated, based on previous findings [26]. To study the functional role of CKLiK in human granulocytes we linked this peptide to the 11-amino acid portion of the HIV tat protein, generating a peptide capable of transducing primary cells [24; 27; 28]. First, the ability of this peptide CKLiK(297-321), to inhibit the kinase activity of CKLiK was investigated. Indeed we could demonstrate a dose-dependent inhibitory effect of CKLiK(297-321) on the ability of CKLiK to phosphorylate the substrate CREMβ (Fig. 5A). This was not observed utilizing a control peptide. Further the effect of this CKLiK(297-321) on the production of reactive oxygen species in human granulocytes was analyzed, since a role for Ca^{2+} in the respiratory burst has been proposed [29]. For optimal activation of the respiratory burst cells need to be pre-activated or "primed" [30]. Therefore, cells were first treated with GM-CSF or PAF, and initiation of the respiratory burst was induced by fMLP. ROS production was dramatically inhibited by the CKLiK(297-321) in a concentration dependent manner (Fig. 5b), while no inhibition was observed with 50 μM tat-control peptide. This indicates a potential role for CKLiK in the regulation of the respiratory burst in human granulocytes.

CKLiK(297-321) inhibits migration of human granulocytes.
Although specific mechanisms by which changes in $[Ca^{2+}]_i$ can regulate migration has not been defined, some aspects of the migration process have been shown to be at least partially controlled by changes in $[Ca^{2+}]_i$ [8; 31]. The effect of CKLiK(297-321) and tat-control peptides on the fMLP induced migration was investigated (Fig. 6A, upper panels). Migration of fMLP stimulated neutrophils on albumine coated coverslips was measured by timelapse imaging during 10 minutes. Migration distance is visualized in figure 6, showing the tracks of 40 cells (upper panels) and the centered tracks (lower panels). Upon fMLP stimulation, neutrophil migration was markedly increased compared to the untreated cells, which showed little or no movement (data not shown). fMLP treated cells migrated with a speed of 6.71±0.48 μm/minute (Fig. 4B). Pre-incubation with CKLiK(297-321) strongly inhibited fMLP-induced migration, as demonstrated by a reduction of the speed to 1.51±0.36 μm/minute. From the tracks in the lower panel of Fig. 6A it can be concluded that most cells incubated with CKLiK(297-321) demonstrated a

completely abrogated migration. Treatment with the tat-control peptide showed no significant inhibition. These results demonstrate a role for CKLiK in granulocyte migration behavior.

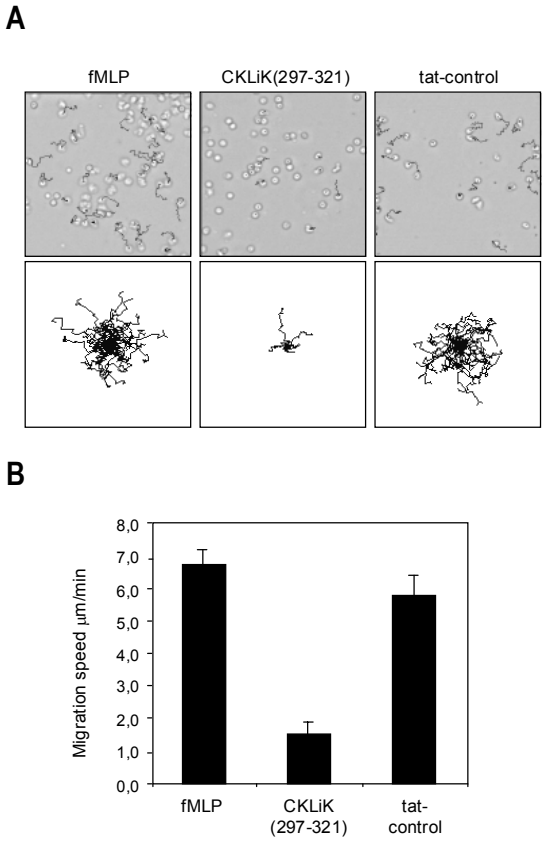

Figure 6: *CKLiK(297-321) abrogates fMLP induced migration.*
Migration of granulocytes was monitored by time lapse analysis. Cells were first allowed to attach to albumine coated coverslips for 15 minutes. Granulocytes were incubated as indicated with 50 µM CKLiK(297-321) or 50 µM tat control peptide. **(A)** Neutrophil migration was induced by fMLP (10^{-8}M) and imaged every 20 seconds for 10 min. Migration tracks of individual cells are shown per cell (A, upper panel) and centered (A, lower panel) **(B)** Average migration speed of at least three independent experiments are calculated and expressed as µm per minute ± SD.

Discussion

In this report the protein expression, regulation and function of the recently identified protein kinase CKLiK in primary human granulocytes was analyzed. Comparison of expression levels between neutrophils and eosinophils shows higher CKLiK protein levels in human eosinophils. However, neutrophils derived by differentiation of hematopoietic stem cells also demonstrate a high CKLiK expression (Fig. 3A). Low expression of CKLiK in neutrophils might be due to proteolytic breakdown, since these cells contain greater protease activity then peripheral blood eosinophils [32]. Immunecomplex kinase assays of CKLiK in human granulocytes, confirm the Ca^{2+} and calmodulin dependency expected of this protein kinase (Fig. 3B). We observed a higher basal level of CKLiK activity in human neutrophils compared to eosinophils. This could indicate that CKLiK is already activated in unstimulated neutrophils or that there is a low Ca^{2+}/calmodulin- independent activity of CKLiK in human neutrophils. Alternatively, isolation of neutrophils might induce some artificial cellular activation [33]. For CaMKII and CaMKV a Ca^{2+}-independent activity has been demonstrated [34]. For the closely related CaMKIV it has been suggested that phosphorylation of Thr196 by CaMKK resulted in a Ca^{2+}-independent activity [17]. However, for CaMKI this could not be demonstrated [10]. Our previous findings with co-transfection studies of CKLiK and CaMKK indeed demonstrated a small Ca^{2+}-independent activity [9]. PAF and fMLP are both inflammatory mediators that can trigger a rise in $[Ca^{2+}]_i$, by binding to their respective G-protein-coupled receptors. PAF in eosinophils and fMLP in neutrophils are potent inducers of granulocyte effector functions. Here we demonstrate that stimulation of granulocytes with these inflammatory mediators results in induction of CKLiK kinase activity (Fig. 4). This activation of CKLiK parallels the transient rise of Ca^{2+} observed upon fMLP stimulation, and is in line with the observed Ca^{2+}-dependent CKLiK activation.

Terminally differentiated cells such as granulocytes are difficult to manipulate by conventional transfection methodologies. Thus far, most studies that describe the role of intracellular signaling pathways in granulocyte functioning have been performed using pharmacological inhibitors. Recently, we demonstrated that eosinophils could be transduced with dominant negative Ras or active Rho linked to an 11 amino sequence (YGRKKRRQRRR) from the human immunodeficiency virus (HIV) Tat protein [28 24]. This sequence has been found to be one of the most efficient domains at allowing proteins to traverse cell membranes, probably in a receptor-independent fashion [35-37]. When expressed as a N-terminal peptide linked to an unrelated protein, this sequence renders the fusion protein capable of entering cells in a time- and concentration- dependent manner into multiple cell types [35; 38; 39]. In order to investigate a role for CKLiK, we generated an inhibitory peptide containing amino acids 297 to 321 of CKLiK linked to the tat protein transduction domain. A similar, but distinct sequence derived from CaMKI and CaMKII has previously been shown to have an inhibitory effects on the kinase activity of CaMKI and CaMKII respectively [26; 40]. We were able to demonstrate a potent inhibitory effect of the CKLiK peptide on CKLiK kinase activity (Fig. 5A). Based on the crystal structure of CaMKI and mutation analysis it has been suggested that in an inactive state, the autoinhibitory domain (AID) of CKLiK acts as a pseudo-substrate by binding to the catalytic domain [11]. Indeed, the autoinhibitory domain of CKLiK is located in the sequence of the peptide, suggesting a mechanism by which this peptide can block the catalytic domain of CKLiK. Additionally the AID and the CBD are in critically close proximity and probably are overlapping [26], which indicates that this peptide may also act as a competitor of CKLiK for calmodulin binding.

Production of reactive oxygen species by the NADPH oxidase is a critical granulocyte effector function required for the elimination of invading pathogens. The importance of the NADPH oxidase is demonstrated in patients suffering from chronic granulomatous disease, which is caused by a defect in one of components of the NADPH oxidase [41]. In resting cells, the subunits of NADPH oxidase are localized at the membrane of specific granules ($gp91^{phox}$ and $p22^{phox}$) and in the cytoplasm ($p47^{phox}$ and $p67^{phox}$ and $p40^{phox}$) [3; 42]. In response to stimulation with inflammatory mediators, such as fMLP the cytosolic subunits and the small GTPases p21Rac and p21Rap are activated and interact with the membrane-bound subunits to form an activated enzyme complex. It has been described that formation of ROS in human granulocytes requires intracellular Ca^{2+} [6; 29]. However, the mechanism by

which Ca^{2+} is linked to the regulation of the respiratory burst is still unclear. Here, we demonstrate that incubation of granulocytes with CKLiK(297-321) resulted in reduced ROS production in human granulocytes stimulated with fMLP (Fig. 5B). One of the possible mechanisms by which CKLiK can influence the respiratory burst is via the activation of the small GTPases Rac or Rap1. Rac has been found to be necessary in the respiratory burst [43], whereas Rap1 co-purifies with flavocytochrome b558, but is not necessary for cell-free reconstitution of oxidase activity [44]. Both Rac and Rap1, have been shown to be activated by stimulation with the calcium ionophore ionomycin, or thapsigargin, which both elevate $[Ca^{2+}]_i$ [45; 46]. However, in Ca^{2+}-depleted cells GTP loaded active of Rac and Rap1, could still be observed. It has been described that Rap1 can be phosphorylated directly by CaMKIV *in vitro*, suggesting a link between Ca^{2+}/calmodulin dependent signaling mechanism and small GTPase signaling [47]. Therefore, it might be possible that CKLiK exerts a similar effect towards Rap1 in human granulocytes. Also, several findings support the involvement of Ca^{2+} in the activation of Rac. For example, in guinea pig neutrophils Rac GTP-loading could be inhibited by calmodulin inhibitors [48]. Furthermore, there are some indications that several guanine exchange factors (GEFs), which activate small GTPases by the exchange of GDP to GTP, can be activated in a Ca^{2+}-dependent mechanism. For Rac, two exchange factors have been identified: Vav and Tiam [49; 50]. Vav is predominantly expressed in hematopoietic cells, and is activated by tyrosine phosporylation, which excludes a direct role for CKLiK. Tiam1 on the other hand, has been shown to be phosphorylated by CaMKII, which results in translocation of Tiam1 to the membrane [51; 52]. It is still not clear which exchange factor is involved in the activation of Rac2 in the NADPH complex in human granulocytes and there are contradictory findings concerning the Ca^{2+}-dependent activation of Rac. It is still a possibility that CKLiK regulates the activity or the translocation of a GEF involved in small GTPase activation. Another potential role for CKLiK in regulating the respiratory burst involves the phosphorylation of $p47^{phox}$. Membrane translocation in intact cells is dependent on phosphorylation of multiple serine residues of $p47^{phox}$ [53]. $p47^{phox}$ phosphorylation occurs on multiple serine residues after neutrophil stimulation with fMLP [54]. These include some potential CKLiK phosphorylation sites For $p47^{phox}$, as was identified by consensus site comparison (data not shown). Activation of the respiratory burst in granulocytes requires priming for optimal activation [30]. In this study, we primed granulocytes with either GM-CSF or PAF. PAF is known to induce a Ca^{2+} signal and able to activate CKLiK (Fig. 4), whereas GM-CSF does not induce a rise of $[Ca^{2+}]_i$. We found, no differences in the inhibitory effect of CKLiK(297-321) when cells were either primed with GM-CSF or PAF (data not shown), indicating that CKLiK is probably not involved in the priming process, but rather is directly involved in the induction of the ROS production by fMLP.

Treatment of neutrophils with CKLiK(297-321) also demonstrated a dramatic inhibitory effect on neutrophil migration on albumin-coated surfaces. Several aspects of neutrophil migration have been suggested to be Ca^{2+}-dependent. Neutrophil movement on vitronectin and fibronectin, substrates that are encountered in the connected tissue stroma, is abrogated in Ca^{2+}-buffered conditions [7]. On vitronectin this loss of motility is due to the clustering of $\alpha v\beta 3$ integrin in the rear of the Ca^{2+}-buffered cells, and this could be mimicked by addition of inhibitors of the Ca^{2+}/calmodulin dependent phosphatase calcineurin. However the $[Ca^{2+}]_i$-sensitive motility of neutrophils on fibronectin, which is predominantly mediated by $\alpha 5\beta 1$, was not effected by calcineurin inhibitors [55; 56]. From these studies, an endocytotic recycling of integrins in motile neutrophils has been suggested. A role for CKLiK in Ca^{2+}-dependent recycling of integrins during migration is a possibility. The most abundant integrins found in neutrophils are the β2 integrins. Blocking these integrins inhibits spreading and motility on glass surfaces, fibrin and fibronectin [56-58], suggesting an important role in adhesion. In our study, pretreatment of neutrophils with CKLiK(297-321) before surface attachment, demonstrated less adherent cells (data not shown). This might be an indication for a role of CKLiK in β2-mediated attachment. Furthermore, a role for Ca^{2+} in rear release has been suggested. During migration neutrophils have to break adhesive contacts with the substratum at the rear of the cell and establish new contacts at the leading edge. Buffering of $[Ca^{2+}]_i$ results in impaired uropod retraction (data not shown, [7; 56; 59]). Myosin II activation was found to be necessary for uropod retraction [8]. This was suggested to be mediated by myosin light chain kinase (MLCK), since inhibitors against this kinase demonstrated a similar phenotype as cells buffered of $[Ca^{2+}]_i$. MLCK belongs to the family of Ca^{2+}/calmodulin dependent kinases similar as CKLiK, which might suggest a similar role for CKLiK. Additionally, we recently demonstrated a role for RhoA in the detachment of the uropod during migration [24]. We found that a dominant negative form of the small GTPase RhoA, and inhibition of the downstream kinase ROCK, abrogated granulocyte migration. ROCK leads to activation of the light chain of myosin II and probably works cooperatively with MLCK. As mentioned above several guanine exchange factors (GEFs) for

the small Rho GTPases are Ca^{2+}- dependent, and thus a role for CKLiK in this process is a possibility. Since the family of small Rho GTPases is involved in rearrangement of the cytoskeleton, migration and respiratory burst [60], it is possible that regulation of Rho GEFs could be a general mechanism by which CKLiK regulates these processes.

Our data are consistent with the hypothesis that CKLiK is a Ca^{2+}-calmodulin dependent kinase that couples G-protein coupled receptor functioning to granulocyte functionality. This, together with the restricted expression of this kinase, might make it an interesting drug target.

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Chapter 6

Identification of cytokine-regulated genes in human leukocytes *in vivo*

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J Allergy Clin Immunol., 2000, 105(4),760-768

Abstract

Background: Human polymorphic nuclear granulocytes (PMNs) such as neutrophils and eosinophils play a critical role in mediating inflammatory responses to microbial and parasitic infection. Exposure of these leukocytes to cytokines leads to an amplification of granulocyte effector functions by a mechanism termed "priming". While many studies have investigated the effects of granulocyte priming, little is known concerning the molecular mechanisms that lead to this phenomenon.

Objective: The purpose of this study was to identify potential markers for granulocyte priming and thus also to gain further insight into the pathogenesis of inflammatory responses.

Methods: We have utilized a modified differential display technique, Random Arbitrary Primed (RAP)-PCR to identify genes regulated during the priming of human PMNs by GM-CSF *in vitro*. Genes identified were validated by Northern blot analysis of *in vitro* and *in vivo* primed leukocytes.

Results: Several genes were identified and their expression characterized *in vitro*. One of these genes, 5-lipoxygenase activating protein (FLAP), was also found to be upregulated in leukocytes isolated after allergen challenge of allergic asthmatic patients.

Conclusion: The use of differential display technology is a rapid and effective means of identifying genes whose expression is regulated by priming *in vitro* and *in vivo*. Further analysis will lead to a better understanding of the priming phenotype and may provide further insight into the pathology of inflammatory processes.

Introduction

Human polymorphic nuclear granulocytes such as neutrophils and eosinophils play a critical role in mediating inflammatory responses to microbial and parasitic infection [1]. At the site of infection, granulocytes participate in the inflammatory reaction by phagocytosis, intracellular killing of bacteria, production of inflammatory mediators and release of pre-formed cytotoxic enzymes and proteins. Environmental factors can quickly amplify these granulocyte functions by a mechanism termed "priming" which is independent of protein synthesis [2-4]. Priming refers to a process by which the response of cells to a subsequent (activating) stimulus is amplified if these cells were previously exposed to a (priming) stimulus. This process is an important regulatory mechanism whereby phagocytes can be controlled while responding to the local conditions of their environment. For example, prior exposure of human eosinophils to either the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) or the inflammatory mediator platelet-activating factor (PAF) will greatly enhance the ability of these cells to be activated by subsequent exposure to opsonized particles [5;6]. While many studies have analyzed the effects of granulocyte priming, little is known concerning the molecular mechanisms that lead to this phenomenon. Recent studies have demonstrated the cytokine-mediated activation of various intracellular signaling pathways in human granulocytes, while the use of specific pharmacological inhibitors has implicated some of these events in the regulation of cell effector functions [7-12]. It is however unclear whether cytokine may also result in changes in granulocyte gene expression that might correlate with this phenomenon. Indeed, cytokine-induced regulation of gene expression in human granulocytes and the functional consequences thereof have not been studied in any detail.

In an attempt to gain further insight into the pathogenesis of inflammatory responses and also to identify potential markers for granulocyte priming, we have utilized the technique of differential-display [13]. This PCR-based technique is allows the identification and cloning of genes that are differentially expressed in cells under pre-determined conditions. The advantage of this technique lies in its ease of operation and the ability to process many samples at one time unlike previous methodologies such as subtractive hybridization. Utilizing a modified differential display technique, Random Arbitrary Primed (RAP)-PCR [14], we have analyzed the expression of genes either up- or down-regulated in human PMNs after treatment with physiological concentrations of GM-CSF. Several known genes were identified *in vitro* and their regulation by GM-CSF was confirmed by Northern blotting. Furthermore, we demonstrate that one of the genes identified, 5-lipoxygenase activating protein (FLAP), was also up regulated *in vivo* in peripheral blood leukocytes after allergen challenge.

These data confirm that differential-display technology can indeed be utilized to gain further insight into the mechanisms of granulocyte priming and pathogenesis of inflammatory reactions.

Materials and Methods

Isolation of human granulocytes.

Blood was obtained from a healthy donor with no history of allergy or asthma . There was no relevant clinical history (no allergy, no parasitic infection, no indications of other eosinophilic diseases). However, a persistent (>10 years) finding blood of slight leukocytosis (~$8x10^9$ leukocytes/liter) and 30% eosinophils in the granulocyte fraction was observed. Apart from the elevated amount of eosinophils the remainder of the leukocyte population was within the normal range. Total leukocytes $7.3x10^9$/litre: 2% basophils, 18% eosinophils, 34% neutrophils, 35% lymphocytes and 11% monocytes.

Mononuclear cells were removed by centrifugation over Ficoll-paque (density 1,077/cm^3) , 1000xg, 20 min at RT. The lower cell layer was collected and the remaining erythrocytes were lyzed by incubation in isotonic 115mM ammonium chloride solution (pH 7.4) on ice for 20 min. After centrifugation the granulocytes were incubated in RPMI-1640 (Life Technologies, Paisley, UK) containing 0.5% HSA (wt/vol) for 30 min at 37°C. Cells were washed with PBS and half of the cells were stimulated with GM-CSF (10^{-10} M) for 3 hours in RPMI-medium supplemented with 25mM Hepes, 8% fetal calf serum (FCS, Hyclone), 1 mg/ml penicillin and 1 mg/ml streptomycin in a CO_2 incubator (5% CO_2, 37°C).

RNA isolation.

Total RNA was isolated as follows: 10^8 cells were resuspended in 500 µl sterile PBS and 10 µl of RNAsin (Promega, Madison, WI) was added. The cells were lyzed in 20 ml of GIT solution (6M guanidine thiocyanate, 25mM sodium citrate, 0.5% N'Lauroyl sarcosine, 100mM β-mercaptoethanol). One volume of sodium acetate (2M, pH 4.0) was added and an equal volume of phenol followed by vortexing. Half of the total volume of chloroform/iso-amylalcohol was added to the solution and vortexed. The solution was incubated on ice for 15 min and centrifuged at 4°C for 20 min at 10.000xg. The upper phase was extracted with half volume of chloroform/iso-amylalcohol and centrifuged for 2 min. RNA was precipitated with an equal volume of 2-propanol and 200mg of carrier glycogen at -20 °C for overnight. Samples were centrifuged at 81000 rpm for one hour, re-suspended in a small volume and RNA was re-precipitated with ethanol. Poly A^+ mRNA was extracted by Oligo-dT affinity purification using Dynabeads Oligo(dT)25 (Dynal, Oslo, Norway). cDNA was synthesized using a ProStar First Strand RT-PCR kit as described manufacturers protocol (Stratagene).

RAP PCR.

The differential display PCR reactions were performed with 1 µl of cDNA, 1x Goldstar reaction buffer, 1.5mM $MgCl_2$, 200ng primer, 2mM dNTPs, 1µCi of [^{33}P]dATP and 4 units of Goldstar Taq polymerase (Eurogentec, Seraing, Belgium) per 20 µl reaction. Cycles were performed as follows: 3 min 96°C, 2x (1 min 96°C, 5 min 37°C, 5 min 37°C to 72°C), 30x (1 min 96°C, 2 min 50°C, 5 min 72°C) and 10 min 72°C. The annealing temperature was increased stepwise from 37°C to 50°C allowing the arbitrary primer to bind over multiple homologous regions. From the PCR reaction, 5µl was mixed with 5µl of 96% formamide loading buffer (0.05% (wt/vol) xylene cyanol, 0.05% (wt/vol) bromophenol blue) and boiled for 2 min. The amplified cDNAs were loaded on a 6% sequencing gel and subjected to electrophoresis. Gels were vacuum dried and autoradiographed with Biomax MR film (Eastman Kodak, Rochester, NY) at -70°C. The dried gel and autoradiogram were orientated and cDNA bands of interest were excised and immersed in 100µl TE. DNA was eluted by twice freezing and thawing in liquid N_2 and vortexing. Samples were extracted twice with phenol-chloroform-isoamylalcohol (25:24:1) and once with chloroform-iso-amylalcohol. cDNA was re-amplified by using 5-10µl in a standard 50µl PCR reaction and utilizing the primer used for the original differential display (annealing temperature, 50°C). The mixture was separated by agarose gel electrophoresis, bands were excised, purified with Glassmax (Life Technologies Inc., Paisley, UK) and cloned in pGEM-T (Promega, Madison, WI) or PCR-script (Stratagene, La Jolla, CA). Plasmid DNA was isolated from 5 to 8 colonies per excised band and inserts sequenced.

Northern blotting analysis.

PMNs (97% neutrophils; <3% eosinophils) were isolated from blood obtained from healthy volunteers from the Red Cross Blood Bank, Utrecht, The Netherlands, as described above. Cells were incubated in the presence or absence of GM-CSF (10^{-10} M) or TNFα (100 U/ml) in RPMI-medium (Life Technologies Inc., Paisley, UK) supplemented with 25mM Hepes, 8% fetal calf serum, 1mg/ml penicillin and 1mg/ml streptomycin in a CO_2 incubator (5% CO_2, 37°C). Cells were washed twice with PBS and total RNA was isolated using the acid-phenol method [15]. 20µg RNA was lyophilized and separated on a 0.8% agarose formaldehyde gel. RNA was transferred to Hybond-N membranes (Amersham, Rainham, UK) by capillary transfer using 10x SSC (1.5M sodium chloride, 0.15M sodium citrate) overnight. The blots were washed and baked for 2 hours at 80°C. Differential display fragments were used as probes and were labeled with α[^{32}P]-dCTP by Rediprime random priming according to manufacturer protocol (Amersham, Rainham, UK). Blots were hybridized overnight at 42°C, washed subsequently with 2x SSC/0.1% SDS, 1x SSC/0.1% SDS, 0.1x SSC/0.1% SDS, and exposed to film (Kodak, Rochester, NY). A 1.4kb cDNA fragment of the human glyceraldehyde-3-phosphate dehydrogenase gene was used to control for equal loading.

Allergen challenge.

Patients used for this study had a history of periodic wheeze and demonstrated a reversibility of more than 15% in forced expiratory volume in one second (FEV_1), 15 minutes after inhalation of 400µg salbutamol from a pressurized metered dose inhaler attached to an aerosol chamber (Volumatic®, Allen and Hanburys, Greenford, UK). None of

them had used oral corticosteroids in maintenance dose during 12 months before the study. All subjects were clinically stable at the time of the study and had no evidence of systemic or respiratory disease. On the day of the experiment, the FEV_1 value was >60% predicted. Furthermore, patients were required to show an increased bronchial hyperresponsiveness to histamine with a provocative concentration causing a 20% fall in FEV_1 of less than 4.0mg.mL^{-1} (PC_{20}<4.0mg.ml^{-1}), and a fall in FEV_1 within 1 hour after allergen inhalation challenge of at least 20%. All patients had blood eosinophilia (>5%) and raised levels of total serum IgE and specific IgE antibodies to either house dust mite or cat. Patient data is summarized in Table 1.

Bronchial provocation with allergen was performed by 2 min inhalations (tidal breathing) with 10 min intervals. Allergen provocation was preceded by an initial aerosol of buffer (2.5mg/ml $NaHCO_3$ with 5mg/ml NaCl, 0,03% HAS and 0.5% phenol) to which none of the patients reacted with a fall in FEV_1. Increasing concentrations of antigen aerosol were delivered through a nebulizer (model 646, de Vilbiss Co, Somerset, PA). House dust mite antigen (10,000 biological units/ml; Allergy Laboratory Copenhagen, Denmark) and cat (10,000 biological units/ml; Allergy Laboratory Copenhagen, Denmark) were diluted from stock solution and the dilution used for the first inhalation was calculated from the skin-prick test and the pre-antigen PC_{20} for histamine according to Cockcroft *et al.* [16]. Inhalations were performed by stepwise doubling the dose of antigen until the FEV_1 fell at least 20% from baseline values. Blood was collected at various time points after allergen challenge as indicated via an intravenous catheter and was anti-coagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4) and kept at 4 °C. Total leukocytes were isolated after isotonic lysis of the erythrocytes in ice-cold ammonium chloride solution containing 155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA (pH 7.2). Total RNA isolation, blotting and hybridization were performed as previously described.

Table 1: *PC_{20} and effects of allergen inhalation challenge on lung function*

				Fall in PEF from baseline (%)	
Allergen	PC20 histamine (mg.mL-1)	Specific IgE (kU.L-1)	Fall in FEV1 from baseline <20 min (%)	<20 min	3-8 hrs
Der p	0.21	57	29	28	30
Cat	0.16	59	27	18	13

Allergen inhalation challenge was performed with house dust mite (Dermatophagoides pteronyssinus; Der p) or cat allergen. After PC_{20} allergen challenge, perecntage fall in FEV_1 from baseline within 20 min and percentage fall in PEF from baseline within 20 min and 3-8 hrs were measured. PC_{20}: provocative concentration producing a 20% fall in FEV1; BU: biological units; PEF: peak expiratory flow.

Results

Method of differential screening for cytokine induced genes

Little is currently known concerning the regulation of genes expressed in response to cytokine-challenge of human granulocytes. Genes that are regulated by cytokines may be utilized as potential markers for pre-activation or "priming" of these phagocytes. Furthermore, these genes may provide greater insight into the pathogenesis of inflammatory disorders. In order to identify differentially expressed genes several approaches have been previously been utilized. For example, subtractive hybridization or differential hybridization is an approach based on removing the common transcripts between different cell types or tissues, leaving the specific transcripts for further manipulation and analysis. These are mainly qualitative methods and extremely time consuming. Secondly, nuclear run-on transcription has been applied to analyze changes in the level of expression of mRNAs but this method can only be applied to the detection of changes in the expression of known genes.

More recently a novel method of analyzing gene expression, termed differential display, has been described [13]. This technique provides a sensitive and flexible approach to the identification of genes that are differentially expressed at the mRNA level. Differential display is based up on selective reverse transcription of expressed mRNAs and subsequent amplification using PCR in which a radioactive label is included. For the PCR reaction an anchored oligo-dT primer is utilized to selectively amplify a subset of mRNAs. The amplified, labeled PCR-fragments can then be separated and compared on a polyacrylamide gel with cell specific band patterns. A

modification of this technique termed Random Arbitrary Primed (RAP)-PCR has been utilized in this study [14], and the procedure is represented schematically in Figure 1. In this modification, a single arbitrary primer is utilized in the PCR cycles. Such an arbitrary primer can be selected with the criteria that it should be approximately 18bp long and have roughly a 50% (A/T):(G/C) ratio and have an annealing temperature of around 50^{O}C (see Table 2 and Ref 14). Primers were also selected that generated between 20-50 bands per lane. This primer is allowed to anneal to the reverse-transcribed PMN cDNA at low temperature, allowing it to act as both a forward and reverse primer. It thus tends to select a subset of cDNAs with somewhat palindromic 5'- and 3'-sequences. A disadvantage of the original anchored oligo-dT method was the identification of many 3'-untranslated regions of genes, which are difficult to analyze. The RAP-PCR protocol does not restrict primer annealing to these untranslated regions and thus provides greater opportunities to amplify the coding regions of differentially expressed genes. This leads to a more rapid analysis of the identification of the cloned gene. Primers were tested for their suitability in this assay and were selected by two criteria, the length should be around 18 base pairs and the annealing temperature should be above 50°C. Arbitrary primers were subsequently chosen that met these criteria and are listed in Table 2.

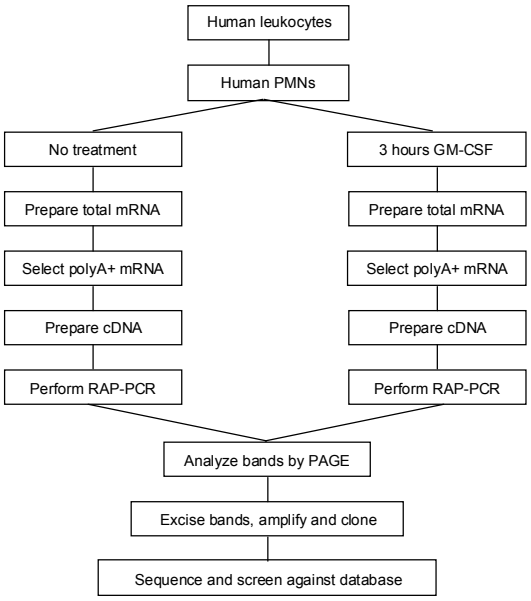

Figure 1: *Flow diagram of RAP-PCR methodology.*
Human PMNs are isolated from 500ml whole blood and cultured in the presence or absence of GM-CSF. Total RNA is isolated, poly-A+ mRNA and cDNA prepared and subjected to RAP-PCR analysis. Up- or down-regulated bands were excised, cloned and sequenced. See Materials and methods for full-details.

Table 2: *Sequences of primers utilized for differential display reactions.*

Primer	Sequence	Length (bp)
1	GGCCAGATGCATGTTCAA	18
2	CACAAGGTGGCAGGATGT	18
3	CTGTCGCCAGAGAAGATG	18
4	GGAAGCCTTCTCTCTCC	17
5	GGAGTCCTGTCTCCTCCG	18
6	GTCTCGACCGGCACCAAG	18
7	CTGCAGCTTCCGTTCTCA	18

Identification of novel GM-CSF induced genes in human PMNs

RAP-PCR was performed on RNA isolated from human granulocytes treated with or without GM-CSF (10^{-10}M) for 3 hours, as described in the Materials and Methods. Samples were analyzed by acrylamide gel electrophoresis as shown in Figure 2. Around 30 fragments were found to be clearly differentially regulated and most of them represented up-regulation in the presence of GM-CSF. Each differentially expressed band was excised from the gel and amplified with the original primer used for differential display reactions. The fragments obtained were cloned and partially sequenced. These sequences were then screened against available nucleotide databases and the results of the sequence comparisons are outlined in Tables 3 and 4.

Interestingly, Five-Lipoxygenase Activating Protein (FLAP), an important regulator of the leukotriene synthesis was identified as a gene up regulated by GM-CSF [17]. Leukotrienes are potent lipid mediators of inflammatory responses and have been implicated in the pathophysiology of both acute and chronic inflammatory diseases including asthma. A second potentially interesting product was the Immunoglobulin-Like Transcript-2 (ILT-2). ILT-2 has a large cytoplasmic tail which contains putative ITIMs (Immunoreceptor Tyrosine-based Inhibitory Motif) similar to those found in Killer cell Inhibitory Receptors [18]. These tyrosine-based motifs implicate ILT-2 in inhibitory signaling pathways. Furthermore, the transcription factor c-fos was identified. c-Fos is transiently induced by a wide variety of extracellular stimuli [19]. Ferritin, another gene identified, is the major iron-storage protein in eukaryotes, also has a potential role in the regulation of myelopoiesis and furthermore, has been implicated as a tumor marker [20].

Although we found several previously described genes many novel sequences were also identified that, at this time, have no identity to either known genes or human Expressed Sequence Tags (EST) sequences (Table 4). The percentage homology of these clones with EST clones is given along with the length of the insert obtained after cloning the RAP-PCR product. Extensive work is now required to determine the identity of these coding sequences and to correlate their expression with cytokine priming.

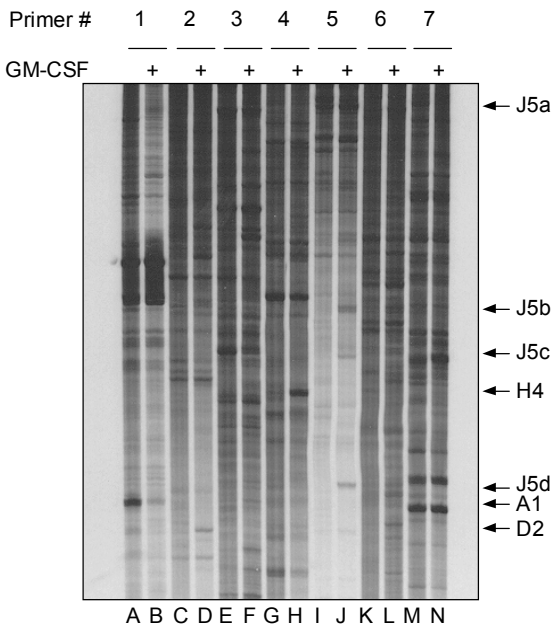

Figure 2: *Identification of differentially regulated transcripts in PMNs after GM-CSF treatment.*

Human PMNs were isolated as indicated in the Materials and Methods. Cells were incubated with or without GM-CSF (10^{-10}M) for 3 hours. Poly A^+ mRNA was isolated and cDNA prepared for RAP-PCR analysis. Seven different arbitrary primers were utilized as indicated. Lanes are labeled A-N to aid description. Indicated are the bands that were isolated, amplified, purified, cloned and sequenced (see Table 3).

Table 3: *cDNAs isolated and cloned from RAP-PCR reactions*

Band Code	Fragment size (bp)	Identity
A1	500	PECAM-1
D2	500	ADP/ATP Carrier Protein
H4	900	FLAP
J5a	550	Ferritin Heavy Chain
J5b	250	ILT-2
J5c	250	c-Fos
J5d	300	Cytochrome Oxidase-1

ILT-2, Ig-Like transcript-2

Table 4: *Novel cDNAs cloned from RAP-PCR*

Clone	Size	Primer	Sequence	EST	Identity	Strand
13	500	1	caagggacgtttgcatgagtccatggtatgttcaggtgtccaagagtgaa	AA470449	71/104 (68%)	Minus/Plus
20	750	1	aagactgagtggctgggtgcagtggctcacacctgtaatctcagtatttt	THC83029	130/158 (82%)	Minus/Plus
36	600	1	gcaaggtttgacaagcggcttacaaaattcctaaattcttcacaatcacc	THC280785	93/147 (63%)	Plus/Plus
42	450	1	ggcacagcacacactgggcaatggatggaaggagactggcccaaggaac	W23538	46/60 (76%)	Plus/Plus
79	350	4	gctgctctctgtctctttctctgctcaaggcttaaggccgtgtctcccca	THC287677	53/73 (72%)	Minus/Plus
90	500	5	agaggctcaaaaagcagccgggcctgaatttcattctgtttccaaagaga	THC266464	61/92 (72%)	Minus/Plus
93	600	5	cctgcctgtccccagcaccgcctggctcacgtcaccctggtaattgctcc	THC101232	98/158 (62%)	Minus/Plus
99	350	5	ttgaggttgcgtctgttagtagtatagtgatgccagcagctaggactggg	THC197030	101/141 (78%)	Minus/Plus
101	500	5	ggacagacaggaaatcgctgtgcagggcccctgcctgtgcccagcaccgc	THC254052	54/73 (73%)	Plus/Plus
103	400	5	gtcctcatggcctaggcagctgcagccgccagacagtgagcccggacaca	R81083	50/67 (74%)	Plus/Plus
113	450	5	gatccattcgtccatggatggatacttaagttgattccatatttcagtta	AA084052	94/124 (75%)	Minus/Plus
122	650	2	cagattggcttcttagtaatacatttgagtttctttcatggcttgacatc	AA205247	65/85 (76%)	Minus/Plus
129	650	5	tgattggcccctgggattcttcctccttgcctcctctccctgggttcatc	THC292692	97/148 (65%)	Plus/Plus
139	600	5	gtcctcagggcctaggcacctgcagccgccagacagtgagcccggacaca	AI095822	97/152 (63%)	Minus/Plus
148	500	6	tcaatacaggtagtctgatgactaacgagagggaacataaggaccctatg	AA558722	65/91 (71%)	Minus/Plus
238	300	2	gtcatcgttgaggctcaggtcggtcggataccgccaactggaacaccctg	EST185621	62/87 (71%)	Minus/Plus
244	650	2	aggatgtccagcccatatgaggagcttgttgaattgaattgcgtggagac	EST68741	49/70 (70%)	Plus/Plus
247	550	2	ggatgtgggacaaagtcaaagattattattaagctctaaggttttaatgt	THC267228	131/176 (74%)	Minus/Plus
249	600	2	aggatgtgtgtagcaacaggagctctcgttcacggctgctgggaacgcaa	THC150192	90/127 (70%)	Minus/Plus
255	650	2	gaccctcagagggacctagaaaagctggtccctccagtccctttgaggca	THC269356	57/84 (67%)	Plus/Plus
256	700	2	gtgccaggccttaggtcataccagacttggcaggggttgaggatcgaggg	THC203964	68/94 (72%)	Minus/Plus
262	400	3	ctgtcgccagagaagatgcccactttatgaataagcagaattaactacat	THC270838	120/123 (97%)	Plus/Plus
273	400	4	ctgtcgccagagaagatgtttatttgatgtaacaaaacaatacagttagt	THC254502	120/125 (96%)	Minus/Plus
286	600	5	ggacagacaggaaatcgctgtgcagggcccctgcctgtccccagcaccgc	AA502694	46/64 (71%)	Minus/Plus
294	350	5	gggcatgttgctcccactcaccagcttggcccatcacagagcttctccc	THC282228	71/104 (68%)	Plus/Plus
311	650	7	atattgattcagataggcataatctattcatttttcataatgaaatgtat	THC212219	137/157 (87%)	Minus/Plus
314	450	7	gggagctaaggagaaccatcatatgtgtccatctcttccgcatttacatg	THC232302	114/154 (74%)	Minus/Plus
320	400	7	cttccgttctcaggagttggccctgtggttttggtgaaaactctggcctt	THC271399	73/112 (65%)	Minus/Plus

Data were obtained by screening the Institute for Genome Research's expresed sequence tag (EST) databases, which can be found at http:/www.tigr.com

Confirmation of GM-CSF-mediated induction of genes in human PMNs

To confirm that the genes isolated were indeed differentially expressed, Northern blots of total RNA from human PMNs (97% neutrophils; <3% eosinophils) incubated with GM-CSF were probed with selected cDNAs. Figure 3a shows a strong up-regulation of the FLAP gene after 3 hours treatment with GM-CSF. Similarly, ILT-2 mRNA was also rapidly induced after 3 hours. Since c-fos mRNA is known to be very rapidly induced by extracellular stimuli [21], we prepared a northern blot including a 30 minutes GM-CSF time point. Indeed, c-*fos* expression was extremely rapidly and transiently up regulated, being optimally expressed after 30 min. Interestingly while c-*fos* appears to be both rapidly and transiently up-regulated, as is characteristic of immediate early genes, both FLAP and ILT-2 are slower, and levels remain high for at least 3 hours. Data was confirmed in at least three independent donors. These data demonstrate that the genes identified as being differentially expressed by the RAP-PCR technique are indeed upregulated in human PMNs stimulated with GM-CSF.

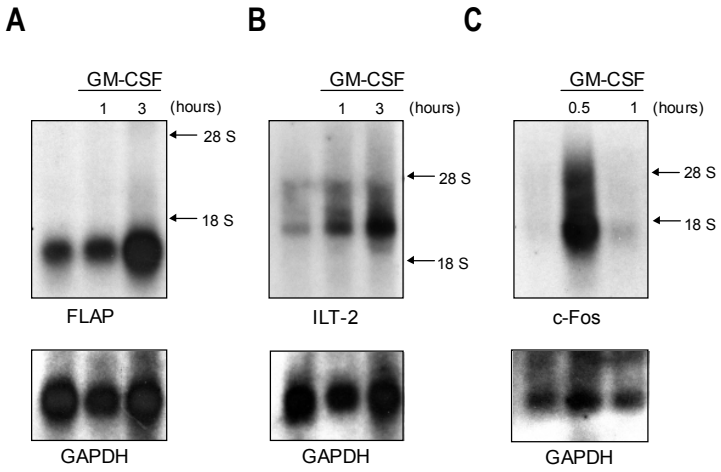

Figure 3: *GM-CSF induces the expression of RAP-PCR products in human PMNs.*
Human PMNs were incubated for the indicated times with GM-CSF (10^{-10}M). 20 μg of total RNA per lane was used for northern blotting and blots were hybridized with the isolated RAP-PCR fragments (upper panels). **(A)** FLAP (Five-Lipoxygenase Activating Protein) **(B)** ILT-2 (Immunoglobulin-Like Transcript-2) and **(C)** c-Fos. All blots were reprobed with GAPDH (Glyceraldehyde Phosphate Dehydrogenase) to correct for equal loading (lower panels). The positions of 28S and 18S RNA are indicated.

FLAP is rapidly induced in human PMNs by both GM-CSF and TNFα

To analyze the duration and extent of FLAP expression in human PMNs in detail, we prepared RNA from cells stimulated for up to 6 hours with GM-CSF. Interestingly, while FLAP expression is rapidly induced, it remains high for at least 6 hours (Fig. 4A). Since the "lifespan" of human neutrophils is rather short, even in the presence of cytokines, it appears that once FLAP is upregulated, it remains highly expressed until the granulocyte apoptotic program is induced. Thus, FLAP provides a somewhat permanent marker for the activation status of these phagocytes. To determine if FLAP may be induced by other inflammatory stimuli and thus a general marker of inflammation, we also stimulated human PMNs with TNF-α. Again, the expression of FLAP in PMNs treated with TNF-α was increased and remained high for at least 6 hours (Fig. 4B). This suggests that at least some of the GM-CSF-stimulated mRNAs are also markers of a common pool of inflammatory cytokine-induced genes.

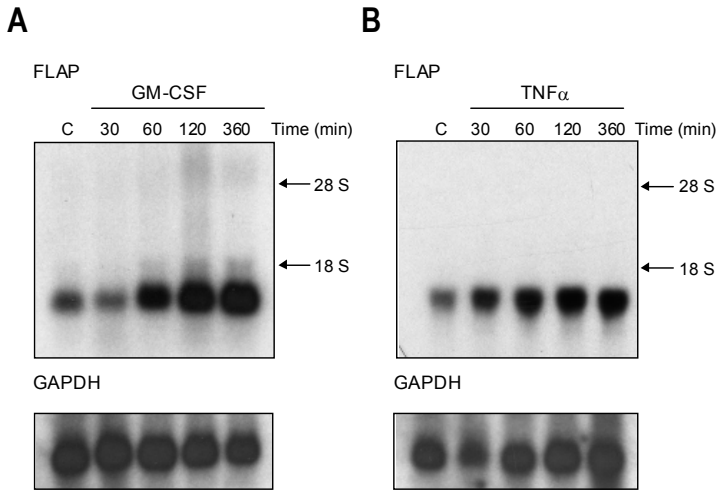

Figure 4: *FLAP mRNA expression is induced in GM-CSF and TNF-α stimulated PMNs.*
Northern blots were prepared from RNA of PMNs stimulated with **(A)** GM-CSF (10^{-10}M) and **(B)** TNF-α (100 U/ml) for the indicated time points. 20 μg of total RNA per lane was loaded and blots were probed with the RAP-PCR fragment of FLAP. The positions of 28S and 18S RNA are indicated.

Allergen challenge induces FLAP-expression in peripheral blood leukocytes in vivo

We wished to determine whether the increase in FLAP-expression we observed by *in vitro* stimulation of PMNs could also be observed *in vivo*. To do this, we analyzed FLAP-expression in the total leukocyte population from allergic asthma patients after allergen inhalation challenge. Total leukocytes were utilised and not PMNs for several reasons: (1) it is not possible to isolate sufficient amounts of eosinophils and neutrophils from 10ml of blood to obtain enough mRNA for northern blotting. (2) Isolation of cells takes at least 2 hrs (neutrophils) or 4 hrs (eosinophils). These rather long isolation times can interfere with the detection of fast and transient-induced genes. (3) By using total leukocytes, aspecific priming caused by the isolation procedures was minimized. Blood was collected after allergen challenge and leukocytes isolated as indicated in detail in the Materials and Methods. Northern blots of RNA from two patients who were allergic to either house-dust mite or cat were probed for FLAP mRNA expression. The methodology used for allergen challenge is detailed in the Materials and Methods. The expression of FLAP *in vivo* was also very rapidly induced, reaching a maximum level in both patients between 30 min and 1 hour, before decreasing to basal levels within 4-5 hours (Figure 5). This decrease to basal levels is in contrast to what was observed in isolated PMNs stimulated *in vitro* with cytokines (see Figure 4). This discrepancy is likely to reflect the fact that in these *in vivo* experiments the primed leukocytes will migrate out of the peripheral blood after several hours. These data demonstrate that gene expression identified *in vitro* by cytokine challenge of human PMNs reflects the *in vivo* situation after induction of an inflammatory response.

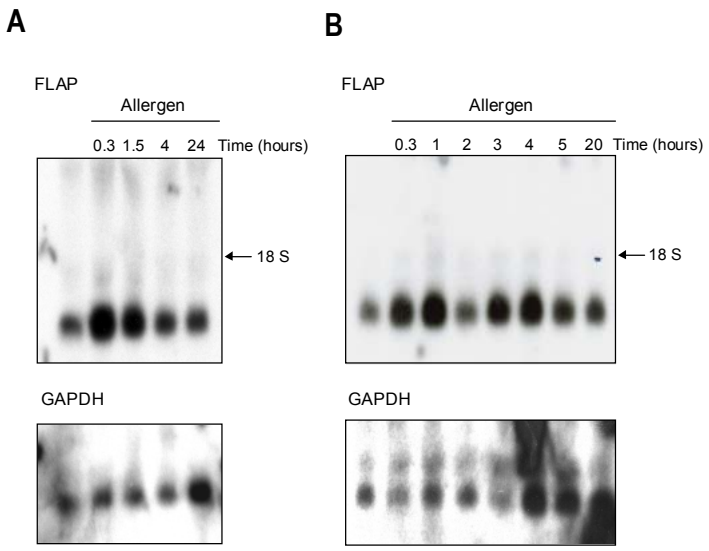

Figure 5: *FLAP expression is increased in leukocytes isolated from allergic asthmatics after allergen inhalation challenge.*
Total RNA was prepared from total white cell fractions of blood taken from patients before (control) and after allergen challenge at the times indicated. **(A)** House-dust mite allergen (10.000 biological units/ml) and **(B)** Cat allergen (10.000 biological units/ml) were utilized. Inhalations were performed as previously described and samples were collected after the FEV_1 was at least 20% below baseline values. 10μg of total RNA per lane was loaded and blots were probed with the differential display fragment of FLAP (upper panels). Blots were subsequently re-probed with GAPDH to correct for equal loading (lower panels).

Discussion

Phagocytes (granulocytes and monocytes) in the peripheral blood are characterized by a non-primed phenotype that is refractory to activation in the context of cytotoxic responses [2-4]. This is a safeguard for the inappropriate activation of these cells in the peripheral blood. In severe clinical conditions, such as septic shock, uncontrolled activation of phagocytes can lead to multi organ failure and death. At inflammatory loci, such as bronchial tissue in allergic asthma, eosinophils are attracted to the site of inflammation and become activated [1]. The process of extravasation of inflammatory cells to these loci is a tightly controlled multistep process. The first step involves the interaction of phagocytes in the peripheral blood by cytokines liberated from the inflammatory locus. This results in pre-activation or priming of cells, which is essential for their subsequent extravasation and the eventual activation of cytotoxic responses upon interaction with activating stimuli. Therefore, the process of priming is critical for regulation of phagocyte effector functions *in vivo*. This multistep paradigm of phagocyte activation predicts that cytokine-mediated priming of peripheral blood granulocytes is a result of a local inflammatory reaction in a given organ. Indeed, several studies have shown that inflammatory cells are characterized by a primed phenotype in the peripheral blood of patients with allergic inflammatory conditions [22-25]. Understanding the mechanisms of priming might be useful in the development specific approaches to inhibit this phenomenon thereby preventing inflammatory cells becoming optimally activated. Moreover, this *in vivo* primed phenotype of inflammatory cells in the peripheral blood might be used to develop strategies to measure this phenotype as read out for the severity of inflammatory processes localized in tissues. This diagnostic approach has been followed by Venge and colleagues who have measured the release of eosinophil cationic protein by eosinophils in the blood of allergic asthmatics ex-*vivo* [26;27]. This phenomenon occurs during clotting of blood in samples of patients with various inflammatory diseases in particular allergic asthma [28]. The ability of eosinophils to release ECP under these conditions can be considered as an indirect measure of eosinophil priming *in vivo*. We have tried to measure priming of isolated eosinophils as a measure of changes in inflammatory processes in the lung during anti-inflammatory treatment (unpublished data.). Although PAF-induced eosinophil chemotaxis is inhibited by this type of treatment, functional eosinophil responses such as chemotaxis and activation of the respiratory burst cannot be routinely used for this type of study. The methodology is too laborious and the data are too sensitive for variations in processing of the cells.

Despite recognition of the importance of priming, very little is currently known as to the precise molecular mechanisms of this phenomenon. Therefore, we set out experiments to find new determinants of cytokine-induced priming utilizing the technique of differential display. This powerful PCR based technique has enabled us to define several mRNAs that were either up- or down regulated during GM-CSF treatment of PMNs *in vitro* (see Table 3). While utilizing RAP-PCR will not lead to the identification of *all* genes regulated by cytokine priming it does result in the identification of a large subset of genes. In this article alone we describe around 25 unknown genes that must now be subsequently characterized (Table 4). Interestingly, two of the genes identified, ferritin-H and cytochrome oxidase, were also identified as allergen induced genes in monocytes isolated from mononuclear cells of patients with allergic asthma [29]. Ferritin-H plays an important role in iron storage and iron sequestration [20]. Our data suggest that in human PMNs, GM-CSF may play a role in regulating iron homeostasis through regulating the expression of ferritin-H. Indeed it has been reported, in primary human myoblasts, that TNFα induces ferritin-H mRNA independently of cellular iron concentrations and known iron-dependent regulatory pathways [30]. This suggests that regulation of iron homeostasis by inflammatory cytokines might be a general response of cells. Increased sequestration of iron by increasing the levels of ferritin-H might serve the beneficial functions of, (a) making iron unavailable to invading organisms or, (b) reducing free-radical tissue damage. The latter may play an important role in phagocyte functioning, protecting cells against damage during the killing of microbial organisms by superoxide production.

We have also identified the transcription factor c-fos as a rapidly upregulated gene in PMNs (Fig. 3c). This transcription factor is a member of a family of immediate early response genes whose expression is regulated by a variety of cytokines, growth factors and hormones [19]. Once expressed, it is then able to bind and activate the promoters of a diverse array of cellular genes. Thus, GM-CSF induced c-fos expression in human neutrophils may be responsible for rapidly "switching on" relevant target genes needed for initiating a correct inflammatory response.

Another GM-CSF regulated gene identified is the immunoglobulin-like transcript 2 (ILT-2; Fig. 3b), a transmembrane protein that is expressed on many myeloid and lymphoid cells [18]. This receptor binds MHC class I molecules and delivers a negative signal that inhibits killing by NK and Tcells, as well as Ca^{2+}-mobilization in B cells triggered through the B-cell antigen receptor [31]. Cytokine regulated expression of this gene has not previously reported neither has its expression in neutrophils. Our finding that ILT-2 can be upregulated by GM-CSF suggest a potentially novel mechanism for regulating cellular activation during an immune response.

5-lipoxygenase activating protein (FLAP) was identified as a gene potently upregulated in neutrophils treated with GM-CSF and TNFα (Fig. 4). This protein is a critical component in the synthesis of leukotrienes [17]. Leukotrienes are secreted by neutrophils, eosinophils, mast cells and macrophages and potently stimulate bronchoconstriction and mucous secretion, and cause vasodilatation with increases in postcapillary permeability [32]. It is thus interesting that through differential display we were able to identify FLAP as a marker of primed granulocytes *in vitro*. The consequences of FLAP upregulation are thus likely to be an increased inflammatory response.

Differential display was utilized to find determinants of priming in the peripheral blood of allergic asthmatics. The most prominent signal *in vitro* was found with the FLAP mRNA on Northern blots from cytokine treated granulocytes. To confirm that genes identified *in vitro* were also relevant *in vivo*, mRNA was prepared from total leukocytes isolated from allergic asthmatic patients before and after allergen challenge. Total leukocytes were chosen over isolated PMNs, due to the relatively long time of isolation and the sensitivity of these granulocytes for aspecific pre-activation during isolation [33]. As can be seen from Figure 5, FLAP mRNA is rapidly induced after allergen challenge and this increase is transient. These findings are consistent with a model that inflammatory blood cells encounter priming signals in the peripheral blood. These cytokines modulate the transcription of several genes including the induction of the FLAP gene. Moreover, these cells become much more responsive to chemotactic factors [34]. These primed cells are destined to leave the bloodstream and extravasate into the tissues leaving the unprimed cells in the peripheral blood. The observation that FLAP is also upregulated *in vivo* validates our chosen methodology. Further *in vivo* analysis of both the known and unknown genes identified in our screen may well lead to the identification of novel markers of inflammation as well as further insight into immune regulation.

Concluding, differential display enables the characterization of genes that are differentially transcribed in response to cytokines and are relevant for the pathogenesis of inflammatory diseases. This occurs *in vitro* and *in vivo* in patients with symptomatic asthma. Characterization of novel genes will enable us to find new markers of inflammatory diseases and will provide valuable information on the processes that occur early in the sequence eventually leading to activation of inflammatory cells in the tissues.

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Chapter 7

General Discussion

Neutrophils mature in the bone marrow to become functional phagocytotic cells. The main function of neutrophils is to destroy invading microbial pathogens in order to protect the host from infections. During inflammation chemoattractants and cytokines bind to specific receptors expressed on the neutrophil surface resulting in the initiation of specific granulocyte effector functions, such as priming, adhesion, migration, respiratory burst and degranulation. Correct control of all these processes is necessary for efficient functioning of the neutrophil. Dysregulation or defects in neutrophil functioning can lead to immune failure or inflammatory diseases. Therefore, it is of importance to understand the downstream signaling events activated by cytokines and chemoattractants that regulate neutrophil maturation and function.

The work described in this thesis work has focussed on the intracellular signaling pathways induced by inflammatory mediators that are involved in maturation and functioning of human neutrophils. An ex-*vivo* differentiation model was developed to study granulopoiesis, and the roles of PI3K and MEK were investigated using specific pharmacological inhibitors. Here, the results of this work are discussed with respect to cell fate decisions.

Two approaches were used to investigate granulocyte functioning. Random arbitrary primer PCR was applied to identify genes in granulocytes that were regulated by GM-CSF treatment. This technique was found to be effective for identifying genes regulated *in vitro* as well as *in vivo*. Secondly, degenerate PCR technique was used to identify novel kinases in human granulocytes. In this way, a novel granulocyte specific kinase was cloned whose possible function(s) are discussed in this section.

Neutrophil maturation

Granulopoiesis is a complex process by which hematopoietic stem cells (HCS) mature into fully differentiated functionally competent granulocytes. This process is associated with the control of proliferation and differentiation, which are regulated by external stimuli including cytokines and growth factors as well as interactions with stromal cells present in the bone marrow.

Maturation: Communication between transcription factors and cell cycle regulating factors

Specific transcription factors are expressed during granulopoiesis and some of them, such as CCAAT/enhancer binding protein α (C/EBPα) and PU.1 are key regulators of early gene expression [1]. These transcription factors regulate expression of proteins involved in the maturation process, including the GM-CSF receptor and the G-CSF receptor, as well as granule proteins such as myeloperoxidase (MPO) and lysozyme [2]. Recent studies have demonstrated a role for additional transcription factors in the control of granulopoiesis. For example, C/EBPε is expressed predominantly at the late stage of granulocyte differentiation and contributes to induction of transcription of a number of key genes, including lactoferrin [3-5]. Finally, several of more ubiquitously expressed transcription factors, such as STATs, c-myc and Mad1 are known to play important roles in granulopoiesis as demonstrated by gene knockout and overexpression studies [1]. A schematic representation of the expression of several transcription factors during granulopoiesis and the phenotype of the respective null mutant mice is illustrated in Figure 1. The regulation of hematopiesis by transcription factors is complicated by the fact that multiple transcription factors can work cooperatively. For example, C/EBPα, c-myb and PU.1 cooperate to activate the murine neutrophil elastase (NE) promoter, and probably cooperate to activate the highly homologous proteinase-3 and azurocidin genes as well [6; 7]. Hematopoietic transcription factors can also influence each other's expression. Inducible activation of C/EBP is described to elevate PU.1 mRNA levels, in both 32Dcl3 myeloid cells and the Ba/F3 B cell line [2], suggesting that C/EBPα acts directly on the PU.1 promoter. In addition, both C/EBPα and PU.1 can directly affect their own promoters, resulting in enhanced expression [8-10]. These findings demonstrate the complexity underlying the mechanisms by which transcription factors can regulate granulopoiesis.

Apart from transcription factors, evidence has been accumulating concerning the role of cell cycle regulatory proteins in the myeloid differentiation process. Terminal differentiation appears to correlate with upregulation of the cell cycle inhibitor $p27^{kip1}$ (Fig. 1). It has been demonstrated that retinoic acid induced differentiation of U937, HL60 and NB40 cells is associated with upregulation of $p27^{kip1}$ and down regulation of cyclin E [11]. In addition, 32D cells differentiated towards neutrophils by G-CSF treatment have increased 27^{kip1} levels [12]. Furthermore, the down regulation of the transcription factor c-myc as referred to earlier, has been associated with differentiation. Many c-myc target genes are involved in cell cycle progression or protein translation [13], suggesting a counter balance between differentiation and proliferation. This hypothesis is supported by the fact that murine $p27^{kip1}$-deficient myeloid progenitors showed increased proliferation as well as reduced differentiation [12]. Furthermore, the retinoblastoma protein (Rb) is down regulated in granulocyte differentiation, whereas it is upregulated during erythroid and monocytic differentiation. This suggests that Rb functions as a switch between monocytic and granulocytic lineages [14; 15].

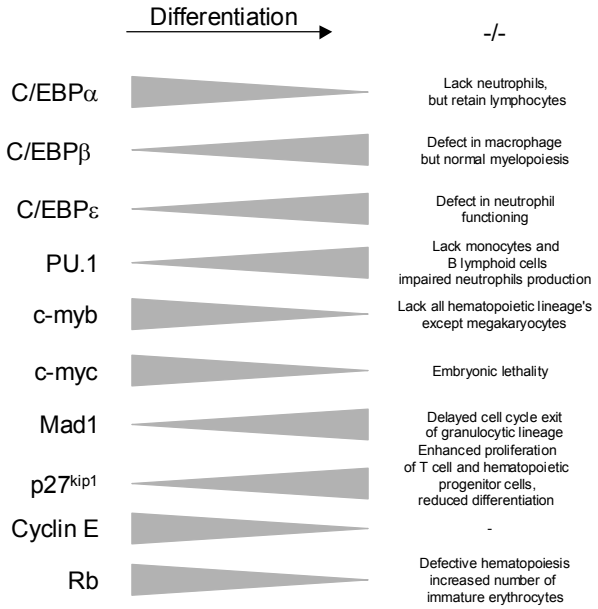

Figure 1: *Expression of transcription factors and cell cycle regulators during differentiation of human granulocytes and phenotype of respective knockout mice.*

The role of PI3K and MEK in the regulation of transcription factors and cell cycle regulators

In the bone marrow, stromal cells produce cytokines such as SCF, FLT3L, IL-3, GM-CSF and G-CSF, which support the maturation of hematopoietic stem cells. These cytokines then regulate the expression or activation of transcription factors necessary for differentiation as described above [1; 16]. Binding of cytokines to their receptor results in the activation of multiple signaling molecules, including PI3K and MEK. In contrast to the established role of transcription factors in granulopoiesis, surprisingly little is known about the role of signaling molecules.

For the research described in this thesis, an ex-*vivo* differentiation model has been developed to study the role of PI3K and MEK in the maturation process of $CD34^+$ HCS towards human neutrophils (Chapter 2). Inhibition of PI3K was found to abrogate granulocyte maturation in this model. This might suggest that PI3K is critical for the regulation of hematopoietic transcription factors. Indeed, in B-cells it has been described that PI3K can positively regulate PU.1 activity via phosphorylation, which is mediated by protein kinase B (PKB) [17]. For C/EBPα an inhibitory effect of PI3K on the phosphorylation of two threonine residues (222 and 226) has been observed, which is mediated by inhibition of GSK-3 activity [18]. It is tempting to speculate that GSK3-induced phosphorylation of C/EBPα targets it for degradation, as is proposed for c-myc (see further on). Furthermore, an active form of Ras has been shown to activate C/EBPα, through to phosphorylation of serine 248, which is a protein kinase C (PKC) consensus site (Behre G JBC, manuscript in press). Since it has been reported that PI3K can activate PKC isoforms via 3'-phosphoinositide-dependent kinase-1 (PDK1) [19], PI3K may regulate C/EBPα phosphorylation, dephosphorylation or both during granulocyte maturation.

Proliferation of the HCS was affected by inhibition of both PI3K and MEK. PI3K inhibition resulted in a complete cell cycle arrest within 24 hours, while inhibition of MEK resulted in a three-fold inhibition of proliferation. Several cell cycle regulating proteins have been described to be regulated by PI3K and MEK. ERK, which acts downstream of MEK, has been found to phosphorylate c-myc leading to stability of this protein [20]. PI3K has also been demonstrated to inhibit degradation of c-myc, which is likely to occur through the PI3K-dependent inhibition of

GSK3. Cyclin D1 levels might also be stabilized by PI3K, since a similar mechanism as for c-myc has been described for this protein [21]. For both MEK and PI3K a role in the regulation transcription of cyclin D1 and $p27^{kip1}$ has been suggested [22-25]. PI3K-induced down regulation of $p27^{kip1}$ is at least in part regulated via phosphorylation and inactivation of the Forkhead family of transcription factors [26 27].

These findings (depicted in Figure 2) suggest that PI3K may regulate proliferation and differentiation through multiple mechanisms. (i) By inhibition of degradation of factors involved in differentiation, such as c-myc, and possibly cyclin D1 and C/EBPα and/or (ii) by regulation of transcriptional activity by the phosphorylation of transcription factors, such as Forkheads, PU.1 and possibly C/EBPα. MEK seems to cooperate with PI3K to stabilize factors predominantly involved in the cell cycle regulation such as cyclin D1 and c-myc, and/or down regulate cell cycle inhibitors, such as $p27^{kip1}$. Further work is required to define the precise mechanisms by which PI3K and MEK regulate granulopoiesis.

Notably, during the early stage of differentiation progenitor cells enlarge to become promyelocytes. In *Drosophila melanogaster*, PI3K has been demonstrated to be involved in the control of cell, organ and body size [28]. Furthermore, expression of a constitutively active form of PI3K in mammals resulted in increased hart size, due to an increase in the size of the individual myocytes, rather than altering the cell numbers [29]. It is interesting to speculate that PI3K activity may be involved in the increase in size of hematopoietic stem cells.

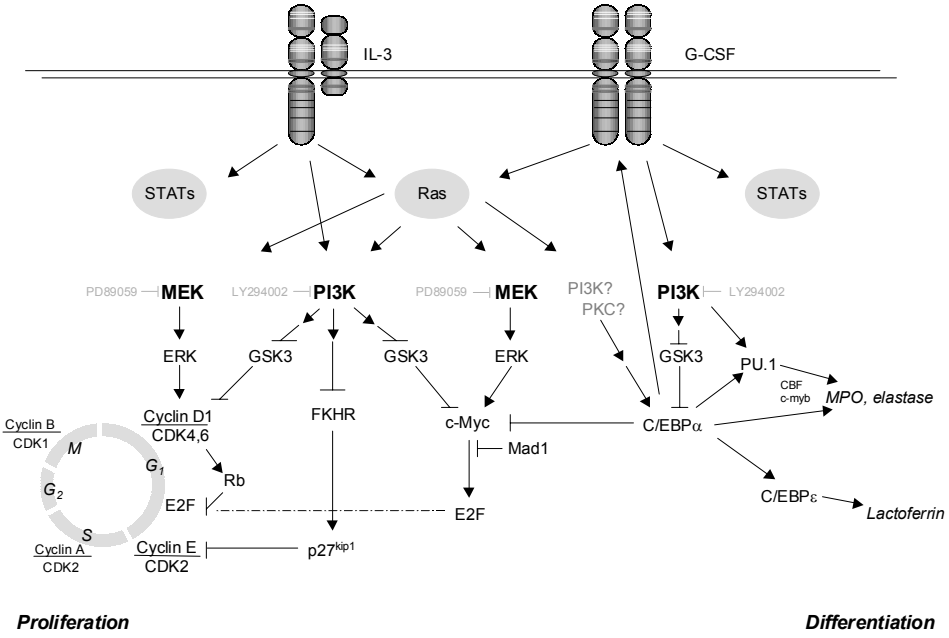

Figure 2: *Schematic representation of the role of PI3K and MEK in regulating transcription factors and cell cycle regulators.*

Cell fate decisions: Divide or Differentiate?

Multiple signals are involved in the regulation of differentiation. However, one question remains. What makes a cell decide to differentiate rather than proliferate? Thus far, two models have been proposed. (i) The "inductive model" favors the hypothesis that differentiation is controlled by specific cytokine receptors. For example, activation of the G-CSF receptor specially drives a progenitor cell to the neutrophilic lineage. However, C/EBPα that is important for the neutrophilic lineage can upregulate G-CSF receptor expression. This suggests that the decisions to differentiate have to be made earlier. (ii) The "stochastic model" favors the hypothesis that stem cells are already intrinsically committed to differentiate within the correct microenvironment. However, if a progenitor cell is already "programmed" for its differentiation fate, why are so many factors differentially regulated during differentiation? It

might be considered that early differentiation is a result of stochastic processes, in which the "correctly programmed" cells then go on to survive, proliferate and differentiate. During late maturation the "inductive model" seems to be dominant resulting in terminal differentiation. This process is controlled by specific receptors, such as the G-CSF receptor for the neutrophilic lineage. When proliferation is accelerated by intrinsic defects, or by dysfunction of the components regulating the inductive process, leuko-proliferative disorders such as leukemia might develop (Fig 3).
This still does not explain what drives a cell to decide to differentiate or proliferate. There are some indications that immature cells leave the bone marrow and differentiate *in situ*, where specific cytokines are present to induce the last step of differentiation. Further studies are required on how signaling molecules mediate the early and/or late maturation by regulating transcription factors, cell cycle regulating factors, or even adhesion receptors are of importance to elucidate the mechanisms controlling hematopoiesis.

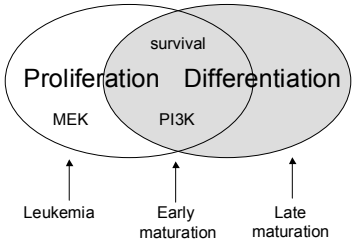

Figure 3: *Cell fate decisions, proliferation, differentiation, or both.*
In early maturation proliferation and differentiation can occur concomitantly. During late maturation, differentiation might be dominant resulting in terminal differentiation correlated with a decreased proliferation. PI3K possible plays a critical role in the early maturation, since both proliferation and differentiation are inhibited. A role for MEK is observed only the proliferation process.

Identification a novel kinase in human granulocytes

Human granulocytes are cells with specific effector functions that ensure efficient killing of invading microorganisms. Since these cells have such specific functions we speculated that unidentified cell-type specific signaling molecules involved in these processes might be present in these cells. A degenerate PCR technique was used to identify novel kinase(s) in human granulocytes. In our search for these granulocyte specific kinases, the Ca^{2+}/calmodulin-dependent Kinase I-Like Kinase (CKLiK) was identified (Chapter 3). CKLiK belongs to the family of Ca^{2+}/calmodulin dependent kinases, which include CaMKI, CaMKII, CaMKIV and CaMKK. Although several of these kinases have a wide tissue distribution, most studies are performed in neuronal cells [30 31]. CKLiK was demonstrated to be predominantly expressed in primary granulocytes, and its activity was induced by stimulation of G-protein coupled receptors expressed on these cells (Chapter 3). Since CKLiK activity is dependent on Ca^{2+}, a role for CKLiK downstream of activated GPCRs and their induced Ca^{2+} signal has been proposed in this thesis.

Role of $[Ca^{2+}]_i$ and CKLiK in granulocyte effector functions

Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) have been associated with multiple functions of human granulocytes [32]. Ca^{2+} release in neutrophils has been suggested to be regulated in two different ways. Activation of GPCRs have been shown to result in Ca^{2+} release from a central store located near the nucleus [33; 34]. Alternatively, cross-linking of integrins results in Ca^{2+} release from peripheral Ca^{2+} stores. These peripheral stores can relocate towards an area near the plasma membrane [35]. Chemoattractants, which activate GPCRs, can thus give rise to release of Ca^{2+} from the central store, generating a global Ca^{2+} signal, which is in turn followed by a influx of extracellular Ca^{2+} to refill the store [36]. Cross-linking of integrins results in release of the locally stored Ca^{2+} near the plasma membrane generating a local Ca^{2+} signal [37]. These different modes of Ca^{2+} signaling may be linked to the induction of specific granulocyte effector functions in response to inflammatory factors, as is suggested in Figure 4. A model for the Ca^{2+} mediated CKLiK activation is depicted in Figure 5.

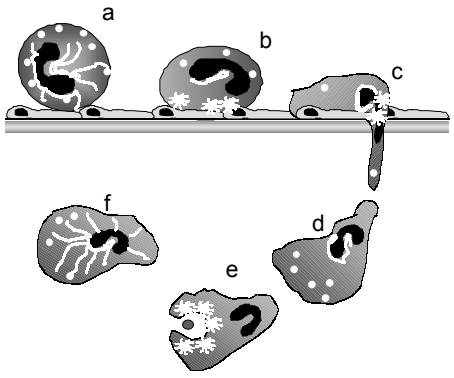

Figure 4: *Hypothetical model of controlling granulocyte function by the release of Ca^{2+} from central fixed stores and peripheral mobile stores*
(a) When a neutrophil first encounters a physiological stimulus, for example PAF on the endothelial cell, cytosolic free Ca^{2+} is released from the central Ca^{2+} store, with accompanying global Ca^{2+} influx. Integrin upregulation is consequently signaled equally around the cell surface. This initial Ca^{2+} signal would thus lack directionality, because unpolarized nature of the cell and the central location of the Ca^{2+} release site. (b) When integrins become engaged on encountering adhesion molecules such as ICAM-1, cytosolic free Ca^{2+} is then released from the peripheral stores without influx of extracellular Ca^{2+}, and so a localized Ca^{2+} signal is generated. (c) This directional Ca^{2+} signal leads to cell shape changes in the required direction, in readiness for migration. (d) During migration, the local stores translocate to the integrin- engaging surface providing a Ca^{2+} signal at the leading edge of the cell. (e) The mobile Ca^{2+} storages will also be strategically placed for liberation of Ca^{2+} at the required site for NADPH oxidase activation. This would results in the NADPH oxidase being activated locally at the phagosome by cross-linking stimuli. Global activation of the respiratory burst and degranulation can also be triggered by, for example, bacterial formlylated peptides. Note that GPCR activation results in Ca^{2+} release from the central stores and may be involved in a and f. Cross-linking stimuli, which induces release from the peripheral mobile stores, may be involved in b, c, and e. (Figure is adapted from Davies *et al*).

Role of CKLiK in respiratory burst

Inhibition of calmodulin or CKLiK activity resulted in a reduced ROS production in human neutrophils stimulated with fMLP. It has been described that formation of ROS in human granulocytes requires intracellular Ca^{2+} [38; 39]. However, the mechanism by which Ca^{2+} is linked to the induction of the respiratory burst is still unclear. In chapter 4 and 5 it was discussed that CKLiK could effect ROS activity by influencing the activity of exchange factors (GEFs) for the small GTPases Rac2 or Rap1, or by the phosphorylation of $p47^{phox}$ (see Fig 5). $p47^{phox}$ as well as these small GTPases are important for the generation of an

active NADPH oxidase complex, necessary for the production of ROS in human granulocytes. Tiam1, a GEF for Rac, has been shown to be phosphorylated by CaMKII, which results in translocation of Tiam1 to the membrane [40; 41]. After neutrophil stimulation with fMLP, $p47^{phox}$ is phosphorylated on multiple serine residues [42]. Some of these serine residues are potential CKLiK phosphorylation sites, as was identified by consensus site comparison. In addition to this, cross-linking of β2 integrins can also significantly stimulate ROS production in human neutrophils [43], suggesting a role for these integrins in the respiratory burst. Indeed, leukocytes from patients with leukocyte adhesion deficiency (LAD) who are characterized by deficient or defective β2 integrins, exhibit besides a perturbed migration an impaired respiratory burst [44]. As mentioned above cross-linking of integrins can induce Ca^{2+} release from the locally present Ca^{2+} stores, which in turn can activate downstream signaling events, such as possibly the activation of CKLiK (Fig. 5).

Role of CKLiK on neutrophil migration

Neutrophil migration over albumin coated surfaces was perturbed when cells were treated with inhibitors for calmodulin and CKLiK (Chapter 4 and 5). Several phases of neutrophil migration have been suggested to be Ca^{2+}-dependent. Movement on several substrates is abrogated in Ca^{2+}-buffered conditions, which was associated with clustering of integrins at the rear of the cell [45]. Based on this observation, an endocytotic recycling of integrins in motile neutrophils, back to the leading edge of the migrating cell has been suggested. Another study demonstrated that inhibition of CaMKII resulted in a high affinity state of β1 integrins [46], therefore a role for CaMKII on β1-detachment can be speculated. A similar role for CKLiK might be possible. However, besides an abrogated migration in the presence of the CKLiK inhibitor, we also observed a reduced number of adhered cells. This would rather suggest a role for CKLiK in the enhancement of integrin activity. In parallel, increased integrin activity was observed in T cells that express constitutively active Rap1 [47]. Therefore, it is tempting to speculate a link between CKLiK and Rap1. Furthermore, Ca^{2+} has also been implicated in rear release of the cell during migration. Buffering of $[Ca^{2+}]_i$ results in impaired uropod retraction [48]. This force-mediated retraction in neutrophils was found to be dependent on the phosphorylation of myosin II. Phosphorylation of the myosin light chain can be mediated by the Ca^{2+}/calmodulin dependent myosin light chain kinase (MLCK) and Rho kinase (ROCK), which acts downstream of Rho [48; 49]. Although these are defined processes, a role for CKLiK in rear release cannot be excluded (Fig. 5).

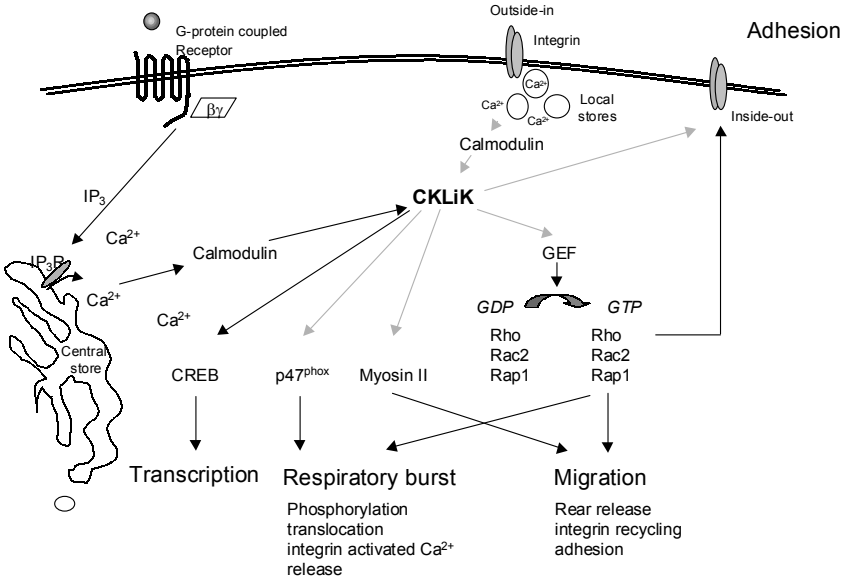

Figure 5: *Possible mechanisms by which CKLiK regulates granulocyte effector functions.*
Activation of GPCRs leads to Ca^{2+} release from the central store. Ca^{2+} binding by calmodulin results in a conformational change leading to binding to and thereby activation of CKLiK. This can lead to the activation of possible downstream targets such as guanine exchange factors of the Rho family members, intergrins, $p47^{phox}$, myosin II or CREB. A possible second mechanism in which CKLiK might be activated is via integrin cross-linking, since this also induce Ca^{2+} release.

Downstream targets of CKLiK

Since CKLiK can regulate both respiratory burst and migration it might be speculated that CKLiK has a general role in neutrophil signaling through direct activation of multiple target proteins. The closely related CaMKI has been demonstrated to be a multifunctional kinase, since it can phosphorylate several target molecules [31]. Thus far, CKLiK has only been shown to phosphorylate the transcription factor CREB (Fig. 5). However, the effect of CKLiK inhibition on migration and respiratory burst cannot be directly linked with CREB phosphorylation. Possible additional targets of CKLiK phosphorylation are myosin II and/or $p47^{phox}$ (Fig. 5). Another explanation for a general effect of CKLiK is that it can target a limited number of molecules, which have a broad function of them selves. Two potential candidates are the small GTPases (Rac, Rho and Rap1), and the integrins (Fig. 5), since both groups of molecules can effect many cellular processes as is discussed above [50 51].

PI3K and granulocyte functions

Chemoattractants and chemokines activate many additional signaling pathways, besides increased $[Ca^{2+}]_i$. PI3K is critically important, since inhibition of this kinase leads to a reduced granulocyte effector functions as already discussed in the introduction of this thesis. It is interesting to note that we did not observe an effect of PI3K inhibition on the migration of neutrophils on albumin coated surfaces. Recently it has been described that murine PI3Kγ-/- mice showed frequently directional changes and failed to orient toward a gradient [52]. This discrepancy might be explained by the fact that our assays are not based on a chemotactic gradient. Additionally, PI3K is suggested to be necessary to colocalize F-actin and protein kinase B, a downstream target of PI3K, in the leading edge of the cell [52]. In Chapter 4 we observed convergence of a calmodulin sensitive pathway with PKB. Since we demonstrated a dramatic inhibition of migration using a calmodulin inhibitor, it could be speculated that phosphorylation on PKB is critical for controlling the migration responses at the leading edge of the cell. It would be of interest to study the effects of direct inhibition of PKB on migration. The calmodulin sensitive phosphorylation of PKB is probably not mediated by CKLiK since we did not observe PKB activation in the presence of active CKLiK (Chapter 3). This suggests that besides CKLiK other CaMKs might play a role in regulating signaling in human granulocytes. Indeed, we demonstrated by PCR that more family members are present in these cells (Chapter 4). Therefore, it is important to explore the role of other CaMKs in human granulocytes.

Concluding remarks

Neutrophil maturation and neutrophil functioning are controlled by multiple signal transduction pathways activated by several stimuli. The work in this thesis demonstrates the importance of the PI3K and MEK pathways in human neutrophil differentiation. The use of an ex-*vivo* differentiation model will allow us to study the role of further signaling molecules important for maturation, for example GSK-3. Furthermore, since these stem cells can be transduced with (inducible) active or dominant-negative cDNAs we will be able to study the role of a specific signaling molecule at different stages of maturation in detail. More insight into the genes that are involved in the maturation process may also be obtained by the use of RAP-PCR or by microarray analysis. By identifying a Ca^{2+}/calmodulin-dependent kinase expressed predominantly in human granulocytes, we provide a novel mechanism for specific Ca^{2+}-induced signaling in these cells. We demonstrate that several important granulocyte effector functions are dramatically decreased by the inhibition of calmodulin and CKLiK. Besides the role of CKLiK in respiratory burst and migration, CKLiK might also have an effect on transcription. We demonstrated that CKLiK could activate the transcription factor CREB and that an active truncated form of CKLiK translocates to the nucleus. Identification of direct target proteins of CKLiK in future research is imperative to gain insight into the specificity of Ca^{2+}-mediated CKLiK signaling. Possibly then, CKLiK might be suitable as a therapeutic drug target since it is specifically expressed in a subset of myeloid cells.

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Nederlandse samenvatting voor niet-ingewijden

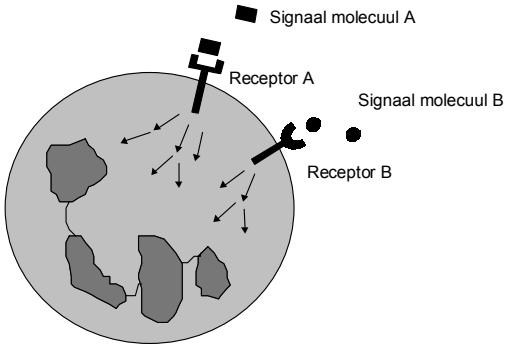
Naast rode bloedcellen bevat bloed ongeveer 1% witte bloedcellen die belangrijk zijn voor ons immuunsysteem. Dagelijks worden miljarden van deze cellen aangemaakt in het beenmerg, die na uitrijping in de bloedbaan terechtkomen. Het grootste deel van de witte bloedcellen worden gevormd door de neutrofiele-granulocyten (oftewel neutrofielen), die zeer herkenbaar zijn door hun gelobde kern. Deze cellen zorgen ervoor dat lichaamsvreemde stoffen (zoals bijvoorbeeld bacteriën) direct herkend en opgeruimd worden. Zij zijn dus zeer belangrijk bij een eerste afweer tegen binnendringende micro-organismen in het lichaam.

Als het lichaam door bacteriën geïnfecteerd is, worden er signalen afgegeven die door neutrofielen opgemerkt worden. Deze signalering leidt ertoe dat de neutrofielen geactiveerd worden, uit de bloedbaan treden en in de richting van de infectie migreren. Daar aangekomen, kunnen neutrofielen de bacteriën aanvallen en vernietigen. Zij kunnen dit o.a. doen door toxische stoffen uit te scheiden. Vervolgens worden de aangetaste bacteriën opgenomen door de neutrofielen en in zijn geheel afgebroken.

Omdat deze cellen zo reactief zijn, kan het gevaarlijk zijn als een van de bovengenoemde processen ontregeld is. Bijvoorbeeld, als neutrofielen teveel toxische stoffen uitscheiden, kan naast beschadiging van bacteriën ook het lichaamseigen weefsel worden aangetast. Verder kan ook door disregulatie een chronische ontsteking ontstaan die eveneens slecht is voor het omliggende weefsel.

Het is dus van groot belang dat alle processen die betrokken zijn bij de activatie van neutrofielen nauwkeurig gereguleerd worden. Signalen die op de plaats van ontsteking worden afgegeven, kunnen door neutrofielen opgemerkt worden omdat zij op hun oppervlak sensoren hebben. (Fig. 1). Deze sensoren worden receptoren genoemd. Omdat er veel soorten signalen zijn, zijn er ook veel soorten receptoren en ieder signaal heeft een eigen receptor. Deze signaal-receptor-interactie functioneert als een sleutel-slotprincipe waardoor ieder signaal een unieke receptor bindt (Fig. 1). Als het signaal molecuul (sleutel) interactie aan kan gaan met de receptor (slot) kan in de cel weer een specifiek signaal gegeven worden. Juist deze signalen die in de cel veroorzaakt worden (intracellulaire signalen), zijn bestudeerd en beschreven in dit proefschrift.

De vraagstelling hierbij was, welke signalen een rol spelen bij het functioneren van neutrofielen. Dus bijvoorbeeld, welke intracellulaire signalen zijn belangrijk voor de verplaatsing van neutrofielen naar de plek van ontsteking? Of, welke intracellulaire signalen zijn betrokken bij de uitscheiding van de toxische stoffen? Maar ook de intracellulaire signalen die betrokken zijn bij de rijping (differentiatie) van stamcellen uit het beenmerg naar volwassen neutrofielen zijn bestudeerd.



Figuur 1: *Schematische weergave van het tot stand komen van intracellulaire signalen in een neutrofiele granulocyt.*
Signaal moleculen (bijvoorbeeld vrijgekomen bij een ontsteking) passen in de juiste receptor. Hierdoor vindt er signaaloverdracht plaats van buiten de cel naar binnen (intra) de cel. Door de binding van een signaalmolecuul aan zijn receptor kunnen vele intracellulaire signalen geactiveerd worden, zoals is weergegeven met de pijltjes binnen de cel. Activatie van intracellulaire signalen resulteert uiteindelijk in een respons van de cel, zoals bijvoorbeeld, migratie naar de plaats van ontsteking, maar ook het tot expressie brengen van bepaalde genen. De rol van verschillende intracellulaire signalen in het functioneren van neutrofiele granulocyten is in dit proefschrift bestudeerd.

In hoofdstuk 2 wordt een modelsysteem beschreven voor de differentiatie van stamcel naar neutrofiele granulocyt. Hiertoe worden stamcellen geïsoleerd uit navelstrengbloed. In dit systeem is de rol van intracellulaire signalering in het differentiatie proces bestudeerd. Dit is gedaan door gebruik te maken van farmacologische remmers die specifieke intracellulaire signalen blokkeren. Remming van één van deze specifieke signalen (het phosphatidylinositol 3 kinase signaal) heeft als gevolg dat stamcellen niet meer kunnen uitrijpen tot volwassen neutrofielen. Dit geeft dus aan dat dit intracellulaire signaal van belang is voor het normale differentiatieproces.

Omdat neutrofielen een zeer specifieke functie hebben (namelijk aanvallen en elimineren van bacteriën), dachten we dat er ook specifieke intracellulaire signalen in deze cellen aanwezig konden zijn. In hoofdstuk 3 wordt het resultaat beschreven van de identificatie van een nieuwe granulocyt specifieke kinase. Het is bekend dat kinases belangrijke intracellulaire signaleringsmoleculen zijn. Het nieuwe geïdentificeerde en granulocyt specifieke kinase heeft een sterke overeenkomst met een andere kinase, namelijk het "Ca^{2+}/calmodulin-dependent kinase I" (CaMKI). We laten zien dat het nieuwe kinase geactiveerd wordt door Ca^{2+} (calcium) en calmoduline, net zoals het kinase waarop deze lijkt. We hebben het dan ook CaMKI Like Kinase, CKLiK, genoemd.

Doordat bleek dat CKLiK alleen in neutrofielen voorkomt en gereguleerd wordt door Ca^{2+} en calmoduline, hebben we onderzoek gedaan naar de rol van calmoduline in het functioneren van neutrofielen. Hierbij is gebruik gemaakt van een specifieke farmacologische remmer voor calmoduline. Hoofdstuk 4 beschrijft dat remming van calmoduline resulteert in een blokkade van neutrofiel migratie en de productie van toxische stoffen.

Vervolgens is de rol van het nieuwe gekarakteriseerde kinase CKLiK in het functioneren van neutrofielen bestudeerd. Hierbij is gebruik gemaakt van een stof die het CKLiK signaal kan remmen. Uit de resultaten die beschreven zijn in hoofdstuk 5 blijkt dat CKLiK een belangrijke rol speelt bij de migratie en de zuurstofradicaal productie.

In hoofdstuk 6 is getracht om op basis van DNA-patronen een geactiveerde neutrofiel te herkennen. Bij geactiveerde cellen worden bepaalde genen actief aangezet en andere weer actief uitgezet. FLAP is een van die genen die wordt aangezet. Dat dit daadwerkelijk een gen is dat verhoogde expressie vertoont tijdens activatie is aangetoond in witte bloedcellen van allergische astmatische patiënten na een allergeen provocatie.

Samenvattend zijn in dit proefschrift bevindingen gedaan die meer inzicht geven in de intracellulaire signalering van humane neutrofielen. Verder kan gespeculeerd worden over CKLiK als een toekomstig drugtarget.

Dankwoord

Beste vrinden en vage kennissen,

Natuurlijk wil ik iedereen ontzettend bedanken. Dus het komt mooi uit dat een proefschrift ook een dankwoord bevat. Paul, jij bent degene die ik als eerste wil bedanken, want je bent enorm belangrijk voor mij geweest tijdens mijn AIO periode. Je gaf me veel vrijheid en volledige steun wanneer ik dat nodig had. Daarnaast heb je me enorm geïnspireerd, en mede daardoor heb ik zoveel technieken toegepast in mijn onderzoek. Ik zal je humor, speelsheid (dit is toch een goede eigenschap van echte wetenschappers) en je muziekcollectie missen.

Leo, jij bent geen manager maar een "inspirator". Je volle lach en continue vraag: "Wat is nu je hypothese?", brengen de beste ideeën naar boven. Bedankt voor je brainstorm sessies. Jan-Willem, bedankt voor de vrijheid die ik kreeg om dit onderzoek te doen.

Labgenoten, door jullie heb ik het (bijna) altijd naar me zin op het lab, dus dat is wel een bedankje waard. Laurien, zoals je zelf al zei zijn er weinig dingen die we niet weten van elkaar. Je bent een geweldige meid, en ik denk dat het rollabje van jouw wel een succes wordt. Cornelieke, bedankt voor het zijn van een goede vriendin (en voor hoofdstuk 6). Moeilijke beslissingen heb jij een beetje makkelijker gemaakt. Ik vind het fijn dat je mijn paranimf wil zijn. Caroline, je was een stille student maar wel een goede. Hoofdstuk 4 is een heel groot deel aan jou te danken. Hanneke, jij hebt me het laatste halve jaar enorm geholpen. Zonder jou zou het boekje niet zijn geworden zoals het nu is. Je begreep precies wat ik wilde, ook al kwam het in halve zinnen (door de stress) uit mijn keel. Kei bedankt.

Pascale, ook al zit je nu in San Franciso, je (e-mail) verhalen blijven fantastisch (en vaag). Jou als ex-pipeteerbuurvrouw te hebben was erg gezellig. Ook al kon 80% het ene oor in en het andere oor uit (maar dat heb ik wel met meerdere mensen). Evert, je eeuwige geduld, interessante discussies (de eerste 20 minuten) en onbegrip voor gehaaste mensen (dan vertrek je toch iets eerder!) hebben mij van tijd tot tijd goed geholpen. Je was een gezellige pipeteerbuurman, (vooral als je een tijdreeks deed). Kim, als Duitse Groninger ben je toch aardig ingeburgerd in ons Utrechtse labje. Ik heb al veel lol aan je beleefd, omdat je in de meeste grapjes trapte, die de rest al kon dromen. Jammer dat ik nu ga, maar we gaan nog wel borrelen en bijkletsen op z'n tijd hè? Miranda, bedankt voor al die keren dat ik met je mee mocht rijden, en netjes thuis afgezet werd, na een avondje borrelen. Ik vind het leuk dat je kunt blijven en met jouw inzet kom je er wel. Belinda, samen door de laatste promotie stress, alhoewel ik denk ik iets meer stress had dan jij. Hoe krijg je dat toch voor elkaar? Madelon, de eerste dag al kwam je me op de hoogte stellen van het wel en wee op het lab. Maar ook voor goed advies over wat dan ook, kon (kan) ik altijd bij je terecht. Leuk dat je weer in Utrecht bent (wanneer gaan we nu mozaïeken of is kleine jantje weer een excuus). Lisa, heerlijk dat Italiaanse temperament van jou. Bedankt voor het mee opzetten van de neutrofiel differentiatie, dat was een hele klus. Verder de bende van beneden, Jan, Deon, Corneli en Lei, bedankt voor jullie ondersteuning met betrekking tot microscopen, de FACS, etc. Natuurlijk mag ik ook niet Bart en Erik-jan vergeten de twee meest enthousiaste artsen onderzoekers. Alhoewel Erik-Jan liever niet genoemd wilde worden (sorry Erik-Jan, nu sta je er toch drie keer in)

Marcela, thanks for hassling over and over again the people in Colombia to send my peptide. It just arrived in time. I wish you al the best with finishing your phD. Mike, thanks for your "gezelligheid" in the lab and you're definitely not a typical American, although, there are some things… just kidding. It was great to have you around. Good luck in the future.

Dan nog de ex-lab genoten; Niels (altijd even enthousiast, bedankt voor het leuke weekend in Boston. Jacqueline, samen begonnen met het moleculair werk op lab 1, 't was gezellig bedankt voor al je tips. We moeten snel weer eens wat afspreken. Rolf, Thamar, Eric, Annelien (hoe is ie, ben je nu al getrouwd of gaat dat nog gebeuren), Andy, Carolien, Paula, Nadia, Jaap-Jan, Philip, Humberto, Marja, bedankt voor jullie gezelligheid in het lab.

Dorien, ons hele leven zijn we al hartsvriendinnen. Je weet dat je een belangrijke plaats in mijn leven hebt en daarom vind ik het fijn dat je naast me staat tijdens mijn promotie. Kus (ook aan René en Rens).

Mam, zonder jou had ik het niet zo ver geschopt. Bedankt voor al je stimulatie en steun mijn hele leven lang. Pap, de nuchterheid die ik van jou heb kwam soms goed te pas als ik weer eens in een dip zat. Bedankt voor al het vertouwen in mij.

Jeu en Wilma, Pascal en Marisa. Thuis basis nummer 2. Het is fijn om te weten dat ik altijd bij jullie terecht kan met wat dan ook. Wilma, mooi hè, de omslag. Bedankt.

Rob, beste vriend, lekkere vent, steunpilaar, vervelend mannetje, mijn man. Ik hou van je, je bent mijn rots in de branding.

Curriculum Vitae

De schrijfster van dit proefschrift werd op 22 augustus 1971 te Wijchen geboren. Na het behalen van het HAVO diploma aan de Dukenburg College in Nijmegen is zij in 1989 begonnen met de Hogere Laboratorium opleiding in Eindhoven. In januari 1994 ronde zij deze studie af met als afstudeerrichting moleculaire microbiologie en immunologie (Dr. W.A. Buurman. Afdeling Algemene Heelkunde, Universiteit Maastricht). Vervolgens is zij in 1994 begonnen aan de verkorte opleiding Biologie aan de Universiteit Utrecht. In maart 1997 haalde zij het doctoraal examen met als hoofdvak moleculaire celbiologie (Prof. Y. Guisez, Prof. J. Tavernier en Dr. G. Plaetinck, Roche research te Gent, België). Het in dit proefschrift beschreven onderzoek werd uitgevoerd in de periode maart 1997 tot juli 2002 onder begeleiding van Dr. P.J. Coffer, Prof. Dr. L Koenderman en Prof. Dr. J-W. J Lammers bij de afdeling Longziekten in het Universitair Medisch Centrum te Utrecht. Vanaf September 2002 zal zij werkzaam zijn als "scientist" bij het biotechnologisch bedrijf Genmab te Utrecht.