Systemic neutrophil activation in COPD

Analysis of gene expression profiles and intracellular signaling

Evert Nijhuis

Systemic neutrophil activation in COPD

Analysis of gene expression profiles and intracellular signaling

Systemische activatie van neutrofiele granulocyten in COPD
Analyse van gen expressie profielen en signaal transductie

(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 6 mei 2003 des middags te 16.15 uur

door

Evert Herman Jan Nijhuis
Geboren op 14 oktober 1974 te Almelo

Promotores: Prof. dr. L. Koenderman
Prof. dr. J-W. J. Lammers

Co-promotor: Dr. P. J. Coffer

Department of Pulmonary Diseases , Heart Lung Center Utrecht, University Medical Center, Heidelberglaan 100, 3584 CX, Utrecht

Print: FEBODRUK BV, Enschede

The research described in this thesis was performed at the Department of Pulmonary Diseases, University Medical Center, Utrecht, The Nederlands and was financially supported by a research grant from the Dutch Asthma Foundation (AF 97.68)

Publication of this thesis was financially supported by:
Department of Pulmonary Diseases, Utrecht; Faculty of Medicine (University of Utrecht)

ISBN: 90-393-3351-3

“Niet omdat de dingen moeilijk zijn durven wij niet,
maar omdat wij niet durven zijn de dingen moeilijk”

Seneca

Contents

Chapter 1

General Introduction

1.0 The role of granulocytes in chronic inflammatory diseases

Human granulocytes develop in the bone marrow from myeloid precursor cells that, when terminally differentiated, are released into the peripheral blood. There are three types of granulocytes: neutrophils, eosinophils and basophils. In this thesis, we have focused on eosinophils and neutrophils, the body's first line of defense against invading microorganisms, and are critical effector cells in both innate and adaptive immunity (1-3). While neutrophils are critical effector cells in the killing and removal of bacteria (1,2), eosinophils are involved in the killing of parasitic worms (4).

A consequence of granulocyte activation *in vivo* is the ability to cause tissue damage, as is often seen at inflammatory loci. Correct functioning of granulocytes and a controlled clearance of these cells contributes to a proper inflammatory response. This is required to prevent greater tissue damage than necessary, and enables repair of the tissue over time. Long term and/or uncontrolled activation of granulocytes, however, is thought to play an important role in the pathogenesis of several inflammatory diseases. These range from severe acute diseases, for example adult respiratory stress syndrome (5,6,7) to chronic inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma (8,9). Allergic asthma is characterized by an increase predominantly of eosinophils in the airways, whereas neutrophils accumulate in this compartment in COPD. This thesis focuses on the role of peripheral blood neutrophils in systemic inflammation observed in COPD.

1.1 COPD

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible airflow limitation. Airflow limitation results from of aspects of two closely interrelated diseases of the respiratory system, chronic bronchitis and emphysema (8,10,11). Chronic bronchitis is a chronic inflammatory disease in the airways and is characterized by an excessive airway mucus secretion leading to a persistent, productive cough (10). In addition, there may also be narrowing of the large and small airways causing dyspnea in these patients. In emphysema there is a permanent destruction of the alveoli, due to the irreversible degradation of proteins like elastin in the lung. This leads the to loss of structure of alveolar walls, and decreased elasticity of the alveolar tissue. Loss of elastin also causes collapse and narrowing of the bronchioles, which in turn limits expiratory airflow (10). Most patients with COPD suffer from both pathologic entities, but the relative extent of emphysema and obstructive bronchitis within individual patients can vary. Smoking is the major risk factor to develop COPD in the western world (12). In general COPD develops in older individuals with a long smoking history and 15-20% of all smokers will develop clinically symptomatic COPD during their lifetime(13).

Lung function tests and chest radiographs are generally used for routine diagnosis of COPD and monitoring of airway obstruction. Because of their simplicity and

reproducibility, forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), the ratio of these two parameters, and the absence of reversibility of airway obstruction are the main parameters for monitoring COPD (11). Lung function studies have demonstrated that the FEV_1 declines slowly with age even in healthy non-smokers, but this gradual loss of function will have no significant clinical effects, due to normal excess of the lung capacity (Fig. 1)(14). In smokers, however, lung function tends to decrease much more rapidly. If a smoker stops smoking before serious COPD develops, the rate of decline of the FEV1 may return to almost normal in individuals (15). Other risk factors for COPD are continuous exposure to occupational dusts and chemicals, infections, and outdoor and indoor air pollution (11). Since not all smokers will develop COPD, there must be additional risk factors involved and it is also thought that genetic factors will be important. Data suggest that imbalances between proteases and anti-proteases and oxidants and anti-oxidants contribute to the pathogenesis of the disease. Supporting this view, there is a genetic variant of COPD, which is caused by a deficiency of alpha-1-antitrypsin (AAT) (16). AAT protects for the destruction of elastin by elastases, such as neutrophil elastase. If individuals with a genetic deficiency of AAT also smoke, they usually will develop symptoms of COPD by the time they reach early middle age.

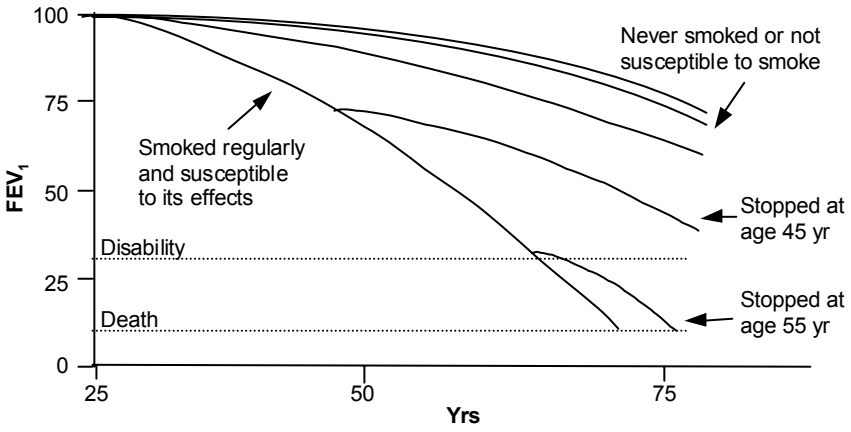

Figure 1: Presumed effect of smoking cessation on decrease in forced expiratory volume at 1 second (FEV_1). FEV_1 of individuals who have never smoked or are not susceptible to smoke were compared with patients who stopped smoking at different stages in life. Disability occurs at ~30% FEV1, and death occurs at ~10%, as indicated by the dotted horizontal lines. Adapted from Fletcher et al. (14).

The course of the disease can be divided into four different clinical stages according to the classification of the GOLD guidelines (Table 1) (11). Stage 0 ("at risk") refers to patients with chronic cough and sputum expectoration, but without any abnormality of the lung function as measured by FVC and FEV_1. Stage I patients ("mild") have mild airflow obstruction, defined as a ratio of FEV_1 to FVC of less than 0.7, but an FEV1 that is still, greater than 80%. Stage II ("moderate") patients with moderate airflow limitation are subdivided in group A, which contain patients with an FEV1 between 50 and 80%, and in group B, which exists of patients with a FEV1 between 30 and 50%. Usually in stage II, patients begin to experience also dyspnea especially upon exertion, and exacerbations of symptoms. Stage III patients have severe airflow limitation with an FEV1 less than 30% and at this stage quality of life is severly impaired. Exacerbations are typical in moderate to severe stages of COPD and are charcterized by increased dyspnea, cough, and sputum production lasting days to a few weeks (17,18). These exacerbations may have several inciting factors, including viral or bacterial infections, or common pollutants, and probably are associated with increased inflammatory changes within the respiratory tract. These exacerbations can be life threatening, cause frequent hospitalization and will have a negative influence on the prognosis of a COPD patient. Moreover comorbidity like right heart failure may aggravate symptoms.

Table 1. Classification of COPD according to the GOLD guidelines (10)

Class	Characteristics
0: At risk	normal lung function chronic symptoms (cough, sputum)
I: Mild	FEV_1/FVC < 70%; $FEV_1 \geq 80\%$ predicted. (With or without symptoms of cough, sputum)
II: Moderate	FEV_1/FVC <70%; A: FEV_1 80-50% predicted B: FEV_1 50-30% predicted
III:Severe	FEV_1/FVC <70%; FEV_1<30% predicted or FEV1 <50% predicted plus: respiratory failure or clinical signs of right heart failure

FEV_1: Forced expiratory volume in one second
FVC: Forced vital capacity

Recently, the definition of COPD has been extended and is now: "a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases" (11). Thus it is now generally accepted that COPD is associated with an abnormal inflammation in the airways. It is thought that this abnormal inflammatory response is mediated by increased levels of cytokines/chemokines, such as TNFα and IL-8, as found in bronchial alveolar lung (BAL) fluid and sputum (19-21). Furthermore, increased levels of neutrophils, monocytes and CD8+ cytotoxic lymphocytes are found in the lungs (22). Their recruitment is likely to be mediated by the increased levels of cytokines/chemokines in the lungs (23-26). Indeed, studies have measured neutrophil activation in the lungs of COPD patients (21,23,27,28). This implicates an important role for neutrophils in the pathogenesis of COPD. The identification of increased concentrations of soluble TNF receptors, which are thought to counteract TNFα, in the plasma of COPD patients suggests that the local inflammation in the airways communicates with the systemic compartment. Indeed, studies have reported that neutrophils in the peripheral blood are characterized by a primed phenotype in COPD patients (29,30). These findings suggest that the activation of neutrophils already starts in the circulation. However, it is not clear which mediators are involved and whether the activation of neutrophils is correlated with the severity of the disease. Therefore, it is important to investigate the molecular mechanisms responsible for neutrophil activation in peripheral blood, including type of stimuli, regulation of intracellular signaling pathways, and changes in the transcriptional program of these cells, for understanding the role of neutrophils in the pathogenesis of COPD.

2.0 Granulocytes and their cellular functions

As previously mentioned, granulocytes kill and remove microorganisms through specific cellular functions, such as phagocytosis, release of cytotoxic enzymes and proteins by the process degranulation, and production of toxic oxygen metabolites initiated by a membrane bound NADPH-oxidase (1). Furthermore, granulocytes also release pro-inflammatory cytokines and bioactive lipids. These mediators are involved in maintaining inflammatory reactions by recruitment of leukocytes, increasing the lifespan of granulocytes and enhancing cellular effector functions of granulocytes.

2.1 Migration

Granulocytes circulate through the body in the blood surveying for invading microorganisms. A rapid transition between a circulating non-adherent state and an adherent state allows them to migrate into the inflamed tissue (Fig. 2). This first step of entering organs or tissue, involves tethering and rolling of leukocytes along the endothelial cell layer (Fig. 3). The tether and rolling interactions are mediated by

granulocyte L-selectin, and by E- and P-selectins expressed on activated endothelial cells (31-34). During this tethering step, granulocytes respond to ligands, such as chemokines released or expressed on the membrane of endothelial cells. This results in activation of integrin mediated sustained, firm adhesion, spreading, and diapedesis (35). Defective neutrophilic recruitment to inflamed sites in leukocyte adhesion deficient (LAD) patients and in adhesion molecules-deficient mice demonstrates that migration indeed requires selectins and the interactions of integrins, such as CD11b/CD18, with the endothelium (36,37). The next step of migration towards the site of inflammation requires the migration of the granulocytes through the endothelial layer. This process, called diapedesis, requires modifications of endothelial cell-to-cell adherent junctions. Two adhesion molecules, including platelet endothelial cell adhesion molecule-1 (PECAM-1) and junction adhesion molecule (JAM) have been shown to be involved in this process (38,39). Migration within the tissue is mediated by a gradient of immobilized, rather than soluble chemoattractants. These chemoattractants, produced by bacteria, or by various stromal and epithelial cells of inflamed tissue, are bound to extracellular matrix components. This process of migration is defined as haptotaxis. Signals delivered by "end target-derived" chemoattractants, such as formyl peptides (released by bacteria), or complement C5a (produced in their immediate surrounding), are thought to be dominant and therefore override "regulatory cell-derived" attractants, such as bioactive peptides or chemokines (40,41). This allows granulocytes, recruited by endothelial-derived chemoattractants, to migrate away from the endothelial agonist source towards their final target with a tissue.

2.2 Phagocytosis, degranulation and respiratory burst

Through the processes described above, granulocytes are mobilized to sites of inflammation, where they can destroy invading microorganisms. To accomplish this, granulocytes possess an impressive array of "microbicidal weaponary". Once the microorganism is ingested by phagocytosis, the invading microorganism can be destroyed by proteolytic enzymes stored in special granules, and by reactive oxygen species, produced by the NADPH oxidase complex in the so called "respiratory burst".

Ganulocyte phagocytosis is mediated by two different receptor classes, Fc gamma receptors (FcRs), such as FcγIIA and FcαRI, and complement receptors (CRs), such as CR3 (CD11b/CD18 integrin) (42,43). CR3 binds C3bi on complement-opsonized targets, whereas FcγRs and FcαRI bind to immunoglobulin G (IgG) and IgA coated targets, respectively. Phagocytosis by these receptor types is driven by reorganization of filamentous actin (F-actin), but the mechanisms of uptake appear to be different. FcR mediated uptake is accompanied by pseudopod extension and membrane ruffling, whereas complement-opsonized targets "sink into" the cell, producing few protrusions. Signaling

pathways mediating phagocytosis by these two classes of receptors are also different (reviewed in (44).

Degranulation into the phagolysosome or in the extracellular space is an important event for the killing of microorganisms, since inherited granule deficiencies, such as Chediak-Higashi syndrome (CHS), are marked by frequent and life-threatening bacterial infections (45). The contents of granules, which mainly consist of proteolytic enzymes and other antimicrobial proteins, directly effect the killing of microorganisms. Four types of granules in neutrophils are characterized by the contents and order of appearance during maturation (1,46-48). Of the granules, the azurophil granules appear first in neutrophils and contain myeloperoxidase, serine proteases, and bactericidal proteins. Later in differentiation, specific granules containing lactoferrin and collagenase emerge, followed by the tertiary granules containing gelatinase. A fourth type of granule, called secretory vesicles, appear at the stage of terminal neutrophil differentiation. Secretory vesicles contain mainly serum proteins and therefore the origin is thought to be endocytic.

The activation of the respiratory burst is mediated by the NADPH oxidase, which is a complex of cytosolic and membrane proteins (49) (Fig. 3). The cytosolic proteins are $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and small GTPases Rac and Rap1A, and the flavocytochrome b_{558} complex, containing $p22^{phox}$, and $gp91^{phox}$. Upon activation of neutrophils, $p47^{phox}$ becomes phosphorylated, resulting in the translocation of the $p40^{phox}/p47^{phox}/p67^{phox}$ complex to the membrane, where it interacts via multiple binding sites with flavocytochrome b_{558} (50,51). The active oxidase also requires the translocation of Rac (52), which occurs simultaneously, but independent, of the translocation of the $p40^{phox}/p47^{phox}/p67^{phox}$ complex (53-56). Rac is thought to support NADPH oxidase activity only in its GTP-bound active form (57), however this remains a point of controversy (58,59). The generation of superoxide anions via the activation of the oxidase complex is followed by the production of many types of reactive oxidant species (ROS), such as H_2O_2 and $HO^{\bullet}$. In turn, myeloperoxidase (MPO) amplifies the toxic potential of H_2O_2 by producing hypochlorous acid (HOCl). The generation of ROS together with the granule contents are critical in the killing of microorganisms. Genetic defects in the phox genes (p22-, p47- ,p67- ,gp91phox) results in an immune deficiency: chronic granulomatous disease (CGD) (60). This disease is associated with persistent infections as a result of the inability of their phagocytes to destroy invading microorganisms. One exception to this is the normal killing of microorganisms that themselves produce significant quantities of H_2O_2 (e.g. pneumococci). The severe clinical picture observed in CGD clearly demonstrates the vital importance of NADPH oxidase in host defense against invading microorganisms.

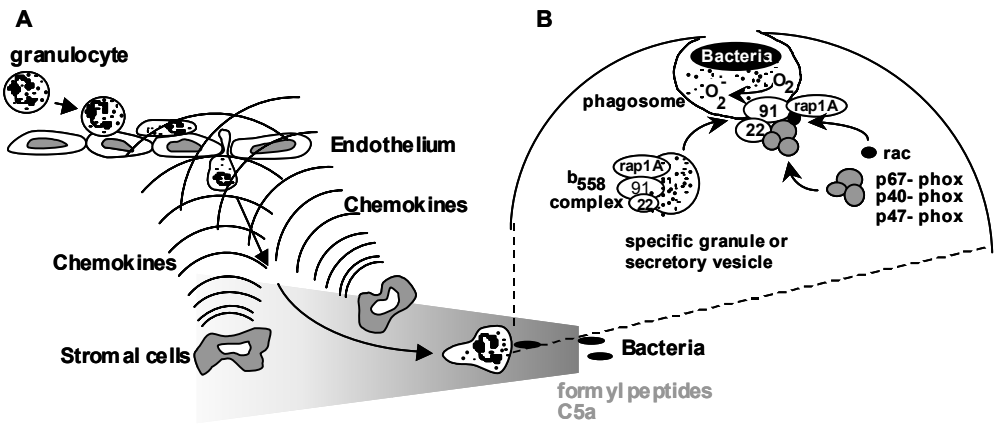

Figure 2: Schematic view of the granulocyte migration towards the site of inflammation and subsequent respiratory burst. (A) Neutrophils roll, adhere and spread to the endothelium, which release cytokines and chemoattractants. Subsequently, cells move through the endothelium and into the tissues by responding to successive combinations of chemoattractant gradients. The direction of granulocyte movement is first guided by the steepest local chemoattractant gradient, and is then regulated by successive receptor desensitization and attraction by secondary distant agonists. Finally, end target attractants dominate over regulatory cell-derived agonists. (adapted from Witko-sarsat et al (1). **(B)** Upon full activation of granulocytes, the membrane localized flavo-cytochrome b558 complex and the cytoplasmic complex fuse with each other in the membrane of the phagosome, resulting in the activation of the NADPH oxidase.

2.3 Production of inflammatory molecules

In recent years it has become obvious that the contribution of neutrophils to host defence extends well beyond their traditional effector functions, such as phagocytosis, degranulation and production of reactive oxygen species. Indeed, several studies have demonstrated that neutrophils not only synthesize proteins that directly participate in their effector functions, but that these cells can also produce a broad range of pro- and anti-inflammatory molecules (Table 2) (61,62). Neutrophils produce mainly pro-inflammatory molecules, such as cytokines (e.g. TNFα, IL-1β, IFN-α, G-CSF and IL-12) and chemokines (e.g. IL-8, MIP-1α, MIP-1β and GRO-α). Anti-inflammatory cytokines expressed by neutrophils are IL1-RA and TGFβ. In addition, neutrophils can express membrane receptors (such as IL1-R2, which binds to IL-1α/β) that bind ligand without activating the cells (63,64). Expression of these receptors can protect the cell and its neighbors against the pro-inflammatory action of the ligand. Recently is has been demonstrated that there are also soluble forms of receptors, such as sTNFRs and IL-1R2 which bind agonist and counteract its function (63,65). Thus, neutrophils are able to produce a striking diversity of inflammatory molecules, suggesting that they not only play

a direct role in killing microorgansims, but also contribute to the regulation of immune reactions. Indeed, whereas the production of inflammatory mediators by neutrophils is less than that produced by monocytes, the amount of neutrophils in the peripheral blood is much higher, and at the site of inflammation, neutrophils are the first cells to be recruited and largely predominate over monocytes (66).

The production of inflammatory molecules is induced by a broad range of agents, including cytokines (TNFα, IL-1α/β, GM-CSF and IFNγ) and bacterial products (LPS) (61,67-69). The pattern of inflammatory molecules generated greatly differs depending on the stimulating agent, and for some cytokines co-stimulation is required, e.g. IFNγ plus LPS in the case of IL-12 expression (61). In addition, co-stimulation can enhance or inhibit regulation of inflammatory molecules. For instance, IFNγ enhances, whereas IL-10 inhibits LPS induced gene expression of inflammatory molecules. Thus the interaction of neutrophils with a given agonist(s) will result in a production of a characteristic pattern of inflammatory mediators. Investigation of gene and/or protein expression in neutrophils may reveal the agents that these cells have encountered.

Gene expression of many inflammatory molecules depends on the activation of the transcription factor NF-κB. Among the wide range of neutrophil agonists, TNFα, LPS and PMA were found to be strong activators of NF-κB, whereas IL-1β, LTB4, fMLP and PAF are weaker stimuli (70). In contrast, GM-CSF, G-CSF, and IFNγ are not thought to induce NF-κB activation in neutrophils (70,71). These stimuli have in common that they activate the STAT transcription factor (72), however it is not clear whether STATs are critically involved in regulating transcription of inflammatory molecules by these stimuli.

Table 2. Expression of inflammatory molecules by neutrophils *in vitro*

Pro-inflammatory	Anti-inflammatory
cytokines	
Tumor necrosis factor-α	cytokines
IL-1α, IL-1β	IL-1 receptor antagonist (IL-1RA)
IL-12	transforming growth factor-β (TGFβ)
Granulocyte colony-stimulating factor (G-CSF)	sReceptors
Interferon-α, (IFNα), IFNβ	sIL-1R2
chemokines	
IL-8	
Growth-related gene product-α (GRO-α)	
Macrophage inflammatory protein-1α (MIP1α), MIP1β	
Cytokine-induced chemoattractants (CINK)	
others	
Fas ligand	
vascular endothelial growth factor (VEGF)	
Hepatocyte growth factor (HGF)	

Adapted from Cassatella et al (59)

2.4 Priming

The capacity for killing microorganisms can also result in the ability to cause host tissue damage inappropriately. Therefore regulation of neutrophil activation is a tightly controlled process as is depicted schematically in Figure 3. Normally, resting neutrophils in the peripheral blood are poorly responsive to many physiological activators in the context of cytotoxicity (73-75). To fully activate granulocytes, a pre-activation step is necessary. This pre-activation step is generally referred as "priming" (3,76,77). Priming is the amplification of granulocyte functional responses by prior exposure of cells to a priming agent. The priming agent itself, however, does not need to cause a noticeable functional response, except at very high concentrations (76). Thus priming alters these cells from a "non-responsive" to a "responsive" state. Priming agents are mostly "regulatory cell-derived" mediators, such as bioactive peptides, chemokines or cytokines (73-75,78,79), Products of bacteria, or bacteria covered with immunoglobulins, or complement factors are the final activators of the effector functions (80,81). This allows granulocytes to activate the killing machine only in the presence of pathogens. At the moment it is still not precisely clear, which intracellular molecules allow the distinction between a priming and an activation signal, since both priming and final activation signals can activate similar signaling pathways. Apart from the activation of specific signaling pathways, both the level and kinetics of the activation of signaling molecules might be important in modulation of granulocyte functioning.

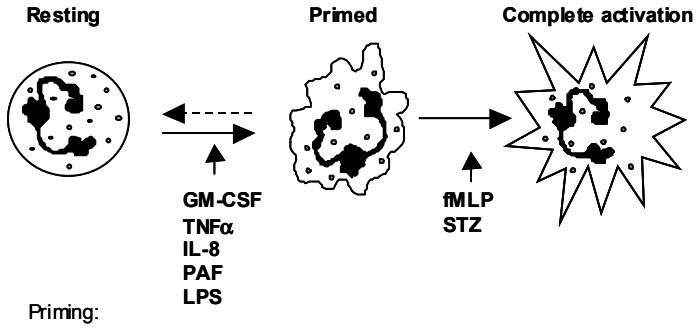

Figure 3: Granulocyte priming and activation. Neutrophil activation can be modelled as a two step activation mechanism. Agents such as PAF, GM-CSF or TNFα, can bring the cell in a pre-activated or primed state, which appears to be a reversible event. Subsequent exposure of a primed cell to an activating stimulus pushes the cell irreversibly to a fully activated state, whereas non-primed cells are poorly responsive for the activation stimulus.

3.0 Membrane receptors

Granulocytes display multiple structurally different membrane-associated receptors that can regulate cellular functioning. Major groups of receptors are cytokine receptors, G-protein coupled receptors (GPCRs), integrins, Fc receptors, and Toll-like receptors.

The cytokine receptor family is divided into 4 classes: (a) the hematopoietin receptor family, termed class I, which is further divided into IL-3, IL-2, IL-6, and growth hormone subfamilies; (b) the interferon receptor family or called class II; (c) Tumor necrosis receptor family; (d) immunoglobulin superfamily receptors, such as IL-1 (82). Most of the cytokine receptors are single transmembrane-domain receptors. The receptors of the IL-3 subfamily, including IL-3, IL-5, and GM-CSF, consist of a cytokine specific α-chain, which binds ligand with low affinity, and a common β-chain (83). Heterodimerization of these receptors in the presence of ligand results in a conformational change, followed by the activation of associated tyrosine kinases, which mediate further downstream signaling. Receptors the TNF subfamily, such as the TNF receptors p55 and p75, which bind TNFα, and the lymphotoxin receptors, form homotrimers upon ligand binding (84). Subsequently signaling proteins, such as TRADD, RIP and TRAF2 are recruited to the cytoplasmic tail of the receptor by a 60 amino-acid domain known as the "death domain". This complex can in turn activate caspases (85) and via other proteins, such as NIK, the transcription factor NF-κB in granulocytes (70,80). NF-κB mediate survival, while activation of caspases has been implicated in inducing apoptosis (86-88). This dual function of TNFα might explain the controversial function of TNFα in survival, since TNFα was reported by some to have pro-apoptotic and by others to have anti-apoptotic effects on neutrophils (89-92). A recent study reported that the outcome of TNFα on survival depends on the concentration of TNFα, suggesting that the balance between these pathways is different at distinct concentrations of TNFα (93). Furthermore, it is likely that other stimuli can influence this balance.

Generally Fc receptors are single transmembrane-domain molecules that require crosslinking for activation, and are expressed on lymphoid and myeloid cells. Fc receptors can be divided into two classes. The first class participates in the translocation of plasma Ig across epithelial surfaces (poly-Ig receptor,FcRn). The second class of Fc receptors are expressed by leukocytes and are involved in immunoglobulin specific activation of cellular functions, such as degranulation, cytokine production, phagocytosis and respiratory burst (94,95). Most Fc receptors of this second class exist as complexes of a ligand-binding α-chain and accessory subunits (β, γ, or ζ-subunits). These subunits, but also FcγRII receptors A and C contain immunoreceptor tyrosine-based activation motifs (ITAMs) (96). Phosphorylation of ITAMs results in further activation of downstream signaling pathways via the activation of tyrosine kinases (44,95,97). Besides Fc receptors containing ITAMS, there also Fc receptors including FcγRIIB that contain immunoreceptor tyrosine-based inhibition motifs (ITIMS), which requires

phosphorylation to obtain its inhibitory activity (95). Co-engagement of an inhibitory Fc receptor with an ITAM containing receptor leads to phosphorylation of the ITIM and subsequently inhibits the ITAM triggered response.

The G-protein coupled seven transmembrane glycoproteins, also called serpentine receptors, include receptors for complement C5a, formylpeptides, PAF, leukotriene B4, and receptors for C-X-C or α-chemokines such as receptors for IL-8. All these ligands share the property of being chemoattractants for granulocytes. Binding of these chemoattractants to their receptors induce a conformational change of the cytoplasmic tail of the receptor resulting in G-protein activation. In an inactive state, G-proteins form a complex consisting of an α- and a βγ-subunit, whereas upon GPCRs activation, the α subunit binds to GTP, resulting in dissociation of the βγ subunit (98). Subsequently, release of these G-protein subunits results in activation of a variety of downstream signaling pathways.

4.0 Signaling pathways regulating granulocyte effector functions

A large amount of research has been performed over the last decade to understand the molecular mechanisms responsible for activation of neutrophil effector functions, including not only the type of stimuli causing activation, but also unraveling components of the signaling pathways responsible for the priming and activation of effector functions (Fig. 4). Apart from the activation of signaling pathways, both, duration and localization of signaling molecules are important in modulation of granulocyte functioning.

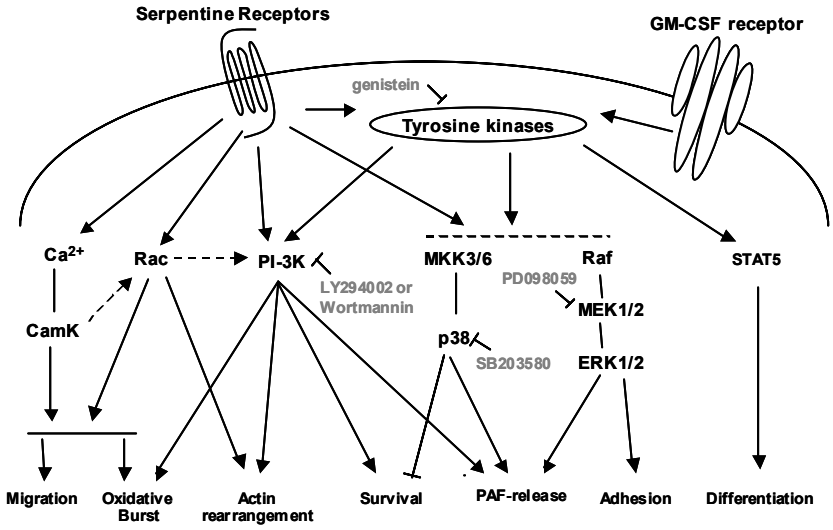

Figure 4: Simplified model of signaling pathways involved in cytokine and GPCRs induced granulocyte functioning. Specific inhibitors are depicted in grey.

4.1 Small GTPases

Many receptors are able to activated small GTPases. The Ras superfamily, which includes Ras, Rap1 and Ral (99); and the Rho family, including Rho, Rac, and Cdc42 (100) belong to the family of small GTPases. All small GTPases act as molecular switches, alternating between a GTP bound and GDP bound states in the relay of signals from receptors. When bound to GTP, a conformational change occurs, which unmasks structural motifs, in particular the so called effector loop, which can then mediate interactions with target proteins or effector molecules. This GDP/GTP cycle is tightly regulated by three main classes of proteins: (a) proteins that facilitate the exhange of the hydrolyzed GDP for a fresh GTP, guanosine nucleotide exchange factors (GEFs); (b) those that enhance the intrinsic hydrolysis activity of the GTPase, (GAPs); (c) and those that inhibit the dissociation of the GDP from the GTPase, GDP dissociation inhibitors (GDIs) (101). In granulocytes it has been reported that, both Ras and Rap1 are activated by many distinct types of receptors, whereas Ral and the Rho family of GTPases are mainly activated by GPCRs, integrins, and Fc receptors, but not by cytokine receptors (102-104). Ras is known to activate the RAF-MEK-ERK pathway in many cell types as well in eosinophils. Using dominant negative Ras, a study showed that Ras attenuates IL-5 mediated cell viability in eosinophils (105). Rap1 has been shown to be present in the NADPH complex, possibly playing a role in correct targeting or assembly of this complex (102). Rac has three isoforms, Rac1, Rac2, and Rac 3 (100). Rac1 is ubiquitously expressed, whereas Rac2 is highly restricted to hemopoietic cells and is the most abundant Rac protein in neutrophils. In neutrophils, Rac GTPases are activated by GPCRs but not by the receptors of the cytokines of the GM-CSF/IL-3/IL-5 family. Rac has been shown to be present NADPH oxidase complex of human neutrophils, where it is thought to be critical in the production of oxygen radicals, by stabilizing the complex (56). A distinction between Rac/Cdc42 and Rho signaling has been reported for their role in phagocytosis. Cdc42 and Rac mediate FcγRs phagocytosis, whereas CR3 mediated phagocytosis requires Rho activity (Cox 1997;Caron 1998; Massol 1998). Recently, a role of Rho in the process of uropod detachment or rear release during migration has been demonstrated in granulocytes (106). Thus co-ordination of the activity of these three GTPases might be necessary for optimal migration of these cells.

4.2 Intracellular Ca^{2+}

Upon triggering of many plasma membrane receptors such as GPCRs, intergrin and Fc receptors, phospholipase C (PLC)β/γ is activated, which catalyzes the conversion of phosphatidylinositol 4,5-biphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (107,108). DAG activates protein kinase C, whereas IP3 induces Ca^{2+} release from intracellular stores. The increase of intracellular Ca^{2+} ($[Ca^{2+}]i$) is later followed by an influx of extracellular Ca^{2+}. The effect of increased $[Ca^{2+}]i$ is mediated by

EF-hand motif containing proteins, such as calmodulin (109). Binding of Ca^{2+} to calmodulin leads to a conformational change, resulting in the ability to bind to and activate proteins that contain a camodulin binding domain (CBD) (110). An important group of proteins that are activated by Ca^{2+}/calmodulin are the Ca^{2+}/calmodulin-dependent protein kinases (111). Using an inhibitor of calmodulin, it has been demonstrated that calmodulin is involved in neutrophil migration and generation of superoxide (112). Recently a new calmodulin-dependent kinase, termed CKLiK, was indentified, which is predominantly expressed in human granulocytes (113). Furthermore, a role for Ca^{2+} has been suggested in the priming process, since a transient increase in $[Ca^{2+}]i$, by ionomycin, is in itself sufficient to prime granulocytes (114). However, changes in $[Ca^{2+}]i$ is per se a requirement, since priming induced by PAF, which increases the level of $[Ca^{2+}]i$, is only partially inhibited under $[Ca^{2+}]i$-buffering conditions (7). Furthermore, other priming agents such as cytokines of the GM-CSF/IL-3/IL-5 family, do not induce a increase in $[Ca^{2+}]i$ (115).

4.3 Tyrosine kinases

Tyrosine kinases have been demonstrated to mediate downstream signaling of several receptors, such as those of the GM-CSF/IL-3/IL-5 family, the G-protein coupled receptor (GPCR) agonists such as PAF and fMLP as well as Fc receptors such as FcγRII (116-121). Receptor mediated activation of tyrosine kinases results in the induction of intracellular kinase cascades which can amplify and modulate the initial response. In neutrophils several protein tyrosine kinases have been identified, including Janus Kinases (JAKs) and the Src kinase family. Heterodimerization of cytokine receptors, such as those of the GM-CSF/IL-3/IL-5 family (118,122), results in bringing two JAK2 proteins into close proximity, enabling cross-phosphorylation and activation of the JAK2 proteins themselves as well as the phosphorylation of the cytoplasmic tail of the β-chain. Subsequently other signaling proteins, such as Src kinases and Signal Transducers and Activators of Transcription (STAT) transcription factors can bind to the β-chain, resulting in activation of further signaling pathways. Phosphorylation of STAT proteins by JAKs results in dimerization and subsequently STAT proteins translocate to the nucleus to induce the expression of target genes. This pathway is important for linking many cytokines to gene regulation, but it has not been known whether it is directly linked to regulation of granulocyte effector functions.

Of the Src kinase family, several members such as Lyn, Hck and Fgr are expressed in neutrophils (123,124). These Src kinases have also been shown to be activated by various cytokine and G-protein coupled receptors (121,125,126). Src kinases are kept in an inactive state by the phosphorylation of a C-terminal tyrosine residue (Tyr 507, of c-Src) (Fig. 5) (127). Phosphorylation of this site results in binding of the C-terminal region with its own SH2 domain and thereby blocking the SH2 and SH3 domain in binding substrates

and keeping the kinase domain obscured. Activation of Src kinases is mediated by dephosphorylation of this site. In addition, Src kinase are phosphorylated at the tyrosine residue 416 (of c-Src), which enables further activation of Src kinases. In human neutrophils little is known about the role of Src kinases in downstream signaling pathways and effector functions. However by using knock-out mice deficient in the different Src kinases, several studies have shown that Src kinases are involved in degranulation (128), and migration (129).

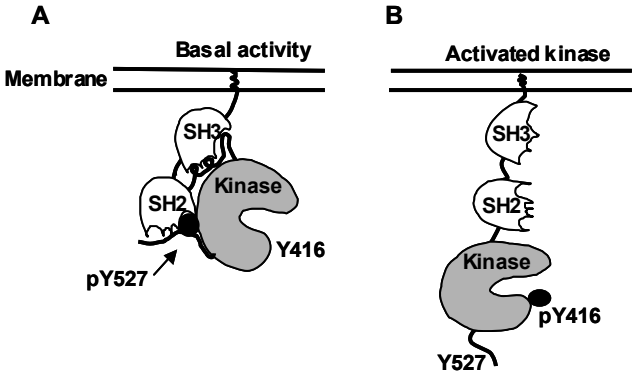

Figure 5: **Regulation of Src kinase activity. (A)** Closed autoinhibited state; **(B)** open state induced either by dephosphorylation of the C-terminal Tyr-527 or by interactions of the SH3 and SH2 domains with the Src protein partners. This makes Tyr-416 accessible for phosphorylation, resulting in enhanced activation.

4.4 Mitogen Activated Protein (MAP) Kinases

One family of tyrosine phosphorylated proteins that has been demonstrated to be activated by many inflammatory mediators are the mitogen activated protein kinases, or MAP kinases. There are three distinct groups of MAP kinases (130): extracellular signal-related protein kinases (ERKs) activated by a large variety of agonist, c-Jun amino-terminal kinase/stress-activated protein kinases (JNK/SAPKs) and the p38 MAPK family. The best charaterized MAPK pathway consists of Raf, MEK1/2 and ERK1/2 module (131). This cascade is regulated by the small GTPase Ras. While ERK is activated by diverse stimuli, the other two groups are more specifically activated be environmental stress and pro-inflammatory cytokines. However, in neutrophils both the pathways ERK and p38 MAPK are activated by diverse stimuli, such as LPS, fMLP, GM-CSF, PAF and IL-8, whereas TNFα, is a stong activator of p38 MAPK, but weakly activates ERK in neutrophils (80,132-135). Inhibitory studies of MEK1/2 have shown that this pathway regulates PAF release by serum treated zymosan, whereas variable effects has been shown about the role

of ERK1/2 in migration and fMLP induced superoxide production (78,134,136-138). In addition a role for ERK has been shown in regulating neutrophil survival (139). A partial decrease in superoxide production and stress induced apoptosis has been reported in the presence of p38 inhibitors (140-142). Activation of JNK has only been demonstrated in adherent neutrophils and is dependent on beta2 integrin binding (91).

4.5 Phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (PKB)

Another signal transduction pathway that appears to play a critical role in both priming and activation of granulocyte effector functions involves PI3K (143). At the membrane, PI3K phosphorylates phophoinositides at the D-3 position. Class I PI3Ks form hetereodimers composed of a regulatory unit and a catalytic subunit. Several isoforms of both the adaptor and catalytic subunit have been identified. The best characterized catalytic subunits are the p85 adaptor subunit and the catalytic subunit p110α/β, which are activated by tyrosine kinase, whereas the p101 adaptor subunit together with the catalytic subunit p110γ are thought to be regulated only by G-protein activation. In neutrophils, many receptors, including cytokine and G-protein coupled receptors are able to activate PI3K either via tyrosine kinases and/or G-proteins (80,144). Utilizing specific pharmacological inhibitors of PI3K, wortmannin and LY294002, ourselves and others have demonstrated that this kinase plays a critical role in several neutrophil cellular functions including the respiratory burst, survival, and migration (80,145-147). Protein kinase B (PKB) is one of the major downstream targets of PI3K and is also activated in granulocytes in response to both cytokine and G-protein coupled receptors. It has been shown in several cell types that PKB can phosphorylate the pro-apoptotic Bcl-2 family member BAD, which in turn prevents its association with anti-apoptotic Bcl-2 family members, resulting in prolonged survival (148,149). In neutrophils, the PI3K inhibitor LY294002 blocked GM-CSF dependent PKB and BAD phosphorylation (150). Thus it might be that this PKB/BAD pathway is also involved in regulating neutrophil survival. Furthermore, PI3K is involved in other cellular functions of granulocytes, such as the respiratory burst. It has been proposed for example that PI3K can mediate the phosphorylation of the p47phox, a component of the NADPH oxidase complex (151).

5.0 Scope of this thesis

Granulocytes are thought to play an important role in the maintenance and progression of chronic diseases, such as COPD, allergic asthma, and rheumatoid arthritis. Despite the importance of granulocytes in these diseases surprisingly little is known about the processes occurring *in vivo* that regulate inappropriate granulocyte functioning. In this thesis, gene expression of neutrophils has been analyzed *in vitro* and *in vivo*, to characterize chronic systemic inflammation and identify mediators that are playing a role in the pathogenesis of COPD. To understand the finely tuned process of neutrophil functioning *in vivo*, it is also necessary to define components of the signaling pathways responsible for the priming and activation of effector functions in granulocytes. In this thesis, several research questions were addressed, concerning granulocyte functioning *in vitro* and *in vivo*.

Are changes in gene expression of peripheral blood neutrophils a result of the pathogenetic mechanisms underlying the development of COPD and can these gene profiles be used to define penotypes and severity of the disease?

Exposure of neutrophils to cytokines or chemokines, results in their activation and changes in gene expression profiles. In Chapter 2, we utilise gene array technology and real time reverse trancriptase polymerase chain reaction to define the gene expression profiles of peripheral blood neutrophils of COPD patients compared with in vitro stimulated neutrophils. In Chapter 3, we use the same techniques, to investigate differences between peripheral blood neutrophils of COPD patients during a stable phase of their disease and during an exacerbation. Furthermore, we investigated the kinetics of activation of peripheral blood neutrophils in COPD patients in a longitudial setting.

What is the role of Src kinases and small GTPase Rac in granulocyte functioning?

To address this question, we use pharmacological inhibitors and a recently developed technique of protein transduction. In Chapter 5 we investigate the role of Src kinases in neutrophil functioning, whereas in Chapter 6 the role of Rac is investigated in peripheral blood eosinophil functioning by introducing a constitutively active mutant into these cells. In Chapter 7, we describe an ELISA technique developed for non-radioactive semi-quantitative analysis of the activation state of several kinases in adherent and suspension cells using phosphospecific antibodies.

References

1. Witko-Sarsat, V., P. Rieu, B. Descamps-Latscha, P. Lesavre, L. Halbwachs-Mecarelli. 2000. Neutrophils: molecules, functions and pathophysiological aspects. *Lab. Invest.* 80:617-653.
2. Haslett, C., J. S. Savill, L. Meagher. 1989. The neutrophil. *Curr. Opin. Immunol.* 2:10-18.
3. Coffer, P. J., and L. Koenderman. 1997. Granulocyte signal transduction and priming: cause without effect? *Immunol. Lett.* 57:27-31.
4. Gleich, G. J., and C. R. Adolphson. 1986. The eosinophilic leukocyte: structure and function. *Adv Immunol* 39:177-253.
5. Lesur, O., Y. Berthiaume, G. Blaise, P. Damas, E. Deland, J. G. Guimond, R. P. Michel. 1999. Acute respiratory distress syndrome: 30 years later. *Can. Respir. J.* 6:71-86.
6. Botha, A. J., F. A. Moore, E. E. Moore, B. Fontes, A. Banerjee, V. M. Peterson. 1995. Postinjury neutrophil priming and activation states: therapeutic challenges. *Shock* 3:157-166.
7. Roos, D., M. de Boer, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz, H. Middleton-Price. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood* 87:1663-1681.
8. Barnes, P. J. 2000. Chronic obstructive pulmonary disease. *N. Engl. J. Med.* 343:269-280.
9. Lacoste, J. Y., J. Bousquet, P. Chanez, T. Van Vyve, J. Simony-Lafontaine, N. Lequeu, P. Vic, I. Enander, P. Godard, F. B. Michel. 1993. Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J. Allergy Clin. Immunol.* 92:537-548.
10. Martinez, F. J. 1998. Diagnosing chronic obstructive pulmonary disease. The importance of differentiating asthma, emphysema, and chronic bronchitis. *Postgrad. Med.* 103:112-2, 125.
11. Pauwels, R. A., A. S. Buist, P. M. Calverley, C. R. Jenkins, S. S. Hurd. 2001. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am. J. Respir. Crit. Care Med.* 163:1256-1276.
12. Scanlon, P. D., J. E. Connett, L. A. Waller, M. D. Altose, W. C. Bailey, A. S. Buist. 2000. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am. J. Respir. Crit. Care Med.* 161:381-390.
13. Rennard, S. I., and D. M. Daughton. 2000. Smoking cessation. *Chest* 117:360S-364S.
14. Fletcher, C., and R. Peto. 1977. The natural history of chronic airflow obstruction. *Br. Med. J.* 1:1645-1648.
15. Scanlon, P. D., J. E. Connett, L. A. Waller, M. D. Altose, W. C. Bailey, A. S. Buist. 2000. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am. J. Respir. Crit. Care Med.* 161:381-390.
16. Stockley, R. A. 2000. Alpha-1-antitrypsin deficiency: what next? *Thorax* 55:614-618.
17. Wedzicha, J. A. 2002. Exacerbations: etiology and pathophysiologic mechanisms. *Chest* 121:136S-141S.
18. Anthonisen, N. R., J. Manfreda, C. P. Warren, E. S. Hershfield, G. K. Harding, N. A. Nelson. 1987. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann. Intern. Med.* 106:196-204.
19. Keatings, V. M., P. D. Collins, D. M. Scott, P. J. Barnes. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am. J. Respir. Crit. Care Med.* 153:530-534.
20. Vernooy, J. H., M. Kucukaycan, J. A. Jacobs, N. H. Chavannes, W. A. Buurman, M. A. Dentener, E. F. Wouters. 2002. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am. J. Respir. Crit. Care Med.* 166:1218-1224.
21. Yamamoto, C., T. Yoneda, M. Yoshikawa, A. Fu, T. Tokuyama, K. Tsukaguchi, N. Narita. 1997. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 112:505-510.

22. Pesci, A., M. Majori, A. Cuomo, N. Borciani, S. Bertacco, G. Cacciani, M. Gabrielli. 1998. Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease. *Respir. Med.* 92:863-870.
23. Keatings, V. M., and P. J. Barnes. 1997. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am. J. Respir. Crit. Care Med.* 155:449-453.
24. Saetta, M., S. Baraldo, L. Corbino, G. Turato, F. Braccioni, F. Rea, G. Cavallesco, G. Tropeano, C. E. Mapp, P. Maestrelli, A. Ciaccia, L. M. Fabbri. 1999. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 160:711-717.
25. O'Shaughnessy, T. C., T. W. Ansari, N. C. Barnes, P. K. Jeffery. 1997. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am. J. Respir. Crit. Care Med.* 155:852-857.
26. Saetta, M., G. Turato, F. M. Facchini, L. Corbino, R. E. Lucchini, G. Casoni, P. Maestrelli, C. E. Mapp, A. Ciaccia, L. M. Fabbri. 1997. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. *Am. J. Respir. Crit. Care Med.* 156:1633-1639.
27. Pesci, A., B. Balbi, M. Majori, G. Cacciani, S. Bertacco, P. Alciato, C. F. Donner. 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur. Respir. J.* 12:380-386.
28. Aaron, S. D., J. B. Angel, M. Lunau, K. Wright, C. Fex, N. Le Saux, R. E. Dales. 2001. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 163:349-355.
29. Koenderman, L., D. Kanters, B. Maesen, J. Raaijmakers, J. W. Lammers, J. de Kruif, T. Logtenberg. 2000. Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. *J. Leukoc. Biol.* 68:58-64.
30. Noguera, A., S. Batle, C. Miralles, J. Iglesias, X. Busquets, W. MacNee, A. G. Agusti. 2001. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax* 56:432-437.
31. Walcheck, B., J. Kahn, J. M. Fisher, B. B. Wang, R. S. Fisk, D. G. Payan, C. Feehan, R. Betageri, K. Darlak, A. F. Spatola, T. K. Kishimoto. 1996. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. *Nature* 380:720-723.
32. Kuijper, P. H., H.I., J. A. van der Linden, J. W. Lammers, J. J. Sixma, J. J. Zwaginga, L. Koenderman. 1997. Neutrophil adhesion to fibrinogen and fibrin under flow conditions is diminished by activation and L-selectin shedding. *Blood* 89:2131-2138.
33. Lawrence, M. B., and T. A. Springer. 1993. Neutrophils roll on E-selectin. *J. Immunol.* 151:6338-6346.
34. Patel, K. D., K. L. Moore, M. U. Nollert, R. P. McEver. 1995. Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. *J. Clin. Invest.* 96:1887-1896.
35. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76:301-314.
36. Springer, T. A., W. S. Thompson, L. J. Miller, F. C. Schmalstieg, D. C. Anderson. 1984. Inherited deficiency of the Mac-1, LFA-1, p150,95 glycoprotein family and its molecular basis. *J. Exp. Med.* 160:1901-1918.
37. Sligh, J. E. J., C. M. Ballantyne, S. S. Rich, H. K. Hawkins, C. W. Smith, A. Bradley, A. L. Beaudet. 1993. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U. S. A.* 90:8529-8533.
38. Williams, L. A., I. Martin-Padura, E. Dejana, N. Hogg, D. L. Simmons. 1999. Identification and characterisation of human Junctional Adhesion Molecule (JAM). *Mol. Immunol.* 36:1175-1188.
39. Muller, W. A., S. A. Weigl, X. Deng, D. M. Phillips. 1993. PECAM-1 is required for transendothelial migration of leukocytes. *J. Exp. Med.* 178:449-460.
40. Foxman, E. F., E. J. Kunkel, E. C. Butcher. 1999. Integrating conflicting chemotactic signals. The role of memory in leukocyte navigation. *J. Cell Biol.* 147:577-588.

41. Kitayama, J., M. W. Carr, S. J. Roth, J. Buccola, T. A. Springer. 1997. Contrasting responses to multiple chemotactic stimuli in transendothelial migration: heterologous desensitization in neutrophils and augmentation of migration in eosinophils. *J. Immunol.* 158:2340-2349.
42. Allen, L. A., and A. Aderem. 1996. Mechanisms of phagocytosis. *Curr. Opin. Immunol.* 8:36-40.
43. Newman, S. L., L. K. Mikus, M. A. Tucci. 1991. Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. *J. Immunol.* 146:967-974.
44. Kwiatkowska, K., and A. Sobota. 1999. Signaling pathways in phagocytosis. *Bioessays* 21:422-431.
45. Barak, Y., and E. Nir. 1987. Chediak-Higashi syndrome. *Am J Pediatr Hematol Oncol* 9:42-55.
46. Gullberg, U., N. Bengtsson, E. Bulow, D. Garwicz, A. Lindmark, I. Olsson. 1999. Processing and targeting of granule proteins in human neutrophils. *J. Immunol. Methods* 232:201-210.
47. Borregaard, N. 1997. Development of neutrophil granule diversity. *Ann. N. Y. Acad. Sci.* 832:62-68.
48. Borregaard, N., and J. B. Cowland. 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 89:3503-3521.
49. Babior, B. M. 1999. NADPH oxidase: an update. *Blood* 93:1464-1476.
50. Babior, B. M., J. D. Lambeth, W. Nauseef. 2002. The neutrophil NADPH oxidase. *Arch. Biochem. Biophys.* 397:342-344.
51. DeLeo, F. R., and M. T. Quinn. 1996. Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J Leukoc Biol* 60:677-691.
52. Quinn, M. T., C. A. Parkos, L. Walker, S. H. Orkin, M. C. Dinauer, A. J. Jesaitis. 1989. Association of a Ras-related protein with cytochrome b of human neutrophils. *Nature* 342:198-200.
53. Abo, A., E. Pick, A. Hall, N. Totty, C. G. Teahan, A. W. Segal. 1991. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* 353:668-670.
54. Knaus, U. G., P. G. Heyworth, T. Evans, J. T. Curnutte, G. M. Bokoch. 1991. Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. *Science* 254:1512-1515.
55. Heyworth, P. G., B. P. Bohl, G. M. Bokoch, J. T. Curnutte. 1994. Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. *J. Biol. Chem.* 269:30749-30752.
56. Bokoch, G. M., and B. A. Diebold. 2002. Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood* 100:2692-2696.
57. Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knaus, R. J. Ulevitch, G. M. Bokoch. 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* 270:23934-23936.
58. Bromberg, Y., E. Shani, G. Joseph, Y. Gorzalczany, O. Sperling, E. Pick. 1994. The GDP-bound form of the small G protein Rac1 p21 is a potent activator of the superoxide-forming NADPH oxidase of macrophages. *J. Biol. Chem.* 269:7055-7058.
59. Di-Poi, N., J. Faure, S. Grizot, G. Molnar, E. Pick, M. C. Dagher. 2001. Mechanism of NADPH oxidase activation by the Rac/Rho-GDI complex. *Biochemistry* 40:10014-10022.
60. Vergnaud, S., M. H. Paclet, J. el Benna, M. A. Pocidalo, F. Morel. 2000. Complementation of NADPH oxidase in p67-phox-deficient CGD patients p67-phox/p40-phox interaction. *Eur J Biochem* 267:1059-1067.
61. Cassatella, M. A. 1999. Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* 73:369-509.
62. Scapini, P., J.A., S. Gasperini, F. Calzetti, F. Bazzoni, M. A. Cassatella. 2000. The neutrophil as a cellular source of chemokines. *Immunol. Rev.* 177:195-203.
63. Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J. G. Giri, S. K. Dower, J. E. Sims, A. Mantovani. 1993. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 261:472-475.

64. Mantovani, A., M. Muzio, P. Ghezzi, C. Colotta, M. Introna. 1998. Regulation of inhibitory pathways of the interleukin-1 system. *Ann. N. Y. Acad. Sci.* 840:338-351.
65. Bemelmans, M. H., L. J. van Tits, W. A. Buurman. 1996. Tumor necrosis factor: function, release and clearance. *Crit. Rev. Immunol.* 16:1-11.
66. Cassatella, M. A. 1995. The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* 16:21-26.
67. Fujishima, S., A. R. Hoffman, T. Vu, K. J. Kim, H. Zheng, D. Daniel, Y. Kim, E. F. Wallace, J. W. Larrick, T. A. Raffin. 1993. Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-alpha, and IL-1 beta. *J. Cell Physiol.* 154:478-485.
68. Malyak, M., M. F. J. Smith, A. A. Abel, W. P. Arend. 1994. Peripheral blood neutrophil production of interleukin-1 receptor antagonist and interleukin-1 beta. *J. Clin. Immunol.* 14:20-30.
69. McColl, S. R., R. Paquin, C. Menard, A. D. Beaulieu. 1992. Human neutrophils produce high levels of the interleukin 1 receptor antagonist in response to granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J. Exp. Med.* 176:593-598.
70. McDonald, P. P., A. Bald, M. A. Cassatella. 1997. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* 89:3421-3433.
71. Chaturvedi, M. M., M. Higuchi, B. B. Aggarwal. 1994. Effect of tumor necrosis factors, interferons, interleukins, and growth factors on the activation of NF-kappa B: evidence for lack of correlation with cell proliferation. *Lymphokine Cytokine Res.* 13:309-313.
72. McDonald, P. P., C. Bovolenta, M. A. Cassatella. 1998. Activation of distinct transcription factors in neutrophils by bacterial LPS, interferon-gamma, and GM-CSF and the necessity to overcome the action of endogenous proteases. *Biochemistry* 37:13165-13173.
73. Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, A. M. Waltersdorph. 1986. Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* 136:4220-4225.
74. Weisbart, R. H., L. Kwan, D. W. Golde, J. C. Gasson. 1987. Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. *Blood* 69:18-21.
75. Ingraham, L. M., T. D. Coates, J. M. Allen, C. P. Higgins, R. L. Baehner, L. A. Boxer. 1982. Metabolic, membrane, and functional responses of human polymorphonuclear leukocytes to platelet-activating factor. *Blood* 59:1259-1266.
76. Swain, S. D., T. T. Rohn, M. T. Quinn. 2002. Neutrophil priming in host defense: role of oxidants as priming agents. *Antioxid. Redox. Signal.* 4:69-83.
77. Downey, G. P., T. Fukushima, L. Fialkow, T. K. Waddell. 1995. Intracellular signaling in neutrophil priming and activation. *Semin. Cell. Biol.* 6:345-356.
78. Coffer, P. J., N. Geijsen, L. M'Rabet, R. C. Schweizer, T. Maikoe, J. A. Raaijmakers, J. W. Lammers, L. Koenderman. 1998. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem. J.* 329:121-130.
79. Dewald, B., and M. Baggiolini. 1985. Activation of NADPH oxidase in human neutrophils. Synergism between fMLP and the neutrophil products PAF and LTB4. *Biochem Biophys Res Commun* 128:297-304.
80. Coffer, P. J., N. Geijsen, L. M'Rabet, R. C. Schweizer, T. Maikoe, J. A. Raaijmakers, J. W. Lammers, L. Koenderman. 1998. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem. J.* 329:121-130.
81. Koenderman, L., A. T. Tool, D. Roos, A. J. Verhoeven. 1990. Priming of the respiratory burst in human eosinophils is accompanied by changes in signal transduction. *J. Immunol.* 145:3883-3888.
82. Foxwell, B. M., K. Barrett, M. Feldmann. 1992. Cytokine receptors: structure and signal transduction. *Clin Exp Immunol* 90:161-169.

83. Geijsen, N., L. Koenderman, P. J. Coffer. 2001. Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family. *Cytokine. Growth Factor. Rev.* 12:19-25.
84. Bazzoni, F., and B. Beutler. 1996. The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med.* 334:1717-1725.
85. Gupta, S. 2002. A decision between life and death during TNF-alpha-induced signaling. *J. Clin. Immunol.* 22:185-194.
86. Karin, M., and A. Lin. 2002. NF-kappaB at the crossroads of life and death. *Nat. Immunol.* 3:221-227.
87. Datta, S. R., A. Brunet, M. E. Greenberg. 1999. Cellular survival: a play in three Akts. *Genes Dev* 13:2905-2927.
88. Li, Q., and I. M. Verma. 2002. NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* 2:725-734.
89. Murray, J., J. A. Barbara, S. A. Dunkley, A. F. Lopez, O. Van, X, A. M. Condliffe, I. Dransfield, C. Haslett, E. R. Chilvers. 1997. Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* 90:2772-2783.
90. Takeda, Y., H. Watanabe, S. Yonehara, T. Yamashita, S. Saito, F. Sendo. 1993. Rapid acceleration of neutrophil apoptosis by tumor necrosis factor-alpha. *Int. Immunol.* 5:691-694.
91. Avdi, N. J., J. A. Nick, B. B. Whitlock, M. A. Billstrom, P. M. Henson, G. L. Johnson, G. S. Worthen. 2001. Tumor necrosis factor-alpha activation of the c-Jun N-terminal kinase pathway in human neutrophils. Integrin involvement in a pathway leading from cytoplasmic tyrosine kinases apoptosis. *J. Biol. Chem.* 276:2189-2199.
92. Niwa, M., A. Hara, Y. Kanamori, D. Hatakeyama, M. Saio, T. Takami, H. Matsuno, O. Kozawa, T. Uematsu. 2000. Nuclear factor-kappaB activates dual inhibition sites in the regulation of tumor necrosis factor-alpha-induced neutrophil apoptosis. *Eur. J. Pharmacol.* 407:211-219.
93. van den Berg, J. M., S. Weyer, J. J. Weening, D. Roos, T. W. Kuijpers. 2001. Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. *J. Leukoc. Biol.* 69:467-473.
94. Hulett, M. D., and P. M. Hogarth. 1994. Molecular basis of Fc receptor function. *Adv. Immunol.* 57:1-127.
95. Ravetch, J. V., and J. P. Kinet. 1991. Fc receptors. *Annu. Rev. Immunol.* 9:457-492.
96. Daeron, M. 1997. Structural bases of Fc gamma R functions. *Int. Rev. Immunol.* 16:1-27.
97. van Egmond, M., C. A. Damen, A. B. van Spriel, G. Vidarsson, E. van Garderen, J. G. van de Winkel. 2001. IgA and the IgA Fc receptor. *Trends. Immunol.* 22:205-211.
98. Bourne, H. R. 1997. How receptors talk to trimeric G proteins. *Curr Opin Cell Biol* 9:134-142.
99. Bos, J. L. 1998. All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *EMBO J* 17:6776-6782.
100. Aspenstrom, P. 1999. The Rho GTPases have multiple effects on the actin cytoskeleton. *Exp. Cell Res.* 246:20-25.
101. Bos, J. L. 2001. Glowing switches. *Nature* 411:1006-1007.
102. M'Rabet, L., P. Coffer, F. Zwartkruis, B. Franke, A. W. Segal, L. Koenderman, J. L. Bos. 1998. Activation of the small GTPase rap1 in human neutrophils. *Blood* 92:2133-2140.
103. M'Rabet, L., P. J. Coffer, R. M. Wolthuis, F. Zwartkruis, L. Koenderman, J. L. Bos. 1999. Differential fMet-Leu-Phe- and platelet-activating factor-induced signaling toward Ral activation in primary human neutrophils. *J Biol Chem* 274:21847-21852.
104. Geijsen, N., S. van Delft, J. A. Raaijmakers, J. W. Lammers, J. G. Collard, L. Koenderman, P. J. Coffer. 1999. Regulation of p21rac activation in human neutrophils. *Blood* 94:1121-1130.
105. Hall, D. J., J. Cui, M. E. Bates, B. A. Stout, L. Koenderman, P. J. Coffer, P. J. Bertics. 2001. Transduction of a dominant-negative H-Ras into human eosinophils attenuates extracellular signal-regulated kinase activation and interleukin-5-mediated cell viability. *Blood* 98:2014-2021.
106. Alblas, J., L. Ulfman, P. Hordijk, L. Koenderman. 2001. Activation of Rhoa and ROCK are essential for detachment of migrating leukocytes. *Mol. Biol. Cell* 12:2137-2145.

107. Downey, G. P., T. Fukushima, L. Fialkow. 1995. Signaling mechanisms in human neutrophils. *Curr Opin Hematol* 2:76-88.
108. Dawson, A. P. 1997. Calcium signalling: how do IP3 receptors work? *Curr Biol* 7:R544-R547.
109. Lewit-Bentley, A., and S. Rety. 2000. EF-hand calcium-binding proteins. *Curr Opin Struct Biol* 10:637-643.
110. Chin, D., and A. R. Means. 2000. Calmodulin: a prototypical calcium sensor. *Trends Cell Biol* 10:322-328.
111. Soderling, T. R. 1996. Structure and regulation of calcium/calmodulin-dependent protein kinases II and IV. *Biochim Biophys Acta* 1297:131-138.
112. Verploegen, S., C. M. van Leeuwen, H. W. van Deutekom, J. W. Lammers, L. Koenderman, P. J. Coffer. 2002. Role of Ca2+/calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions. Comparison with the role of phosphotidylinositol-3 kinase. *Eur. J. Biochem.* 269:4625-4634.
113. Verploegen, S., J. W. Lammers, L. Koenderman, P. J. Coffer. 2000. Identification and characterization of CKLiK, a novel granulocyte Ca(++)/calmodulin-dependent kinase. *Blood* 96:3215-3223.
114. Koenderman, L., M. Yazdanbakhsh, D. Roos, A. J. Verhoeven. 1989. Dual mechanisms in priming of the chemoattractant-induced respiratory burst in human granulocytes. A Ca2+-dependent and a Ca2+-independent route. *J Immunol* 142:623-628.
115. van der, B. T., P. T. Kok, J. A. Raaijmakers, A. J. Verhoeven, R. G. Kessels, J. W. Lammers, L. Koenderman. 1993. Cytokine priming of the respiratory burst in human eosinophils is Ca2+ independent and accompanied by induction of tyrosine kinase activity. *J. Leukoc. Biol.* 53:347-353.
116. van der, B. T., D. Kanters, A. T. Tool, J. A. Raaijmakers, J. W. Lammers, A. J. Verhoeven, L. Koenderman. 1998. Cytokine-induced protein tyrosine phosphorylation is essential for cytokine priming of human eosinophils. *J. Allergy Clin. Immunol.* 101:103-109.
117. Luttrell, L. M., Y. Daaka, R. J. Lefkowitz. 1999. Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* 11:177-183.
118. van der, B. T., E. Caldenhoven, D. Kanters, P. Coffer, J. A. Raaijmakers, J. W. Lammers, L. Koenderman. 1995. Interleukin-5 signaling in human eosinophils involves JAK2 tyrosine kinase and Stat1 alpha. *Blood* 85:1442-1448.
119. Thelen, M., and S. A. Didichenko. 1997. G-protein coupled receptor-mediated activation of PI 3-kinase in neutrophils. *Ann N Y Acad Sci* 832:368-382.
120. al-Shami, A., W. Mahanna, P. H. Naccache. 1998. Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b. *J. Biol. Chem.* 273:1058-1063.
121. Corey, S., A. Eguinoa, K. Puyana-Theall, J. B. Bolen, L. Cantley, F. Mollinedo, T. R. Jackson, P. T. Hawkins, L. R. Stephens. 1993. Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src-related tyrosine kinase(s) in human myeloid derived cells. *EMBO J.* 12:2681-2690.
122. Caldenhoven, E., T. B. van Dijk, A. Tijmensen, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, R. P. de Groot. 1998. Differential activation of functionally distinct STAT5 proteins by IL-5 and GM-CSF during eosinophil and neutrophil differentiation from human CD34+ hematopoietic stem cells. *Stem Cells* 16:397-403.
123. Willman, C. L., C. C. Stewart, T. L. Longacre, D. R. Head, R. Habbersett, S. F. Ziegler, R. M. Perlmutter. 1991. Expression of the c-fgr and hck protein-tyrosine kinases in acute myeloid leukemic blasts is associated with early commitment and differentiation events in the monocytic and granulocytic lineages. *Blood* 77:726-734.
124. Yamanashi, Y., S. Mori, M. Yoshida, T. Kishimoto, K. Inoue, T. Yamamoto, K. Toyoshima. 1989. Selective expression of a protein-tyrosine kinase, p56lyn, in hematopoietic cells and association with production of human T-cell lymphotropic virus type I. *Proc. Natl. Acad. Sci. USA* 86:6538-6542.

125. Yan, S. R., L. Fumagalli, G. Berton. 1995. Activation of p58c-fgr and p53/56lyn in adherent human neutrophils: evidence for a role of divalent cations in regulating neutrophil adhesion and protein tyrosine kinase activities. *J. Inflamm.* 45:297-311.
126. Welch, H., and I. Maridonneau-Parini. 1997. Lyn and Fgr are activated in distinct membrane fractions of human granulocytic cells. *Oncogene* 15:2021-2029.
127. Tatosyan, A. G., and O. A. Mizenina. 2000. Kinases of the Src family: structure and functions. *Biochemistry (Mosc.)* 65:49-58.
128. Mocsai, A., E. Ligeti, C. A. Lowell, G. Berton. 1999. Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. *J. Immunol.* 162:1120-1126.
129. Lowell, C. A., and G. Berton. 1998. Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the Src-family kinases Hck and Fgr. *Proc. Natl. Acad. Sci. USA* 95:7580-7584.
130. Robinson, M. J., and M. H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr Opin Cell Biol* 9:180-186.
131. Cobb, M. H., and E. J. Goldsmith. 1995. How MAP kinases are regulated. *J Biol Chem* 270:14843-14846.
132. Suzuki, K., M. Hino, F. Hato, N. Tatsumi, S. Kitagawa. 1999. Cytokine-specific activation of distinct mitogen-activated protein kinase subtype cascades in human neutrophils stimulated by granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-alpha. *Blood* 93:341-349.
133. McLeish, K. R., C. Knall, R. A. Ward, P. Gerwins, P. Y. Coxon, J. B. Klein, G. L. Johnson. 1998. Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF-alpha and GM-CSF. *J Leukoc Biol* 64:537-545.
134. Knall, C., G. S. Worthen, G. L. Johnson. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases.
135. Yuo, A., E. Okuma, S. Kitagawa, F. Takaku. 1997. Tyrosine phosphorylation of p38 but not extracellular signal-regulated kinase in normal human neutrophils stimulated by tumor necrosis factor: comparative study with granulocyte-macrophage colony-stimulating factor. *Biochem. Biophys. Res. Commun.* 235:42-46.
136. Zu, Y. L., J. Qi, A. Gilchrist, G. A. Fernandez, D. Vazquez-Abad, D. L. Kreutzer, C. K. Huang, R. I. Sha'afi. 1998. p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. *J. Immunol.* 160:1982-1989.
137. Rane, M. J., S. L. Carrithers, J. M. Arthur, J. B. Klein, K. R. McLeish. 1997. Formyl peptide receptors are coupled to multiple mitogen-activated protein kinase cascades by distinct signal transduction pathways: role in activation of reduced nicotinamide adenine dinucleotide oxidase. *J. Immunol.* 159:5070-5078.
138. Downey, G. P., J. R. Butler, H. Tapper, L. Fialkow, A. R. Saltiel, B. B. Rubin, S. Grinstein. 1998. Importance of MEK in neutrophil microbicidal responsiveness. *J. Immunol.* 160:434-443.
139. Klein, J. B., M. J. Rane, J. A. Scherzer, P. Y. Coxon, R. Kettritz, J. M. Mathiesen, A. Buridi, K. R. McLeish. 2000. Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J. Immunol.* 164:4286-4291.
140. Lal, A. S., A. D. Clifton, J. Rouse, A. W. Segal, P. Cohen. 1999. Activation of the neutrophil NADPH oxidase is inhibited by SB 203580, a specific inhibitor of SAPK2/p38. *Biochem. Biophys. Res. Commun.* 259:465-470.
141. Nick, J. A., N. J. Avdi, S. K. Young, C. Knall, P. Gerwins, G. L. Johnson, G. S. Worthen. 1997. Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J. Clin. Invest.* 99:975-986.

142. Frasch, S. C., J. A. Nick, V. A. Fadok, D. L. Bratton, G. S. Worthen, P. M. Henson. 1998. p38 mitogen-activated protein kinase-dependent and -independent intracellular signal transduction pathways leading to apoptosis in human neutrophils. *J Biol Chem* 273:8389-8397.
143. Vanhaesebroeck, B., S. J. Leevers, G. Panayotou, M. D. Waterfield. 1997. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* 22:267-272.
144. Tilton, B., M. Andjelkovic, S. A. Didichenko, B. A. Hemmings, M. Thelen. 1997. G-Protein-coupled receptors and Fcgamma-receptors mediate activation of Akt/protein kinase B in human phagocytes. *J Biol Chem* 272:28096-28101.
145. Kodama, T., K. Hazeki, O. Hazeki, T. Okada, M. Ui. 1999. Enhancement of chemotactic peptide-induced activation of phosphoinositide 3-kinase by granulocyte-macrophage colony-stimulating factor and its relation to the cytokine-mediated priming of neutrophil superoxide-anion production. *Biochem J* 337 (Pt 2):201-209.
146. Sasaki, T., J. Irie-Sasaki, R. G. Jones, A.J., W. L. Stanford, B. Bolon, A. Wakeham, A. Itie, D. Bouchard, I. Kozieradzki, N. Joza, T. W. Mak, P. S. Ohashi, A. Suzuki, J. M. Penninger. 2000. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 287:1040-1046.
147. Hirsch, E., V. L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, M. P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287:1049-1053.
148. Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, M. E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91:231-241.
149. del Peso, L., M. Gonzalez-Garcia, C. Page, R. Herrera, G. Nunez. 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278:687-689.
150. Klein, J. B., M. J. Rane, J. A. Scherzer, P. Y. Coxon, R. Kettritz, J. M. Mathiesen, A. Buridi, K. R. McLeish. 2000. Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* 164:4286-4291.
151. Didichenko, S. A., B. Tilton, B. A. Hemmings, K. Ballmer-Hofer, M. Thelen. 1996. Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase. *Curr Biol* 6:1271-1278.

Chapter 2

Analysis of systemic inflammation in COPD by gene profiling in peripheral blood neutrophils

Evert HJ. Nijhuis*, Erik-Jan D. Oudijk*, Michael D. Zwank, Ed van de Graaf, J.J. Mager, Jan-Willem J. Lammers, Paul J. Coffer and Leo Koenderman

*These authors contributed equally to this study

Submitted

Abstract

Neutrophils are thought to play an important role in the inflammatory response in COPD by activation of their cytotoxic machinery in the lung and airway tissue. In addition, these cells are able to synthesize *de novo* several inflammatory mediators. Therefore, the study of gene expression profiles of peripheral blood neutrophils from COPD patients may provide unique insights into the mechanisms of activation of neutrophils *in vivo*.

Gene expression profiling of peripheral blood neutrophils of moderate to severe COPD patients was performed by gene array and real time reverse transcriptase-polymerase chain reaction analysis. Expression of many inflammatory genes was regulated indicating that these cells were in an activated state. These genes included cytokines (e.g. IL-1β), chemokines (e.g. IL-8) and adhesion molecules (e.g. ICAM-1). These genes were also regulated in neutrophils *in vitro* by pro-inflammatory mediators such as TNFα and GM-CSF. Disease severity as measured by the forced expiratory volume in one second (FEV_1) in COPD patients correlated with expression of several of these genes, including IL-1β (r = -0.540; p = 0.008), MIP1β (r = -0.583; p = 0.003), CD83 (r = -0.514; p =0.012), IL-1R2 (r = -0.546; p = 0.007) and IL-1RA (r = -0.612; p = 0.002). These data are consistent with a hypothesis that progression of COPD is associated with the activation of neutrophils in the systemic compartment. *De novo* expression of inflammatory mediators by peripheral blood neutrophils implicate a pro-inflammatory role for these cells in the pathogenesis of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the airways, which is characterized by the presence of chronic airflow obstruction and an abnormal inflammatory response (1). It is thought that this abnormal inflammatory response is mediated by increased levels of cytokines and/or chemokines, such as tumor necrosis factor (TNF)-α and Interleukin (IL)-8, which are found in elevated levels in bronchoalveolar lavage (BAL) fluid and sputum of COPD patients (2-4). Furthermore, increased levels of neutrophils, monocytes and CD8+ cytotoxic lymphocytes are found in the pulmonary compartment (5-9). This recruitment of inflammatory cells is likely to be mediated by the increased levels of cytokines and/or chemokines in the tissue. Several studies have focused on this in COPD by measuring granulocyte activation products at the site of inflammation (4, 6, 10, 11).

In contrast to this local inflammation, little is known concerning the systemic inflammatory response in COPD. The identification of soluble TNF receptors, which are thought to counteract the function of TNFα, in the plasma of COPD patients, suggests that the local inflammation communicates with the systemic compartment (3). Unfortunately, direct measurement of many inflammatory mediators such as TNFα is difficult in the peripheral blood because of their short half-life, binding to soluble receptors, and renal clearance (12-14). A few studies have reported that neutrophils in the peripheral blood of COPD patients exhibit characteristics of a primed phenotype (15, 16). Further evidence of activation of inflammatory cells in the systemic compartment is suggested by a study showing an increased expression of Mac-1 in circulating neutrophils (17). These findings suggest that the activation of neutrophils already starts in the circulation. The underlying pathogenetic mechanisms remain, however, to be determined.

Novel methods that allow monitoring of neutrophil activation and analysis of inflammatory mediators, are important for the characterization of the systemic inflammatory response in COPD. Ourselves an others have shown that *in vitro* priming of neutrophils with for example GM-CSF is associated with the expression of specific gene profiles (18, 19). Thus investigation of gene expression profiles of peripheral blood neutrophils of COPD patients in comparison with *in vitro* cytokine-induced gene profiles of neutrophils could be a powerful tool for characterization of chronic systemic inflammation in COPD.

In this study the activation status of peripheral blood neutrophils was examined through analysis of cytokine-regulated gene profiles *in vitro* using gene arrays. Gene profiles of neutrophils from severe stable COPD patients exhibit characteristics of cells, which are activated by pro-inflammatory mediators. Further investigation of differentially expressed genes, such as several IL-1 family members, MIP1β and CD83 in mild and severe COPD patients demonstrated that expression of these genes in peripheral blood neutrophils correlate with the severity of COPD.

Materials and Methods

Reagents

Formyl-methionyl-leucy-lphenylalanine (fMLP) and LPS were purchased from Sigma (St. Louis, MO, USA). Recombinant human GM-CSF was from Genzyme (Boston, MA, USA). Recombinant human TNFα was purchased from Boehringer Mannheim (Germany). IL-8 was purchased from Peprotech (Rocky Hill, NJ, USA). Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). RPMI 1640 medium with Glutamax was purchased from Life Technologies (Breda, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden).

Isolation of human neutrophils

Blood was obtained from healthy volunteers and COPD patients. Mixed granulocytes were isolated from 50-100 ml (normal volunteers) or 20 ml blood (COPD patients), which was anti-coagulated with 0.32% sodium citrate as described before (20). In short, blood was diluted 2.5:1 with PBS containing 0.32% sodium citrate and human pasteurized plasma-protein solution (4 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Pague. The erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 minutes at 37°C in HEPES buffered RPMI 1640 medium, supplemented with L-glutamine and 0.5% HSA. All preparations contained >97% neutrophils.

Subjects

Twenty three patients with clinically stable moderate to severe COPD were included in the study. COPD was defined according to the criteria of the GOLD guidelines (1). Inclusion criteria were: FEV_1 < 80% and a reversibility of less than 10% or less than 200 ml after inhalation of a β2-agonist and a FEV_1/FVC ratio of less than 70% of the predicted value. A history of other concomitant confounding diseases such as diabetes mellitus, lung carcinoma, thyroid, cardiovascular disease and bronchiectasis were exclusion criteria for the study. Furthermore, patients with an exacerbation due to a respiratory tract infection or other respiratory complaints during the 4 weeks prior to the study or patients with a history of asthma or atopy were excluded from the study. The mean age of the patient group was 64.0 ±2.3 (range 40-79) and their mean FEV_1 was 51.7 ± 3.8% of predicted (range 19.1%-79.3%). Fourteen of the 23 patients met the criteria for moderate COPD (class IIA according to the GOLD guidelines), whereas the other 9 patients suffered from moderate to severe COPD (class IIb and III). All patients had a smoking history of more than 10 years (pack-years: mean 40 ±4.1, range 14-76). All patients were treated with inhaled long-acting β2-agonists (b.i.d) and additional bronchodilating agents if needed,

none of them used oral glucocorticosteroids. The study was approved by the Medical Ethics Committee of the University Medical Center, Utrecht, and informed consent was obtained from all patients.

RNA isolation and cDNA synthesis.

RNA isolation was performed as previously described (18). Briefly, 2×10^7 cells were washed with ice-cold PBS and lyzed in 0.4 mL of GIT solution (6 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, 0.5% N´-lauroyl sarcosine, 100 mmol/L β-mercaptoethanol). After storage at –20°C, phenol extraction was performed followed by ethanol precipitation. Total RNA was resuspended in a small volume of H_20 and treated with DNAse for half an hour. After DNAse treatment RNA was again extracted with phenol, precipitated with ethanol and RNA was then eluted in 10 µl of RNAse-free H_2O. cDNA was synthesized using MMLV reverse transcriptase and oligo(dT) primers. Samples containing 1 µg of total RNA were heated for 3 minutes at 65°C and quickly chilled on ice. A mixture of 25 µl containing 20 µg/ml oligo(dT) primers, 5 µl of 5x RT buffer, 20 mM DTT, 2 mM each deoxynucleoside triphosphate, 0.8 U/µl of RNAse inhibitor and 400 units of MMLV reverse transcriptase was added and incubated at 37 °C for 90 minutes, followed by RT inactivation for 10 min at 65°C. cDNA was stored at -20°C before further use. All reagents used for cDNA synthesis were obtained from Life Technologies (Breda, The Netherlands)

Gene array analysis

Isolated human neutrophils were incubated in RPMI 1640 supplemented with 0.5% HSA for 15 minutes and stimulated for 3 hours at 37°C, followed by RNA isolation as described above. RNA was isolated from neutrophils, which were isolated from 10 ml of blood of COPD patients. RNA was treated with DNAse to avoid contamination with genomic DNA and subsequently analyzed by agarose gel electrophoresis to verify the integrity of the RNA preparations. PolyA+ RNA was extracted with magnetic beads coated with poly dT using the Atlas Pure Total RNA Labeling System (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's protocol. Forty microgram of total RNA from neutrophils stimulated with or without cytokines and 20 µg of neutrophils from COPD patients or healthy volunteers were used to perform a gene array with. The gene array screen was performed with the Atlas Human Hematology/Immunology Array #7737-1 (Clontech, Palo Alto, CA, U.S.A.) using reagents supplied and manufactures protocol. In short, cDNA probes were generated using specific primers for the genes on the array. Reverse transcription of the isolated polyA+ RNA was performed with radioactive γ^{32}P-dATP. The two arrays were incubated overnight with the probe at 68 °C. The following day, arrays were washed and membranes analyzed by autoradiography. The spots on the arrays were quantitated using a PhosphorImager and ImageQuant software

(Amersham Biosciences, Uppsala, Sweden). Values were corrected for background and housekeeping genes.

Real time PCR

Primers and probes for CD83 were selected using Primer Express version 1.0 (Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands). For CD83, a 72 bp (nt 67-139) fragment was amplified using the forward primer 5'-TCCTGAGCTGCGCCTACAG-3' and the reverse primer 5'-AAGTCCACATCTTCGGAGCAA-3' (Amersham Biosciences, Uppsala, Sweden). The TaqMan probe for CD83, 5'-TCACCTCCGGCGTCGCGG-3' was labeled with the fluorescent reporter dye 6-carboxy fluorescein (FAM) at the 5' end and the quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at the 3' end (Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands). A 25-µl PCR was performed using 0.1 µl of cDNA, 12.5 µl of TaqMan universal PCR master mix containing 6-carboxyrhodamine (ROX) as a passive reference (PE Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands), 800 nM of each CD83 primer and 150 nM of CD83 probe.

All the other genes were analyzed by real time pcr using SYBR green I. For β-actin, a 174 bp fragment was amplified using the forward primer 5'-AGCCTCGCCTTTGCCGA-3' and the reverse primer 5'-CTGGTGCCTGGGGCG-3' as described by Kreuzer et al. (21). All other primers were designed using Primer 3 software of the whitehead institute/MIT center for genome Research (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and are listed in table 1. Real time PCR with SYBR green I was performed in a 25-µl mixture containing 0.1 µl of cDNA, 12.5 µl of SYBR Green PCR master mix containing ROX as a passive reference (PE Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands) and 400 nM of each primer. Amplification and detection of both Taqman and SYBR green I pcr were performed with an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands) under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C to activate AmpliTaq Gold DNA polymerase, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The signal of the dye was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. Results were normalized for the housekeeping gene β-actin and GAPDH. A reference sample of cDNA on every 96 wells plate allowed correction of differences between plates. Results were expressed as fold regulation.

Table 1. Sequences of primers used for real time PCR (SYBR green)

Target sequence	Forward primer	Reverse primer
GAPDH	agaaggctggggctcattt	gaggcattgctgatgatcttg
MIP-1β	ccatgaagctctgcgtgact	agcccattggtgctgagag
IL-1β	gcttattacagtggcaatgaggat	ggtggtcggagattcgtagc
IL-1RA	gaagatgtgcctgtcctgtgtc	cgcttgtcctgctttctgttc
IL-1R2	caggaggactctggcacctac	tgagatgaacggcaggaaag
IL-8	agctctgtgtgaaggtgcagtt	gggtggaaaggtttggagtatg
IL-8R1	tcaagtgccctctagctgttaagt	tcttcagtttcagcaatggtttg

Statistics

Unless otherwise noted, all data are expressed as the mean ± SE, and differences between values were compared by the paired t-test. Differences between study groups were compared using the Mann-Whitney U test. Statistical significance was defined as $p<0.05$

Results

Analysis of gene expression profiles in peripheral blood neutrophils from patients with stable COPD

We investigated peripheral blood neutrophil activation in moderate to severe COPD patients utilizing a gene array approach. Atlas gene arrays (Atlas Human Hematology/Immunology filters) were hybridized with radioactive labeled cDNA synthesized from peripheral blood neutrophils isolated from COPD patients. This type of analysis requires many neutrophils, which have typically a low yield of mRNA, complicating the use of gene arrays for cohorts of COPD patients. Therefore, we decided to identify genes differently regulated in two moderate to severe COPD patients, and subsequently validate these findings by real time reverse transcriptase-polymerase chain reaction (RT-PCR) in a larger cohort. Gene expression analysis of these two COPD patients compared to healthy volunteers indicated that 16 genes were regulated in both patients, while 21 genes were regulated in at least one of the two (Table 2). Approximately half of the regulated genes encoded for chemokines/cytokines and cell surface receptors. Increased expression of IL-8, IL-1β, IL-1RA, and ICAM-1, which are known to be upregulated in neutrophils after stimulation with pro-inflammatory mediators, such as TNFα, GM-CSF or LPS (22), suggest that these peripheral blood neutrophils exhibited an activated phenotype.

Table 2. Genes differently expressed in neutrophils of patients with COPD

Description	GENBANK number	COPD1 fold-change	COPD2 fold-change
Chemokine/cytokine receptors			
IL-8R1	M68932	-5,4	-16,2
IL-1R2	X59770	-5,1	-24,9
Chemokines/cytokines			
IL1-β	K02770	20,5	29,5
IL-1RA	M63099	12,8	5,1
IL-8	Y00787	12,1	3,8
MIP-1β	J04130	11,2	20,9
lymphotoxin-β	L11015	-17,6	-9,7
Surface antigens			
ICAM1	J03132	3,2	3,5
CD58 antigen (LFA3);	Y00636	-9,0	-5,0
T-lymphocyte maturation-associated protein MAL	M15800	13,1	
CDW50 antigen (ICAM3)	X69711	-6,1	-3,4
CD83 antigen	Z11697	34,8	
Transcription factors			
STAT5A/B	U47686	2,8	
interferon-stimulated gene factor 3 gamma subunit	M87503	-2,7	-5,2
interferon regulatory factor 7 (IRF7)	U73036	-7,2	-4,0
Protein kinases/ oncogenes			
c-Raf1	X03484	-7,3	-4,0
EVI2B protein	M60830	-2,5	-2,9
neutrophil S100 protein	X97859	-2,5	-8,0
SLP-76; 76-kDa tyrosine phosphoprotein	U20158	4,1	
Others			
heat shock 60-kDa protein (HSP60)	M34664	-6,1	-3,4
lymphocyte cytosolic protein 1 (LCP1)	M22300	3,6	

TNFα and/or GM-CSF induce differential expression of inflammatory genes in human neutrophils

To gain further insight into the mechanisms of neutrophil activation we evaluated differential gene expression in neutrophils activated with GM-CSF and TNFα *in vitro*. Both cytokines have been implicated in the pathogenesis of chronic inflammatory disease including COPD (2, 23, 24). The receptors of these two cytokines are structurally different and activate the transcriptional machinery by distinct means.

Isolated peripheral blood neutrophils were stimulated in the absence or presence of these cytokines for three hours. Total RNA was isolated and PolyA+ RNA was prepared and used for generating radioactively labeled cDNA. This was hybridized with Atlas gene array filters as described in Material and Methods (Atlas Human Hematology/Immunology filters). A representative gene array of each condition is shown in Figure 1. The lower row of each gene array represent housekeeping genes, which were used as an internal reference.

Unstimulated neutrophils of normal donors expressed 17 % (68 of 402 genes) of the spotted genes. Exposure of neutrophils to the cytokines TNFα or GM-CSF and the combination of TNFα and GM-CSF resulted in changes in expression of 15, 18, and 19 genes respectively relative to control (Table 3). These genes belong to several different functional classes, but the majority encoded for cytokines/chemokines and cell surface receptors. Among the cytokines and chemokines, IL-8, IL-1β, and IL-1RA were clearly upregulated by both TNFα and GM-CSF. On the other hand MIP-1β was only induced by TNFα, and lymphotoxin-beta by GM-CSF. Moreover, this analysis also revealed that TNFα and GM-CSF both induced pro-inflammatory genes such as IL-1β and IL-8, and anti-inflammatory genes such as IL-1RA and IL1R2.

Of the regulated cell surface antigens, most were upregulated by both TNFα and GM-CSF except for CD83 and CD69, which were only upregulated by TNFα or GM-CSF, respectively. The anti-apoptotic Bcl-2 family member A1 is involved in regulating cell survival, and expression was already high directly after isolation, decreasing after 3 hours at 37°C in the absence of cytokines. Both TNFα and GM-CSF, maintained the high expression.

The combination of TNFα and GM-CSF resulted in regulation of almost all genes, which are regulated by TNFα and GM-CSF individually, however, some genes were regulated only by the individual cytokines. MIP-1β, CD83, IL-8R1, and ALP were only regulated by TNFα, while GM-CSF alone regulated RAR-alpha, BTG1, lymphotoxin-alpha, CD69, MNDA, STAT5A/B, and vav2 (Table 4). Comparison of these cytokine profiles with the regulated genes of the COPD patients shows that three out of four TNFα induced genes

are similarly regulated *in vivo* in COPD patients. Of the GM-CSF induced genes, none of these seven genes were regulated in these COPD patients.

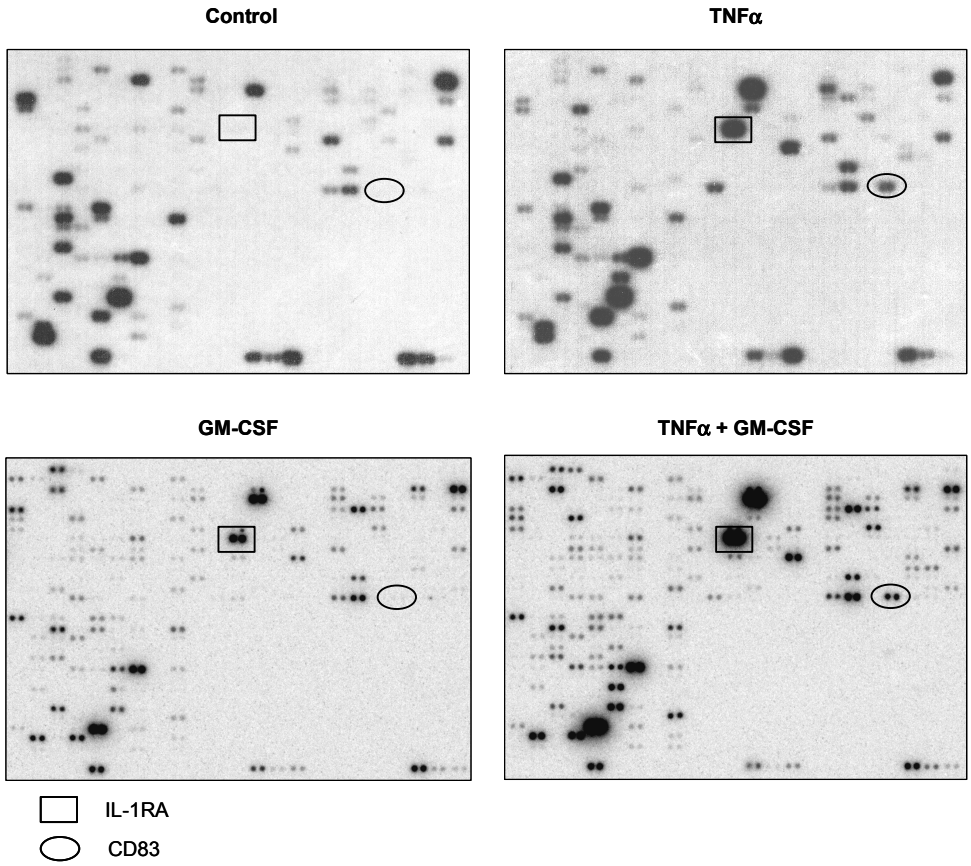

Figure 1: Gene arrays analysis of TNFα stimulated peripheral blood neutrophils. Neutrophils were incubated without or with TNFα (100 U/ml) and/or GM-CSF (0.1 nM) for 3 hours *in vitro*. RNA was isolated and used for generating radioactively labeled cDNA, which was hybridized with Atlas array filters (Atlas Human Hematology/Immunology filters). CD83 and ICAM-1 were boxed with a circle and square respectively. Membranes were analyzed using a PhosphorImager and Image Quant software. All genes of the array were spotted in duplo.

Table 3. Genes regulated by several stimuli in neutrophils

Description	GENBANK number	TNFα fold-change	GM-CSF fold-change	TNFα +GM-CSF fold-change
Chemokine/cytokine receptors				
IL8 receptor alpha	M68932	-8.6		-6.4
IL1R-beta	X59770	11.5*	13.6	4.4
Blood disorder proteins				
antileukoproteinase 1 (ALP)	X04470	5.1		
retinoic acid receptor alpha (RAR-alpha)	M73779		15.0	
B-cell translocation gene 1 (BTG1)	X61123		-10.3	-9.2*
NOTCH 1	M73980	-4.5	-6.4	-5.8
Chemokines/cytokines				
IL-1β	K02770	13.0*	27.3*	
IL-1RA	M63099	106.7	98.5	227.6
IL-8	Y00787	7.1	9.3	22.2
MIP-1β	J04130	6.0		4.1*
lymphotoxin-β	L11015		3.2	
Surface antigens				
ICAM1	J03132	15.4	5.0	18.1
CD14 antigen	M86511	2.8	9.5*	4.5*
CD44 antigen	M59040	8.7	21.6	16.6
CD58 antigen	Y00636	3.4	6.7*	3.5*
CD83 antigen	Z11697	12.5		10.3*
CD69 antigen	L07555		17.4	8.2
Transcription factors				
MNDA	M81750		-4.4	-11.8*
STAT5A/B	U47686		-5.7	
fli1 proto-oncogene	M93255	-3.1	-6.9	-16.3*
Protein kinases/oncogenes				
vav2	S76992		12.0	10.7
SLP-76	U20158	3.3	15.2	15.1
ras-like small GTPase TTF	Z35227			8.2
Apoptosis				
BCL2-related protein-A1 (BCL2-A1)	U29680	13.9	4.7	19.2*

* regulated in one of the two performed gene arrays

Table 4. Specific cytokine regulated genes of neutrophils

TNFα (fold-change)		GM-CSF (fold-change)		TNFα +GM-CSF (fold-change)		TNFα and/or GM-CSF	
MIP-1β	(6.0)	RAR-alpha	(15)	TTF	(8.2)	IL-1R2	(+)
CD83 antigen	(12.5)	BTG1	(-10.3)			NOTCH 1	(-)
IL-8R1	(-8.6)	lymphotoxin-β	(3.2)			Fli1-proto-ocogene	(-)
ALP	(5.1)	CD69 antigen	(17.4)			IL-1β	(+)
		MNDA	(-4.4)			IL-1RA	(+)
		STAT5A/B	(-5.7)			IL-8	(+)
		vav2	(12.0)			ICAM1	(+)
						CD14 antigen	(+)
						CD44 antigen	(+)
						CD58 antigen	(+)
						SLP-76	(+)
						Bcl2-A1	(+)

Modulation of inflammatory genes in neutrophils by different inflammatory mediators

We utilized several physiological stimuli to analyze the expression of inflammatory genes, such as CD83, IL-8R1 and MIP-1β in peripheral blood neutrophils *in vitro*. Expression of these genes was measured by a real time RT-PCR as described in Materials and Methods. Specific primers were used to amplify only cDNA generated from mRNA and not genomic DNA. Gene expression was normalized for β-actin and GAPDH expression. Analysis revealed that IL-8 and fMLP did not induce any of the genes investigated, whereas TNFα, GM-CSF, LPS and the combination TNFα/GM-CSF modified upregulated of IL-1RA and IL-1β, (Fig 2 and Table 5). IL-1R2 was down-regulated by all stimuli. Similar to the data obtained with the gene arrays CD83 and MIP-1β were upregulated by TNFα and LPS and not by GM-CSF.

Table 5. Genes regulated (fold-change (log2)) by several stimuli in neutrophils

	TNFα	GM-CSF	IL-8	fMLP	TNFα + GM-CSF	LPS
CD83	4.0	-3.4	-2.7	-2.2	1.9	4.8
MIP-1β	4.4	-0.6	-0.8	0.8	5.0	6.7
IL-1RA	4.9	4.7	-0.7	-0.2	7.7	5.6
IL-1β	2.1	0.8	-1.0	-1.4	4.3	3.8
IL-1R2	0.0	1.3	-0.7	-1.5	2.8	1.3
IL-8	0.4	0.2	-0.6	-0.3	2.4	4.1
IL-8R1	-3.2	-3.7	-1.0	-2.2	-3.8	-2.7

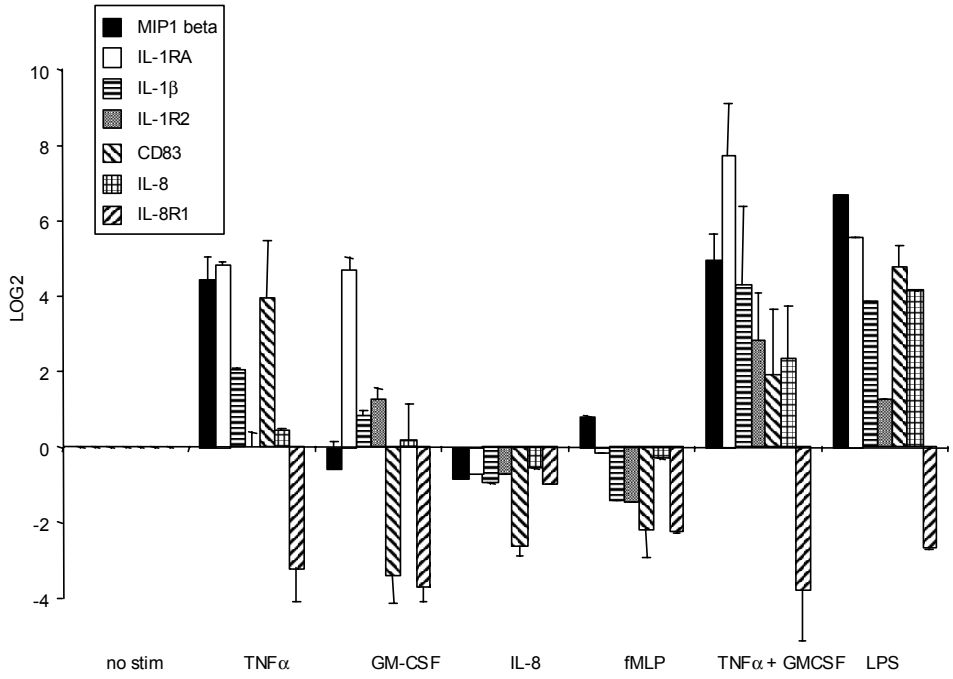

Figure 2: Gene expression in peripheral blood neutrophils, stimulated by several mediators *in vitro*. Peripheral blood neutrophils are incubated with several physiological stimuli for 3 hours *in vitro* at 37°C (TNFα, 100U/ml; GM-CSF, 0.1 nM; IL-8, 10 nM; fMLP, 1 μM; LPS, 10 ng/ml). Expression of indicated genes were measured by real time RT-PCR. Results are expressed as LOG2 fold change ±S.E.M. (n=3).

Expression of TNFα and GM-CSF regulated genes in patients with stable COPD

Investigation of the expression of genes preferentially induced by specific cytokines will help to determine which mediators play a role in the activation of neutrophils in patients with COPD. cDNA was prepared from total RNA from healthy volunteers and from the aforementioned COPD patients and used for real time RT-PCR analysis of indicated genes (Fig. 3). Data of the moderate COPD patients (GOLD class IIa) indicated that only the IL-8R1 gene was significantly decreased compared to healthy volunteers. Neutrophils of the more severe COPD patients showed a significant increase of CD83, MIP-1β, and IL-1β compared to healthy volunteers. Furthermore, CD83, IL-1β, MIP-1β, but also IL-1RA were significantly increased in severe COPD patients compared to mild COPD patients. This suggests that a pro-inflammatory mediator such as TNFα is involved in systemic activation of neutrophils from patients with severe COPD.

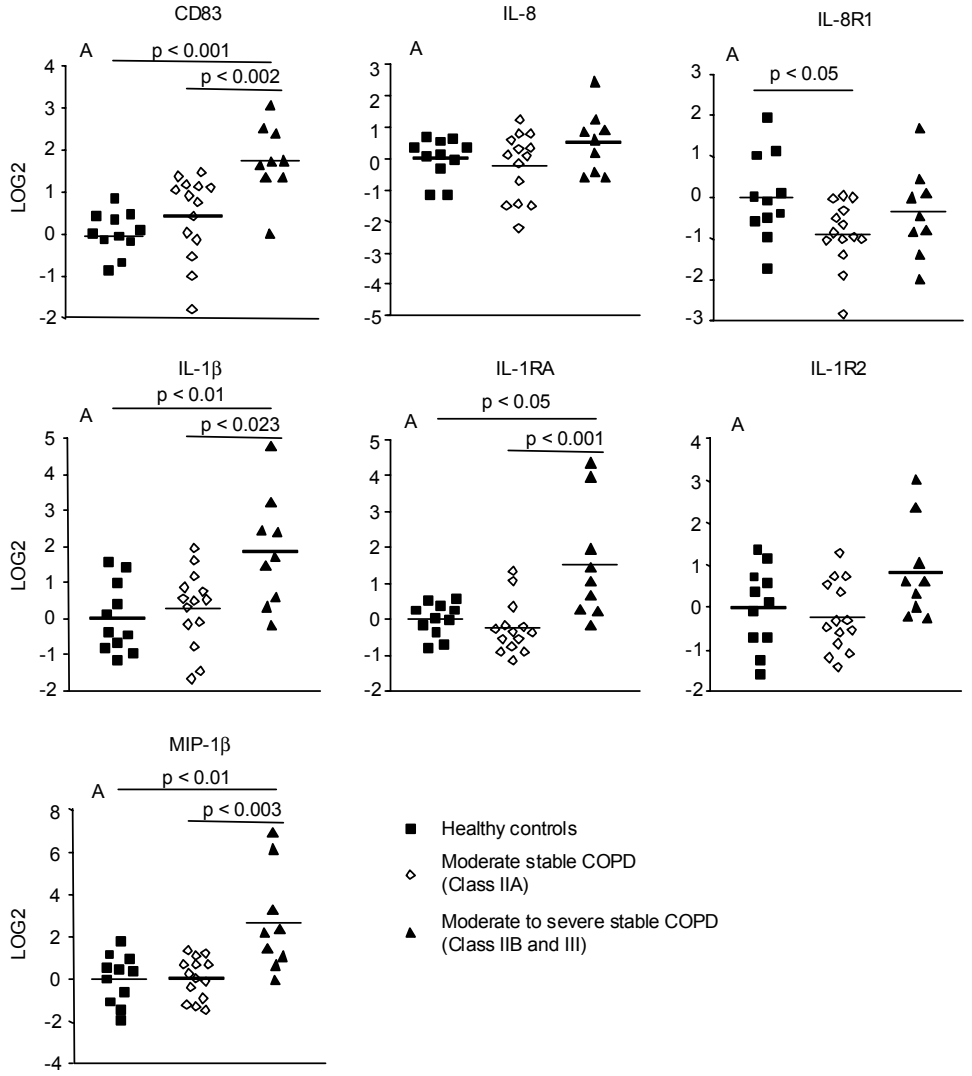

Figure 3: Gene expression analysis in peripheral blood neutrophils of COPD patients. Peripheral blood neutrophils were isolated from healthy volunteers (Filled squares), moderate (class IIA) COPD patients (open diamonds), and moderate to severe (class IIB and III) stable COPD patients (filled triangles). Expression of indicated genes were measured using real time RT-PCR and expressed as LOG2 fold-change compared to the group of healthy controls. Differences between the study groups were compared using the Mann-Whitney U test.

TNFα regulated genes correlate with severity of disease in patients with stable COPD

Our findings that patients with severe stable COPD had an increased expression of CD83, MIP-1β and IL-1RA compared to mild stable COPD patients suggest that these genes might correlate with the severity of COPD. Indeed, these genes, CD83 (r = -0.51), MIP-1β (r = -0.58) and IL-1RA (r = -0.61), but also IL-1β (r = -0.54) and IL-R2 (r = -0.546) correlated with the severity of COPD as measured by FEV_1 (Fig 4). In contrast, no correlation between FEV_1 and the expression of IL-1β, IL-8 and IL-8R1 was found (results not shown).

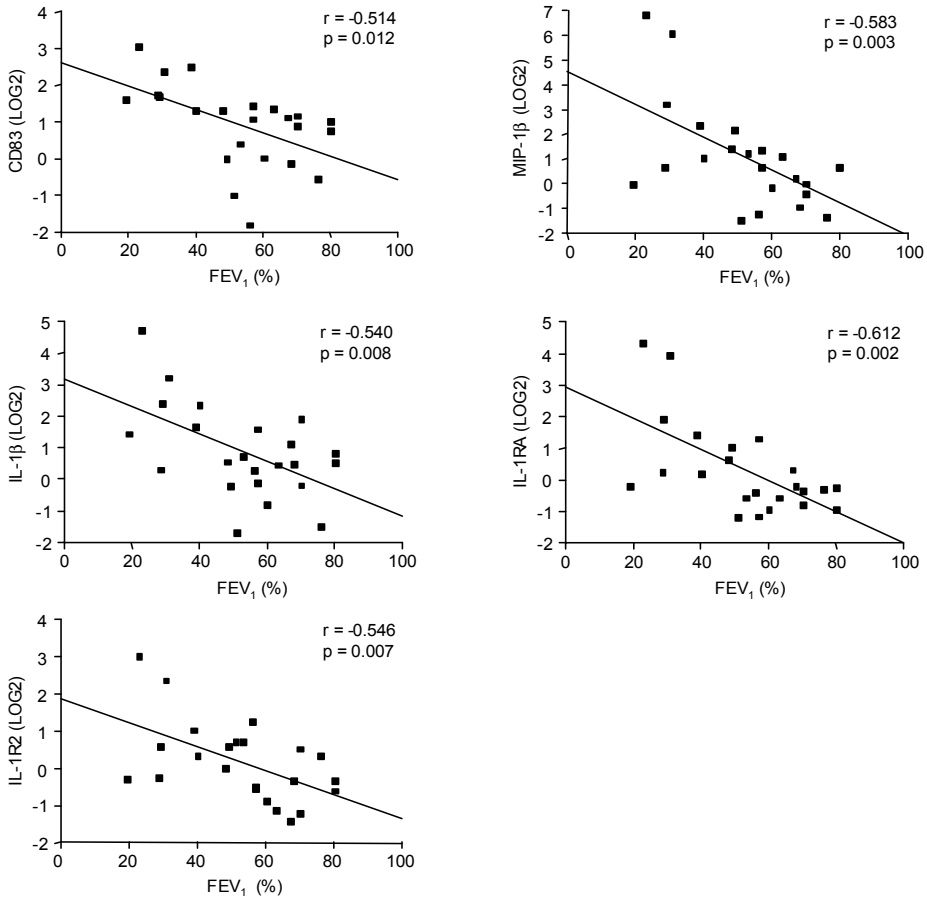

Figure 4: Correlation of gene expression in peripheral blood neutrophils of COPD patients with FEV_1 (% predicted). Expression of indicated genes were measured using real time RT-PCR and expressed as LOG2 fold-change compared to the group of healthy controls. Correlations between the FEV_1 and expression of indicated genes in neutrophils from stable COPD patients were evaluated using Pearson's rank correlation analysis.

Discussion

Peripheral blood neutrophils of COPD patients exhibit an activated phenotype.

Activation of neutrophils plays an important role in the pathogenesis of several chronic inflammatory diseases such as COPD and rheumatoid arthritis (25, 26). Therefore, investigation of gene expression profiles of peripheral blood neutrophils of COPD patients could provide a unique insight into the molecular mechanisms modulating chronic systemic inflammation. Furthermore, these gene profiles compared with *in vitro* cytokine-induced gene profiles of neutrophils from normal donors can be used as fingerprints for mediators that are critical in disease pathogenesis. In this study the activation status of peripheral blood neutrophils was examined through gene array analysis and real time RT-PCR.

Gene expression analysis of peripheral blood neutrophils from patients with severe stable COPD indicated that peripheral blood neutrophils exhibited an activated phenotype. Several genes, including IL-1β, IL-1RA, IL-8, ICAM1, CD44, CD69, and MIP1β which have been previously reported to be regulated by pro-inflammatory mediators, such as TNFα, GM-CSF or LPS (Fig. 2)(22, 27-31), were clearly regulated in neutrophils from moderate to severe COPD patients (Class IIB and III). Upregulation of CD83, which is described as a specific marker for dendritic cells, has recently been described to also be expressed on neutrophils *in vitro* by TNFα and *in vivo* by acute infection (32, 33). These data are consistent with hypothesis that circulating neutrophils in severe COPD patients have encountered pro-inflammatory mediators.

Comparison of cytokine induced gene expression profiles of neutrophils with the gene expression profiles of neutrophils isolated from patients with stable severe COPD revealed that gene expression profiles of circulating neutrophils from COPD patients were very similar to that of *in vitro* TNFα or LPS, but not that of GM-CSF stimulated neutrophils of normal donors. The gene profile of neutrophils from COPD patients is not identical to that of individual mediators such as TNFα or LPS. This demonstrates that it is unlikely that dominant single mediators drive systemic inflammation in COPD but rather a combination of inflammatory mediators. Indeed, a combination of inflammatory mediators can lead to enhancement (Fig. 2) or inhibition of transcription of genes (see Chapter 3). For instance, IFNγ enhances the production of cytokines/chemokines of neutrophils stimulated by LPS, whereas IL-10 inhibits the production of inflammatory mediators of (27). In addition, the kinetics of gene expression is important. Genes that are expressed late upon activation might not be found in peripheral blood, but only in the tissues.

Investigation of expression of several of these genes, including IL-1β, IL-1RA, IL-8, CD83 and MIP-1β, in patients with different stages of COPD, revealed that these genes were significantly increased in the more severe COPD patients (Class IIb and III) and a

significant negative correlation was present between the extent of the gene expression and the lung function. This was not a generalized finding, since the expression of several genes did not demonstrate this correlation. These data show a close correlation between disease status in the pulmonary tissue and the systemic inflammatory reaction as measured in the peripheral blood. The status of systemic inflammation can thus be measured as an additional read-out for the health status and may also helpful for the prognosis of a COPD patient.

Gene expression of peripheral blood neutrophils of COPD patients show upregulation of functional groups of genes

Among the regulated genes of severe COPD patients, several cytokines and chemokines were upregulated. It has been described that besides the traditional functions, such as phagocytosis, degranulation and production of superoxide, neutrophils can express a variety of inflammatory mediators (reviewed by (27)). These findings suggest that circulating neutrophils not only participate in airway damage in COPD patients, but also participate in the regulation of systemic inflammation. In addition, it is likely that these cells can also regulate local inflammation after migration into the airways of COPD patients. Here they can act in a pro-and anti-inflammatory fashion depending on the mix of genes that is transcribed (see below).

Other genes that are regulated, such as CD58, CD44, ICAM3, ICAM1, and CD69 point at a putative change in neutrophil adhesion properties in these COPD patients. The change in adhesion properties is in agreement with data showing that peripheral blood neutrophils of COPD patients have increased levels of Mac1 (17). Thus a change in adhesion properties of neutrophils might facilitate the recruitment of cells into the lung tissue of COPD patients, and contributes to the sequestration of neutrophils in the airways of COPD patients.

Balance between anti- and pro-inflammatory mediators

TNFα, GM-CSF but also LPS can induce the expression of pro-inflammatory mediators in neutrophils and, thereby, enhance the inflammatory response. However, at the same time TNFα, GM-CSF and LPS downregulated the responsiveness of neutrophils and bystander cells towards other pro-inflammatory mediators. IL-1RA and IL-1R2 are natural inhibitory proteins for IL-1β. IL-1RA exerts its inhibitory action by binding to IL-1 receptors without triggering any intracellular signaling responses, whereas IL-1R2 binds IL-1β without giving a biological response (34, 35). The upregulation of IL-1RA and IL-1R2 in neutrophils *in vivo* is likely involved in dampening of the inflammatory response initiated by IL-1β. Similarly, TNFα or GM-CSF down-regulated the expression of IL-8R1 which is likely to result in reduced responsiveness to this pro-inflammatory chemokine. Other studies have shown that systemic inflammation can cause functional down-

regulation of serpentine receptors *in vitro* and *in vivo* (36-38). Thus activated neutrophils can not only induce an inflammatory response, but also an anti-inflammatory response, generating a negative feedback loop. The outcome of these two processes may be determined by differences in the levels and kinetics of the responses. Thus neutrophils are not only effector cells, but might also play a role in the regulation of the duration of an inflammatory reaction.

Interesting is that the balance between the pro-inflammatory mediator IL-1β and the anti-inflammatory mediators IL-1RA and IL-1R2 is disturbed in patients with severe stable COPD compared to *in vitro* stimulation of neutrophils by inflammatory mediators. Real time RT-PCR analysis showed that the expression of IL-1RA is much higher than that of IL-1β in neutrophils stimulated with TNFα (Fig. 2), compared to neutrophils from severe stable COPD patients (Fig. 3). Apparently, the regulation of natural inhibitory genes is disturbed in peripheral blood neutrophils from severe COPD patients. In addition, expression of IL-1R2 is not upregulated in these COPD patients, while *in vitro* GM-CSF or LPS induced the expression of IL-1R2. Thus the balance of expressed genes, has been shifted towards a pro-inflammatory state. In contrast to rheumatoid arthritis, in COPD little is known concerning the function of IL-1 family proteins. In the rheumatoid synovium, an imbalance exists in the pro- and anti-inflammatory mediators since the relative levels of production of IL-1RA are not adequate to effectively block the pro-inflammatory effects of IL-1 (39). Injection of IL-1RA in the synovium, significantly reduced the signs and symptoms of rheumatoid arthritis at 24 weeks (40). So the imbalance between anti- and pro-inflammatory genes, including the IL-1 family, of circulating neutrophils of COPD patients, might therefore also contribute to the development of a chronic inflammatory response in COPD.

In conclusion, this study indicates that activated neutrophils can be found in the peripheral blood of stable COPD patients and this activation correlates with the severity of the disease. Inflammatory mediators such as TNFα, are likely involved in the activation of neutrophils in these stable COPD patients. Interestingly, there is an imbalance in expression of pro- and anti-inflammatory mediators, including the IL-1 family, in neutrophils of the more severe COPD patients. This imbalance towards a pro-inflammatory response may contribute to the systemic inflammation observed in these COPD patients. Greater understanding of these mechanisms by fine-tuning analysis of the gene profiles may increase our understanding of the systemic inflammation in COPD and may result in better markers for disease staging.

References

1. Pauwels, R. A., A. S. Buist, P. M. Calverley, C. R. Jenkins, S. S. Hurd. 2001. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am. J. Respir. Crit. Care Med.* 163:1256-1276.
2. Keatings, V. M., P. D. Collins, D. M. Scott, P. J. Barnes. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am. J. Respir. Crit. Care Med.* 153:530-534.
3. Vernooy, J. H., M. Kucukaycan, J. A. Jacobs, N. H. Chavannes, W. A. Buurman, M. A. Dentener, E. F. Wouters. 2002. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am. J. Respir. Crit. Care Med.* 166:1218-1224.
4. Yamamoto, C., T. Yoneda, M. Yoshikawa, A. Fu, T. Tokuyama, K. Tsukaguchi, N. Narita. 1997. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 112:505-510.
5. Pesci, A., M. Majori, A. Cuomo, N. Borciani, S. Bertacco, G. Cacciani, M. Gabrielli. 1998. Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease. *Respir. Med.* 92:863-870.
6. Keatings, V. M., and P. J. Barnes. 1997. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am. J. Respir. Crit. Care Med.* 155:449-453.
7. Saetta, M., S. Baraldo, L. Corbino, G. Turato, F. Braccioni, F. Rea, G. Cavallesco, G. Tropeano, C. E. Mapp, P. Maestrelli, A. Ciaccia, L. M. Fabbri. 1999. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 160:711-717.
8. O'Shaughnessy, T. C., T. W. Ansari, N. C. Barnes, P. K. Jeffery. 1997. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am. J. Respir. Crit. Care Med.* 155:852-857.
9. Saetta, M., G. Turato, F. M. Facchini, L. Corbino, R. E. Lucchini, G. Casoni, P. Maestrelli, C. E. Mapp, A. Ciaccia, L. M. Fabbri. 1997. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. *Am. J. Respir. Crit. Care Med.* 156:1633-1639.
10. Pesci, A., B. Balbi, M. Majori, G. Cacciani, S. Bertacco, P. Alciato, C. F. Donner. 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur. Respir. J.* 12:380-386.
11. Aaron, S. D., J. B. Angel, M. Lunau, K. Wright, C. Fex, N. Le Saux, R. E. Dales. 2001. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 163:349-355.
12. Bemelmans, M. H., L. J. van Tits, W. A. Buurman. 1996. Tumor necrosis factor: function, release and clearance. *Crit. Rev. Immunol.* 16:1-11.
13. Diez-Ruiz, A., G. P. Tilz, R. Zangerle, G. Baier-Bitterlich, H. Wachter, D. Fuchs. 1995. Soluble receptors for tumour necrosis factor in clinical laboratory diagnosis. *Eur. J. Haematol.* 54:1-8.
14. Takabatake, N., H. Nakamura, S. Abe, S. Inoue, T. Hino, H. Saito, H. Yuki, S. Kato, H. Tomoike. 2000. The relationship between chronic hypoxemia and activation of the tumor necrosis factor-alpha system in patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 161:1179-1184.
15. Noguera, A., S. Batle, C. Miralles, J. Iglesias, X. Busquets, W. MacNee, A. G. Agusti. 2001. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax* 56:432-437.
16. Koenderman, L., D. Kanters, B. Maesen, J. Raaijmakers, J. W. Lammers, J. de Kruif, T. Logtenberg. 2000. Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. *J. Leukoc. Biol.* 68:58-64.

17. Noguera, A., X. Busquets, J. Sauleda, J. M. Villaverde, W. MacNee, A. G. Agusti. 1998. Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 158:1664-1668.
18. Pals, C. M., S. A. Verploegen, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, P. J. Coffer. 2000. Identification of cytokine-regulated genes in human leukocytes in vivo. *J. Allergy Clin. Immunol.* 105:760-768.
19. Yousefi, S., P. R. Cooper, B. Mueck, S. L. Potter, G. Jarai. 2000. cDNA representational difference analysis of human neutrophils stimulated by GM-CSF. *Biochem. Biophys. Res. Commun.* 277:401-409.
20. Coffer, P. J., N. Geijsen, L. M'Rabet, R. C. Schweizer, T. Maikoe, J. A. Raaijmakers, J. W. Lammers, L. Koenderman. 1998. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem. J.* 329:121-130.
21. Kreuzer, K. A., U. Lass, O. Landt, A. Nitsche, J. Laser, H. Ellerbrok, G. Pauli, D. Huhn, C. A. Schmidt. 1999. Highly sensitive and specific fluorescence reverse transcription-PCR assay for the pseudogene-free detection of beta-actin transcripts as quantitative reference. *Clin. Chem.* 45:297-300.
22. Fujishima, S., A. R. Hoffman, T. Vu, K. J. Kim, H. Zheng, D. Daniel, Y. Kim, E. F. Wallace, J. W. Larrick, T. A. Raffin. 1993. Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-alpha, and IL-1 beta. *J. Cell Physiol.* 154:478-485.
23. Balbi, B., C. Bason, E. Balleari, F. Fiasella, A. Pesci, R. Ghio, F. Fabiano. 1997. Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *Eur. Respir. J.* 10:846-850.
24. Culpitt, S. V., D. F. Rogers, P. Shah, C. De Matos, R. E. Russell, L. E. Donnelly, P. J. Barnes. 2002. Impaired Inhibition by Dexamethasone of Cytokine Release by Alveolar Macrophages from COPD Patients. *Am. J. Respir. Crit. Care Med.*
25. Stockley, R. A. 2002. Neutrophils and the pathogenesis of COPD. *Chest* 121:151S-155S.
26. Edwards, S. W., and M. B. Hallett. 1997. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol. Today* 18:320-324.
27. Cassatella, M. A. 1999. Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* 73:369-509.
28. Malyak, M., M. F. J. Smith, A. A. Abel, W. P. Arend. 1994. Peripheral blood neutrophil production of interleukin-1 receptor antagonist and interleukin-1 beta. *J. Clin. Immunol.* 14:20-30.
29. McColl, S. R., R. Paquin, C. Menard, A. D. Beaulieu. 1992. Human neutrophils produce high levels of the interleukin 1 receptor antagonist in response to granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. *J. Exp. Med.* 176:593-598.
30. Mandi, Y., Z. Nagy, I. Ocsovski, G. Farkas. 1997. Effects of tumor necrosis factor and pentoxifylline on ICAM-1 expression on human polymorphonuclear granulocytes. *Int. Arch. Allergy Immunol.* 114:329-335.
31. Wang, J. H., D. M. Sexton, H. P. Redmond, R. W. Watson, D. T. Croke, D. Bouchier-Hayes. 1997. Intercellular adhesion molecule-1 (ICAM-1) is expressed on human neutrophils and is essential for neutrophil adherence and aggregation. *Shock* 8:357-361.
32. Yamashiro, S., J. M. Wang, D. Yang, W. H. Gong, H. Kamohara, T. Yoshimura. 2000. Expression of CCR6 and CD83 by cytokine-activated human neutrophils. *Blood* 96:3958-3963.
33. Iking-Konert, C., C. Wagner, B. Denefleh, F. Hug, M. Schneider, K. Andrassy, G. M. Hansch. 2002. Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent expression in acute bacterial infections and chronic inflammatory disease. *Clin. Exp. Immunol.* 130:501-508.
34. Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J. G. Giri, S. K. Dower, J. E. Sims, A. Mantovani. 1993. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 261:472-475.

35. Carter, D. B., M. R. J. Deibel, C. J. Dunn, C. S. Tomich, A. L. Laborde, J. L. Slightom, A. E. Berger, M. J. Bienkowski, F. F. Sun, R. N. McEwan. 1990. Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. *Nature* 344:633-638.
36. Jawa, R. S., G. A. Quaid, M. A. Williams, C. M. Cave, C. T. Robinson, G. F. Babcock, M. A. Lieberman, D. Witt, J. S. Solomkin. 1999. Tumor necrosis factor alpha regulates CXC chemokine receptor expression and function. *Shock* 11:385-390.
37. Khandaker, M. H., L. Xu, R. Rahimpour, G. Mitchell, M. E. DeVries, J. G. Pickering, S. K. Singhal, R. D. Feldman, D. J. Kelvin. 1998. CXCR1 and CXCR2 are rapidly down-modulated by bacterial endotoxin through a unique agonist-independent, tyrosine kinase-dependent mechanism. *J. Immunol.* 161:1930-1938.
38. Soejima, K., S. Fujishima, H. Nakamura, Y. Waki, M. Nakamura, H. Matsubara, S. Tasaka, K. Sayama, A. Ishizaka, M. Kanazawa. 1997. Downmodulation of IL-8 receptors, type A and type B, on human lung neutrophils in vivo. *Am. J. Physiol.* 273:L618-L625.
39. Arend, W. P. 2001. Cytokine imbalance in the pathogenesis of rheumatoid arthritis: the role of interleukin-1 receptor antagonist. *Semin. Arthritis Rheum.* 30:1-6.
40. Schiff, M. H. 2000. Role of interleukin 1 and interleukin 1 receptor antagonist in the mediation of rheumatoid arthritis. *Ann. Rheum. Dis.* 59 Suppl 1:i103-i108.

Chapter 3

Exacerbations of COPD correlate with down-regulation of cytokine-inducible genes in peripheral blood neutrophils

Evert HJ. Nijhuis*, Erik-Jan D. Oudijk*, Michael D. Zwank, Ed van de Graaf, Jan-Willem J. Lammers, Paul J. Coffer and Leo Koenderman1

*These authors contributed equally to this study

Submitted

Abstract

Chronic obstructive pulmonary disease (COPD) is associated with systemic inflammation, which is in part mediated by increased levels of inflammatory cytokines/chemokines and by (pre)activation of peripheral blood neutrophils. To investigate systemic inflammation during acute exacerbations of the disease, we analyzed the expression of cytokine-induced inflammatory genes in peripheral blood neutrophils from frequently exacerbating COPD patients during a stable phase of the disease compared to an exacerbation. Data were obtained by using real time reverse transcriptase-polymerase chain reaction.

The expression of several pro-inflammatory genes, including CD83, IL-1β, IL-1RA, IL-8 and MIP-1β was increased in patients with moderate to severe COPD (Gold class IIa and III). In contrast to our expectation the expression of these genes was actually decreased in peripheral blood neutrophils during an exacerbation of the disease to levels similar to that of neutrophils obtained from normal donors. This was most evident for CD83 and, therefore, we utilized this marker for an *in vitro* mechanistic study. TNFα induced CD83 mRNA in a dose and time dependent manner in neutrophils *in vitro*. Interestingly, CD83 was dramatically down-regulated by simultaneous incubation of neutrophils with granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFα. This effect was dose dependent and optimal at 100 pM GM-CSF. This GM-CSF-induced effect is independently of IκBα phosphorylation. Thus, acute exacerbations of COPD are associated with a clear down-regulation of inflammatory genes in circulating neutrophils, which might be caused by a change in the levels of systemic inflammatory mediators.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the airways, which is characterized by the presence of chronic airflow obstruction and chronic inflammation in the lower airways. Several studies have monitored local inflammation in COPD by measuring granulocyte activation at the site of inflammation (1-3). Additional studies have measured cytokine levels in the sputum to monitor disease progress (4-6).
In contrast to this relatively well-studied local inflammation, little is known concerning the systemic inflammatory response in COPD. A number of studies provide evidence that local inflammation is reflected by cytokine release into the systemic circulation. However, several inflammatory mediators such as tumor necrosis factor (TNF)-α are difficult to detect in the peripheral blood because of their short half-life, binding to soluble receptors, and renal clearance from the peripheral blood (7-9). Recent studies showed that levels of soluble TNF-receptor (sTNF-R)55 and sTNF-R75 were increased in the peripheral blood of patients with COPD, which is thought to reflect TNFα induced inflammation (9-11). Other studies reported increased levels of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) in the serum of COPD patients (12), which are indirect measurements for pre-activation of granulocytes. Indeed, initial data have shown that inflammatory cells in the peripheral blood of patients with chronic pulmonary diseases are characterized by a primed phenotype (13-15). Despite the increasing evidence that granulocytes are activated in the peripheral blood from patients with COPD, little is known regarding the identity of the inflammatory mediators and the type and kinetics of the inflammatory response. Even less is known regarding systemic inflammation during exacerbations of COPD.
In this study the activation status of peripheral blood neutrophils was examined as read-out for the chronic inflammatory response during acute exacerbations of COPD. This was achieved by analysis of the expression of inflammatory genes in peripheral blood neutrophils *in vivo* and *in vitro* using real time RT-PCR. We compared gene profiles of neutrophils, stimulated with the pro-inflammatory cytokine TNFα *in vitro* with the gene profiles of neutrophils isolated from patients with COPD before and at time of exacerbation of their disease. Pro-inflammatory genes such as CD83, IL-1β and MIP-1β were upregulated in neutrophils obtained from severe COPD patients. These genes are typically induced by TNF *in vitro* (see Chapter 2). Surprisingly, these genes were down-regulated in peripheral blood neutrophils during acute exacerbation of the disease. These findings demonstrate a change in systemic inflammation during an acute exacerbation of COPD.

Materials and Methods

Reagents

Formyl-methionyl-leucyl-phenylalanine (fMLP) and LPS were purchased from Sigma (St. Louis, MO, USA). Recombinant human GM-CSF was from Genzyme (Boston, MA, USA). Recombinant human TNFα was purchased from Boehringer Mannheim (Mannheim, Germany). IL-8 was purchased from Peprotech (Rocky Hill, NJ, USA). Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). RPMI 1640 medium with Glutamax was purchased from Life Technologies (Breda, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Anti-phospho-IκBα (ser32) was from New England Biolabs (Bevery, MA, USA). Actin (I-19) polyclonal antiserum was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Subjects

Six patients were enrolled into the study with unstable moderate to severe (class IIb and III) COPD according to the inclusion criteria of the GOLD guidelines (16). All patients had in the year prior to the inclusion to the study at least two exacerbations requiring hospital admission. Patients also had to meet the following additional inclusion criteria: a FEV_1 < 80% and a reversibility of the airway obstruction of less than 10% or less than 200 ml after inhalation of a β2-agonist and a FEV1/FVC of less than 70%. A history of other concomitant confounding diseases such as diabetes mellitus, lung carcinoma, cardiovascular disease and bronchiectasis were excluded from the study. Also patients with a history of asthma or atopy were excluded. Patients were on standardized therapy with a muscnismic receptor antagonist (b.i.d), long acting β2-agonist (b.i.d.), inhalated cortico-steroids if neccecary, and they were allowed to use a short acting β2-agonist for rescue medication. These COPD patients were followed for nine months and disease status was monitored every two weeks during a visit to the out patient clinic. During every visit a medical history, Borg score (17), lung function (FEV_1 and FVC), and routine laboratory tests were performed. Blood samples were taken during these visits to investigate peripheral blood neutrophil gene expression during an exacerbation(s) before treatment of the exacerbation and during stable phases of their disease. The presence of an acute disease exacerbation was diagnosed according to Anthonisen (18), being an increase of dyspnoea, cough, or sputum production resulting in hospital admission. During exacerbations patients were treated with antibiotics (levofloxacin 500 mg once a day for fourteen days) and glucocorticosteroids intravenously (Di-Adreson F-aquosum 50 mg for 7 days). A stable phase was defined as at least 4 weeks before or after an acute exacerbation and no presence of a respiratory tract infection or respiratory complaints during 4 weeks prior or after sampling. The mean age of the patient group was 63.3 ±4.9

(range 48-78).The mean FEV1 was 29.8 ± 3.4% (range 19.1%-39.9%). Eleven healthy subjects with a normal lung function were enrolled as controls. The study was approved by the Medical Ethics Committee of the University Medical Center, Utrecht, and informed consent was obtained from all subjects.

Isolation of human neutrophils

Blood was obtained from healthy volunteers and COPD patients. Mixed granulocytes were isolated from 50-100 ml blood (normal volunteers) or 20 ml (COPD patients), which was anti-coagulated with 0.32% sodium citrate. Blood was diluted 2.5:1 with PBS containing 0.32% sodium citrate and human pasteurized plasma-protein solution (4 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Paque for 20 minutes at 2000 rpm. The erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 minutes at 37°C in Hepes buffered RPMI 1640 medium, supplemented with L-glutamine and 0.5% HSA. All preparations contained >97% neutrophils.

RNA isolation and cDNA synthesis.

RNA isolation was performed as previously described (19). Briefly, 2×10^7 cells were washed with ice-cold PBS and lyzed in 0.4 mL of GIT solution (6 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, 0.5% N´-lauroyl sarcosine, 100 mmol/L β-mercaptoethanol). After storage at –20°C, phenol extraction was performed followed by ethanol precipitation. Total RNA was resuspended in a small volume of H_20 and treated with DNAse for half an hour. After DNAse treatment RNA was again extracted with phenol, precipitated with ethanol and RNA was then eluted in 10 µl of RNAse-free H_2O. cDNA was synthesized using MMLV reverse transcriptase and oligo(dT) primers. Samples containing 1 µg of total RNA were heated for 3 minutes at 65°C and quickly chilled on ice. A mixture of 25 µl containing 20 µg/ml oligo(dT) primers, 5 µl of 5x RT buffer, 20 mM DTT, 2 mM each deoxynucleoside triphosphate, 0.8 U/µl of RNAse inhibitor and 400 units of MMLV reverse transcriptase was added and incubated at 37 °C for 90 minutes, followed by RT inactivation for 10 min at 65°C. cDNA was stored at -20°C before further use. All reagents used for cDNA synthesis were obtained from Life Technologies (Breda, The Netherlands)

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

CD83 gene was analyzed by Taqman real time pcr as described in Chapter 2. All the other genes were analyzed by real time pcr using SYBR green I (Chapter 2). Amplification and detection of both Taqman and SYBR green I pcr were performed with an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands) under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C to

activate AmpliTaq Gold DNA polymerase, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The signal of the dye was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. Results were normalized for the housekeeping gene β-actin and GAPDH. A reference sample of cDNA on every 96 wells plate allowed correction of differences between plates. Results were expressed as fold regulation.

Western blotting

Cytosolic extracts were prepared by the same method as for obtaining nuclear extracts in the section EMSA. Samples were normalized for protein content and boiled for 5 min at 95°C in the presence of Laemmli sample buffer. Proteins were analyzed on 10% SDS-polyacrylamide gels and transferred to Immobilon-P. The blots were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 5% BSA for 1 hour followed by incubation with polyclonal anti-phospho-IκBα (ser32) (1/2000) in hybridization buffer with 0.5% BSA for 1 hours at room temperature. Second antibody was incubated in hybridization buffer for 1 hour. The western blots for hybridization with anti-actin were blocked in hybridization buffer containing 5% non-fat milk for 1 hour followed by incubation with anti-actin (1/1000) in hybridization buffer with 0.5% non-fat milk for 2 hours at room temperature. Second antibody was incubated in hybridization buffer containing 0.5% non-fat milk for 1 hour. Detection of all western blots was performed by enhanced chemiluminescence (ECL; Amersham, UK).

Statistics

Unless otherwise noted, all data are expressed as the mean ± SE, and differences between values were compared for significance by the paired t-test. Differences between study groups were compared using the Mann-Whitney U test. The Wilcoxon signed rank test was used for comparisons within an individual between their stable phase and exacerbation. Statistical significance was defined as $p<0.05$

Results

Exacerbations of COPD are associated with down-regulation of cytokine inducible genes in peripheral blood neutrophils.

Neutrophils isolated from patients with moderate to severe COPD appeared to have a markedly increased expression of several inflammatory genes, compared to cells obtained from normal control donors (see Chapter 2). These genes are typically induced by pro-inflammatory cytokines, such as TNFα. We tested the hypothesis that exacerbations of COPD would be reflected in a more pronounced expression of these genes, since increased levels of inflammatory cytokines have been found in the airways of patients with acute exacerbations of COPD as compared to a stable phase of their disease (1). We focused on a small set of regulated genes, including IL-1β, IL-1RA, IL-8, IL-8R1, MIP1β and CD83 to analyze their expression during stable phases and during exacerbations of COPD patients. cDNA was prepared from total RNA of cells from healthy volunteers and of above mentioned COPD patients, and was used for real time RT-PCR analysis of the indicated genes (Fig 1). Pro-inflammatory genes such as CD83, MIP-1β, and IL-1β were significantly increased in severe COPD patients compared to healthy volunteers (Fig 1, see also Chapter 2).

Interestingly, neutrophils isolated from these patients were characterized by a clear and significant decrease in CD83, MIP-1β, IL-1β, and IL-8 expression at the time of an exacerbation of their disease compared to the stable phase (Fig.1). The expression of IL-1RA and IL-1β returned to the level of healthy volunteers. Expression of CD83, MIP-1β and IL-8 was reduced to a level even lower than that of healthy volunteers. Not all analyzed genes were down-regulated in neutrophils from COPD patients with an exacerbation. For example the IL-8R1 gene was not significantly influenced.

Kinetics of TNFα induced CD83 expression in neutrophils in vitro.

The results obtained with CD83 were the most striking of all genes analyzed. To gain more insight into the mechanism underlying this phenomenon, we studied expression of CD83 under different conditions in vitro. We and others have shown that this gene is specifically induced by inflammatory mediators that activate the transcription factor NFκb, including TNFαand LPS (see Chapter 2,(20-24). We first set out experiments to study the characteristics of TNFα-induced activation of neutrophils in terms of expression of CD83 mRNA in these cells. Neutrophils were stimulated with TNFα for various periods of time to analyze the kinetics of TNFα-mediated upregulation of CD83 expression. CD83 expression was measured by real time RT-PCR as described in Materials and Methods. Specific primers were used to amplify only cDNA generated from CD83 mRNA and not genomic DNA. CD83 expression was normalized for β-actin

expression. Increased CD83 expression was already observed within 30 minutes of TNFα stimulation, and was further elevated over time (Fig. 2A). Maximal increase of CD83 expression was reached between 2-3 hours. Next, neutrophils were incubated with increasing concentrations of TNFα for 3 hours to determine the dose dependency of CD83 induction (Fig. 2B). A significant increase of CD83 expression of 7- and 14-fold was found at a concentration of 10 and 100 U/ml, respectively. These data demonstrate that the increase in CD83 mRNA expression by TNFα is time and concentration dependent. These findings are consistent with a role for cytokines such as TNFα in systemic inflammation in COPD, but do not shed light on the down-regulation of CD83 mRNA in neutrophils from COPD patients during an acute exacerbation of their disease.

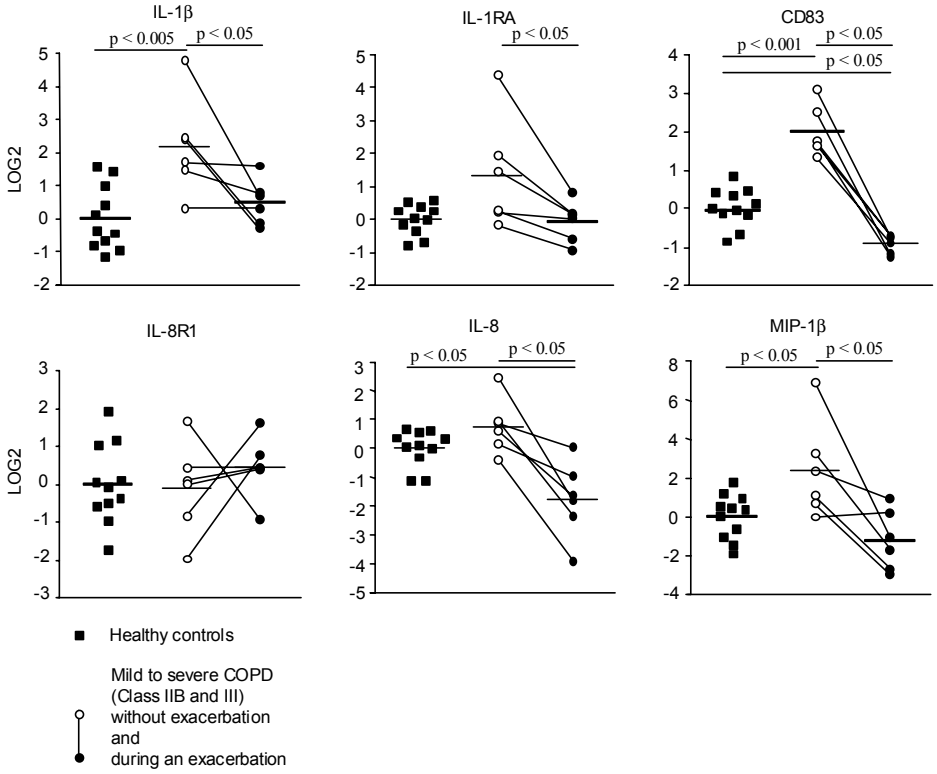

Figure 1: Gene expression analysis in peripheral blood neutrophils of COPD patients. Peripheral blood neutrophils were isolated from healthy volunteers (Filled squares) and moderate to severe (class IIB and III) COPD patients during their stable phase (open circles) and at time of exacerbation (filled circles). A line connected the stable phase and exacerbation of the same patient. Expression of indicated genes were measured using real time RT-PCR and expressed as LOG2 fold-change compared to the group of healthy controls. Differences between the study groups were compared using the Mann-Whitney U test. Differences between stable phase and exacerbation were compared using the Wilcoxon signed rank test.

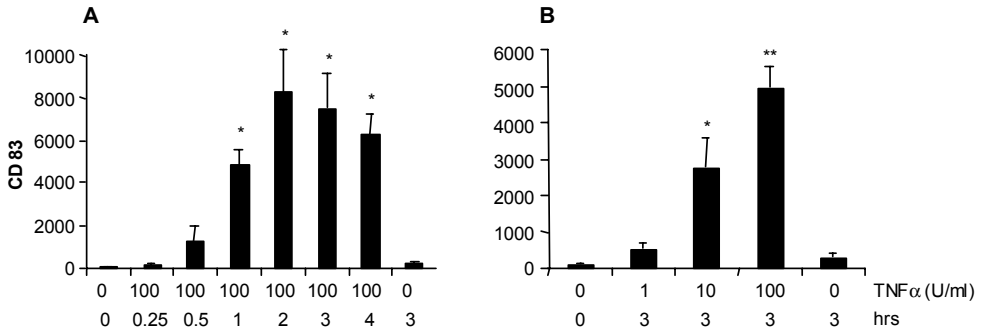

Figure 2: TNFα induced CD83 expression is time and dose dependent. Peripheral blood neutrophils were stimulated with TNFα (100U/ml) for several time points **(A)** or with several concentrations of TNFα for 3 hours *in vitro* **(B)**. RNA was isolated and real time RT-PCR for CD83 was performed. Results were normalized for the housekeeping gene β-actin. Results were expressed as relative levels of CD83 ±S.E.M. (n=4). For statistical analysis the paired T-test was used (*$p<0.05$ versus 3 hrs no stimulation; **$p<0.001$ versus 3 hrs no stimulation).

GM-CSF down-regulates TNFα-induced CD83 mRNA in neutrophils

Besides TNF-α, a role for GM-CSF in the pathogenesis of COPD has been proposed (4, 25, 26). Therefore, we designed experiments to study the effect of co-stimulating neutrophils with GM-CSF and TNFα on the expression of CD83 mRNA. In Chapter 2 we demonstrated that TNFα but not GM-CSF could induce CD83 expression. Neutrophils were stimulated with increasing concentrations of TNFα in the presence or absence of GM-CSF (0.1 nM). RNA was isolated and CD83 expression was determined by real time RT-PCR (Fig 3A). Co-incubation with GM-CSF caused a dramatic inhibition of TNFα (100 U/ml) induced CD83 expression in human neutrophils. This inhibition was complete in neutrophils stimulated with 1 or 10 U/ml TNFα.

Neutrophils were subsequently stimulated with TNFα and increasing concentrations of GM-CSF to determine the dose dependency of the inhibition. As shown in Figure 3B, increasing concentrations of GM-CSF results in decreasing TNFα induced CD83 expression. Complete inhibition of 10 U/ml of TNFα induced CD83 expression was obtained at a concentration of 0.1 nM GM-CSF. These findings demonstrate that a combination of pro-inflammatory cytokines can result in down-regulation of a pro-inflammatory gene.

GM-CSF-induced down-regulation of CD83 mRNA is not associated by changes in NFκB signaling

As previously mentioned both activators of CD83, TNFα and LPS (see Chapter 2), are strong activators of the transcription factor NF-κB in neutrophils (27). In addition, TNFα induced CD83 expression in T-cells is NF-κB dependent (20, 21). Therefore, we investigated whether the effect of GM-CSF might be mediated through inhibition of NF-κB signaling. As a read out for this signaling pathway, the phosphorylation state of IκBα was studied. In an unphosphorylated state, IκB associates with NF-κB, retaining it in the cytoplasm and subsequently preventing translocation of NF-κB to the nucleus. TNFα but not GM-CSF stimulation was sufficient to induce phosphorylation of IκBα in neutrophils (and thus activation of NFκB) (Fig 3C). Stimulation of neutrophils with TNFα in the presence GM-CSF resulted in IκBα phosphorylation to a similar level as TNFα alone. Thus, the inhibitory effect of GM-CSF on TNFα-induced CD83 expression is independent of IκBα phosphorylation in human neutrophils.

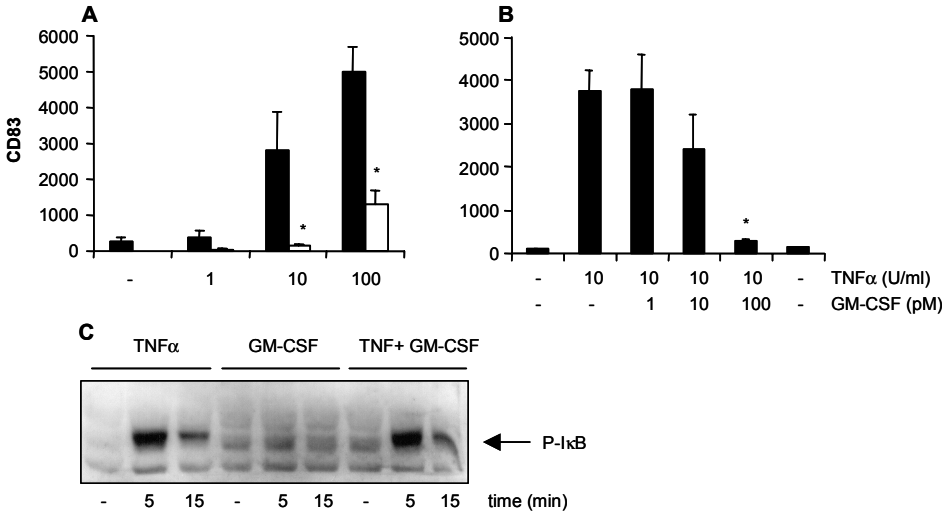

Figure 3: **TNFα induced CD83 expression in peripheral blood neutrophils is inhibited by GM-CSF. (A)** Isolated neutrophils were incubated with increasing concentrations of TNFα (as indicated) and simultaneously without (black bars) or with GM-CSF (0.1 nM) (empty bars) for 3 hours *in vitro*. CD83 expression was measured by performing real time RT-PCR and results are expressed as relative levels of CD83 ±S.E.M. (n=4). For statistical analysis the paired T-test was used.*$p<0.05$. **(B)** Neutrophils were incubated with TNFα (10 U/ml) and simultaneously with increasing concentrations of GM-CSF for 3 hours *in vitro*. Results are expressed as relative levels of CD83 ± S.E.M. (n=4). For statistical analysis the paired T-test was used.*$p<0.05$ versus TNFα (10 U/ml). **(C)** Neutrophils were incubated with TNFα (10 U/ml) and/or GM-CSF (10^{-10} M) for the times indicated. After stimulation, cytosolic extracts were prepared, normalized for protein content and analyzed by SDS-PAGE followed by Western blotting with anti-phospho-IκBα (ser 32) or anti-actin. Data is representative of three independent experiments.

Discussion

At chronic inflammatory loci, such as bronchial tissue in COPD, neutrophils migrate to the site of inflammation and can become activated even in the absence of microbial derived triggers. Several studies have monitored inflammatory diseases by measuring granulocyte activation at the site of inflammation (1-3). In contrast to local inflammatory sites, little is known about systemic inflammation associated with granulocyte activation in peripheral blood in COPD. Furthermore, it is not clear which inflammatory mediators are playing a role in the activation of granulocytes in the systemic compartment. The analysis of gene profiles of cytokine-induced genes in neutrophils (*in vitro*) can be used as an indication for the action of these cytokines *in vivo*. Indeed, we have found several differentially expressed genes in neutrophils from the blood of patients with moderate to severe COPD compared to normal donors (see Chapter 2).

Here we focused on a set of genes that were found to be regulated *in vivo* in COPD patients, and are also induced by cytokines in neutrophils *in vitro* (see Chapter 2). The expression of CD83, MIP-1β, and IL-1β were significantly enhanced in peripheral blood neutrophils from COPD patients in a stable phase of their disease (Fig. 1). However, to our surprise, the expression of CD83, MIP-1β, IL-1β, and IL-8 was dramatically down-regulated in peripheral neutrophils from COPD patients during an acute exacerbation of their disease (Fig. 1). This unanticipated result may have important consequences concerning the insight into the mechanisms of the chronic systemic inflammatory response in COPD. Apparently, either a quantitative (excess of inflammatory signals) and/or a qualitative (different inflammatory mediators) change reflects itself in a gene profile that fits more with a reduced inflammatory phenotype. It is tempting to speculate that neutrophils act as pro-inflammatory cells at the onset of an inflammatory reaction, but can have a more anti-inflammatory function at later stages of an acute exacerbation. This altered neutrophil phenotype might then be more involved in dampening the inflammatory reaction. Support for this hypothesis is the finding that peripheral blood neutrophils are found to be primed directly after post-injury multiple organ failure, but become unresponsive to several pro-inflammatory chemoattractants after 48 hours (28, 29)

TNFα is thought to be an important cytokine in the pathogenesis of COPD, has been implicated in many chronic inflammatory diseases (30) and is a strong activator of neutrophils (31). Besides TNFα, GM-CSF has also been implicated in the pathogenesis of COPD (25, 26). Interestingly, GM-CSF induces a distinct profile of expressed genes in neutrophils compared to TNFα (see Chapter 2). Not much is known regarding GM-CSF in systemic inflammation, but we did not find clear indications of specific GM-CSF induced genes in neutrophils isolated from the peripheral blood of COPD patients (see Chapter 2). Based on these findings we designed experiments to study the down-regulation of CD83 mRNA in an *in vitro* setting. We focused on TNFα-induced CD83

expression in neutrophils *in vitro* as a model for processes occurring *in vivo* in patients with COPD.

Time course experiments showed that CD83 expression was significantly induced by TNFα after one hour and was maximal after three hours. Concentrations between 1 and 10 U/ml of TNFα were sufficient to induce CD83 mRNA expression. These concentrations are likely to be found near chronic inflammatory sites (4, 11, 32). The expression of CD83 induced by TNF is likely to be mediated by NFκB, since mediators such as LPS and TNFα, that are well known activators of NFκB, induce CD83 in neutrophils (26), whereas mediators that do not activate NFκB in neutrophils (like GM-CSF and IL-8) do not increase CD83 mRNA. In addition, a recent study provides evidence that NF-κB mediates TNFα induced CD83 expression in Jurkat T cells (20).

The effect of a combination of pro-inflammatory cytokines, TNF and GM-CSF, revealed the surprising observation that GM-CSF inhibits TNFα induced CD83 expression. This finding is consistent with the hypothesis that cytokines such as GM-CSF can cross-modulate the expression of pro-inflammatory genes in neutrophils *in vivo*. Indeed, GM-CSF levels were markedly increased in serum and sputum from chronic bronchitic patients with an exacerbation, as compared with patients under baseline conditions (25). The precise molecular mechanism by which GM-CSF inhibits CD83 expression remains to be determined. However, our IκBα phosphorylation data demonstrated that GM-CSF is inhibiting CD83 expression independently of IκBα phosphorylation.

Another explanation for the down-regulation of genes at the time of exacerbation may be that the "hyper" activation of neutrophils causes enhanced homing to inflammatory loci. This hypothesis is supported by studies describing that circulating neutrophils are (pre)activated during the first 24 hours after major trauma and that a subsequent activation of these cells during this period can lead to enhanced sequestering in the organs and induction of an intense, cytotoxic, inflammatory response resulting in multiple organ injury (33, 34).

Interestingly, the periods of exacerbation were characterized by neutrophilia rather than neutropenia (results not shown). Previous studies have also demonstrated an increase of neutrophils in the airways (25, 35-37). A consequence of this is that neutrophils obtained during an exacerbation in the periphery were likely recently released from the bone marrow. These neutrophils may not have encountered pro-inflammatory mediators such as TNFα. This might also explain why the expression of some genes decreases to below that of healthy volunteers. Most likely these processes occur simultaneously. The resulting phenotype of neutrophils obtained from patients during an exacerbation of COPD is the result of a very delicate balance between recruitment of neutrophils from the bone marrow, cross-modulation by multiple cytokines and enhanced homing to inflammatory sites.

In conclusion, our data indicate that combinations of cytokines *in vivo* can result in the production of specific gene profiles dependent on the combination and concentrations of cytokines. This suggests that cytokine/chemokine induced gene expression profiles may provide novel insights into the mechanisms of systemic inflammatorion in COPD in terms of type of inflammatory response, participation of unique combinations of cytokines and kinetics of the presence of different phenotypes of leukocytes in the peripheral blood. Thorough analysis of these gene profiles will allow characterization of different stages of COPD and possibly better timing of appropriate therapy.

References

1. Aaron, S. D., J. B. Angel, M. Lunau, K. Wright, C. Fex, N. Le Saux, R. E. Dales. 2001. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 163:349-355.
2. Keatings, V. M., and P. J. Barnes. 1997. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am. J. Respir. Crit. Care Med.* 155:449-453.
3. Pesci, A., B. Balbi, M. Majori, G. Cacciani, S. Bertacco, P. Alciato, C. F. Donner. 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur. Respir. J.* 12:380-386.
4. Keatings, V. M., P. D. Collins, D. M. Scott, P. J. Barnes. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am. J. Respir. Crit. Care Med.* 153:530-534.
5. Yamamoto, C., T. Yoneda, M. Yoshikawa, A. Fu, T. Tokuyama, K. Tsukaguchi, N. Narita. 1997. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 112:505-510.
6. Dentener, M. A., E. C. Creutzberg, A. M. Schols, A. Mantovani, C. van't Veer, W. A. Buurman, E. F. Wouters. 2001. Systemic anti-inflammatory mediators in COPD: increase in soluble interleukin 1 receptor II during treatment of exacerbations. *Thorax* 56:721-726.
7. Bemelmans, M. H., L. J. van Tits, W. A. Buurman. 1996. Tumor necrosis factor: function, release and clearance. *Crit. Rev. Immunol.* 16:1-11.
8. Diez-Ruiz, A., G. P. Tilz, R. Zangerle, G. Baier-Bitterlich, H. Wachter, D. Fuchs. 1995. Soluble receptors for tumour necrosis factor in clinical laboratory diagnosis. *Eur. J. Haematol.* 54:1-8.
9. Takabatake, N., H. Nakamura, S. Abe, S. Inoue, T. Hino, H. Saito, H. Yuki, S. Kato, H. Tomoike. 2000. The relationship between chronic hypoxemia and activation of the tumor necrosis factor-alpha system in patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 161:1179-1184.
10. Takabatake, N., H. Nakamura, S. Abe, T. Hino, H. Saito, H. Yuki, S. Kato, H. Tomoike. 1999. Circulating leptin in patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 159:1215-1219.
11. Vernooy, J. H., M. Kucukaycan, J. A. Jacobs, N. H. Chavannes, W. A. Buurman, M. A. Dentener, E. F. Wouters. 2002. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am. J. Respir. Crit. Care Med.* 166:1218-1224.
12. Fiorini, G., S. Crespi, M. Rinaldi, E. Oberti, R. Vigorelli, G. Palmieri. 2000. Serum ECP and MPO are increased during exacerbations of chronic bronchitis with airway obstruction. *Biomed. Pharmacother* 54:274-278.
13. Warringa, R. A., H. J. Mengelers, P. H. Kuijper, J. A. Raaijmakers, P. L. Bruijnzeel, L. Koenderman. 1992. In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. *Blood* 79:1836-1841.
14. Koenderman, L., D. Kanters, B. Maesen, J. Raaijmakers, J. W. Lammers, J. de Kruif, T. Logtenberg. 2000. Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. *J. Leukoc. Biol.* 68:58-64.
15. Noguera, A., S. Batle, C. Miralles, J. Iglesias, X. Busquets, W. MacNee, A. G. Agusti. 2001. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax* 56:432-437.
16. Pauwels, R. A., A. S. Buist, P. M. Calverley, C. R. Jenkins, S. S. Hurd. 2001. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am. J. Respir. Crit. Care Med.* 163:1256-1276.

17. Wilson, R. C., and P. W. Jones. 1991. Long-term reproducibility of Borg scale estimates of breathlessness during exercise. *Clin. Sci. (Lond)* 80:309-312.
18. Anthonisen, N. R., J. Manfreda, C. P. Warren, E. S. Hershfield, G. K. Harding, N. A. Nelson. 1987. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann. Intern. Med.* 106:196-204.
19. Pals, C. M., S. A. Verploegen, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, P. J. Coffer. 2000. Identification of cytokine-regulated genes in human leukocytes in vivo. *J. Allergy Clin. Immunol.* 105:760-768.
20. McKinsey, T. A., Z. Chu, T. F. Tedder, D. W. Ballard. 2000. Transcription factor NF-kappaB regulates inducible CD83 gene expression in activated T lymphocytes. *Mol. Immunol.* 37:783-788.
21. Berchtold, S., P. Muhl-Zurbes, E. Maczek, A. Golka, G. Schuler, A. Steinkasserer. 2002. Cloning and characterization of the promoter region of the human CD83 gene. *Immunobiology* 205:231-246.
22. Iking-Konert, C., C. Wagner, B. Denefleh, F. Hug, M. Schneider, K. Andrassy, G. M. Hansch. 2002. Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent expression in acute bacterial infections and chronic inflammatory disease. *Clin. Exp. Immunol.* 130:501-508.
23. Lyakh, L. A., G. K. Koski, W. Telford, R. E. Gress, P. A. Cohen, N. R. Rice. 2000. Bacterial lipopolysaccharide, TNF-alpha, and calcium ionophore under serum-free conditions promote rapid dendritic cell-like differentiation in CD14+ monocytes through distinct pathways that activate NK-kappa B. *J. Immunol.* 165:3647-3655.
24. Yamashiro, S., J. M. Wang, D. Yang, W. H. Gong, H. Kamohara, T. Yoshimura. 2000. Expression of CCR6 and CD83 by cytokine-activated human neutrophils. *Blood* 96:3958-3963.
25. Balbi, B., C. Bason, E. Balleari, F. Fiasella, A. Pesci, R. Ghio, F. Fabiano. 1997. Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *Eur. Respir. J.* 10:846-850.
26. Culpitt, S. V., D. F. Rogers, P. Shah, C. De Matos, R. E. Russell, L. E. Donnelly, P. J. Barnes. 2002. Impaired Inhibition by Dexamethasone of Cytokine Release by Alveolar Macrophages from COPD Patients. *Am. J. Respir. Crit. Care Med.*
27. McDonald, P. P., A. Bald, M. A. Cassatella. 1997. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. *Blood* 89:3421-3433.
28. Botha, A. J., F. A. Moore, E. E. Moore, B. Fontes, A. Banerjee, V. M. Peterson. 1995. Postinjury neutrophil priming and activation states: therapeutic challenges. *Shock* 3:157-166.
29. McCall, C. E., L.M., K. LaRue, R. N. Guzman, S. L. Cousart. 1993. Tolerance to endotoxin-induced expression of the interleukin-1 beta gene in blood neutrophils of humans with the sepsis syndrome. *J. Clin. Invest.* 91:853-861.
30. Beutler, B. A. 1999. The role of tumor necrosis factor in health and disease. *J. Rheumatol.* 26 Suppl 57:16-21.
31. Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, A. M. Waltersdorph. 1986. Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* 136:4220-4225.
32. Eid, A. A., A. A. Ionescu, L. S. Nixon, V. Lewis-Jenkins, S. B. Matthews, T. L. Griffiths, D. J. Shale. 2001. Inflammatory response and body composition in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 164:1414-1418.
33. Botha, A. J., F. A. Moore, E. E. Moore, F. J. Kim, A. Banerjee, V. M. Peterson. 1995. Postinjury neutrophil priming and activation: an early vulnerable window. *Surgery* 118:358-364.
34. Partrick, D. A., E. E. Moore, D. A. Fullerton, C. C. J. Barnett, D. R. Meldrum, C. C. Silliman. 1999. Cardiopulmonary bypass renders patients at risk for multiple organ failure via early neutrophil priming and late neutrophil disability. *J. Surg. Res.* 86:42-49.
35. Selby, C., E. Drost, S. Lannan, P. K. Wraith, W. MacNee. 1991. Neutrophil retention in the lungs of patients with chronic obstructive pulmonary disease. *Am Rev Respir Dis JID - 0370523* 143:1359-1364.

36. Crooks, S. W., D. L. Bayley, S. L. Hill, R. A. Stockley. 2000. Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. *Eur Respir J JID - 8803460* 15:274-280.
37. Gompertz, S., C. O'Brien, D. L. Bayley, S. L. Hill, R. A. Stockley. 2001. Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. *Eur Respir J JID - 8803460* 17:1112-1119.

Chapter 4

Variable systemic inflammation in COPD visualized by kinetics of priming of leukocytes in peripheral blood

Evert HJ. Nijhuis*, Erik-Jan D. Oudijk* Corneli van Aalst, Michael Zwank, J.J. Mager, Jan-Willem Lammers, Ed van de Graaf, Paul J. Coffer and Leo Koenderman

* These authors contributed equally to this study

Submitted

Abstract

Chronic inflammation of the airways is a hallmark of Chronic Obstructive Pulmonary Disease (COPD). We investigated the kinetics of priming of inflammatory cells in peripheral blood as a consequence of this process.

Priming of leukocytes was determined by measuring the expression of priming epitopes by novel antibodies designated A17 and A27, CD83 mRNA expression and activation of intracellular signaling proteins in a cross sectional and a longitudinal study. Leukocytes were obtained from COPD patients (GOLD class IIa, b and III) and healthy controls. In a longitudinal study of six patients with moderate to severe COPD (GOLD class IIb and III) and six normal controls, the kinetics of priming was determined every two weeks for a period of nine months.

In the cross sectional study, the priming of neutrophils in the context of expression of priming epitopes was not increased in COPD patients (31.6 ± 4.8 AU (A17) and 31.6 ± 5.1 AU (A27) respectively), as compared to healthy controls (29.3 ± 5.2 AU (A17) and 29.7 ± 4.2 AU (A27) respectively). For eosinophils, a slight but significant decrease in priming epithopes A17 was observed in COPD patients (45.7 ± 6.9 AU (A17) and 46.7 ± 6.5 AU (A27) respectively) compared to healthy controls (58.2 ± 5.3 AU (A17) and 61.4 ± 5.2 AU (A27) respectively). The absence of priming of neutrophils was unanticipated and was evaluated in more detail in a small group of severe COPD patients (GOLD class IIb and III). In this group further characteristics of neutrophil priming were evaluated: expression of CD83 mRNA by real time PCR and activation of intracellular signaling molecules. This was studied in a longitudinal setting and all priming markers in combination with clinical characteristics were measured every two weeks during a period of nine months. Priming of neutrophils in this group of severe COPD patients was variable with regard to all priming markers compared with normal controls (coefficient of variation for A27 on neutrophils in COPD patients: 0.50, in healthy controls: 0.24 ($p<0.0001$)). No correlation was found between clinical parameters and priming of peripheral blood neutrophils.

We conclude that patients with advanced COPD show characteristics of a highly variable systemic inflammation, which does not reflect itself in individual clinical symptoms.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is defined as a disease state characterized by irreversible, usually progressive airflow limitation (1). Central in the pathogenesis of COPD is the inflammatory reaction found in the airways and the alveolar structures, which leads to persistent injury. COPD is a slowly progressive disease and the damage to the lungs can be ascribed to years of exposure to noxious particles in cigarette smoke being the most important risk factor for the development of COPD in the western world (1). These particles can evoke direct damage to the lung parenchyma, but are also involved in the initiation of an inflammatory reaction in the lungs of COPD patients (2-4). The induction of this inflammatory reaction is accompanied by an imbalance in protease/antiprotease and oxidative/anti-oxidative mechanisms. These processes can lead to pulmonary damage and are not restricted to COPD (5-7). In the peripheral and central airways of COPD patients but also in `healthy` smokers inflammatory changes are found characterized by an increase in the number of neutrophils, monoyctes and T lymphocytes (3, 8, 9).

Progression of COPD is characterized by a decline in lung function and development of a systemic response. Systemic effects are initiated by the local persistent inflammatory response in the lungs of COPD patients. Mediators originating form the local inflammatory response such as cytokines, chemokines and acute phase proteins are found in the peripheral circulation (10, 11). Activation of peripheral leukocytes, the occurrence of cachexia and muscle wasting are often observed in patients with severe COPD (12, 13).

Sputum and bronchoalveolar lavage (BAL) studies have been performed to gain more insight into the processes of inflammation induced tissue damage in COPD. These studies showed increased levels of pro-inflammatory cytokines and chemokines in the bronchial compartment (10, 14, 15). In addition increased levels of inflammatory cytokines and chemokines as well as their receptors on leukocytes have been observed in the peripheral circulation. These circulating cytokines and chemokines mediate part of the systemic inflammatory reaction and are thought to prime inflammatory cells in the peripheral blood. This will facilitate the margination, extravasation and accumulation of inflammatory cells in to the tissues. Therefore, only partially primed cells can be found in the blood (11). Insight into the mechanism of priming of peripheral blood leukocytes of patients with COPD will contribute to the understanding of the kinetics of the systemic inflammatory processes, which are associated with the disease.

We performed a cross-sectional study of COPD patients to determine whether there was inflammation-associated priming of neutrophils and eosinophils in the peripheral blood compared with cells obtained from normal control donors. An additional longitudinal study was undertaken to determine the kinetics of priming in COPD patients and in

normal controls. We show that systemic neutrophilic inflammation is variable in patients with severe COPD in contrast to mild to moderate patients and healthy controls.

Materials and Methods

Reagents

Recombinant human GM-CSF was from Genzyme (Boston, MA, USA). Recombinant human TNFα was purchased from Boehringer Mannheim (Mannheim, Germany). Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). RPMI 1640 medium with Glutamax was purchased from Life Technologies (Breda, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Polyclonal anti-phospho-PKB (ser473), anti-phospho-ERK1/2 (Thr202/Tyr204).

Study population and design

For the cross-sectional study twenty-three patients with clinically stable, smoking related COPD (class IIa, IIb and III according to the GOLD criteria (1) were recruited from the outpatient clinic of the Heart Lung Center Utrecht. Patients with a FEV1 < 80% and a reversibility of less than 10% or less than 200 ml after administration of an inhaled β-agonist were included. Patients with other concomitant confounding diseases such as diabetes mellitus, lung carcinoma and cardiovascular disease and those with bronciectasis were excluded from the study. Also patients with a history of asthma or atopy and patients with a respiratory tract infection or respiratory complaints during 4 weeks prior to the study were excluded. The mean FEV1 was 51.7 ± 3.8 % (range 19.1% - 79.3%). All patients had a previous history of at least 14 years of smoking (mean 40.0 ± 4.1, range 14 – 76). The mean age of the patients was 64.0 ± 2.3 years (range 40 – 79). Eleven patients were currently smoking, other patients stopped smoking at least one year prior to the inclusion in the study. Regular use of oral glucocortico-steroids was also an exclusion criterion. Patients were treated with a long-acting β2 agonist, inhaled corticosteroids twice a day (when needed) and were prescribed bronchodilating agents if necessary. The priming state of neutrophils and eosinophils in the context of expression of priming epitopes on granulocytes was determined in all participants essentially as described before (11) (see also below).

For the longitudinal study six patients with moderate to severe (class IIb and III) COPD according to the GOLD criteria, were investigated every two weeks during a period of nine months. Inclusion criteria were moderate (class IIB) to severe (class III) COPD, no other concomitant diseases or history of asthma or atopy. In the year prior to the inclusion to the study these patients experienced minimally two exacerbations requiring hospital admission. During every visit medical pulmonary history, severity of dyspnea was determined with the Borg score (16), lung function (FEV_1 and FVC) and routine laboratory tests (Hb, Hct, Leukocyte count, CRP and BSE) were performed. All COPD patients were treated according a standardized protocol with a muscarinic receptor

antagonist (b.i.d.) and a long acting β2-agonist (b.i.d.). As rescue medication a short acting β2-agonist was used. During exacerbations not requiring admittance to the hospital patients were treated with antibiotics (levofloxacin 500 mg once a day for seven days) and oral glucocorticosteroids (prednison 30 mg). Severe exacerbations, requiring hospital admission were treated intravenously with glucocorticosteroids (Di-Adreson-F aquosum 50 mg for 7 days) and antibiotics (levofloxacin 500 mg once a day for fourteen days). Exacerbations were diagnosed based on the major and minor criteria, being an increase in dyspnea, increased sputum volume, or sputum purulence, minor symptoms of exacerbation included cough, wheeze, sore throat, nasal discharge or fever (17, 18) For the diagnosis of an exacerbation the patients required two symptoms for the diagnosis, one of which had to be a major symptom(17). Sputum samples were obtained during exacerbations if possible and checked for bacterial growth. During exacerbations serum was obtained for virus PCR. Eight healthy subjects with a normal lung function were enrolled as controls and followed for six month. In the patient group and in the healthy controls the priming of leukocytes was measured every two weeks and also the expression of CD83 mRNA in neutrophils and activation of signaling molecules. In addition, for monitoring the patients thoroughly clinical parameters like borg score, lung function, medical history and routine laboratory tests (Hb, Hct, Leukocyte count, BSE and CRP) were determined at every visit.

The study was approved by the Medical Ethics Committee of the University Medical Center, Utrecht, and informed consent was obtained from all subjects.

Procedure for staining phagocytes with FITC labeled MoPhabs A17 and A27

Two human monoclonal phage antibodies were isolated from a synthetic bacteriophage antibody library. These phage antibodies recognize epitopes that are upregulated on neutrophils and eosinophils present in whole blood, these epitopes are expressed on the cells at low priming concentration of cytokines such as GM-CSF and TNFα (11). Blood was collected on ice immediately after venapuncture and kept at 4 °C.100µl of FITC-labeled MoPhab (A17 and A27 (11)) was added to 50µl of whole blood and incubated for 90 min on ice. After incubation red cells were lysed in ice cold NH_4Cl. Hereafter, the cells were washed and taken up in PBS/1%HSA, cells were analyzed in a FACS vantage flowcytometer (Becton & Dickinson, Mountain View, CA, USA). The different phagocytes were identified according to their specific side-scatter and forward-scatter signals (14).

Isolation of human neutrophils

Blood was obtained from healthy volunteers and COPD patients. Mixed granulocytes were isolated from 50-100 ml (normal volunteers) or 20 ml blood (COPD patients), which was anti-coagulated with 0.32% sodium citrate as described before (15). In short, blood

was diluted 2.5:1 with PBS containing 0.32% sodium citrate and human pasteurized plasma-protein solution (4 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Pague. The erythrocytes were lyzed in isotonic ice-cold NH_4Cl solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 minutes at 37°C in HEPES buffered RPMI 1640 medium, supplemented with L-glutamine and 0.5% HSA. All preparations contained >97% neutrophils. Isolated human neutrophils were incubated in RPMI 1640 supplemented with 0.5% HSA for 15 minutes and stimulated with or without TNFα (100U/ml) or GM-CSF (10^{-10} M) for 3 hours at 37°C, followed by RNA isolation or protein lysation as described below.

Isolation of Total RNA and PCR

Total RNA form cells was obtained using the method described by Pals et al (19). cDNA was synthesized with use of a Pro Star First Strand reverse transcription-PCR kit as described by the manufacturers protocol (Stratagene, La Jolla, USA). Relative levels of CD83 expression were measured using primers and probes for CD83 designed by the Primer Express(TM) version 1.0 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). cDNA samples were measured by real-time quantitative PCR using a Perkin-Elmer Applied Biosystems Prism 7700 sequence detection system. (For details see also Chapter 2).

Western blotting

Neutrophils (10^6 cells) were pre-incubated with several concentrations of inhibitor for 20 minutes followed by stimulation for the indicated time points, washed in ice-cold PBS and immediately lysed in Laemmli sample buffer. Total cell lysates were boiled for 5 min at 95°C and analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P. The blots for hybridization with phospho-specific antibodies were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 3% BSA for 1 hour followed by incubation with phospho-specfic antibody (1/1000) in hybridization buffer with 1% BSA for 2 hours at room temperature. Second antibody was incubated in hybridization buffer for 1 hour. The western blots for hybridization with all other antibodies were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 5% non-fat milk for 1 hour followed by incubation with antibody (1/1000) in hybridization buffer with 0.5% non-fat milk for 2 hours at room temperature. Second antibody was incubated in hybridization buffer containing 0.5% non-fat milk for 1 hour. Detection of all western blots was performed by enhanced chemiluminescence (ECL; Amersham, UK).

Statistics

Data are presented as mean ± SEM. Comparison of data was performed with using the Mann-Whitney U-test. Coefficient of variation was calculated as SD/mean. Probability values of < 0.05 were considered significant.

Results

Priming of peripheral leukocytes in COPD and controls

Peripheral venous blood was obtained from 23 patients with stable COPD (GOLD class IIa-IIb and III) and a group of 23 healthy non-smoking controls. The characteristics of the patients and controls are listed in Table 1. Figure 1 shows the priming of neutrophils and eosinophils recognized by moPhabs A17 and A27 in all COPD patients and in the healthy controls. These two human phage antibodies isolated from a synthetic bacteriophage antibody library recognize epitopes that are upregulated on neutrophils present in whole blood activated with low priming concentrations of cytokines, such as GM-CSF and TNFα (11). Surprisingly no significant differences were observed in the priming of neutrophils between COPD patients and healthy controls. A slight but significant decrease in eosinophil priming was observed in patients with stable COPD. In the COPD study group no relation between current smoking status and priming of granulocytes was observed (results not shown).

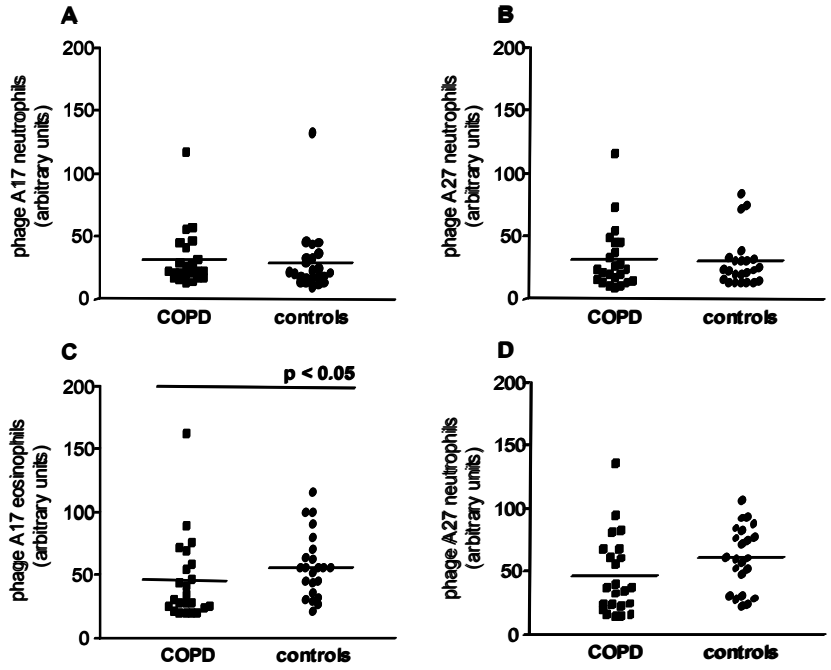

Figure 1. Expression of priming epithopes on neutrophils and eosinophils recognized by the phage antibodies A17 and A27 in patients with COPD and healthy controls. Comparison of the priming of neutrophils (COPD ■, controls □) (panels **A** and **B**) and eosinophils (COPD ●, controls ○) (panels **C** and **D**) in peripheral blood of all COPD patients irrespective of the smoking status and healthy non-smoking controls. The priming was measured with two directly labeled monoclonal phage antibodies defined A17 (**A** and **C**) and A27 (**B** and **D**). The statistic analysis was performed by the Mann-Whitney U-test.

Table 1. Patient characteristics of the cross-sectional part of the study

	COPD	Controls
number	23	23
Age, yr	62.8 ± 11.1	33.3 ± 6.4
Sex, M/F	14/9	11/10
FEV % predicted	52.9 ± 3.8	100.9 ± 2.5
VC % predicted	81.3 ± 3.5	100.7 ± 1.9
pack years	38.0 ± 4.7	-

Variable neutrophil priming in stable COPD

During the follow-up period the priming of neutrophils was highly variable in COPD patients compared to healthy controls (Fig. 2). The characteristics of the patients and controls are listed in Table 2. Figure 2 shows representative examples of the neutrophil priming in the follow-up period of three patients compared to three healthy controls. The priming of neutrophils expressed by moPhab A17 and A27 was highly variable in all six patients. No relation with individual clinical data, such as signs of exacerbations, bacterial or viral infections, colonization, increase in dyspnea and sputum production could be found. Table 4 shows the variation coefficient of leukocyte priming over a period of nine months. The variation coefficient for neutrophil priming is significantly increased in the COPD patients compared to the healthy controls.

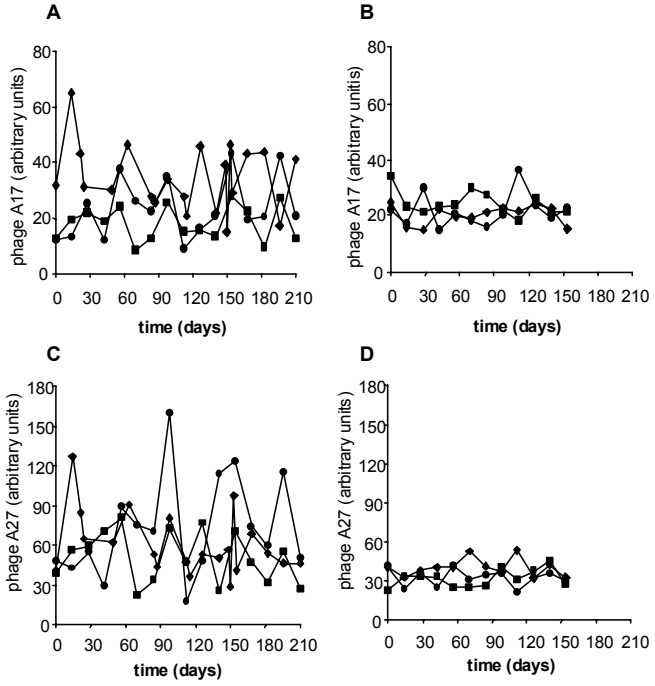

Figure 2. Highly variable expression of priming epithopes in neutrophils during follow-up of patients with COPD. Representative example of the neutrophil priming measured every two weeks in three patients with COPD (panels **A** and **C**) compared to three healthy controls (panels **B** and **D**). The monoclonal phage antibody named A17 was used in panels **A** and **B**, whereas the monoclonal phage antibody, A27, was used in panels **C** and **D**. No relation with clinical symptoms was observed; one patient shown in the graph, marked with closed circles (•) had no signs of a clinical exacerbation during the follow-up period.

Table 2. Patient characteristics of the longitudinal study

	COPD	Controls
number	6	8
age, yr	62.1 ± 4.3	34.8 ± 2.6
sex M/F	4/2	5/3
FEV % predicted	30 (19 –40)	101 (94-113)
VC % predicted	63 (46-88)	100 (86-113)
pack years	42.5 ± 6.0	-

Table 3. Coefficient of variation in COPD cohort compared to the healthy controls

	COPD cohort (SD/mean)	Control cohort (SD/mean)
A17 neutrophil	0.37 ($p < 0.05$)	0.25
A27 neutrophil	0.50 ($p< 0.0001$)	0.24
A17 eosinophil	0.49 ($p < 0.05$)	0.31
A27 eosinophil	0.54 ($p< 0.005$)	0.37

Expression of CD83 mRNA is variable in neutrophils obtained from COPD patients

Expression levels of CD83 in neutrophils can be induced by stimulation of neutrophils with cytokines such as TNFα (Chapter 2)(20). PCR was performed to determine CD83 mRNA expression in neutrophils and was visualized by agarose gel electrophoresis, as well as by real time quantitative PCR (Fig. 3 A and B). The expression of CD83 mRNA on neutrophils isolated from patients showed a high, but variable expression during the follow-up period. The two patients analyzed had no signs of an exacerbation during the observation period for CD83 shown in Figure 3. As control CD83 expression is shown in isolated neutrophils from a healthy donor unstimulated and stimulated with TNFα.

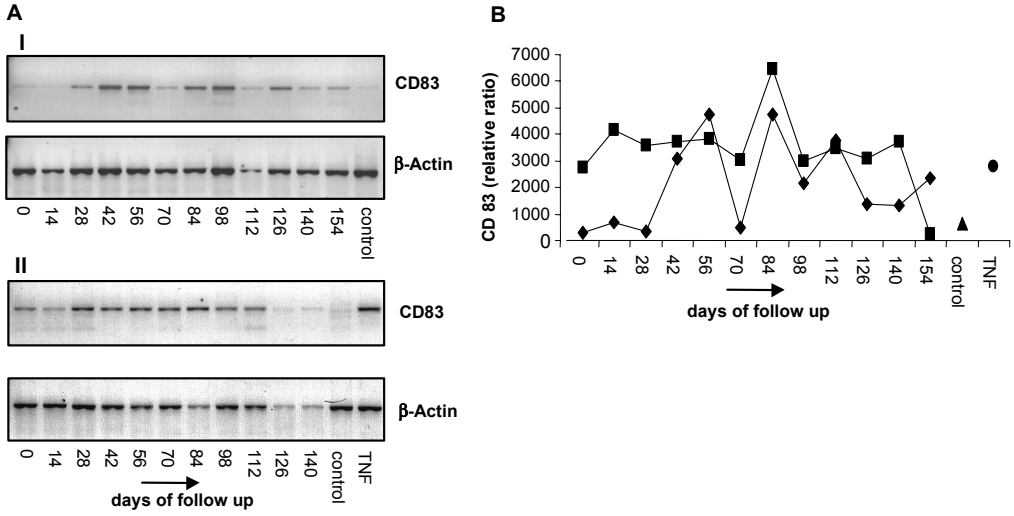

Figure 3: CD83 mRNA expression in peripheral blood neutrophils of COPD patients. In panel **A**, CD83 mRNA expression in neutrophils, during the follow-up of two patients, is visualized by gel electrophoresis, in panel **B** the quantitative expression of CD83 mRNA is measured via real-time PCR. In both panels the expression of CD83 in a healthy control is shown and also after stimulation of neutrophils with TNF (10 U/ml). Both patients had no clinical signs of an exacerbation during the period of CD83 mRNA measurement shown in the panels.

Variable activation of PKB and ERK1/2 in neutrophils of COPD patients

Some chronic inflammatory diseases are characterized by constitutive activation of intracellular signaling pathways in peripheral blood granulocytes (21). We evaluated the phosphorylation/activation status of two kinases ERK1/2 and Protein Kinase B (PKB). Both kinases require phosphorylation for activation (22). Application of phosphospecific antibodies allows the study of activation of these kinases by Western Blotting. As can be seen in Figure 4 these kinases are activated in neutrophils obtained from COPD patients. Again a high, but variable pattern of activation of ERK/PKB was found in COPD. Particularly PKB can be highly phosphorylated during the course of the disease, and phosphorylation of this kinase in non-cytokine treated cells from COPD patients can be as high as that found in control cells *in vitro* after cytokine stimulation.

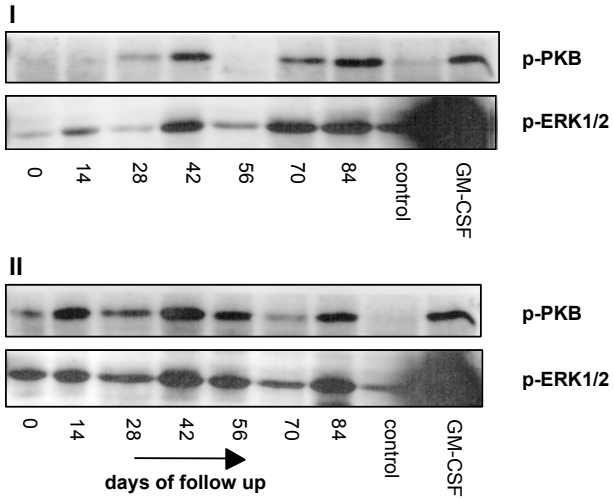

Figure 4: Phosphorylation of PKB and ERK1/2 in neutrophils of COPD patients. A representative example of the phosphorylation of PKB and ERK1/2 in two patients with COPD during the follow-up period is shown in **I** and **II**. Human neutrophils were isolated from peripheral blood of patients with COPD. Cells were lysed and western blotting was performed as described in Materials and Methods. Phosphospecific antibodies were used for ERK1/2-p and PKB-p. Also shown is kinase phosphorylation in neutrophils obtained form a healthy control and after stimulation with GM-CSF (10^{-10} M).

Discussion

The present study was undertaken to investigate chronic and systemic inflammation in COPD patients in a unique context. Analysis of leukocyte priming in peripheral blood by inflammatory mediators is an elegant method to study the kinetics of these processes. In this study we showed highly variable priming of neutrophils in patients with moderate to severe COPD, which is unrelated to individual clinical symptoms. This is in line with a hypothesis that the systemic inflammatory response in the airways of COPD patients is highly variable (18). Apparently clinical symptoms are not a good measure for the degree of systemic inflammation. In addition, this response is not very sensitive for cortico-steroids, which is in line with the findings that chronic treatment of COPD with these drugs has a limited effect (23-25).

The inflammatory changes observed in COPD patients include increased numbers of mucosal macrophages and sputum neutrophils (26, 27). The initial inflammatory insult is usually mediated by cigarette smoke. These inflammatory processes can be generated directly via cigarette smoke induced chemokine release by fibroblasts and resident tissue macrophages, and indirectly via the oxidative stress and proteases-induced damage (28). Chemokines are thought to contribute to inflammatory cell recruitment to the lungs and are, therefore, involved in the inflammatory processes in the lung parenchyma (3). These processes can occur in all smoking individuals (29) and the inflammation is to a large extent reversible upon smoking cessation (30). In patients with COPD, however, several studies showed a continuing inflammatory response (10, 26). The development of COPD in a minority of `healthy` smokers is caused by a switch to a chronic and exaggerated inflammatory response to noxious particles in cigarette smoke. More follow-up research in COPD patients as well as in `healthy` smokers has to be performed to elucidate the mechanisms underling the switch to persistent inflammation in COPD patients.

It is tempting to speculate that the continuous activation of neutrophils is critical for the occurrence of a chronic irreversible inflammatory process observed in COPD. These cells are pre-activated in the peripheral blood of more severe COPD patients (see Chapter 2) and are found in enhanced numbers in the tissue of COPD patients (31, 32). Previous studies on peripheral blood neutrophils isolated from COPD patients showed an increase in respiratory burst and the increased expression of adhesion molecules (33). Studies with bronchial biopsies and sputum induction also showed an increase in the number of neutrophils and degranulation products of neutrophils in patients with COPD (32, 34-36). This methodology for studying neutrophils and neutrophil function in COPD has many disadvantages, the induction of sputum, bronchial lavage and biopsies in patients with moderate to severe COPD (classes IIb and III, GOLD guidelines) are often not possible (37).

Despite the importance ascribed to the neutrophil in COPD, surprisingly we found no difference in the priming of peripheral neutrophils of patients as measured by expression of priming epitopes compared to controls. However, it could not be ruled out that expression of surface markers is not sensitive enough to sense alterations in peripheral blood neutrophils induced by low amounts of cytokines, such as TNFα and/or IL-8. Gene expression profiles in neutrophils with gene array technology in patients with COPD revealed an increase in mRNA's for pro-inflammatory genes such as CD83 (38). CD83 is a dendritic cell marker, which can be induced in neutrophils activated with TNF-α and IFN-γ (39). Previous studies performed in our laboratory showed an increase in CD83 mRNA expression in neutrophils obtained from COPD patients. In addition, a positive correlation was observed between neutrophil CD83 expression and FEV1 (see Chapter 2). These data demonstrate that an increase in neutrophil activation coincides with deterioration of COPD. Therefore, a longitudinal study was set up to study the kinetics of expression of inflammatory markers to obtain a better insight into inflammatory processes in patients with COPD. This longitudinal study was performed with six patients with moderate to severe COPD. Despite the best clinical treatment the neutrophil priming during this follow-up period in patients was variable and unrelated to individual clinical symptoms (Fig. 2). A similar variability was also observed for CD83 expression in neutrophils during follow-up of these patients (Fig. 3). Detailed analysis showed that the expression of CD83 mRNA significantly decreased during a clinical exacerbation of COPD (see Chapter 3). Apart from this finding we observed in this study a high but variable expression of CD83, also outside periods of a clear clinical exacerbation. This variability shows that the chronic systemic inflammatory process in COPD patients cannot be considered as a "stable" process.

It is tempting to speculate that the high variability in neutrophil priming is related to a discontinuous production of pro-and anti-inflammatory cytokines/chemokines (see also Chapter 3). To explore this hypothesis we evaluated the function of two protein kinases that are activated by both chemokines and cytokines. We have previously reported that hyperactivation of PKB in eosinophils of patients with asthma coincided with the enhanced priming status of these cells (21). Application of phosphospecific antibodies allows the study the activation status of these kinases by Western Blot analysis (22). In neutrophils stimulation with cytokines such as GM-CSF results in the phosphorylation of ERK1/2 and PKB (see figure 4 and (40-42)). In neutrophils from patients with COPD these signaling pathways were, and remained, activated after isolation and, increased phosphorylation of both PKB and ERK1/2 was observed. Again the pattern of activation was variable. The activation of both kinases has important consequences for granulocytes, particularly activation of PKB points at prolonged activation of the PI-3 kinases signaling pathway. This pathway has been shown to be essential in several granulocyte responses including inside-out control of Fc-receptors and integrins (40), priming of cytotoxicity,

enhanced survival (43) and priming of adhesion and migration (22, 44). It is tempting to speculate that neutrophils from more severe COPD are activated in a cycle of high/low priming that reflects itself in a variability of priming markers. High priming might be associated with enhanced homing, activation and survival. Low priming on the other hand might be associated by an anti-inflammatory phenotype (see Chapter 2).

References

1. Pauwels, R. A., A. S. Buist, P. M. Calverley, C. R. Jenkins, S. S. Hurd. 2001. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 163:1256-1276.
2. Hunninghake, G. W., and R. G. Crystal. 1983. Cigarette smoking and lung destruction. Accumulation of neutrophils in the lungs of cigarette smokers. *Am Rev Respir Dis* 128:833-838.
3. Sato, E., S. Koyama, A. Takamizawa, T. Masubuchi, K. Kubo, R. A. Robbins, S. Nagai, T. Izumi. 1999. Smoke extract stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activities. *Am J Physiol* 277:L1149-L1157.
4. Rennard, S. I. 1999. Inflammation and repair processes in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 160:S12-S16.
5. Rahman, I., and W. MacNee. 1996. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 21:669-681.
6. Churg, A., K. Zay, S. Shay, C. Xie, S. D. Shapiro, R. Hendricks, J. L. Wright. 2002. Acute cigarette smoke-induced connective tissue breakdown requires both neutrophils and macrophage metalloelastase in mice. *Am J Respir Cell Mol Biol* 27:368-374.
7. Repine, J. E., A. Bast, I. Lankhorst. 1997. Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Respir Crit Care Med* 156:341-357.
8. Bergmann, S., R. Siekmeier, C. Mix, W. Jaross. 1998. Even moderate cigarette smoking influences the pattern of circulating monocytes and the concentration of sICAM-1. *Respir Physiol* 114:269-275.
9. Masubuchi, T., S. Koyama, E. Sato, A. Takamizawa, K. Kubo, M. Sekiguchi, S. Nagai, T. Izumi. 1998. Smoke extract stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activity. *Am J Pathol* 153:1903-1912.
10. Vernooy, J. H., M. Kucukaycan, J. A. Jacobs, N. H. Chavannes, W. A. Buurman, M. A. Dentener, E. F. Wouters. 2002. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am J Respir Crit Care Med* 166:1218-1224.
11. Koenderman, L., D. Kanters, B. Maesen, J. Raaijmakers, J. W. Lammers, J. de Kruif, T. Logtenberg. 2000. Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. *J Leukoc Biol* 68:58-64.
12. Wouters, E. F., E. C. Creutzberg, A. M. Schols. 2002. Systemic effects in COPD. *Chest* 121:127S-130S.
13. Wouters, E. F. 2002. Chronic obstructive pulmonary disease * 5: Systemic effects of COPD. *Thorax* 57:1067-1070.
14. Saetta, M., G. Turato, F. M. Facchini, L. Corbino, R. E. Lucchini, G. Casoni, P. Maestrelli, C. E. Mapp, A. Ciaccia, L. M. Fabbri. 1997. Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. *Am J Respir Crit Care Med* 156:1633-1639.
15. Traves, S. L., S. V. Culpitt, R. E. Russell, P. J. Barnes, L. E. Donnelly. 2002. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* 57:590-595.
16. Wilson, R. C., and P. W. Jones. 1989. A comparison of the visual analogue scale and modified Borg scale for the measurement of dyspnoea during exercise. *Clin. Sci. (Lond)* 76:277-282.
17. Anthonisen, N. R., J. Manfreda, C. P. Warren, E. S. Hershfield, G. K. Harding, N. A. Nelson. 1987. Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease. *Ann. Intern. Med.* 106:196-204.
18. Wedzicha, J. A. 2002. Exacerbations: etiology and pathophysiologic mechanisms. *Chest* 121:136S-141S.

19. Pals, C. M., S. A. Verploegen, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, P. J. Coffer. 2000. Identification of cytokine-regulated genes in human leukocytes in vivo. *J Allergy Clin Immunol* 105:760-768.
20. Yamashiro, S., J. M. Wang, D. Yang, W. H. Gong, H. Kamohara, T. Yoshimura. 2000. Expression of CCR6 and CD83 by cytokine-activated human neutrophils. *Blood* 96:3958-3963.
21. Bracke, M., G. E. van de, J. W. Lammers, P. J. Coffer, L. Koenderman. 2000. In vivo priming of FcalphaR functioning on eosinophils of allergic asthmatics. *J Leukoc Biol* 68:655-661.
22. Curnock, A. P., M. K. Logan, S. G. Ward. 2002. Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology* 105:125-136.
23. Dalal, S., K. Imai, B. Mercer, Y. Okada, K. Chada, J. M. D'Armiento. 2000. A role for collagenase (Matrix metalloproteinase-1) in pulmonary emphysema. *Chest* 117:227S-228S.
24. Culpitt, S. V., W. Maziak, S. Loukidis, J. A. Nightingale, J. L. Matthews, P. J. Barnes. 1999. Effect of high dose inhaled steroid on cells, cytokines, and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 160:1635-1639.
25. Barnes, P. J. 2000. Inhaled corticosteroids are not beneficial in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 161:342-344.
26. Turato, G., A. Di Stefano, P. Maestrelli, C. E. Mapp, M. P. Ruggieri, A. Roggeri, L. M. Fabbri, M. Saetta. 1995. Effect of smoking cessation on airway inflammation in chronic bronchitis. *Am J Respir Crit Care Med* 152:1262-1267.
27. Rutgers, S. R., D. S. Postma, N. H. ten Hacken, H. F. Kauffman, T. W. van Der Mark, G. H. Koeter, W. Timens. 2000. Ongoing airway inflammation in patients with COPD who Do not currently smoke. *Chest* 117:262S.
28. Buckley, C. D., D. Pilling, J. M. Lord, A. N. Akbar, D. Scheel-Toellner, M. Salmon. 2001. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol 32* 22:199-204.
29. Churg, A., J. Dai, H. Tai, C. Xie, J. L. Wright. 2002. Tumor necrosis factor-alpha is central to acute cigarette smoke-induced inflammation and connective tissue breakdown. *Am J Respir Crit Care Med* 166:849-854.
30. Van Tiel, E., P. H. Peeters, H. A. Smit, N. J. Nagelkerke, A. J. Van Loon, D. E. Grobbee, H. B. de-. 2002. Quitting smoking may restore hematological characteristics within five years. *Ann Epidemiol* 12:378-388.
31. Maestrelli, P., M. Saetta, C. E. Mapp, L. M. Fabbri. 2001. Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 164:S76-S80.
32. Pesci, A., B. Balbi, M. Majori, G. Cacciani, S. Bertacco, P. Alciato, C. F. Donner. 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* 12:380-386.
33. Noguera, A., S. Batle, C. Miralles, J. Iglesias, X. Busquets, W. MacNee, A. G. Agusti. 2001. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax* 56:432-437.
34. Stockley, R. A. 2002. Neutrophils and the pathogenesis of COPD. *Chest* 121:151S-155S.
35. Fiorini, G., S. Crespi, M. Rinaldi, E. Oberti, R. Vigorelli, G. Palmieri. 2000. Serum ECP and MPO are increased during exacerbations of chronic bronchitis with airway obstruction. *Biomed Pharmacother* 54:274-278.
36. Stockley, R. A. 1999. Neutrophils and protease/antiprotease imbalance. *Am J Respir Crit Care Med* 160:S49-S52.
37. Saetta, M. 1999. Airway inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 160:S17-S20.
38. Iking-Konert, C., C. Wagner, B. Denefleh, F. Hug, M. Schneider, K. Andrassy, G. M. Hansch. 2002. Up-regulation of the dendritic cell marker CD83 on polymorphonuclear neutrophils (PMN): divergent

expression in acute bacterial infections and chronic inflammatory disease. *Clin. Exp. Immunol.* 130:501-508.

39. Yamashiro, S., H. Kamohara, J. M. Wang, D. Yang, W. H. Gong, T. Yoshimura. 2001. Phenotypic and functional change of cytokine-activated neutrophils: inflammatory neutrophils are heterogeneous and enhance adaptive immune responses. *J Leukoc Biol* 69:698-704.
40. Bracke, M., E. Nijhuis, J. W. Lammers, P. J. Coffer, L. Koenderman. 2000. A critical role for PI 3-kinase in cytokine-induced Fcalpha-receptor activation. *Blood* 95:2037-2043.
41. Fuhler, G. M., A. L. Drayer, E. Vellenga. 2003. Decreased phosphorylation of protein kinase B and extracellular signal-regulated kinase in neutrophils from patients with myelodysplasia. *Blood* 101:1172-1180.
42. Gomez-Cambronero, J., and C. Veatch. 1996. Emerging paradigms in granulocyte-macrophage colony-stimulating factor signaling. *Life Sci.* 59:2099-2111.
43. Nijhuis, E., J. W. Lammers, L. Koenderman, P. J. Coffer. 2002. Src kinases regulate PKB activation and modulate cytokine and chemoattractant-controlled neutrophil functioning. *J Leukoc Biol* 71:115-124.
44. Coffer, P. J., J. Jin, J. R. Woodgett. 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* 335 (Pt 1):1-13.

Chapter 5

Src kinases regulate PKB activation and modulate cytokine and chemoattractant controlled neutrophil functioning

Evert Nijhuis, Jan-Willem J. Lammers, Leo Koenderman, and Paul J. Coffer

Journal of Leukocyte Biology, 2002, 71, 115-124

Abstract

Tyrosine phosphorylation is thought to be critical in the regulation of neutrophil functioning and members of the Src-family of tyrosine kinases have recently been shown to be regulated in activated granulocytes. We have utilized a specific pharmacological inhibitor of Src-kinases, pyrazolpyrimidine 1 (PP1), to evaluate the role of Src-kinases in cytokine/chemoattractant induced regulation of neutrophil function. PP1 inhibits PKB phosphorylation, but not STAT5 phosphorylation or the activation of MAP kinases by either fMLP or GM-CSF. Pre-treatment of neutrophils with PP1 and also with the PI3K inhibitor LY294002 resulted in a strong inhibition of fMLP induced superoxide production and cytokine-mediated survival, but not fMLP induced migration. Interestingly, the kinetics of inhibition of actin polymerization and the respiratory burst are very similar. While initiation of both processes was not effected, sustained activation was inhibited by PP1. Taken together, our results demonstrate a critical role for Src-kinases in regulating neutrophil cytotoxic effector functioning through PI3K-PKB.

Introduction

Neutrophils are critical effector cells in the killing and removal of microorganisms through the regulation of specific effector functions (1). The recruitment of neutrophils from peripheral blood to the inflammatory locus is mediated by several processes, consisting of rolling, and later firm adhesion to the vascular endothelium, followed by transmigration through the endothelium and migration to the specific site (2). At the inflammatory locus, the killing of microorganisms is mediated by different cytotoxic effector mechanisms including phagocytosis, release of cytotoxic proteins, and production of toxic oxygen metabolites initiated by a membrane bound NADPH-oxidase (3). Furthermore, these leukocytes are also involved in maintaining inflammatory reactions by the release of cytokines and bioactive lipids.

A consequence of activation of neutrophils is the ability to cause tissue damage during inflammatory reactions and, therefore, the activity of neutrophils must be tightly controlled. For this reason the activation of neutrophils occurs in a multistep process. Resting neutrophils in the peripheral blood are poorly responsive to many activators including naturally occurring formyl peptides such as fMet-Leu-Phe (fMLP). However, when these cells are exposed to several pre-activating or "priming" agents such as cytokines, effector functions such as the respiratory burst, phagocytosis and degranulation are greatly enhanced (4,5). To understand the mechanism by which specific agents activate or prime granulocytes it is important to define components of signaling pathways responsible for the activation of effector functions in granulocytes.

Recently several intracellular signal transduction cascades have been found to be activated in human neutrophils in response to cytokines such as GM-CSF, as well as G-protein coupled receptor (GPCR) agonists such as PAF and fMLP. One family of proteins that has been demonstrated to be activated by many priming agents are the mitogen activated protein kinases or MAP kinases (6-10). There are three distinct groups of MAP kinases: extracellular signal-related protein kinases (ERKs) activated by a large variety of agonist, stress-activated protein kinases (JNK/SAPKs) and the p38 MAPK family. Another signal transduction pathway that appears to play a critical role in both priming and activation of granulocyte effector functions involves the lipid kinase phosphatidylinositol 3-kinase (PI3K). Utilizing specific inhibitors of PI3K, wortmannin and LY294002, ourselves and others have recently demonstrated that this kinase plays a critical role in several neutrophil effector functions including the respiratory burst and migration (7,11-13).

Despite the fact that in neutrophils cytokine and G-protein coupled receptors can activate the signaling cascades mentioned above, little is known about the specific mechanism by which these receptors initiate these cascades. During the last few years increasing evidence shows that tyrosine kinases are involved in transducing signals from both cytokine and G-protein coupled receptors to downstream signal cascades (14). In granulocytes, it has been shown that tyrosine kinases are involved in cytokine and G-

protein coupled receptor agonist signaling (15-18). In neutrophils several protein tyrosine kinases have been identified, including Janus Kinases (JAKs) and the Src kinase family. The JAKs, which are activated by cytokine receptors but not by G-protein coupled receptors, phosphorylate the STAT transcription factors (19-21). This pathway is important for linking many cytokine receptors to gene regulation but is not involved in activation of cytotoxic mechanisms. Of the family of Src kinases, several members like Lyn, Hck and Fgr are expressed in neutrophils (22,23). These Src kinase members have also been shown to be activated by several cytokine and G-protein coupled receptors (18,24,25). Data from cell lines and knock-out mice show that Src kinases are involved in many cellular processes including differentiation, adhesion/spreading, migration, apoptosis, gene transcription and cell cycle (26,27).

The development of a pharmacological Src family-selective tyrosine kinase inhibitor, PP1, has allowed the investigation of the role of this tyrosine kinase family (28). In this study we have utilized this inhibitor to evaluate the role of Src-kinases in cytokine and chemoattractant signaling regulating neutrophil functions. Our results demonstrate that Src kinases are involved in PKB but not in MAPK activation in neutrophils. Furthermore, we show that Src kinases play a role in fMLP-induced superoxide production, migration, actin polymerization and cytokine mediated survival.

Materials and Methods

Reagents and Antibodies

N-formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma (St. Louis, MO, USA). Recombinant human GM-CSF (2.5 x 10^8 U/mg) was from Genzyme (Boston, MA). Human serum albumin (HSA) and human pasteurised plasma-protein solution (40 g/L) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Hepes buffered RPMI 1640 medium with L-Glutamine and Hyclone were purchased from Life Technologies, Breda, The Netherlands. Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Polyclonal anti-phospho-PKB (ser473), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Thr182) and anti-phospho-STAT5 (Tyr694) antibodies were from New England Biolabs (Westburg, Leusden, The Netherlands). ERK-1 (C-16), ERK-2 (C-14), Hck (N-30) and c-Fgr (N-47) polyclonal antisera were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti-Lyn was purchased from Transduction Laboratories (Lexington, KY). Anti-PKB anti-sera has been described previously (29). The inhibitors PP1, PD098059, and LY294002 were from Biomol (Plymouth, PA).

Isolation of human neutrophils

Blood was obtained from healthy volunteers. Mixed granulocytes were isolated from 50-100 ml blood, which was anti-coagulated with 0.32% sodium citrate as described before (7). Blood was diluted 1.4 times with phosphate-buffered saline (PBS) containing 0.32% sodium citrate and 10% human pasteurized plasma-protein solution (40 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Pague for 20 minutes at 2000 rpm. The erythrocytes were lysed in isotonic ice-cold NH_4CL solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 minutes at 37°C in Hepes buffered RPMI 1640 medium Technologies, supplemented with L-glutamine and 0.5% HSA. All preparations contained >97% neutrophils. Before stimulation, neutrophils were resuspended in incubation buffer (20 mM Hepes pH 7.4, 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% (v/v) HSA) for 15-30 minutes at 37°C.

Lyn, Hck and Fgr kinase activity

Neutrophils were isolated as described above and incubated at 37°C for 30 minutes. After stimulation with fMLP, cells (5×10^6 cells) were lysed in 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholaat, 1 mM Na_3VO_4, 1% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF and benzamidine. Lysates were pre-cleared for 30 minutes at 4°C with protein G-sepharose and

subsequently, Lyn, Hck and Fgr kinase was immunoprecipitated with 1 μg of Lyn, Hck or Fgr antibody for 1 hour at 4°C on a rotating-wheel. To immunoprecipitate Lyn monoclonal antibody, protein G-sepharose was added for a further 1 hour at 4°C, while protein A-sepharose was used to immunoprecipitate Hck and Fgr antibodies. After washing twice with lysis-buffer and twice with wash buffer (25 mM Tris pH 7.5, 150 mM NaCl and 0.1 mM Na_3VO_4), precipitates were incubated in 30 μl kinase buffer (20 mM Hepes pH 7.5, 10 mM $MnCl_2$, 1 μM rATP, and 0.3 μCi [γ^{32}P]ATP) with increasing concentrations of PP1 for 2 minutes at room temperature. The Hck and Fgr kinase reactions contained 10 μg of enolase. Reaction was stopped by the addition of 5x Laemmli sample buffer and boiled for 5 minutes. Samples were separated by electrophoresis on 15% SDS-polyacrylamide gels. Kinase activity was detected by autoradiography.

MAP kinase activity in vitro

MAP kinase activity was measured as described before (7). In short, neutrophils were isolated as described above and incubated at 37°C for 30 minutes. After pre-treatment with PP1 or PD98059 and stimulation with fMLP or GM-CSF, cells (5×10^6 cells) were lysed in 50 mM Tris-Cl pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and benzamidine. Lysates were pre-cleared for 30 minutes at 4°C with protein A-sepharose and subsequently, MAP kinase was immunoprecipitated with 1 μg of both ERK-1/ERK-2 or p38 polyclonal antisera for 1 hour at 4°C on a rotating-wheel. Protein A-sepharose was then added for a further 1 hour at 4°C. After washing twice with lysis-buffer, samples were washed twice with kinase buffer (30 mM Tris-Cl pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM rATP, 10 μg myelin basic protein and 0.3 μCi [γ^{32}P]ATP) without ATP and MBP. Precipitates were then incubated in 25 μl kinase buffer for 20 minutes at room temperature. Reaction was stopped by the addition of 5x Laemmli sample buffer. Samples were separated by electrophoresis on 15% SDS-polyacrylamide gels. MBP phosphorylation was detected by autoradiography.

Western blotting

Neutrophils (10^6 cells) were pre-incubated with several concentrations of inhibitor for 20 minutes followed by stimulation for the indicated time points, washed in ice-cold PBS and immediately lysed in Laemmli sample buffer. Total cell lysates were boiled for 5 min at 95°C and analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P. The blots for hybridization with phospho-specific antibodies were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 3% BSA for 1 hour followed by incubation with phospho-specfic antibody (1/1000) in hybridization buffer with

1% BSA for 2 hours at room temperature. Second antibody was incubated in hybridization buffer for 1 hour. The western blots for hybridization with all other antibodies were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 5% non-fat milk for 1 hour followed by incubation with antibody (1/1000) in hybridization buffer with 0.5% non-fat milk for 2 hours at room temperature. Second antibody was incubated in hybridization buffer containing 0.5% non-fat milk for 1 hour. Detection of all western blots was performed by enhanced chemiluminescence (ECL; Amersham, UK).

F-actin measurement

Fluorescent F-actin staining was performed as described previously (30). In short, neutrophils (2.5 x 10^6 cells/ ml) were stimulated for the indicated time points and subsequently fixed and permeabilized with ice-cold 3% formaldehyde in PBS, containing 100 μg/ ml lysophosphatidylcholine for 10 minutes at room temperature. F-actin was stained with 30 U/ml NBD-phalloidin for 30 minutes at room temperature. The intracellular fluorescence was determined by FACS-analysis (FACSvantage, Becton Dickinson, San Jose, CA), by measuring a total cell count of 5000 cells per sample.

Measurement of neutrophil migration

Neutrophil migration was measured using a modification of the method according to Boyden as described before (7); using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). Chemoattractants or incubation buffer (30 μl) were added to the lower compartments. Two filters were placed between lower and upper compartments. The lower filter had a pore width of 0.45 μM (Millipore, Bedford, MA), while the upper filter (cellulose nitrate) had a pore width of 8 μM (thickness 150 μM; Sartorius, Gottingen, Germany). Before use the filters were soaked in incubation buffer. Neutrophils were placed in the upper compartment (25 μl of 2 x 10^6 cells/ml). The chambers were subsequently incubated for 1.5 hours at 37°C. The upper filters were removed, fixed in butanol/ethanol (20/80% v/v) for 10 minutes and stained with Weigert solution (composition: 1% v/v haematoxylin in ethanol mixed with a 70 mM acidic $FeCl_3$ solution at 1:1 ratio). The filters were dehydrated with ethanol, made transparent with xylene and fixed upside down. All migratory responses were quantified with an image analysis system (Quantimet 570C, Leica Cambridge Ltd.) using Quantimet 570 Control Software (QUIC version 2.02) together with custom made software. An automated microscope, Leitz DMRXE (Leica, Weitzlar, Germany) was used to step through the filters in the Z direction with 16 intervals of 10 μM. Neutrophils were counted at each level and the total migration to each level was calculated. The results are expressed as migration index, which is calculated by the cumulative migration of all intervals (μm) divided by the total number of cells multiplied by the amount of migrated cells. The mean of four randomly chosen points on each filterspot was calculated.

Measurement of NADPH-oxidase activation

Superoxide was measured by cytochrome c reduction according to a modified method described by Pick and Mizel (31). In short, neutrophils (4 x 10^6 cells/ml) were pre-incubated for 5 min at 37^{O}C in incubation buffer. Subsequently, neutrophils were pre-incubated with inhibitors and/or cytokines for the indicated periods of time. Hereafter, the cells (200 µl) were transferred to a microtitre plate in a thermostat-controlled microtitre plate reader (340 ATTC; SLT LabInstruments, Austria), mixed with cytochrome c (75 µM) and the incubation was continued for 5 min, the plates shaken every 3 seconds. The cells were then stimulated with fMLP (1 µM) and cytochrome c reduction measured every 12 seconds as an increase in absorbance at 550 nm.

Oxygen consumption was measured as describe before (32). In short, granulocytes were resuspended (3×10^6 cells/ml) in the incubation buffer and pre-incubated with GM-CSF (10^{-10}M) for 30 min. After incubation, cells were brought in a stirred and thermostated airtight vessel and inhibitor was added for 5 min. Subsequently, fMLP (1µM) was added to activate the respiratory burst and oxygen consumption was continually measured with an oxygen probe (Yellow Springs Instrument) for several minutes.

Measurement of neutrophil apoptosis

Apoptosis of neutrophils was measured by analyzing Annexin V-FITC binding (Alexis; Kordia bv, The Netherlands). In short, freshly isolated neutrophils (0.5×10^6/ml) were resuspended in Hepes buffered RPMI medium supplemented with 8% Hyclone serum. After treatment with inhibitors/cytokines, cells were incubated for the indicated times at 37 °C. At the end of incubation, cells were stored at 4°C until the last incubation time point had been reached. Subsequently, cells were washed with ice-cold PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Annexin V-FITC (1/40) was added to the cells and incubated for 10 min at room temperature. After washing, cells were resuspended in binding buffer containing 1 µg/ml propidium iodide. The fluorescence was determined by FACS-analysis (FACSvantage, Becton Dickinson, San Jose, CA), by measuring a total cell count of 10000 cells per sample.

Results

PP1 inhibits Lyn and Hck kinase activity but not STAT5 phosphorylation in neutrophils

To analyze the role of Src kinases in neutrophil signaling and effector functions we utilized the recently identified inhibitor pyrazolpyrimidine (PP1) (28). This compound has been described to specifically inhibit Src kinases including Fyn and Lck but not ZAP-70, JAK2 and EGF-R protein kinases. As previously mentioned, several members of the Src kinase family including Lyn, Hck and Fgr are expressed in neutrophils and have been shown to be activated by several cytokine and G-protein coupled receptors (18,22,23). Furthermore, a recent study found that the sensitivity of the various members of the Src kinase family to PP1 was different (33). To characterize the inhibition of Lyn, Hck and Fgr by PP1 we stimulated neutrophils with fMLP and an immunocomplex kinase assay was performed in the presence of different concentrations of PP1. Whereas Lyn and Hck demonstrated kinase activity, no Fgr kinase activity could be found in fMLP stimulated neutrophils (data not shown). As shown in Figure 1A and 1B, increasing concentrations of PP1 resulted in a concentration-dependent decrease in Lyn and Hck kinase activities.

Recently it has been suggested that Src kinases are critically involved in the activation of STAT transcription factors in many cell types (34). c-Src was found to be involved in IL-3 mediated activation of STAT3 and it has been shown that Lyn can enhance STAT5 activation (35,36). To investigate whether Src kinases may be involved in STAT activation in neutrophils, cells were pre-incubated with increasing concentrations of PP1 and subsequently stimulated with GM-CSF. After stimulation, samples were analyzed by performing a western blot utilizing an activation-specific phospho-STAT5 antibody. Figure 1C shows no inhibition of STAT5 phosphorylation by PP1 at a concentration of 50 μM PP1, demonstrating that Src kinases are not involved in GM-CSF induced STAT5 phosphorylation in neutrophils. This observation also establishes that PP1 does not aspecifically inhibit other tyrosine kinases.

Activation of MAPKs by GM-CSF or fMLP is not inhibited by PP1

Stimuli such as GM-CSF and fMLP have been described to transiently activate MAPKs in neutrophils (6-10,37). Whereas Src kinases have been proposed to be important regulators of MAPKs in several cell types (38-41), very little is known about the role of Src kinases in neutrophils. To determine whether fMLP and GM-CSF activate MAPKs via Src kinase we performed two different assays to measure the activation state of MAPKs, an *in vitro* kinase assay (Fig. 2A and C) and western blotting using phospho-specific antibodies to ERK1/2 and p38 (Fig. 2B and D respectively). Before stimulation, neutrophils were pre-incubated with increasing concentrations of PP1 for 20 minutes. As shown in Figure 2 neither ERK1/2 nor p38 kinase activities were inhibited by the highest concentration of 50

μM PP1 in both the *in vitro* kinase assay as well as the western blots using activation-specific phospho-antibodies against p38 and ERK1/2. However a specific inhibitor of MEK (PD098059), the upstream activator of ERK1/2, completely blocks ERK1/2 kinase activity. Thus in neutrophils neither the GM-CSF nor fMLP receptors require Src kinases in the regulation of ERK1/2 and p38 MAPK activation.

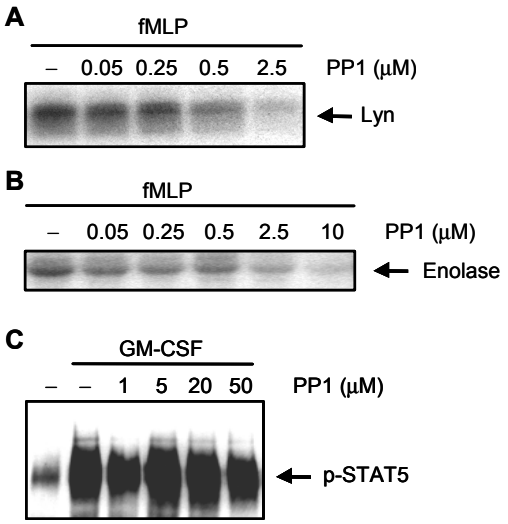

Figure 1: Src-kinase inhibitor PP1 inhibits Lyn and Hck kinase activity in vitro but not STAT5 phosphorylation in neutrophils. Neutrophils were stimulated with fMLP (10^{-6} M) for 2 min, lysed and immunoprecipitated with a Lyn or Hck antibody. Kinase assays were performed in the presence of increasing concentrations of PP1 as indicated. **(A)** Autophosphorylation of Lyn was detected by autoradiography. **(B)** Kinase activity of Hck was measured using enolase as substrate. Phosphorylation of enolase was detected by autoradiography. **(C)** Neutrophils were pre-treated with DMSO or increasing amounts of PP1 before stimulation with GM-CSF (10^{-10} M). Protein lysates were analyzed by SDS-PAGE followed by Western blotting with anti-phospho-STAT5. Data is representative of three independent experiments.

A role for Src kinases in GM-CSF- and fMLP-induced PKB activation

Recent studies have shown that both cytokines and chemoattractants activate PI3K which plays a critical role in regulating a variety of neutrophil effector functions (7,12,13,42-45). A role for tyrosine kinases in activation of the PI3K has also been proposed in neutrophils (17,18,46). To investigate the role of Src kinases in PI3K activation in neutrophils, we used the phosphorylation status of PKB, a downstream target of PI3K (29,47), as a measurement of PI3K activation. Firstly, we analyzed the ability of fMLP and GM-CSF to induce PKB activation in neutrophils using an activation-specific phospho-antibody against serine 473 of PKB. Figure 3A shows a transient phosphorylation of PKB upon either GM-CSF or fMLP stimulation. fMLP induced PKB phosphorylation is much stronger and more rapid than by GM-CSF, similar to the kinetics of MAPK and PI3K activation by these two stimuli (7,18). As shown in Figure 3B, increasing concentrations of PP1 resulted in a decrease in both GM-CSF and fMLP induced PKB phosphorylation with a half-maximal inhibition of approximately 1-5 μM of PP1 and a complete block at 5-20 μM of PP1 respectively. Addition of the specific PI3K inhibitor LY294002 (48), also completely blocks GM-CSF and fMLP induced PKB phosphorylation, indicating that PKB

activation requires PI3K. Furthermore we have found that PP1 does not directly inhibit PKB kinase activity (data not shown). Taken together, our data demonstrate that Src kinases are critical in activation of the PI3K-PKB signaling.

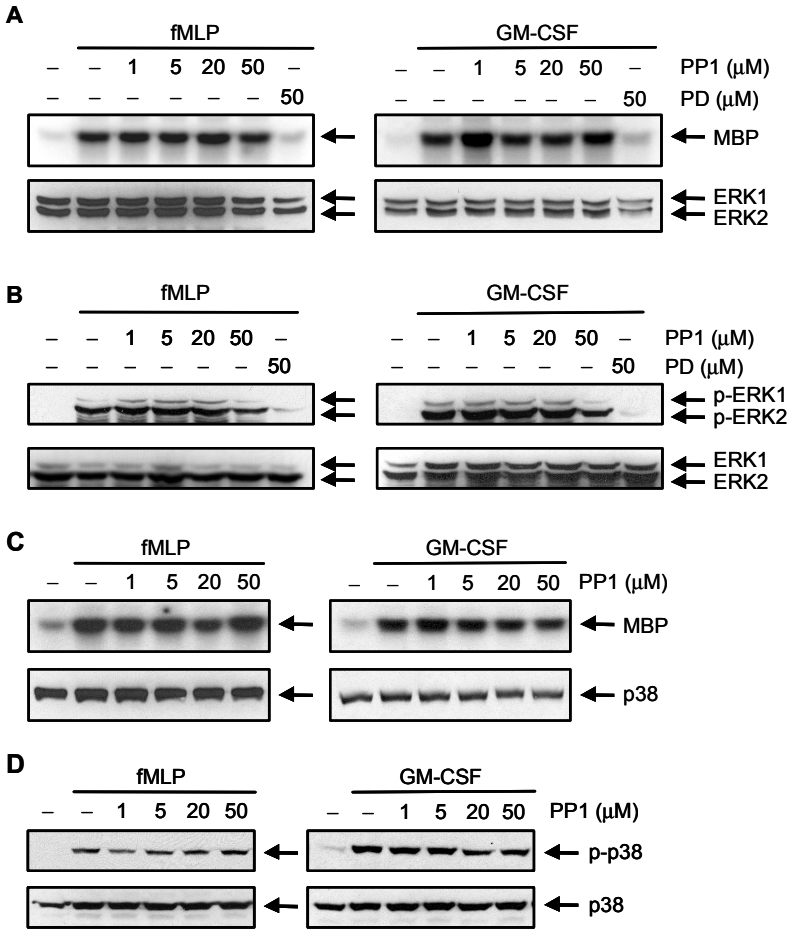

Figure 2: Activation of ERK1/2 and p38 MAPK by fMLP and GM-CSF do not require Src kinases. Freshly isolated neutrophils were pre-incubated with DMSO, PD-98059 (50 μM) or increasing concentrations of PP1 for 20 min and subsequently stimulated with GM-CSF (10^{-10} M) and fMLP (10^{-6} M) for 10 and 2 min respectively. **(A and C)** After stimulation, $5x10^6$ cells were lysed and $1/25^{th}$ was used for Western blotting with anti-ERK1/2 and anti-p38 MAPK antibodies to confirm as equal amount of protein. The remainder of the sample was used for immunoprecipitation with a mixture of ERK1 and ERK2 (1:1) antibodies or with p38 MAPK antibody. Kinase activity of both ERK1/2 and p38 MAPK was measured for 20 minutes at room temperature using Myelin Basic Protein (MBP) as substrate. Phosphorylation of MBP was detected by autoradiography. **(B and D)** Neutrophils (10^6 cells) were treated as above and immediately lysed in Laemmli sample buffer. Proteins were analyzed by SDS-PAGE followed by Western blotting with indicated antibodies. Data is representative of three independent experiments.

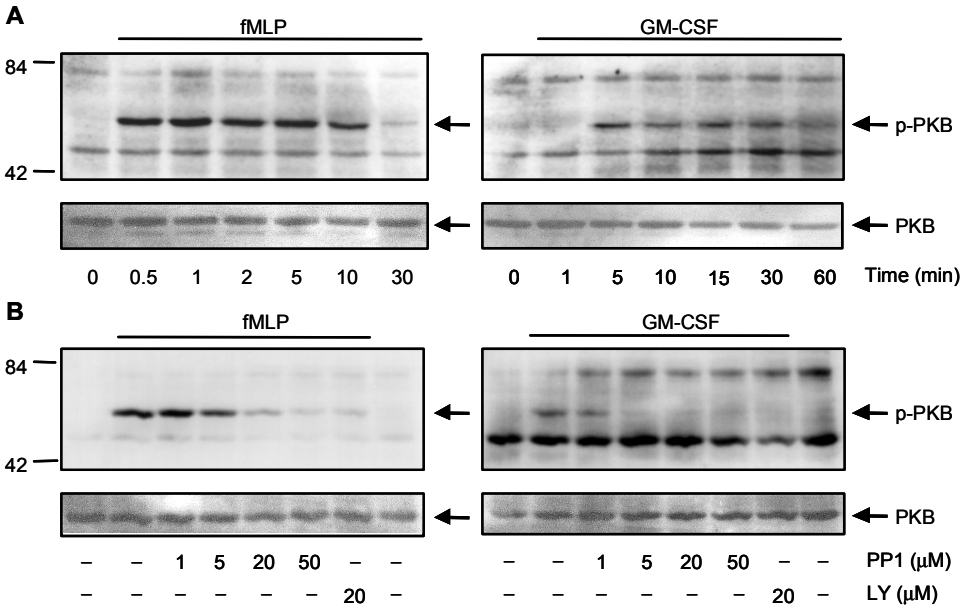

Figure 3: Src kinases are critical for fMLP and GM-CSF induced PKB activation. (A) Isolated neutrophils were incubated with GM-CSF (10^{-10} M) and fMLP (10^{-6} M) for the times indicated. After stimulation, cells were immediately lysed in Laemmli sample buffer and samples were analyzed by SDS-PAGE followed by Western blotting with anti-phospho-PKB (ser 473) or with anti-PKB. **(B)** Neutrophils were pre-treated with DMSO, increasing concentrations of PP1 or 20 μM of LY294002 followed by stimulation with GM-CSF (10^{-10} M) and fMLP (10^{-6} M) for 10 or 2 min respectively. All samples were analyzed as previous described. Data is representative of three independent experiments.

Src-kinase activity is required for activation of the respiratory burst in neutrophils

Stimulation of neutrophils with the chemotactic peptide fMLP induces the rapid formation of microbicidal oxidants, a process termed the respiratory burst and is dependent on prior priming of cells with cytokines, chemoattractants or lipopolysaccharide (1,4,5,7). Previously we have shown a role for PI3K but not ERK in this process (7). Taken this finding together with our result that PKB but not ERK activation is inhibited by PP1, suggests a possible role for Src kinases in fMLP-induced superoxide production. We analyzed the effect of pre-incubation of neutrophils with various concentrations of PP1 on the fMLP induced respiratory burst using GM-CSF as a priming agent.

As shown in Figure 4A, GM-CSF and fMLP individually do not activate the respiratory burst in neutrophils, whereas neutrophils treated with GM-CSF prior to fMLP activation show a large increase in superoxide production which is markedly decreased by both 50 μM PP1 and 20 μM LY294002. Neutrophils incubated with increasing concentrations PP1 show that the maximum decrease of superoxide production is already reached at 5 μM of

PP1 (Fig. 4B). The same decrease in fMLP-induced respiratory burst was observed in cells first primed with GM-CSF, followed by treatment with different concentrations of PP1 before finally being activated with fMLP (Fig. 4C). This demonstrates that indeed Src kinases are involved in superoxide production, but it is not apparent whether Src kinases are critical for GM-CSF mediated neutrophil priming. Interestingly, PMA, which strongly induces the respiratory burst independently of priming, is not inhibited by 50 μM of PP1 (Fig. 4D). This demonstrates that under certain conditions, activation of the respiratory burst can occur independently of Src-kinases.

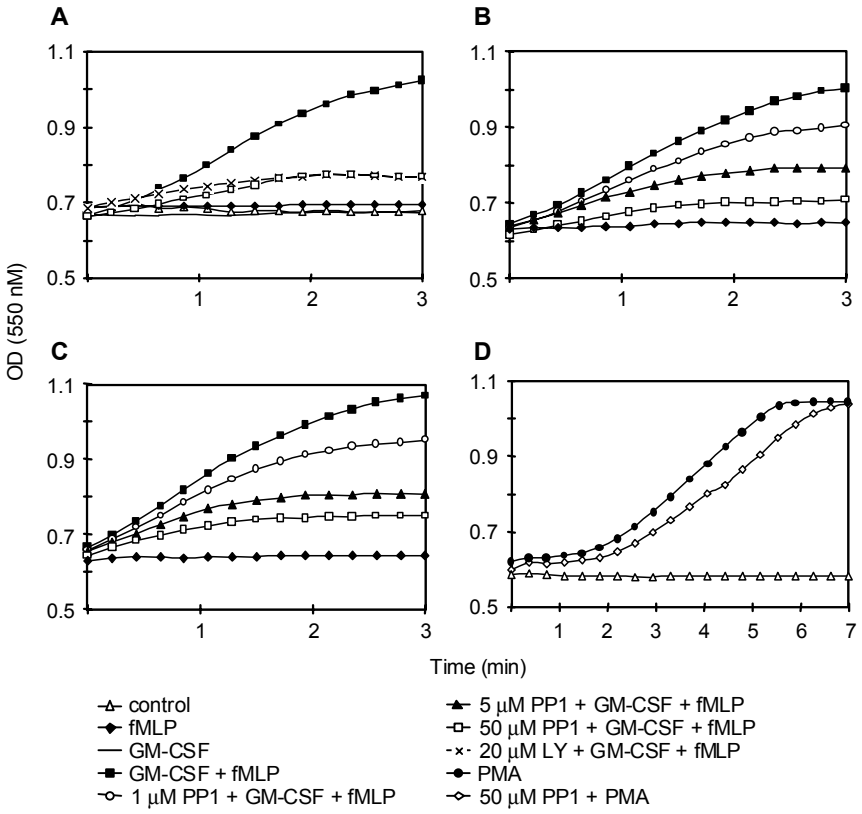

Figure 4: PP1 inhibits fMLP induced respiratory burst, but not PMA stimulated respiratory burst. (A and B) Neutrophils were pre-incubated with DMSO, increasing amounts of PP1 or 20 μM of LY294002 for 20 min before incubation of GM-CSF (10^{-10} M) for 30 min followed by stimulation with fMLP (10^{-6}). **(C)** Neutrophils were incubated with GM-CSF (10^{-10} M) for 30 min, before treatment with DMSO or increasing amounts of PP1 for 20 min followed by stimulation with fMLP (10^{-6}). **(D)** Neutrophils were pre-incubated with DMSO or with 50 μM PP1 for 20 min followed by stimulation with PMA (0.1 μg/ml). Superoxide production was monitored continuously by measurement of cytochrome c reduction. Results are expressed as the OD at a wavelength of 550 nm and are representative of four independent experiments.

Src kinases are not involved in fMLP induced neutrophil migration

To reach the site of infection, chemoattractants guide neutrophils to the inflammatory locus by a process termed chemotaxis. In order to investigate the role of Src kinases in neutrophil migration we utilized a modification of the method described by Boyden with a 48-well microchemotaxis chamber where the stimuli was placed in the lower chamber and migratory activity was measured as described previously (7). To induce migration we used the chemoattractant fMLP, which is able to potently induce neutrophil migration (Fig. 5A).

To determine the role of Src kinases in neutrophil migration, we pre-incubated neutrophils with various concentrations of PP1 before performing Boyden-chamber migration assays. As is shown in Figure 6B, fMLP induced migration is not blocked by PP1. Similar results were also obtained by incubating the cells with 20 μM of LY294002 (Fig. 5B).

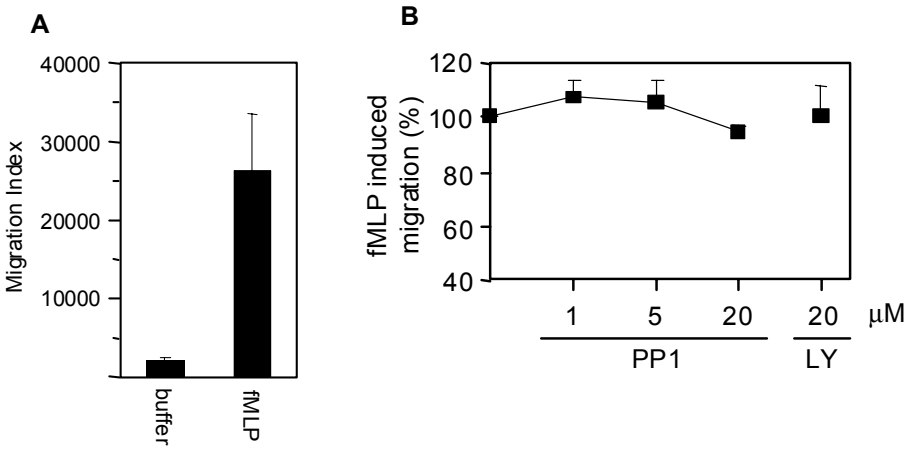

Figure 5: Src kinases are not involved in fMLP induced neutrophil migration. Neutrophil migration was monitored in microchemotaxis Boyden chambers in response to buffer or fMLP (10^{-8} M) as described in the Materials and Methods. Stimulus was placed in the lower compartment and cells were left to migrate for 1.5 hours at 37 °C. Neutrophils were pre-incubated with DMSO **(A)**, increasing amounts of PP1 ***(B)*** or 20 μM of LY294002 **(B)**. Results are expressed as migration index ±S.E.M. (n=3)

PP1 and LY294002 prevent GM-CSF mediated neutrophil survival

The life span of circulating neutrophils is relatively short compared to other leukocytes and cannot be dramatically extended (49). However, several stimuli can delay apoptosis including GM-CSF and LPS (50), whereas other stimuli, such as TNF-α, accelerate neutrophil apoptosis (51,52). In several cell lines it has been shown that the PI3K-PKB pathway plays an important role in cell survival (53). Since, we have demonstrated in this

study that Src kinases regulate PI3K-PKB activity it is reasonable to conclude that Src kinases might also play a role in inhibiting neutrophil apoptosis. To investigate the role of Src kinase and PI3K in apoptosis of neutrophils, we incubated freshly isolated neutrophils for 20 min with various concentrations of PP1 or 20 μM of LY294002 before addition of GM-CSF. To measure apoptosis we utilized Annexin-V, which binds to phosphatidylserine (PS), present on the outer leaflet of the plasma membrane only in those cells which have initiated an apoptotic program. In Figure 6A, dot plots are shown in which the lower left corner represents living cells and the lower right corner represents early apoptotic cells. Few apoptotic cells were observed in freshly isolated neutrophils whereas after 12 hours approximately 50% of the cells were apoptotic. Stimulating cells with GM-CSF results in a decrease of the number of apoptotic cells from 50% to 20%, while PP1 completely blocks this GM-CSF rescue (Fig. 6A). In Figure 6B we analyzed different time points of apoptosis using several concentrations of PP1. Figure 6B shows that neutrophils initiate the apoptotic program after 4-8 hours of isolation and that this process is not blocked but delayed by GM-CSF for approximately 4 hours. This delay is completely blocked with PP1 at a concentration of 20 μM, as well as with 20 μM of LY294002. Neutrophils treated with PP1 alone give similar kinetics for apoptosis as neutrophils without treatment (data not shown).

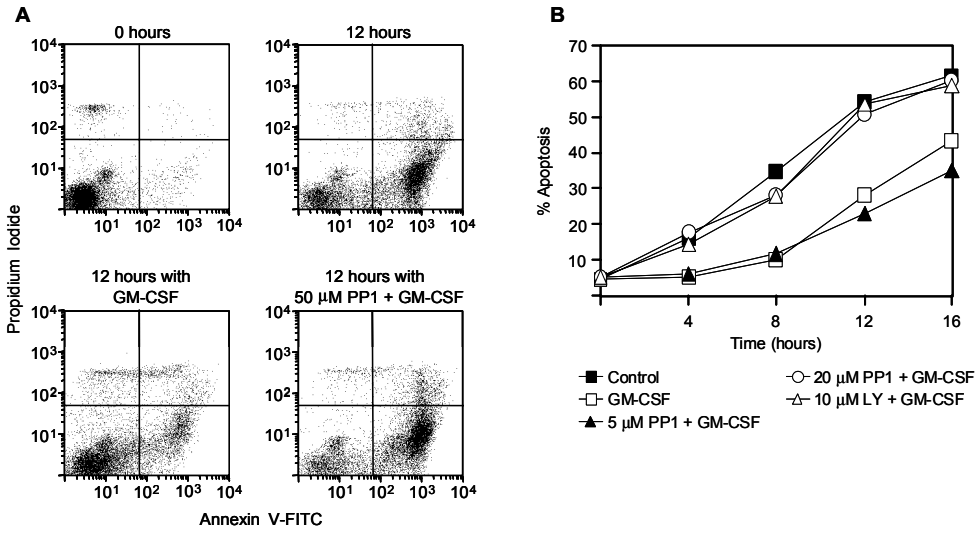

Figure 6: Src-kinases and PI3K are involved in GM-CSF mediated survival. Neutrophils were pre-incubated for 20 min with DMSO or increasing concentrations of PP1 before adding GM-CSF. Apoptosis of the cells was measured using Annexin-V in combination with propidium iodide followed by FACS analysis. Cells positive for Annexin-V represent cells in apoptosis. FACS results are presented in dot plots **(A)** or **(*B*)** cells positive for Annexin-V were plotted in a graph. Results of the graph are expressed as percentage apoptosis. (n=3)

PP1 inhibits fMLP induced actin polymerization in neutrophils with the similar kinetics as for respiratory burst

Cytoskeletal rearrangement, which involves changes in levels of filamentous actin (F-actin) is considered to be an essential event for several neutrophil effector functions including the respiratory burst and migration. In neutrophils, chemoattractants like fMLP and PAF induce a rapid increase in relative F-actin content (54,55). To determine whether Src kinases may also be involved in actin polymerization in neutrophils, cells were pre-incubated with different concentrations of PP1 and subsequently stimulated with fMLP. Actin polymerization was determined by measuring F-actin content using NBD-phalloidin followed by FACS analysis as described in Materials and Methods. Cells pre-treated with 20 μM PP1 demonstrate a similar rapid increase of F-actin as control cells, however the decline in F-actin, which normally occurs after several minutes of fMLP stimulation, occurs much more rapidly (Fig. 7A). This suggests a possible role for Src-kinases in regulating the depolymerization reaction.

A link between actin polymerization and correct functioning of the NADPH oxidase has recently been proposed (56). Furthermore, we also observe inhibition of both F-actin polymerization and superoxide production by PP1 (Fig. 4 and 7A). Utilizing an oxygen probe we analyzed the detailed kinetics of fMLP induced oxygen consumption (Fig. 7B). Interestingly, similar to actin polymerization, while the overall respiratory burst is reduced dramatically, the initial phase of activation appears to be unaffected. Although indirect this supports a link between sustained actin polymerization and activation of the respiratory burst complex.

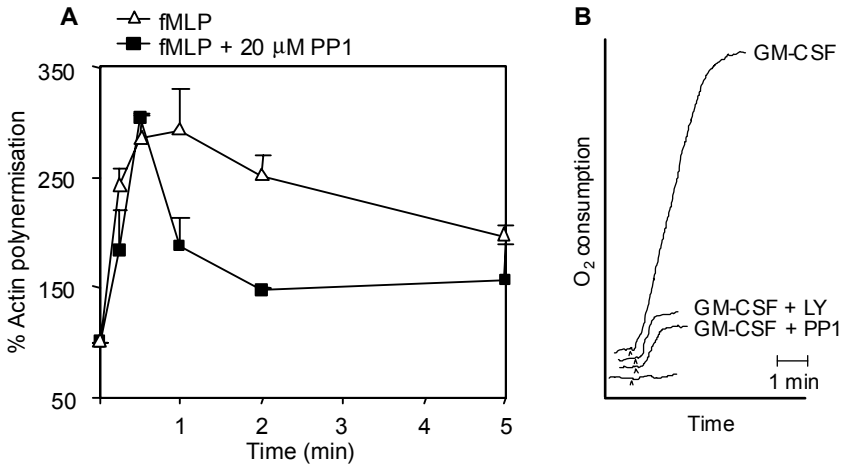

Fig. 7: Kinetics of PP1 inhibition of F-actin polymeriza-tion and oxygen consumption in neutrophils. (A), Human neutrophils were pre-treated with DMSO or PP1 (20 µM) for 20 min before stimulation with fMLP (1µM). At the indicated time-points cells were fixed an F-actin staining was performed followed by FACS analysis. The results are expressed as the relative F-actin content ±S.E.M. (n=3) **(B)**, Respiratory burst was measured as oxygen consumption by neutrophils as described in Materials and Methods. Cells were stimulated with GM-CSF (10^{-10}M) for 30 min before the addition of DMSO, 50 µM of PP1 or 20 µM of LY294002 for 5 min. Subsequently, fMLP (1µM) was added to activate the respiratory burst and oxygen consumption was continually measured for several min. ∧ Represents addition of fMLP

Discussion

During the last few years increasing evidence has demonstrated that tyrosine kinases are involved in the coupling between cytokine and G-protein coupled receptor activation of agonist effector functions in granulocytes (57-59). Among the tyrosine kinases, the family of Src kinases are activated upon stimulation of several cytokine and G-protein coupled receptors in neutrophils (18,24) and therefore might play an important role in neutrophil signaling and effector functions. Data obtained utilizing knock-out mice of different Src family members demonstrate that these kinases are involved in degranulation (60), and migration (61) in human neutrophils, whereas other studies have shown a role of Lyn in superoxide production (33) and in regulation of neutrophil survival by cytokines (62). Furthermore a link between Src kinases and PI3K (18) (63) and p38 MAPK (64) has been proposed in human neutrophils. Recently a specific Src family kinase inhibitor called PP1 has been developed (28). By using this inhibitor we have been able to determine the role of Src kinase in regulating human neutrophil signal transduction and effector functions.

Recent studies show that PP1 can inhibit several members of the Src kinase family including Fyn, Lck, Lyn, slightly Fgr, but not Hck *in vitro* (28) (33). In our study we found that Lyn kinase was slightly more sensitive to PP1 than Hck kinase (Fig. 1A and 1B). The finding that Hck is also inhibited is in contrast with a previous study (33). However in this study only 2 μM PP1, was utilized, whereas we found inhibition of Hck kinase activity between 2.5 and 10 μM PP1. Previous studies have shown that neutrophils stimulated with GM-CSF have enhanced phosphorylation of STAT1, STAT3 and STAT5 (65,66). Whereas Src kinases have recently been implicated in cytokine induced STAT activation in various cell types (34-36,67), little is known in neutrophils. In this study we have demonstrated that Src kinases are apparently not involved in GM-CSF induced STAT5 phosphorylation in human neutrophils (Fig. 1C). This also demonstrates that PP1 is not a general tyrosine kinase inhibitor since cytokine-induced tyrosine phosphorylation of STAT is not perturbed. Importantly, responses such as fMLP induced migration and also PMA activated respiratory burst, are not sensitive for the inhibitor PP1, indicating that PP1 is not simply toxic to these cells.

Both the cytokine GM-CSF and G-protein coupled receptor agonist fMLP are able to transiently activate ERK, p38 and PI3K-PKB signaling cascades in neutrophils (6-9,17,18,37,68). Currently little is known about the specific upstream signals by which receptors activate these cascades. In Figure 2 and 3B we demonstrate that PP1 inhibits PKB phosphorylation but not ERK1/2 and p38 MAPK activities upon both fMLP and GM-CSF stimulation. Our observation that PP1 does not inhibit ERK, correlates well with data showing that in neutrophils the tyrosine kinase inhibitors, genistein and erbstatin, and also PP1 do not inhibit activation of the small GTPase p21ras whose activity is critical as a regulator of ERK (69,70). In the case of activation of p38, opposing data has been published showing that fMLP stimulated neutrophils from $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice are

unable to activate p38 MAPK (64). This contradictory finding is difficult to reconcile with our data but may be due to functional differences between murine and human derived neutrophils. The inability of $Hck^{-/-}Fgr^{-/-}Lyn^{-/-}$ mice to activate p38 could also be through a variety of alternative mechanisms not directly linked to receptor mediated stimulation. Furthermore, the same study also demonstrates a partial inhibition of p38 MAPK phosphorylation with PP1 in human neutrophils. An alternate explanation for this opposing result might be due to the different concentrations of fMLP used in these assays.

We have also demonstrated that both fMLP and GM-CSF require Src kinases to activate the PI3K effector kinase PKB. It is likely that activation PI3K is mediated by Src kinases since pre-incubation of cells with either PP1 or the PI3K inhibitor LY294002 inhibited PKB activation. This is supported by data showing that a member of the Src family kinases, Lyn, can bind to the GM-CSF receptor and to the p85 subunit of PI3K in neutrophils (18,71). The observation that Src kinases are involved in fMLP-mediated activation of PKB is supported by data showing that in neutrophils stimulated with agonists of G-protein-coupled receptors, PI3K is activated through a genistein-sensitive target, presumably a protein tyrosine kinase (16,46).

The observation that Src kinases are involved in PI3K-PKB activation together with previous data showing that PI3K is involved in several neutrophil effector functions, also predicts a role for Src kinases in these processes (42). Indeed, we observed a decrease of fMLP-induced respiratory burst with PP1 incubated either before or after GM-CSF priming, but we did not observe inhibition of the PMA induced response. This demonstrates that Src kinases are involved in fMLP activation of the respiratory burst but that these kinases are not essential for the assembly of the NADPH oxidase *per se*. Furthermore, we found a similar inhibition of fMLP induced respiratory burst with LY249002 (Fig. 4A), suggesting that Src kinases may act through PI3K-PKB to regulate fMLP induced respiratory burst.

Again supporting a functional link between Src kinases and PI3K-PKB signaling, we demonstrated that both PP1 and LY294002 inhibit GM-CSF mediated survival (Fig. 6). This data is supported by a study demonstrating a role of Lyn in GM-CSF delayed apoptosis (62) and by a recent study showing that the PI3K inhibitor LY294002 blocked GM-CSF dependent PKB and BAD phosphorylation in neutrophils (72). It has been shown in several cell types that PKB can phosphorylate the pro-apoptotic Bcl-2 family member BAD, which in turn prevents its association with anti-apoptotic Bcl-2 family members by association with 14-3-3 proteins, resulting in prolonged survival (73,74). Thus it is tempting to speculate that Src-kinases act through PKB to inhibit pro-apoptotic Bcl-2 family members resulting in a delay in neutrophil apoptosis.

The effector functions, migration and superoxide production, involve cytoskeletal rearrangement requiring actin polymerization. Previous studies have shown that fMLP induces actin polymerization in neutrophils in suspension (54,75). Here we demonstrate that while the rapid induction of F-actin formation is not inhibited by PP1, the decline in

F-actin content is more rapid in PP1 treated neutrophils (Fig. 7A). This suggests that Src kinases may play a role in the stabilization and duration of actin polymerization. This finding might be considered in contrast with our data that Src kinases and PI3K do not affect fMLP induced migration, which also involves cytoskeletal rearrangement. The role of PI3K in regulating migration however is still controversial. Whereas some studies, including utilization of PI3Kγ knockout mice, show that PI3Ks play an important role in neutrophil migration (12,13,45,76), other studies demonstrate that PI3K inhibitors do not inhibit fMLP induced migration (77,78). Furthermore, the small GTPase p21Rac, which is an upstream regulator of actin polymerization, superoxide production and migration (79,80), does not appear to require Src-kinases or PI3Ks in neutrophils (76,81). This feature might be cell type specific since in several cell lines p21Rac is regulated by PI3K. Thus it might be that Src-kinases and PI3K/PKB act in parallel with p21Rac regulating actin depolymerization and superoxide production in neutrophils. The kinetics of PP1 inhibition of actin polymerization (Fig. 7A) are very similar to that of inhibition of the respiratory burst, see also Figure 4 and 7B. In both cases, PP1 does not block the initiation of the response, but rather the maintenance of the signal. One model might be that Rac is involved in initiating of actin polymerization and superoxide production, whereas Src kinases and PI3K/PKB are necessary in maintaining these responses. A recent report has shown that neutrophil NADPH oxidase could be deactivated by actin depolymerization agents in a cell-free system, suggesting that actin filaments, which grow during the activation of NADPH oxidase, prolong the lifetime of the oxidase (56). This might indicate a mechanism by which Src kinases act via PI3K/PKB to regulate fMLP-induced respiratory burst through maintenance of actin polymerization.

The difference in sensitivity of the respiratory burst and GM-CSF delayed apoptosis and also between fMLP and GM-CSF activation of PKB might suggest that different Src kinases are involved in these processes. Therefore we tested the sensitivity of Lyn and Hck kinases to PP1. However the difference between the sensitivity of Lyn and Hck to PP1 is too small to draw any definitive conclusions. Furthermore the sensitivity of Lyn and Hck to PP1 is probably different whether PP1 is added to an *in vitro* kinase assay compared to adding PP1 to cells, as has been suggested by others (28). This difference may be attributed in part to permeability of PP1 and its distribution within the cell.

The finding that Src kinases are involved in PKB phosphorylation but not in MAPK activation together with the comparison of data of the Src kinase inhibitor PP1 with those of PI3K inhibitor LY294002, suggests that Src kinases and PI3K-PKB act in the same pathway that is responsible for GM-CSF induced survival, prolonging the fMLP induced respiratory burst and regulating F-actin polymerization. Thus the regulation of Src kinase family members by both cytokine and chemoattractant receptors provides a critical upstream control point modulating human neutrophil function.

References

1. Haslett, C., Savill, J.S., Meagher, L. (1989) The neutrophil. Curr. Opin. Immunol. 2, 10-18.
2. Hallett, M.B. (1997) Controlling the molecular motor of neutrophil chemotaxis. Bioessays 19, 615-621.
3. Segal, A.W., Abo, A. (1993) The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem. Sci. 18, 43-47.
4. Downey, G.P., Fukushima, T., Fialkow, L., Waddell, T.K. (1995) Intracellular signaling in neutrophil priming and activation. Semin. Cell. Biol. 6, 345-356.
5. Coffer, P.J., Koenderman, L. (1997) Granulocyte signal transduction and priming: cause without effect? Immunol. Lett. 57, 27-31.
6. Torres, M., Hall, F.L., O'Neill, K. (1993) Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation of two distinct mitogen-activated protein-kinases. J. Immunol. 150, 1563-1577.
7. Coffer, P.J., Geijsen, N., M'Rabet, L., Schweizer, R.C., Maikoe, T., Raaijmakers, J.A., Lammers, J.W., Koenderman, L. (1998) Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. Biochem. J. 329, 121-130.
8. Waterman, W.H., Sha'afi, R.I. (1995) Effects of granulocyte-macrophage colony-stimulating factor and tumour necrosis factor-alpha on tyrosine phosphorylation and activation of mitogen-activated protein kinases in human neutrophils. Biochem. J. 307, 39-45.
9. McLeish, K.R., Knall, C., Ward, R.A., Gerwins, P., Coxon, P.Y., Klein, J.B., Johnson, G.L. (1998) Activation of mitogen-activated protein kinase cascades during priming of human neutrophils by TNF-alpha and GM-CSF. J. Leukoc. Biol. 64, 537-545.
10. Thompson, H.L., Marshall, C.J., Saklatvala, J. (1994) Characterization of two different forms of mitogen-activated protein kinase kinase induced in polymorphonuclear leukocytes following stimulation by N-formylmethionyl-leucyl-phenylalanine or granulocyte-macrophage colony-stimulating factor. J. Biol. Chem. 269, 9486-9492.
11. Kodama, T., Hazeki, K., Hazeki, O., Okada, T., Ui, M. (1999) Enhancement of chemotactic peptide-induced activation of phosphoinositide 3-kinase by granulocyte-macrophage colony-stimulating factor and its relation to the cytokine-mediated priming of neutrophil superoxide-anion production. Biochem. J. 337, 201-209.
12. Sasaki, T., Irie-Sasaki, J., Jones, R.G., Oliveira, d.S.A., Stanford, W.L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T.W., Ohashi, P.S., Suzuki, A., Penninger, J.M. (2000) Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. Science 287, 1040-1046.
13. Hirsch, E., Katanaev, V.L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., Wymann, M.P. (2000) Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. Science 287, 1049-1053.
14. Luttrell, L.M., Daaka, Y., Lefkowitz, R.J. (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. Curr. Opin. Cell Biol. 11, 177-183.
15. van der Bruggen, T., Kanters, D., Tool, A.T., Raaijmakers, J.A., Lammers, J.W., Verhoeven, A.J., Koenderman, L. (1998) Cytokine-induced protein tyrosine phosphorylation is essential for cytokine priming of human eosinophils. J. Allergy Clin. Immunol. 101, 103-109.
16. Thelen, M., Didichenko, S.A. (1997) G-protein coupled receptor-mediated activation of PI 3-kinase in neutrophils. Ann. N Y Acad. Sci. 832, 368-382.
17. Al-Shami, A., Bourgoin, S.G., Naccache, P.H. (1997) Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. I. Tyrosine phosphorylation-dependent stimulation of phosphatidylinositol 3-kinase and inhibition by phorbol esters. Blood 89, 1035-1044.

18. Corey, S., Eguinoa, A., Puyana-Theall, K., Bolen, J.B., Cantley, L., Mollinedo, F., Jackson, T.R., Hawkins, P.T., Stephens, L.R. (1993) Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src-related tyrosine kinase(s) in human myeloid derived cells. EMBO J. 12, 2681-2690.
19. Darnell, J.E.J., Kerr, I.M., Stark, G.R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415-1421.
20. Bromberg, J., Darnell, J.E.J. (2000) The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19, 2468-2473.
21. Coffer, P.J., Koenderman, L., de Groot, R.P. (2000) The role of STATs in myeloid differentiation and leukemia. Oncogene 19, 2511-2522.
22. Willman, C.L., Stewart, C.C., Longacre, T.L., Head, D.R., Habbersett, R., Ziegler, S.F., Perlmutter, R.M. (1991) Expression of the c-fgr and hck protein-tyrosine kinases in acute myeloid leukemic blasts is associated with early commitment and differentiation events in the monocytic and granulocytic lineages. Blood 77, 726-734.
23. Yamanashi, Y., Mori, S., Yoshida, M., Kishimoto, T., Inoue, K., Yamamoto, T., Toyoshima, K. (1989) Selective expression of a protein-tyrosine kinase, p56lyn, in hematopoietic cells and association with production of human T-cell lymphotropic virus type I. Proc. Natl. Acad. Sci. USA 86, 6538-6542.
24. Yan, S.R., Fumagalli, L., Berton, G. (1995) Activation of p58c-fgr and p53/56lyn in adherent human neutrophils: evidence for a role of divalent cations in regulating neutrophil adhesion and protein tyrosine kinase activities. J. Inflamm. 45, 297-311.
25. Welch, H., Maridonneau-Parini, I. (1997) Lyn and Fgr are activated in distinct membrane fractions of human granulocytic cells. Oncogene 15, 2021-2029.
26. Corey, S.J., Anderson, S.M. (1999) Src-related protein tyrosine kinases in hematopoiesis. Blood 93, 1-14.
27. Thomas, S.M., Brugge, J.S. (1997) Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. 13, 513-609.
28. Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., Connelly, P.A. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J. Biol. Chem. 271, 695-701.
29. Burgering, B.M., Coffer, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376, 599-602.
30. Schweizer, R.C., Welmers, B.A., Raaijmakers, J.A., Zanen, P., Lammers, J.W., Koenderman, L. (1994) RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5. Blood 83, 3697-3704.
31. Pick, E., Mizel, D. (1981) Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J. Immunol. Methods 46, 211-226.
32. van der Bruggen, T., Kok, P.T., Raaijmakers, J.A., Verhoeven, A.J., Kessels, R.G., Lammers, J.W., Koenderman, L. (1993) Cytokine priming of the respiratory burst in human eosinophils is Ca2+ independent and accompanied by induction of tyrosine kinase activity. J. Leukoc. Biol. 53, 347-353.
33. Yan, S.R., Novak, M.J. (1999) Src-family kinase-p53/ Lyn p56 plays an important role in TNF-alpha-stimulated production of O2- by human neutrophils adherent to fibrinogen. Inflammation 23, 167-178.
34. Smithgall, T.E., Briggs, S.D., Schreiner, S., Lerner, E.C., Cheng, H., Wilson, M.B. (2000) Control of myeloid differentiation and survival by Stats. Oncogene 19, 2612-2618.
35. Chaturvedi, P., Reddy, M.V., Reddy, E.P. (1998) Src kinases and not JAKs activate STATs during IL-3 induced myeloid cell proliferation. Oncogene 16, 1749-1758.

36. Chin, H., Arai, A., Wakao, H., Kamiyama, R., Miyasaka, N., Miura, O. (1998) Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. Blood 91, 3734-3745.
37. Yuo, A., Okuma, E., Kitagawa, S., Takaku, F. (1997) Tyrosine phosphorylation of p38 but not extracellular signal-regulated kinase in normal human neutrophils stimulated by tumor necrosis factor: comparative study with granulocyte-macrophage colony-stimulating factor. Biochem. Biophys. Res. Commun. 235, 42-46.
38. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S.A., Schlessinger, J. (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature 383, 547-550.
39. Wan, Y., Kurosaki, T., Huang, X.Y. (1996) Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors. Nature 380, 541-544.
40. Singh, R.A., Sodhi, A. (1999) Expression and activation of RAS and mitogen-activated protein kinases in macrophages treated in vitro with cisplatin: regulation by kinases, phosphatases and Ca2+/calmodulin. Immunol. Cell Biol. 77, 356-363.
41. Hayashi, T., Umemori, H., Mishina, M., Yamamoto, T. (1999) The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. Nature 397, 72-76.
42. Arcaro, A., Wymann, M.P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem. J. 296 (Pt 2), 297-301.
43. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., Ui, M. (1994) Blockage of chemotactic peptide-induced stimulation of neutrophils by wortmannin as a result of selective inhibition of phosphatidylinositol 3-kinase. J. Biol. Chem. 269, 3563-3567.
44. Vlahos, C.J., Matter, W.F., Brown, R.F., Traynor-Kaplan, A.E., Heyworth, P.G., Prossnitz, E.R., Ye, R.D., Marder, P., Schelm, J.A., Rothfuss, K.J. (1995) Investigation of neutrophil signal transduction using a specific inhibitor of phosphatidylinositol 3-kinase. J. Immunol. 154, 2413-2422.
45. Knall, C., Worthen, G.S., Johnson, G.L. (1997) Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. Proc. Natl. Acad. Sci. USA 94, 3052-3057.
46. Ptasznik, A., Prossnitz, E.R., Yoshikawa, D., Smrcka, A., Traynor-Kaplan, A.E., Bokoch, G.M. (1996) A tyrosine kinase signaling pathway accounts for the majority of phosphatidylinositol 3,4,5-trisphosphate formation in chemoattractant-stimulated human neutrophils. J. Biol. Chem. 271, 25204-25207.
47. Didichenko, S.A., Tilton, B., Hemmings, B.A., Ballmer-Hofer, K., Thelen, M. (1996) Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase. Curr. Biol. 6, 1271-1278.
48. Vlahos, C.J., Matter, W.F., Hui, K.Y., Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. 269, 5241-5248.
49. Haslett, C. (1997) Granulocyte apoptosis and inflammatory disease. Br. Med. Bull. 53, 669-683.
50. Lee, A., Whyte, M.K., Haslett, C. (1993) Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J. Leukoc. Biol. 54, 283-288.
51. Ward, C., Dransfield, I., Chilvers, E.R., Haslett, C., Rossi, A.G. (1999) Pharmacological manipulation of granulocyte apoptosis: potential therapeutic targets. TiPS 20, 503-509.
52. Murray, J., Barbara, J.A., Dunkley, S.A., Lopez, A.F., Van, O., X, Condliffe, A.M., Dransfield, I., Haslett, C., Chilvers, E.R. (1997) Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. Blood 90, 2772-2783.
53. Datta, S.R., Brunet, A., Greenberg, M.E. (1999) Cellular survival: a play in three Akts. Genes Dev. 13, 2905-2927.

54. Howard, T.H., Oresajo, C.O. (1985) The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution, and the shape of neutrophils. J. Cell. Biol. 101, 1078-1085.
55. Shalit, M., Dabiri, G.A., Southwick, F.S. (1987) Platelet-activating factor both stimulates and "primes" human polymorphonuclear leukocyte actin filament assembly. Blood 70, 1921-1927.
56. Tamura, M., Kanno, M., Endo, Y. (2000) Deactivation of neutrophil NADPH oxidase by actin-depolymerizing agents in a cell-free system. Biochem. J. 349, 369-375.
57. Naccache, P.H., Gilbert, C., Caon, A.C., Gaudry, M., Huang, C.K., Bonak, V.A., Umezawa, K., McColl, S.R. (1990) Selective inhibition of human neutrophil functional responsiveness by erbstatin, an inhibitor of tyrosine protein kinase. Blood 76, 2098-2104.
58. Yousefi, S., Green, D.R., Blaser, K., Simon, H.U. (1994) Protein-tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils. Proc. Natl. Acad. Sci. USA 91, 10868-10872.
59. McColl, S.R., DiPersio, J.F., Caon, A.C., Ho, P., Naccache, P.H. (1991) Involvement of tyrosine kinases in the activation of human peripheral blood neutrophils by granulocyte-macrophage colony-stimulating factor. Blood 78, 1842-1852.
60. Mocsai, A., Ligeti, E., Lowell, C.A., Berton, G. (1999) Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck. J. Immunol. 162, 1120-1126.
61. Lowell, C.A., Berton, G. (1998) Resistance to endotoxic shock and reduced neutrophil migration in mice deficient for the Src-family kinases Hck and Fgr. Proc. Natl. Acad. Sci. USA 95, 7580-7584.
62. Wei, S., Liu, J.H., Epling-Burnette, P.K., Gamero, A.M., Ussery, D., Pearson, E.W., Elkabani, M.E., Diaz, J.I., Djeu, J.Y. (1996) Critical role of Lyn kinase in inhibition of neutrophil apoptosis by granulocyte-macrophage colony-stimulating factor. J. Immunol. 157, 5155-5162.
63. Yu, W., Cassara, J., Weller, P.F. (2000) Phosphatidylinositide 3-kinase localizes to cytoplasmic lipid bodies in human polymorphonuclear leukocytes and other myeloid-derived cells. Blood 95, 1078-1085.
64. Mocsai, A., Jakus, Z., Vantus, T., Berton, G., Lowell, C.A., Ligeti, E. (2000) Kinase pathways in chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein kinase activated by Src family kinases. J. Immunol. 164, 4321-4331.
65. Al-Shami, A., Mahanna, W., Naccache, P.H. (1998) Granulocyte-macrophage colony-stimulating factor-activated signaling pathways in human neutrophils. Selective activation of Jak2, Stat3, and Stat5b. J. Biol. Chem. 273, 1058-1063.
66. Brizzi, M.F., Aronica, M.G., Rosso, A., Bagnara, G.P., Yarden, Y., Pegoraro, L. (1996) Granulocyte-macrophage colony-stimulating factor stimulates JAK2 signaling pathway and rapidly activates p93fes, STAT1 p91, and STAT3 p92 in polymorphonuclear leukocytes. J. Biol. Chem. 271, 3562-3567.
67. Kazansky, A.V., Kabotyanski, E.B., Wyszomierski, S.L., Mancini, M.A., Rosen, J.M. (1999) Differential effects of prolactin and src/abl kinases on the nuclear translocation of STAT5B and STAT5A. J. Biol. Chem. 274, 22484-22492.
68. Ding, J., Vlahos, C.J., Liu, R., Brown, R.F., Badwey, J.A. (1995) Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. J. Biol. Chem. 270, 11684-11691.
69. Zheng, L., Eckerdal, J., Dimitrijevic, I., Andersson, T. (1997) Chemotactic peptide-induced activation of Ras in human neutrophils is associated with inhibition of p120-GAP activity. J. Biol. Chem. 272, 23448-23454.
70. M'Rabet, L., Coffer, P.J., Wolthuis, R.M., Zwartkruis, F., Koenderman, L., Bos, J.L. (1999) Differential fMet-Leu-Phe- and platelet-activating factor-induced signaling toward Ral activation in primary human neutrophils. J. Biol. Chem. 274, 21847-21852.
71. Adachi, T., Pazdrak, K., Stafford, S., Alam, R. (1999) The mapping of the Lyn kinase binding site of the common beta subunit of IL-3/granulocyte-macrophage colony-stimulating factor/IL-5 receptor. J. Immunol. 162, 1496-1501.

72. Klein, J.B., Rane, M.J., Scherzer, J.A., Coxon, P.Y., Kettritz, R., Mathiesen, J.M., Buridi, A., McLeish, K.R. (2000) Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. J. Immunol. 164, 4286-4291.
73. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91, 231-241.
74. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., Nunez, G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278, 687-689.
75. Wang, D.H., Berry, K., Howard, T.H. (1990) Kinetic analysis of chemotactic peptide-induced actin polymerization in neutrophils. Cell Motil Cytoskeleton 16, 80-87.
76. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.V., Wu, D. Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction.
77. Thelen, M., Uguccioni, M., Bosiger, J. (1995) PI 3-kinase-dependent and independent chemotaxis of human neutrophil leukocytes. Biochem. Biophys. Res. Commun. 217, 1255-1262.
78. Harakawa, N., Sasada, M., Maeda, A., Asagoe, K., Nohgawa, M., Takano, K., Matsuda, Y., Yamamoto, K., Okuma, M. Random migration of polymorphonuclear leukocytes induced by GM-CSF involving a signal transduction pathway different from that of fMLP.
79. Hall, A. (1998) G proteins and small GTPases: distant relatives keep in touch. Science 280, 2074-2075.
80. Ambruso, D.R., Knall, C., Abell, A.N., Panepinto, J., Kurkchubasche, A., Thurman, G., Gonzalez-Aller, C., Hiester, A., deBoer, M., Harbeck, R.J., Oyer, R., Johnson, G.L., Roos, D. (2000) Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. Proc. Natl. Acad. Sci. USA 97, 4654-4659.
81. Geijsen, N., van Delft, S., Raaijmakers, J.A., Lammers, J.W., Collard, J.G., Koenderman, L., Coffer, P.J. (1999) Regulation of p21rac activation in human neutrophils. Blood 94, 1121-1130.

Chapter 6

Intracellular modulation of FcαRI function by Rac

Evert HJ. Nijhuis, Madelon Bracke, Hanneke van Deutekom, Deon Kanters, Jan van der Linden, Leo Koenderman, and Paul J. Coffer

In preparation

Abstract

The small GTPase Rac has been implicated in various cellular processes, including cytoskeletal reorganisation, chemotaxis, proliferation and the production of reactive oxygen species. However, little is known about the precise role of Rac in human granulocyte functioning. Here we have investigated downstream Rac-dependent functions by introducing a constitutively active Rac(Q61L) into human eosinophils. Rac(Q61L) fused to a Tat protein-transduction domain entered cells rapidly and with a high efficiency. Introduction of Rac(Q61L) into eosinophils did not itself lead to the induction or enhancement of superoxide production or migration. However, we found that Rac(Q61L) induced IgA binding to the Fcα Receptor (CD89), a process normally requiring cytokine mediated inside-out signaling. This finding was confirmed in Ba/F3 cells stably overexpressing constitutively active Rac(G12V). Since the regulation of ligand binding to Fc receptors on eosinophils is similar to that of integrins, we also investigated the activation of integrins by Rac(Q61L). However, STZ binding, which is mainly mediated by the CR3 integrin, was not increased by Rac(Q61L). Utilizing the technique of fluorescence recovery after photobleaching, we further demonstrated that that the lateral mobility of the FcαRI in the plasma membrane is decreased by constitutively active Rac or IL-3. This is not due to a decrease of the rate of diffusion, but to an increase in the immobile pool of FcαRI. These results show that Rac activation in eosinophils is not itself sufficient for priming or activation of superoxide production, but is sufficient for the modulation of FcαRI resulting in enhanced ligand binding.

Introduction

Granulocytes are critical effector cells for the killing and removal of microorganisms through specific effector functions, such as phagocytosis, degranulation, and activation of the respiratory burst (1). A consequence of granulocyte activation is the ability to cause tissue damage, as is often seen at inflammatory loci. Therefore, the regulation of granulocyte activation must be a tightly controlled, multistep process. Resting granulocytes in the peripheral blood are poorly responsive to many physiological activators (2-4). When these cells encounter pre-activating mediators, effector functions are greatly potentiated in a process generally referred to as "priming" (5, 6). Genetic defects, such as hereditary chronic granulomatous disease (CGD), resulting from a defect in the oxidase system (7, 8) and leukocyte adhesion deficiency (LAD) results in abnormal neutrophil functioning and immunodeficiency (9). Patients with disorders of neutrophil functioning suffer from severe infections, which shows the importance of correct granulocyte functioning for normal host defence.

To function appropriately in response to extracellular signals, granulocytes use a plethora of intracellular signal transduction pathways to link receptors for extracellular stimuli with effectors in the cytoplasm and nucleus. The Rho family of GTPases, members of the Ras superfamily of small signalling molecules, has been implicated in the control of neutrophil functioning. One of the members of the Rho family, Rac, has been implicated in several granulocyte effector functions (10-12). Rac has three isoforms, of which Rac1 is ubiquitous expressed, Rac2 is predominantly expressed in hematopoietic cells, and Rac3 in heart, placenta, brain, kidney and pancreas. Utilising overexpression of dominant-negative and constitutively active mutants in cell lines, Rac has been implicated in cellular processes including migration, superoxide production, cytoskeletal reorganisation and proliferation (13-18).

Until recently, the inability to manipulate granulocytes by standard molecular biological techniques, due to their short-life and quiescent state, has made it difficult to investigate the specific role of signalling pathways eosinophils and neutrophils. The role of Rac in primary cells has thus mainly been investigated in Rac-deficient animal models, which have implicated Rac2 in neutrophil migration and respiratory burst (11, 14). Furthermore, evidence suggesting for a role of Rac in migration and respiratory burst in human neutrophils has been provided by analysis of a patient with neutrophil immunodeficiency as a result of a Rac2 mutation, resulting in production of a dominant-negative protein (19, 20). We have used a recently developed technique of protein transduction for elucidating the role of Rac in eosinophil functioning.

Protein transduction methodology has previously been used with success in eosinophils (21, 22) to investigate the role of signal transduction molecules in effector functions. The protein transduction domain of the HIV Tat protein (YGRKKRRQRRR) (23-26), was fused to the gene coding for an activating Rac with point mutation (Q61L), resulting in a

constitutively active Rac (27, 28). Incubation of this fusion protein with eosinophils resulted in Tat-Rac(Q61L) rapidly entering eosinophils (within 5 minutes) and efficienct (±99%). Introduction of Tat-Rac(Q61L) into eosinophils was not sufficient to activate or enhance superoxide production or migration. Interestingly, constitutively active Rac alone resulted in enhanced IgA binding to FcαR, a process that normally requires prior cytokine-stimulation. In contrast, Rac was not found to be involved in CR3 integrin (CD11b/CD18) activation. These findings demonstrate a novel and specific function for Rac in eosinophils.

Materials and methods

Reagents

Human serum albumin (HSA) was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Purified human serum IgA (>20 mg/ml) was obtained from Cappel (Malvern, PA). It contained no detectable trace of IgG, IgM or non-immunoglobulin serum proteins. Recombinant human IL-5 was a gift from Dr M. McKinnon, GlaxoSmithKline (Stevanage, UK). Recombinant mouse IL-3 was produced in COS cells (29). STZ was prepared as described (30). PAF and 3-amino propyl-triethoxysilane was purchased from Sigma (St. Louis, MO, USA). Tat-Rac(Q61L) vector was a kind gift of Dr. S. Dowdy (Howard Hughes Medical Institute, Dept. of Pathology, Washington University School of Medicine, St Louis, Missouri 63110, USA)

Isolation of human eosinophils

Blood was obtained from healthy volunteers. Mixed granulocytes were isolated from 50-100 ml blood, which was anti-coagulated with 0.32% sodium citrate as described before (31). Blood was diluted 1.4 times with a PBS2+ buffer (phosphate-buffered saline PBS containing 0.32% sodium citrate and 10% human pasteurised plasma-protein solution (40 g/L)). Mononuclear cells were removed by centrifugation over Ficoll-Pague. The erythrocytes were lysed in isotonic ice-cold NH_4CL solution followed by centrifugation at 4°C. Granulocytes were washed with PBS2+ buffer and eosinophils were purified from granulocytes by negative immunomagnetic selection with anti-CD16-conjugated microbeads (MACS;Miltenyi Biotec,(32)). To avoid mononuclear cell contamination also anti-CD3- and anti-CD14-conjugated microbeads were added to the granulocyte suspension. Purity of eosinophils was >97%. Before stimulation, eosinophils were resuspended in incubation buffer (20 mM Hepes pH 7.4, 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% (v/v) HSA) for 15-30 minutes at 37°C.

Tat-Rac(Q61L) protein isolation

A polyhistidine-tagged Tat-RacQ61L construct was expressed in BL21 bacteria (Novagen, Madison, WI). Expression and isolation of Tat-RacQ61L was performed similarly as has been described by Hall et al (21), except that the isolation of the protein was performed under non-reducing conditions. Briefly, expression of Tat-RacQ61L in bacteria was induced by IPTG for 3-4 hours at 37 C, before the bacteria pellet was solubilized in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1mM PMSF, pH 8.0). Lysozyme (1mg/ml) and DNAse I (5 µg/ml) was added to the bacteria solution and incubated for 30 minutes on ice. Solution was sonicated for 6-8

times for 10 seconds each, and centrifuged twice at 15 000 RPM for 20 minutes. Supernatant containing the fusion protein was added to Ni-NTA beads and rotated for 30 minutes at 4°C. Beads were incubated with lysis buffer, twice with wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.5% Tween-20, 10% glycerol, pH 8.0) and 3-4× with wash buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.5% Tween-20, 10% glycerol, pH 8.0). Protein was eluted from the beads with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, 0.5% Tween-20, 10% glycerol, pH 8.0). LPS was removed from the protein elution by a method described previously (33). In short, 0.1 volume of LPS removal buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol, 10% Triton X-114, pH to 7.5) was added and rotated at 4°C for 10 minutes. After transferring the solution to 37°C for 5 minutes, solution was centrifuged at 3000 RPM for 5 minutes. Upper layer was transferred to a new tube and the procedure was repeated 4× and twice with the LPS removal buffer without Triton X-114. Imidazole, and last traces of Triton X-114, were removed from the resultant protein solution by dialysing twice against PBS (pH 7.4) containing 10% glycerol for at least 4 hours at 4ºC each time, using Slide-A-Lyzer dialysis cassette (extra strength), 3-12ml, MW cut off is 10 kDa, (Pierce). Protein concentration was measured and purity of Tat-RacQ61L was approximately 95%, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Coomassie blue staining. Protein expression of the Tat-RacQ61L protein was confirmed by SDS-PAGE and immunoblotting using anti-HA and anti-Rac antibodies. Protein solution was aliquoted and frozen at –80°C

Generation of stable transfectants

Ba/F3 cells were cultured at a cell density of 10^5-10^6 cells/ml in RPMI 1640 supplemented with 8% Hyclone serum (Gibco) and recombinant mouse IL-3. Ba/F3_FcαRI cells, expressing FcαRI-VSV, were generated as previously described by Bracke et al (34). Furthermore, FcαRI was tagged with EGFP and transfected in Ba/F3 cells to generate stable Ba/F3_FcαRI_EGFP cells. Subsequently Ba/F3_FcαRI cells were used for transfection of pLXSN (neo) containing the Rac(G12V) mutant. Cells were cultured with mouse IL-3 and 500 mg/ml G418 (Bhoeringer Mannheim, Germany) to select resistance. Stable cells were grown continually on mouse-IL-3, G418 and hygromycin. Expression of FcαRI was analyzed regularly by FACS.

Transduction of eosinophils and Ba/F3 cells by Tat-RacQ61L

Tat-RacQ61L, or a control protein (bovine serum albumin, BSA), was labeled with FITC (1:3 wt/wt) in PBS with 100 mM $NaHCO_3$ (pH9.0) for 1 hour at room temperature. Excess of free FITC was removed by dialysis with PBS. Eosinophils and Ba/F3 cells were attached to poly-L-lysine coated coverslips and incubated in incubation buffer containing 10 μg/ml FITC labeled Tat-Rac61L or BSA for 5 min. After washing the coverslips with

incubation buffer without FITC-labeled proteins, cells were fixed with 1% of paraformaldehyde. Presence of FITC-labeled protein in eosinophils and Ba/F3 cells was measured by confocal laser scanning microscopy.

IgA binding assays

IgA-binding assays were performed either with purified human eosinophils or with cytokine starved Ba/F3_FcαRI cells as described previously (34). Briefly, for IL-3 starvation, Ba/F3_FcαRI cells were washed twice with PBS and left in medium (RPMI 1640 with 0.5% HY clone) without IL-3 for 4 hrs. Prior to performing a binding assay, Ba/F3_FcαRI cells or purified eosinophils were washed with Ca^{2+}-free incubation buffer containing 0.5 mM EGTA and brought to a concentration of 8×10^6 cells/ml. Ba/F3_FcαRI cells (0.4×10^6 cells) were incubated with Tat-Rac(Q61L) (20 μg/ml) or with IL-3 for 15 min, whereas eosinophils were activated with either Tat-Rac(Q61L) (20 μg/ml), PAF (10^{-6}M) or IL-5 (10^{-9}M) for 15, 5 and 15 min respectively at 37°C. After stimulation of the cells, dynabeads pre-coated with serum IgA (10 mg/ml), were added in a ratio of 3.5 beads/cell and incubated for 20 min for PAF, and 30 min for Tat-Rac(Q61L), IL-5, or IL-3 treated cell. After incubation, cells were resuspended vigorously and IgA-binding was evaluated under a microscope. All cells that had bound two beads or more were defined as rosettes. One hundred cells (bound and unbound to beads) were counted and the amount of beads bound to rosettes was designated as the rosette index.

Fluorescence recovery after photobleaching (FRAP) assay

Ba/F3_FcαRI-YFP cells were cytokine starved and attached to poly-L-lysine coated coverslips. Cells were incubated with 20 μg/ml Tat-Rac61L, with IL-3 (1:1000) or with incubation buffer for 15 min at 37°C. FRAP was performed on a Zeiss LSM510 META confocal microscope equipped with an Argon/ML laser, and images were acquired with standard YFP settings (512 x 512, 16 bit deep 2x line-averaged images). All FRAP experiments were performed at 37°C. In each individual experiment, about 20 cells were partially bleached using with the following protocol. The first two frames were taken as pre-bleach images, and following bleaching for 40 iterations with maximal laser power, time lapse sequences of 50 images with an interval of 3.2 seconds were taken. Analysis was done with image analysis software (Optimas 6.1, Media Cybernetics, Silver Spring, Maryland U.S.A.) using custom written macros. Background bleaching during the course of imaging was corrected for bleaching profiles were fitted with Sigma plot (version 4.0, SPSS Inc, Chicago, Illinois U.S.A.) using the following equation: $y = y_0 + a(1 - e^{-bx})$ where *a* is the asymptotic plateau and *b* the recovery time constant.

Migration assay

Migration experiments were performed as described previously (22). Glass coverslips (0.3 mm) were treated with 3-amino propyl-triethoxysilane, washed with PBS, blocked with 0.5% glutaraldehyde for 30 min at room temperature and coated with fibronectin (100 μg/ml). Eosinophils, suspendend in incubation buffer, were allowed to attach to the coverslip for 15 minutes at 37°C. Medium was removed and the cells were washed twice with incubation buffer. The coverslip was inverted in a droplet of incubation buffer containing PAF (10^{-7}M) and sealed with a mixture of beeswax, paraffin and pertroleum jelly (1:1:1, wt/wt/wt). Cell tracking was monitored by time-lapse microscopy at 37 C and analyzed by custum-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 capturing an image every 20 seconds.

STZ binding assay

STZ binding was measured as previously described (35). STZ particles were stained green fluorescent with FITC (1000:1, wt/wt) in a buffer containing 100 mM $NaHCO_3$ (pH 8.5) for 30 minutes at room temperature. Eosinophils (4×10^6 cells/ml) were resuspended in incubation buffer. The FITC coupled STZ particles retained the same ability to stimulate the respiratory burst as did the orginal STZ particles (data not shown). Eosinophils were resuspended in the incubation buffer (3×10^6 cells/ml) and brought in a stirred and thermostated airtight vessel. Cells were stimulated with Tat-Rac(Q61L) (20 μg/ml), or with PAF (10^{-6}M) for 15 and 5 min respectively. Subsequently, STZ-FITC (1mg/ml) was added and after 5 min a 100 μl cell aliquot was taken, fixed with ice-cold paraformaldehyde (1% w/v) and analyzed in a FACScan flowcytometer (Becton Dickinson). Eosinophils were gated from the free STZ-FITC particles and fluorescent eosinophils (that had bound at least one particle) were expressed as percentage of total number of eosinophils. This percentage is an underestimation of the amount of cells that actually have bound an STZ-FITC particle, because an aggregate of cells (bridged by STZ-FITC particles) is detected as a single double-coloured event.

Measurement of NADPH-oxidase activation

Oxygen consumption was measured as previously described (36). In brief, granulocytes were resuspended in the incubation buffer (3×10^6 cells/ml) and transferred to in a stirred and thermostated airtight vessel. Cells were stimulated with Tat-Rac(Q61L) (20 μg/ml), with PAF (10^{-6}M) or with incubation buffer for 15, 15 and 5 min respectively. Subsequently, STZ (1mg/ml) was added to activate the respiratory burst, and oxygen consumption was continually measured with an oxygen probe (Yellow Springs Instrument) for several minutes.

Results

Transduction of cells with FITC-labeled Tat-Rac(Q61L)

Until recently, it was not possible to utilize standard molecular biological techniques to investigate the function of specific signaling molecules in human granulocytes. Recently, however, the novel technique of protein-transduction has been utilized to introduce protiens into eosinophils at an efficiency of more than 95%. Proteins are fused to 11 amino acids of the HIV Tat protein, a protein transduction domain known to allow fusion-proteins to enter mammalian cells in a receptor independent way (37). We utilized Rac(Q61L) a constitutively active form of Rac, which was fused with the Tat-protein transduction domain. This was expressed in bacteria, and isolated as previously described (21). Due to protein aggregation during the isolation, we adapted the method and performed the isolation under non-reducing conditions (see materials and methods). To determine whether Tat-Rac(Q61L) fusion protein entered cells, protein was labeled with FITC and incubated with eosinophils, or a bone-marrow derived B-cell line Ba/F3, for 5 minutes at 37°C. Localization of FITC labeled Tat-Rac(Q61L) was analyzed by confocal microscopy. As shown in Figure 1, both eosinophils and Ba/F3 cells, incubated with FITC-labeled Tat-Rac(Q61L), demonstrated strong intracellular fluorescence. FITC labeled Tat-Rac(Q61L) entered cells in a rapid manner, as has previously been reported (21). In addition, transduction of these cells by FITC labeled Tat-Rac(Q61L) was maintained for at least 90 minutes (data not shown). In contrast, incubation of eosinophils or Ba/F3 cells with FITC-bovine serum albumin, not linked to the Tat peptide, resulted in no intracellular staining, demonstrating only the Tat-linked fusion protein are capable of entering cells (Fig. 1; upper panels).

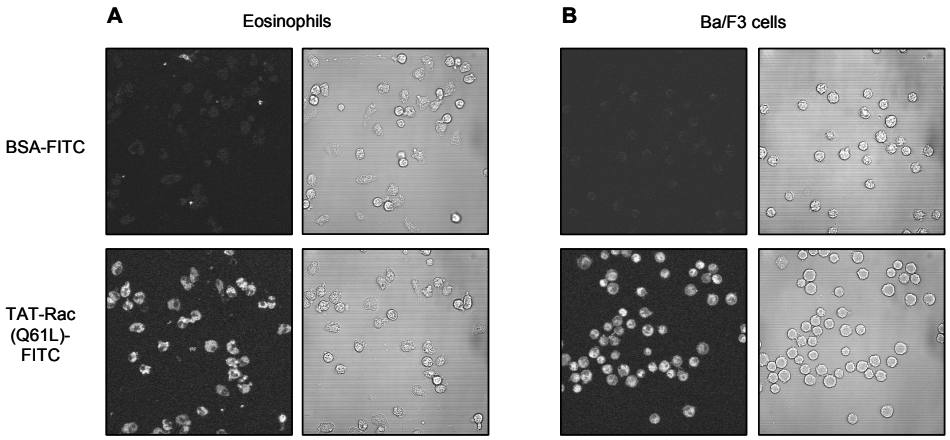

Figure 1: Transduction of eosinophils and Ba/F3 cells by Tat-Rac(Q61L). Isolated human eosinophils **(A)** or Ba/F3 cells **(B)** were incubated for 5 minutes with 10 µg/ml FITC labeled BSA or Tat-Rac(Q61L) proteins. Fluorescence of cells was analyzed by confocal laser scanning microscopy.

Eosinophil superoxide production and migration in the presence of constitutively active Tat-Rac(Q61L) protein.

Rac has been implicated in several granulocyte effector functions including superoxide production (10, 11, 14). Here we investigated the role of Rac in superoxide production by transduction of eosinophils with constitutively active (Q61L) Rac. Eosinophils were incubated with Tat-Rac proteins for 15 minutes at 37°C before stimulation. Superoxide production was initiated by STZ, and could be primed by pre-incubation with PAF for 2 minutes (38). PAF is a strong activator of Rac proteins, but does not activate the oxidase complex itself (31). Superoxide production was measured by oxygen consumption using an oxygen probe. Although superoxide production was induced by STZ and this was enhanced by PAF, transduction of eosinophils with Tat-Rac(Q61L) alone was not sufficient to induce superoxide production, nor enhance STZ induced superoxide production (Fig. 2A).

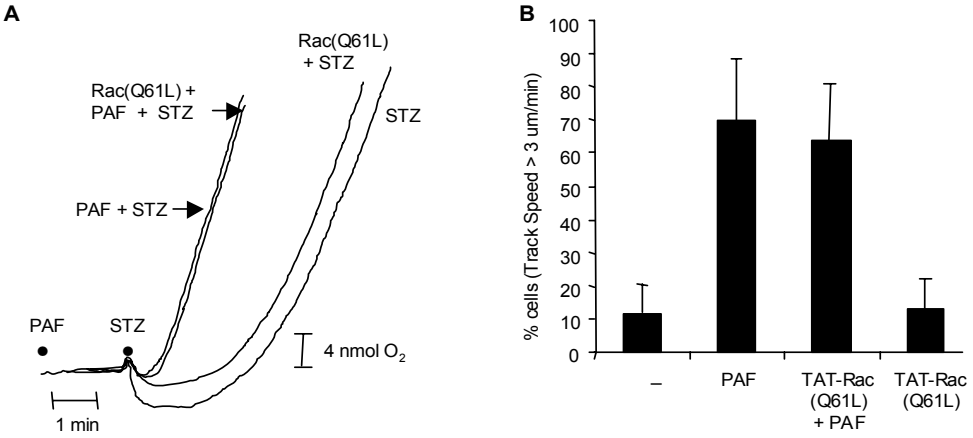

Figure 2: Rac activation in eosinophils is not sufficient to induce or enhance respiratory burst and migration. (A) Respiratory burst was measured as oxygen consumption by eosinophils as described in Materials and Methods. Cells were pre-incubated with Tat-Rac(Q61L) protein for 15 minutes at 37°C and stimulated with or without PAF (10^{-6} M) for 2 minutes 37°C. Subsequently, STZ (1mg/ml) was added to activate the respiratory burst and oxygen consumption was continually measured for several min. The results shown is representative for 3 experiments. • Represents addition of PAF or STZ. **(B)** Migration of eosinophils was monitored by time lapse analysis. Cells were incubated with Tat-RacQ61L (20 µg/ml) and allowed to attach to fibronectine coated coverslips for 15 minutes. Neutrophil migration was induced by PAF (10^{-7}M) and imaged every 20 seconds for 10 minutes. Migration of eosinophils with a migration speed greater than 3 µm/min were expressed as percentage of total number of eosinophils ±SD. (n=3)

Rac proteins are known to regulate actin polymerization in a variety of cells, resulting in cell spreading and the formation of lamellipodia. Furthermore neutrophils of Rac2-deficient mice displayed significant defects in chemotaxis (14). We investigated the role of constitutively active Rac(Q61L) in PAF induced migration of eosinophils. Migration of eosinophils on fibronectin coated coverslips was measured by timelapse imaging for 10 minutes. Pre-treatment of eosinophils with Tat-Rac(61L) proteins had no effect on the migration of either the unstimulated cells, nor the PAF activated cells (Fig. 2B). These findings demonstrate that Rac alone was unable to induce or prime the superoxide burst or migration in eosinophils.

Rac modulates FcαRI function

Previously we have reported that disruption of the cytoskeleton by cytochalasin D treatment prevents cytokine induced binding of IgA in Ba/F3 cells (ectopically expressing FcαRI) and human peripheral blood eosinophils (39). Since Rac is thought to be involved in regulating cytoskeletal organization, we hypothesized that Rac might thus be involved in IgA binding. First, we investigated the effect of constitutively active Rac in Ba/F3_FcαRI cells, which has been proven to be a good model to study cytokine induced IgA binding. Ba/F3_FcαRI cells were cytokine starved, incubated with Tat-Rac(Q61L) protein (20 μg/ml) or with IL-3 for 15 minutes. After incubation with IgA coated beads for 30 minutes, IgA binding was scored under the microscope. IgA binding was strongly induced by IL-3 as has been reported previously (34). Ba/F3 cells transduced with Tat-Rac(Q61L), resulted in strong IgA binding without prior-stimulation of cells with cytokines (Fig. 3A). These findings were confirmed by using Ba/F3_FcαRI cells ectopically expressing a constitutively active Rac(G12V), which had similar characteristics as Rac(Q61L) (27, 28). Furthermore, a role for Rac in regulating IgA binding of eosinophils was analysed using Tat-Rac(Q61L) protein. In these cells, IL-5 strongly induces the IgA binding, whereas PAF is less potent. Similar to Ba/F3_FcαRI cells, Tat-Rac(Q61L) also activated IgA binding in eosinophils (Fig. 3B). The induction of IgA binding to eosinophils by Rac(Q61L) was less than for IL-5, but similar to that of PAF. These findings demonstrated that Rac alone is sufficient to induce IgA binding.

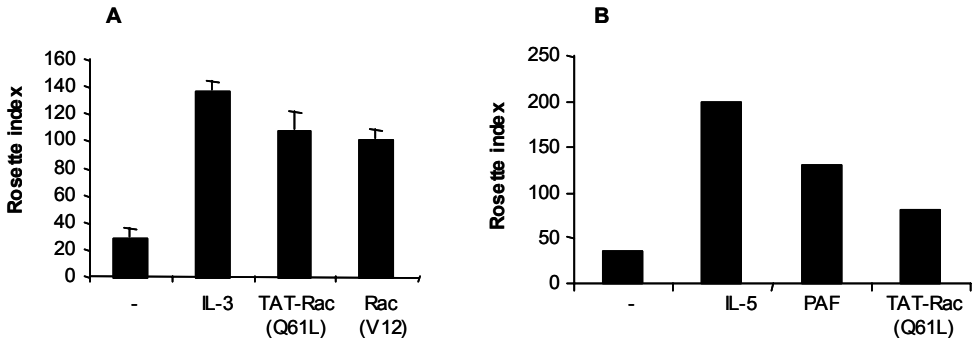

Figure 3: **Constitutively active Rac induces IgA binding in eosinophils and Ba/F3_FcαR cells. (A)** Cytokine-starved Ba/F3_FcαRI cells were incubated with Tat-Rac(Q61L) protein (20 μg/ml; 15 min), with IL-3 (1:1000; 15 min) or with incubation buffer alone at 37°C. Furthermore, IgA binding of Ba/F3_FcαRI stably overexpressing Rac(G12V) gene was determined. **(B)** Isolated eosinophils were incubated with Tat-Rac(Q61L) protein (20 μg/ml; 15 min), with IL-5 (10^{-9} M; 15 min), with PAF (10^{-6} M; 5 min) or with incubation buffer alone at 37°C. Binding of IgA beads to these cells was measured and results were expressed as rosette index (number of beads of a rosette/100 cells).

Rac activity is not sufficient for modulating CR3 integrin (CD11b/CD18) function.

Similarly to Fc receptors, interactions of integrins with the cytoskeleton are thought to be important for their (in)activation (40, 41). Our data thus suggest that it might be possible that Rac proteins, are also involved in regulating integrin function. Here we investigated the binding of STZ particles by eosinophils. This binding is mainly mediated by CR3 (CD11b/CD18) (42). Although we showed in Figure 2 that Rac(Q61L) protein was not sufficient to activate or prime STZ-stimulated superoxide production in eosinophils, it was still possible that Rac can regulate STZ binding to eosinophils. As previously reported, PAF strongly induced STZ binding to eosinophils (35), but Rac(Q61L) had no effect on STZ binding to unstimulated eosinophils (Fig. 4). This demonstrates that Rac alone was unable to activate CD11b and CD18 integrins and the effects we observed in FcαRI function suggest a distinct mechanism between CD11b/CD18 and FcαRI functioning.

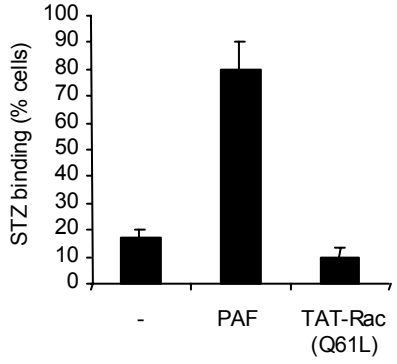

Figure 4: Effect of constitutively active Rac on STZ binding of eosinophils. Isolated human eosinophils were pre-incubated with Tat-Rac(Q61L) protein for 15 minutes at 37°C and stimulated with or without PAF (10^{-6} M) for 2 minutes 37°C. After adding FITC labeled STZ (1mg/ml) for 5 min, eosinophils were analyzed for STZ binding by flow cytometer. Eosinophils bound to STZ were expressed as percentage of total number of eosinophils ±S.D. (n=3)

Rac regulates FcαRI lateral mobility in the plasma membrane.

The results described above demonstrate that Rac is able to modulate FcαRI function. To investigate this process in more detail, we measured the lateral mobility of FcαRI in the plasma membrane using fluorescence recovery after photobleaching (FRAP). Ba/F3 cells were generated ectopically expressing FcαRI fused to yellow fluorescent protein (YFP). Cells were cytokine starved, pre-incubated with Tat-Rac(Q61L) protein for 15-30 minutes and subsequently stimulated with or without IL-3 for 15 minutes. The percentage of the recovery of fluorescence after bleaching is significantly diminished in cells stimulated with IL-3 (52.3 ±7.3%) or transduced with Tat-Rac(Q61L) (52.5 ±7.7%) compared to unstimulated cells (69.4 ±7.9%). The recovery time constant was only slightly increased for IL-3 (0,064 ±0.003) and Tat-Rac(Q61L) (0,062 ±0.002) compared to unstimulated cells (0.056 ±0.004). These findings demonstrated that IL-3 and dominant-active Rac reduced the lateral mobility of FcαRI by increasing the immobile pool of FcαRI.

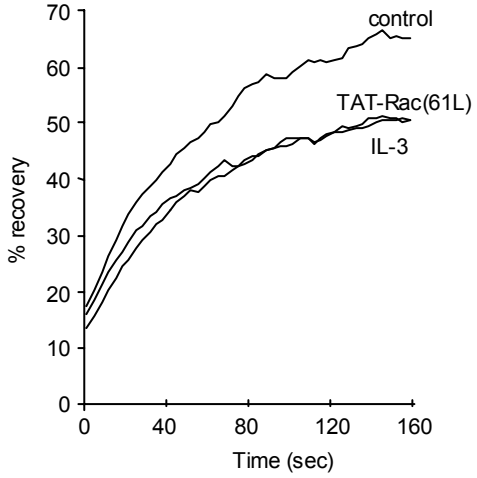

Figure 5: Mobility of FcαRI is reduced by Rac activation. Ba/F3 cells overexpressing FcαRI tagged with EGFP were attached to coverslips and incubated with Tat-Rac(Q61L), IL-3 (1:1000) or with incubation buffer for 15 min at 37°C. In each independent experiment 20 cells were used for FRAP measurements. FRAP curve and time constants were analyzed using Sigma plot. (n=3)

Discussion

Granulocytes are critical in the first line of host defense against invading microorganisms. This requires activation of a series of effector functions, including degranulation, phagocytosis and respiratory burst to accomplish the killing and removal of pathogens. The small GTPase Rac of the Rho-family has been previously implicated in regulating these processes (10, 11). However, most of these studies have utilized *in vitro* systems, cell lines or knock-out mice, and has focused on neutrophils, whereas the role of Rac in eosinophils is largely unknown.

We have investigated the role of Rac in primary human peripheral blood eosinophils. Overexpression of a constitutively active Rac (Q61L) in eosinophils, using the recently developed technique of protein transduction (21, 24, 37) has allowed us for the first time to manipulate Rac function in primary human granulocyte function. Due to aggregation of the proteins during isolation, we modified the protein isolation method performing the isolation under non-reducing conditions. Incubation of Tat-Rac(Q61L) with eosinophils resulted in fusion protein entering eosinophils rapidly and efficiently (±99%) and confirmed the success of protein transduction in eosinophils (21, 22).

In this study we demonstrated that Rac activation alone is not sufficient to prime or activate superoxide production and migration in eosinophils. Although several studies have suggested that Rac plays an important role in these processes, none of these have investigated whether Rac alone is sufficient to modulate eosinophil functioning. As shown by others, the presence and activation of Rac is necessary for the fMLP induced respiratory burst in neutrophils (13, 19, 20). We showed here that Rac alone is not sufficient to induce or prime fMLP induced superoxide production (Fig. 2). Thus both fMLP induced migration and respiratory burst are dependent on Rac activation, but this activation alone is not enough to trigger these processes. Indeed, it has been reported that triggering of other signaling pathways, including Ca^{2+}/Calmodulin and PI3K, are necessary in the activation of these processes (31, 44-46).

Further investigation of the role of Rac in both Ba/F3_FcαRI cells and eosinophils demonstrated that Rac modulates FcαRI function (Figure 3). The regulation of ligand binding to Fc receptors and integrins is very similar. The affinity for extracellular ligands of both receptors are modulated in response to intracellular signals or so-called “inside-out” signalling (40, 47, 48). Furthermore, the cytoplasmic tails of both receptors are targets for the modulation of the external receptor affinity state (39, 40). However, introducing constitutively active Rac in eosinophils demonstrated that Rac alone was insufficient to modulate CR3 integrin function (Figure 4).

A possible mechanism by which Rac activated FcαRI is through modulating the cytoskeleton, which is crucial for correct functioning of FcαRI (39). Rac proteins are known to regulate actin rearrangement in a variety of cells, resulting in cell spreading and the formation of lamellipodia (15). Cytoskeletal rearrangement is also thought to play an

important role in modulation of integrin function (40, 41). Since Rac did not modulate integrin function it suggest that beside cytoskeletal rearangement, other pathways are necessary to modulate FcαR or CD11b/CD18 functioning. An explanation might be that Rac regulates FcαRI function through the cytoplasmic tail directly. Mutation of a C-terminal serine (Ser263) to alanine (S263A) in FcαR, results in constitutive binding of IgA, whereas mutation to an aspartic acid, which mimics the phosphorylation of this residue, results in only a weak binding of IgA, even after cytokine stimulation (39). In addition, PI3K and p38 have been implicated in cytokine-induced activation of FcαRI. Thus it might be that Rac, which has been described to act upstream of PI3K and p38 in some cell systems (49), regulates FcαRI function via PI3K and p38(50). The presence of highly conserved tyrosine, threonine and serine residues in the integrin cytopasmic tails suggest that phosphorylation has also the potential to regulate integrin function. CD11b is constitutively phosphorylated, whereas the CD18 becomes phosphorylated after activation (51, 52). Threonine phosphorylation of CD18 has been recognised as being important for the regulation of CD11/CD18 avidity (53). Thus the phosphorylation of the cytoplasmic tails of FcαRI and CR3 through specific signalling pathways might allow the regulation of these receptors independently of each other.

The mechanism of FcαRI modulation by cytokines and Rac was further investigated by determining the lateral membrane mobility of FcαRI using FRAP methodology. We were able to demonstrate that cytokine activation of FcαRI lead to decreased mobility, caused by reduction of a mobile pool of FcαRI, but not by a lower diffusion rate. Introduction of Rac in these cells caused a similar decrease of the mobile fraction of FcαRI. These data suggest that decreased mobility of FcαRI results in enhanced IgA binding. A likely mechanism for these findings is that the decreased mobility is associated with the forming of “clusters” of FcαRI receptor, and thereby increasing avidity for ligands. However, it is also possible that FcαRI affinity is also modulated. Further investigation is needed to unravel the specific role of Rac in these processes.

In the present report, we showed that Rac activation is not in itself sufficient for activation or priming of the migration and respiratory burst in eosinophils. Interestingly we have identified a new role for Rac in eosinophils as mediator of FcαRI function. The observation that Rac mediates FcαRI ligand binding but not CR3, indicates that Fc receptor and integrin activation are not regulated by the same inside-out signalling system. Greater understanding of these mechanisms will result in a better insight into the regulation of leukocyte activation. This information might provide novel approaches to the design of anti-inflammatory therapies.

References

1. Witko-Sarsat, V., P. Rieu, B. Descamps-Latscha, P. Lesavre, L. Halbwachs-Mecarelli. 2000. Neutrophils: molecules, functions and pathophysiological aspects. *Lab. Invest.* 80:617-653.
2. Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, A. M. Waltersdorph. 1986. Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* 136:4220-4225.
3. Weisbart, R. H., L. Kwan, D. W. Golde, J. C. Gasson. 1987. Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. *Blood* 69:18-21.
4. Ingraham, L. M., T. D. Coates, J. M. Allen, C. P. Higgins, R. L. Baehner, L. A. Boxer. 1982. Metabolic, membrane, and functional responses of human polymorphonuclear leukocytes to platelet-activating factor. *Blood* 59:1259-1266.
5. Downey, G. P., T. Fukushima, L. Fialkow, T. K. Waddell. 1995. Intracellular signaling in neutrophil priming and activation. *Semin. Cell. Biol.* 6:345-356.
6. Coffer, P. J., and L. Koenderman. 1997. Granulocyte signal transduction and priming: cause without effect? *Immunol. Lett.* 57:27-31.
7. Dinauer, M. C., and S. H. Orkin. 1992. Chronic granulomatous disease. *Annu. Rev. Med.* 43:117-124.
8. Roos, D., M. de Boer, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz, H. Middleton-Price. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood* 87:1663-1681.
9. Arnaout, M. A., H. Spits, C. Terhorst, J. Pitt, R. F. 3. Todd. 1984. Deficiency of a leukocyte surface glycoprotein (LFA-1) in two patients with Mo1 deficiency. Effects of cell activation on Mo1/LFA-1 surface expression in normal and deficient leukocytes. *J. Clin. Invest.* 74:1291-1300.
10. Dinauer, M. C. 2003. Regulation of neutrophil function by Rac GTPases. *Curr. Opin. Hematol.* 10:8-15.
11. Gu, Y., and D. A. Williams. 2002. RAC2 GTPase deficiency and myeloid cell dysfunction in human and mouse. *J. Pediatr. Hematol. Oncol.* 24:791-794.
12. Bokoch, G. M. 2000. Regulation of cell function by Rho family GTPases. *Immunol. Res.* 21:139-148.
13. Kim, C., and M. C. Dinauer. 2001. Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. *J. Immunol.* 166:1223-1232.
14. Roberts, A. W., C. Kim, L. Zhen, J. B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J. D. Pollock, J. B. Borneo, G. B. Bradford, S. J. Atkinson, M. C. Dinauer, D. A. Williams. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity.* 10:183-196.
15. Aspenstrom, P. 1999. The Rho GTPases have multiple effects on the actin cytoskeleton. *Exp. Cell Res.* 246:20-25.
16. Yang, F. C., S. J. Atkinson, Y. Gu, J. B. Borneo, A. W. Roberts, Y. Zheng, J. Pennington, D. A. Williams. 2001. Rac and Cdc42 GTPases control hematopoietic stem cell shape, adhesion, migration, and mobilization. *Proc. Natl. Acad. Sci. U. S. A.* 98:5614-5618.
17. Gabig, T. G., C. D. Crean, P. L. Mantel, R. Rosli. 1995. Function of wild-type or mutant Rac2 and Rap1a GTPases in differentiated HL60 cell NADPH oxidase activation. *Blood* 85:804-811.
18. Dorseuil, O., A. Vazquez, P. Lang, J. Bertoglio, G. Gacon, G. Leca. 1992. Inhibition of superoxide production in B lymphocytes by rac antisense oligonucleotides. *J. Biol. Chem.* 267:20540-20542.
19. Ambruso, D. R., C. Knall, A. N. Abell, J. Panepinto, A. Kurkchubasche, G. Thurman, C. Gonzalez-Aller, A. Hiester, M. deBoer, R. J. Harbeck, R. Oyer, G. L. Johnson, D. Roos. 2000. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc. Natl. Acad. Sci. U. S. A.* 97:4654-4659.
20. Williams, D. A., W. Tao, F. Yang, C. Kim, Y. Gu, P. Mansfield, J. E. Levine, B. Petryniak, C. W. Derrow, C. Harris, B. Jia, Y. Zheng, D. R. Ambruso, J. B. Lowe, S. J. Atkinson, M. C. Dinauer, L.

Boxer. 2000. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood* 96:1646-1654.

21. Hall, D. J., J. Cui, M. E. Bates, B. A. Stout, L. Koenderman, P. J. Coffer, P. J. Bertics. 2001. Transduction of a dominant-negative H-Ras into human eosinophils attenuates extracellular signal-regulated kinase activation and interleukin-5-mediated cell viability. *Blood* 98:2014-2021.
22. Alblas, J., L. Ulfman, P. Hordijk, L. Koenderman. 2001. Activation of Rhoa and ROCK are essential for detachment of migrating leukocytes. *Mol. Biol. Cell* 12:2137-2145.
23. Nagahara, H., A.M., E. L. Snyder, A. Ho, D. G. Latham, N. A. Lissy, M. Becker-Hapak, S. A. Ezhevsky, S. F. Dowdy. 1998. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. *Nat. Med.* 4:1449-1452.
24. Fawell, S., J. Seery, Y. Daikh, C. Moore, L. L. Chen, B. Pepinsky, J. Barsoum. 1994. Tat-mediated delivery of heterologous proteins into cells. *Proc. Natl. Acad. Sci. U. S. A.* 91:664-668.
25. Chen, L. L., A. D. Frankel, J. L. Harder, S. Fawell, J. Barsoum, B. Pepinsky. 1995. Increased cellular uptake of the human immunodeficiency virus-1 Tat protein after modification with biotin. *Anal. Biochem.* 227:168-175.
26. Vives, E., P. Brodin, B. Lebleu. 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272:16010-16017.
27. Xu, X., D. C. Barry, J. Settleman, M. A. Schwartz, G. M. Bokoch. 1994. Differing structural requirements for GTPase-activating protein responsiveness and NADPH oxidase activation by Rac. *J. Biol. Chem.* 269:23569-23574.
28. Xu, X., Y. Wang, D. C. Barry, S. J. Chanock, G. M. Bokoch. 1997. Guanine nucleotide binding properties of Rac2 mutant proteins and analysis of the responsiveness to guanine nucleotide dissociation stimulator. *Biochemistry* 36:626-632.
29. Caldenhoven, E., T. van Dijk, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, R. P. de Groot. 1995. Activation of the STAT3/acute phase response factor transcription factor by interleukin-5. *J. Biol. Chem.* 270:25778-25784.
30. Goldstein, I. M., D. Roos, H. B. Kaplan, G. Weissmann. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* 56:1155-1163.
31. Coffer, P. J., N. Geijsen, L. M'Rabet, R. C. Schweizer, T. Maikoe, J. A. Raaijmakers, J. W. Lammers, L. Koenderman. 1998. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem. J.* 329:121-130.
32. Hansel, T. T., I. J. De Vries, J. M. Carballido, R. K. Braun, N. Carballido-Perrig, S. Rihs, K. Blaser, C. Walker. 1992. Induction and function of eosinophil intercellular adhesion molecule-1 and HLA-DR. *J. Immunol.* 149:2130-2136.
33. Aida, Y., and M. J. Pabst. 1990. Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J. Immunol. Methods* 132:191-195.
34. Bracke, M., E. Nijhuis, J. W. Lammers, P. J. Coffer, L. Koenderman. 2000. A critical role for PI 3-kinase in cytokine-induced Fcalpha-receptor activation. *Blood* 95:2037-2043.
35. Blom, M., A. T. Tool, D. Roos, A. J. Verhoeven. 1992. Priming of human eosinophils by platelet-activating factor enhances the number of cells able to bind and respond to opsonized particles. *J. Immunol.* 149:3672-3677.
36. Pick, E., and D. Mizel. 1981. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *J. Immunol. Methods* 46:211-226.
37. Schwarze, S. R., A. Ho, A. Vocero-Akbani, S. F. Dowdy. 1999. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 285:1569-1572.

38. Koenderman, L., A. T. Tool, D. Roos, A. J. Verhoeven. 1990. Priming of the respiratory burst in human eosinophils is accompanied by changes in signal transduction. *J. Immunol.* 145:3883-3888.
39. Bracke, M., J. W. Lammers, P. J. Coffer, L. Koenderman. 2001. Cytokine-induced inside-out activation of FcalphaR (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. *Blood* 97:3478-3483.
40. Hughes, P. E., and M. Pfaff. 1998. Integrin affinity modulation. *Trends. Cell Biol.* 8:359-364.
41. Hynes, R. O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell* 110:673-687.
42. Blom, M., A. T. Tool, P. T. Kok, L. Koenderman, D. Roos, A. J. Verhoeven. 1994. Granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3), and IL-5 greatly enhance the interaction of human eosinophils with opsonized particles by changing the affinity of complement receptor type 3. *Blood* 83:2978-2984.
43. Gardiner, E. M., K. N. Pestonjamasp, B. P. Bohl, C. Chamberlain, K. M. Hahn, G. M. Bokoch. 2002. Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. *Curr. Biol.* 12:2029-2034.
44. Nijhuis, E., J. W. Lammers, L. Koenderman, P. J. Coffer. 2002. Src kinases regulate PKB activation and modulate cytokine and chemoattractant-controlled neutrophil functioning. *J. Leukoc. Biol.* 71:115-124.
45. Hirsch, E., V. L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, M. P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287:1049-1053.
46. Verploegen, S., C. M. van Leeuwen, H. W. van Deutekom, J. W. Lammers, L. Koenderman, P. J. Coffer. 2002. Role of Ca2+/calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions. Comparison with the role of phosphotidylinositol-3 kinase. *Eur. J. Biochem.* 269:4625-4634.
47. Tozer, E. C., P. E. Hughes, J. C. Loftus. 1996. Ligand binding and affinity modulation of integrins. *Biochem. Cell Biol.* 74:785-798.
48. Ginsberg, M. H., X. Du, E. F. Plow. 1992. Inside-out integrin signalling. *Curr. Opin. Cell Biol.* 4:766-771.
49. Keely, P. J., J. K. Westwick, I. P. Whitehead, C. J. Der, L. V. Parise. 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 390:632-636.
50. Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knaus, R. J. Ulevitch, G. M. Bokoch. 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* 270:23934-23936.
51. Chatila, T. A., R. S. Geha, M. A. Arnaout. 1989. Constitutive and stimulus-induced phosphorylation of CD11/CD18 leukocyte adhesion molecules. *J. Cell Biol.* 109:3435-3444.
52. Buyon, J. P., S. G. Slade, J. Reibman, S. B. Abramson, M. R. Philips, G. Weissmann, R. Winchester. 1990. Constitutive and induced phosphorylation of the alpha- and beta-chains of the CD11/CD18 leukocyte integrin family. Relationship to adhesion-dependent functions. *J. Immunol.* 144:191-197.
53. Valmu, L., and C. G. Gahmberg. 1995. Treatment with okadaic acid reveals strong threonine phosphorylation of CD18 after activation of CD11/CD18 leukocyte integrins with phorbol esters or CD3 antibodies. *J. Immunol.* 155:1175-1183.

Chapter 7

A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein

Henri H. Versteeg, Evert Nijhuis, Gijs R. van den Brink, Maaike Evertzen, Gwenda N. Pynaert, Sander J.H. van Deventer, Paul J. Coffer, and Maikel P. Peppelenbosch

Biochemical Journal, 2000, 350, 717–722

Abstract

Assaying activation of signal transduction is laborious and does not allow the study of large numbers of samples, essential for high-throughput drug screens or for large groups of patients. Using phosphospecific antibodies, we have developed ELISA techniques enabling non-radioactive semi-quantitative assessment of the activation state of p42/p44 MAPK, p38 MAPK, Protein Kinase B and the transcription factor CREB in 96 well plates. This assay has been termed PACE (Phosphospecific Antibody Cell-based ELISA) and was successfully used for both adherent and suspension cells. Various stimuli induced dose-dependent enzymatic activity of which the kinetics closely correlated with those measured via classical methodology. Using PACE we have now characterised for the first time the concentration-dependent effects of various inflammatory prostaglandins on CREB phosphorylation in macrophages. Hence PACE is a straightforward and novel technique enabling the large-scale analysis of signal transduction.

Introduction

Cells must continuously adapt to external stimuli. The molecular details of most of the underlying signalling pathways have now partially been clarified and frequently involve the sequential by phosphorylation of the molecules involved. Probably the best-characterised cascade is the signalling pathway initiated by active p21Ras leading to the sequential activation of Raf, MEK and p42/p44 MAPK (1-4). Other signalling cascades, analogous to this pathway, lead to the phosphorylation and stimulation of p38 MAPK and SAPK/JNK, which both show significant sequence homology to p42/p44 MAPK. Aberrant control of these signal transduction pathways have been implicated in a variety of pathological conditions and are therefore an interesting target for both drug discovery as well as for research into the alterations of signal transduction in primary material isolated from individual patients.

Unfortunately, current methods for assaying activation of p38 MAPK, p42/p44 MAPK, or SAPK/JNK, such as Western Blotting and in-gello kinase assays are labour intensive and do not allow the study of large numbers of samples. The situation has improved recently with the advent of phosphospecific antibodies, which recognise the phosphorylated forms of proteins but not their unphosphorylated counterparts. However, analysis still requires SDS-PAGE followed by Western Blotting and this will typically not allow simultaneous loading of more as 20 samples. Therefore, this technique is still not suited for applications as drug discovery or the testing of large numbers of patients, and alternatives are called for.

The same situation holds true for other signal transduction components, such as c-Akt/PKB, (5,6) which constitutes an important cellular survival signal (7). Detection of c-AKT/PKB activity remains dependent on kinase assays or Western Blotting. Similarly, cAMP-dependent transactivation of CRE-containing promoters through protein kinase A and phosphorylation of CREB (8,9) remains a difficult target for high-throughput screening.

The above-mentioned considerations prompted us to develop an ELISA-based assay for measuring the activation of specific signalling pathways. We employed phosphospecific antibodies, which recognise the activated state of signalling components but not the unphosphorylated, non-active form. We used these antibodies to determine immunoreactivity of cells grown in 96 wells plates exposed to various stimuli and we have thus created the PACE (Phosphospecific Antibody Cell-based ELISA). As PACE and similar ELISA-based techniques provide a non-radioactive semi-quantitative read-out of signal transduction pathways which is suited for the investigation of large numbers of samples and allows high-throughput screening, we suggest that the assessment of signal transduction by PACE will become the technique of choice for rapid analysis.

Materials and Methods

Materials and cell culture

fMLP, poly-L-lysine, OPD and BSA were from Sigma (St. Louis, MO, USA). HSA was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Polyclonal antibody against p44/42 MAPK was from UBI (Lake Placid, NY, U.S.A.) and polyclonal anti-phospho-p44/42 MAPK, anti-phospho-p38 MAPK, anti-phospho-PKB/c-Akt and anti-phospho-CREB antibodies were from New England Biolabs (Beverly, MA, U.S.A.) as were all in-vitro kinase assay kits. Swine-anti-rabbit peroxidase-conjugated antibody and Goat-anti-rabbit were from DAKO. PD098059 was from Biomol (Plymouth, PA). Murine macrophages clone 4/4 (10) were cultured according to routine procedures. Blood was obtained from healthy volunteers. Mixed granulocytes were isolated as described earlier (11).

Cell-based ELISA for adherent cells

Cultured murine macrophages were seeded in 96 wells at 50,000 cells/cm^2. When appropriate, the cells were serum-starved for 4 hours and stimulated for 10 minutes. After stimulation, the cells were fixed with 4% formaldehyde in PBS for 20 minutes at room temperature and washed three times with PBS containing 0.1% Triton X-100 (PBS/Triton). Endogenous peroxidase was quenched with 0.6 % H_2O_2 in PBS/Triton for 20 minutes and three times washed (PBS/Triton), blocked with 10% FCS in PBS/Triton for 1 hour and incubated overnight with various dilutions of primary antibody in PBS/Triton containing 5% BSA at 4°C. Next day, cells were washed 3 times with PBS/Triton for 5 minutes and incubated with secondary antibody (peroxidase-conjugated goat-anti-rabbit, dilution 1:100) in PBS/Triton with 5% BSA for 1 hour at room temperature and washed thrice with PBS/Triton for 5 minutes and 2 times with PBS. Subsequently the cells were incubated with 50 µl of a solution containing 0.4 mg/ml OPD, 11.8 mg/ml $Na_2HPO_4.2H_2O$, 7.3 mg/ml $C_6H_8O_7.H_2O$ (citric acid) and 0.015% H_2O_2 for 15 minutes at room temperature in the dark. The reaction was stopped with 25 µl of 1 M H_2SO_4 and the $OD_{490/650}$ was measured and standard error of the mean was determined (error bars in figures).

Cell-based ELISA for non-adherent cells

A 96-wells plate (Nunc. Maxisorp) was coated with 10µg/ml poly-L-lysine for 30 minutes at 37°C. After two washes with sterile PBS, 1.5×10^5 granulocytes were plated into each well and incubated for 30 minutes at 37°C. Granulocytes were stimulated with fMLP and fixed with 8% formaldehyde in PBS for 20 minutes at room temperature, followed by three washes with PBS/Triton. Quenching was performed with 1% H_2O_2 and 0.1% Azide

in PBS/Triton for 20 minutes and cells were further treated as described for adherent cells with the exception of the use of a 1:500 dilution of the secondary antibody (peroxidase-conjugated swine-anti-rabbit) in PBS/Triton with 5% BSA for 1 hour at room temperature.

Crystal-violet Cell quantification assay

After the peroxidase reaction, the cells were washed twice with PBS-triton followed by 2× with demi water. After drying the wells for 5 minutes, 100 µl of crystal violet solution (0.04% crystal violet in 4% (v/v) ethanol/water) was added for 30 minutes at room temperature. Subsequently, the cells were washed at least 3 times with demineralised water and 100 µl of 1% SDS solution was added and incubated on a shaker for 1 hour at room temperature. Finally, the absorbance was measured at 595 nm with an ELISA reader.

In-vitro kinase assays

Murine macrophages were grown in either 6-well plates (p42/p44 MAPK assay), or 60 mm dishes (p38 MAPK and PKB assay) and serum starved for 16 hrs. After appropiate stimulation cells were treated according to the protocol of the manufacturer.

Western Blotting

40 µl of cell lysate or kinase reaction mix was loaded onto SDS-PAGE and blotted onto PVDF membranes. After blocking and washing, bands were visualised using the appropriate phosphospecific antibodies (1:1000), peroxidase-conjugated goat-anti-rabbit (1:2000), and enhanced chemiluminescence.

Results and Discussion

Development of a p42/p44 MAPK phosphorylation assay in 96 wells plates

The activation p42/p44 MAPK is accomplished by phosphorylation of the protein on threonine^{183}/tyrosine^{185} and threonine^{202}/tyrosine^{204}, respectively by MEK (4) and commercial antibodies recognising phosphorylated p42/p44 MAPK with little cross reactivity to the unphosphorylated form of MAPK or other cellular proteins are available (Fig. 1). We analysed how these antibodies would perform when used for an ELISA-based assay. Therefore, 4/4 macrophages were challenged with different concentrations of TPA, a potent activator of MAPK in these cells. Subsequently, the cells were fixed, blocked and washed (see experimental procedures) and incubated with different concentrations of anti-phospho p42/p44 MAPK antibody followed by further routine ELISA procedures, finally culminating in oxidation of OPD by a peroxidase reaction and determination of absorbance at 490/650 nm. We have termed this procedure PACE (Phoshospecific Antibody Cell-based ELISA). As evident from figure 1, in unstimulated cells or in the absence of primary or secondary antibody, little enzymatic activity is detected. Increasing concentrations of TPA, however, yield increased levels of enzymatic product (Fig. 1A), the dose response curve observed in the ELISA being in strict accordance with the dose-response curve of TPA-enhanced MAPK activity as observed with Western Blots or in-vitro kinase assays (Fig. 1B and C). We found that at a 1:250 antiphospho p42/p44 MAPK dilution combined low background with high sensitivity. The specificity of PACE was further established by the observation that PD98059, which inhibits activation of MEK, (12) blocked the TPA-induced increase in absorbance (not shown). We concluded that PACE is a sensitive and specific method for determining p42/p44 MAPK activation.

PACE is useful for primary isolated suspension cells

In the PACE-assay described above, the immunoreactive substance, i.e. phosphorylated p42/p44 MAPK, is immobilised via formaldehyde fixation of cells grown in 96 wells tissue culture plates. To adapt the assay for cell types which do not naturally attach to tissue culture plastic, we adhered primary-isolated human granulocytes to poly-l-lysine-coated ELISA plates. When tested in PACE according to the procedures employed for 4/4 macrophages we encountered a high background signal due to endogenous peroxidase activity. Inclusion of 0.1 % sodium azide was sufficient to quench this activity. Concentration and temporal-dependent, PD98059-sensitive, fMLP-induced enzymatic product formation was readily detected (Fig. 2). The p42/p44 MAPK activation of FMLP closely matched that observed in primary-isolated human granulocytes using classical kinase assays or Western Blot analysis (11). Thus PACE is a specific and sensitive method for detection of p42/p44 MAPK phosphorylation in both adherent and suspension

cells. Furthermore, these results show that this assay can also be useful for primary non-transformed cells.

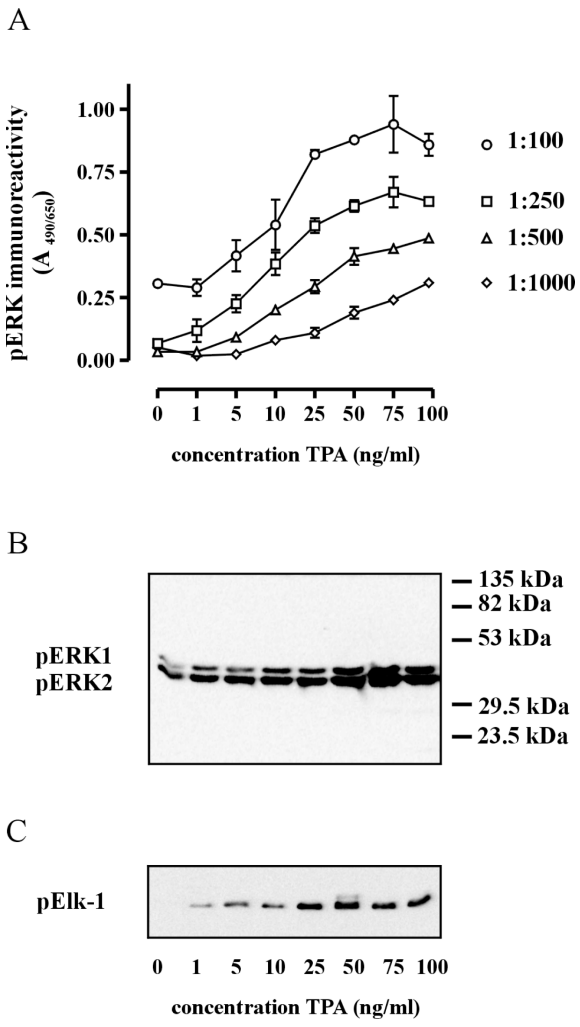

Figure 1 Results of a p42/p44 MAPK PACE. (A) 4/4 cells were grown in a 96 wells plate, stimulated with the indicated concentrations of TPA and subjected to PACE procedures. Results obtained with different concentrations of a polyclonal anti-phospho p42/p44 MAPK antibody as a primary antibody are shown. Phosphorylation and activity of MAPK was determined by analysis on Western Blot **(B)** and an in-vitro kinase assay **(C)** as well.

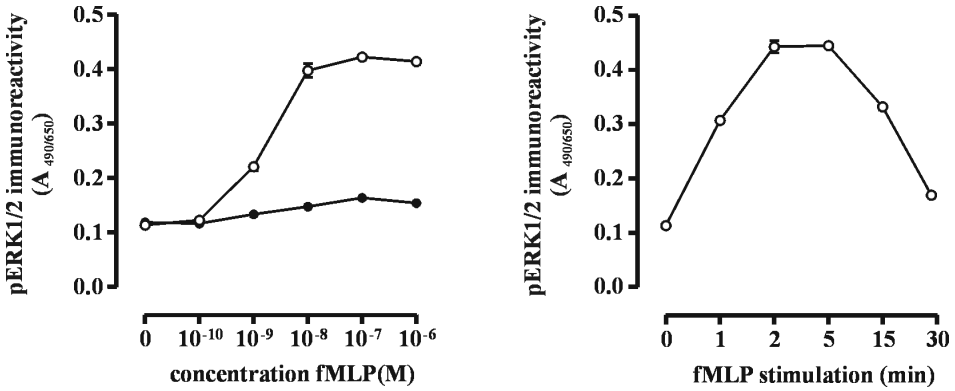

Figure 2: fMLP-mediated p42/p44 MAPK activation in neutrophils measured by PACE. Human neutrophils were isolated from peripheral blood, adhered to poly-l-lysine-coated 96 wells plates and stimulated for 2 min with fMLP or PD98059 and fMLP (left panel). The right panel shows the effects of 10^{-7} M fMLP for different time periods. After stimulation p42/p44 MAPK PACE was performed

Development of a PACE for p38 MAPK, PKB/c-Akt, and CREB

We investigated whether phosphospecific antibodies directed against other signal transduction components are applicable for PACE. Macrophages were challenged with different concentrations of LPS, a potent activator of p38 MAPK. Subsequently, the cells were fixed and incubated with different concentrations of anti- phosphothreonine[180]/ phosphotyrosine[182] p38 MAPK antibody, followed by further routine ELISA procedures. Again little enzymatic product formation was detected in the absence of either the primary or secondary antibody or in the absence of LPS. Increasing concentrations of LPS, however, produced a dose-dependent increase in immunoreactivity (Fig. 3A). We found that at an anti-phospho p38 MAPK antibody dilution of 1:250 combined low background with high sensitivity. The PACE-detected increase in phosphorylated p38 MAPK corresponded to that observed using Western Blotting and to activity in in-vitro kinase assays (Fig. 3C). Although the phosphospecific p38 MAPK antibody is reported to have cross reactivity with other stress-activated MAPKs, we only detected one band on Western Blot.

We also tested a phosphoserine 473-specific PKB/c-Akt antibody in PACE. Macrophages were stimulated with the PKB activator insulin. As shown in Figure 4(A), we observed a dose-dependent stimulation of enzymatic product formation, in agreement with the insulin-dependent increase of PKB/c-Akt activity observed in both Western Blotting (which also showed the specificity of the antibody for PKB/c-Akt; Fig. 4B) and in-vitro kinase assays (Fig. 4C). The optimal dilution of primary antibody (1:100) was higher than those observed with the p42/p44 MAPK and p38 MAPK PACE. The specificity, however,

of the PKB/c-Akt PACE was confirmed by the sensitivity of the insulin-induced signal to wortmannin.

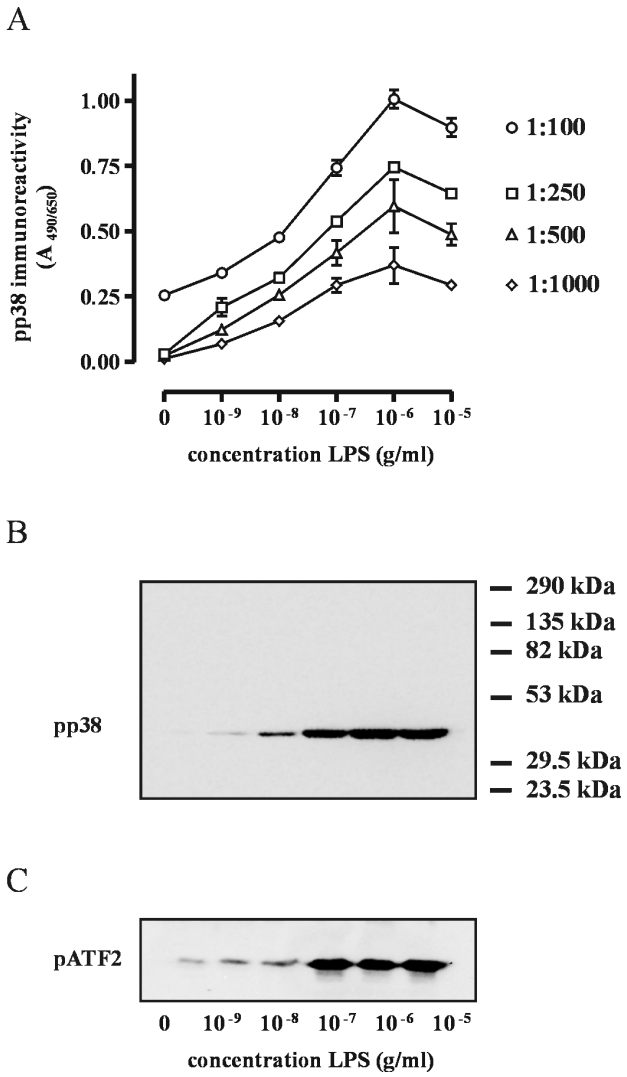

Figure 3: **Results of a p38 MAPK PACE. (A)** 4/4 cells were grown in a 96 wells plate and stimulated with LPS, followed by PACE procedures. Results obtained with different concentrations of a polyclonal anti-phospho p38 MAPK antibody are shown. Analysis of p38 MAPK on Western Blot **(B)** and an in-vitro kinase assay **(C)** is also shown.

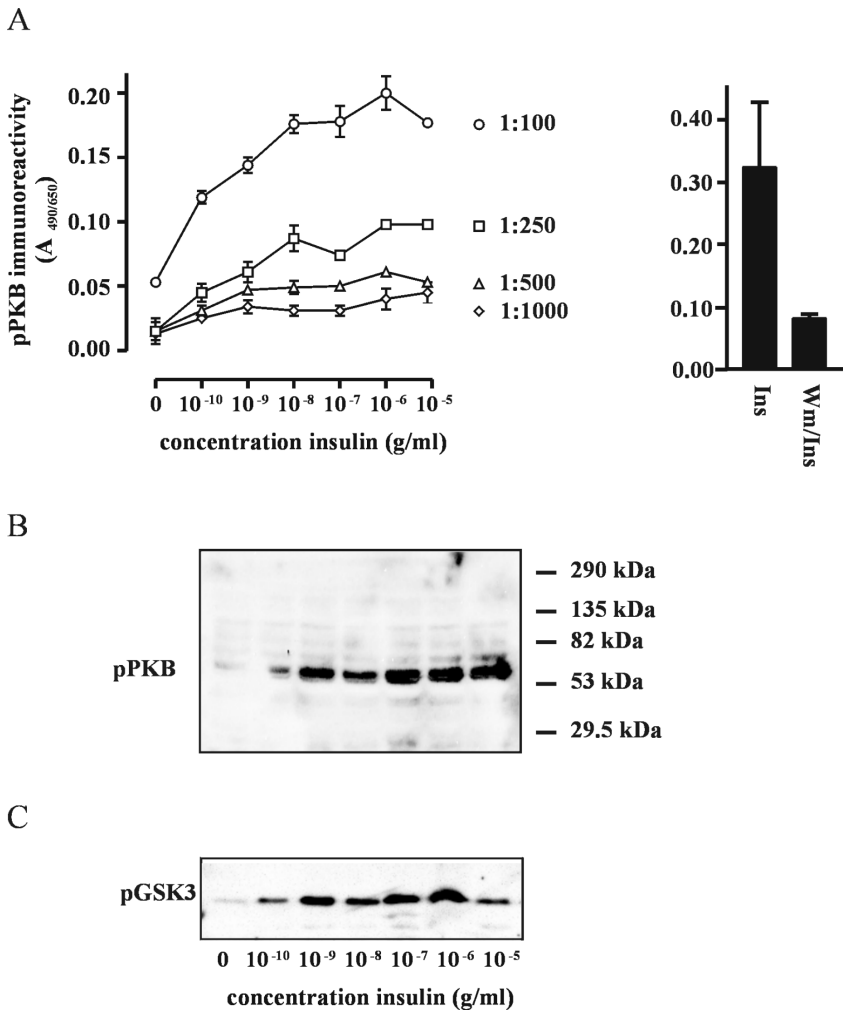

Figure 4: Results of a PKB/c-Akt PACE. 4/4 cells grown in a 96 wells plate were stimulated with insulin followed by PACE procedures. Results with different concentrations of a anti-phospho PKB/c-Akt antibody are shown **(A)**. The effect of wortmannin on insulin-stimulated cells was also determined (right panel). Phosphorylation and activity of PKB determined by Western Blot **(B)** and in-vitro kinase assay **(C)** are also depicted.

Finally we tested a phosphoserine 133 CREB antibody in PACE. To this end 4/4 macrophages were stimulated with prostaglandin E_2, prostaglandin $F_{2\alpha}$ and prostaglandin I_2, since various prostaglandins have been reported to mediate CREB-phosphorylation (13-16), but the effects of these important inflammatory mediators on CREB phosphorylation in macrophages have as yet not been assessed. As shown in Figure 5(A), prostaglandin E_2 resulted in CREB phosphorylation in macrophages as detected by Western Blot (the antibody also cross reacts with ATF1). After stimulation, a CREB-PACE was performed, whereas cAMP production was measured in a parallel experiment. Prostaglandin $F_{2\alpha}$ did not induce cAMP production nor did it lead to the production of enzymatic product in a PACE assay (Fig. 5B). In contrast, prostaglandin E_2 and prostaglandin I_2 gave rise to a significant increase in cAMP production and CREB immunoreactivity in PACE. Increases in cAMP formation did not completely correlate with increases in CREB phosphorylation at lower concentrations of prostaglandins, but this was attributed to cAMP independent phosphorylation of CREB (14). We concluded that PACE is a convenient method to determine the phosphorylation state of important signal transduction elements and is applicable for a variety of different phosphospecific antibodies against both protein kinases and transcription factors.

Correcting for differences in cell number

The validity of the PACE assay critically depends on two factors: the loading of equal amounts of cells in different wells and the quality of the primary antibody. We investigated, therefore, whether it is possible to correct for the former factor using crystal violet staining after the PACE procedure. As shown in Figure 6, crystal violet staining works well under these conditions and provides the opportunity to correct the signals measured in PACE for cell number, as evident by the reduction in variability in the assay. With the respect to the latter factor, the antibodies employed may react with other cellular proteins. This is not a major problem if this antibody reactivity is not different between stimulated and unstimulated cells as this will only result in some additional background signal. If, however, immunoreactivity is induced by stimulation of the cells, a problem may arise. Western Blotting of TPA-stimulated cells using the anti phospho p42/p44 antibody reveals, after prolonged exposure, some faint induced bands apart from the immunoreactivity at 42 and 44 kD, and these bands may contribute to the signal measured in ELISA. Nevertheless, the enhanced chemiluminescence used to detect antibody binding is not a very linear technique, is very sensitive, and saturates quickly. The contribution of phospho p42/p44 MAPK to the signal measured in PACE may therefore be even larger than estimated from Western Blot. Indeed, one of the major advantages of PACE over Western Blotting is that the enzymatic reaction underlying PACE is of a more linear nature and therefore produces quantitatively more accurate results.

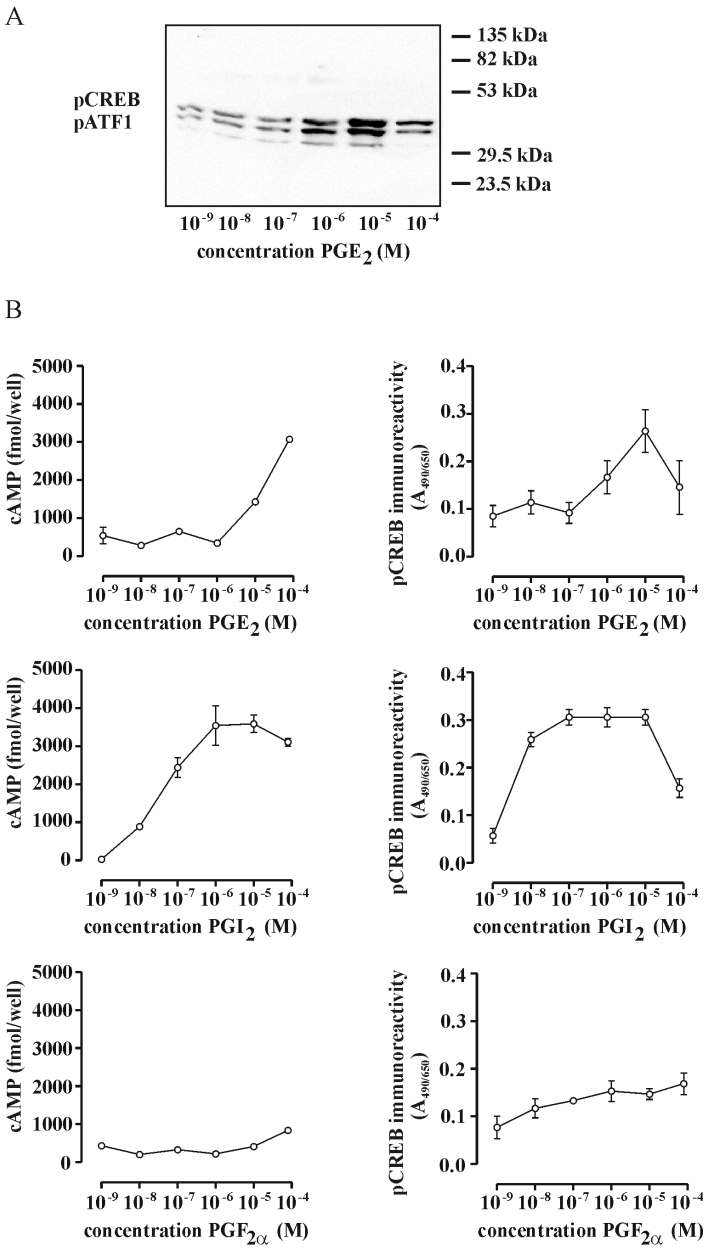

Figure 5: Results of prostaglandin treatment on cAMP production and CREB. (A) 4/4 cells were grown in 60 mm dishes and stimulated with PGE_2. CREB phosphorylation was subsequently measured by Western Blotting, using phospho-specific CREB antibodies. **(B)** Effects of PGE2, PGI2 and $PGF_{2\alpha}$ on cAMP production and CREB phosphorylation, measured using a commercial enzyme-linked immuno assay for cAMP (Amersham) and PACE.

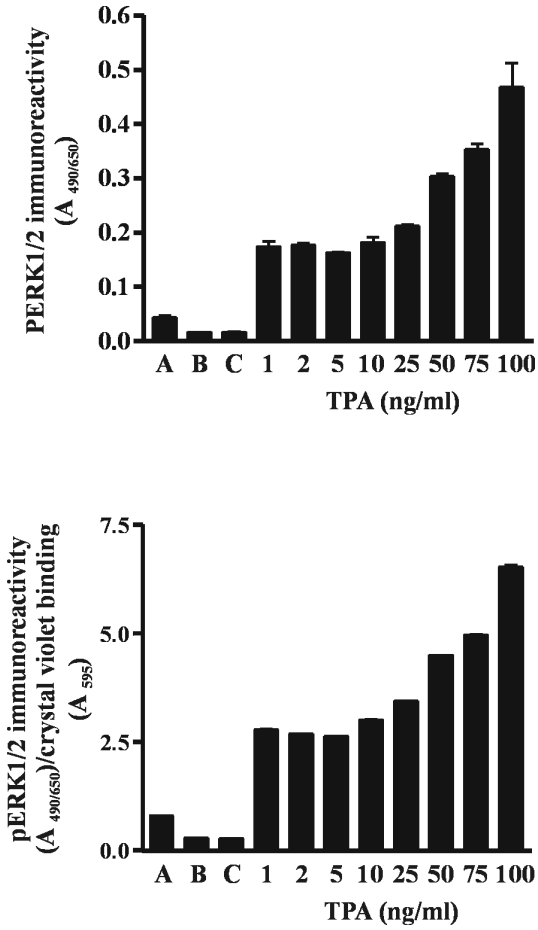

Figure 6: Correcting for differences in cell number by staining the wells with crystal violet after the PACE procedure. 4/4 cells were stimulated with TPA and subjected to PACE (upper panel). Subsequently cell number in the individual wells was determined using crystal violet staining and measuring absorbance at 595 nm, and the ratios were calculated (lower panel). The bars indicated by A represent background signal

References

1: Dent, P., Haser, W., Haystead, T. A., Vincent, L. A., Roberts, T. M., and Sturgill, T. W. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro, Science 257 (1992): 1404-1407.

2: Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. Raf-1 activates MAPK-kinase, Nature 358 (1992), 417-421.

3: Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J.. Activation of the MAPK pathway by the protein kinase raf, Cell 71 (1992): 335-342.

4: Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAPK), EMBO J. 10 (1991): 885-892.

5: Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. Mechanism of activation of protein kinase B by insulin and IGF-1, EMBO J. 15 (1996): 6541-6551.

6: Burgering, B. M. Th, and Coffer, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction, Nature 376 (1995): 599-602.

7: Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N., and Hay, N. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal, Gen. Dev. 11 (1997): 701-713.

8: Gonzalez, G. A., and Montminy, M. R. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133, Cell 59 (1989): 675-680.

9: Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G., Green, M. R., and Goodman, R. H. Nuclear protein CBP is a coactivator for the transcription factor CREB, Nature 370 (1994): 223-226.

10: Desmedt, M., Rottiers, P., Dooms, H., Fiers, W., and Grooten, J.. Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses, J. Immunol. 160 (1998): 5300-5308.

11: Coffer, P. J., Geijsen, N., M'Rabet, L., Schweizer, R. C., Maikoe, T., Raaijmakers, J. A., Lammers, J. W., and Koenderman, L.. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function, Biochem. J. 329 (1998): 121-130.

12: Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R.. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo, J. Biol. Chem. 270 (1995): 27489-27494.

13: Won, J. S., Suh, H. W., Kim, Y. H., Song, D. K., Huh, S. O., Lee, J. K., and Lee, K. J. Prostaglandin E2 increases proenkephalin mRNA level in rat astrocyte-enriched culture, Brain Res. Mol. Brain Res. 60 (1998): 203-214.

14: McNulty, S., Ross, A. W., Shiu, K. Y., Morgan, P. J., and Hastings, M. H. Phosphorylation of CREB in ovine pars tuberalis is regulated both by cyclic AMP-dependent and cyclic AMP-independent mechanisms, J. Neuroendocrinol. 8 (1996): 635-645.

15: Chin, J. H., Okazaki, M., Frazier, J. S., Hu, Z. W., and Hoffman, B. B.. Impaired cAMP-mediated gene expression and decreased cAMP response element binding protein in senescent cells, Am. J. Physiol. 271 (1996): C362-371.

16: Thomas, J. M., Frazier, J. S., Hu, Z. W., and Hoffman, B. B. Phosphorylation of cyclic AMP response element-binding protein and induction of c-fos gene expression on withdrawal from chronic treatment with carbachol in NG108-15 cells, Mol.Pharmacol. 48 (1995): 593-600.

Chapter 8

General Discussion

1 Sytemic inflammatory response in COPD

COPD is associated with an abnormal inflammatory response, which is likely to be reversible during the first stages of COPD. Eventually the process becomes irreversible during further progression of the disease (Fig 1A). The inflammatory response in COPD is thought to be partly mediated by increased numbers of neutrophils in the peripheral blood and airways of these patients (1-4). Neutrophil activation has been demonstrated in BAL fluid, sputum and in the peripheral blood of COPD patients (1, 5-7). Full activation of neutrophils results in release of cytotoxic enzymes by degranulation and production of reactive oxygen species by a membrane bound NADPH-oxidase. These harmful molecules are thought to contribute to the damage of the lung tissue that gradually develops in COPD patients. The recruitment and subsequent activation of neutrophils in the tissue is likely to be orchestrated by the increased levels of cytokines/chemokines, such as IL-8 and TNFα, in the airways. These inflammatory mediators are strong activators of neutrophils and can be produced by epithelial cells, which are exposed to cigarette smoke (8-10). A direct role for cigarette smoke in the activation of neutrophils has also been suggested (11). Leaking of the cytokines/chemokines from the airways to the peripheral blood is thought to mediate the priming/activation of the peripheral blood neutrophil. These findings point to an important role for neutrophils in the pathogenesis of COPD. To gain further insight in the systemic inflammatory processes in COPD, we studied the activation of neutrophils from the peripheral blood of COPD patients by analysis of gene expression profiles.

In Chapter 2 we demonstrated that gene expression profiles of peripheral blood neutrophils from moderate to severe COPD (Class IIB and III) patients exhibited an "activated" phenotype. These findings are in agreement with data from others demonstrating that neutrophils from COPD patients show some features of activation (6). Furthermore we demonstrate that activation of neutrophils correlates with the severity of COPD, suggesting that the progress of COPD is accompanied by an increasing systemic response (Fig 1A). The increase in systemic inflammation is, however, only significant in the more severe patients (class IIB and III). This finding suggest that systemic inflammation is probably initiated in moderate COPD patients (class II). This hypothesis is supported by a previous study demonstrating an enhanced neutrophilic response in COPD patients (class II), but not in healthy smokers (5). However, a start of a systemic response in the COPD classes 0 and I can not be excluded.

A recent report demonstrated that levels of IL-8 and sTNF-R55 in the airways correlated with the severity of COPD (12, 13). The hypothesis that the local inflammation communicates with the systemic compartment via cytokines/chemokines would also suggest that the levels of cytokines/chemokines in the peripheral blood correlate with the severity of COPD. These findings have so far not been substantiated. Levels of systemic inflammatory mediators are likely too low to be measured due to a short half-life and

clearance in the peripheral blood (14). Another explanation is suggested in Chapter 4. Here it was shown that neutrophil activation is variable within individual COPD patients compared to healthy volunteers. Assuming that neutrophil activation can be used as a read out of the systemic inflammation, this response cannot be considered as a stable, but more as a variable process, with a production of pro- and anti-inflammatory cytokines/chemokines. So the variability in cross sectional studies of COPD might be caused by a continuous imbalance between different inflammatory processes in the neutrophils.

Besides the fluctuations of the systemic inflammatory response in the stable phase, a consistent change in systemic inflammatory response was demonstrated in COPD patients with an acute exacerbation (Chapter 3). Peripheral neutrophils from COPD patients with an acute exacerbation showed no "activation phenotype" compared to the stable phase. This change in neutrophil activation might be caused by a change in levels and/or combination of inflammatory mediators (Chapter 3). This may result in the migration of neutrophils to the tissue, leaving mainly unstimulated or recently matured neutrophils from the bone marrow in the peripheral blood. These findings suggest that inflammatory responses in mild to moderate to severe COPD patients are continually changing. These changes result not in clear clinical symptoms in moderate COPD patients due to excess of lung capacity. In more severe COPD patients, in which the excess of lung capacity is not large enough to compensate for changes in inflammatory responses, these changes can lead to an exacerbation of the disease (Fig. 1C).

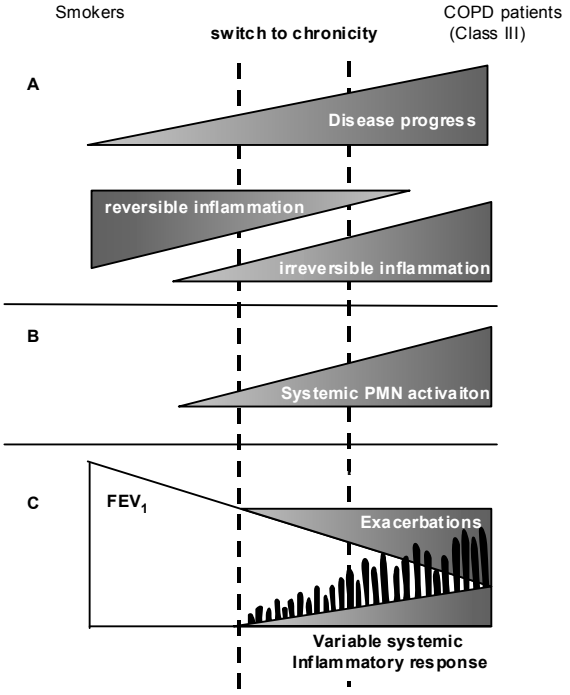

Fig. 1: Model for disease progression in COPD. **(A)** The subclincial stage of COPD which can last up to 20 years or more is characterized by a mainly reversible inflammation (GOLD severity stages at risk to mild COPD), which gradually becomes irreversible due to a chronic inflammatory reaction. **(B)** During the progression of COPD a systemic activation of neutrophils is initiated in the moderate stage of COPD. **(C)** The high but variable activation of neutrophils represents changes in the systemic inflammation. Hyperactivation of neutrophils and a subsequent sequestering in the airways represent a change in the sytemic and local inflammation. This change can lead to acute exacerbations in severe COPD patients, if the decrease in the lung function cannot be compensated by excess lung capacity.

Similar findings for the activation and migration of circulating neutrophils were found in patients with multiple organ failure (MOF). A two step model has been suggested for this disease, in which circulating neutrophils are highly primed in the first step due to a major trauma (15). The underlying mechanisms of this priming are not clear, but there are indications that increased production of several cytokines/chemokines are involved (15-18). Priming of circulating neutrophils results in some sequesteration in organs, but sequesteration alone is not sufficient for the development of organ injury (16, 19, 20). The priming of neutrophils last for about 24 hours and during this time, neutrophils are responsive to additional stimuli, such as surgery, resulting in enhanced migration to the organs and subsequently full activation. This full activation is required to cause tissue damage. It is tempting to speculate that in more severe COPD patients circulating neutrophils are in a primed state, which will lead to sequesteration to the airways. This priming of neutrophils makes these cells prone to activation by additional stimuli, such as an infection, leading to hyperactivation of the neutrophils with subsequent lung damage. Although infection is a cause of exacerbation in many COPD patients (21) not all exacerbations are associated with viral or bacterial infections (22), suggesting that other factors are present to induce exacerbations.

In addition, hyperactivation of neutrophils during an exacerbation might also lead to migration of neutrophils to other tissue than the lungs which might lead to injury of these organs. This might explain why COPD patients often develop systemic effects, such as muscle wasting and weight loss during later stages of their disease. Thus activation of circulating neutrophils can be useful as a read-out for variable systemic inflammatory responses. Further analysis of longitudinal studies of the expression profiles of neutrophils and other cells are required to define the changes in systemic inflammatory responses in COPD patients. This will result in a better understanding of the pathogenesis of COPD.

The finding that neutrophils themselves can express several inflammatory cytokines/chemokines (Chapter 2) (23) suggests that neutrophils are not only effector cells, but that these cells can also act as pro-inflammatory immune cells. Indeed, several inflammatory genes regulated in patients with COPD correlate with the severity of the disease. Since the increase in gene expression of inflammatory cytokines/chemokines in peripheral blood neutrophils correlated with the severity of COPD, and is particularly prominent in severe COPD patients (Chapter 2), it is tempting to speculate that the peripheral neutrophils play a role in the switch to a chronic more severe disease.

An explanation for the underlying mechanisms of this hypothesis might be a change in the balance of expression of anti- and proinflammatory mediators by circulating neutrophils. Indeed, Chapter 2 we reported that expression of the anti-inflammatory mediator IL-1RA is much higher than that of the pro-inflammatory mediator IL-1β in neutrophils stimulated with TNFα, compared to neutrophils from severe stable COPD patients. Apparently, the balance between the IL-1β and IL-1RA is disturbed in patients with severe stable COPD. Thus it might be that in the reversible smoking induced inflammatory reaction, neutrophils are activated by cytokine/chemokines and control the inflammatory reaction by expression of large amounts of IL-1RA (Fig.2A). In an abnormal inflammatory reaction, such as that observed in severe COPD patients, neutrophils cannot produce enough anti-inflammatory molecules leading to a self-perpetuating inflammatory response (Fig. 2B). It would also be interesting to investigate the activation of monocytes in COPD patients, since these cells can express large amounts of inflammatory mediators. This model is supported by data from another chronic inflammatory disease where neutrophils play a role in the chronic phase of rheumatoid arthritis. In contrast to COPD, in rheumatoid arthritis much more is known concerning the function of IL-1 family proteins. It has been shown that in rheumatoid synovium an imbalance exists in the pro- and anti-inflammatory mediators since the levels of production of IL-1RA are inadequate to effectively block the pro-inflammatory effects of IL-1 (24). Injection of IL-1RA in the synovium, significantly reduced the signs and symptoms of rheumatoid arthritis at 24 weeks (25). Recently it has been suggested that soluble IL-1R2, which is increased on day one of an acute exacerbation of COPD may may be associated with the clinical improvement (26). Thus applying an anti-IL-1 therapy to COPD patients might improve clinical symptoms. It would be necessary to investigate the expression of pro- and anti-inflammatory mediators by neutrophils in a acute or reversible inflammatory response, since our data on normal inflammation is based on *in vitro* data. Further development and thorough investigation of gene expression profiles of neutrophils, and other inflammatory cells might give further insight in the underlying mechanism of chronic inflammation.

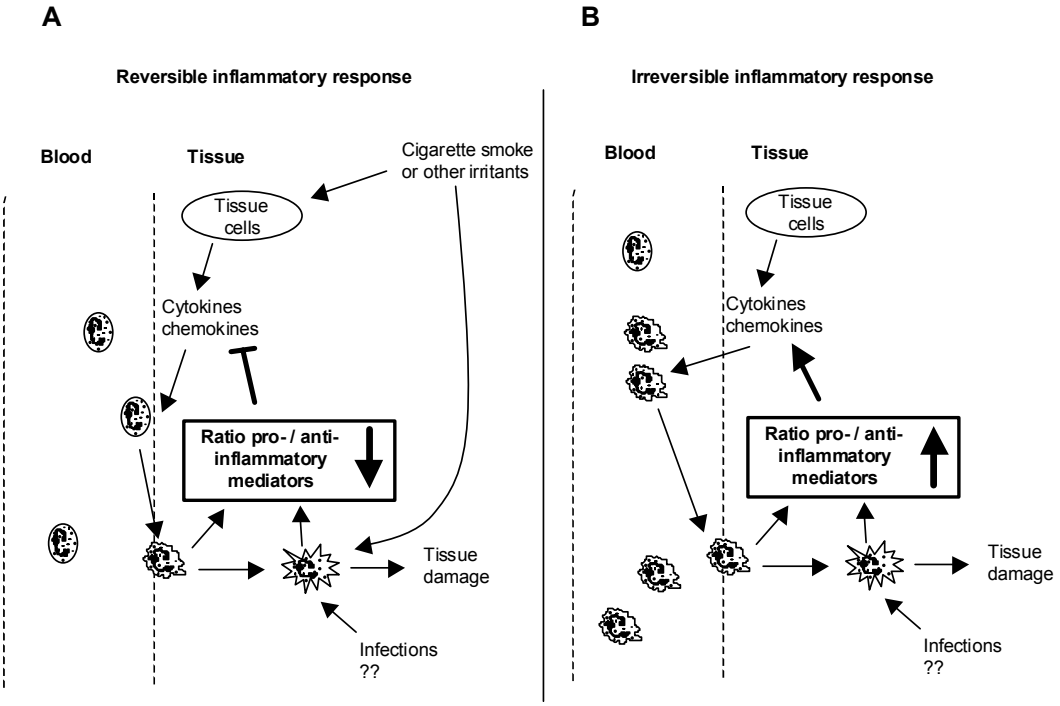

Fig. 2: Model for the switch between reversible to irreversible inflammation in COPD. (A) Release of cytokine/chemokines by tissue cells by smoke inhalation, results in the recruitment and pre-activation of neutrophils and other inflammatory cells to the tissue. Full activation of neutrophils by stimuli such as infection or unknown factors results to degranulation and production of reactive oxygen species, leading to tissue damage. The inflammation is controlled by the production of anti-inflammatory mediators produced by (pre)activated neutrophils and other inflammatory cells, resulting in a temporal or reversible inflammation. **(B)** Irreversible inflammation is characterized by its independence on smoke inhalation. Long term reversible inflammation in the tissue leads to a decrease in the expression of anti-inflammatory mediators by (pre)activated neutrophils, resulting in an increase in pro-inflammatory mediators in the tissue. This loss of control, leads to an increase of pro-inflammatory mediators, which might leak into the peripheral blood and (pre)activated inflammatory cells. These cells in turn produce more pro- than anti-inflammatory mediators, and thereby enhancing the chronic effects. Thus the loss of control on the increase of pro-inflammatory mediators lead to a positive feedback loop for the tissue and other inflammatory cells, resulting in an irreversible inflammation.

2 Function of signaling proteins in granulocytes

Granulocytes are critical effector cells in killing invading microorganisms. The killing is mediated by specific effector functions, such as phagocytosis, degranulation and production of reactive oxygen species. Correct control of these effector functions is necessary, since abnormal regulation, or a defect in these processes can lead to severe infections or inappropriate tissue damage. The control of these processes is mediated by inflammatory mediators, such as cytokines and chemoattractants. Upon binding to their receptors, these mediators trigger a large variety of intracellular signaling molecules, resulting in the correct functioning of the granulocytes. Understanding of the activation of intracellular signaling molecules and their role in granulocyte functioning is important, as it might provide novel insights in regulating and controlling inflammation. In this thesis we have focused on two distinct signaling molecules, Src kinase and the small GTPase Rac, which are known to be activated by diverse stimuli in granulocytes, however their precise role in granulocyte functioning is not clear.

2.1 Role of Src kinases in neutrophil function

Src kinases were one of the first kinases families identified, however it has only been in the last decade that advances have been made in understanding the regulation and function of these proteins. It is has been well established that Src kinases are involved in diverse signal transduction pathways, regulating a broad spectrum of physiological responses, including cell cycle, cell proliferation, differentiation, adhesion and survival (reviewed by (27)). Although several Src kinases, including Lyn, Hck, and Fgr are activated by both cytokine and GPCRs, little is known about their role in granulocyte functioning. It might be that the Src kinases play a role in regulating distinct signaling events between the chemokine and cytokine receptors. In Chapter 5, we investigated the role of Src kinase using a pharmacological inhibitor. In several other cell types, Src kinases have been implicated in the regulation of various intracellular signaling pathways. In neutrophils, we found that Src kinases mediate both fMLP and GM-CSF induced activation of PKB, but not that of p38MAPK and ERK1/2 (Fig. 3). These findings are supported by a report showing that Src kinases are not involved in fMLP induced Ras activation (28). Investigation of several neutrophil functions, revealed that Src kinases as well as PI3K are involved in the superoxide production, survival and f-actin polymerization (Chapter 5) (29). These findings demonstrate that Src kinases mediate both fMLP and GM-CSF induced activation of PKB, probably through PI3K activation. This is supported by data showing that a member of the Src kinase family, Lyn, associates with both the GM-CSF receptor and the p85 subunit of PI3K in neutrophils (30, 31). Src kinases are thought to recruit Class I PI3Ks (p110$\alpha/\beta/\delta$) via their adaptor subunits, (p85), which contain Src homology-2 (SH2) domains that are able to bind to phosphorylated tyrosine residues (32). The observation that Src kinases are involved in fMLP-mediated activation of PKB is

supported by data showing that in neutrophils stimulated with agonists of G-protein-coupled receptors, PI3K is activated through a genistein-sensitive target, presumably a protein tyrosine kinase (33-35). However, other reports have suggested that GPCRs act solely via PI3Kγ to activate PKB in neutrophils (36, 37). PI3Kγ, which is associated with the adaptor molecule p101 (38), is stimulated by G-protein βγ subunits (39, 40), which do not interact with SH2 domain containing adaptors, excluding tyrosine kinases from direct activation of PI3Kγ. In addition, recent data also demonstrate that PI3Kγ can be activated by pathways independent of G-proteins such as those initiated by exposure of neutrophil to LPS (41). Furthermore, it has recently been shown that the G-proteins Gαs and Gαi directly stimulate the kinase activity of c-Src, Lck, and Hck (42, 43). Since the GPCRs, such as fMLP receptor or IL-8 receptor, are coupled to Gαi proteins (44), this suggests that the activation of Src kinases by fMLP may be directly mediated by G-proteins. Taken together, these findings suggest that besides the direct regulation of PI3K by G-proteins, GPCRs can also regulate PI3K via Src kinases. So the regulation of PI3K by GPCRs is more complicated than previously thought. In conclusion, Src kinases are important signaling molecules, linking GPCRs and cytokines to PI3K/PKB, in neutrophils. This pathway is necessary, but not sufficient for activation of the respiratory burst, since GM-CSF, which does not activate the respiratory burst, also activates this pathway.

The finding that both inhibitors of Src kinases and PI3K inhibit GM-CSF mediated survival suggest that GM-CSF may act through Src kinases, and subsequently via PI3K, to regulate neutrophil lifespan (Chapter 5). In several cell types, activation of the PI3K/PKB pathway has been shown to be associated with cell survival. One of the mechanisms by which PI3K/PKB can mediate cell survival, is via phosphorylation of the pro-apoptotic Bcl-2 family member BAD. Phosphorylation promotes its association with the cytoplasmic protein 14-3-3, which in turn prevents the association of BAD with anti-apoptotic Bcl-2 family members, effecting prolonged survival (45, 46). This data is supported by a recent study in neutrophils demonstrating a role for Lyn in GM-CSF delayed apoptosis (47) and by studies showing that the PI3K inhibitor LY294002 blocked GM-CSF dependent PKB and BAD phosphorylation in neutrophils (48, 49). GM-CSF induced survival is also mediated by reducing the levels of BAD mRNA, which is also sensitive to PI3K inhibition (49). It has been shown in other cell types that cytokines can inhibit the transcription factor FKHR-LI via PI3K/PKB pathway, resulting in reduced expression of pro-apoptotic members, such as Bim (50). It would be interesting whether transcription of BAD is also under control of FKHR-L1. Besides downregulation of pro-apoptotic proteins, cytokines also upregulate the expression of anti-apoptotic proteins. The major cytokine induced anti-apoptotic proteins found in neutrophils are Mcl-1 and A1 (51, 52). However it is unclear how cytokines regulate the expression of these anti-apoptotic proteins in neutrophils. Thus cytokines induce survival in neutrophils by several mechanisms, in which the Src/PI3K/PKB pathway play central role.

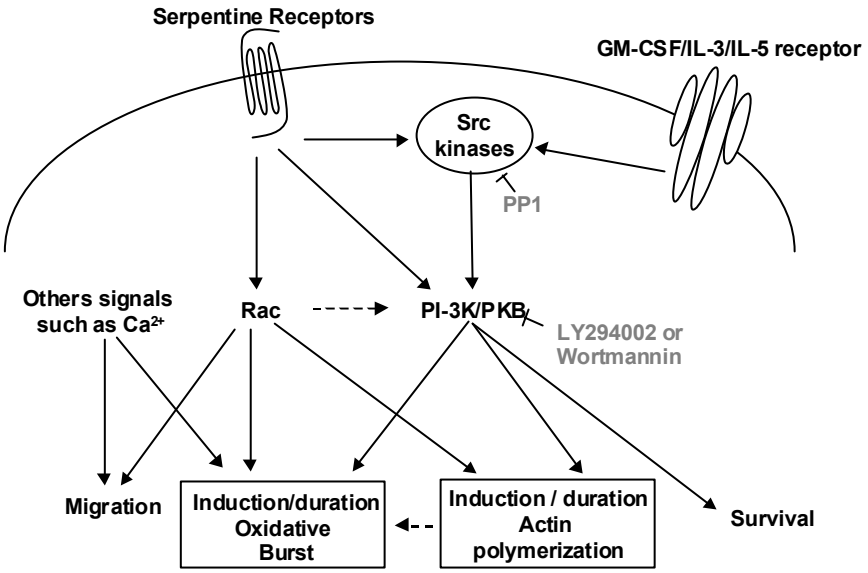

Fig. 3: Possible mechanisms by which Src kinases regulate neutrophil functioning.
GPCRs and cytokine receptors, such as GM-CSF/IL-3/IL-5 receptors, can both activate PI3K/PKB pathway via Src kinases and thereby modulate several neutrophil functions including production of superoxide, survival and actin polymerization. Actin polymerization and production of superoxide is also modulated by Rac, which is involved in the induction of these processes, while Src kinases mediate the duration.

Furthermore, we found that Src kinases may act through PI3K-PKB to regulate fMLP induced respiratory burst, since a similar inhibition of fMLP induced respiratory burst with inhibitors of PI3K and Src kinase were found (Chapter 5). These findings are in agreement with data showing that PI3K is involved in the superoxide production in neutrophils (29, 36, 37, 53). It has been shown that PI3K and subsequent PKB activation is involved in the phosphorylation of $p47^{phox}$, which is required for the correct translocation of NADPH oxidase components (54, 55). Recently it has been reported that the phox proteins, such as $p40^{phox}$ and $p47^{phox}$, are able to bind to the products of PI3K including phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P(2)) and phosphatidylinositol-3-phosphate (PtdIns(3)P), respectively (56, 57). Thus Src kinases may act through PI3K/PKB to regulate the phox proteins and thus modulate superoxide production. Besides the phox proteins, Src kinases might regulate fMLP-induced respiratory burst through maintenance of actin polymerization (Chapter 5). The kinetics of inhibitors of Src kinases and PI3K on the inhibition of actin polymerization are very similar to that of inhibition of the respiratory burst. In both cases, the inhibitors do not block the initiation of the response, but rather the maintenance of the signal. This is

further based on a recent report showing that neutrophil NADPH oxidase could be deactivated by actin depolymerization agents in a cell-free system, suggesting that actin filaments, which grow during the activation of NADPH oxidase, prolong the lifetime of the oxidase complex (58). In addition, the membrane cytoskeleton is involved in recruiting Rac, $p47^{phox}$ and $p67^{phox}$ during oxidase activation (59). These findings suggest a role for Rac, which is known to be involved in actin polymerization, however in neutrophils it has been shown that fMLP induced Rac activation is not mediated by Src kinases or PI3K (60). Thus it might be that Rac and Src/PI3K/PKB act in parallel regulating fMLP induced actin polymerization and superoxide production, whereby Rac is inducing the processes and Src/PI3K/PKB is mediating the stabilization and duration (Fig. 3).

2.2 Role of Rac in granulocyte function

In contrast to Src kinases, Rac is activated by GPCRs and not by cytokine receptors. Most of the Rac data are based on cell lines using ectopic over-expression of mutants of Rac or are from mice deficient in Rac (61-66). Disadvantages of these methods are that transformed cell lines, are not truly representative of primary cells and that Rac may also influence the embryonic development of mice, or the differentiation of granulocytes. The only data concerning a role of Rac in human granulocytes are from a patient bearing a dominant negative mutant of Rac2 (67, 68). Several processes such as fMLP induced migration and respiratory burst are diminished in neutrophils from these patients (67). In human neutrophils, however is not known which cellular functions can be mediated by activation of Rac alone.

In Chapter 6 we investigated the role of Rac in eosinophil functioning, by transducing these cells with a constitutively active mutant of Rac. We found that activation of Rac is not required for activation or enhancement of the respiratory burst. We also showed that constitutively active Rac did not induce or enhance the superoxide production induced by STZ. This is supported by reports showing that although superoxide production in response to fMLP, or fMLP/PAF and IgG-opsonized particles stimulation was greatly diminished in neutrophils from patients bearing the dominant negative Rac2 or in Rac2 deficient mice, opsonized zymosan and PMA induced superoxide production was almost normal (62, 63, 67, 68). In addition, PMA stimulation results in a strong activation of the respiratory burst without activating Rac in neutrophils (60). These findings suggest that the requirement of Rac in superoxide production is not absolute and is selective for specific signaling pathways. Although studies have shown that Rac is present in the NADPH oxidase complex, conflicting data exist on the precise role of activation of Rac in the superoxide production (69-72). It might be that some stimuli use the activation of Rac to enhance the superoxide burst, while others pathways only need the presence of Rac in

the NADPH complex and regulated the stability of the NADPH complex through alternative means.

We observed that Rac activation is sufficient to modulate IgA binding, to eosinophils, whereas STZ binding, which is mainly dependent on CD11b/CD18 intergrins, is not sensitive. Furthermore, we could demonstrate that FcαRI lateral mobility in the membrane was diminished by activation of Rac. Our findings suggest that decreased lateral mobility of FcαRI, results in clustering of the FcαRI and subsequent enhanced IgA binding. Although it is likely that the enhanced IgA binding by activation of Rac is the result of an increase in avidity, a subsequent change in affinity cannot be excluded. Furthermore, these findings demonstrate that functioning of FcαRI and CD11b/CD18, can be modulated by distinct inside-out signaling pathways. Functioning of both receptors are known to be dependent on cytoskeletal rearangements (73, 74), which has been reported to be mediated by Rac via F-actin polymerisation (75). However, our data suggest that besides cytoskeletal rearangement, an alternative pathway is necessary to modulate receptor functioning. Indeed, the function of FcαRI, is also modulated by the phosphorylation of the intracellular tail. Mutation of a C-terminal serine (Ser263) to alanine (S263A) in FcαR, results in constitutive IgA binding, whereas mutation to an aspartic acid (S263D), which mimics the phosphorylation of this residue, greatly reduced IgA binding, even after cytokine stimulation (73). Besides the regulation of FcαRI by phosphorylation, phosphorylation also has the potential to regulate integrin function, since the presence of highly conserved tyrosine, threonine and serine residues in the integrin cytopasmic tails. CD11b is constitutively phosphorylated, whereas the CD18 becomes phosphorylated after activation (76, 77). Furthermore it has been reported that threonine phosphorylation of CD18 plays a role in CD11b/CD18 avidity (78). For β1 integrin it has been shown similarly to FcαRI that phosphorylation of a serine residue leads to inactivation of the integrin (79). Thus, although phosphorylation of β1 integrin and FcαRI leads to similar regulation of both receptors, the pathway mediating the phosphorylation of FcαRI is modulated by a distinct mechanism than that of modulating integrin functioning.

Inhibitor studies implicated PI3K and p38, respectively, in IL-3 and IL-4 induced activation of FcαRI. In addition, both IL-3 and IL-4 mediate IgA binding via PKC, which has been shown to act downstream of PI3K in regulating IgA binding (80). Thus it might be that IL-3 and IL-4 signalling converge at the level of PKC. Possibly Rac, which has been described to act upstream of PI3K and p38 in some other cell systems (81), regulates FcαRI function via PI3K and p38 (82) or that Rac acts in parallel with PI3K and p38 to modulate PKC. The involvement of PI3K in activation of FcαRI, might also suggest a role for Src kinases in this process (Chapter 5). In neutrophils, there is no link between Rac and Src kinases, since a pharmacological inhibitor of Src kinases did not inhibit GPCRs induced activation of Rac, and cytokines cannot induced Rac activation (60),

however we can not exclude the possibility that Rac is upstream of Src kinases. In addition, it has been described that Src kinases are physical associated with the FcαR (83), and play a role in FcαRI induced release of IL-1RA and IL-8. Although this finding suggests a role for Src kinases downstream of FcαRI, it would be interesting to investigate whether the Src kinases that are bound to the FcαRI are also involved in linking cytokine signaling with FcαRI functioning.

Of interest are the observations that Rac is involved in NAPDH oxidase activation upon stimulation of FcγR, but not with serum treated zymosan, which stimulate neutrophils mainly via CD11b/CD18 (Chapter 6) (62). Consistent with these observations, in macrophages, Rac and Cdc42 regulate the uptake of Ig-opsonized particles via FcγR, whereas Rho, and not Rac or Cdc42, plays a prominent role in CD11b/CD18-mediated phagocytosis (84). These findings together with our finding that Rac is modulating the FcαRI functioning and not CD11b/CD18, suggest an important and specific role for Rac in the functioning of Fc receptors (Fig. 4). To make the picture more complete, it would be interesting to determine whether Rho is modulating CD11b/CD18 integrin avidity.

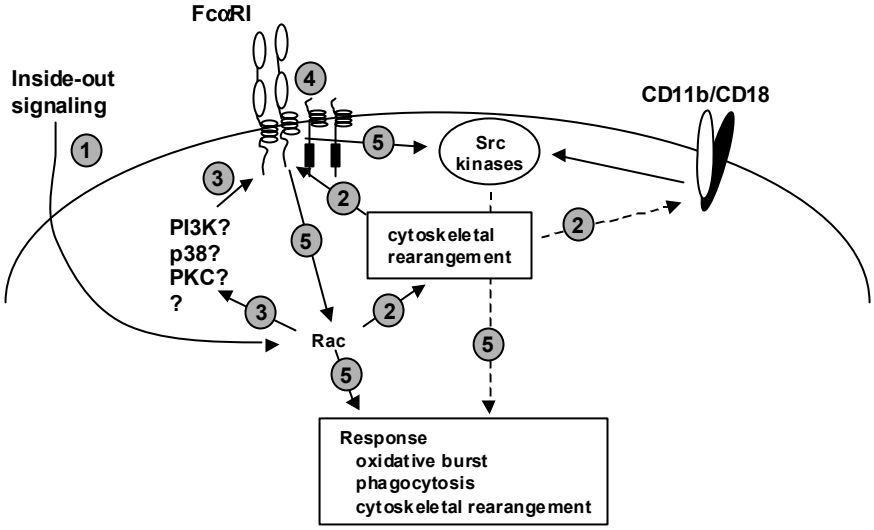

Fig. 4: Rac mediated inside out signaling. (1) Inside out signaling can be mediated via Rac, which modulates FcαRI functioning via two distinct pathways. **(2)** One pathway is through regulation of cytoskeletal rearangement, which is involved in both FcαRI and integrin functioning. Besides this general pathway, **(3)** Rac mediates FcαRI functioning via a specific pathway, which might be mediated by PI3K, p38 MAPK, PKC or by other signaling proteins. **(4)** Activation of both pathways leads to a decrease in lateral membrane mobility, resulting in clustering of the FcαRIs and subsequent enhanced binding of IgA, followed by activation of FcαRI. **(5)** Activation of FcαRI leads to activation of Rac and Src kinases, which mediate FcαRI responses such as production of superoxide and phagocytosis.

Concluding remarks

In this thesis, we have investigated via molecular/cellular tools, including gene array and real time RT-PCR, the activation of peripheral blood neutrophils in COPD patients (Chapter 2-4). These findings demonstrate that investigation of the transcriptional program of cells give insight into their activation state, and thus on the processes, in which they play an important role. Here we demonstrate that the activation state of neutrophils can be determined through various approaches including gene expression profiling, activation of intracellular signaling pathways and priming. In addition in Chapter 7 a new methodology was developed to determine activation of intracellular signalling pathways in a high-throughput assay. Understanding the molecular mechanisms regulating cellular function under distinct conditions (Chapter 5 and 6) is important to define the processes underlying activation of inflammatory cells. Furthermore, understanding how granulocyte function is perturbed can provide new therapeutic drug targets, that will restore proper neutrophil functioning. The ideal therapy would prevent unnecessary tissue damage while preserving the adaptive and benefical aspects of the inflammatory response.

References

1. Pesci, A., B. Balbi, M. Majori, G. Cacciani, S. Bertacco, P. Alciato, C. F. Donner. 1998. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur. Respir. J.* 12:380-386.
2. Pesci, A., M. Majori, A. Cuomo, N. Borciani, S. Bertacco, G. Cacciani, M. Gabrielli. 1998. Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease. *Respir. Med.* 92:863-870.
3. Terashima, T., M. E. Klut, D. English, J. Hards, J. C. Hogg, S. F. van Eeden. 1999. Cigarette smoking causes sequestration of polymorphonuclear leukocytes released from the bone marrow in lung microvessels. *Am. J. Respir. Cell Mol. Biol.* 20:171-177.
4. Bosken, C. H., J. Hards, K. Gatter, J. C. Hogg. 1992. Characterization of the inflammatory reaction in the peripheral airways of cigarette smokers using immunocytochemistry. *Am. Rev. Respir. Dis.* 145:911-917.
5. Noguera, A., S. Batle, C. Miralles, J. Iglesias, X. Busquets, W. MacNee, A. G. Agusti. 2001. Enhanced neutrophil response in chronic obstructive pulmonary disease. *Thorax* 56:432-437.
6. Noguera, A., X. Busquets, J. Sauleda, J. M. Villaverde, W. MacNee, A. G. Agusti. 1998. Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 158:1664-1668.
7. Keatings, V. M., and P. J. Barnes. 1997. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am. J. Respir. Crit. Care Med.* 155:449-453.
8. Masubuchi, T., S. Koyama, E. Sato, A. Takamizawa, K. Kubo, M. Sekiguchi, S. Nagai, T. Izumi. 1998. Smoke extract stimulates lung epithelial cells to release neutrophil and monocyte chemotactic activity. *Am. J. Pathol.* 153:1903-1912.
9. Sato, E., S. Koyama, A. Takamizawa, T. Masubuchi, K. Kubo, R. A. Robbins, S. Nagai, T. Izumi. 1999. Smoke extract stimulates lung fibroblasts to release neutrophil and monocyte chemotactic activities. *Am. J. Physiol.* 277:L1149-L1157.
10. Dubar, V., P. Gosset, C. Aerts, C. Voisin, B. Wallaert, A. B. Tonnel. 1993. In vitro acute effects of tobacco smoke on tumor necrosis factor alpha and interleukin-6 production by alveolar macrophages. *Exp. Lung Res.* 19:345-359.
11. Koethe, S. M., J. R. Kuhnmuench, C. G. Becker. 2000. Neutrophil priming by cigarette smoke condensate and a tobacco anti-idiotypic antibody. *Am. J. Pathol.* 157:1735-1743.
12. Vernooy, J. H., M. Kucukaycan, J. A. Jacobs, N. H. Chavannes, W. A. Buurman, M. A. Dentener, E. F. Wouters. 2002. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am. J. Respir. Crit. Care Med.* 166:1218-1224.
13. Keatings, V. M., P. D. Collins, D. M. Scott, P. J. Barnes. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am. J. Respir. Crit. Care Med.* 153:530-534.
14. Bemelmans, M. H., L. J. van Tits, W. A. Buurman. 1996. Tumor necrosis factor: function, release and clearance. *Crit. Rev. Immunol.* 16:1-11.
15. Botha, A. J., F. A. Moore, E. E. Moore, F. J. Kim, A. Banerjee, V. M. Peterson. 1995. Postinjury neutrophil priming and activation: an early vulnerable window. *Surgery* 118:358-364.
16. Lee, W. L., and G. P. Downey. 2001. Neutrophil activation and acute lung injury. *Curr. Opin. Crit. Care* 7:1-7.
17. Hoch, R. C., R. Rodriguez, T. Manning, M. Bishop, P. Mead, W. C. Shoemaker, E. Abraham. 1993. Effects of accidental trauma on cytokine and endotoxin production. *Crit. Care Med.* 21:839-845.
18. Hensler, T., S. Sauerland, B. Bouillon, M. Raum, D. Rixen, H. J. Helling, J. Andermahr, E. A. Neugebauer. 2002. Association between injury pattern of patients with multiple injuries and circulating levels of soluble tumor necrosis factor receptors, interleukin-6 and interleukin-10, and polymorphonuclear neutrophil elastase. *J. Trauma.* 52:962-970.
19. Webster, R. O., G. L. Larsen, B. C. Mitchell, A. J. Goins, P. M. Henson. 1982. Absence of inflammatory lung injury in rabbits challenged intravascularly with complement-derived chemotactic factors. *Am. Rev. Respir. Dis.* 125:335-340.
20. Anderson, B. O., R. S. Poggetti, P. F. Shanley, D. D. Bensard, J. M. Pitman, D. W. Nelson, G. J. Whitman, A. Banerjee, A. H. Harken. 1991. Primed neutrophils injure rat lung through a platelet-activating factor-dependent mechanism. *J. Surg. Res.* 50:510-514.

21. Wedzicha, J. A. 2002. Exacerbations: etiology and pathophysiologic mechanisms. *Chest* 121:136S-141S.
22. Aaron, S. D., J. B. Angel, M. Lunau, K. Wright, C. Fex, N. Le Saux, R. E. Dales. 2001. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 163:349-355.
23. Cassatella, M. A. 1999. Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* 73:369-509.
24. Arend, W. P. 2001. Cytokine imbalance in the pathogenesis of rheumatoid arthritis: the role of interleukin-1 receptor antagonist. *Semin. Arthritis Rheum.* 30:1-6.
25. Schiff, M. H. 2000. Role of interleukin 1 and interleukin 1 receptor antagonist in the mediation of rheumatoid arthritis. *Ann. Rheum. Dis.* 59 Suppl 1:i103-i108.
26. Dentener, M. A., E. C. Creutzberg, A. M. Schols, A. Mantovani, C. van't Veer, W. A. Buurman, E. F. Wouters. 2001. Systemic anti-inflammatory mediators in COPD: increase in soluble interleukin 1 receptor II during treatment of exacerbations. *Thorax* 56:721-726.
27. Thomas, S. M., and J. S. Brugge. 1997. Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* 13:513-609.
28. M'Rabet, L., P. J. Coffer, R. M. Wolthuis, F. Zwartkruis, L. Koenderman, J. L. Bos. 1999. Differential fMet-Leu-Phe- and platelet-activating factor-induced signaling toward Ral activation in primary human neutrophils. *J Biol Chem* 274:21847-21852.
29. Coffer, P. J., N. Geijsen, L. M'Rabet, R. C. Schweizer, T. Maikoe, J. A. Raaijmakers, J. W. Lammers, L. Koenderman. 1998. Comparison of the roles of mitogen-activated protein kinase kinase and phosphatidylinositol 3-kinase signal transduction in neutrophil effector function. *Biochem. J.* 329:121-130.
30. Corey, S., A. Eguinoa, K. Puyana-Theall, J. B. Bolen, L. Cantley, F. Mollinedo, T. R. Jackson, P. T. Hawkins, L. R. Stephens. 1993. Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src-related tyrosine kinase(s) in human myeloid derived cells. *EMBO J.* 12:2681-2690.
31. Adachi, T., K. Pazdrak, S. Stafford, R. Alam. 1999. The mapping of the Lyn kinase binding site of the common beta subunit of IL-3/granulocyte-macrophage colony-stimulating factor/IL-5 receptor. *J. Immunol.* 162:1496-1501.
32. Vanhaesebroeck, B., S. J. Leevers, G. Panayotou, M. D. Waterfield. 1997. Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* 22:267-272.
33. Thelen, M., and S. A. Didichenko. 1997. G-protein coupled receptor-mediated activation of PI 3-kinase in neutrophils. *Ann N Y Acad Sci* 832:368-382.
34. Ptasznik, A., E. R. Prossnitz, D. Yoshikawa, A. Smrcka, A.E., G. M. Bokoch. 1996. A tyrosine kinase signaling pathway accounts for the majority of phosphatidylinositol 3,4,5-trisphosphate formation in chemoattractant-stimulated human neutrophils. *J Biol Chem* 271:25204-25207.
35. Ptasznik, A., A. Traynor-Kaplan, G. M. Bokoch. 1995. G protein-coupled chemoattractant receptors regulate Lyn tyrosine kinase.Shc adapter protein signaling complexes [published erratum appears in J Biol Chem 1995 Oct 13;270(41):24622]. *J. Biol. Chem.* 270:19969-19973.
36. Hirsch, E., V. L. Katanaev, C. Garlanda, O. Azzolino, L. Pirola, L. Silengo, S. Sozzani, A. Mantovani, F. Altruda, M. P. Wymann. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287:1049-1053.
37. Sasaki, T., J. Irie-Sasaki, R. G. Jones, A.J., W. L. Stanford, B. Bolon, A. Wakeham, A. Itie, D. Bouchard, I. Kozieradzki, N. Joza, T. W. Mak, P. S. Ohashi, A. Suzuki, J. M. Penninger. 2000. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 287:1040-1046.
38. Stephens, L. R., A. Eguinoa, H. Erdjument-Bromage, M. Lui, F. Cooke, J. Coadwell, A. S. Smrcka, M. Thelen, K. Cadwallader, P. Tempst, P. T. Hawkins. 1997. The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* 89:105-114.
39. Stoyanov, B., S. Volinia, T. Hanck, I. Rubio, M. Loubtchenkov, D. Malek, S. Stoyanova, B. Vanhaesebroeck, R. Dhand, B. Nurnberg. 1995. Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science* 269:690-693.
40. Stephens, L., A. Smrcka, F. T. Cooke, T. R. Jackson, P. C. Sternweis, P. T. Hawkins. 1994. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein beta gamma subunits. *Cell* 77:83-93.
41. Yum, H. K., J. Arcaroli, J. Kupfner, R. Shenkar, J. M. Penninger, T. Sasaki, K. Y. Yang, J. S. Park, E. Abraham. 2001. Involvement of phosphoinositide 3-kinases in neutrophil activation and the development of acute lung injury. *J. Immunol.* 167:6601-6608.

42. Ma, Y. C., J. Huang, S. Ali, W. Lowry, X. Y. Huang. 2000. Src tyrosine kinase is a novel direct effector of G proteins. *Cell* 102:635-646.
43. Gu, C., Y. C. Ma, J. Benjamin, D. Littman, M. V. Chao, X. Y. Huang. 2000. Apoptotic signaling through the beta -adrenergic receptor. A new Gs effector pathway. *J. Biol. Chem.* 275:20726-20733.
44. Arai, H., C. L. Tsou, I. F. Charo. 1997. Chemotaxis in a lymphocyte cell line transfected with C-C chemokine receptor 2B: evidence that directed migration is mediated by betagamma dimers released by activation of Galphai-coupled receptors. *Proc Natl Acad Sci U S A* 94:14495-14499.
45. Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, M. E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91:231-241.
46. del Peso, L., M. Gonzalez-Garcia, C. Page, R. Herrera, G. Nunez. 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278:687-689.
47. Wei, S., J. H. Liu, P. K. Epling-Burnette, A. M. Gamero, D. Ussery, E. W. Pearson, M. E. Elkabani, J. I. Diaz, J. Y. Djeu. 1996. Critical role of Lyn kinase in inhibition of neutrophil apoptosis by granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 157:5155-5162.
48. Klein, J. B., M. J. Rane, J. A. Scherzer, P. Y. Coxon, R. Kettritz, J. M. Mathiesen, A. Buridi, K. R. McLeish. 2000. Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* 164:4286-4291.
49. Cowburn, A. S., K. A. Cadwallader, B. J. Reed, N. Farahi, E. R. Chilvers. 2002. Role of PI3-kinase-dependent Bad phosphorylation and altered transcription in cytokine-mediated neutrophil survival. *Blood* 100:2607-2616.
50. Dijkers, P. F., R. H. Medema, J. W. Lammers, L. Koenderman, P. J. Coffer. 2000. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* 10:1201-1204.
51. Moulding, D. A., C. Akgul, M. Derouet, M. R. White, S. W. Edwards. 2001. BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. *J Leukoc Biol* 70:783-792.
52. Moulding, D. A., J. A. Quayle, C. A. Hart, S. W. Edwards. 1998. Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood* 92:2495-2502.
53. Kodama, T., K. Hazeki, O. Hazeki, T. Okada, M. Ui. 1999. Enhancement of chemotactic peptide-induced activation of phosphoinositide 3-kinase by granulocyte-macrophage colony-stimulating factor and its relation to the cytokine-mediated priming of neutrophil superoxide-anion production. *Biochem J* 337 (Pt 2):201-209.
54. Dang, P. M., C. Dewas, M. Gaudry, M. Fay, E. Pedruzzi, M. A. Gougerot-Pocidalo, J. el Benna. 1999. Priming of human neutrophil respiratory burst by granulocyte/macrophage colony-stimulating factor (GM-CSF) involves partial phosphorylation of p47(phox). *J Biol Chem* 274:20704-20708.
55. Didichenko, S. A., B. Tilton, B. A. Hemmings, K. Ballmer-Hofer, M. Thelen. 1996. Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase. *Curr Biol* 6:1271-1278.
56. Ellson, C. D., S. Gobert-Gosse, K. E. Anderson, K. Davidson, H. Erdjument-Bromage, P. Tempst, J. W. Thuring, M. A. Cooper, Z. Y. Lim, A. B. Holmes, P. R. Gaffney, J. Coadwell, E. R. Chilvers, P. T. Hawkins, L. R. Stephens. 2001. PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40(phox). *Nat Cell Biol* 3:679-682.
57. Kanai, F., H. Liu, S. J. Field, H. Akbary, T. Matsuo, G. E. Brown, L. C. Cantley, M. B. Yaffe. 2001. The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *Nat Cell Biol* 3:675-678.
58. Tamura, M., M. Kanno, Y. Endo. 2000. Deactivation of neutrophil NADPH oxidase by actin-depolymerizing agents in a cell-free system. *Biochem. J.* 349:369-375.
59. el Benna, J., L. P. Faust, B. M. Babior. 1994. The phosphorylation of the respiratory burst oxidase component p47phox during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. *J Biol Chem* 269:23431-23436.
60. Geijsen, N., S. van Delft, J. A. Raaijmakers, J. W. Lammers, J. G. Collard, L. Koenderman, P. J. Coffer. 1999. Regulation of p21rac activation in human neutrophils. *Blood* 94:1121-1130.
61. Li, S., A. Yamauchi, C. C. Marchal, J. K. Molitoris, L. A. Quilliam, M. C. Dinauer. 2002. Chemoattractant-stimulated Rac activation in wild-type and Rac2-deficient murine neutrophils: preferential activation of Rac2 and Rac2 gene dosage effect on neutrophil functions. *J. Immunol.* 169:5043-5051.
62. Kim, C., and M. C. Dinauer. 2001. Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. *J. Immunol.* 166:1223-1232.

63. Roberts, A. W., C. Kim, L. Zhen, J. B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J. D. Pollock, J. B. Borneo, G. B. Bradford, S. J. Atkinson, M. C. Dinauer, D. A. Williams. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity.* 10:183-196.
64. Gu, Y., and D. A. Williams. 2002. RAC2 GTPase deficiency and myeloid cell dysfunction in human and mouse. *J. Pediatr. Hematol. Oncol.* 24:791-794.
65. Gabig, T. G., C. D. Crean, P. L. Mantel, R. Rosli. 1995. Function of wild-type or mutant Rac2 and Rap1a GTPases in differentiated HL60 cell NADPH oxidase activation. *Blood* 85:804-811.
66. Dorseuil, O., A. Vazquez, P. Lang, J. Bertoglio, G. Gacon, G. Leca. 1992. Inhibition of superoxide production in B lymphocytes by rac antisense oligonucleotides. *J. Biol. Chem.* 267:20540-20542.
67. Ambruso, D. R., C. Knall, A. N. Abell, J. Panepinto, A. Kurkchubasche, G. Thurman, C. Gonzalez-Aller, A. Hiester, M. deBoer, R. J. Harbeck, R. Oyer, G. L. Johnson, D. Roos. 2000. Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc. Natl. Acad. Sci. U. S. A.* 97:4654-4659.
68. Williams, D. A., W. Tao, F. Yang, C. Kim, Y. Gu, P. Mansfield, J. E. Levine, B. Petryniak, C. W. Derrow, C. Harris, B. Jia, Y. Zheng, D. R. Ambruso, J. B. Lowe, S. J. Atkinson, M. C. Dinauer, L. Boxer. 2000. Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. *Blood* 96:1646-1654.
69. Miyano, K., H. Fukuda, K. Ebisu, M. Tamura. 2003. Remarkable Stabilization of Neutrophil NADPH Oxidase Using RacQ61L and a p67(p)(hox)-p47(p)(hox) Fusion Protein. *Biochemistry* 42:184-190.
70. Heyworth, P. G., U. G. Knaus, J. Settleman, J. T. Curnutte, G. M. Bokoch. 1993. Regulation of NADPH oxidase activity by Rac GTPase activating protein(s). *Mol. Biol. Cell* 4:1217-1223.
71. Bromberg, Y., E. Shani, G. Joseph, Y. Gorzalczany, O. Sperling, E. Pick. 1994. The GDP-bound form of the small G protein Rac1 p21 is a potent activator of the superoxide-forming NADPH oxidase of macrophages. *J. Biol. Chem.* 269:7055-7058.
72. Di-Poi, N., J. Faure, S. Grizot, G. Molnar, E. Pick, M. C. Dagher. 2001. Mechanism of NADPH oxidase activation by the Rac/Rho-GDI complex. *Biochemistry* 40:10014-10022.
73. Bracke, M., J. W. Lammers, P. J. Coffer, L. Koenderman. 2001. Cytokine-induced inside-out activation of FcalphaR (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. *Blood* 97:3478-3483.
74. Schoenwaelder, S. M., and K. Burridge. 1999. Bidirectional signaling between the cytoskeleton and integrins. *Curr. Opin. Cell Biol.* 11:274-286.
75. Hall, A. 1998. Rho GTPases and the actin cytoskeleton. *Science* 279:509-514.
76. Chatila, T. A., R. S. Geha, M. A. Arnaout. 1989. Constitutive and stimulus-induced phosphorylation of CD11/CD18 leukocyte adhesion molecules. *J. Cell Biol.* 109:3435-3444.
77. Buyon, J. P., M. R. Philips, J. T. Merrill, S. G. Slade, J. Leszczynska-Piziak, S. B. Abramson. 1997. Differential phosphorylation of the beta2 integrin CD11b/CD18 in the plasma and specific granule membranes of neutrophils. *J. Leukoc. Biol.* 61:313-321.
78. Valmu, L., and C. G. Gahmberg. 1995. Treatment with okadaic acid reveals strong threonine phosphorylation of CD18 after activation of CD11/CD18 leukocyte integrins with phorbol esters or CD3 antibodies. *J. Immunol.* 155:1175-1183.
79. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69:11-25.
80. Bracke, M., E. Nijhuis, J. W. Lammers, P. J. Coffer, L. Koenderman. 2000. A critical role for PI 3-kinase in cytokine-induced Fcalpha-receptor activation. *Blood* 95:2037-2043.
81. Keely, P. J., J. K. Westwick, I. P. Whitehead, C. J. Der, L. V. Parise. 1997. Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* 390:632-636.
82. Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knaus, R. J. Ulevitch, G. M. Bokoch. 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J. Biol. Chem.* 270:23934-23936.
83. Gulle, H., A. Samstag, M. M. Eibl, H. M. Wolf. 1998. Physical and functional association of Fc alpha R with protein tyrosine kinase Lyn. *Blood* 91:383-391.
84. Caron, E., and A. Hall. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* 282:1717-1721.

Nederlandse samenvatting

Een aanhoudende ontsteking als reactie op ingeademde externe prikkels, zoals bij roken, wordt gezien als een belangrijke kenmerk voor chronisch obstructief longlijden (COPD). Deze ontsteking wordt onderhouden door een continue migratie van witte bloedcellen naar het longweefsel, waarbij speciale extracellulaire signaal eiwitten (cytokines en chemokines) betrokken zijn. Neutrofiele granulocyte is één van die witte bloed cellen die naar het longweefsel migreert en waarvan men denkt dat het daar een rol speelt in weefsel beschadiging. Neutrofiele granulocyten zijn belangrijke cellen voor het immuunsysteem omdat ze het lichaam verdedigen tegen micro-organismen. Ze kunnen namelijk bacteriën onschadelijk maken door ze “op te eten”. Dit “opeten” wordt ook wel fagocytose genoemd. Tijdens fagocytose komen veel schadelijke stoffen vrij die bedoeld zijn voor het vernietigen van de bacteriën. Bij onjuiste productie van deze schadelijke stoffen kunnen deze cellen ook veel schade toebrengen aan weefsels. Daarom bestaat de volledige activatie van deze cellen uit twee stappen. Tijdens de eerste stap worden de cellen in staat van paraatheid gebracht (pre-activatie), waarna een tweede activatie (bijvoorbeeld een infectie) resulteert in fagocytose en productie van toxische stoffen (zie ook Fig. van hoofdstuk 1).

In dit proefschrift is onderzocht of circulerende neutrofiele granulocyten zijn geactiveerd in patiënten met COPD. Neutrofiele granulocyten kunnen na activatie verscheidene nieuwe eiwitten maken, zoals cytokines, chemokines, intracellulaire signaal eiwitten en eiwitten die in de wand van de cel zitten. Dus onderzoek naar de expressie van deze eiwitten kan duidelijk maken of deze cellen geactiveerd zijn. Eventuele expressie patronen zouden meer inzicht kunnen geven door welke stimuli deze cellen zijn geactiveerd. Daarnaast zouden deze expressie patronen ook meer inzicht kunnen geven in de ernst van COPD. Het maken van nieuwe eiwitten gebeurt via de processen transcriptie en translatie. Van het DNA dat als een mal fungeert, wordt een tijdelijke copie gemaakt tijdens het proces transcriptie. Deze tijdelijk copie van een gen wordt messenger RNA genoemd en fungeert weer als mal voor het maken van het desbetreffende eiwit. Dit proces wordt translatie genoemd. Activatie van deze cellen is onderzocht doormiddel van messenger RNA expressie patronen van de neutrofiele granulocyten.

Hoofdstuk 2 toont aan dat neutrofiele granulocyten, gestimuleerd met enkele cytokines, verschillende gen expressie patronen laten zien. Dus aan de hand van deze gen expressie patronen zouden we kunnen bepalen op welke manier neutrofiele granulocyten zijn geactiveerd in COPD patiënten. Inderdaad, de gen expressie patronen van neutrofiele granulocyten van ernstige COPD patiënten vertonen een patroon van activatie die overeenkomt met die van neutrofiele granulocyten geactiveerd met signaal eiwitten zoals TNFα of LPS. Deze activatie is significant bij de meer ernstige COPD patiënten en correleert met de ernst van COPD. Uit het gen expressie patroon van neutrofiele granulocyten blijkt dat deze cellen ook cytokines en chemokines kunnen produceren,

waardoor deze cellen naast het aanbrengen van weefselschade ook de ontstekings reactie kan reguleren. Deze regulatie wordt bepaald door een balans van remmende en stimulerende cytokines/chemokines. Het lijkt dat deze balans in ciruclerende neutrofiele granulocyten van COPD patiënten is verstoort in het nadeel van remmende cytokines.chemokines. Door deze mindere productie van ontstekings remmende cytokines zouden neutrofiele granulocyten een rol kunnen spelen bij de handhaving van de ontsteking. Verder blijkt dat naast deze verhoogde activatie van neutrofiele granulocyten, de activatie variabel is binnen een patiënt (Hoofdstuk 4). Dit is vooral zichtbaar bij een een acute aanval (exacerbatie) van COPD (Hoofdstuk 3). Hierbij verandert het gen expressie patroon van neutrofiele granulocyten vergeleken met de meer stabiele periode. In plaats van een verhoogde activatie tijdens de meer stabiele periode van COPD, lijken de neutrofiele granulocyten weer vrij normaal tijdens een exacerbatie. Een verklaring kan zijn dat de geactiveerde neutrofiele granulocyten migreren naar de luchtwegen door hyperactivatie, terwijl de cellen die achterblijven in het bloed bestaan uit niet geactiveerde cellen of uit net geproduceerde cellen door het beenmerg. Het is bewezen door andere studies dat de instroom van neutrofiele granulocyten, maar ook dat van andere witte bloed cellen, toeneemt tijdens een exacerbatie van COPD. Verder is het waarschijnlijk dat hyperactivatie van neutrofiele granulocyten vooral in ernstige COPD patiënten, die al een lage longfunctie hebben, lijdt tot een verdere daling in longfunctie. Deze daling in longfunctie zal dan eerder tot ernstige klachten lijden of te wel resulteren in een exacerbatie, terwijl bij een mildere COPD patiënt, deze daling in longfunctie nog niet zal resulteren in ernstige klinische symptomen of een exacerbatie. Dus het volgen van de activatie van neutrofiele granulocyten kan helpen bij een beter diagnose van een exacerbatie en kan leiden tot een betere afstemming van het toedienen van medicatie.

Verder hebben we de activatie van intracellulaire signaal eiwitten door cytokines en chemokines in granulocyten vergeleken met de functie van deze cellen. Cytokines en chemokines activeren beide de intracellulaire signaal eiwitten, Src kinases, die op hun beurt een ander signaal eiwit, Protein Kinase B (PKB) kunnen activeren (Hoofdstuk 5). Activatie van Src kinases door cytokines en chemokines spelen een belangrijke rol in het functioneren van neutrofiele granulocyten. Cytokines en chemokines reguleren de overleving van neutrofiele granulocyten en zijn belangrijk voor het maken van schadelijke stoffen zoals zuurstof radicalen. Een ander intracellulair signaal eiwit, Rac, wordt ook geactiveerd door chemokines, maar niet door cytokines. In Hoofdstuk 6 hebben we gevonden dat activatie van Rac resulteert in een verandering in binding van een extracellulair signaal eiwit, IgA. Het is bekend dat binding van IgA resulteert in fagocytose en productie van toxische stoffen door deze cellen. Dus activatie van Rac maakt de cel veel gevoeliger voor IgA oftewel Rac pre-activeert de cel zodat IgA de cel volledig kan activeren.

Verder is er in samenwerking met het Academisch Medisch Centrum te Amsteram een nieuwe techniek ontwikkeld om op een snelle en eenvoudige manier de activatie van

intracellulaire signaal eiwitten te kunnen meten (Hoofdstuk 7). Door deze techniek is het een stuk eenvoudiger om veel patiënten te screenen op de activatie van intracellulaire signaal eiwitten. De combinatie van de activatie van intracellulaire signaal eiwitten en de gen expressie patronen zou een nog beter inzicht kunnen geven op welke manier cellen zijn geactiveerd.

Samenvattend kan gezegd worden dat naast een beter inzicht in de rol van deze cellen in abnormale ontstekings reacties, zoals die zich afspeelt bij COPD, het ook belangrijk is om het onderliggende mechanisme van activatie van granulocyten te begrijpen. Aan de hand van deze informatie kunnen nieuwe specifieke remmers worden ontwikkeld, die eventuele weefsel schade zouden kunnen voorkomen.

Dankwoord

Tja het is dan zover. Na deze woorden te hebben geschreven is het helemaal af. Vier jaar lijken aan het begin nog zo lang, maar vele mensen en natuurlijk ook het project zelf hebben ertoe bijgedragen dat deze jaren voorbij vlogen. Soms sneller dan ik kon bijbenen (letterlijk in het begin van deze periode en figuurlijk later).

Leo en Paul, samen vormden jullie voor mij een goed begeleiders team. Elk vanuit zijn eigen vakgebied, stuurden en holpen jullie mij om mijn eigen weg te vinden in de wereld van het fundamentele en klinisch gericht onderzoek. Paul, eerst dacht je dat ik het maar een week zou uithouden als AIO, maar gelukkig was dit maar een grap, want ik had deze AIO jaren bij jouw niet willen missen. Ik heb enorm veel van je geleerd (behalve, gelukkig, die "chronische" moeheid). Bedankt voor de vrijheid, enthousiasme en de vele discussies. Leo, je wist me altijd weer te inspireren door je eeuwige optimisme, je interesse en vele discussies. Veel plezier in Australië.

Jan-Willem en Ed, al waren jullie niet direct betrokken bij mijn proeven, jullie maakten het wel mogelijk dat ik onderzoek kon doen aan COPD. Jan-Willem ook nog bedankt voor het snelle nakijken van mijn proefschrift.

Verder wil ik graag al mijn collega's bedanken voor hun interesse, discussies, adviezen en natuurlijk de vele borrels. Van de boven verdieping wil ik graag mijn "oude" pipetteerbuurvrouw, Sandra, bedanken voor het advies om op het grote lab te gaan pipeteren, het leren van verscheidene technieken en natuurlijk de vele bakkies koffie. Erik-jan, ik was nog niet weg of je had mijn plekkie al ingenomen. Bedankt voor al het werk dat je hebt verzet voor de patienten studies en veel succes met je vervolg studie. We zullen elkaar nog wel vaker tegen komen. Mike, I would like to thank you for all the hard work (RNA isolations, pcr etc) you have done. It was a nice time and I still miss your humor and our discussions. Cornelieke, bedankt voor de vele tips en het in orde houden van de boven verdieping, wat soms een hele kunst is. Miranda, we zijn elkaar al vaker tegen gekomen (studie, stages etc), bedankt voor de vele discussies en op naar een nieuw schaatsseizoen (dit laatste geldt ook voor Erik-Jan en Monique). Marcela, thanks for a great time at the molecular course in Spetses and good luck with finishing your PhD. Hanneke bedankt voor de laatste experimenten, die hoofdstuk 6 meer inhoud hebben gegeven. Kim, met jou en Erik-Jan in de buurt was er altijd wel wat te beleven, bedankt voor de gezelligheid.

De beneden verdieping: Laurien, als mede Twentenaar hebben we nogal eens veel plezier gehad om het Twentse dialect (loat oe'n kop nich gek maakn). Deon, Jan en Corneli, bedankt voor de ondersteuning bij de microscopen en de FACS en mede dankzij jullie kon hoofdstuk 6 uitgebreid worden. Madelon, bedankt voor de IgA insteek in Hoofdstuk 6. Lei, bedankt voor alle adviezen over afval verwerking. Bart en Willem, bedankt voor jullie interesse en discussies en veel succes met jullie projecten.

Verder wil ik nog alle ex-labgenoten (die nog niet genoemd waren) bedanken voor de vele discussies en tips: Belinda, Niels, Pascale, Rolf, Jacqueline (bedankt voor de vele tips over de migratie assay), Ramon, Annelien, Andy, Paula, Nadia en Boudewijn.

Van de afdeling Virologie wil ik graag Pauline, Dorien, Loek en Michiel bedanken voor hun hulp bij het opzetten van real time PCR technologie binnen mijn project.
Uit Twente wil ik graag mij kameraden bedanken voor de vele feestjes, die mij weer op andere gedachten dan het werk konden brengen (’t spiet me doonders, me’r vor oeleu is mien book nig kats in ’t plat). Oud-huis genoten, bedankt voor de gezelligheid na mijn werktijd.
Beste Monique, zus(je) en paranimf, je bent voornamelijk verantwoordelijk dat ik in deze wereld van wetenschap ben beland. Ik vraag me nog steeds af of het toeval was of dat er toch een genetische factor een rol speelt. Bedankt voor je steun en advies.
Pa en ma, bedankt dat ik de kans heb gekregen om verder te studeren en dat ik zelf de keuze had om te kunnen doen wat ik graag wilde. Helaas pa, ik ben niet de agrarische kant op gegaan, want tijdens mijn eerste jaar biologie vond ik de plantenkant zo’n gestuntel dat ik toen besloot dat dat niks voor mij was. René, mijn grote broer, vroeger was je voor mij het grote voorbeeld, alleen is later mijn interesse een andere kant opgegaan (die van mijn zus dus). Ik bewonder je wilskracht in het voortzetten van het bedrijf (ik zou het niet kunnen). In de toekomst hoop ik af en toe wat tijd vrij te kunnen maken om net als vroeger weer eens te helpen op zaterdag.
Verin, zonder jouw zou ik de laatste tijd niet zijn door gekomen. Je maakte me bewuster van dingen waar ik niet zo snel bij stil sta. Hoewel dit leidde tot enige conflicten, ben ik er nu een stuk beter uitgekomen. Bedankt voor je steun.

Evert

Curriculum Vitae

De schrijver van dit proefschrift werd op 14 oktober 1974 geboren te Almelo. Na het behalen van zijn Atheneum B diploma aan het Twents Carmellyceum in Oldenzaal begon hij in 1993 met de studie Biologie aan de Universiteit Utrecht.
In augustus 1998 haalde hij het doctoraal examen met als hoofdvakken moleculaire biologie (Dr. A. Thomas, afdeling moleculaire biologie, Universiteit Utrecht) en immunologie (Dr. M. Sims, afdeling immunologie, Glaxo Wellcome in Stevenage, England).
In de periode van september 1998 tot augustus was hij werkzaam als assistent in opleiding bij de afdeling Longziekten van het Universitair Medisch Centrum te Utrecht. Onder begeleiding van Dr. P. Coffer, Prof. L. Koenderman en Prof. Dr. J-W.J. Lammers heeft hij gewerkt aan een project gefinancierd door het Nederlands Astma Fonds.
Momenteel is hij werkzaam als post-doctoraal onderzoeker bij de afdeling Dermatologie/Allergologie van het Universitair Medisch Centrum te Utrecht