Pro- and Anti-inflammatory Regulation of β2 Integrin Signalling in Human Neutrophils

Veronika Patcha Brodin
Cover image: Fluorescence image of differentiated HL60 cells phagocytosing yeast. Shown in the image; F-actin depicted in red, and yeast in grey.

© Veronika Brodin
All rights reserved

ISSN: 0345-0082
ISBN: 978-91-85715-22-0

The published articles are reprinted with the permission from the publishers

Printed by LiU-Tryck, Linköping, Sweden 2007
To Greger, Ella, and Alexander

...and all the curious people out there
ABSTRACT

The body is under constant attack from pathogens trying to slip by our immune defence. If the barrier is breached, invading pathogens enter the tissues and cause inflammation. During this process neutrophils, constituting the first line of defence, leave the bloodstream and seek out and kill the invading pathogens. The mechanisms leading to activation of receptors on neutrophils must be closely orchestrated. Pro- and anti-inflammatory substances can influence the outcome of the inflammation process by affecting the involved players. If not well balanced, inflammatory diseases, such as atherosclerosis and rheumatoid arthritis, can be the outcome.

The aim of this thesis was to elucidate the effect of pro- (fMLP, Leukotriene B₄, and Interleukin-8) and anti- (lipoxins, aspirin and statins) inflammatory substances on the β₂ integrins, mediating adhesion of neutrophils both under “normal” conditions and during coronary artery disease. More specifically, the effect of these substances on the β₂ integrins were studied in regard to: i) the activity (i.e. affinity and avidity) of β₂ integrins, ii) the signalling capacity of β₂ integrins (i.e. detected as release of arachidonic acid, and the production of reactive oxygen species, and iii) the signal transduction mediated by the β₂ integrins (i.e. phosphorylation of Pyk2).

The pro-inflammatory substances belong to the family of chemoattractants that induces transmigration and chemotaxis. A hierarchy exists between the different family members; the end-target chemoattractants (e.g. fMLP) being more potent than intermediary chemoattractants (e.g. IL-8 and LTB₄). It was found that intermediary chemoattractants regulate β₂ integrins by mainly affecting the avidity of β₂ integrins. End-target chemoattractants on the other hand, affected the β₂ integrins by increasing the avidity and the affinity, as well as their signalling capacity.

The anti-inflammatory substances used in this study were the exogenous aspirin and statins, and the endogenous lipoxins. In the presence of aspirin, stable analogues of lipoxin (i.e. epi-lipoxins) are formed in a trans-cellular process. Lipoxin inhibited the signalling capacity of β₂
integrins mediated by intermediary chemoattractants, as well as the signal transduction induced by end-target chemoattractants. Moreover, the signalling capacity of β2 integrins in neutrophils from patients suffering from coronary artery disease (CAD) was impaired. Arachidonic acid, the precursor for both pro- and anti-inflammatory eicosanoid, induced an increase in the β2 integrin activity (both affinity and avidity), but had no effect on the signal transduction.

In conclusion, different “roles” were observed for end-target and intermediary chemoattractants in the regulation of β2 integrins. The inhibitory effects of the anti-inflammatory lipoxins support earlier studies suggesting that these agents function as “stop signals” in inflammation. This is also confirmed by our findings in CAD patients, who have elevated levels of epi-lipoxins due to aspirin treatment. Moreover, Pyk2 was identified as a possible target for the inhibitory effect of anti-inflammatory drugs.
PREFACE

This thesis is based on the following papers, referred to by their Roman numerals (I-V):


II) **Patcha Brodin V** and Särndahl E: Lipoxin A₄ inhibits the fMet-Leu-Phe-induced, but not β2 integrin-induced activation of the non-receptor tyrosine kinase Pyk2 in human leukemia cells (HL-60). Manuscript 2007, submitted

III) **Patcha Brodin V** and Särndahl E: Inside-out regulated, β2 integrin-induced release of arachidonic acid in Human Leukemia 60 cells. Manuscript 2007, submitted


<table>
<thead>
<tr>
<th>CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT ............................................................................................. 2</td>
</tr>
<tr>
<td>PREFACE .................................................................................................. 4</td>
</tr>
<tr>
<td>CONTENTS ............................................................................................... 6</td>
</tr>
<tr>
<td>ABBREVIATIONS ....................................................................................... 8</td>
</tr>
<tr>
<td>INTRODUCTION .......................................................................................... 8</td>
</tr>
<tr>
<td>ADHESION ............................................................................................... 12</td>
</tr>
<tr>
<td>THE ACTIN CYTOSKELETON ........................................................................ 13</td>
</tr>
<tr>
<td>INTEGRINS ............................................................................................... 14</td>
</tr>
<tr>
<td>Integrin activation: affinity, avidity, and valency .................................. 16</td>
</tr>
<tr>
<td>B2 integrin ligation ............................................................................. 18</td>
</tr>
<tr>
<td>INTEGRINS, PARTNERS IN SICKNESS AND IN HEALTH .................................. 20</td>
</tr>
<tr>
<td>INTEGRINS, MATRIX ADHESIONS AND ASSOCIATED PROTEINS ....................... 21</td>
</tr>
<tr>
<td>- Cytoskeletal proteins associated with adhesion complexes .................. 21</td>
</tr>
<tr>
<td>- Proteins involved in mediating signal transduction induced by integrins 23</td>
</tr>
<tr>
<td>CELL MOTILITY ......................................................................................... 25</td>
</tr>
<tr>
<td>REGULATION OF ADHESION SIGNALLING BY LIPID METABOLITES ............... 26</td>
</tr>
<tr>
<td>- Phospholipase A2 ............................................................................. 26</td>
</tr>
<tr>
<td>- Arachidonic acid and pro-inflammatory eicosanoids ............................... 27</td>
</tr>
<tr>
<td>- Anti-inflammatory lipoxins .................................................................. 28</td>
</tr>
<tr>
<td>PHAGOCYTOSIS ......................................................................................... 29</td>
</tr>
<tr>
<td>- The phagocytic process ..................................................................... 29</td>
</tr>
<tr>
<td>- Degranulation ................................................................................... 30</td>
</tr>
<tr>
<td>AIMS OF THE INVESTIGATION ................................................................. 31</td>
</tr>
<tr>
<td>METHODS .................................................................................................. 33</td>
</tr>
<tr>
<td>NEUTROPHILS ......................................................................................... 33</td>
</tr>
<tr>
<td>[3H]ARACHIDONIC ACID-LABELLING AND RELEASE .................................... 34</td>
</tr>
<tr>
<td>CELL STIMULATION ................................................................................ 34</td>
</tr>
<tr>
<td>- Activation by chemotactic factors .................................................... 34</td>
</tr>
<tr>
<td>- Integrin ligation ................................................................................. 34</td>
</tr>
<tr>
<td>FLOW CYTOMETRY .................................................................................. 35</td>
</tr>
<tr>
<td>- Expression of B2-integrins ................................................................ 37</td>
</tr>
<tr>
<td>- B2 integrin affinity ........................................................................... 37</td>
</tr>
<tr>
<td>- Binding of soluble ICAM-I ................................................................. 38</td>
</tr>
<tr>
<td>FLUORESCENCE MICROSCOPY ............................................................ 38</td>
</tr>
<tr>
<td>SINGLE PARTICLE TRACKING .................................................................. 38</td>
</tr>
<tr>
<td>CONFocal SCANNING LIGHT MICROSCOPY ........................................... 39</td>
</tr>
<tr>
<td>IMAGE ANALYSIS ................................................................................... 40</td>
</tr>
<tr>
<td>- Clustering of integrins ...................................................................... 40</td>
</tr>
<tr>
<td>- Granule distribution .......................................................................... 40</td>
</tr>
<tr>
<td>INTRODUCTION OF RECOMBINANT PROTEINS INTO HUMAN NEUTROPHILS ... 40</td>
</tr>
<tr>
<td>HIV TAT PROTEINS ............................................................................... 41</td>
</tr>
<tr>
<td>- TAT transduction .............................................................................. 41</td>
</tr>
<tr>
<td>IMMUNOPRECIPITATION AND WESTERN BLOT ....................................... 42</td>
</tr>
<tr>
<td>PHAGOCYTOSIS AND PRODUCTION OF REACTIVE OXYGEN SPECIES ........ 42</td>
</tr>
<tr>
<td>STATISTICAL ANALYSIS ...................................................................... 42</td>
</tr>
<tr>
<td>RESULTS .................................................................................................. 44</td>
</tr>
<tr>
<td>THE EFFECT OF PRO- AND ANTI-INFLAMMATORY CHEMOTACTANTS ON THE ACTIVATION STATE OF THE B2 INTEGRINS ................................................................. 44</td>
</tr>
<tr>
<td>Abbreviation</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>ATL</td>
</tr>
<tr>
<td>BSA</td>
</tr>
<tr>
<td>CAD</td>
</tr>
<tr>
<td>COX</td>
</tr>
<tr>
<td>CR3</td>
</tr>
<tr>
<td>CRP</td>
</tr>
<tr>
<td>C3b</td>
</tr>
<tr>
<td>C3bi</td>
</tr>
<tr>
<td>DMSO</td>
</tr>
<tr>
<td>EDTA</td>
</tr>
<tr>
<td>EGTA</td>
</tr>
<tr>
<td>ECM</td>
</tr>
<tr>
<td>F-actin</td>
</tr>
<tr>
<td>FcR</td>
</tr>
<tr>
<td>FSC</td>
</tr>
<tr>
<td>FAK</td>
</tr>
<tr>
<td>FITC</td>
</tr>
<tr>
<td>fMLP</td>
</tr>
<tr>
<td>GAP</td>
</tr>
<tr>
<td>GDI</td>
</tr>
<tr>
<td>GDF</td>
</tr>
<tr>
<td>GDP</td>
</tr>
<tr>
<td>GEF</td>
</tr>
<tr>
<td>G-protein</td>
</tr>
<tr>
<td>GPCR</td>
</tr>
<tr>
<td>GTP</td>
</tr>
<tr>
<td>HA</td>
</tr>
<tr>
<td>HETE</td>
</tr>
<tr>
<td>HL60</td>
</tr>
<tr>
<td>HPETE</td>
</tr>
<tr>
<td>HRP</td>
</tr>
<tr>
<td>HSA</td>
</tr>
<tr>
<td>ICAMs</td>
</tr>
<tr>
<td>IgG</td>
</tr>
<tr>
<td>IL-8</td>
</tr>
<tr>
<td>LAD</td>
</tr>
<tr>
<td>LFA-1</td>
</tr>
<tr>
<td>LOX</td>
</tr>
<tr>
<td>LTs</td>
</tr>
</tbody>
</table>
LTB₄  leukotriene B₄
LX    lipoxin
LXA₄  lipoxin A₄
MAPK  mitogen activated protein kinase
MAPTA/AM 1,2-bis-5-methyl-amino-phenoxy-ethane-N,N,N´-tetra-acetoxyethyl acetate
MFI   mean fluorescence intensity
NADPH nicotinamide adenine dinucleotide phosphate
PAF   platelet activating factor
PFA   paraformaldehyde
PGs   prostaglandins
PI-3 K phosphatidylinositol-3 Kinase
PKC   protein kinase C
PLA₂  phospholipase A₂
cPLA₂ cytosolic PLA₂
sPLA₂ secretory PLA₂
PMA   phorbol 12-myristate acetate
PMB   polymyxin B
PMN   polymorphonuclear neutrophils
PTKs  protein tyrosine kinases
Pyk2  proline-rich tyrosine kinase 2
ROS   reactive oxygen species
S.E.M. scanning electron microscopy
SOD   superoxide dismutase
SPT   single particle tracking
SSC   side scatter
Tat   transcriptional activator of transcription of HIV
TAT   transduction domain of HIV Tat (11 aa)
TNF   tumour necrosis factor
It was the darkest of times. Evil forces thrived in the world. An enemy invaded the land. But there was hope. The "White Army" secretly prepared for war on the islands of the Red River. Armed with powerful weapons, they formed the “first line of defence”. Brave soldiers breached the Endothelial Wall in the quest to seek out the vile enemy. The climb in the ECM-Mountains was steep and dangerous. The tracks were easily followed, the foul smell of the invaders lingering in the air. Finally, the enemy camps were in reach. Equipped with deadly toxins, the soldiers attacked. The battle went on for days. Darkness prevailed, but in the end hope returned. Out-numbered, the enemy perished. The land slowly began to heal, and soon no signs of invasion were seen.

Once again the inhabitants of the land lived in peace and harmony, knowing that somewhere out there...the quiescent White Army... awaits the next attack...
INTRODUCTION

Adhesion

The ability to adhere is crucial for many cellular processes. The adhesive interactions are usually transient, but can become static. During embryogenesis, organs are formed by static, homotypic adhesion between cells. In fertilization, the sperm needs to bind to the egg cell for new life to begin. During inflammation, white blood cells (e.g. neutrophils) adhere to, and squeeze between the endothelial cells lining the blood vessels, and seek out invading pathogens. At the site of infection, the white blood cells must adhere to the bacteria in order to engulf them. Many different adhesion receptors are involved in these processes. Cadherins form the static connections between cells. Selectins mediate the initial, loose attachment of neutrophils to the endothelium. Integrins are involved in both firm adhesion to the endothelium, adhesion to extracellular matrix (ECM) during chemotaxis, and during phagocytosis in the adhesion between cells and pathogens during phagocytosis (Fig. 1) (reviewed in (1)).
The inflammatory process. During inflammation, P- and E-selectins are upregulated on the activated endothelium. L-selectins on neutrophils bind loosely to ligands on the endothelial cells, and rolling of the cells on the blood vessel is initiated. Integrins are upregulated on neutrophils upon stimulation with chemokines/chemoattractants or by selectin-mediated rolling (1). Integrins adhere firmly to the endothelium upon binding to ICAMs (reviewed in (2)). After locating “gaps” between endothelial cells, neutrophils, following a gradient of chemoattractants (+), squeeze through the endothelial layer (diapedesis) and migrate towards the site of infection (chemotaxis). Pathogens opsonized with complement factors (C3b/C3bi) or antibodies are engulfed via complement receptor (CR)-mediated or Fc-receptor (FcR)-mediated phagocytosis. Reactive oxygen species and enzymes degrade and kill the intracellular pathogens.

The actin cytoskeleton

Reorganization of the cytoskeleton is an essential process during adhesion, chemotaxis and phagocytosis. Actin filaments form a three-dimensional network called the actin cytoskeleton, which stabilizes the cell. The actin cytoskeleton is remodelled during cell migration, the filamentous-actin (F-actin) being polymerized at the plus (barbed) end, and depolymerized into globular-actin (G-actin) at the minus (pointed) end (Fig. 2). Polymerization of actin is dependent of ATP and monovalent or divalent ions e.g. potassium (K+) and magnesium (Mg^{2+}) (reviewed in (3)). Cellular movement by so called “amoeba-like” migration requires constant turnover of extracellular adhesive contacts, which are intracellularly connected to the cytoskeleton. In order to move the cell body forward, a lamellipodium is extended at the leading edge and attached to the substratum. Simultaneously, the adhesive connections in the back of the cell are disconnected and the cell body is retracted (Fig. 2).
Figure 2. Cellular migration. A) During migration, the leading edge extends forward and attaches to the extracellular matrix (ECM). In order to retract the cell body, the adhesive connections must be severed at the trailing edge (uropod) of the cell. B) The actin cytoskeleton is composed of monomeric, globular actin (G-actin) i) polymerizing into actin filaments. Polymerization of filamentous actin (F-actin) ii) occurs at the plus end, whereas depolymerization occurs at the minus end of the filaments.

Integrins

Integrins are transmembrane cell-surface receptors mediating adhesion to the ECM, and to other cells or pathogens. Integrins consist of a α-subunit and a β-subunit, non-covalently bound to each other. Today, 18 α-, and 8 β-subunits have been identified, which can be combined into at least 24 different integrin receptors (4) (Fig. 3), all having a specific function, as shown by the phenotypes of knockout mice (reviewed in (5)).
Figure 3. The integrin superfamily. One α- together with one β-subunit constitutes an integrin receptor. Also shown are the alternative designations for β2 integrins. Integrins containing an I-domain are marked with an asterisk.

The integrin receptors consist of a large extracellular, ligand-binding domain, a transmembrane domain, and a short cytoplasmic tail (Fig. 4). The CD11/CD18 integrins (β2 integrins), solely expressed on leukocytes, share the same β-subunit combined with four different α-subunits (CD11a, -b, -c, -d). The CD11b/CD18 integrins are the most abundant β2 integrins on neutrophils.

In order to participate in cellular processes such as wound healing, cell differentiation, immune responses, and cell migration, the integrins can not be constitutively active. The ligand-binding affinity is closely regulated via conformational changes (Fig. 4) (5). Inactive integrins have a bent conformation, folded at the genu, with the headpiece located 5 nm above the membrane (6, 7). When activated, the unfolded integrins project 20 nm above the membrane, and are accessible for ligand binding (8). It should be noted, however, that ligand binding has been observed already for the “bent” conformation of αvβ3- integrins (9), suggesting that the activation model does not apply to all integrins.
Figure 4. The structure of integrins. The conformational changes in integrins containing an I-domain. In a low affinity state, the α- and β-integrin tails are in a closed, bent conformation. Upon priming (or ligand binding), the integrin receptor "legs" straighten up in a “switchblade”-like motion (10) and separate; a mechanism associated with increased affinity. The separation of the legs, accompanied by conformational changes allows for binding of cytoplasmic proteins and signalling. Adapted from (6, 11-14).

Integrin activation: affinity, avidity, and valency

Besides the induction of conformational changes of integrins (affinity change), clustering of the receptors on the plasma membrane, together with the association of integrins and the cytoskeleton, are additional ways of activating the integrin receptors (avidity regulation). However, if a conformational change of the integrins is the cause or the result of ligand binding is under debate (15, 16).

Changes in affinity and avidity are brought about via an inside out mediated-mechanism, induced by activation of other membrane receptors, e.g. receptors for growth factors, cytokines and chemoattractants. Inside-out signalling, in which the cytoplasmic regions of β2 integrins are key players, is perceived as conformational changes in the extracellular region of the integrins, mediated from the cytoplasmic side by the signal transduction induced by other
active receptors. The proteins affecting the inside-out signalling should possess the ability to directly associate with the integrins (Table 1), or be involved in the signal transduction resulting in the activation of integrin-associated molecules (reviewed in (17)). Changes in affinity and avidity put the integrins in a different activation state, so called “primed state”, giving the cells a more “readily activated” character.

The terminology surrounding integrin activation is vague. Recently, the term valency has been introduced (14). Valency regulations include changes in the local density, or in the ability of the receptor and ligand to move, which alter the possible number of adhesive bonds that can be formed. Affinity regulations are explained as changes in integrin affinity due to conformational changes. (14). The avidity is considered as the total strength of cellular adhesive interactions resulting from the affinity, valency, and the total number of formed bonds (18). In this thesis, the term valency is not applied, instead affinity and avidity will be used (Fig. 5).
Figure 5. Schematic illustration of integrin activation regulated by affinity and avidity changes. Upon activation, the number of integrin receptors is increased by upregulation from intracellular stores. When the cytoskeletal connections are broken, integrin receptors move laterally on the plasma membrane and form clusters, thereby accumulating the number of low-affinity binding integrins in one area, resulting in increased binding/adhesion. The ligand binding of integrins can also be strengthened by altering the connection between receptors and the cytoskeleton. Affinity is increased by a conformational change in the integrin receptor, resulting in augmented ligand binding.

**β2 integrin ligation**

Besides being adhesion receptors, integrins cross-talk with other receptors on the plasma membrane, and transmit signals into the cell interior. The extracellular domain of β2 integrins binds to the ECM, or to counter receptors on neighbouring cells. The cytoplasmic domain interacts with cytoskeletal proteins, forming a physical link between ECM and the cytoskeleton, thereby regulating the signalling capacity of the receptor. Ligation of the receptor induces a signal that propagates within the cell, a process called outside-in signalling. For example, besides inducing the activation of adhesion kinases localized to focal complexes, ligation of integrins can activate mitogen activated protein kinase (MAPK), GTP-binding proteins (G-proteins), polymerization of F-actin, and induce release of Ca^{2+}.
ICAM-1, was one of the first molecules to be identified as a ligand for leukocyte integrins (19). ICAMs belong to the immunoglobulin superfamily consisting of five members: ICAM 1-5 (reviewed in (20, 21)). In addition to ICAMs, β2 integrins bind a vast number of molecules including; fibrinogen, collagen I, and C3bi. In T-cells, the binding of soluble ICAM (sICAM), corresponding to increased integrin affinity, is induced by divalent ions such as Mg$^{2+}$ and Manganese (Mn$^{2+}$) (16, 22), (23). The integrin receptors contain multiple binding sites for calcium (Ca$^{2+}$) in their extracellular domains, and Ca$^{2+}$ is another ion regulating integrin activation/ligand binding. Surprisingly, Ca$^{2+}$ has a dual role in both inducing adhesion, by integrin clustering (24), and inhibiting adhesion. Actually, Ca$^{2+}$ has to be removed in order for Mg$^{2+}$ to induce changes in affinity (23).

Table 1. A summary of proteins interacting with integrin cytoplasmic domains. 
Adapted from (25).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Integrin tail</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filamin</td>
<td>β1A, β2, β7</td>
<td>(26, 27)</td>
</tr>
<tr>
<td>Talin</td>
<td>β1-5, αllb, (β7)</td>
<td>(26, 28)</td>
</tr>
<tr>
<td>α-actinin</td>
<td>β2, β1</td>
<td>(29)</td>
</tr>
<tr>
<td>Radixin</td>
<td>β2</td>
<td>(30)</td>
</tr>
<tr>
<td>CIB</td>
<td>αllb</td>
<td>(31)</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>α</td>
<td>(32)</td>
</tr>
<tr>
<td>FAK (p125)</td>
<td>β</td>
<td>(33)</td>
</tr>
<tr>
<td>ILK (p59)</td>
<td>β</td>
<td>(34)</td>
</tr>
<tr>
<td>Pyk2</td>
<td>β2</td>
<td>(35)</td>
</tr>
<tr>
<td>Paxillin</td>
<td>β1, β2, β3, α4, α9</td>
<td>(33, 36, 37)</td>
</tr>
<tr>
<td>Cytohesin-1, and -3</td>
<td>β2</td>
<td>(38)</td>
</tr>
<tr>
<td>β3-Endonexin</td>
<td>β3</td>
<td>(39)</td>
</tr>
<tr>
<td>ICAP-1</td>
<td>β1</td>
<td>(40, 41)</td>
</tr>
<tr>
<td>Rack1</td>
<td>β1, β2, β3</td>
<td>(42)</td>
</tr>
<tr>
<td>Eps8</td>
<td>β1a</td>
<td>(43)</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>β1</td>
<td>(44)</td>
</tr>
<tr>
<td>IRS-1</td>
<td>αβ3</td>
<td>(45)</td>
</tr>
<tr>
<td>Phospholipase Cγ</td>
<td>β1</td>
<td>(46)</td>
</tr>
<tr>
<td>14-3-3 β</td>
<td>β1</td>
<td>(47)</td>
</tr>
<tr>
<td>Annexin-V</td>
<td>β5</td>
<td>(48)</td>
</tr>
</tbody>
</table>
Integrins, partners in sickness and in health

Leukocytes are key players in inflammation and thereby also potential targets for anti-inflammatory drugs. Normally, non-adherent leukocytes circulate in the blood, become activated, and transmigrate through the endothelium into the tissues. Unregulated accumulation of leukocytes in target organs or tissues may result in diseases such as asthma, rheumatoid arthritis, atherosclerosis, multiple sclerosis and Crohn’s disease. Moreover, the elevated number of neutrophils in circulation increases the risk of developing cardiovascular disease. Data from patients with leukocyte adhesion deficiency (LAD) and integrin knockouts, confirm the central role of integrin signalling during inflammation. LAD-I is an inherited immunodeficiency in which the expression of β2 integrins is diminished or lost (49). LAD patients suffer from impaired inflammatory responses, defects in T cell proliferation, and skin infections (reviewed in (5)). Other LAD deficiencies have been identified, where the expression of β2 integrins is normal, but the function is impaired, probably due to defective “inside-out signalling” (50, 51). In the last decade, neutrophils have been identified as active participants in the inflammatory process of atherosclerosis, which in turn is a major risk factor in the development of coronary artery disease (CAD).
Integrins, matrix adhesions and associated proteins

Integrins and their binding partners (summarized in Table 1) are organized in multi-protein complexes termed “matrix adhesions”. They are highly dynamic and difficult to study as isolated organelles. The matrix adhesions include focal complexes, focal adhesions (also designated focal contacts), fibrillar adhesions and podosomes (52). Focal complexes are small (100 nm), dot-like structures usually concentrated in membrane protrusions of migrating cells. Focal complexes can mature into larger focal adhesions (1 μm) found mainly in resting cells or in areas with low motility (52). Fibrillar adhesions are formed in cells adhering to fibronectin via α5β1, and do not contain vinculin or paxillin (53). Podosomes are formed in osteoclasts or cells of hematopoietic origin e.g. neutrophils (54-56).

Cytoskeletal proteins associated with adhesion complexes

Talin was the first protein identified as a cytoplasmic binding partner to integrins (28). Talin is a cytoskeletal protein that participates in the activation of integrins, formation of adhesion matrices, in connecting integrin receptors to the cytoskeleton (reviewed in (57)), and in β2 mediated phagocytosis (58). Talin binds with high affinity to the cytoplasmic tails of β integrins via the N-terminal, FERM (i.e. protein 4.1, ezrin, radixin, moiesin) domain (26), and to actin via the C-terminal domain. Recently, an alternative splicing product of talin, talin2, has been identified (59). The function of talin2 remains to be elucidated. In neutrophils, the connection between CD11a/CD18 and talin is disrupted upon activation, following an association of the integrin receptors with α-actinin (60), the cytoskeletal protein that crosslink F-actin. Paxillin is another adaptor protein important for the assembly and functions of matrix adhesions. Paxillin, together with talin, is one of the first proteins recruited to focal contacts (61).

Rho GTPases and cytoskeletal rearrangements

The regulation of the cytoskeletal rearrangements induced by chemoattractants is mediated by the family of small GTPases. In neutrophils, research has mainly focused on the members Rho, Rac, and Cdc42, which become transiently activated by N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (62-65). The transition of the GTPases between inactive, GDP-bound and active, GTP-bound state is regulated by guanine nucleotide exchange factors
(GEFs), and GTPase activating proteins (GAPs) (reviewed in (52)). GEFs catalyze the exchange of GDP for GTP during activation of the GTPases, and GAPs stimulate the low intrinsic hydrolytic activity (Fig. 6). The activation of GTPases is further regulated by guanine nucleotide dissociation inhibitors (GDIs) which prevent exchange of GDP to GTP, and hence must be dissociated from the GTPases during activation (66).

**Figure 6. The activation model of Rho GTPases.** The transition between active/inactive state is modulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). The dissociation of RhoGDI and RhoGTPase is mediated by GDI displacement factors (GDFs).

Post-translational lipid modifications at the C-terminal part target the GTPases to different membranes/compartments (67), thereby exerting different effects on the cytoskeletal rearrangements. In neutrophils, Rac is located in the front of the cells, mediating the formation of leading edge and F-actin assembly (68, 69). The primary role of Rac is to induce protrusive force during cell motility. Integrin ligation induces the translocation of inactive Rac and Cdc42 from the cytoplasm to the membrane, and mediates the dissociation of RacGDI (70). β2 integrin-ligation activates Rac and Cdc42 in neutrophils (71), and both Rac and Cdc42 are translocated to the plasma membrane in an integrin-dependent manner (70). Cdc42 localizes to the leading edge of chemoattractant-stimulated neutrophils, and becomes activated by p21-activated kinase (PAK) and the RacGEF, PIXα. Rho regulates myosin II-mediated retraction of the tail in monocytes and neutrophils through its effector Rho kinase.
(ROCK) (72, 73). Rho-H has been proposed to negatively regulate CD11a/CD18 avidity (74). Rac and Rho are concentrated in lipid rafts (75), (76), and Rac co-localizes strongly with GM1 (77, 78).

**Rap1 and RAPL**

Recently Rap1, a small GTPase belonging to the family of Ras-like GTPases has emerged as an important regulator of integrin-mediated adhesion (79). Activated Rap1 induces increased affinity and clustering of CD11a/CD18 in lymphocytes (80). Rap1 also regulates the interaction between talin and integrins (81). In macrophages, Rap1 regulates complement-mediated phagocytosis (82). Using yeast-two-hybrid screening, the Rap1-binding proteins RAPL and Riam (Rap1-interacting adaptor molecule) have been identified (83-85). RAPL is highly expressed in lymphocytes, and associates with the GTP-bound Rap1. Both RAPL and Riam contain Ras-association domains but their function needs to be elucidated.

**Proteins involved in mediating signal transduction induced by integrins**

**The protein tyrosine kinases**

Plasma membrane receptors lacking intrinsic protein tyrosine kinase activities, *i.e.* integrins, recruit non-receptor tyrosine kinases in order to mediate signals (86-92). The focal adhesion kinase (FAK) belongs to the family of non-receptor tyrosine kinases and plays a central role in integrin-mediated signalling (93). The FERM domain, located at the N-terminus of FAK, binds β1 integrin cytoplasmic tails. At the C-terminus, FAK contains a focal adhesion targeting (FAT) domain functioning as binding site for talin (94), paxillin (95) and p190Rho-GEF (96). FAK-mediated tyrosine phosphorylation of Y12 in the α-actinin binding domain negatively regulates the interaction between α-actinin and actin (97). FAK-deficient cells show delayed spreading, reduced adhesion turnover (98), as well as decreased Rac activity and loss of lamellipodia (99, 100). Over-expression of FAK on the other hand, increases the directional motility of cells (101).

Another member of the of non-receptor tyrosine kinase family is the proline-rich tyrosine kinase 2 (Pyk2; also designated CAKβ, RAFTK, FAK2, or CADTK) identified in 1995
Pyk2 and FAK share high overall amino acid identity (48%) (102), and display 60% identity in the catalytic domain, 31% in the N-terminal part, and 45% at the C-terminus (102). Pyk2 is expressed mainly in the central nervous system and in cells of hematopoietic origin (35, 103, 104). Two spliced isoforms of Pyk2 have been described, characterized by the presence or absence of an exon coding for a 42 amino acid insert in the C-terminal region (105, 106). Pyk2-H, the spliced variant lacking the exon, is expressed in hematopoietic cells, and the unspliced form is predominant in the brain (105). FAK has been detected in cell lysates of human neutrophils although it does not appear to be activated by cell adhesion in these cells (107, 108). Differentiated HL60 cells do not express FAK (35). These data suggest that FAK has a less certain role in integrin signalling in human neutrophils/HL60 cells (109), and we therefore propose Pyk2 to be the focal adhesion protein tyrosine kinases involved in integrin mediated-signal transduction in these cells.

The central catalytic domain of Pyk2 is flanked by non-catalytic regions on both sides. The major autophosphorylation site is Tyr-402 in the N-terminal domain, which acts as a binding site for the SH2 domain of Src (110). The activation of Pyk2 occurs in multiple steps. The initial autophosphorylation of Tyr-402 (88, 111) mediates binding of Src-kinases which in turn phosphorylate Tyr-580, thereby enhancing the enzymatic activity of Pyk2 (112, 113). The proceeding phosphorylation of Pyk2 involves both Src-dependent and independent signalling pathways (110, 111, 114). The C-terminal domain of Pyk2 contains two proline-rich motifs that function as sites for SH3-mediated protein-protein interactions (115-120). Several proteins associate with Pyk2, including the focal adhesion proteins; paxillin (121), and p130Cas (122), Src-kinases (102), the ARF-GAP protein PAP (123), and the Nirs (124). Thus, Pyk2 not only functions as a link between heterotrimeric G-protein-coupled receptors and signalling pathways (125, 126), but also has an essential role in the transfer of signals from the adhesion receptors to regulators of the cytoskeleton, i.e. serves as a scaffold in the formation/activation of focal complexes.
Cell motility

The capacity of cells to sense changes in the external environment during migration towards a gradient of chemotactic substances (i.e. chemotaxis), is essential in inflammation. Neutrophils are one of the fastest moving cells; migrating up to 10-20 μm per minute, and represent a good model system for investigating chemotaxis. Chemotaxis is initiated by binding of chemoattractants to G protein-coupled receptors (GPCRs). GPCRs belong to a large family of seven transmembrane spanning receptor proteins that activate intracellular heterotrimeric G proteins consisting of α-, β-, and γ-subunit. Upon activation, the GDP bound to the α-subunit is exchanged for GTP. GTP induces to dissociation of the β/γ and α subunit, and regulates down-stream effectors, thereby amplifying the signal (Fig. 6).

The chemoattractants inducing chemotaxis are N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) released by bacteria, the arachidonic acid metabolite leukotriene B₄ (LTB₄), complement factor 5a (C5a), platelet activating factor (PAF), and the chemokine interleukin-8 (IL-8) (127). A hierarchy exists between the different chemoattractants in their potency of inducing chemotaxis, where end-target chemoattractants e.g. fMLP are more potent compared to intermediary chemoattractants e.g. IL-8. In a milieu of different chemotactic gradients, the cells will favour end-target before intermediary chemoattractants (128). Moreover, different signalling pathways are activated by intermediary, versus end-target chemoattractants during neutrophil chemotaxis. The chemotactic response to end-target chemoattractants involves p38 MAPK and CD11b/CD18, whereas the signalling pathways activated by intermediary chemoattractants are dependent on PI-3K and CD11a/CD18 (128, 129).
Figure 7. The signalling pathways activated by heterotrimeric G-proteins. Chemoattractants, binding to seven transmembrane spanning, G protein-coupled receptors (GPCRs), activate heterotrimeric G proteins. Upon GTP binding, the α- and β/γ-subunits dissociate and mediate the activation of downstream effector proteins. Adapted from (130).

**Regulation of adhesion signalling by lipid metabolites**

**Phospholipase A2**

To date, 22 genes encoding different phospholipase A2 (PLA2) proteins have been identified in mammals (131). The superfamily of PLA2 can be divided, according to their biochemical properties into 4 subfamilies: i) the Ca^{2+}-dependent secreted enzymes (sPLA2), ii) the Ca^{2+}-dependent cytosolic enzymes (cPLA2), iii) the Ca^{2+}-independet cytosolic enzymes (iPLA2), iv) and the platelet-activating factor acetyl hydrolases (PAF-AH) (132). It is generally accepted that the activity of PLA2 is regulated by phosphorylation. Several kinases regulate the activity of PLA2, including PKC and MAPK (133).

The plasma level of sPLA2 is increased in patients with inflammatory or autoimmune diseases such as rheumatoid arthritis, septic shock, Crohn’s disease, and ulcerative colitis. In
neutrophils, the group IIA sPLA$_2$ stimulates release of LTB$_4$ (134), and increases the expression of CD11b/CD18 by promoting translocation from secretory vesicles to the plasma membrane (135). The unique trait for iPLA$_2$ is the lack of requirement for calcium for their enzymatic activity. iPLA2 is involved in regulation of cell physiology and metabolic homeostasis, but is not a major player in inflammatory processes (136). Instead cPLA$_2$ and sPLA$_2$ appear to be important for induction of inflammation.

**Arachidonic acid and pro-inflammatory eicosanoids**

In addition to regulation by protein-protein interactions, the signalling pathways mediated in adherent and migrating leukocytes are heavily influenced by lipid mediators. Lipid mediators have been found to enhance the β2-integrin mediated adhesion to fibrinogen and C3bi (137). Moreover, β2-mediated phagocytosis induces the release of arachidonic acid (AA) (138). Arachidonic acid is a polyunsaturated fatty acid hydrolyzed by PLA$_2$ from the sn-2 position of glycerophospholipids (139). Upon release, arachidonic acid *per se* can influence cellular processes, *e.g.* actin bundling (140), degranulation, and superoxide production (141), or become metabolized by either cyclooxygenases (COX) to prostaglandins and thromboxanes, or by lipoxygenases (LOX) to form leukotrienes and lipoxins. The oxygenation of arachidonic acid by 5-LOX, results in the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE), a precursor for leukotrienes and lipoxins (Fig. 8). Various eicosanoids (AA metabolites, *e.g.* 5-LOX metabolites), can regulate cell adhesion, as well as other processes involving cytoskeletal reorganization (142, 143). Activation of PLA$_2$ and AA *per se*, increase the expression of β2-integrins on the plasma membrane of neutrophils (144). cPLA$_2$ and AA have also been shown to regulate the superoxide production in phagocytes (145, 146). In addition, the arachidonic acid binding protein S100A8/A9, has been identified as a novel binding partner for the NADPH-oxidase subunits p67$^{phox}$ and Rac (146).
Figure 8. The arachidonic acid metabolism. Arachidonic acid (AA) is hydrolyzed by phospholipase A$_2$ (PLA$_2$) at the sn-2 position of glycerophospholipids. The lysophospholipid formed can be metabolized into platelet activating factor (PAF). AA is metabolized via the cyclooxygenase (COX) pathway to prostaglandins (PGs) and thromboxanes (TXs), or via lipoxygenase (LOX) pathways to leukotrienes (LTs), and lipoxins (LX).

Anti-inflammatory lipoxins

Lipoxins are eicosanoids derived during a transcellular process by the combined action of 5-LOX, and 12-LOX or 15-LOX (147). Lipoxins function as innate “stop signals” that control inflammation processes (148) mainly by inhibiting the chemotactically induced signalling pathways. Lipoxins inhibit the LTB$_4$ and fMLP-induced chemotaxis (149), adhesion and transmigration (150), expression of β2 integrins on neutrophils (151), and cytokine formation (152). Lipoxins bind to G-protein-coupled seven-transmembrane spanning receptors. The lipoxin receptor (ALX) belongs, together with fMLP- and LTB$_4$-receptors, to the same cluster of GPCRs (153).

In the presence of aspirin 15-epi lipoxins are formed, which are more potent than the native lipoxins (153). Due to the rapid conversion of lipoxins to inactive compounds (154), stable
Lipoxin analogues (LXa) have been synthesized (155). These analogues are almost as potent as the aspirin-triggered lipoxins (ATL) (reviewed in (156)), and are therefore useful tools in elucidating the signalling pathways induced by adhesion and chemotactic stimuli. The lipoxin analogues inhibit PMN transmigration across epithelia and block PMN adhesion (155). Lipoxins redistribute myosin IIA and Cdc42 in macrophages, and are implicated to regulate phagocytosis and apoptosis (157) in a non-phlogistic manner.

**Phagocytosis**

Phagocytosis is defined as cellular engulfment of particles larger than 0.5 μm. Professional phagocytes are cells mainly focusing on phagocytosis *i.e.* neutrophils, eosinophils, basophils, monocytes, and macrophages. In order for phagocytosis to take place, the cells must first localize, recognize and bind the particle. Recognition is mediated via specific receptors on the plasma membrane. Two sets of receptor types are activated during phagocytosis, namely Fc-receptors (FcR) and complement receptors (CR) both mediating distinct signalling pathways. Particles opsonized with IgG-antibodies are captured and engulfed by FcγR located on F-actin-containing protrusions called pseudopodia. FcγR-mediated phagocytosis is dependent on Ca²⁺ and actin reorganization. The FcγR-mediated signalling cascade involves the activation of various proteins including: PLC, PKC, PI-3K, Rac, Cdc42, PLA₂ and MAPK (reviewed in (158)). The CR-mediated phagocytosis on the other hand, does not involve active formation of pseudopodia and is thus less dependent on calcium. The complement receptor 3 (CR3), identical to CD11b/CD18 integrin, binds C3bi-opsonized particles. An early event in CR-mediated phagocytosis is tyrosine phosphorylation and activation of proteins such as: paxillin, RhoGTPases, Pyk2, PLC, and vav (103, 107, 121, 159, 160).

**The phagocytic process**

Besides adhesion, migration, and aggregation, the phagocytic process also includes engulfment of the particle, degranulation and bacterial killing. Killing of microorganisms located in the phagosome, involves generation of reactive oxygen species (ROS) including superoxide anion produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Superoxide anion produced by NADPH-oxidase, is converted by superoxide
dismutase (SOD) and catalase to \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O} \). In the presence of myeloperoxidase and chloride, \( \text{H}_2\text{O}_2 \) forms the toxic hypochloric acid.

NADPH-oxidase consists of several membrane associated and cytosolic components, one of them being the RhoGTPase Rac (161). Cdc42 has emerged as a competitor of Rac2 for the binding to NADPH-oxidase (162, 163). The actin cytoskeleton plays an important role in regulating the assembly and stabilization of the NADPH-oxidase (164, 165). Assembly of NADPH-oxidase occurring at the membrane of intracellular components induces intracellular ROS production, whereas activation of NADPH-oxidase at plasma membrane leads to extracellular release of ROS.

**Degranulation**

Neutrophils are equipped with vesicles containing proteolytic enzymes and bactericidal peptides to combat ingested bacteria. Four populations of granules are found in neutrophils namely  

i) primary (azurophil) granules,  
ii) secondary (specific), peroxidase negative granules,  
iii) tertiary gelatinase granules, and  
iv) secretory vesicles (reviewed in (166)). The different granules are mobilized following a hierarchy, and differ in aspects of content and function. The azurophilic granules contain for example myeloperoxidase, defensins, lysozymes, and CD63. Certain receptors are also stored in granules and are upregulated upon stimulation. The \( \beta_2 \) integrins and receptors for fMLP and IL-8, localize to specific granules and/or secretory vesicles (166). HL60 cells lack specific granules and the upregulation of for example \( \beta_2 \) integrins is therefore impaired (167). Translocation of granules, and fusion with phagosome is mediated by both the actin- and microtubulin network (168). The internalization of the particle induces polymerization of F-actin at the site of ingestion. The F-actin surrounding the phagosome must be degraded to allow endosome/lysosome to fuse with the phagosomal membrane. Proteins implicated in the phagosomal maturation are SNAREs, rab 5, rab7, and PKC (166, 168). Despite being activated during phagocytosis, neither Rac nor Cc42 has been found to localize to the plasma membrane in their active form. Since small GTPases regulate actin turnover, the involvement of activate Rac and Cdc42 in phago-lysosomal formation could be expected.
AIMS OF THE INVESTIGATION

The aim of this thesis was to shed light on the regulation of the β2 integrin-mediated signalling in human neutrophils. To achieve this, pro-inflammatory (i.e. fMLP, IL-8 and LTB₄) substances, and anti-inflammatory endogenous- (i.e. lipoxin, epi-lipoxin), and exogenous substances (i.e. statin, aspirin) were employed. The more specific aims were to understand:

i) The regulatory effect of pro- and anti-inflammatory substances on the β2 integrin-activation state (avidity vs. affinity) of human neutrophils from healthy donors and from patients suffering from coronary artery disease (CAD).

ii) The regulatory effect of the pro- and anti-inflammatory substances, on the signalling capacity of β2 integrins (i.e. the release of AA, and production of ROS), in healthy donors and in patients suffering from coronary artery disease (CAD).
iii) The effect of the pro- and anti-inflammatory substances on the β2 integrin-mediated signal transduction (phosphorylation of Pyk2) in neutrophils.

iv) In addition, the aim was to establish methods for investigating the proteins involved in the regulation of β2 integrin-mediated signalling, e.g. western blot, immunoprecipitation, immunofluorescence, single particle tracking, and TAT-transduction.
METHODS

Some of the methods used in this thesis will be discussed briefly herein.

Neutrophils

When studying blood cells, different approaches are available. One is to use freshly isolated neutrophils from blood donors. The limited usage of neutrophils lies in the availability of the cells and in the limited life span of the neutrophils once isolated from whole blood. The status of the blood cells from donors can also vary between experiments, due to individual donor differences. Moreover, the neutrophils are affected by the isolation procedure, and the use of whole blood is therefore preferable for some assays. However, when interpreting the results, one must keep in mind that the different cell types in whole blood could influence each other. In his study, whole blood was used when analyzing neutrophils from patients suffering from stable Coronary Artery Disease (CAD), due to the limitation in blood sample volumes.

Thirty patients with angiographically verified stable CAD were recruited. Each patient was matched, regarding age and gender, with a clinically healthy control subject, randomly selected from the population register. Patients were excluded if they were >65 years old, had severe heart failure, immunologic disorders, neoplasm disease, evidence of acute or recent (<2 months) infection, recent major trauma, surgery or revascularization procedure, or treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin).

Another approach when studying neutrophils is to use commercially available cell lines, which can be differentiated into neutrophil-like cells. The cells have a more stable phenotype compared to freshly isolated neutrophils. However, the tumour cells may have some inherent defects. In this thesis, we have used both freshly isolated human neutrophils, and a promyelocytic cell line i.e. Human Leukemia 60 (HL60). The HL60 cells can be differentiated into neutrophils, monocytes and macrophages depending on the differentiation agent used. When grown in suspension in the presence of DMSO for 6-7 days (169), the HL60 cells become neutrophil-like and acquire the ability to adhere, migrate, and
phagocytose like the peripheral blood neutrophils. Neutrophil-like HL60 cells do not contain specific granules and lack the ability to up-regulate some receptors on the plasma membrane. This can be used as tool when studying certain cellular processes in neutrophils, such as the activation state (affinity / avidity) of β2 integrins.

To investigate the role of lipid mediators in β2 integrin signalling, the cells were incubated with[^3]H]labelled arachidonic acid ([^3]H]AA). Isolated neutrophils were incubated for 2h at 37°C, whereas HL60 cells were labelled for the final 18-24 hours of the cell-differentiation period. During the experiments, human serum albumin (HSA) was added in order to trap the released fatty acids and prevent further metabolism (170). The reaction was stopped by centrifugation and the radioactivity of the supernatant was measured in a β-counter.

Cell stimulation

Activation by chemotactic factors
During inflammation, neutrophils navigate along a gradient of chemotactic factors e.g. fMLP, LTB₄, and IL-8. fMLP is an end-target chemoattractant released by the bacteria at the site of infection, and is considered more potent than the endogenously formed intermediary chemoattractants LTB₄ and IL-8 (128). In this study, the effect of intermediary- and end target chemoattractants on β2 integrin signalling was investigated both in differentiated HL60 cells and in human neutrophils from healthy donors and CAD patients.

Integrin ligation

Activation by antibody-coated Pansorbins®
The pansorbins® are heat-hardened Staphylococcus aureus, expressing protein A on the surface. The Fc-part of the antibody will bind to protein A, giving rise to a pansorbin particle coated with antibodies exposing the Fab-part outwards, in contrast to opsonized particles (Fig. 9). By using pansorbins, receptors on the plasma membrane of cells kept in suspension can be directly activated without the interference from other adhesion receptors. This is a good model system for β2 integrin ligation, since the particles do not mediate spreading or
phagocytosis. One advantage of using Pansorbins® is that the unspecific antibody-interactions are reduced, since the Fc-part of the antibody is coupled to the Pansorbin® and can not interact with Fc-receptors.

![Diagram](image)

**Figure 9.** A) The difference between antibody-coated and antibody-opsonized particles. Anti-β2 integrin antibodies coupled to pansorbins® interact with β2 integrin receptors via their Fc-part, giving rise to receptor mediated signalling. B) Pansorbins® opsonized with IgG antibodies are recognized by the receptors through the Fc-part of the antibody, inducing Fc receptor-mediated phagocytosis and subsequent signalling.

**Integrin ligation in adherent cells**

In order to verify the results observed by anti-β2 integrin-pansorbins, cells were allowed to adhere to the β2-integrin ligand, fibrinogen. Using the adhesion assay enabled us to study the signalling pathways induced by β2 integrin ligation and reorganization of the cytoskeleton, and to elucidate the need for “cellular priming”, *i.e.* integrin activation, prior to adhesion. As a control, cells were plated on BSA-coated culture dishes.

**Flow cytometry**

Flow cytometry is a quantitative approach, used to analyze the total fluorescence of individual cells/particles ranging from 0.5 μm to 40 μm in size, in a single cell suspension. Intracellular molecules and membrane markers can be stained with different fluorochromes and analyzed simultaneously in the flow cytometer (Fig. 10). The cells pass through a light source one at a time. Light is scattered upon passing through the cells and the fluorochromes are exited to a
higher energy state. This energy released in form of emitted light, is detected together with the scattered light by several detectors. Light passing through the cells is detected as forward scatter (FSC) and is relative to the cell size. The more granular the cells, the more the light is scattered to the sides, detected as side scatter (SSC). By plotting FCS against SSC, different cell populations can be visualised and a region of interest established, so called gating. Therefore, when analyzing different blood cells by flow cytometry, whole blood can be analyzed directly in the flow cytometer. The cell population of interest is then visualized in a frequency histogram and the mean fluorescence intensity (MFI) can be analyzed by statistics.

Figure 10. The principles of flow cytometry. In brief; cells suspended in a carrier solution, pass a light source one at a time, and the fluorochromes become excited to a higher energy state. The light emission is detected together with the scattered light, enabling the selection of a region of interest (i.e. gating). Fluorescence (FL1) is plotted in a frequency histogram and the mean fluorescence intensity (MFI) is calculated for the selected population.

In this study, the FITC-fluorescence of individual cells was determined by flow cytometry, using a Becton Dickinson FACSCalibur (Becton Dickinson, San Jose, CA/USA). Mean
fluorescence values, from a minimum of 5000 cells/sample, were determined. Gating was performed by using forward scatter versus side scatter (excluding cell debris). Autofluorescence of unstained cells was routinely analyzed and subtracted from the fluorescence values of the stained cells. Unspecific binding was analyzed by isotype antibodies.

Expression of β2-integrins

Prior to investigating the activation state of the β2 integrins, it was important to quantify the number of integrin receptors on the plasma membranes of HL60 cells. If no receptors are upregulated upon stimulation, increased integrin affinity should be due to an activation of β2 integrins and not be dependent of an increased number of receptors on the plasma membrane. In brief: Cells were pre-incubated with anti and pro-inflammatory stimuli alone or in combination. Stimulation was stopped by ice-cold PFA, washed and incubated with primary anti-CD18 antibodies, followed by incubation with secondary, FITC-conjugated antibodies.

In human neutrophils, β2 integrins are stored in granules and become expressed on the membrane upon stimulation. The basal expression of β2 integrins on human neutrophils from healthy donors and CAD patients was therefore investigated. Due to limitations in blood volume from patients, the flow cytometry assay was adjusted by analysing neutrophils from whole blood.

β2 integrin affinity

One way of investigating the activation state of the integrins is to measure affinity of the integrin receptors. Two high-affinity antibodies; CBRM 1/5 and MAb24 were used in this study. CBRM 1/5 is a monoclonal antibody recognizing the ligand binding domain (i.e. I-domain) on CD11b integrins (171), whereas MAb24 recognizes a high affinity epitope on the α-subunit of both the CD11a and CD11b integrins (172). In order to measure affinity of β2 integrins, the antibodies had to be present prior to stimulation of the cells, probably due to destruction of the recognition epitopes by PFA. The cells were pre-incubated with the CBRM 1/5 antibody for one minute prior to stimulation, fixed in PFA and incubated with FITC-
conjugated secondary antibodies. Alternatively, a FITC-conjugated CBRM 1/5 antibody was used.

**Binding of soluble ICAM-1**

The β2 integrin-ligand ICAM-1, binds to the receptor when the ligand binding domain is exposed upon increased affinity. In order to verify the results obtained by the high affinity antibody with a more biological read-out, we investigated the binding of soluble ICAM-1 (s-ICAM) to the β2 integrins (173) by flow cytometry.

**Fluorescence microscopy**

The fluorescence microscope was used to evaluate the lateral movement of β2 integrins during the Single Particle Tracking experiments, and to analyze the distribution of F-actin in HL60 cells during phagocytosis. In contrast to flow cytometry, which gives the total amount of fluorescence in a cell, the fluorescence microscope can be used to determine the distribution of fluorescence in the cell.

**Single Particle Tracking**

The Single Particle Tracking (SPT) technique was used to measure the movement of individual β2 integrins on the plasma membrane of neutrophils. By conjugating β2 integrin-specific antibodies to fluorescent latex beads (174), the lateral movement of integrin receptors can be monitored in a fluorescence microscope (Fig. 11). In short: neutrophils were plated on poly-L-lysine-coated cover slips and stimulated. The movement of individual antibody-conjugated beads was recorded for 30 seconds and the diffusion coefficient (D) was calculated.
Figure 11. The principles of the single particle tracking method. Cells are stimulated and allowed to settle on poly-L-lysine coated cover slips. The cells are incubated with specific antibodies conjugated to fluorescent latex beads, and movement of the fluorescent particles is monitored in a fluorescence microscope. The diffusion coefficient, equivalent to lateral movement of the receptor, is determined by computer software.

Confocal Scanning Light Microscopy

By using Confocal Scanning Light microscopy (CSLM), the fluorescence from a thin optical section in a cell (a focal plane) can be analyzed. In contrast to ordinary fluorescence microscopy, CSLM gives a high resolution image of a chosen focal plane in a cell, simultaneously eliminating the light coming from out-of-focus regions above and below that plane. CSLM was used to analyze clustering of β2 integrin receptors on the plasma membrane, the actin distribution around the phagosome, and the distribution of CD63-positive granules in HL60 cells.
Image analysis
To analyze the results obtained by CSLM different imaging analysis procedures were used.

Clustering of integrins
In order to quantify the size of the integrin clusters from a confocal microscope image, the β2 integrins on individual cells were traced manually. The fluorescence was plotted as a histogram in which the peaks were equivalent to clusters of integrins. This allowed us to quantify the number of small and large peaks i.e. small/large β2 integrin clusters.

Granule distribution
The effect of TAT-proteins on the distribution of CD63 positive granules was analyzed by CSLM. To more objectively evaluate granule movement in living cells, confocal microscope images of cells loaded with LysoTracker®Red DND 99 were analyzed. Software was used to evaluate the accumulation-, number-, and distribution of granules in the cells.

Introduction of recombinant proteins into human neutrophils
Transfection is a method used to introduce recombinant DNA into cells using eukaryotic expression vectors. The most common transfection methods are electroporation, liposome-based transfection (e.g. Lipofectamine), retroviral gene transfer, cationic polymer-based system (e.g. DEAE-dextran), and the use of chemicals such as CaPO₄ (175, 176). DNA can also be directly injected into a cell, by so called microinjection. When working with neutrophils, one quickly discovers the obstacles lying ahead. Neutrophils have a limited life span, and contain granules filled with degrading enzymes. The production of stable transfectants is therefore impossible. To circumvent this, cell lines can be used for transfection. Initially in this project, efforts were made to transfec neutrophils and HL60 cells by various transfection approaches (i.e. electroporation, DEAE-dextran, retrovirus, and lipofectamine) without success, hence other methods to introduce recombinant proteins were established.
HIV Tat proteins

The human immunodeficiency virus-1 (HIV-1) contains a transcriptional activator of transcription (Tat) necessary for the replication of the virus (reviewed in (177)). In 1988, it was shown that the HIV-tat protein (86 amino acids) could cross cellular membranes and accumulate in the nucleus and induce gene activation (178, 179). Different molecules linked to Tat, e.g. FITC, DNA and proteins, translocate along with the Tat-peptide into the cytoplasm. The uptake of tat-proteins into the cell remains unclear, but is proposed to take place via a receptor/transporter- and endocytosis- independent pathway. Tat-proteins have also been suggested to directly penetrate the membranes (180), via a process facilitated by, but not dependent of, denaturation and refolding of the proteins (181, 182).

Nevertheless, Tat-mediated protein transduction has emerged as a useful tool to insert molecules into cells, in which transfection protocols are not applicable.

TAT transduction

In this study, we used a pTAT- HA vector (181) containing an amino-terminal, transduction domain of HIV Tat (11 aa), termed TAT. The pTAT-HA vector contains the gene for six histidines (used for affinity purification) and a hemagglutinin (HA) epitope (used for detection of transduced proteins) linked to a TAT-sequence, followed by a multiple cloning site (Fig. 12). Initially, human neutrophils were transduced with TAT-fusion proteins, but due to rapid degradation of the proteins, differentiated HL60 cells were used instead. After optimization of the transduction conditions, the cells were transduced with 200 nM TAT-fusion proteins for 30 min at 37ºC in calcium free-KRG. The transduction efficiency was 65% or more.

![Diagram of pTAT-HA vector](image)

**Figure 12. Characterization of the pTAT-expression vector.**

The pTAT-HA vector contains six histidines (His6), followed by 11 amino acid transduction domain of HIV-Tat (TAT), and a hemagglutinin (HA) epitope linked to a multiple cloning site (MCS). Adapted from (181).
**Immunoprecipitation and Western blot**

When studying activation of intracellular proteins different approaches can be used. The first step involves mechanical (e.g. sonication, homogenization), or chemical (e.g. Triton-X 100, saponine) disturbance of the plasma membrane integrity in order to access the intracellular proteins. The proteins of interest can be “fished out” by specific antibodies conjugated to sepharose beads (i.e. immunoprecipitation), and subjected to SDS-PAGE and Western Blot. During SDS-PAGE, proteins are electrically separated on a polyacrylamide gel according to size and charge, transferred to nitrocellulose membrane and incubated with antibodies (Western Blot; WB).

The advantages of using immunoprecipitation instead of whole cell lysate is that i) the number of “bands” on the gel is limited giving a “purer” blot, ii) due to amplification of the proteins larger quantities can be loaded on the gel, iii) associated proteins can be detected after stripping and re-probing with additional antibodies.

In this study, we have used anti-Pyk2 antibodies together with phosphotyrosine antibodies, to immunoprecipitate Pyk2 and screen for phosphorylated Pyk2 proteins.

**Phagocytosis and production of reactive oxygen species**

Different approaches can be used to quantify phagocytosis. The amount of fluorescent conjugated particles, associated to the cells can be quantified by flow cytometry. Using light microscopy, phagocytosis can be estimated directly. Phagocytosis, especially FcR-mediated, has been shown to be a good model system for studying adhesion signalling (reviewed in (183)), and was therefore used as a biological read-out to evaluate the involvement of Cdc42 in this study. The effect of GTPases on phagocytosis and phagocytosis-mediated signalling was evaluated by light and fluorescence microscopy.

**Statistical analysis**

Student’s t-test was used to calculate the significance of results displaying a Gaussian distribution. When data lacked Gaussian distribution, a non parametric test was chosen i.e. Mann Whitney test when comparing two groups, or Kruskal-Wallis one-way analysis of variance by ranks together with Dunn’s post test. All the statistics were calculated using
GraphPad InStat version 3.0.1 (www.graphpad.com).* represents p < 0.05, ** represents p < 0.01, and *** p < 0.001.
RESULTS

The effect of pro- and anti-inflammatory chemoattractants on the activation state of the β2 integrins

The activation state of β2 integrins on neutrophils from healthy donors, mediated by pro-inflammatory chemoattractants

One aim of this thesis was to elucidate the effect of pro-inflammatory substances e.g. fMLP, LTB₄, and IL-8 on the activation state of β2 integrins in human neutrophils.

Upon activation, the connection between integrin receptors and the cytoskeleton is momentarily broken (60), allowing lateral movement of the receptors in the plasma membrane (184). β2 integrins cluster on the membrane and form focal complexes to which structural and signalling molecules are recruited, thereby enhancing the avidity of the receptor for its ligand, and the signalling capacity. Analysis of the mobility and clustering of integrin receptors on the plasma membrane showed that the chemoattractant LTB₄ induced an increase in β2 integrin mobility and the formation of both small and large clusters (Paper I, and Fig. 14). The chemoattractant fMLP on the other hand, was less potent compared to LTB₄ in mediating lateral mobility of the integrins and induced formation of predominantly small β2 integrin-clusters on neutrophil-like HL60 cells (Paper I, and summarized in Fig.14).

In order to determine the affinity status of β2 integrins, two α-integrin antibodies were used i) an antibody recognizing a high affinity epitope on the α-subunit of both CD11a and CD11b integrins (MAb24) (172), and ii) antibodies recognizing the ligand binding domain (I-domain) on CD11b integrins (CBRM 1/5). It has been disputed whether the results obtained by these antibodies correlate with ligand binding or not (185). Therefore, our results were confirmed by measuring the binding of soluble ICAM (s-ICAM), a natural ligand to β2 integrins. The binding studies confirmed the results on β2 integrin affinity showing that fMLP, but not LTB₄, induced an affinity change of the β2 integrins (Paper I).

Lately, arachidonic acid, the pre-cursor of both pro-and anti-inflammatory eicosanoids, has emerged as a mediator of inflammatory responses. In HL60 cells, arachidonic acid induced a small
release of $[^3\text{H}]\text{AA}$ and mediated an increase in β2 integrin affinity (Paper III). Arachidonic acid also induced an increase in the avidity of β2 integrins in differentiated HL60 cells (Fig. 13, unpublished results), supporting a role of arachidonic acid as mediator of the activation of β2 integrins.

![Graph showing the effect of arachidonic acid on β2 integrin avidity](image)

**Figure 13. The effect of arachidonic acid on the β2 integrin avidity in differentiated HL60 cells.** Differentiated HL60 cells were stimulated with 20 μM or 100 μM of arachidonic acid (AA) for various time-points. The cells were fixed in PFA for 15 minutes and incubated with NKLI16 antibodies recognizing clustered integrins. Increase in integrin avidity was evaluated by flow cytometry. Data are expressed as % of control from one experiment.

The effect of anti-inflammatory substances on the activation state of β2 integrins

The anti-inflammatory, exogenous lipoxin analogues (LXa) were used as a tool for elucidating the signalling pathways that regulate the β2 integrin activation and to investigate the effect of anti-inflammatory substances *per se*. LXa inhibited the formation of small fMLP-mediated clusters by half, but had no effect on the LTB$_4$-mediated formation of small clusters (Paper I). The large, LTB$_4$-mediated β2 integrin clusters were sensitive to LXa-treatment. LXa had no effect on the fMLP-induced, β2 integrin affinity (Paper I and summarized in Fig. 14). In conclusion, LXa seems to regulate the activity of β2 integrins mainly by affecting the avidity of the adhesion receptors.
The activation state of \( \beta_2 \) integrins on neutrophils from patients with stable CAD

After analyzing \( \beta_2 \) integrin mediated signalling in under normal conditions, the signalling capacity of neutrophils from patients suffering from stable coronary artery disease (CAD) was investigated. Circulation of leukocytes and extravasation to the tissues is one part of the normal inflammatory response. Abnormal accumulation of leukocytes in target tissues is connected to a variety of diseases such as: rheumatoid arthritis, multiple sclerosis and atherosclerosis (186). The activation state of \( \beta_2 \) integrins was investigated by quantifying the number of \( \beta_2 \) integrins on the plasma membrane as well as the affinity of the \( \beta_2 \) integrin receptors on neutrophils from CAD patients compared to age and gender matched healthy donor subjects. The medication administered to all CAD patients consists of a low dose...
aspirin combined with statins. Statins affect a number of signalling molecules important for adhesion (187), and aspirin induces the formation of endogenous, anti-inflammatory substances, i.e. 15-epi lipoxins, earlier found to inhibit the avidity state of the β2 integrins (Paper I). Our study showed that there was no difference between patients and control subjects, in neither the expression-, or in the affinity of β2 integrins during basal conditions (Paper V). The lack of a primed status of neutrophils shows that the therapy of today is sufficient in maintaining the neutrophils in a non-reactive state, thereby limiting progress of the disease.

The effect of pro- and anti-inflammatory substances on the signalling capacity of β2 integrins

Signalling capacity, as measured by the release of arachidonic acid

After investigating the activation state of β2 integrins on neutrophils from healthy donors and CAD patients, the aim was to look more closely at the signalling capacity of the β2 integrin receptors.

Arachidonic acid (AA) is a poly-unsaturated lipid incorporated in cell membranes, which is released upon stimulation and metabolized into eicosanoids. In other words, arachidonic acid is a precursor for both pro-inflammatory (e.g. LTB₄) and anti-inflammatory (lipoxins) eicosanoids. Starting this investigation, AA had been found to be released in HeLa cells adherent to collagen (188). In macrophages on the other hand, CR3-mediated (β2 integrin-mediated) phagocytosis did not activate arachidonate release (189). We found that in HL60 cells kept in suspension, ligation of β2 integrins by pansorbins induced a release of [³H]AA (Paper III, and summarized in Fig. 15A). fMLP by itself, induced a substantial release of [³H]AA, and enhanced the release mediated by β2 integrin-ligation probably due to inside-out signalling (Paper III, and summarized in Fig. 15A). Depletion of calcium affected the AA release, probably due to the fact that calcium is necessary for the activation of PLA₂ and for the activation of β2 integrins per se (Paper III). The intermediary chemoattractant LTB₄ on the other hand, did not induce any release of [³H]AA in differentiated HL60 cells (Fig. 15B, unpublished results, and summarized 15A).
Figure 15. The effect of fMLP and LTB\textsubscript{4} on the release of arachidonic acid in differentiated HL60 cells. A) A schematic illustration on the effect of fMLP and LTB\textsubscript{4} on the \(\beta_2\) integrin-mediated release of arachidonic acid in HL60 cells. In contrast to LTB\textsubscript{4}, both fMLP and \(\beta_2\) integrin-pansorbins induce the release of [\textsuperscript{3}H]AA. Pre-incubation of fMLP potentiates the fMLP-mediated release of AA. Data are presented in Paper III. B) The release of [\textsuperscript{3}H]AA in differentiated HL60 cells. HL60 cells were incubated with [\textsuperscript{3}H]AA for 18-24 hours. The cells were washed and pre-incubated for 1 minute prior to stimulation with fMLP (100 nM) or LTB\textsubscript{4} (100 nM) for 5 minutes. The stimulation was stopped by centrifugation, and the radioactivity of supernatant was measured in a \(\beta\)-scintillation counter. For more details see Materials and methods section in Paper III. Data shown are expressed as mean \(\pm\) SEM from 4 separate experiments, run in duplicates.

Arachidonic acid, released via active \(\beta_2\) integrins and by fMLP, could become further metabolized into eicosanoids, or affect biological processes \textit{per se}. Plasma levels of free arachidonic acid vary between 6-50 \(\mu\)M (190), but can under pathological conditions increase to both 100 \(\mu\)M (malaria) and 500 \(\mu\)M (brain ischemia) (191). A low concentration of arachidonic acid (20 \(\mu\)M) had no effect on cellular morphology and F-actin-content in HL60 cells (Fig. 16 and 17, unpublished results). Cells exposed to 100 \(\mu\)M of arachidonic acid on the other hand, displayed a more round morphology with extensive fillipodia formation (Fig. 16, unpublished results), and increased level of F-actin (Fig. 17, unpublished results).
Figure 16. The effect of arachidonic acid on the cellular morphology of HL60 cells. Differentiated HL60 cells were pre-incubated with arachidonic acid (AA) prior to plating for 15 minutes on glass slides pre-coated with IgG-opsonized yeast. Incubation was stopped by addition of ice-cold PFA, followed by permeabilization and staining of F-actin in PBS supplemented with lysophosphatidylcholine and Alexa 594-conjugated phalloidin. Panels A) show HL60 cells during spreading and chemotaxis, and B) during phagocytosis. The data shown are representative of two independent experiments.

Figure 17. The effect of arachidonic acid on the amount of F-actin in differentiated HL60 cells. Differentiated HL60 cells were stimulated with 100 μM arachidonic acid (AA) or 100 nM fMLP and fixed in PFA for 15 minutes. F-actin was labelled with FITC-conjugated phalloidin and evaluated by flow cytometry. Data are expressed as median values from two independent experiments.
The signalling capacity of $\beta_2$ integrins, measured by production of reactive oxygen species

The signalling capacity of $\beta_2$ integrins was determined by analyzing the effect of the pro-inflammatory chemoattractants LTB$_4$ and IL-8, on the C3bi- ($i.e.\ \beta_2$ integrin) mediated ROS-production in neutrophils from healthy donor subjects. Compared to the $\beta_2$ integrin-mediated intracellular release, the chemoattractants mainly induced an extracellular production of ROS (Paper V, and summarized in Fig. 18A).

It has been proposed that the progress of disease is due to a primed state of neutrophils. However, the signalling capacity of neutrophils from CAD patient was decreased compared to healthy donors, $i.e.\ $the neutrophils from CAD patients were less prone to produce ROS when challenged with C3bi-opsonized yeast or PMA (Paper V, and summarized in Fig. 18B). We hypothesize that the impaired response to C3bi-opsonized yeast in CAD-patients could result from decreased adhesion brought about by a decreased $\beta_2$ integrin avidity due to the anti-inflammatory medication administered to CAD patients.
Figure 18. A schematic illustration over the production of reactive oxygen species in human neutrophils. A) The chemoattractants; LTB$_4$ and IL-8, mainly induce extracellular production of reactive oxygen species (ROS) in neutrophils from healthy donor subjects. The production mediated by β2 integrins on the other hand, is predominantly intracellular. B) The β2 integrin-mediated production of ROS is impaired in human neutrophils from CAD patients, which are given statins and aspirin. For more details see paper V.

The effect of pro- and anti-inflammatory substances on the signal transduction mediated by β2 integrins

After investigating the mobility, clustering, and affinity of β2 integrins, the focus was turned to their signal transduction. This process involves β2 integrin-mediated scaffolding of cytoskeletal proteins and signalling molecules, and utilizes the cytoskeleton as a matrix for the interactions. One of the signalling events activated by ligation of β2 integrins is phosphorylation of proteins mediated by protein tyrosine kinases. When this investigation was initiated in 1998, FAK had been detected in cell lysates of human neutrophils, but was not activated by adhesion in these cells. Another member of the family of non-receptor protein tyrosine kinases, Pyk2, was expressed in hematopoietic cells (104). The aim was therefore to investigate the role of Pyk2 in β2 integrin-mediated signalling in HL60 cells. The strong
tyrosine phosphorylation of Pyk2 induced by fMLP and ligation of β2 integrins in cells kept in suspension was mediated by Src and PKC (Paper II, and summarized in Fig 19) (35, 103). The chemoattractant LTB₄ on the other hand, did not induce phosphorylation of Pyk2 at any given time or concentration. Adhesion to fibrinogen also induced phosphorylation of Pyk2 in HL60 cells, although to a lesser extent compared to β2 pansorbins (Paper II). This can be explained by the ability of the pansorbins to cross-link integrin receptors in the plasma membrane, thereby mimicking clustering/scaffolding of the integrins. Moreover, pre-incubation with fMLP or LTB₄ prior to adhesion, increased the integrin-mediated Pyk2 phosphorylation, most probably due to pre-activation of β2 integrin receptors by inside-out signalling (Paper II, and summarized in Fig. 19). LXa inhibited fMLP-induced phosphorylation of Pyk2 in cells kept in suspension, but did not affect the β2 integrin mediated Pyk2 phosphorylation in cells adherent to fibrinogen (Paper II). Our results concerning the effect of fMLP and LTB₄ on the lateral mobility, clustering, affinity and signalling capacity of β2 integrins (summarized in Fig. 14), support the idea that different chemoattractants activate distinct signalling pathways during β2 integrin mediated signalling (128, 129). Lipoxins emerge as potential anti-inflammatory drugs, and Pyk2 may be a suitable target for the action of these drugs.
Figure 19. Schematic illustration about the effect of chemotactic stimuli on β2 integrin-mediated adhesion in neutrophils. The effect of fMLP and LTB4 on the phosphorylation of Pyk2 in HL60 cells in suspension. Ligation of β2 integrins by pansorbins induces a phosphorylation of Pyk2 in differentiated HL60 cells. The chemoattractant fMLP phosphorylates Pyk2. Due to the strong activation of Pyk2 by β2-pansorbins, it is impossible to evaluate the effect of fMLP on the pansorbin-mediated phosphorylation of Pyk2. Pre-incubation with the anti-inflammatory lipoxin analogues (LXa) inhibits fMLP-mediated activation of Pyk2. Data are presented in detail in paper III.
Since arachidonic acid was found to mediate the activation status of β2 integrins (Paper III), the effect of arachidonic acid on the phosphorylation of Pyk2 was investigated. Stimulation with arachidonic acid (100 μM) had no effect on the phosphorylation of Pyk2 (Fig. 20, unpublished results).

![Figure 20.](image)

**Figure 20. The effect of arachidonic acid on the phosphorylation of Pyk2 in differentiated HL60 cells.** Serum starved HL60 cells were stimulated with arachidonic acid (AA; 100 μM) or fMLP (100 nM) for 2.5 minutes and lysed in ice-cold lysis buffer. The cells were centrifuged at 12000 x g for 10 minutes, and Pyk2 in the supernatant was immunoprecipitated (IP) by anti-Pyk2 antibodies (#600) together with protein A sepharose. The samples were subjected to SDS-PAGE, and western blot (WB). After blocking, the membranes were incubated with phosphotyrosine antibodies (4G10). In order to ensure proper loading, membranes were stripped and incubated with anti-Pyk2 antibodies.

These results are supported by our finding that the AA-metabolite, LTB₄ does to induce phosphorylation of Pyk2 (Paper II), in likeness of its precursor AA. Additional experiments are however needed to understand the effect of arachidonic acid on the activation of β2 integrin mediated signalling.

**Additional methods to study proteins important for the regulation of β2 integrins**

In order to claim a protein to be of importance for a cellular process, the effect of inhibition of the protein must be investigated. Pyk2 was earlier proposed to be a protein involved in the signalling mediated by β2 integrins. No specific inhibitors of Pyk2 were commercially available when this study was initiated, but the effect of tyrosine kinase inhibitors suggested involvement of Pyk2 in β2 integrin-mediated signalling (Paper II). A way to introduce constitutively active and dominant negative, recombinant forms of Pyk2 was therefore
needed. Transfection of neutrophils/HL60 cells by Lipofectamine, DEAE-dextran, electroporation, and retro-viral approach was however unsuccessful, hence other transfection methods needed to be established.

**TAT-protein transduction**

The TAT-transduction method which utilizes short sequence of HIV-Tat protein expressed as a fusion protein was exploited. Due to the small size of Cdc42 (approx. 25kDa), it was considered as a suitable protein for optimizing the transduction method in neutrophils and differentiated HL60 cells (Paper IV). Moreover, the fact that Cdc42 is activated by both β2-integrin ligation and stimulation with both fMLP and LTB₄ in human neutrophils (data not shown), and that G-proteins are involved in the activation of Pyk2, made Cdc42 highly interesting to investigate.

Enzymatic degradation of TAT-Cdc42 fusion proteins in human neutrophils forced us to focus on differentiated HL60 cells. After optimizing the procedure, differentiated HL60 were successfully transduced with TAT-Cdc42 (Paper IV).

As a biological read-out, the effect of constitutively active- (V12) and dominant negative- (N17) forms of TAT-Cdc42 on IgG-mediated phagocytosis was analyzed. Staining of F-actin by phalloidin, revealed that cells transduced with constitutively active TAT-Cdc42 displayed a pronounced accumulation of F-actin, both at the leading edge of the cell and around the phagosome (Paper IV, and in Fig. 23), which was also confirmed by scanning electron microscopy experiments (Paper IV). TAT-N17Cdc42 on the other hand, had no effect on the F-actin content. None of the TAT-proteins affected phagocytosis of IgG-opsonized yeast *per se* (Paper IV), despite the fact that TAT-N17Cdc42 inhibited chemotaxis to a small extent (20% reduction, Fig. 21, unpublished results).
Figure 21. fMLP-induced chemotaxis of TAT-Cdc42 transduced HL60 cells. Differentiated HL60 cells, non-transduced were transduced with constitutively active (V12) and dominant negative (N17) TAT-Cdc42 proteins for 30 minutes. Three wells were cut out in an agarose gel casted in a petri-dish. HL60 cells were applied to the mid-well, fMLP in one well (A) and buffer in the other well (B). Cells were allowed to migrate towards the chemoattractant for one hour. After fixation with PFA, the cells were stained with Giemsa, and migration was evaluated in a light microscope, using an ocular containing a ruler. Chemotaxis was evaluated as migration index (a/b), where a represents the migration against a chemotactic gradient and b the spontaneous migration of cells. Data are shown as mean ± SEM for migration (% of non transduced cells) of three separate experiments run in triplicates.

The presence of an F-actin ring around the phagosome seemed to correlate with the lack of CD63 positive granules (Paper IV) and was therefore subjected to further investigation. To analyze translocation of CD63 positive granules, cells were loaded with Lysotracker® and the movement of granules was recorded (Paper IV). Using quantitative image analysis, the following scenario unravelled. In non-transduced cells, the ingested particle is located in a phagosome surrounded by a transient ring of F-actin. Constitutively active Cdc42 induced the formation of a thick F-actin ring affecting phagolysosome maturation. The F-actin has to be dissolved to allow fusion of granules with the phagosome. Cdc42 is also involved in the actual translocation of granules to the phagosome, since dominantly negative Cdc42 resulted in non-aggregated granules, randomly dispersed in the cell (Paper IV, and summarized in Fig. 22).
**Figure 22. A schematic illustration on the effect of active/inactive Cdc42.** In control (non-transduced) cells the F-actin surrounding the phagosome is depolymerized during phago-lysosome formation, allowing fusion of granules with the phagosomal membrane. Transduction of cells with constitutively active (V12) form of Cdc42 induces an accumulation of F-actin around the phagosome, impeding granule-phagosome fusion. In cells transduced with dominant negative Cdc42 (N17), the appearance of the F-actin ring around the phagosome resembles control cells, but translocation of granules to the phagosome is impaired. The results summarized in this figure are presented in detail in paper IV.

**Transduction of HL60 cells with TAT-Pyk2**

After optimizing the transduction method by using TAT-Cdc42, we were interested in transducing the cells with TAT-Pyk2. By then, TAT-Pyk2 had successfully been transduced into neutrophils (192). Differentiated HL60 cells were transduced with C-terminal part of Pyk2, earlier shown to inhibit ROS production in neutrophils (192). It proved difficult to transduce TAT-Pyk2 proteins into differentiated HL60 cells; probably due to the larger size of TAT-Pyk2-fusion proteins compared TAT-Cdc42 proteins, and further optimization is necessary. Nevertheless, protein transduction of HL60 cells with CT-Pyk2 had no effect on the fMLP-mediated induction of β2 integrin affinity, nor on the β2- and fMLP-mediated release of [³H]AA (data not shown); indicating that Pyk2 is not involved in these processes.
Figure 23. The regulatory effect of pro- and anti-inflammatory mediators on the β2 integrin-induced signalling in neutrophils. Prior to adhesion, integrin receptors are activated by chemokines and chemotactic factors. The pro-inflammatory chemoattractants fMLP and LTB₄, mediate an increase in the lateral mobility and clustering of β2 integrins. The affinity of β2 integrins, on the other hand, is mainly increased by fMLP, and is not sensitive to treatment with the anti-inflammatory lipoxin analogue (LXa). Also the β2 integrin-mediated tyrosine phosphorylation of Pyk2 is insensitive to LXa treatment. Besides inducing phosphorylation of Pyk2, ligation of fMLP- and β2 integrin-receptors (R) releases arachidonic acid (AA). Moreover, pre-incubation of fMLP augments the β2-mediated release of AA. AA per se induces an increase in β2 integrin affinity and avidity and increases the amount of F-actin. Pre-incubation with the anti-inflammatory lipoxin analogues (LXa) or treatment with aspirin, inhibits fMLP-mediated activation of Pyk2, and the β2-mediated release of ROS. The data summarized in this figure are presented in detail in Papers I-V, and as unpublished observations.
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

When investigating the regulatory effect of pro- and anti-inflammatory substances on the β2 integrin-mediated signalling in neutrophils, more questions were raised concerning:

i) The activation state of the β2 integrins (i.e. affinity, avidity).
   - Does Pyk2 modulate the β2 integrin affinity/avidity?
   - Does Cdc42 mediate in the activation of β2 integrins?
   - What is the roll of small GTPases (e.g. Rac, Cdc42, and Rap1) in the regulation of β2 integrin affinity?

ii) The signalling capacity of β2 integrins (i.e. AA release and ROS production). More specifically, the questions would be:
   - Does the anti-inflammatory lipoxin (LXa) affect the release of AA?
   - Does IL-8 induce release of AA?
   - How is the AA-release mediated in CAD patients?
   - Does AA per se induce the release of ROS?
   - Is the β2 integrin mediated release of ROS dependent of Pyk2 activation?

iii) The signal transduction mediated by β2 integrins (i.e. Pyk2 phosphorylation). The more specific questions would be:
   - Which tyrosine phosphorylation sites on Pyk2 are activated upon stimulation with the pro-inflammatory chemoattractants LTB₄, and fMLP?
   - What is the effect of LXa on these phosphorylation sites?
   - Does Pyk2 associate with β2 integrins prior or after stimulation with pro- and anti-inflammatory substances? Does α-actinin/talin associate with Pyk2 in β2 integrin-stimulated HL60 cells? In other words, is Pyk2 a scaffolding protein during β2 integrin-mediated signalling?
   - What effect do dominant negative- and constitutively active forms of Cdc42 have on the activation of Pyk2?

The questions are never-ending…

So, to my fellow scientists out there: I turn over the baton to you!
ACKNOWLEDGEMENTS

Many people have contributed in the making of this thesis, and I am grateful for all the support I received along the way!

Detta är ett av de få tillfällen man har här i livet att verkligen tacka alla som har betytt något. Så nu får ni stå ut med att det blir några sidor. Jag har ju haft turen att träffa så många trevliga människor och lära känna så många nya vänner genom åren!

Jag vill speciellt tacka:

Min handledare, Eva Särndahl. Tack för allt stöd och all uppmuntran genom åren! Tack för att du efter alla år (11) ser till att upprätthålla mitt intresse för forskning. Det är så mycket man kan lära av dig: att vara en bra pedagog, att framgångsrikt skriva en forskningsplan och söka anslag, och att inte ge upp i första taget! Jag har haft så kul när vi har rest tillsammans, trots förlorat bagage och alla konstiga åkommor…

Tack för att du delat med dig av ditt liv och din familj, det har varit roligt att få se dina barn växa upp. För att du alltid bryr dig och alltid ställer upp (ibland på bekostnad av din familj…tack familjen!). Mest av allt vill jag tacka dig för att du har ”släppt mig lös” på lab och att du (nästan) aldrig säger nej till mina vilda idéer! Du har under dessa år inte bara varit min handledare utan också blivit en väldigt god vän och van…och vad mer kan man önska?

Olle Stendahl, min mentor och på den sista tiden också min biträdande-handledare.

Tack för att du engagerar dig, delar med dig av din stora kunskap, kommer med så bra synpunkter och tack för att du alltid tar dig tid! Tack för att du är med oss i med och motgång... och för att du blev nästan tusen gånger gladare än jag när manuset gick in till slut…

Pia Druid: Den bästa som finns! Tack för allt genom åren, inte bara all laborationsmässig hjälp (som har varit super...”utan dig är vi intet”) utan också för allt ”skitsnack” och alla härliga skratt. Tack för att du har brytt dig om och för att du ringt mig varje vecka under mina barnlediga år!

Jane Wigren: Tack för att du såg till att labbet inte blev alltför ödsligt när Eva och Pia tillfälligt ”emigrerade”. Du är alltid så trevlig och hjälpsam och din korrekturläsning höjer engelskan till nya nivåer. Vi syns vid kanalen i sommar (jag fika…du löpa…!)

Liselotte Ydrenius: Vad vi har haft kul genom åren! Hotten-Totten, tack för att din humor (om än konstigare än min) och dina humörsvångningar. Det var min stora uppgift att retas med dig tills du skrattade… jag lyckades för det mesta. Jag saknar det hasande ljudet av dina tofflor!

Martin Tinnerfelt Winberg, min ”lillebror” på labbet. Tack för allt skoj, för att du har stått ut med alla ”tjuvnyp” och för att du gillar att kramas!

Sailesh Surapureddi, my sweetheart! Thanks for all the fun in the lab, for all the great meals and for introducing me to “Pidgin English”.

Maria Lerm, för att du alltid hjälper till och alltid stöttar.

Alla våra studenter genom åren: Ida, Iida, Anna, Martin, Annelie, Calle, Malin, Pernilla, Emma, Hanna och alla andra…tack för att ni bidragit till att höja stämningen på lab.

Några av mina rumskamrater genom åren: Jesper Svartz, Kristina Loinder och Tobias Strid.
Tänk vad mycket man får reda på i doktorandrummet! Tack för att jag fick störa er hela tiden. Även om vetenskap kan vara kul att diskutera, så finns det ju andra saker här i livet som jag hellre pratar om…som ni har märkt. Jesper, som också har varit NIR-doktorand, har jag haft turen att få åta många lyxiga hotellfrukostar med i Lund. Vi har haft en massa skoj på alla kurser och konferenser! Det finns bara en som du! På gränsen till för mycket…men sååå rolig!

Mina andra rumskamrater Johan Paulsson, Sofia Nyström och nyligen Sebastian Schultz. Johan, ”the rising star over there”, du är inte bara trevlig, och smart…du är ju snygg också…jag kommer att sjunga om dina biceps varje dag när du har åkt. Sofia, en av mina ”medbrottslingar” på Cellbiologen sedan länge. Skönt att ha någon som vad jag menar när jag pratar om ”gamla tider”. Jag kommer att sakna dina monologer och att hitta dina nycklar och plånböcker överallt…Sebastian, tack för att du såg till att jag fick nya ”bevingade” kompisar på labbet.


Peter Strålfors, mannen vars äsiker jag oftast inte delar (det har nog inte undgått någon?!). Tack för att du alltid har engagerat dig och brytt dig om hur det går för mig och för att du tar dig tid! Jag kommer att sakna våra ”diskussioner”…Vem ska nu säga emot dig?

Gunilla Westermark, tack för alla sena diskussioner och för att du alltid delar med dig av din gourmet mat! Jana Sponarova Jano, thanks for your friendship. I’m sorry that I am so bad at Czech. Perhaps you can find someone to speak to at the party…?

Mats Söderström och Sven Hammarström. Mats, tack för all hjälp genom åren…datorer har ett eget liv! Sven, tack för att du delar med dig av din stora kunskap och för att du tog dig tid att läsa både manus och delar av avhandlingen.

Lärarna på Medicinsk Bilogi T3: Mats Lindahl, Tony Forslund och Margareta Lindroth. Mats och Tony, det har varit kul att få ”bestämma lite” i termingsgruppen. Hoppas att ni hittar något annat offer som vill läsa alla ”uppsatser”. Margareta, alltid så glad och så kunnig! Du är bäst!…och söt i din nya frisyr…

De övriga på Cellbiologen, Mikrobiologen, och Farmakologen: Lotta Ericsson, Anna Berg, Cecilia Trinks, Peter Pålsson Maria Lerm, ”flödesexperten” Tony Forslund, Torbjörn Bengtsson, Maria Forsberg, Åsa Holm, Charlotte Immerstrand, Birgitta Rasmusson och alla ni andra.

Anna, nu när jag kommer att ha all tid i världen så måste vi faktiskt se till att träffas!

Alla mina vänner från Biomedicinska forskarskolan: Charlotte Anna, Petra, Karin, Hans, Johan, Jacob, Ylva, Marcus, och så naturligtvis Torbjörn och Lillemor! Tack för ett roligt år!...jag fick ”kexet” till slut!

Mina vänner från NIR: speciellt Maria, Lisa, Veronica och Cecilia. Vi har haft så kul när vi har bott på Patienthotellet i Lund! Tack till Mark Hickery och Maria Olofsson för ett superbra jobb!

All the people at the Ludwig Institute for Cancer Research in Uppsala: the “Boss” Calle Heldin, Ivan, Inga, my great friends Ninna, Jill, Sofia and Eva G. The rest of “the gang” Tobias, Olli, Johan, my room-mate Anders, Ann-Sofie, Andree, and of course cykelfixaren Ulf. Thank you for taking care of me and for all the fun during the late lab-nights. My supervisor Ivan Dikic, thank you for introducing me to the realities of science and the “importance” of being focused and dedicated…

The people “over there” in Indianapolis: Fred Pavalko, Sarah Sawyer and all the rest. Fred, thank you for taking such good care of me during my stay! Your never failing enthusiasm, encouragement and kindness will not be forgotten. I had a great time! Thank you for giving me a ride to the lab every morning. Sarah, thank you for being such a good friend and for helping me spending my money at great restaurants and in book shops! All the rest of the people at the Indiana University School of Medicine who I had the fortune to meet…I miss you all!

Patrik Lindblom, utan din hjälp hade vi inte kunnat få iväg några artiklar! Och tack för all super-god mat genom åren!

Förutom forskning, så har man ju faktiskt andra intressen här i livet. Därför finns det en hel del människor som jag vill tacka!


Karin Stenkula och Johan Tengelin. Tack för all vänskap. Alla goda middagar (ett återkommande tema!) och alla skratt. Ihop med yrvärdet Karin kan man inte annat än skratta! Karin, tack för att du har delat mitt ”livsfärliga” hästintresse genom åren… Synd att ni har flyttat till Örebro…men vem vet vi kanske flyttar efter?!

Charlotte och Björn Immerstrand. Charlotte, den mest positiva människan jag någonsin träffat!...förutom din man Björn kanske. Det är alltid så trevligt att umgås med er och era barn. Vi ser fram emot att plaska i poolen i sommar också!

Christel Winell för allt roligt vi hade på labbet och för att jag fick vara en del av din familj under en ganska lång tid…

Josefin Axelsson (numera Perman), ”biologen” som övergav mig till förmån till läkarlinjen. Det var ju så tråkigt att du slutade, men idag är jag glad att vi har en egen ”familjeläkare”. Tack för alla våra långa telefonsamtal genom åren, för allt fikande och shopande. Det var kul att titta på tv (läs ER) tillsammans per telefon… Hoppas att jag får träffa guldklippen snart!

”Mahjong-gänget” : Camilla Wohlgemuth, Annika Silvervarg och Kristina Pieslinger. Tack för alla Mahjong dagar, med chips, dipp, en massa prat och lite spelande…tänk att jag inte har vunnit en hel omgång en enda gång, trots att vi har spelat i sex (!) år! Camilla, tack för att du har varit min vän sedan den dagen jag knackade på hemma på Lövudden och ville låna böcker…Man blir så glad av att träffa dig!

Alla våra trevliga grannar. Tack för att ni ser till att vi inte vill flytta ifrån Berg!

Ulrika och Peter Bergman, tack för att ni hjälper oss så mycket med allt! Passar våra barn och bjuder på god mat. Jag uppskattar det! Tack Ulrika för att du utan att tveka skjutsade in mig till jobbet när jag behövde referenser och för att du släpar ut mig på promenader så att jag får ”röra på fläsket”.

Karin och Magnus Seger, våra ”jobbiga” grannar tvärs över som alltid fixar på huset, i trädgården eller är ute och motionerar…suck! Karin tack för alla kaffepauser, goda kakor, middagar och allt ”skitsnack”…jag behövde det när jag skrev! Vilken tur att du håller mig uppdaterad på allt som händer i Berg! Tack för att ni inte har ”gjort er av med” våra katter ännu…

Sist, men inte minst så måste jag få tacka mina nära och kära.

Mina svärföreldrar Inger och Lars, tack för att ni har hjälpt oss så mycket genom åren.

My family in the Czech republic. I hope to see you soon!

Min familj som ibland kan likna ”the Fockers”…
Min mamma Rumenka som alltid sätter sig i bilen och kommer om jag behöver det. Tack mamma för att du alltid stöttar mig i det jag vill göra. Jag hoppas att jag kan uppostra mina barn lika bra som du har gjort! Stig, som är bra på allt! Vår egen handy-man som fick en massa barn på köpet när han gifte in sig i vår familj.

Min lillebror Daniel (inte så liten nu för tiden). En duktig musiker, designer, helt enkelt skitbra på att flyga modellflygplan…och att ragga tjejer på nätet. Jag är stolt över dig!

Mina barn, Ella och Alexander. För all er villkorslösa kärlek!


The research presented in this thesis was supported by the following grants:

- Ph.D. student fellowship from the Network for Inflammation research (NIR) funded by the Swedish foundation for Strategic Research (SSF)
- Fellowship from the Swedish Heart & Lung Foundation
- Swedish Research Council
- King Gustaf V Memorial Foundation
- Swedish Medical Society
- Medical County Council of Östergötland
- Lars Hierta Foundation
REFERENCES


58. Lim, J., A. Wiedemann, G. Tzircotis, S. J. Monkelley, D. R. Critchley, and E. Caron. 2007. An essential role for talin during alpha(M)beta(2)-mediated...


S100A8/A9 promotes NADPH oxidase activation by interaction with p67phox and Rac-2. *Faseb J* 19:467-469.


Mg(2+). *Biochim Biophys Acta* 1510:270-277.


