Regulation of Leukocyte Integrin Binding to Ig-Family Ligands

DIVISION OF BIOCHEMISTRY AND BIOTECHNOLOGY
DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

LIISA UOTILA

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Regulation of leukocyte integrin binding to Ig-family ligands

Liisa Uotila

Division of Biochemistry and Biotechnology
Department of Biosciences
Faculty of Biological and Environmental Sciences
and
Integrative Life Sciences Doctoral Program
University of Helsinki

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in the lecture hall B2 of Viikki B-building (VI B LS 2), Latokartanonkaari 7, on 21st November at 12 noon.

Helsinki 2014
Supervisor  Carl Gahmberg, professor
Division of Biochemistry and Biotechnology
Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki

Reviewers  Jorma Keski-Oja, professor
Translational Cancer Biology Research Program
Haartman Institute
University of Helsinki

                Manuel Patarroyo, professor
Department of Dental Medicine
Karolinska Institutet
Stockholm, Sweden

Opponent  Francisco Sánchez-Madrid, professor
Servicio de Inmunología, Hospital de la Princesa
Instituto de Investigación Sanitaria de la Princesa
Universidad Autónoma de Madrid
Madrid, Spain

Custos  Jukka Finne, professor
Division of Biochemistry and Biotechnology
Department of Biosciences
Faculty of Biological and Environmental Sciences
University of Helsinki

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

ISBN 978-951-51-0338-3 (paperback)
ISSN 2342-3161 (print)

ISBN 978-951-51-0339-0 (PDF)
ISSN2342-317X (Online)
http://ethesis.helsinki.fi

Layout: Tinde Päivärinta/PSWFolders Oy
Hansaprint Oy, Vantaa, Finland 2014
Le plus grand bonheur de l’étude consiste à trouver les raisons soi-même.
René Descartes
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*These authors have contributed equally to the work

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Contributions

I  LU performed protein purification, cell adhesion assays, flow cytometry and solid phase ELISA assays together with AT, MS and EI and participated in planning the experiments, analysing the results and writing the manuscript with other authors.

II LU planned and performed or supervised the experiments together with EI, AT and MV and participated in analysing the data and writing the article with EI.

III LU planned the experiments and performed most experiments, analysed the data and wrote the manuscript together with CGG.

IV LU planned the experiments together with MG, performed the experiments together with FJ, LSH, EM and MG, supervised LSH’s work and wrote the article together with MG and CGG.
Abbreviations

AP-1 Activator protein-1 transcription factor  
Arf6 ADP-ribosylation factor 6  
BCR B cell receptor  
BSA Bovine serum albumin  
CalDAG-GEF1 Ca²⁺ and diacylglycerol-regulated guanine nucleotide exchange factor 1  
c-Cbl Casitas B-lineage lymphoma  
CD Cluster of differentiation, a nomenclature system for surface antigens  
Cdc42 Cell division control protein 42 homolog  
CNS Central nervous system  
CR4 Complement receptor 4 (leukocyte integrin αXβ2, CD11c/CD18, p150.95)  
DC-SIGN Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin  
Del-1 Developmental endothelial locus-1  
DLC-1 Deleted in liver cancer-1  
ECM Extracellular matrix  
Emp Erythroblast-macrophage protein  
ERM Ezrin, radixin and moesin  
FA Focal adhesion  
FAK Focal adhesion kinase  
FERM domain 4.1 protein, ezrin, radixin, moesin domain  
Fn Fibronectin  
Foxp1 Forkhead fox protein P1  
GEF Guanine-nucleotide exchange factor  
Hb Hemoglobin  
ICAM Intercellular adhesion molecule  
IL-2 Interleukin-2  
ILK Integrin-linked kinase  
IP3 Inositol trisphosphate  
LFA-1 Lymphocyte function-associated antigen-1 (leukocyte integrin αLβ2, CD11a/CD18)  
Lu Lutheran blood group glycoprotein  
LW Landsteiner-Wiener blood group antigen  
Mac-1 Macrophage-1 antigen (leukocyte integrin αMβ2, CD11b/CD18, CR3/complement receptor 3)  
MBP Myelin basic protein  
MHC Major histocompatibility complex  
MS Multiple sclerosis  
NK Natural killer cell  
PH domain Plexin homology domain  
PI3K Phosphoinositide 3-kinase  
PLC Phospholipase C  
PMN Polymorphonuclear leukocyte/granulocyte  
PSI Plexin-semaphorin-integrin  
PtdIns(4,5)P₂ Phosphatidylinositol 4,5-bisphosphate  
Rac1 Ras-related C3 botulinum toxin substrate 1  

Rap1  Ras-related protein 1
RAPL  Regulator of adhesion and cell polarization enriched in lymphoid tissues or
RASSF5
RBC  Red blood cell
RhoA  Ras homolog gene family, member A
SDF-1α  Stromal-cell derived factor-1α
SLP76  SH2 domain-containing leukocyte phosphoprotein of 76kDa or lymphocyte
cytosolic protein 2
SYK  Spleen tyrosine kinase
TCR  T cell receptor
Tiam1  T-cell lymphoma invasion and metastasis-inducing protein 1
TLR  Toll-like receptor
VCAM-1  Vascular cell adhesion molecule-1
VLA-4  Very late antigen-1
wt  wild type

Three-letter coding for amino acids is used throughout the text

<table>
<thead>
<tr>
<th>Amino acid</th>
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Summary

Under normal physiological conditions blood cells are usually non-adhesive, but for many of their functions, they need to stick to other cells or to extracellular matrix (ECM). For this purpose, all the haematopoietic cells (red blood cells or RBC or erythrocytes, white blood cells or leukocytes and platelets) carry on their surface, or in intracellular stores, a variety of adhesion molecules. These molecules intermediate multiple divergent functions, such as old red cell removal from the circulation, white blood cell migration to the site of infection, blood coagulation or phagocytosis of an invading pathogen by a macrophage. The adhesion molecules on blood cells have many requirements that they need to fulfill in order to maintain a physiological system: they need to stay in an inactive, non-binding state for most of the time, and to be activated and become adhesive only when needed. In addition, they should specifically recognise their binding partners or ligands, as unnecessary binding could lead for example to clogging of the blood vessels, autoimmune diseases or allergic reactions. Still one important feature of blood cell adhesion is the ability to let go and release the adhesion, when the cell needs to move forward or continue patrolling the circulation etc.

The aim of this work was to elucidate the molecular mechanisms behind these adhesion events and, especially to characterize the regulation of certain adhesion molecules. The work was initiated by analysis of the interactions between intercellular adhesion molecule-4 (ICAM-4) and leukocyte integrins. ICAM-4 is a protein belonging to the immunoglobulin superfamily (Ig-SF) of adhesion molecules and it is expressed only on red blood cells and their precursors. It binds to the members of the leukocyte integrin family that are expressed on white blood cells. We discovered a new binding partner for ICAM-4, the leukocyte integrin CR4 (complement receptor 4) and identified the amino acids that are involved in the binding. We then investigated which part of the leukocyte integrin is responsible for the binding, and which divalent cations are needed for it. Comparison of the binding sites of different members of the leukocyte integrin family on ICAM-4 reveals similarities and differences between the family members. The interactions between ICAM-4 and leukocyte integrins are probably needed when red cells develop in the bone marrow or when they are removed from the circulation by spleen macrophages.

An essential question emerged how the phosphorylation of the intracellular part of CR4 regulates its adhesion. The exact phosphorylation site was then identified, and the effects of this phosphorylation on the known different functions of CR4 were pinpointed. We found out that the phosphorylation is required for cell adhesion and phagocytosis of cells expressing CR4. Phosphorylation was also needed for correct activation of CR4 through an inside-out activation pathway, whereas it was not required for signalling events initiated by leukocyte integrin ligand binding, in the so called outside-in activation.

These observations inspired analysis of the interplay between lymphocyte function-associated antigen-1 (LFA-1) or complement receptor 4 (CR4) and very late antigen-4 (VLA-4) that was characterized in the context of T cells. We noticed that LFA-1 activation induced an activation cascade that downregulated the adhesion of VLA-4 to its ligand vascular cell adhesion molecule-1 (VCAM-1) that is expressed on activated endothelium. To understand these events we characterised the molecular mechanisms that mediate this transdominant inhibition between two integrins in a given cell.
These analyses of blood cell adhesion from different directions cover the regulation of cell adhesion at several levels: the structural features of the binding partners, the phosphorylation and the intracellular signalling cascades preceding and following the phosphorylation and the transdominant inhibition between different integrins in the same cell.
REVIEW OF THE LITERATURE

1 Introduction to blood cell adhesion

Cell adhesion is one of the most fundamental phenomena in the lifespan of multicellular organisms, whether it is a human, mouse or malaria parasite. Adhesion is needed in the first steps of development of a multicellular organism as well as in growth, differentiation, immune reactions and neuron differentiation and in many other crucial events along the life course of an individual. Adhesive events are also involved in various pathological states, e.g. cancer metastasis and pathogen infection.

Stable as well as reversible adhesive events are needed for tissue formation and single cell interactions depending on cell type and function. In blood cells, inducible adhesion is especially significant as the cells need to change their behaviour from non-adherent by-passers to active, adhesive players binding to ECM, other blood cells or to endothelium, often in less than seconds.

The molecules responsible for the adhesive events can be divided into different groups or families according to their size, structure and functions. There are some common traits that all the adhesion molecules share, such as the overall structure that consists of three major domains: the intracellular part associated to the cytoskeleton, the transmembrane part, and the extracellular region that is usually considerably larger than the other parts, and is responsible for the recognition of and binding to external ligands. Traditionally the adhesion molecules responsible for haematopoietic cell adhesions have been divided in three large families: the immunoglobulin (Ig) superfamily, integrins and selectins. Also other molecule families, e.g. cadherins, are participating in the adhesion in many tissues.

In the current work the focus will be largely on the adhesion events of erythrocytes and leukocytes, and on molecules belonging to the Ig and integrin families. I will especially discuss the roles of erythrocyte ICAM-4 and leukocyte CR4 in the context of red blood cells and leukocytes, respectively. I will also introduce the most important mechanisms contributing to the regulation of blood cell adhesion, such as phosphorylation, intracellular ligand binding and transdominant regulation by other integrins in the very same cell.

1.1 Interactions of erythrocytes with other cells and ECM

The life cycle of red blood cells is strictly regulated: red blood cells develop in the bone marrow and after maturation and nuclear extrusion the cells move into the blood stream and circulate for about 120 days. The senescent cells are removed from the circulation in a process called erythrophagocytosis that is carried out by spleen macrophages. The role of red blood cells, the transport and exchange of gases, is generally considered to be disturbed by excessive cell adhesion. However, red blood cells express many molecules that possess adhesive properties or that belong to established adhesion molecule families. The novel functions or molecular mechanisms mediated by these adhesion molecules are starting to get elucidated.

1.1.1 Erythropoiesis

Red blood cells (RBC) develop and mature in specific anatomical structures of the bone marrow called erythroblastic islands (figure 1). There the maturing erythroblasts surround the central macrophage and form close contacts with each other, with the central macrophages and with the extracellular matrix. Erythroblastic islands were found in the 1950’s by Marcel Bessis. The RBCs mature in several steps that may be recognised by morphology or behaviour of the cells, finally
leading to massive hemoglobin (Hb) production and nucleus extrusion. The maturation of the erythroblasts is guided by the erythropoietin (Epo) hormone that is synthesised in the kidneys (Bonsdorff & Jalavisto 1948), and also by the interactions between erythroblast, the central macrophage and the extracellular matrix (Mohandas & Prenant 1978, Sadahira & Mori 1999, Chasis & Mohandas 2008, Bessis 1958).

The interactions between central macrophages and the developing erythroblasts are essential for the proper and sufficiently rapid development of erythrocytes. These adhesions, and the downstream signalling cascades, lead to gene expression changes, protection from apoptosis, enhanced proliferation, cytoskeleton reorganisation and nucleus extrusion of the developing erythroblast. Macrophages participate in the erythropoiesis also by producing cytokines as well as by providing iron for the developing erythroblast and phagocytosing the extruded nucleus in the final stages of erythrocyte maturation. The adhesion molecules participating in the interactions have been extensively studied and their significant roles in the multiple steps of red cell maturation are being dissolved (Chasis & Mohandas 2008).

The surface expression of different adhesion molecules and other cell surface molecules in human and murine cells has been measured at different stages of erythrocyte development. Interesting differences have been detected. The red cell precursors express many adhesion molecules (e.g. β1 and β2 integrins and intercellular adhesion molecules, ICAMs), but the expression of these adhesion receptors is largely lost when the cells develop and move into circulation (Bony et al 1999, Southcott et al 1999, Liu et al 2010). One of the most important adhesion molecules is Erythroblast-macrophage protein (Emp) that is a 30 kDa transmembrane protein expressed on both erythroblasts and central macrophages. It promotes homophilic Emp-Emp adhesion between the two cell types, which enhances nucleus extrusion and inhibition of apoptosis (Hanspal & Hanspal 1994, Hanspal et al 1998). The developing erythroblasts also
express VLA-4 integrin and the macrophages express its ligand VCAM-1. The interaction between these two molecules is needed for the integrity of the erythroblastic island (Hamamura et al. 1996, Sadahira et al. 1995). Another prominent adhesion molecule involved in the erythroblastic island formation is ICAM-4 that has been reported to bind to $\alpha_v$ integrins on central macrophage and providing stability to the island structure (Lee et al. 2006).

ECM proteins, especially fibronectin (Fn) and laminin are also possibly involved in the regulation processes leading to erythroblast terminal differentiation and guiding the mature RBC to the circulation. The integrins VLA-4 and VLA-5, which are fibronectin receptors, are expressed on erythroblasts (Hanspal 1997). Lutheran blood group glycoprotein (Lu) on red cells, in turn can bind to laminins (El Nemer et al. 1998).

1.1.2 **Erythrophagocytosis, removal of senescent red cells**

After 120 days in the circulation, the senescent erythrocytes are removed from the circulation primarily by spleen macrophages and the liver in an action called erythrophagocytosis (reviewed in Antonelou 2010). The mechanisms how senescent red cells are recognised by splenic macrophages are poorly understood. Several changes contributing to the removal appear in red blood cells during ageing. The major changes observed in senescent erythrocytes include changes in size, density, volume and morphology of the cells, loss of membrane asymmetry, desialylation of membrane sialoglycoconjugates, formation of senescent cell antigens and increase in the membrane-bound immunoglobulins and complement component C3b. The microvesiculation of the red cell plasma membrane is increased in the older red cells, leading to loss of membrane and its constituents and decreasing the membrane flexibility but, on the other hand, enabling the disposal of denatured proteins. Probably the most important player in the senescence signalling is oxidative stress. It causes changes in Band 3 cell surface protein, creating neo-antigens (or senescence antigens) that are recognised by autologous IgG and complement component C3, and leads to phagocytosis of the red cells by macrophages that are able to bind these molecules. Another consequence of oxidative stress is the activation of pro-apoptotic components (especially caspase 3). Hemoglobin as well is modified and the interactions with plasma membrane components like Band 3 and cytoskeletal spectrin are enhanced, increasing the deformability of the cells. Many hypotheses for the ultimate erythrophagocytosis signal have been put forward, but none have proven to be exclusively responsible for the senescence signals (Aminoff et al. 1992, Bratosin et al. 1998, Antonelou et al. 2010).

1.1.3 **Pathological conditions of red blood cell adhesion**

In addition to the physiological situations described above, red cells may become too adherent in some pathological conditions. In sickle cell anaemia, a mutation in the Hb gene leads to expression of HbS, a poorly soluble and easily precipitating and polymerising form of Hb. Due to the long strands of Hb, the RBC become sickle-shaped and may block the microcirculation, causing hypoxia and acute pain episodes in different tissues. Molecules responsible for the increased adhesion of sickled cells to endothelium appear to be red cell VLA-4 (binding to endothelial VCAM-1), red cell Lu (binding to ECM laminin $\alpha_5$) and ICAM-4 (binding to endothelial $\alpha_v$ integrins) (Zennadi et al. 2012, Wautier & Wautier 2013).

Other pathophysiological conditions where erythrocytes display increased adhesion to the endothelium include e.g. diabetes mellitus and polycytemia vera. In malaria, the *Plasmodium*
Review of the literature

*falciparum*–infected red cells adhere also abnormally to the endothelium, which facilitates the dissemination of the parasite (Wautier & Wautier 2013).

### 1.2 Leukocyte adhesion

Leukocytes or white blood cells are the cornerstone of a functional immune system, participating in both adaptive and innate immune functions. Adhesion events are essential in virtually all events associated with immunological reactions. Together the leukocytes form a functional immune system that can protect the system against foreign pathogens. Leukocytes are divided in several subgroups. Lymphocytes may be further divided in antibody-producing B cells, T cells that assist in the activation and regulation of B cells (T helper cells) or participate in infected cell killing (cytotoxic T cells), and natural killer (NK) cells. Monocytes are phagocytes, able to bind and engulf infected or apoptotic cells. They also give rise to macrophages and dendritic cells, when escaping the blood stream. Granulocytes form a group of three cell types; eosinophils that take care of parasites and participate in allergic reactions, basophils that release histamine for the good and the bad, and neutrophils that are the first cells to arrive to sites of inflammation in large numbers, starting to engulf bacteria and fungi.

#### 1.2.1 The immunological synapse

The immunological or immune synapse (figure 2) is a structural entity formed between a T cell and a professional antigen presenting cell (APC). It is needed for the development of T cell responses in the lymph nodes, where T cells are activated for proliferation and differentiation in order to generate effector and memory T cells. The intracellular events leading to this outcome are initiated when a T cell receptor (TCR) recognises and binds to a bacterial or other foreign peptide presented on the surface of an APC. The signalling pathways involved show a branched, rather than a top-down network of molecules interacting with and activating each other, and lead to the numerous functions of T cells.

The immunological synapse was described at the end of the last millennium (Dustin et al 1998). Another name used for the immunological synapse is supramolecular adhesion complex (SMAC, Monks 1998). It consists of two distinctive, ring-like molecular assemblies, called cSMAC (central SMAC) and pSMAC (peripheral SMAC). TCR, co-receptors CD4 and CD8 as well as additional molecules involved in T cell activation are clustered in the cSMAC, whereas especially LFA-1 integrin can be found in the pSMAC (Monks et al 1998). LFA-1 activation and subsequent binding to its ligand (ICAM-1) is required for the formation of a stable immune synapse. The sustained interaction between the two cells, in turn, allows the prolonged signalling needed for the appropriate T cell activation and proliferation. A third area, the distal SMAC (dSMAC) with concentrated negative regulators such as CD45, has been described later (Huppa & Davis 2003, Springer & Dustin 2012).

The binding of TCR to the peptide associated with the major histocompatibility complex (MHC) leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic moieties of CD3γ, CD3δ, CD3ε and CD3ζ polypeptide chains. The kinase responsible for CD3 ITAM phosphorylation is Lck that is brought to the vicinity of TCR by CD4, a co-receptor binding to the MHC molecule. ITAM phosphorylation leads to binding of the zeta-chain associated protein kinase of 70 kDa (ZAP70) to the phospho-ITAMs and its subsequent phosphorylation by Lck. Activated ZAP-70 in turn phosphorylates the linker for activation of T cells (LAT). LAT is a scaffold protein bound to the plasma membrane which,
when activated, recruits many other signalling proteins and offers a base for the construction of a LAT signalosome. From this multimolecular complex the signalling continues further, branching in directions that result in the variety of outcomes of the T cell activation, such as integrin activation and cell adhesion, gene expression and actin reorganisation (Huppa & Davis 2003, Dustin 2009, Brownlie & Zamoyska 2013).

Recent studies have shown further interesting aspects of the immune synapse and its structure. The observation that the immune synapse, and especially the centrosome structure and intracellular vesicular transport, of a cytotoxic T cell (CTL) encountering tumor or virally infected cells are surprisingly similar to the structure of primary cilia, has led to new insights into the regulation of vesicular transport (Finetti & Baldari 2013). Like in primary cilia, the hedgehog signalling has been shown to have a vital role in the regulation of the vesicular transport and the killing of target cells by CTL. Interestingly, in contrast to the earlier identified hedgehog signalling pathways, the Indian hedgehog homolog (Ihh) molecule needed for signalling is produced by the T cell itself, and the signalling events are intracellular, without a need for extrinsic signalling molecules (de la Roche et al 2013).

Another interesting feature of the immune synapse is the release of extracellular vesicles or exosomes by the T cell. They are instantly engulfed by the APC but their function remains to be elucidated, although general cell activation seems to occur upon vesicle uptake. In any case, the contents of the vesicles is strictly regulated and controlled and thus, most likely play a role in the cell-cell signalling events required for the immunological processes (Choudhuri et al 2014, Gutierrez-Vazquez et al 2013).

**Figure 2.** Schematic description of the immunological synapse and the molecular distribution within T cell and APC (A) and in the membrane plane of T cell (B). DSMAC, distal SMAC, pSMAC, peripheral SMAC, cSMAC, central SMAC. Adapted from Huppa & Davis 2003.
1.2.2 Extravasation

To perform their immunological functions, the leukocytes need to leave the bloodstream, some of them several times during their life cycle. The exit, called extravasation or transendothelial migration, may take place in the lymph nodes, where lymphocytes meet peptide-MHC complexes on the surface of APCs. Another extravasation site is the site of infection or injury, where the leukocytes move to the inflamed tissue in a well-defined sequence (Hyduk & Cybulsky 2009). The traditional view of the extravasation includes the following steps: rolling, activation, arrest and migration (Dutrochet 1824). Later more refinement to the model has been acquired, but the basic steps of the extravasation cascade remain the same (figure 3).

**Figure 3. Leukocyte extravasation. Essential steps and molecules involved.**

The first step in the transmigration cascade is the activation of endothelial cells by inflammatory cytokines, which leads to rapid expression of chemokines, adhesion molecules and lipid chemoattractants on the luminal surface of the endothelial cells (Bevilacqua 1993).

The next step is rolling of the leukocytes along the surface of the endothelium, mediated by selectins (adhesion molecules that adhere to carbohydrate moieties) and their ligands. L-selectin and P-selectin glycoprotein ligand (PSGL-1) are mostly expressed on the leukocytes, whereas P- and E-selectins are expressed on the inflamed endothelium. Binding of selectins to their ligands induces signalling both in the endothelial cells and in leukocytes, leading to e.g. activation of integrins (Simon et al 2000, Ley et al 2007).

Following these steps, the leukocytes start the integrin-mediated rolling. The integrin thought to be mainly responsible for the monocyte and T cell rolling is VLA-4 and it binds to its ligand VCAM-1, that is strongly expressed on the surface of the inflammation-activated endothelial cells (Imai et al 2010). In neutrophils the rolling is mediated by the selectins and the interactions of lymphocyte function associated antigen-1 (LFA-1) with ICAM-1 and -2 on the endothelium (Hakkert et al 1991, Springer 1990, Gahmberg 1997).
The next step is the high-affinity binding and the rapid arrest of the leukocytes at the site of inflammation. In addition to previously mentioned selectins, also chemokines excreted by the endothelium are able to activate adhesion. Chemokines bind to the G-protein coupled receptors on leukocytes, which results in rapid intracellular signalling events, finally leading to activation of leukocyte $\beta_2$ family integrins and subsequent binding to their ICAM counterreceptors expressed on endothelial cells. After binding to their ligands, the integrins start a signalling cascade in the leukocyte, which causes further reinforcement of the leukocyte-endothelium adhesion through changes in the actin cytoskeleton (Gahmberg 1997, Ley et al 2007, Hogg et al 2011). The effector lymphocytes, contrary to naïve or memory lymphocytes, do not need chemokine induction in order to arrest on the endothelium. Instead, they express high numbers of leukocyte integrins that are easily outside-in activatable (Shulman et al 2011, Lek et al 2013).

Finally the cells start to migrate through the endothelial cell layer, the basement membrane and the pericytes surrounding the vessels in most areas of the body. Crawling cells form protrusions penetrating the endothelial cell layer and show a polarised morphology. In the leading edge, the cell has a structure called lamellipodium that is a site of intensive dynamic actin cytoskeleton reorganisation. Filopodia are structures that extend even further onwards to the direction of the migration from the lamellipodia. In the lamellipodia, constant formation of focal complexes takes place. They are adhesive units consisting of integrins, talin and other associated molecules. In case the focal complex adheres to the endothelium or ECM, it collects around it a structure called focal adhesion (FA), a reasonably stable adhesion site between the migrating cell and the endothelium or ECM. The cell utilises FAs as anchoring sites while dragging the cell body along the surface. In the rear, the cell has a structure called uropod, where FAs are dissolved and the adhesion molecules are recycled to the leading edge or to FAs (Ley et al 2007). Migrating cells use a somewhat different set of adhesion molecules than the ones used in rolling and adhesion, including integrins and proteases. Two routes for the transendothelial migration have been suggested: paracellular (between the cells), where the endothelial cell activation and their contacts with leukocytes induce opening of the interendothelial cell junctions and promote leukocyte migration towards the cell-cell junctions. The other route is transcellular (through the cells) migration that takes place at sites where the thickness of the endothelium has diminished. Chemokines are secreted from intracellular endothelial stores to guide the leukocytes on their way (Shulman et al 2011). Migration is facilitated by a system called vesiculo-vacuolar organelles, which form a channel structure through the endothelial cell, leading the leukocyte to the extracellular space. In order to get through the basement membrane below the endothelial cells, yet another set of integrins and proteases degrading the extracellular matrix is needed. Once in the tissue, the cells follow a gradient of chemoattractants to orient themselves in the tissue (Ley et al 2007).

1.2.3 Phagocytosis

Phagocytosis is a means of the innate immunity system to get rid of invading pathogens and to process them further, if needed, for the antigen presentation to lymphocytes. It is also important in the removal of apoptotic cells from the system. Cells responsible for phagocytosis (phagocytes) are macrophages, granulocytes and dendritic cells. The phagocytic process is initiated when phagocyte cell surface receptors encounter and bind to a microbial or other foreign surface. This leads to the formation of a phagosome, an endocytic vesicle. Most common receptors involved in the phagocytosis are Fc receptors (FcR), toll-like receptors (TLR), complement receptors 1-4 (CRs, of which CR3 and CR4 are leukocyte integrins) and scavenger receptors. Interestingly, the...
type of phagocytosis depends on the receptor responsible for the recognition of the pathogen/foreign structure, due to different signalling pathways associated with different receptor families (figure 4). The Fc-receptors induce a signalling pathway leading to "reaching phagocytosis", whereas complement receptors induce "sinking phagocytosis" (Caron & Hall 1998, van Lookeren Campagne et al 2007, Dupuy & Caron 2008, Underhill & Goodridge 2012).

![Diagram of phagocytosis types](image)

**Figure 4.** Presentation of different phagocytosis types and macropinocytosis. From Underhill & Goodridge 2012. Figure is reprinted with kind permission of the copyright holders.

### 1.2.4 Pathological conditions involving leukocyte adhesion

In many pathological conditions the adhesive ability of the leukocytes, or some subset of them, is altered. Often the leukocyte adhesion is too active, enabling the leukocytes to attack self tissues as they were foreign pathogens. The activated immune cells accumulate in the target tissues in an uncontrolled way, leading to pathological autoimmunity. For example in multiple sclerosis (MS), the activated immune cells are able to penetrate through the blood-brain barrier (BBB) into the brain and the central nervous system. Once there, they induce an inflammatory state and attack the myelin sheath protecting the neurons. This causes lesions in the central nervous system (CNS), leading to symptoms like fatigue, imbalance, loss of mobility, sensory symptoms, visual problems and pain. Other examples of diseases where leukocyte adhesion is impaired are asthma, reperfusion syndromes, arthritis, neuroinflammatory diseases, autoimmune diabetes, organ transplant rejection, psoriasis, Crohn’s disease and ulcerative colitis (Hilden et al 2006, Millard et al 2011).
1.3 Haemostasis and thrombosis

Haemostasis starts when the endothelium is injured and the platelets are activated and start to aggregate and bind to the subendothelium, forming a plug to stop the bleeding. Fibrinogen is spliced into fibrin which forms fibrin fibres and a fibrin clot, where platelets and other cells are trapped, further strengthening the blood clot. While haemostasis is the physiological response to an injury, thrombus is the result of pathological clot formation due to excessive activation of haemostasis (Rasche 2001, Versteeg et al 2013).

2 The Ig-superfamily

Immunoglobulin superfamily (IgSF) of adhesion molecules consists of numerous adhesion molecules containing one or more immunoglobulin (Ig) domain on their extracellular part. Some family members contain also other domains needed for adhesion or other functions.

2.1 ICAM family members are important in leukocyte adhesion

For leukocyte functions, especially the ICAM family as well as VCAM-1 are essential. ICAM molecules all share the common ligands, the β2 family of leukocyte integrins and VCAM-1 binds to α4 integrins VLA-4 (α4β7) and α4β1. The structure of the ICAMs as well as VCAM-1 consists of a various number (2-9) of immunoglobulin (Ig) domains, a transmembrane domain and a short cytoplasmic part. The Ig-domains are held in tight conformation with intra-domain cysteine bridges. In figure 5, the schematic structures of the ICAM-1 to -5 and VCAM-1 along with the possible glycosylation sites are depicted. The ligand binding site is often in the first, outermost domain, but also other domains participate in the binding of some ligands (e.g. D3 of ICAM-3 binds to Mac-1). All the ICAMs as well as VCAM-1 are heavily N-glycosylated on their extracellular domains. ICAMs are also genetically linked so that the genes for all the other ICAMs than ICAM-2 reside on the human chromosome 19p13.2-13.3, the ICAM-2 gene is on chromosome 17q23-25. The VCAM-1 gene is on chromosome 1p21.2 (Cybulsky et al 1991, Gahmberg 1997, Gahmberg et al 2008).
ICAM-1 (CD54) was the first described member of the ICAM-family (Rothlein et al 1986, Patarroyo et al 1987, Marlin & Springer 1987). It is expressed at low levels on resting leukocytes, endothelia and other tissues, but inflammatory stimuli, such as TNF, IFN-γ or bacterial lipopolysaccharide (LPS) induce increased expression (Dustin et al 1986, Nortamo et al 1991). Its expression is relatively low on mature T and B cells, but clearly higher on lymphoblasts (Rothlein et al 1988, Prieto et al 1989). ICAM-1 is involved in various adhesion events during the lifecycle of the leukocytes, such as T cell cytotoxicity and leukocyte extravasation (Shaw et al 1986, Dustin & Springer 1988). It can serve as a receptor for several pathogens that use it to infect host cells (rhinovirus, *Plasmodium falciparum*) (Greve et al 1989, Berendt et al 1989). ICAM-1 extracellular domains adopt an L-like structure that is bent between domains three and four (Kirchhausen et al 1993). It has also been shown to form dimers (Miller et al 1995, Reilly et al 1995). In addition to the integrins, ICAM-1 binds fibrinogen (Fg) which may be used as a cross-linker to bind leukocytes through their Fg-binding integrins (Languino et al 1993). ICAM-1 is also essential in the formation and maintenance of immune synapses, where it binds the LFA-1 integrin expressed on T cells (Monks et al 1998).

ICAM-2 (CD102) was cloned in 1989 (Staunton et al 1989), the protein was characterised in the beginning of the 1990s (de Fougerolles et al 1991, Gahmberg et al 1991) and it is the only ICAM expressed on platelets (Diacovo et al 1994). Its expression is constitutively low on lymphocytes as well as monocytes, whereas on endothelial cells it shows higher expression levels. Its expression level is, however, not inducible by cytokines or other inflammatory stimuli (Nortamo et al 1991, de Fougerolles et al 1991). With two Ig-domains, its molecular weight is 55 kDa. An interesting observation was the discovery of an ICAM-2 derived peptide (called P1) that...
was able not only to inhibit LFA-1 binding to the endothelium, but also to stimulate leukocyte integrins on a number of different cell types (Li et al. 1995, Xie et al. 1995, Kotouvuri et al. 1999).

The crystal structure of the ICAM-2 extracellular domain has been solved and it shows similarities between the ICAM family but differences when compared to VCAM-1 and MadCAM-1 that bind to I-less integrins (Casasnovas et al. 1997, Casasnovas et al. 1999).

ICAM-3 (CD50) has high homology with ICAM-1 on the extracellular domain level but differs in the cytoplasmic domain sequence (de Fougerolles & Springer 1992, de Fougerolles et al. 1993). It is also expressed on T cells and has been reported to be an important ligand of dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) expressed on dendritic cells (Geijtenbeek et al. 2000, Bogoevska et al. 2007). ICAM-3 also binds to αβ integrin (Van der Vieren et al. 1999). Its expression is high on resting leukocytes, but it is not expressed on endothelial cells (Van der Vieren et al. 1995). Its most important roles are suggested to be in the leukocyte-leukocyte interactions like B cell activation by T cells, or T cell activation by APCs (Gahmberg 1997, Hayflick et al. 1998).

ICAM-4 or LW blood antigen is expressed only on erythrocytes and their precursors. Its primary function has remained unclear, but it has been implicated in several adhesive functions of RBC, such as erythropoiesis, erythrophagocytosis and in sickle cell disease and deep vein thrombosis (see chapter 2.2).

ICAM-5 is the latest member of the ICAM family to be discovered (Mori et al. 1987, Tian et al. 1997). It is only expressed in the telencephalon-derived regions of the brain and specifically on the neuronal somas and dendrites (Yoshihara et al. 1994, Benson et al. 1998). It is able to bind to various different receptors, of which LFA-1 was the first to be discovered (Tian et al. 1997, Tian et al. 2000a). Other ligands are presenilin (Annaert et al. 2001), ERM proteins (ezrin, radixin, moesin) (Furutani et al. 2007), vitronectin (Furutani et al. 2012), α-actinin (Nyman-Huttunen et al. 2006) and β integrins (Ning et al. 2013). In addition, it shows homophilic binding (Tian et al. 2000b). The three dimensional structures of first two domains (Zhang et al. 2008) and domains 1-5 (Gahmberg et al. 2014) have been solved. It is important in both neuronal development and immunological functions, and it is the first known negative regulator of spine (dendritic protrusions that may develop into synapses) development (Tian et al. 2009). It also takes part in the maintenance of the immune privilege in the CNS (Tian et al. 2008). Recently, it has been observed to regulate synapse formation through interactions with β integrins (Ning et al. 2013).

### 2.2 ICAM-4

#### 2.2.1 The LW blood group antigen is ICAM-4 (CD242)

LW and Rh blood group antigens were discovered simultaneously and they were mixed up with each other for some time. The blood group antigen was named first as Rh (Landsteiner & Wiener 1940), but was later renamed LW according to its finders (Levine et al. 1963). The RhD antigen was identified as a separate protein of 300 kDa (Gahmberg 1982). The LW blood group consists of LW and LW antigens that can be recognised by anti-LW and anti-LW antibodies (Sistonen et al. 1983). Rh and LW are closely related on a phenotypic level: the amount of LW expressed is dependent on the expression of RhD antigen. RhD cells express more LW, whereas RhD cells have less LW on their surface. Rhnull cells don't express any LW (Sistonen et al. 1983, Giles 1980). LW and Rh –complex has been characterized further and the two proteins were discovered to form a non-covalent complex that is transported to the cell surface together (Mallinson et al.
1986, Bloy et al 1989, Hermand et al 1996). Later, a macrocomplex consisting of ICAM-4/LW, Band 3, Rh and many other red cell membrane proteins, taking care of gas exchange, has been reported (Bruce et al 2003). Most Europeans are of LW(a+b-) phenotype, and only less than 1% are LW(a+b+), but Finns are different: about 5% of the population are LW(a+b+) and 0.1% are LW(a-b+), which is very rare elsewhere (Sistonen et al 1981, Sistonen & Tippett 1982, Sistonen et al 1983). The expression of the LW antigen may temporarily decrease during immunological anomalies and simultaneously anti-LW antibodies may be detected (Chown et al 1971, Perkins et al 1977, Parsons et al 1994, Komatsu & Kajiwara 1996). One case of haemolytic disease of the foetus and newborn (HDFN) has been reported to be caused by anti-LW autoantibodies from the mother (Davies et al 2009).

When the expression of the LW antigen was analyzed at the cDNA level, two different forms were discovered: one of 270 amino acid polypeptide and another of 236 amino acid residues that did not contain the transmembrane and cytoplasmic parts. These two were predicted to be the membrane bound and secreted forms (Mallinson et al 1986, Bailly et al 1994). The full-length LW antigen is a 42 kDa glycoprotein that is connected to the cytoskeleton and associated with Rh expression (Mallinson et al 1986, Bloy et al 1989). It needs intramolecular disulfide bonds to be antigenic (Konigshaus & Holland 1984). The soluble form was reported in mice by (Lee et al 2003) and in humans by (Choi et al 2013). Both forms are expressed only on erythrocytes and their precursors (Bailly et al 1994). ILW has 4 possible N-glycosylation sites and consists of 2 extracellular Ig domains, a transmembrane domain (21 amino acids) and a short cytoplasmic tail of 12 amino acid residues. The difference between LWa and LWb is a one-nucleotide change on the DNA-level (A308G), leading to a Gln70Arg change in the amino acid sequence (Hermand et al 1995). The structure and sequence of LW was demonstrated to be homologous to the intercellular adhesion molecule family, and it could also bind to leukocyte integrins as all the other members of the ICAM family, so LW was renamed again, this time as ICAM-4 (Bailly et al 1994, Bailly et al 1995, Hermand et al 1995, Hermand et al 1996).

2.2.2 ICAM-4 contains two Ig domains

A three dimensional model based on the crystal structure of ICAM-2 revealed the structure of ICAM-4. It consists of two Ig domains (D1 & D2), each of which consists of two β-sheets. The first sheet of D1 consists of strands ABED (in D2: ABE) and the second sheet of strands CFG (D2: CCFG) (Hermand et al 2000). The comparison of the amino acid sequence to other ICAMs revealed interesting differences inside the family. Importantly, Glu34 and Gln73 (numbering from ICAM-1), that are important in other ICAMs adhering to their β2 integrin family ligands, are lacking, and they are replaced by Arg52 and Thr91 in ICAM-4. Replacing these with glutamate and glutamine, respectively, does not improve binding to LFA-1 and even inhibits binding to Mac-1. On the other hand, N-glycosylation at position 48 of ICAM-4 is not present in other ICAMs (Hermand et al 2000). A homologue to human ICAM-4 has been found in mouse red cells (Lee et al 2003).
2.2.3 Ligands and functions of ICAM-4

ICAM-4 can bind to the LFA-1 and Mac-1 leukocyte integrins (Bailly et al 1995). The binding sites of these integrins on ICAM-4 have been characterized by site-directed mutagenesis and the important amino acid residues which participate in the integrin/ICAM-4 interactions have been pinpointed. The studies revealed that the two integrins bind to distinct but overlapping sites on ICAM-4. Interestingly, the outermost domain of ICAM-4 (D1) is enough for binding to LFA-1, whereas both D1 and D2 are needed for binding to Mac-1 (Hermand et al 2000).

ICAM-4 differs notably from other ICAM family members due to its promiscuous nature. Other members of the family bind almost exclusively to β₂ family integrins, whereas ICAM-4 interacts with many different integrins. ICAM-4 binding partners include α₁β₃ on activated platelets (Hermand et al 2003, Hermand et al 2004), α₅β₁ (Hermand et al 2004), α₅β₉, α₄β₇, and VLA-4/α₄β₁ integrins (Spring et al 2001, Lee et al 2003, Mankelow et al 2004), although in other reports the interaction between VLA-4 and ICAM-4 could not be detected (Hermand et al 2004). The integrins capable of serving as ICAM-4 ligands are expressed on a great variety of cell types (leukocytes, platelets, endothelial cells), suggesting a big diversity in the possible roles of ICAM-4.

Erythropoiesis

The interactions between the central macrophage and the maturing erythroblast are considered essential for the formation of the erythroblastic islands and for erythropoiesis. An ICAM-4 knock-out mouse has been made. ICAM-4/α₅ integrin interaction was found to play a role in erythroblastic island formation and erythroblast development (Lee et al 2006). ICAM-4 probably also interacts with the β₂ integrins on the central macrophages (Bailly et al 1995, Hermand et al 2000). The soluble form of ICAM-4 has been detected and it plausibly participates in the
detachment of mature red cells from the erythroidic island central macrophage (Lee et al 2003). ICAM-4 – VLA-4 interactions could be also seen, although not in all occasions, possibly connecting neighbouring erythroblasts (Spring et al 2001, Hermand et al 2004). The expression pattern of ICAM-4 proposes an important function during erythrocyte differentiation, as the ICAM-4 expression rapidly increases in the beginning of erythropoiesis and, after some time, gradually decreases to the level of the mature erythrocyte at the time of reticulocyte stage (Southcott 1999, Bony et al 1999). Similar studies have been conducted in mice and they show an even more pronounced decrease of ICAM-4 during the final maturation of the reticulocytes (Chen et al 2009, Liu et al 2010). An interesting report about in vitro erythropoiesis by Choi et al indicates that ICAM-4 would have a significant role in erythropoiesis in the absence of erythroblast – macrophage contact. ICAM-4 interaction with Deleted in liver cancer-1 (DLC-1, a Rho-GTPase-activating protein) seems to enhance cell survival and nucleus extrusion in these conditions, and the addition of soluble ICAM-4 can induce erythropoiesis in vitro (Choi et al 2013).

**Erythropagocytosis**

The binding of ICAM-4 to leukocyte integrins expressed in macrophages might clarify some of the controversy concerning the recognition and uptake of senescent red blood cells in spleen. Indeed, the phagocytosis of senescent red cells is ICAM-4/β2-dependent (Toivanen et al 2008).

**Haemostasis**

The role of red blood cells in haemostasis has, already in the beginning of the last century, been suggested to be more active than just getting trapped in the thrombus through the fibrin network. In fact, there are some indications of RBCs playing an active role in the formation and/or removal of a blood clot, binding actively to the platelets and leukocytes (Andrews & Low 1999). ICAM-4 is known to bind platelet integrin αIIbβ3 on activated platelets so it may take part in these adhesion events (Hermand 2003). ICAM-4/integrin interactions have also been implicated in deep vein thrombosis, where red cells bind to neutrophils at low shear rates (Goel & Diamond 2002).

**Sickle cell anaemia**

In sickle cell anaemia, ICAM-4 mediates the abnormal adhesion of red cells to endothelium. ICAM-4 on sickled but not on normal cells can be activated by epinephrine (adrenaline) through the adrenergic pathway to mediate adhesion to endothelial cell αvβ3. After epinephrine activation, the atypical activation of ERK1/2 results in activation of PKA and tyrosine kinase p72syk, which in turn leads to phosphorylation of ICAM-4 and its abnormal adhesion to endothelium. In normal red cells the serine phosphorylation of ICAM-4 is negligible (Zennadi et al 2004, Zennadi et al 2007, Zennadi et al 2012). Sickle cells were also found to induce adhesion of leukocytes (lymphocytes and monocytes) to the endothelium, probably by activating them, leading to increased adhesion (Zennadi et al 2008). The sickled red cells have been reported to bind to endothelium-adherent leukocytes in inflamed venules (Turhan et al 2002). Peptides based on the regions of ICAM-4 that bind to αv-integrins expressed on the endothelium, as well as αβ integrin agonists, inhibit sickle red cell-endothelial interactions and vaso-occlusion in the microcirculation (Kaul et al 2006, Finnegan et al 2007).
2.3 VCAM-1

VCAM-1 (CD106), a member of the IgSF, is a cell surface protein expressed by activated endothelial cells and certain leukocytes such as macrophages. VCAM-1 expression is induced by IL-1β, IL-4, TNF-α and IFN-γ (Bevilacqua 1993, Zhang et al 2011, Min et al 2005). VCAM-1 binds to leukocyte integrins VLA-4 and α4β7 (Kilger et al 1997, Newham et al 1997). VCAM-1 binding to VLA-4 supports leukocyte tethering and rolling on the endothelium (Alon et al 1995). VCAM-1 is also expressed in bone marrow and lymph nodes, where it participates in the control of the leukocyte homing (Cook-Mills et al 2011). Contrary to the ICAM family of adhesion molecules, VCAM-1 may be expressed in two different splice variants, one having 7 and one having 6 Ig domains (in the shorter version, D4 is lacking) (Cybulsky et al 1991). The structure of the domains one and two has been solved (Jones et al 1995, Wang et al 1995).

3 Leukocyte integrins

3.1 Introduction

Integrins form a reasonably large and diverse family of adhesion molecules that are heterodimeric type I transmembrane proteins. They have a large extracellular ligand binding domain and a shorter cytoplasmic domain (except β4 that has longer cytoplasmic part) that does not have enzymatic activity, but it offers binding site for numerous intracellular adaptor and scaffold proteins. An integrin molecule consists of an α and a β subunit and to date there are 18 α chains and 8 β chains expressed in humans. Most α chains can combine with several β chains and vice versa, which raises the number of heterodimers known to 24 (see figure 7 for overview of the presently known α/β combinations and the nomenclature). The general functions of the integrins are cell adhesion and migration and participation in the signalling cascades leading to cell differentiation, proliferation and angiogenesis or to programmed cell death (Tan 2012).

Leukocytes express a variety of integrins, depending on their maturation and activation status and the cell type. The principal integrins on leukocytes are the four members of the CD18 (β2) family and VLA-4 (α4β1, CD49d/CD29) (Gahmberg 1997, Chigaev & Sklar 2012, Patarroyo et al 1990). Other integrins expressed exclusively on leukocytes are α4β7 (or LPAM) (Ruegg et al 1992) and αEβ7 (Andrew et al 1996). Leukocytes express also a number of β1 integrins (αβ1-αβ1), the expression of which is not specific to leukocytes.

The functions of integrins are not only to adhere in response to different stimuli, but also to function as a signalling molecules themselves. Integrins have the unique ability to transduce signals in two directions (i.e. inside-out and outside-in). Inside-out activation of integrins happens when a non-integrin receptor (e.g. TCR, chemokine receptor, LPS receptor) gets activated upon ligand binding and starts a signalling cascade involving various cytoplasmic signalling and adapter proteins. Some of these proteins also reach the integrin cytoplasmic parts and bind there, enabling a conformational change leading to ligand binding in the extracellular part of the molecule or integrin clustering on the plasma membrane. Outside-in activation may take place when external ligand binds to integrin (see also chapter 4, Regulation of integrin activity).
3.2 Integrin structure and conformational changes

The integrin extracellular part consists of two polypeptides (α and β) forming one functional unit (see figure 8A for general structure). The α chains may be divided in two categories: nine α chains have an inserted or I domain (also called A domain) in their extracellular region, serving as the ligand-binding site, and the other nine alphas do not have this domain (they bind ligands with their β-chain I-like domain). All integrin alpha chain ectodomains consist of a seven-blade β-propeller, a thigh domain (resembling an Ig-domain) and two calf domains that are each composed of two antiparallel β-sheets. The I domain is inserted between the blades two and three of the β-propeller. The structure of the β chain extracellular moiety is composed of a plexin-semaphorin-integrin (PSI) domain, an I-like domain, a hybrid domain (inside of which the I-like domain is inserted), four cysteine-rich EGF domains and the membrane-proximal β-tail domain (Campbell & Humphries 2011).

Especially in haematopoietic cells (platelets, leukocytes, even erythrocytes), the integrins in the resting cells are in an inactive state, but they need to be rapidly and accurately activated to bind their ligands as well as “turned off” when needed. There are at least two ways to control the ligand binding capacity of a certain cell: regulation of integrin affinity and integrin avidity. Affinity means the capability of a single molecule to bind its ligand and is controlled by the conformation of the integrin. Avidity, on the other hand is the measure of a number of clustered integrins on a given cell to bind to their ligand (Dustin et al 2004).
The conformation of the integrins and their affinity towards the ligands are tightly interconnected. Integrins are considered to adopt three different conformations of the extracellular part: bent conformation, extended with a closed headpiece and extended with an open headpiece (see figure 8). It has been proposed that the intracellular signalling (from e.g. TCR) leads to the separation of the α and β cytoplasmic domains from each other, which transduces the signal to the extracellular part of the integrin, extending the conformation and offering a site for the ligand to bind (Hogg et al 2011, Springer & Dustin 2012). There has been controversy about the existence of the intermediate conformation and its ligand binding abilities and even suggestions of the bent conformation being able to bind ligand (Hogg et al 2011, Springer & Dustin 2012, Feigelson et al 2010). Nevertheless, the extended-open conformation has been unanimously shown to bind ligand with high affinity. The understanding of the relations between integrin structure and function constantly increases as new structures are being resolved.

The crystal structures of many I domains have been solved, like α₅ (Lee et al 1995) α₅ (Qu & Leahy 1995), α₇ (Emshley et al 1997), α₄ (Salminen et al 1999), α₉ (Vorup-Jensen et al 2003) and they show changes in conformations in the presence of different divalent cations (like Mg²⁺ or Mn²⁺), ligands or peptides derived from the ligands. This, indeed, has revealed the importance of the MIDAS (metal-ion dependent adhesion site) sequence DxSxS in the ligand binding of the I-domain containing integrins. The crystal structures of the whole extracellular parts of α₅β₃ (Xiong et al 2001), α₆β₄ (Zhu 2008) CR4/α₃β₇ (Xie 2010) and α₅β₇ (Nagae et al 2012) have also been solved, and negative-stain electron microscopy images of e.g. CR4 (Chen et al 2010) have been obtained. These reveal the importance of the conformational regulation of the integrins. One important feature of ligand binding is the opening of the headpiece that is needed for the high-affinity state of the integrin. Opening of the I-less integrin (α₅β₇) has been resolved in the crystal structure level and it involves eight separate steps (Zhu et al 2013). Headpiece opening

**Figure 8. Integrin extracellular part structure. A. Domain organisation in an α chain I domain containing integrin. B. Three conformations of I-domain containing integrins. Domains are colour-coded as in A. Adapted from Hogg et al 2011.**
of the α chain I domain containing integrins requires interplay between the α I domain and the β chain I-like domain. A conformational change occurs when the α 7 helix of the α I domain is pulled downwards to contact the β I domain. An interaction between a glutamate and the β chain β propeller - I domain interface exposes the ligand-binding site in the I domain of the α chain. Importantly, the β chain hybrid domain swings out to create an overall conformation with open legs (Xie et al 2010).

Integrin intracellular domain structure is of utmost importance in the regulation of integrin functions. This part relays information both from the inside of the cell to the outside, and from the extracellular domain of the integrins towards the inside of the cell. Some common traits in the α and β chains have been reported (figure 9), probably the most important being the GFFKR sequence in the membrane-proximal part of the α chains and the β chain membrane proximal NPxY/F and membrane distal NxxY/F, as well as the serine/threonine-rich sequence between these two (Ylänne 1995). Multiple proteins can bind to these areas (Calderwood et al 2003, Takala et al 2008). The NMR structures of a number of cytoplasmic tails have been obtained: αLβ2 (Bhunia et al), αMβ2 (Chua et al 2011), αXβ2 (Chua et al 2012), αm (Vinogradova et al 2000) and α4 (Chua et al 2013). A comparison of the α chain conformations shows large differences at the C-terminus of the cytoplasmic domains, although the membrane-proximal structures form similar conserved helix structures. The α and β chains make numerous contacts with each other through ionic and hydrogen bonding in the helical area close to the membrane, but the membrane-distal parts vary and the tails move more freely in solution. The α tail, being the longest, makes an exception as it is packed in three helices held together by salt bridges and/or hydrogen bonds. The helices form a large negatively charged surface that is able to bind metal ions (Bhunia et al 2009). These findings suggest that the α chain cytoplasmic parts offer a great deal of variety to the integrin regulation mechanisms and probably determine the specificity of the signalling. The reported phosphorylation sites of αL, αM and αX cytoplasmic tails as well as β2 tail are situated outside the membrane-proximal helices and are thus available for kinases or phosphatases for phosphorylation as well as for other cytoplasmic molecules for interactions (see chapter 4.4 on phosphorylation and 4.2 and 4.3 about cytoplasmic binding partners).

Figure 9. Cytoplasmic sequences of integrins expressed in leukocytes (αL, αM, αX, αD, α4, β1, β2). Reported phosphorylation sites are shown in red and amino acids are numbered. The NPXY/F sequence of β chains (see chapter 4) is shown in blue.
3.3 LFA-1 ($\alpha\beta_2$, CD11a/CD18)

LFA-1 is the most widely expressed integrin on leukocytes and probably the most studied member of the $\beta_2$ integrin family. It was discovered already in the 1980's and later found to belong to the $\beta_2$/CD18 leukocyte differentiation antigen family that consisted of LFA-1, CR3 and p150,95 (Davignon et al 1981, Beatty et al 1983, Sanchez-Madrid et al 1983, Micklem & Sim 1985, Springer & Anderson 1986). It was found to be an adhesion protein in 1985 (Patarroyo et al 1985a). It binds to all the five members of the ICAM-family (Patarroyo et al 1987, Marlin & Springer 1987, Staunton et al 1989, de Fougerolles & Springer 1992, Bailly et al 1995, Tian et al 1997), to functional adhesion molecule-1 (JAM-1) (Ostermann et al 2002), to E-selectin (Kotovuori et al 1993) and to collagen (Lahti et al 2013). Its most relevant functions are to take part in cell-cell interactions between leukocytes and endothelial cells in the extravasation of leukocytes to the tissues at the site of inflammation, between leukocytes and antigen presenting cells in the lymph nodes and between leukocytes and infected target cells to be destroyed (Kavanaugh et al 1991, Scheeren et al 1991, Davignon et al 1981). The role of LFA-1 in the immunological synapse is to offer stabilisation to the cell-cell interaction, but it is also fundamental in the formation of the synapse/SMAC structure (Monks et al 1998, Grakoui et al 1999). LFA-1 in T cells, and especially T cell lines like Jurkat, has been exploited to study various integrin-related signalling, activation and adhesion related issues.

3.4 Mac-1 ($\alpha$M$\beta_2$, CD11b/CD18, CR3)

Mac-1 is expressed on granulocytes, NK-cells and macrophages, and on $\gamma\delta$ T cells (Springer et al 1979, Sanchez-Madrid et al 1983, Graff & Jutila 2007). It was first discovered as an antigen on the surface of macrophages (hence the name macrophage-1 antigen) and later researchers realised it was the same molecule as complement receptor 3, found some time ago (Sanchez-Madrid et al 1983).

Together with two other $\beta_2$ integrins, CR4 ($\alpha_x\beta_2$, CD11c/CD18) and $\alpha$D$\beta_2$ (CD11d/CD18), it forms a quite homologous group, whose amino acid sequence, ligands and functions differ from LFA-1. Nonetheless, Mac-1 shares the common traits of $\beta_2$ family, such as the I domain as the binding site for iC3b and the fact that the binding is temperature and divalent cation-dependent (Ueda et al 1994).

Mac-1 ligand repertoire includes cell adhesion molecules as well as ECM molecules. In addition to complement receptor functions and binding to iC3b (Beller et al 1982), Mac-1 was reported soon after its discovery to mediate granulocyte cell-cell and cell-substrate adhesion, that was unrelated to iC3b binding function (Patarroyo et al 1985b). Other Mac-1 ligands include fibrinogen (Wright et al 1988), ICAM-1 (Diamond et al 1990), ICAM-2 (Xie et al 1995), low density lipoprotein (LDL) receptor (Spijkers et al 2005), MMP9 (Stefanidakis et al 2003) and JAM-3 (Santoso et al 2002). This vast repertoire of ligands probably reflects its various functions. The role and regulation mechanisms of Mac-1 in phagocytosis have been clarified extensively (reviewed in Dupuy & Caron 2008). Insights on Mac-1-mediated phagocytosis may shed a light also on other signalling and regulation mechanisms concerning $\beta_2$ integrins or integrins in general (Lim & Hotchin 2012). Some Mac-1 ligands, like uPAR and its ligand uPA and MMP9 refer to a role in ECM degradation and remodelling (Pluskota et al 2004, Stefanidakis et al 2009).

An interesting feature of Mac-1 that has not been reported for the other family members is its function in negative regulation of the immune system. Many of the findings of these functions have been revealed with the use of Mac-1 knockout mice or cell lines. When studying
the function of β2 integrins in APCs, it was found (Varga et al 2007) that, in fact, CD18-deficient APCs are fully capable of antigen presentation and T cell activation and it seems that the integrins in wt DCs are in an inactive, non-functional state. Interestingly, when DC integrins were activated, T cell activation was inhibited. In macrophages, the suppression of Mac-1 activity led to enhanced antigen presentation, suggesting a repressive role for the Mac-1 in antigen presentation. In another study, Mac-1 on APC was found to suppress Th17 differentiation, which led to peripheral immune tolerance. In the same study, αM−/− cells increased the IL-6 production, leading to differentiation of naïve T cells towards Th17 cells and further inhibition of immune tolerance formation (Ehirchiou et al 2007). The interaction of Mac-1 on DC with ICAM-1 on lymphatic endothelial cells results in diminished coreceptor CD86 expression on DCs and further to decreased T cell proliferation (Podgrabinska et al 2009). Mac-1 is also able to regulate signals coming through TLR. TLR ligand binding leads to activation of Mac-1, which in turn leads to feedback regulation of TLR, leading to repression of TLR signalling and reduced proinflammatory cytokine production (Han et al 2010, Yee & Hamerman 2013).

In systemic lupus erythematosus (SLE), the mutation Arg77His of the αM chain in its β propeller domain (that resides next to the ligand-binding I domain) has been implicated. This mutation leads to a decrease in ICAM-1, ICAM-2 and iC3b binding and phagocytosis. On the other hand, IL-6 release is increased. These findings may indicate that the negative regulation function of Mac-1 is inhibited (MacPherson et al 2011). Mac-1 is involved in MS, binding to myelin basic protein (MBP). It probably leads to phagocytosis and presentation of MBP peptides to T cells, thus eliciting an autoimmune reaction (Bullard et al 2005, Stapulionis et al 2008).

3.5 αDβ2

αDβ2 is the most recently found leukocyte β2 integrin. It is expressed on high level on foamy macrophages in aortic strikes, on certain tissue macrophages and on certain subgroups of circulating CD8+ T cells (Van der Vieren et al 1995, Danilenko et al 1995). Its expression is increased upon monocyte differentiation into macrophages (Noti 2002) and cell activation (Van der Vieren et al 1995). αDβ2 binds ICAM-3 (Van der Vieren et al 1995) and, when expressed on eosinophils, VCAM-1 (Grayson et al 1998). VCAM-1 binds to the I domain (Van der Vieren et al 1995) and, when expressed on eosinophils, VCAM-1 (Grayson et al 1998). VCAM-1 binds to the I domain (Van der Vieren et al 1999). As the high homology with Mac-1 suggests, αDβ2 has been reported to bind to many of Mac-1 ligands, such as vitronectin, fibronectin, plasminogen (Yakubenko et al 2006). It is also involved in the migration of monocytes and macrophages, and the amount of αDβ2 on the cell surface seems to control the retention of the macrophages at the inflammatory sites. The more αDβ2 the cells have on their surface, the tighter is their adhesion (Yakubenko et al 2008).

3.6 VLA-4 (a4β1, CD49d/CD29)

VLA-4 or very late antigen-4 is a β1 family integrin, consisting of α4 (CD49d) and β1 (CD29) polypeptide chains (Hemler et al 1990). Its ligands are VCAM-1 (Chuluyan & Issekutz 1993) and fibronectin. It is expressed on many haematopoietic cell types such as T cells, B cells, monocytes, NK cells, eosinophils and neutrophils as well as on haematopoietic stem and progenitor cells. It plays an important role in the regulation of the hematopoietic events, especially in the release of the maturing cells into the circulation. It is dispensable for the haematopoiesis itself, as cell differentiation or development of the cells does not seem to require VLA-4, but essential for homeostasis, taking care of keeping the immature cells in the bone marrow until they are ready to be released. Even though other α subunits dimerize with β1 (e.g. α5, laminin receptor),
they do not have an effect on homing of adult cells. The functions of VLA-4 are regulated by conformational changes. \( \alpha_4 \) lacks an I domain and is more easily activated to bind ligand than LFA-1 (Imai et al 2010, Chigaev & Sklar 2012).

### 3.7 The \( \beta_7 \) integrins \( \alpha E\beta 7 \) and \( \alpha 4\beta 7 \)

The \( \beta_7 \) integrins \( \alpha E\beta 7 \) and \( \alpha 4\beta 7 \) are also leukocyte-specific. \( \alpha E\beta 7 \) was first described as a fibronectin receptor and soon it was observed to bind also VCAM-1 and MAdCAM. It mediates homing to the mucosal sites, but also leukocyte homotypic binding. \( \alpha_4\beta 7 \) is expressed on the mucosal CD8+ T cells and on some other T cell subsets (such as certain regulatory T cells) and its functions are implicated in the mucosal homing of T cells, due to its ability to bind E-selectin expressed on mucosal endothelial cells. Later \( \alpha_4\beta 7 \) has been implicated in allograft reactions (Ruegg et al 1992, Berlin et al 1993, Hamann et al 1994, Kilshaw & Higgins 2002).

### 3.8 CR4 (\( \alpha X\beta 2 \), CD11c/CD18, p150.95)

p150.95 (nowadays also known as \( \alpha X\beta 2 \)), CD11c/CD18 or complement receptor 4, CR4) was reported some thirty years ago to belong to the “LFA-1, Mac-1, p150.95 family” (Sanchez-Madrid et al 1983, Micklem & Sim 1985, Springer et al 1986, Lanier et al 1985) and later it was found to be the same molecule as the previously described complement receptor 4 (CR4) (Myones et al 1988). Its amino acid sequence was solved some years later and it displayed high similarity with the Mac-1 integrin (Corbi et al 1987).

CR4 is expressed on monocytes, macrophages, dendritic cells, granulocytes and on some subsets of T and B cells (Hogg et al 1986, Keizer et al 1987a, Freudenthal & Steinman 1990, Postigo et al 1991, Miller et al 1986) and it is considered to be a marker of hairy cell leukemia (HCL) cells (Schwarting et al 1985, Miller et al 1987b). It is mobilised from intracellular vesicular stores upon monocyte and granulocyte activation (Springer & Anderson 1986, Miller et al 1987a). The expression was noticed to be partly similar to that of Mac-1, but some differences were discerned. Later the reports on the expression of CR4 on different cell types have shown the distinctive expression pattern of CR4. For example, CR4 is more strongly expressed on tissue macrophages, compared to blood monocytes or neutrophils, whereas Mac-1 is not that heavily expressed on macrophages (Myones et al 1988). The expression level of CR4 has been reported to differentiate mature DC from the immature (O’Doherty et al 1994). CR4 is also expressed on cytotoxic pulmonary T cells after a RSV (respiratory syncytial virus) infection that induce virus-specific (antiviral) cytotoxicity, e.g. production of IFNγ (Beyer et al 2005). In the brain, CR4 is expressed on microglia that are considered to resemble macrophages and have similar functions.

#### 3.8.1 Structure

The extracellular part of CR4 was the first reported structure of an integrin with an \( \alpha \) chain I domain (Xie et al 2010), and it revealed more detailed information about the relations between the structure and the activity of the integrin. It seems that the \( \alpha \) chain I domain is able to adopt three different conformations, and that these conformations can flexibly be combined to the two \( \beta \) chain I domain conformations (Xie et al 2010) (figure 10). The \( \alpha \) chain I domain structure was published earlier and it shows the importance of isoleucine in position 314. This residue is normally in a hydrophobic socket, which locks the I domain in an inactive conformation. When mutated to glycine, an open, ligand-binding conformation is induced. Binding of the iC3b ligand to the I domain is cation (Mg\(^{2+}\)) dependent (Vorup-Jensen et al 2003). Recently, the opening of
the headgroup has been studied in even more detail, and a metastable intermediate that probably
does not exist in vivo, was found. It reveals a hypothetical mechanism of rapid activation of the
conformation through “cocked” conformations of β chain I domain, bound or not bound to an
internal ligand from α chain (Sen et al 2013).

The structure of the intracellular part of CR4 has been solved by NMR. Comparison with
other integrin cytoplasmic parts (LFA-1 and Mac-1, VLA-4, αmβ2) shows some similarities but
also differences between the integrins. The membrane proximal part of αX chain forms an α helix,
interacting on its polar face with the membrane-proximal part of the β2 chain. The rest of the αX
cytoplasmic part forms an irregular loop, part of which folds back to contact the α helix of the
same chain. The C-terminal part of the β2 cytoplasmic part also has a relatively long irregular
conformation (Chua et al 2012).

One important feature of CR4 is its resistance to activation. This was found in experiments
where chicken/human chimeras of CR4 were tested for the binding of iC3b (Bilsland et al 1994).
The difficulty to activate αXβ2 is due to extremely tight intersubunit interaction/restraint between
αX and β2 chains. It has been reported that especially the residues in the N-terminal cysteine-rich
PSI domain (plexin/semaphoring/integrin) and in the C-terminal cysteine-rich EGF-repeats 2
and 3 are involved in these interactions (Zang & Springer 2001).

Figure 10. The structure of αXβ2. The binding sites of some important inhibiting
or activating antibodies are indicated. From Xie et al 2010. Figure is reproduced
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3.8.2 Ligands and functions of CR4

CR4 binds to various cellular, soluble and extracellular matrix (ECM) ligands. The binding sites
of many known antibodies used in functional assays (Lu et al 2001, Xie et al 2010, Chen et al
2010) have been solved, which is of great help in the studies concerning the interplay between
structure and function. Earliest studies on CR4 and the whole “LFA-1, Mac-1, p150.95 family”
described the different roles of the family members. The first described role was in granulocyte and monocyte adhesion, phagocytosis, migration and cytotoxicity (Anderson et al 1986, Keizer et al 1987a, Keizer et al 1987b).

Probably the most important or at least most studied ligand of CR4 is the complement component iC3b. Recognition of this molecule by CR4 mediates the phagocytosis of complement-opsonised particles (Micklem & Sim 1985, Malhotra et al 1986, Myones et al 1988, Bilsland et al 1994, Vorup-Jensen et al 2003, Chen et al 2012). The roles of Mac-1/CR3 and CR4 in complement-mediated phagocytosis have raised questions, as the two quite similar molecules are co-expressed on many cell types. In alveolar macrophages Mac-1 is the major iC3b receptor although CR4 is expressed in higher quantities. Different mobility of Mac-1 (more mobile on the membrane, forming patches) and CR4 (less mobile, uniform staining) was reported, and increased mobility was shown to mediate increased CR4-iC3b binding (Ross et al 1992).

The significance of CR4 in phagocytosis has been verified throughout the last three decades. It is involved in the complement-mediated phagocytosis of *Mycobacterium leprae* by macrophages (Schlesinger & Horwitz 1991) and of *Mycobacterium tuberculosis* by alveolar macrophages of the lung (Hirsch et al 1994). Importantly, uptake and removal of apoptotic cells is partly caused by CR4. Phagocytosis of apoptotic cells is essential for the prevention of autoimmune responses, as it enhances anti-inflammatory cytokine release, blocks inflammatory cytokine release and induces T cell tolerance (Mevorach et al 1998, Pittoni & Valesini 2002). CR4 is also heavily involved in the erythrophagocytosis, the uptake of senescent red cells by spleen macrophages (Toivanen et al 2008).

Other soluble ligands for CR4 are heparin (Diamond et al 1995, Vorup-Jensen et al 2007) and fibrinogen (Loike et al 1991, Ruf & Patscheke 1995, Nham 1999, Lishko et al 2001, Choi & Nham 2002). Fibrinogen expressed by platelets binds CR4 on neutrophils, inducing an oxidative burst (Ruf & Patscheke 1995). Serum albumin has also been reported to be a CR4 ligand, but interestingly only when CR4 is expressed on macrophages, not on monocytes (Brevig et al 2005). Plasminogen (zymogen of plasmin that is an enzyme degrading the fibrin clot) can also interact with CR4 with moderate affinity (Gang et al 2007). Interaction of CR4 with LPS leads to cell activation, signal transduction and transcription factor κB translocation (Ingalls & Golenbock 1995).

CR4 on monocytes also binds type I collagen, which promotes cell adhesion and activation in the initial phase of inflammation (Garnotel et al 2000). Lately CR4 has been shown to interact with collagen IV through its I domain and the GFOGER motif on collagen, which is traditionally a β integrin binding motif. Interestingly, the CR4 I domain binds collagen significantly better than LFA-1 or Mac-1 (Lahti et al 2013).

CR4 and Mac-1 (but not LFA-1) on monocytes bind to denatured proteins (Davis 1992). Negatively charged residues in disintegrated proteins probably serve as a pattern recognition motif for CR4. These findings have led to the hypothesis that the pericellular proteolysis at the leading edge of a migrating neutrophil would produce binding sites for CR4, enabling adhesion needed in migration (Vorup-Jensen et al 2005).

It was found already in the beginning of the 1990s that activated endothelial cells bind to CR4 (Stacker & Springer 1991). Since then, CR4 has been reported to interact with several cellular ligands. These include ICAM-1 and more precisely its fourth domain (Diamond et al 1993, Blackford et al 1996, Frick et al 2005). The binding residues on the ε domain to ICAM-1 have also been characterised (Choi 2010). CR4 also binds to ICAM-2 and VCAM-1 (Sadhu et al 2007) but not to ICAM-3 (de Fougerolles et al 1995). Another reported ligand of CR4 is Thy-1,
a membrane protein involved in cell adhesion and signaling regulation in neurons and T cells (Choi et al 2005). CD23 is an IgE receptor lectin and the binding of CR4 to it induces NO₂ and H₂O₂ production (Lecoanet-Henchoz et al 1995).

Recently, studies on the role of CR4 in hypercholesterolemic mice and its involvement in the development of atherosclerotic plaques have revealed an important role for αXβ2 on activated monocytes and in the formation of atherosclerotic plaques. CR4 binding to VCAM-1 is partly responsible (together with VLA-4) for the capture and transmigration of monocytes to inflamed human aortic endothelial cells. Also delayed-type hypersensitivity reaction induced by SRBC (sheep RBC) in mice was reduced in the presence of anti-αX antibody (Sadhu et al 2007). Hypercholesterolemic (apoE deficient) mice display increased CR4 expression on monocytes when fed with a western high-fat diet. CR4 is needed for these cells to arrest on VCAM-1 and E-selectin in shear flow, as well as for monocyte/macrophage accumulation in atherosclerotic lesions. Lack of CR4 expression decreases atherosclerosis in apoE depleted mice on high-fat diet (Wu et al 2009). In humans, the amount of αXβ2 on the monocytes of healthy subjects is increased after a fatty meal, simultaneously with lipid particle uptake. Monocyte adhesion to VCAM-1 under shear flow depends partly on CR4 (Gower et al 2011) and the engagement of CR4 on monocytes leads to the production of proinflammatory cytokine (Lecoanet-Henchoz et al 1995).

CR4 is also implicated in fat tissue reactions, as there is less adipose tissue inflammation induced by the high-fat diet in the αX deficient than in wt mice (Wu et al 2010).

CR4 seems to be heavily involved in the uptake of antigens to dendritic cells (Sadhu et al 2007, Castro et al 2008, Faham & Altin 2011). In helminth infection CR4 is essential for the development of a correct Th2 response (Phythian-Adams et al 2010). Targeting to CR4 helps in the uptake of antigen also in an artificial situation such as vaccination (Reddy et al 2006). This field of research has become extremely important, as the DC related vaccines are being developed based on the discoveries of Ralph Steinman (Steinman & Banchereau 2007). Promising results with CR4 targeting have been obtained. T cell responses may be induced by targeting the antigen to the CR4 with a single chain fragment (scFv) recognising CR4 (Ejaz et al 2012). Also peptides binding CR4 have been successfully used in eliciting T cell priming and antibody production in mice vaccinated with antigen-containing liposomes engrafted with these peptides (Faham & Altin 2011). It also seems that the Mac-1 and CR4 in general are more relevant in the antigen uptake to DCs than in the immune synapse formation or migration (Grabbe et al 2002, Lammermann et al 2008).

CR4 integrin has also been implicated in the development of experimental autoimmune encephalomyelitis (EAE), which is a murine model of MS (Bullard et al 2007). Expression of CR4 on T cells and other leukocytes is a prerequisite for the development of the disease, but also other integrins such as Mac-1 are involved.

CR4 is also a receptor for some pathogens. *Candida albicans* hyphae (filamentous form, but not the fungal form) has been reported to bind to CR4, and this recognition is important for protection against *C. albicans* infections through Kupffer cells in the liver and microglia in the brain (Jawhara et al 2012). Rotaviruses use CR4 for entry to the host cell (Graham et al 2003). It seems that Herpes simplex virus 1 (HSV-1) exploits CR4 on dendritic cells to promote its infectivity. In fact, depletion of CR4 leads to decreased HSV-1 replication, increased cytokine production, and increased levels of virus-specific CD8+ T cells (Allen et al 2011). One of CR4 ligands is osteopontin, a multifunctional cytokine that is able to opsonize some (Streptococcus and Staphylococcus) bacteria for phagocytosis. This phagocytosis requires the presence of CR4 (Schack et al 2009).
4 Regulation of integrin activity

As discussed earlier, the integrins on leukocytes are responsible for a growing number of reported functions in health and disease. Thus solving the mechanisms of integrin signalling is essential for the understanding of leukocyte behaviour in physiological and clinical conditions. Integrins offer an effective target for therapies against various immunological and other malignancies, autoimmune diseases and invading pathogens, which has induced a lot of research and clinical trials (Hilden et al 2006, Millard et al 2011, Marelli et al 2013).

Because of the fundamental connection between integrin ligand-binding activity and their conformation, many antibodies recognising the different conformations have been developed (Byron et al 2009). They provide an elegant way to study the implications of e.g. different activation methods or the role of the numerous intracellular signalling and adapter molecules in the signalling cascades leading to cell adhesion. Especially they allow the researchers to study two different paths to enhanced ligand binding: increase in a single molecule's ability to bind its ligand (affinity) or increase in the number of integrins at the adhesion site (avidity).

The only endogenous inhibitor of leukocyte functions reported so far is developmental endothelial locus-1 (Del-1) that inhibits LFA-1 and Mac-1 functions. It binds to β2 integrins and is able to outcompete ICAM-1 from LFA-1 binding and inhibit macrophage phagocytosis of iC3-coated RBC (Choi et al 2008, Mitroulis et al 2014). A "natural"inhibitor for Mac-1 called neutrophil inhibitory factor or NIF has been found in canine hookworm. It selectively inhibits Mac-1 dependent eosinophil transmigration in and thus is promising candidate for treatment of allergies (Moyle et al 1994, Schnyder-Candrian et al 2012).

4.1 Affinity/avidity

Integrin-dependent leukocyte adhesion to ligands can plausibly be regulated at two levels. First, the affinity of a single integrin may be enhanced so it can bind ligands better (see chapter 3.1). Second, the amount of ligand-binding integrins at the contact site may be raised due to their movement in the plane of the plasma membrane. It has been suggested that rapid inside-out activation of the integrins would change integrin conformation and increase their affinity, whereas outside-in signalling would cause changes in the actin cytoskeleton, leading to integrin clustering at the ligand binding area and thus reinforcing the adhesion (Dustin et al 2004, Hogg et al 2011). This idea has been endorsed by many reports, especially by the finding that LFA-1 clustering follows and does not precede ligand binding to integrins (Kim et al 2004). Probably the whole picture is more complicated than that. In addition, in different cell types or cells in various stages of maturation, different activation mechanisms may prevail.

It has also been suggested that the inside-out activation and hence the conformational change of the integrin would be transduced by the β chain, and the outside-in signalling, leading to integrin clustering (among other consequences) conveyed through the α chain (Kliche et al 2012). However, for example β2 Thr-Thr-Thr sequence mutation to Ala-Ala-Ala does not change initial binding, but completely abrogates the strengthening of the adhesion (Morrison et al 2013).

Mechanical force (shear force experienced by the leukocytes in the blood flow) has also been implicated in integrin activation. It stimulates conformational activation (Katsumi et al 2005) as well as clustering (Knies et al 2006) of integrins. In the shear-free environment of lymph nodes the VLA-4 and LFA-1 integrins are not able to support adhesion (Woolf et al 2007, Alon & Dustin 2007).
Clustering of the integrins takes place upon dynamic changes of the cytoskeleton, as well as regulation of integrin cytoplasmic tail binding to the actin network through adapter proteins. The release of the integrins from the cytoskeleton increases LFA-1 binding to their ligands due to integrin clustering, not affinity changes (Kim et al 2004).

4.2 Inside-out activation of integrins

Inside-out activation of integrins is a series of events where binding of ligand to non-integrin receptors on the plasma membrane initiates an intracellular signalling cascade leading to integrin activation (figure 11). Inside-out activation may occur upon activation through the TCR, the B cell receptor (BCR), chemokine receptors, selectins, or other molecules. After the initial signal from non-integrin receptor, the signalling cascade continues in the cytoplasm, recruiting adapter and signalling proteins. The signal is further delivered to the intracellular parts of the integrins, which leads to the straightening of the integrin extracellular part from bent to extended conformation and enhancement of ligand binding (Abram & Lowell 2009, Springer & Dustin 2012). The intracellular signalling cascades in immunological synapse, extravasation and phagocytosis were discussed to some extent in chapter 1.2. Although the signalling events after different activation mechanisms most probably resemble each other in all leukocytes, there are differences between cell types, maturation stages, types of activation, integrins in question etc. For example, effector T cells are not dependent on chemokine signalling when adhering to the endothelium in the very beginning of extravasation, whereas naïve T cells need chemokines in order to attach properly. In the next steps of extravasation, also effector cells are dependent on chemokines (Shulman et al 2011, Lek et al 2013). It is also good to keep in mind that many of the studies presented here have been carried out in an experimental setup where a limited number of cell types, activation methods and integrin outcomes have been utilized.

Figure 11. A simplified scheme of pathways involved in integrin inside-out activation.
The final outcome of the inside-out signalling is integrin activation through a conformational change. An essential event for this is the dissociation of integrin α and β cytoplasmic parts. In resting state, they form a salt bridge between each other but after cell activation the separation of the tails occurs. This has quite unanimously been reported to happen upon talin head binding to the β chain, forcing the two tails to separate, further leading to conformational change of the extracellular domain towards an extended, ligand-binding state (Tadokoro et al 2003, Simonson et al 2006).

Before talin binding to the integrin cytoplasmic tail, the signals from several activation routes converge in the formation of multi-protein complexes. These include Ras-related protein 1 (Rap1, or Rap2 in B cells), a small GTPase, that has been shown to be essential for LFA-1 activation, interacting with one of its effector proteins: RIAM (Rap1 interacting adapter molecule) or RAPL (regulator for cell adhesion and polarization enriched in lymphoid tissues) and mammalian Ste20-like kinase (Mst1). Other important mediators for at least LFA-1 activation are constitutively interacting adapter proteins ADAP and SKAP55. LFA-1 and Rap1 with its aforementioned interaction partners have been localised to intracellular vesicles in T cells, and the proper activation of the integrin requires Rap1 activation and the vesicle traffic to the plasma membrane. The exact order of the delivery of the vesicles to the plasma membrane and the activation events is not fully elucidated (Sebzda et al 2002, Shimonaka et al 2003, Mor et al 2009, Bivona et al 2004, Katagiri et al 2006, Kliche et al 2012). These signalling events have been extensively characterized, also in the context of platelet integrin activation, and the same molecules (Rap1, RIAM and talin) regulate also αIibβ3 activity (Calderwood 2004, Han et al 2006).

Rap1 activation to a GTP-bound form is induced by two different Rap1 GEFs (guanine-nucleotide exchange factors, activators of Rap1): CalDAG-GEF1 (Ca2+ and diacylglycerol-regulated guanine nucleotide exchange factor 1) and C3G. Following TCR ligation, PLCγ1 (phospholipase C γ1) is activated and Ca2+ and diacylglycerol (DAG) are generated. These in turn are able to activate CalDAG-GEF1 (Katagiri et al 2004, Ghandour et al 2007, Bergmeier et al 2007). C3G is closely associated to CRKL (CRK-like protein) and needs the help of additional actin-binding complex Arp2/3 for the delivery to the plasma membrane (Nolz et al 2008, Mor et al 2009). Interestingly, CalDAG-GEF1 is not needed for VLA-4 activation (Ghandour et al 2007).

The identity of the Rap1-containing complex in the vesicles has drawn some interest, and at least two different complexes have been described: one with Rap1, RIAM, Mst1 and the ADAP/SKAP55 module, including talin and kindlin3 (the role of talin and kindlin, see below) and another with Rap1, RAPL Mst1 and the ADAP/SKAP55 module. It has been suggested that the first complex is involved in the inside-out activation through β2, whereas the second, αL-interacting complex would be important in outside-in signalling. RAPL is able to interact directly with the αL chain on Lys1097 and Lys 1099, whereas the role of Mst1 contributes to translocating LFA-1 to the leading edge of a migrating cell (Katagiri et al 2003, Katagiri et al 2006, Kliche et al 2012).

Altogether, the importance of Rap1 cannot be denied, but its actions still need clarification. It seems to display slightly different functions in different cell types. In B-cell line BAF, the activation of Rap1 leads to an increase in LFA-1 affinity (Katagiri et al 2000, Tohyama et al 2003), whereas in mouse thymocytes it induces clustering (Sebzda et al 2002). In platelets the activation of Rap1 and subsequent formation of the RIAM-talin complex also increases the affinity, not the avidity of αIibβ3 (Han et al 2006).

In the resting state, filamin, a large, actin-crosslinking protein, is bound to the cytoplasmic part of the β2 chain, and the binding keeps the integrin in a resting, non-adhesive conformation.
Filamin binds only to the non-phosphorylated $\beta_2$ and the phosphorylation of the $\beta_2$ chain on threonine 758 that happens upon cell activation (Valmu & Gahmberg 1995), is a negative regulator of filamin binding. Phosphorylation of the $\beta_2$ does not directly affect talin binding as the binding site does not contain any phosphorylatable residues, but filamin and talin probably compete for the binding of the integrin cytoplasmic tail (Kiema et al 2006). Especially talin could be important for the early steps in the adhesion when it probably competes out the cytoplasmic moiety of the $\alpha_L$ chain from binding to the $\beta_2$, as it has higher affinity towards the $\beta_2$ cytoplasmic tail than $\alpha_L$. Talin binding leads to the separation of the $\alpha_L$ and $\beta_2$ cytoplasmic parts and results in straightening of the integrin conformation. Later, after Thr758 phosphorylation, talin itself is probably outcompeted by the 14-3-3 protein, an adapter protein that binds to the phospho-Thr758 with extremely high affinity (see more in the next chapter about outside-in signalling) (Fagerholm et al 2002, Fagerholm et al 2005, Kiema et al 2006, Takala et al 2008, Bhunia et al 2009). In addition to its role in increasing the integrin affinity by conformational change, talin also influences the clustering of the integrins thus enhancing the avidity of the integrins (Simonson et al 2006).

Talin transfer to the plasma membrane and hence close to the integrin cytoplasmic part, is thought to happen upon binding of the plextin homology (PH) domain of talin to the phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) lipid on the plasma membrane (Martel et al 2001, Anthis et al 2009). PtdIns(4,5)P$_2$ is produced by the action of PIP5K1y87, which is also essential for the high affinity LFA-1 (Bolomini-Vittori et al 2009, Wang et al 2004). PIP5K1y87 itself is activated by phosphatidic acid (PA), which is formed by phospholipase D 1 (PLD1) upon TCR or chemokine receptor activation (Mor et al 2009). Talin has been localised in the mid-cell high-affinity LFA-1 zone (Smith et al 2003), whereas $\alpha$-actinin, another $\beta_2$-binding actin-cytoskeleton-associating protein colocalizes with the intermediate-affinity LFA-1 to the leading edge (Stanley et al 2008). It has been suggested that the binding of $\alpha$-actinin is made possible when talin is cleaved by calpain upon cell activation. This would release LFA-1 from the cytoskeleton, leaving it to move more freely on the plasma membrane e.g. to the site of adhesion. It also uncovers an $\alpha$-actinin binding site. $\alpha$-actinin may then attach the LFA-1 cytoplasmic tail back to the cytoskeleton, supporting formation of integrin clusters (Pavalko & LaRoche 1993, Sampath et al 1998, Stewart et al 1998).

Another important molecule associated in integrin activation is kindlin-3 that binds to integrin cytoplasmic tails. Its absence leads to LADIII (leukocyte adhesion deficiency III), a condition where leukocyte and platelet integrin functions are severely defective (Svensson et al 2009, Malinin et al 2009). Kindlins are a family of three FERM (4.1 protein, ezrin, radixin, moesin) domain- and PH (plextin homology) domain-containing proteins with distinctive expression patterns. Kindlin-3 is expressed only on cells of haematopoietic origin and it binds to $\beta_2$, $\beta_3$ and $\beta_3$ cytoplasmic tails and, more specifically to the membrane-distal NxxF/Y-motif and to a phosphorylatable Thr-Thr-Thr motif on the $\beta_2$ chain. It also interacts with several other, actin-associated proteins such as ILK (integrin-linked kinase), FAK (focal adhesion kinase) and $\alpha$-actinin. Kindlin-3 has recently been found to be essential for the activation of the integrins in haematopoietic cells (Moser et al 2009, Morrison et al 2013, Karakose et al 2010). The role of kindlin-3 in $\beta_2$ integrin activity regulation has been studied in detail and, interestingly, it is involved in some but not all processes in $\beta_2$ integrin activation. A possible mechanism of action is to optimize the association of the LFA-1 tail with talin and possibly other intracellular regulators of integrin activities. Furthermore, kindlin-3 does not leave the crime scene after the initial integrin activation, but continues to be active and takes part also in the outside-in
signalling events. It has an adhesion-strengthening role, probably due to its interactions with the actin cytoskeleton. In T cells it is needed for homing to lymph nodes but not for affinity increase (Morrison et al 2013) and it has also been reported to take part in α6β4-dependent outside-in regulation of Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division control protein 42 homolog (Cdc42) activities through spleen tyrosine kinase (Syk) and proto-oncogene Vav1 (Xue et al 2013).

Most of the data previously cited here has been conducted in the T cell or T cell lines expressing LFA-1 as the only member of β2 family. However, the signalling events have been also studied extensively in the context of Mac-1 dependent phagocytosis. Rap1 is a vital activator of Mac-1 dependent phagocytosis, and its interaction with talin localises both of them in the phagocytic cup. Contrary to LFA-1, Rap1 signalling to Mac-1 has been reported to use only RAPL and not RIAM (Lim et al 2010, Goult et al 2010). The inside-out activation and ligand binding of Mac-1 occurs upon talin head binding, but the rod domain is indispensable for phagocytosis (Lim et al 2007).

As for VLA-4, also other molecules and processes are involved in the activation cascades, but many of the results presented earlier hold true also for VLA-4 signalling (Imai et al 2010). The largest structural difference between VLA-4 and the β2 integrins is that VLA-4 does not contain the α chain I domain that is almost exclusively responsible for the ligand binding to β2 integrins. The lack of I domain, however, seems to increase VLA-4 flexibility and ability to bind ligands in various conformations (Chigaev & Sklar 2012). Another factor that differentiates VLA-4 is the binding of paxillin to the α4 cytoplasmic part. Paxillin is essential for VLA-4 adhesion under shear but not in no-shear conditions (Alon et al 2005). Paxillin-α4 interactions are strongly regulated by the phosphorylation of α4, so that paxillin only binds to non-phosporylated α4 and is released upon phosphorylation. The binding of paxillin decreases cell spreading and migration, whereas its release from the α4 tail enhances spreading. Importantly, a dynamic phosphorylation-dephosphorylation cycle is needed for the proper function of VLA-4 in e.g. cell migration (Han et al 2001, Han et al 2003).

4.3 Outside-in signalling initiated by integrin ligand binding

Outside-in activation of integrins may happen upon ligand binding, activating antibody binding (Campbell & Humphries 2011) or divalent cations (Dransfield et al 1992). Specific signalling cascades are induced upon ligand binding, leading to increase in cell adhesion, spreading and migration, although the outcome depends on the cell type and function. For example in T cells, outside-in signalling is associated with cell proliferation and interleukin-2 (IL-2) production as well as stabilisation of the immune synapse between T cell and APC. In neutrophils the result is different: enhancement of degranulation and activation of NADPH oxidase that enables ROS production that is further used for cytotoxic effects. As for macrophages, the outcome of outside-in signalling consists of cell differentiation and IL-1β mRNA stabilisation (Abram & Lowell 2009).

As previously discussed, it is occasionally difficult to dissect the role of the intracellular signalling and adapter proteins in inside-out and outside-in cascades, also because many molecules are used in both pathways according to the present knowledge. In order to bypass the inside-out route and elicit only outside-in signalling, it is possible to use specific activators such as monoclonal antibodies and divalent cations (Mn²⁺) or integrin ligands. Using these methods,
Review of the literature

many of the following results have been obtained. A schematic model of some of the pathways involved in integrin outside-in signalling is shown in figure 12.

In outside-in signalling, a crucial downstream step is the formation of FA or focal adhesion, a large, dynamic complex consisting of about 150 intracellular proteins. Another important consequence of integrin outside-in signalling are the changes in the assembly of the actin cytoskeleton and changes in integrin avidity.

Figure 12. Pathways of integrin outside-in signalling.

The first steps in outside-in signalling are the phosphorylation events of Src family kinases (SFK). In myeloid cells the SFK involved are called Hck, Fgr and Lyn (Giagulli et al 2006, Baruzzi et al 2008) and in T cells Lck and Fyn (Suzuki et al 2007). SFKs then phosphorylate ITAMs on DAP12 and FcγR, which offer docking sites for the next players in the sequence, the Syk family kinases Syk in myeloid cells or ZAP-70 in T and NK cells (Mocsai et al 2002, Mocsai et al 2003, Epler et al 2000). The reports of the importance of Src and Syk family kinases in migration are controversial and it seems to be dependent on cell type (monocyte / macrophage / neutrophil / DC) and the inflammation site (peritoneum / lung / cremaster muscle) (Baruzzi et al 2008, Lammermann et al 2008).

Talin is needed to obtain the high-affinity integrin. It shows different stages in β尾 tail binding (initial binding in the inside-out signalling and further binding to both NxxF/Y sites and to a membrane-proximal, additional site), leading to a stable, high-affinity conformation (Wegener et al 2007). In VLA-4 outside-in signalling, the role of Zap-70 is to deliver talin to the plasma membrane and to phosphorylate Vav1, which dissociates it from talin. This makes it possible for talin to occupy the β tail and the integrin adopts a full high-affinity conformation (Garcia-Bernal et al 2009).

Rap1 and RAPL play critical roles also in the adhesion strengthening phase of transendothelial migration. RAPL binds to two membrane-proximal lysines (Lys1097 and Lys1099) in the αL chain, and it may participate in the stabilisation of the conformation by keeping the intracellular tails separated. RAPL is suggested to be dispensable for the inside-out
affinity change after chemokine activation, whereas Rap1 is needed also in inside-out activation (Katagiri et al 2003, Hogg et al 2011, Ebisuno et al 2010).

Very important players in the outside-in cascade are the tyrosine kinases FAK and Pyk2 that are situated downstream of SFKs. Upon ligand binding, Src phosphorylates and hence activates FAK. FAK and Pyk2 functions in myeloid cells have been studied and the FAK or Pyk2 deficient cells show similar kind of defects in lamellipodia stabilisation, directed chemotaxis, migration in vivo and cell polarisation (Owen et al 2007, Okigaki et al 2003, Han et al 2003).

Close localisation is a prerequisite for such an activation cascade to take place. In that sense, it is important to decipher how the above-mentioned kinases and the integrins initiating the signalling are brought together. Even though the integrin cytoplasmic parts lack traditional SH2 or SH3 domains needed for Src and Syk kinases to bind, it has been reported that they are attached to the integrins, even in resting cells (Arias-Salgado et al 2003, de Virgilio et al 2004).

The ITAM-phosphorylation and subsequent activation steps are partly similar in outside-in signalling that they are in the TCR- or BCR-mediated inside-out signalling. The integrin tails do not themselves contain ITAM-sequences, but they forward the signal through ITAMs of other cytoplasmic proteins. Further separation of α and β tails is needed for proper outside-in signalling and this might bring the integrin tails to close proximity of ITAM sequences (Zhu et al 2007). Another way to bring integrins close to the ITAMs could be the association of the two molecules in lipid rafts that have been implicated in LFA-1 and VLA-4 signalling (Marwali et al 2003, Krauss & Altevogt 1999, Solomkin et al 2007).

Downstream of SFK and Syk family kinases is the important scaffold protein called SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP76). Its expression has been detected in neutrophils, DCs and platelets (Bezman & Koretzky 2007), and it is probably also involved in immunoreceptor (TCR, BCR, FcR) signalling (Abtahian et al 2006). Also ADAP (T cells) and PRAM-1 (myeloid) are molecules that are considered important in outside-in signalling in neutrophils (Clemens et al 2004, Bezman & Koretzky 2007). The SLP76 and ADAP may be contributing to inside-out signalling in T cells, whereas in myeloid cells they are involved in outside-in signalling.

Yet another protein probably involved in outside-in signalling is cytohesin-1 that becomes phosphorylated after LFA-1 ligation, although it functions in inside-out signalling without being phosphorylated upon TCR activation. It is involved in ERK activation and IL-2 production (Perez et al 2003). The transcription factor JAB-1 (Jun-activating binding protein-1) has also an important role in LFA-1 outside-in activation. When LFA-1 binds to its ligand, JAB-1 is released from the LFA-1 cytoplasmic tail and transferred to the nucleus, where it co-activates the c-Jun transcription factor, activating activator protein-1 (AP-1) and resulting in responses such as IL-2 production (Perez et al 2003). In neutrophils, Mac-1 outside-in signalling leads to IL-8 production (Walzog et al 1999) and in monocytes the transcription repressor forkhead fox protein P1 (Foxp1) is downregulated, leading to M-CSF receptor expression upon Mac-1 stimulation (Shi et al 2001, Shi et al 2004).

In myeloid cells, casitas B-lineage lymphoma protein (c-Cbl) is involved in integrin outside-in signalling, in addition to its other functions as adapter and ubiquitin ligase protein (Caveggion et al 2003). It transfers cytoplasmic phosphoinositide 3-kinase (PI3K) to the plasma membrane (Meng & Lowell 1998). PI3K is needed for firm arrest of VLA-4 and LFA-1 (Hyduk & Cybulsky 2009).

The involvement of the actin cytoskeleton in outside-in signalling is transmitted through modulation of Rho GTPases that are regulated by Vav family of Rho-GEFs. In neutrophils,
the lack of Vav leads to the inability to support binding in shear flow even though the initial attachment is not affected (Graham et al 2007). Vavs regulate also PLCγ and Ca^{2+} signalling from TCR and BCR (Swat & Fujikawa 2005) and from integrins in neutrophils (Graham et al 2007). The most extensively studied Rho GTPases are Rac, Rho and Cdc42, that are needed for polarisation and cellular migration (Ivetic & Ridley 2004).

A specific signalling pathway initiated by LFA-1 activation and leading to Rac1 activation and actin cytoskeleton rearrangements has been recently solved. Activation of LFA-1 induces the phosphorylation of the β_{i} chain on threonine 758 (Valmu & Gahmberg 1995, Hilden et al 2003). This is an important switch in the binding of some cytoplasmic signalling and adapter proteins (Takala et al 2008). The affinity of 14-3-3 proteins is high towards T758-phosphorylated β_{i}, and it is able to outcompete filamin from the binding site. Recruitment of 14-3-3 leads to activation of T-cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1) and further to Rac1 activation and actin cytoskeleton rearrangements (Fagerholm et al 2005, Nurmil et al 2007, Gronholm et al 2011). Similar results have been obtained for β_{i} integrins (O’Toole et al 2011).

Most recent reports have focused on kindlin action in the inside-out signalling of integrins, but recently, more evidence on their role in the outside-in signalling has been gathered. Talin binds to integrin β chains through the membrane-proximal Nxx(Y/F) sequence, causing the separation of α and β cytoplasmic tails, whereas kindlin F3 domain binds to the membrane-distal Nxx(Y/F) (Manevich-Mendelson et al 2009). Kindlin-3 is involved in outside-in signalling of LFA-1 and α_{m}β_{3} by inducing spreading. It also induces microclustering of the integrin using its PH and F3 domains (Feng et al 2012). Kindlin-3 is also required for α_{m}β_{3} mediated firm adhesion (Xue et al 2013). Kindlin-3 is also required for α_{m}β_{3} mediated firm adhesion (Xue et al 2013). Atomic force microscopy studies revealed that the kindlin-3/β_{3} tail TTT –motif interaction is not essential for the initial ligand binding, but is definitely required for the strengthening of the adhesion as well as lymphocyte homing in shear flow, whereas in shear-free environment this interaction is not necessary (Morrison et al 2013).

In phagocytosis, the different phagocytic receptors may induce separate signalling pathways, leading to slightly varied modes of phagocytosis. Fc-receptors use Rac1/Cdc42 to induce “reaching phagocytosis” and complement receptors produce “sinking phagocytosis” through Ras homolog gene family, member A (RhoA) (Caron & Hall 1998, Dupuy & Caron 2008). RhoG binding to the Thr-Thr-Thr motif is also involved (Tzircotis et al 2011). Also Rap1 and RhoA induce outside-in activated phagocytosis (Kim et al 2012).

One aspect of signalling is the difference between the various activation methods. In fact, it seems that the TCR-mediated integrin activation that takes place in the static conditions of the lymph nodes or tissues and the chemokine- or selectin-mediated activation in the shear flow environment lead to different outcomes of integrin activation. According to some reports, the affinity of the integrins does not necessarily change after TCR activation (Stewart & Hogg 1996, Burbach et al 2007) and TCR ligation has been suggested to, in fact, prime the integrins for activation, not directly extend them (and leading to ligand binding even by bent form) (Feigelson et al 2010). The LFA-1 molecules on effector cells differ from the naïve T cells in the leukocyte integrin activation status and mechanisms. In effector cells, there are more LFA-1 integrins expressed than in naïve T cells and, importantly, it shows spontaneous binding to ICAM-1, as opposed to the situation in the naïve cell LFA-1. The strengthening of the adhesion is extremely rapid. The binding of the effector cells to the endothelium is independent of chemokine activation, whereas they do need chemokine signals for transendothelial migration (Shulman et al 2011, Lek et al 2013).
4.4 Phosphorylation

As seen in the previous chapters, the binding of the cytoplasmic adapter and other proteins is essential for the accurate regulation of integrin functions. All the proteins able to bind to the short cytoplasmic integrin tails cannot be present at the same time due to spatial limitations, so there has to be some factors that determine which protein(s) can bind under certain conditions. The cellular localisation of the regulators and the differences between binding affinities are important mechanisms to direct the protein-protein interactions. Another way to regulate the adaptor binding to integrin tails is phosphorylation of the integrin cytoplasmic tails, which may increase or decrease the binding of certain molecules. Most α and β chains of the integrins have been reported to undergo phosphorylation either constitutively or upon cell activation (figure 9) (Fagerholm et al 2004, Gahmberg et al 2009).

4.4.1 β2 integrins

Integrin phosphorylation has been reported already almost 30 years ago, and more specific studies on β2 family phosphorylation showed that the β2 chain is phosphorylated upon cell activation, whereas the α chains (αL, αM, αX) are constitutively phosphorylated. In the initial studies, serine was found to be the predominant phosphorylation target in all polypeptide chains studied. Weak threonine phosphorylation was detected in αX and β2, and β2 showed also tyrosine phosphorylation (Chatila & Geha 1988, Chatila et al 1989, Buyon et al 1990). Later, strong threonine phosphorylation has been detected using phosphatase inhibitors (Valmu & Gahmberg 1995).

The following phosphorylation sites on β2 (CD18) chain have been reported. Tyrosine 735 is phosphorylated after IL-2 treatment in NK cells (Umehara et al 1993), and after collagen binding in neutrophils (Garnotel et al 1995). This phosphorylation is reported to be involved the internalisation of LFA-1 at the rear end of a migrating cell (Tohyama et al 2003). Serine 745 is phosphorylated after phorbol ester treatment that is mimicking PKC activity in the cells (Fagerholm et al 2002). An interesting function of Ser745 phosphorylation is that it releases JAB-1 from LFA-1, leading to transcription of certain genes (see previous chapter) (Perez et al 2003). Serine 756 was initially found to be the major phosphorylation site after activation with phorbol esters but it was reported not to be essential for integrin adhesion to its ligands (Hibbs et al 1991, Fagerholm et al 2002). Later Ser756 has been shown to be essential for Mac-1 dependent phagocytosis through Rap1 and talin recruitment to β2. Interestingly, the talin head alone is able to activate the phosphorylation mutant Mac-1 bearing the Ser756Met mutation. The kinase involved is calmodulin-associated molecule CaMKII (Lim et al 2011).

Phosphorylation of the threonine triplet 758-760 has been under profound investigation and indeed has proven to be essential in leukocyte integrin functions. The Thr-Thr-Thr sequence was early shown to be important for LFA-1 mediated adhesion (Hibbs et al 1991, Peter & O’Toole 1995) but the threonine phosphorylation was ignored as it was so labile that it could not be seen unless phosphatase inhibitors were used (Valmu & Gahmberg 1995). The triplet is phosphorylated after TCR engagement or phorbol ester treatment, but in slightly distinct patterns, and all three threonines cannot be phosphorylated at the same time (Valmu et al 1999, Hilden et al 2003). This Thr-Thr-Thr sequence is associated with actin reorganisation, “postreceptor events” (avidity/clustering), but not in affinity regulation (Peter & O’Toole 1995).

Kinases phosphorylating the β2 chain have been described. The main responsible kinases for Ser745 and Thr758 are PKCδ and PKCβ1/II. Also PKCa and PKCe may phosphorylate these
residues and PKCα is also able to phosphorylate Thr-760. Ser-756 is not directly phosphorylated by any PKC isoform, but is still phosphorylated upon phorbol ester treatment, indicating the involvement, but perhaps indirect, of a PKC (Fagerholm et al 2002).

The cytoplasmic domains of the four α chains of β2 integrins vary in length and sequence, enabling specificity for cytoplasmic interactions and signal transduction. Constitutive phosphorylation of β2 integrin α chains was reported several years ago (Chatila et al 1989, Buyon et al 1990, Valmu et al 1991) and the specific phosphorylation sites and the functions of the phosphorylation of αL and αM have been elucidated (Fagerholm et al 2005, Fagerholm et al 2006). Phosphorylation of αX is dealt in chapter 8.2. αDβ2 phosphorylation has not been reported.

The αL chain is phosphorylated on Ser1140 and the phosphorylation affects LFA-1 ligand binding and activation epitope expression. Approximately 40 % of the total αL chain molecules (in heterodimers) are phosphorylated. TCR engagement and phorbol esters are able to activate also the αL-Ser1140Ala mutant, but when activated with activating antibody MEM83 or Mg2+, the phosphorylation mutant binds ICAM-1 less efficiently. Phosphorylation of the α tail also influences the overall conformation of the extracellular region, which can be studied with antibodies recognising different (extended or extended-open, see chapter 3.2) conformations. The Ser1140Ala-mutated LFA-1 cannot adopt the high-affinity conformation detected with antibody mAb24, an antibody recognising the open, extended conformation (Kamata et al 2002), if the cells are activated with cytokine stromal-cell derived factor-1α (SDF-1α) or ICAM-2. Anyway, talin head domain is able to activate both β2 Thr758Ala and αL Ser1140Ala (Fagerholm et al 2005).

The αM chain has only one cytoplasmic serine (Ser1126), which is constitutively phosphorylated. Phorbol esters or extracellular Mg2+/EGTA treatment are not able to activate the Ser1126Ala mutant to bind to ICAM-1 or ICAM-2. However, the binding to other ligands, such as iC3b and denatured bovine serum albumin (BSA), is not affected. Expression of the conformation-specific activation epitopes recognised by antibodies mAb24 and KIM127 (that binds to extended integrin, (Lu et al 2001) is decreased and the binding to soluble ligands is downregulated by the mutation, showing impaired affinity. Importantly, migration through the endothelium and homing of lymphocytes in vivo are also severely affected in the cells where αM phosphorylation is not possible (Fagerholm et al 2006).

### 4.4.2 VLA-4

The α4 chain of VLA-4 is one of the integrin α chains whose phosphorylation has been studied quite extensively. The phosphorylation of serine 988 by PKA regulates paxillin binding to the α4 cytoplasmic tail and this in turn regulates the functions of VLA-4. Paxillin does not bind to the α4 tail phosphorylated on serine 988. Interestingly, in primary cells and different cell lines the α4 phosphorylation varies a lot (from 0% of some cells to 60% of α4 chains being phosphorylated in Jurkat cell line). Point mutations made on α4 chain revealed very interesting consequences of the α4 phosphorylation. Ser988Asp, which mimics constant phosphorylation, accelerates spreading and inhibits migration, whereas Ser988Ala (incapable of phosphorylation) inhibits spreading and reduces migration. Both mutations are able to bind VCAM and both wt and Ser988Ala are able to undergo outside-in signalling upon VCAM-1 binding, whereas Ser988Asp is not able to induce outside-in signalling. Especially cell migration requires a dynamic phosphorylation – dephosphorylation cycle (Han et al 2001, Han et al 2003). α4 interaction with paxillin is needed for the VLA-4 –VCAM-1 strengthening under flow and for leukocyte recruitment to the sites of inflammation (Alon et al 2005, Feral et al 2006).
Also the spatial regulation of integrin activities may be controlled by phosphorylation. In migrating cells, phospho-α4 is accumulated at the leading edge, and if α4 phosphorylation is blocked (Ser988Ala mutation), migration as well as lamellipodia stability are decreased, even though there are no changes in ligand binding capacity. In Ser988Ala cells that are not able to spread, paxillin and α4 co-cluster at the membrane. On the contrary, in Ser988Asp cells paxillin is located in all parts of the cell and there is no colocalisation with α4 (Goldfinger et al 2003). α4 phosphorylation on another serine, Ser978, induces 14-3-3ζ binding and the formation of a complex consisting of the α4 chain, 14-3-3ζ and paxillin, which is needed for Cdc42 (Rho GTPase) activity on the leading edge and lamellipodia of a migrating cell, and for directed cell movement. Interestingly, α4 interaction with paxillin only is sufficient for the regulation of another Rho GTPase, Rac1 (Deakin et al 2009).

Phosphorylation of the β1 chain has also been studied with point mutations in the cytoplasmic moiety. The phosphorylation of Tyr783 and Tyr795 do not affect integrin functions (Wennerberg et al 1998), but phosphorylation of Ser785 induces integrin localisation to FAs, enhances attachment and inhibits spreading and migration (Barreuther & Grabel 1996, Mulrooney et al 2001). Phosphorylation of threonines 788 and 789 (corresponding to the Thr-Thr-Thr motif in the β1 tail) is needed for attachment to Fn and activation epitope expression (Wennerberg et al 1998). Threonine 788 mutation to aspartic acid, which mimics phosphorylation, induces a constitutively active integrin and increases cell adhesion through an inside-out activation pathway (Nilsson et al 2006). Phosphorylation of threonines 788 and 789 is induced with elevated pressure, which increases β1 affinity towards extracellular matrix components such as collagen, Fn and laminin, whereas serine 785 phosphorylation is not needed for pressure-controlled adhesion (Craig et al 2009). Also tyrosine phosphorylation of β1 is required for migration (Sakai et al 1998b, Sakai et al 1998a).

4.5 Transdominant regulation of integrins

4.5.1 Leukocyte integrins

Integrin transdominant regulation or cross-talk is a phenomenon where the activation of one integrin regulates the functions of another in the very same cell. The first report of mutual regulation of different integrins expressed in the same cells dates back to two decades ago, when van Kooyk et al. noticed that in native T cells and different T cell lines there are large differences in the proportions of LFA-1 and VLA-4 mediated adhesion to activated endothelium. For example, in freshly isolated T cells the binding was equally LFA-1 and VLA-4 dependent, but when further cultured, the equilibrium moved towards LFA-1 dependency. Some of the T cell clones were LFA-1 dependent, some VLA-4, even though both integrins are expressed on all lines examined. Usually LFA-1 is used for adhesion whenever it is available, i.e. expressed, functional and activated, and VLA-4 binding to VCAM-1 only takes place when LFA-1 cannot be activated (van Kooyk et al 1993). Next, Porter & Hogg reported that when LFA-1 is activated, VLA-4 (and VLA-5, to some extent) are inhibited to bind their ligands VCAM-1 and Fn. It is the occupancy of LFA-1 that induces the cross talk (Porter & Hogg 1997).

A somewhat controversial but intriguing aspect was detected when Leitinger and Hogg studied the functions of LFA-1 integrin lacking the I domain, using the Jβ2.7 T cell line. They reported that removal of the I domain induced the expression of the activation epitopes and very efficient activation of VLA-4 and VLA-5. The changes in VLA-4 were not affinity-based, but the
localisation of VLA-4 on the plasma membrane was clearly clustered. The increased adhesion was due to actin rearrangements (Leitinger & Hogg 2000).

Interestingly, a similar of phenomenon, but vice versa (VLA-4 activation increasing LFA-1 activation) was observed in a study by Chan et al. When VLA-4 bound to VCAM-1, LFA-1 binding to ICAM-1 was also enhanced, probably due to clustering of LFA-1 on the cell surface. The activation status of LFA-1 detected by mAb24, did not change and cytochalasin D abolished the increase in binding but did not, however, change the basal binding to ICAM-1. These findings suggest that the VLA-4 activation-dependent increase in LFA-1 ligand binding is dependent on the cytoskeleton and increase in LFA-1 avidity rather than affinity (Chan et al 2000). Interestingly, the phosphorylation of the α₄ chain regulates this transdominant activation effect of VLA-4, because the Ser988Asp mutant (“constant phosphorylation”) that is unable to bind paxillin, is also not capable of signalling to increase the LFA-1/ICAM-1 interactions (Han et al 2003).

Mac-1 has also been shown to be involved in the transdominant regulation of integrins. In neutrophils, the activation of β₁ with an activating antibody leads to activation epitope expression and increased Mac-1-dependent binding to Fn (van den Berg et al 2001). α₅β₃ has also been reported to modulate α₅β₃ ligand binding (Van Strijp et al 1993, Ishibashi et al 1994). The role of Mac-1 in negative regulation of immune responses was dealt with in chapter 3.3. Also pathogens may use the cross-talk to conquer the host cells. In *Bordetella pertussis* (whooping cough) pathogenesis, the bacterium adheres to monocytes/macrophages through a Mac-1 dependent mechanism, but the adhesion seems to be enhanced through a β₁ integrin signalling pathway (Ishibashi et al 1994).

CR4 has also been associated with cross-talk in monocytes. As previously mentioned, CR4 expression is increased on monocytes after a high-fat meal, which enhances, together with VLA-4, monocyte adhesion to aortic endothelium VCAM-1. It has been suggested that the role of CR4 in these monocytes is not necessarily only to bind VCAM-1, but to support the VLA-4/VCAM-1 interactions through cross talk (Gower et al 2011).

### 4.5.2 Other integrins in cross talk

In Glanzmann’s thrombastenia, a mechanism involving integrin cross talk has been suggested. In this disease the platelet aggregation is defective and severe bleeding is detected. In fact, there are two reported pathways that may affect platelet functions. In both models, a mutation (Ser752Pro) in the β₃ chain, that inhibits correct α₂β₁ inside-out activation, affects α₃β₁ integrin binding to collagen. In the model proposed by Riederer and colleagues, binding of fibrinogen to and thus activation of α₄β₁, leads to inhibition of α₅β₁-mediated collagen binding. van de Walle et al on the other hand have reported a positive regulation initiated by ligand binding to α₄β₁ and resulting in enhanced collagen binding by α₅β₁ (Riederer et al 2002, Van de Walle et al 2007, Gonzalez et al 2010).

Early reports indicated that also the ligation of α₅β₁ is able to regulate α₃β₁ functions. Initially, it was shown that when the phagocytosis through α₅β₁ was blocked, α₃β₁-mediated phagocytosis was also inhibited. The mechanism of this cross talk has been studied quite thoroughly and the suppression was first reported to take place through PKC, and later CamKII was shown to be the major signal mediator between the two integrins (Calderwood et al 2004). It seems that CamKII, that is essential for α₅β₁-dependent phagocytosis and migration in a normal situation, is inhibited upon α₅β₁ ligation (Blystone et al 1994, Blystone et al 1995, Blystone et al
\( \alpha_\nu \beta_3 \) integrin ligation has been shown to enhance VLA-4 (\( \alpha_4 \beta_1 \)) dependent migration on VCAM-1, followed by rapid extravasation and migration under inflammatory conditions (Imhof et al 1997). Also LFA-1 binding to ICAM-1 has been reported to be downregulated upon \( \alpha_\nu \beta_3 \) activation (Weerasinghe et al 1998).

Yet another situation where cross-talk has been reported is the fibronectin binding in cells expressing \( \alpha_{\text{in}} \beta_3 \) and \( \alpha_\nu \beta_1 \). When \( \alpha_{\text{in}} \beta_3 \) is not activated, binding takes place through \( \alpha_\nu \beta_1 \). But when \( \alpha_{\text{in}} \beta_3 \) is ligated and its conformation changes, Fn binding is blocked and spreading on Fn is reduced (Diaz-Gonzalez et al 1996).

Recently \( \alpha_\nu \) integrins have been shown to control each other in the specific situation of glaucoma that was studied in human trabecular meshwork cells (HTM). Researchers studied phagocytosis mediated by \( \alpha_\nu \beta_5 \) and FAK, that is critical for the maintenance of the physiological conditions in the eye. Activation of \( \alpha_\nu \beta_5 \) that may happen upon steroid treatments inhibits this phagocytosis and may lead to glaucoma (Gagen et al 2013).

The molecular mechanisms of these cross-talk events have been studied to some extent, but no common factor has been found. For example in some cases the affinity of the target integrin is changed, whereas in others, the enhancement of adhesion is due to avidity changes, i.e. integrin clustering on the cell surface. The signalling cascades leading to inhibition or activation of another integrin have been reported in only some cases. Talin sequestration by the inhibiting integrin has been suggested to be the mechanism of cross talk control in the case of \( \alpha_{\text{in}} \beta_3 \) and \( \alpha_\nu \beta_1 \) integrins (Calderwood et al 2004). In another situation, when \( \beta_1 \) controls laminin binding through \( \alpha_\nu \beta_3 \), talin doesn’t seem to play a restrictive role. Instead, a signalling cascade initiated by PKA activation and leading to inhibitor-1 phosphorylation, inhibition of protein phosphatase-1 and sustained phosphorylation of \( \beta_3 \), has been reported to control the cross-talk (Gonzalez et al 2008).
5 Aims of the study

Adhesion events of blood cells are essential for their function and require constant regulation. The purpose of these studies was to define mechanisms behind the multi-step adhesion events of leukocytes and red blood cells. The specific aims were:

1. To analyse the interactions between erythrocyte ICAM-4 and leukocyte integrin CR4 (αxβ2) and to characterise the binding between ICAM-4 and LFA-1 (αβ2), Mac-1 (αβ2) and CR4 (αβ2).

2. To identify the phosphorylation site of CR4 and characterise the role of the phosphorylation in the functions of the integrin.

3. To determine the molecular mechanisms leading to cross-talk or transdominant regulation between β2 integrins and VLA-4.
6 Experimental procedures

Materials and methods used in this study have been described in detail in the original publications.

<table>
<thead>
<tr>
<th>Experimental procedure</th>
<th>Original publication</th>
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<tr>
<td>Antibodies</td>
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</tr>
<tr>
<td>Cell adhesion assays (shear flow)</td>
<td>IV</td>
</tr>
<tr>
<td>Cell adhesion assays (static)</td>
<td>I II III IV</td>
</tr>
<tr>
<td>Cell lines and cell culture</td>
<td>I II III IV</td>
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<tr>
<td>Co-immunoprecipitation</td>
<td>IV</td>
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<tr>
<td>Erythrophagocytosis assay</td>
<td>II</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>I II III IV</td>
</tr>
<tr>
<td>Immunofluorescence microscopy</td>
<td>IV</td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td>III IV</td>
</tr>
<tr>
<td>Migration assays</td>
<td>IV</td>
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<td>Pep-spot assay</td>
<td>II</td>
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<tr>
<td>Peptide transfections</td>
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<td>Phagocytosis assay</td>
<td>III</td>
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<td>Production of point mutations</td>
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<tr>
<td>Protein production and purification</td>
<td>I II</td>
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<tr>
<td>Protein purification from buffy coat cells</td>
<td>I II</td>
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<td>Radioactive labelling of cells</td>
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<tr>
<td>Solid-phase ELISA assays</td>
<td>I II</td>
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<td>Three-dimensional protein modelling</td>
<td>II</td>
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<tr>
<td>Transfection</td>
<td>III</td>
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</tbody>
</table>
7 Results and discussion

7.1 Interactions of ICAM-4 with leukocyte integrins (I & II)

7.1.1 CR4 is a new ICAM-4 binding partner (II)

β2 integrins LFA-1 and Mac-1 had previously been reported to bind to erythrocyte ICAM-4, but the results concerning the interaction between CR4 and ICAM-4 were controversial. In a previous report, the adhesion of various types of leukocytes (B cells, NK cells and monocytes) to purified LW/ICAM-4 was indeed shown to be partly CR4-dependent, but in the same paper, red cells failed to adhere to purified CR4 integrin (Bailly et al 1995). We set out to study the interaction. In the present paper (II), we used a different purification method for CR4, one that has been shown to yield functionally active CR4 protein (Stacker & Springer 1991). It is possible that the purification procedure used previously was not suitable for preserving integrin activity. This is especially important as CR4 has been shown to be resistant to activation and to preferably stay in the inactive state (Bilsland et al 1994, Zang & Springer 2001). Here, we found that CR4, when purified according to the protocol of Stacker & Springer, or expressed in L cell transfectants, was able to bind to ICAM-4. The interaction was characterized in a variety of experiments. First, adhesion assays with red cells binding to purified CR4 coated on plastic were performed in 96-well plates. This adhesion was ICAM-4 and CR4 specific, as shown with inhibiting antibodies against ICAM-4, αX and β2. Next, L cells stably transfected with ICAMs were allowed to adhere to purified integrins and also the interaction between ICAM-4 transfectants and CR4 was specific (II fig 1). Third, the interactions were analyzed in a solid-phase ELISA assay, where purified proteins were allowed to interact on a 96-well plate. ICAM-4 was found to directly bind to LFA-1, Mac-1 and CR4 in a dose-dependent fashion. Again, the specificity of the interaction was verified with antibody inhibition (II fig 2). Fourth, the adhesion of L cells stably transfected with CR4- to purified ICAM-4 was studied and found to be specific (II fig 3a-b).

The finding that ICAM-4 binds to leukocyte integrin CR4, was in a way a return back to the primary findings (Bailly et al 1995). The fact that CR4 is a ligand for ICAM-4 is not surprising as such, as it shares many ligands with Mac-1 and LFA-1. Anyhow, interesting differences between the binding properties could be discerned when comparing the binding of ICAM-4 to the three β2 integrins, as will be discussed in the following section.

7.1.2 Characterisation of the interactions between ICAM-4 and CR4 (II)

The Ig domains and amino acid residues on ICAM-4 responsible for LFA-1 and Mac-1 binding as well as those needed for interactions with αIIbβ3 and αV-integrins have been determined (Hermand et al 2000, Mankelow et al 2004, Hermand et al 2004). We continued this work and characterised the ICAM-4 domains and amino acid residues responsible for CR4/ICAM-4 interactions. This was studied in an adhesion assay, where CR4-transfectants were allowed to adhere to purified wild type, truncated or point mutated ICAM-4-Fc protein. The results show that CR4 needs both Ig domains (the outermost D1- and the membrane proximal D2-domain) of ICAM-4 for maximal binding, even though domain 2 alone binds CR4 to a lesser extent (II fig 4a). This was also supported by mutation studies, where the amino acids needed for binding were shown to be located in both domains as expected. The following amino acid were found to be essential for binding (see three dimensional construction of ICAM-4 in chapter 2.2.2): Trp19 on the A strand of D1, Arg52 on the C strand of D1, Trp77 on the EF loop of D1 and Thr91,
Trp93 and Arg97 on the G strand of D1. On D2, Glu166 on strand E was needed for efficient binding (II fig 4b).

The domain deletion and amino acid substitution studies were further confirmed using synthetic peptides derived from domains 1 and 2 (P-D1 and P-D2, respectively). The specific peptide sequences were determined based on a PepSpot assay, where purified CR4 integrin selectively bound to two sequences, one from each domain (II fig 6a). Both peptides were 13-mers, and P-D1 contained the Arg52 and P-D2 included the Glu166 that were both found to be essential for the binding in the previous assay. Both peptides bound to CR4-transfected cells and were able to inhibit CR4-transfectant binding to ICAM-4-Fc, although they yielded the maximal inhibition only when used together (II fig 6B-C). The peptides have been illustrated in the model of ICAM-4 figure 13.

Figure 13. Model of ICAM-4 based on ICAM-2 three-dimensional structure. The peptides P-D1 (green) and P-D2 (red) binding to CR4 are shown. From Ihanus et al 2007.

The results with amino acid point mutations in the ICAM-4 extracellular part continue the set of reports where the exact binding sites of different integrins on ICAM-4 have been elucidated (Hermand et al 2000, Mankelow et al 2004, Hermand et al 2004). The domains and residues involved in the binding (table 1) show interesting differences between the different integrins. A model of ICAM-4 is shown in figure 13. All integrins studied, except LFA-1, need both domains of ICAM-4 for maximal binding. All the β2 integrins seem to bind to the CFG-face of the first domain, whereas the binding site of αmβ3 is on a spatially distinct location, in the AB-face of the domain 1. αmβ3 binds to the BE-strands of D1, to an area that is adjacent to, but not much overlapping with the αmβ3 binding site. αβ1 and αβ5 integrin binding sites encompass the AG-face of domain 1 with an input from the B strand. When looking at D2, most binding sites reside in the proximity of D1. The C’E loop of domain 2 is used by Mac-1, αmβ3 and αβ5. αβ1
and $\alpha_v\beta_3$ need Lys118 on the B strand and CR4 needs the Glu166 that resides in the bottom of the E strand, fairly distant from D1.

Table 1. Binding sites of reported ICAM-4 binding integrins on domains 1 and 2 of ICAM-4. (Adapted from Toivanen et al 2008).

<table>
<thead>
<tr>
<th>ICAM-4 domain</th>
<th>Integrin</th>
<th>$\alpha_1\beta_1$</th>
<th>$\alpha_1\beta_2$</th>
<th>$\alpha_1\beta_3$</th>
<th>$\alpha_1\beta_4$</th>
<th>$\alpha_1\beta_5$ &amp; $\alpha_1\beta_5$</th>
<th>ICAM-4 strand/loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W19A</td>
<td>W19A</td>
<td>W19A</td>
<td>F18A</td>
<td>W19A</td>
<td>A-B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q30A</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>K33A</td>
<td>K33A</td>
<td></td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R52A/E</td>
<td>R52A/E</td>
<td>R52A/E</td>
<td>R52A/E</td>
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<tr>
<td></td>
<td></td>
<td>W77A/F</td>
<td>W77A/F</td>
<td>W77A/F</td>
<td>W77A/F</td>
<td>W77A/F</td>
<td>E-F</td>
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<tr>
<td></td>
<td></td>
<td>L80G/F</td>
<td>L80G/F</td>
<td>L80G/F</td>
<td>R92E</td>
<td></td>
<td>F</td>
</tr>
<tr>
<td>D2</td>
<td></td>
<td>R97A/E</td>
<td>R97A/E</td>
<td>R97A/E</td>
<td>R97A/E</td>
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<tr>
<td></td>
<td></td>
<td>K118E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E151A/K</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>T154V</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>E166A</td>
<td></td>
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Especially the CR4 binding site on ICAM-4 encompasses almost the whole length of the molecule, which suggests a reasonably large area of integrin making contact with ICAM-4. This is further confirmed by adhesion assays in the presence of CR4 I domain specific adhesion-blocking antibody 3.9. This antibody efficiently inhibits CR4 binding to D1 of ICAM-4, but it is not as efficient in inhibiting adhesion to D2, suggesting CR4 I domain interaction with D1 of ICAM-4 and another integrin part binding to D2. Another result strengthening this idea is that the peptides from both ICAM-4 domains (P-D1 and P-D2) were needed to obtain maximal inhibition between ICAM-4 and CR4, suggesting two distinct binding sites for ICAM-4 on CR4. Co-crystallisation studies with CR4 and ICAM-4 would be required to confirm this data.

The integrin-binding Ig-domains of ICAM family members share approximately 30% of amino acid homology. When comparing the amino acids and domains responsible for ligand binding in ICAM-4 to the other ICAMs, some interesting observations can be made. First, all the other ICAM family members have a glutamate and a glutamine in positions that are highly important for ligand binding (Glu34 and Gln73 in ICAM-1). However, ICAM-4 has an arginine.
and a threonine in these positions (Arg52 and Thr91), and mutating them “back” to glutamate and glutamine, respectively, does not improve ligand binding (Hermand 2000). It is also interesting to note that these amino acids are not used by all ICAM-4-binding integrins. Second, the integrin binding domains on other ICAMs do not encompass more than one domain, the outermost Ig-domain being the most popular. Only Mac-1 makes an exception as it binds to the third domain of ICAM-3 (Diamond 1991).

### 7.1.3 ICAM-4 binds to β2 integrin I domains (I & II)

The I domain is the major binding site of ligands in all α chain I domain containing integrins. In I-less integrins the β chain I-like domain is critical for ligand binding (see chapter 3.2). ICAM-4 has been reported to bind both to integrins containing the α chain I domain (LFA-1, Mac-1 and CR4) (Hermand et al 2000) and to integrins without I domain (αx, integrins and αxβγ) (Hermand et al 2004, Mankelow et al 2004). In original articles I and II the binding of ICAM-4 to I domains of LFA-1, Mac-1 and CR4 was confirmed. The ICAM-4 binding to LFA-1 and Mac-1 was studied in detail with red cells (I fig 1 and 2), ICAM-transfectants (I fig 4-7) and in a solid phase assay with purified proteins (I fig 10). The binding was shown to be specific, as shown with antibodies against ICAM-4 and the I domains (I figs 1B, 1D, 6 and 7) and by inhibition of the binding with purified proteins (I figs 2, 4 and 11). According to the results obtained in a solid phase assay, the αL I domain is not able to block αM I domain binding to ICAM-4, even though soluble αL I domain inhibits ICAM-4/αL I domain binding to some extent (I fig 11). This suggests that the binding sites on these two integrins are partly overlapping but not identical.

ICAM-1 and ICAM-2 transfectants were also used in the cell adhesion assays, and their binding to LFA-1 and Mac-1 was characterised in a similar way as that of ICAM-4 (I figs 4-7). When comparing the interactions between ICAM-1, ICAM-2 and ICAM-4 to the I domains of LFA-1 and Mac-1, certain distinctions could be made. These differences were studied in adhesion assays performed in the presence of a variety of antibodies against these three ICAMs as well as the I domains of αL and αM. We noticed that the I domains of the two integrins do not necessarily bind to the same region in a certain ICAM molecule, and, similarly, the three ICAMs studied here do not bind to exactly the same site on the I domains (I fig 6 & 7). These results could be explained by relatively low homology between αL and αM sequences (35%, also in I domain area) and between different ICAMs (approximately 30 %) (Harris et al 2000, Gahmberg et al 1997).

The role of the CR4 I domain in binding was verified in an adhesion assay where the active CR4 I domain, containing the activating mutation Ile314Gly efficiently inhibited CR4/ICAM-4 interaction (II fig 3D).

### 7.1.4 Interactions between ICAM-4 and leukocyte integrins require divalent cations (I & II)

As integrin adhesion is cation-dependent (see chapter 3.2), the requirements for cations in integrin-ICAM-4 interactions were studied next. LFA-1 and Mac-1 adhesion to ICAM-4 has previously been shown to be dependent on magnesium and calcium, and to be inhibited with removal of Ca^{2+} by EGTA (Hermand et al 2000). The same cations were shown to be partly responsible for CR4 binding as well, but maximal binding was obtained in the presence of Mg^{2+}, Ca^{2+} and Mn^{2+}. Even Mn^{2+} alone showed higher binding than the combination of Mg^{2+} and Ca^{2+} (II fig 3C). The binding of ICAM-4 transfectants to purified LFA-1 and Mac-1 I domains also
required Mn\(^{2+}\) for maximal binding, (I fig 8), whereas RBC show high levels of adhesion with a combination of Mg\(^{2+}\) and Ca\(^{2+}\) (I fig 9) and also with Mn\(^{2+}\) (data not shown).

These results are in accordance with several previous reports of integrins requiring divalent cations for binding to their ligands. But contrary to the report of Dransfield et al, who showed that Mg\(^{2+}\) or Mn\(^{2+}\) are needed for efficient LFA-1/ICAM-1 interaction whereas Ca\(^{2+}\) inhibited the interaction (Dransfield et al 1992), in our experiments calcium did not show total inhibition of the adhesion. One has to keep in mind that there are differences between the experimental design of these reports. Dransfield and colleagues studied LFA-1 in T cells, whereas Hermand et al and us (Dransfield et al 1992, Hermand et al 2000) performed the experiments with transfected L cells. As for the I domains, the cation-dependency experiments were performed with ICAM-4 transfected cells adhering to purified I domains coated on plastic (I).

It is also important to note that the results of the activating effect of Mn\(^{2+}\) by us and others have been obtained in the presence of more than a thousand-fold concentration of Mn\(^{2+}\) compared to the normal levels in human blood (Milne et al 1990).

**7.1.5 The role of ICAM-4 in erythrophagocytosis (II)**

The *in vivo* function of the interactions between ICAM-4 and leukocyte integrins was studied in an erythrophagocytosis assay, where monocytes were differentiated into macrophages, incubated with RBC, and phagocytosis of RBC was detected. Antibodies against LFA-1, Mac-1 and CR4 as well as anti-ICAM-4 efficiently blocked erythrophagocytosis (II fig 7). Similar studies have later been performed with erythrocytes of different ages. As expected, the oldest red cells were the most prone to be phagocytosed and, in agreement with our results (II), erythrophagocytosis could be inhibited by antibodies against β\(_2\) integrins. Interestingly, different β\(_2\) integrins seemed to be responsible for erythrophagocytosis of RBC of different ages, most efficient inhibitor of senescent RBC phagocytosis being CR4. Younger cells were phagocytosed most efficiently by Mac-1 (Toivanen et al 2008).

**7.1.6 Possible roles of ICAM-4/integrin interactions in red cell life cycle**

Leukocyte integrins LFA-1, Mac-1 and CR4 are clearly important ligands for ICAM-4 and the results from these studies (I & II) and those published later (Toivanen et al 2008) suggest that one of the signals leading to senescent RBC removal by spleen macrophages could be ICAM-4 interaction with CR4 and other β\(_2\) integrins. Even though erythrophagocytosis has been extensively studied (see chapter 1.1.2), a lot of controversy still remains. Perhaps there is no single factor causing it, but rather the recognition and engulfment of senescent RBC is a multi-factor sum of different changes in cell composition. One plausible mechanism could be that the loss of sialic acids from cell surface glycoproteins and thus reduction of the overall negative charge of the RBC could enable the senescent cells to come to a closer contact with the macrophages. This would then further allow more intimate interactions between phagocytosis receptors like CR4 and their ligands on RBC. Further studies on the mechanisms of erythrophagocytosis are required to show the importance of ICAM-4 and leukocyte integrins in these events.

The role of other than β\(_2\)-family binding partners of ICAM-4 should not be omitted when looking for the candidates responsible for ICAM-4 mediated erythrophagocytosis. However, to our knowledge, most of them have not been implicated in phagocytosis. α\(_6β\(_3\) has been shown capable of phagocytosis in the context of eye, but its function there is highly specific, maintaining
homeostasis of trabecular meshwork in order to avoid glaucoma (Gagen et al 2013, Dupuy & Caron 2008).

7.2 Phosphorylation of αX chain is important for CR4 functions

7.2.1 CR4 α-chain is phosphorylated on serine 1158

Phosphorylation of leukocyte integrin alpha chains, although not induced by cell activation, has been shown to be highly important in the functions of integrins LFA-1 and Mac-1 (Fagerholm et al 2005, Fagerholm et al 2006). CR4 has also been shown to be phosphorylated on serine (Chatila et al 1989, Buyon et al 1990), but the exact phosphorylation site has not been identified. In order to specify the site(s) of serine(s) to be phosphorylated, we created serine to alanine point mutations in the two serines of the αx chain cytoplasmic part. These constructs were transfected into COS-1 cells and the cells were labelled with radioactive 32P. Integrins were immunoprecipitated and phosphorylation of the αx chain was detected. The primary phosphorylatable serine revealed to be Ser1158, although possibly some trace phosphorylation could be seen also when Ser1158 was mutated to alanine (Fig 1A). Based on that finding, we made stable transfectants of K562 cells expressing wild type (wt) αxβ2 or the phosphorylation mutant Ser1158Ala-αxβ2 (Fig 1C). The sequences and reported phosphorylation sites of different integrin α chains are shown in figure 9 and, as already mentioned, they are very heterologous in sequence and in structure, especially in the areas of phosphorylation.

7.2.2 αX chain phosphorylation is vital for ligand binding

To study the effect of αx chain phosphorylation on cell adhesion, we performed adhesion assays where K562 cells transfected with wt CR4 or Ser1158Ala-CR4 were allowed to adhere to complement component iC3b coated on plastic. These experiments showed that the Ser1158Ala mutation reduced the adhesion of CR4 transfectants to the background level. Adhesion was CR4-specific as it could be inhibited with antibodies against αx and β2 (Fig 2a). To verify the importance of Ser1158 over the other possible phosphorylation sites, we performed adhesion assays also with HEK293T cells transiently transfected with the Ser1161Ala-mutated CR4. We found that the Ser1161Ala mutation does not disturb adhesion, so Ser1158 seems to be the only or at least the most relevant serine controlling integrin adhesion (Fig 2B). Phosphorylation of these α chains is critical for the functions of LFA-1 and Mac-1, but also differences between the three α chains reported so far can be discerned. In LFA-1, the αL chain phosphorylation affects ligand binding when the cells are activated with activating antibody MEM83 or Mg2+, but not when activated with TCR ligation or phorbol esters. The role of different activation methods will be further discussed in next chapter. Also binding of Mac-1 to ICAM-1 or ICAM-2 is inhibited if the phosphorylation site is mutated to alanine (Ser1126Ala) but it is still able to bind some of its
ligands, such as iC3b and dBSA even without a phosphorylatable serine. Interestingly, binding to these two ligands was totally abolished in cells expressing the phosphorylation-mutant CR4 (III fig 2a). iC3b binding to integrins has been characterised reasonably well, and a very detailed picture of iC3b/CR4 binding has been received with negative-stain electron microscopy (Chen et al 2012). I domain is the major binding site of iC3b on CR4 as it has been reported to be on Mac-1 as well (Diamond et al 1993). However, other regions on Mac-1 may contribute (Xiong et al 2002, Li & Zhang 2003). These findings suggest that Mac-1 and CR4 use different, α-chain phosphorylation-independent and -dependent mechanisms for iC3b binding regulation, respectively. This kind of regulation could enable the cell to use these phagocytosis receptors in different conditions, depending for example on cell activation status.

Also the replacement of the phosphorylatable serine with a negatively charged aspartate results in different outcomes in LFA-1 and CR4. The Ser1140Asp mutation on α chain inhibits adhesion in a similar fashion as the Ser1140Ala-mutation, whereas in the α chain the aspartate seems to mimic phosphorylation and does not affect ligand binding (Fagerholm et al 2005). We also verified the importance of Thr758 of the β chain in CR4 functions, as β-chain phosphorylation in CR4 had not been reported. Phosphorylation of Thr758 (along other phosphorylation sites) has been shown to be crucial for LFA-1 functions (Valmu & Gahmberg 1995), but Ser756 is essential for Mac-1 dependent phagocytosis (Lim et al 2011). And at least Thr758 is needed for CR4 functions.

When comparing the differences, it is worth noting that the studies of LFA-1 and Mac-1 have been made in leukemic T cell line Jurkat or its derivative Jβ2.7 (Weber et al 1997) and the CR4 phosphorylation was studied in K562 cells that are quite primitive erythroid/myelomonocytic precursor cells (Andersson et al 1979).

7.2.3 Effects of αX chain phosphorylation on inside-out and outside-in signalling

To study the effects of Ser1158 phosphorylation on inside-out signalling, we transfected the cells with constitutively active Rap1 (Rap1V12, where amino acid in position 12 has been replaced with a valine) that has been shown to activate LFA-1 (Fagerholm et al 2005). Phosphorylation of Ser1158 was found to be needed for proper inside-out activation of the integrin, as the Rap1V12 did not increase Ser1158Ala adhesion to iC3b (III fig 2D). On the contrary, when studying outside-in signalling, we did not find differences between wild type and phosphorylation-mutant CR4. This was observed in two experiments, first in an adhesion assay where activation was induced with a β2-binding activating antibody. In the second assay Syk phosphorylation, which is known to take place downstream of outside-in activation of integrins, was studied by immunoprecipitation. When phosphorylated Syk was detected, there were no differences between wild type and Ser1158Ala-mutated CR4. So we conclude that the α chain phosphorylation does not affect outside-in signalling from CR4 (III fig 2C & 2F).

The inside-out signalling of LFA-1, when studied with Rap1V12 transfection, showed similar dependence on α-chain phosphorylation as CR4 (Fagerholm et al 2005). Corresponding studies have not been made with Mac-1, nor has the α-chain phosphorylation in signalling events been studied in LFA-1 or Mac-1.

One important tool in integrin activation studies are the activating or activation epitope recognising antibodies. When activated with monoclonal antibody MEM83 (or with Mg2+), LFA-1 binding to ICAM-1 is dependent on α chain phosphorylation, whereas induction of
ligand binding with TCR ligation or phorbol esters, that mimick the functions of PKC, is not αL chain phosphorylation-dependent (Fagerholm et al 2005). Interestingly, the activating antibody CBR LFA-1/2 was able to activate CR4 despite of the phosphorylation status (III fig 2C). Antibodies detecting the integrin activation status used in these studies were mAb24 that recognises the extended, open conformation and KIM127 that binds to extended integrin. LFA-1 αL chain phosphorylation mutant is not able to adopt the fully active conformation detected with mAb24 when activated with ICAM-2 (soluble LFA-1 ligand) or SDF-1α (chemokine) (Fagerholm et al 2005). Phosphorylation mutant Mac-1 (αM-Ser1126Ala) is not able to support maximal binding of either of the two antibodies (Fagerholm et al 2006). Interestingly, CR4 binding to activation epitope recognising antibodies was not greatly affected upon phosphorylation site mutation (data not shown).

On the basis of these and other results, discussed in chapter 4 (Regulation of integrin activity), it is intriguing to search for a molecular mechanism that would explain the roles of α- and β-chain phosphorylations in integrin activation and signalling. Different models for the specific functions of α and β chains and their phosphorylations in the integrin signalling have been proposed. According to Fagerholm et al (Fagerholm et al 2005), the role of αL phosphorylation would be needed for the affinity change and β2 would function as an avidity regulator. On the other hand, it has been suggested that the role of αL-interacting -module would regulate the outside-in activation of the integrin, whereas the ADAP/SKAP55/RIAM-module, interacting with β2 chain, would be needed in the inside-out activation (Kliche et al 2012). When keeping in mind that the affinity changes are thought to happen as a result of inside-out signalling, and that clustering and changes in avidity have been reported to occur after ligand binding (and thus being the outcome of outside-in signalling) (Kim et al 2004) it is clear that more studies are needed to specify the role of intracellular phosphorylations in these events. It is also likely that the phosphorylation and other regulation events of inside-out and outside-in are intertwining, and it may be next to impossible to dissect the activation cascades with this kind of classification.

7.2.4 Effect on αX phosphorylation on phagocytosis

Finally, we set up a phagocytosis assay with PMA-activated K562 cells to elucidate the role of α-chain phosphorylation on phagocytosis. The experiments show that αL chain phosphorylation on Ser1158 is indispensable in CR4-mediated phagocytosis (III fig 2E). As a complement receptor, CR4 has been indicated in pathogen phagocytosis as well as apoptotic cell removal (Schlesinger & Horwitz 1991, Hirsch et al 1994, Mevorach et al 1998). However, the molecular mechanisms involved in CR4 mediated phagocytosis have not been studied in detail. Mac-1 signalling mechanisms have been thoroughly studied and reports (Lim & Hotchin 2012) show similarities but also discrepancies, when compared to LFA-1 signalling cascades, as discussed in chapters 3.4 and 4. Some examples of the differences are the immunosuppressive role of Mac-1, the importance of phosphorylation of β2 chain Ser756, and the independence of RIAM signalling. Instead, Mac-1 uses only RAPL for Rap1-mediated activation. Along these lines, further studies on CR4 signalling mechanisms probably reveal interesting distinctions between the signalling routes used by the Mac-1 and CR4.
7.2.5 Why is this important?

For a long time, CR4 has been considered a marker of DC, but recent studies have shown its role in the adhesion of monocytes to the endothelium after a high-fat meal (Gower et al. 2011), in adipose tissue inflammation associated with obesity (Wu et al. 2010) and in antigen uptake by DC (Ejaz et al. 2012). These functions are involved in atherosclerosis, insulin resistance and, on the other hand, in the exciting development of new therapies against cancer. These are probably some of the major health problems of the modern civilisation. Therefore, it is of high importance to learn the molecular mechanisms involved in the regulation of these events, in order to develop specific, targeted cures against these diseases. Perhaps the highly specific nature of phosphorylation of the leukocyte integrin α-chains could serve as a target for future therapies aimed to activate or inhibit specific leukocyte functions.

7.3 Mechanism of trans-dominant inhibition between β2 integrins and VLA-4 (IV)

Already when studying CR4 phosphorylation we noticed that, instead of inducing adhesion, the expression of functional CR4 on K562 cells inhibited binding to VCAM-1. Interestingly, cells expressing the αX chain phosphorylation mutant (CR αX-S/A) bound strongly to VCAM-1, just like cells not transfected with CR4 (IV fig 3D). This lead to further studies on transdominant inhibition of integrins and we observed that Jβ2.7 cells expressing LFA-1 bound less to VCAM-1 (that is not an LFA-1 ligand) than the cells that did not have LFA-1. Binding of Jβ2.7 cells to VCAM-1 was found to be VLA-4 and VCAM-1 dependent and VLA-4 is the only VCAM-1 receptor in Jβ2.7. Jurkat (of which the Jβ2.7 cells are derived) is a well-known cell line used widely in T cell and leukocyte integrin studies and their signalling cascades and activation methods have been extensively reported. Jβ2.7 are also more prone to adhere, migrate and spread than K562, which makes it easier to detect the outcome of specific activations or inhibitions. All these facts led us to study the transdominant inhibition in Jβ2.7 cells. Trans-dominant inhibition of VLA-4/VCAM-1 binding by LFA-1 activation in T cells was reported already almost twenty years ago (Porter & Hogg 1997), but the mechanism of this regulation have not been elucidated. In the original article IV we propose a mechanism of this regulation.

7.3.1 VLA-4 binding to VCAM-1 is blocked by activated β2 integrins

Jβ2.7, which is an αL chain-deficient derivative of the parental Jurkat T cell line and lacks LFA-1, adhere strongly to VCAM-1 through VLA-4 integrin. This adhesion was inhibited when LFA-1 was expressed in the cells and even more efficiently so, when LFA-1 was activated with chemokine SDF-1α. The inhibition was seen in static adhesion assays as well as in flow adhesion on purified VCAM-1 and on activated endothelium (IV fig 1).

Jurkat cells have been reported to adhere efficiently to VCAM-1 through VLA-4 (Manevich et al. 2007) and this strong adhesion was also seen in the migration assays we performed. Without treatment, the cells lacking LFA-1 were not able to transmigrate in a Transwell assay, but stayed bound on the filter coated with VCAM-1. The strong adhesion was released with antibodies against αL chain of VLA-4 and with the expression of functional LFA-1. Also the retraction of the uropod at the rear of the migrating cell was impaired (IV fig 2).
7.3.2 LFA-1/CR4 phosphorylation is essential for transdominant inhibition

To begin the elucidation of the signalling mechanisms behind this phenomenon, we studied how the α-chain phosphorylation mutant of LFA-1 regulated the VLA-4 adhesion. The cells bearing the Ser1140Ala-mutation in the LFA-1 chain were not able to detach the uropod but left long extension behind them while migrating on VCAM-1, just like the cells lacking LFA-1. In a similar way, the cells expressing the Ser1140Ala-mutant adhered tightly to VCAM-1 thus inhibiting the migration (IV fig 3A-C). As already mentioned, the same phenomenon was seen in CR4-K562 transfectants, where the expression of wt CR4, but not that of α-chain phosphorylation mutant Ser1158Ala-CR4, inhibited the VLA-4-mediated adhesion to VCAM-1 (IV fig 3D).

Interestingly, the effect of αL chain phosphorylation is dependent on the activation method. In cells activated with SDF-1α, the phosphorylation of the αL chain is essential for the transdominant inhibition to occur, whereas activation through TCR and outside-in activation with ligand binding or activating antibody are independent of α-chain phosphorylation (IV fig 4A-B). To find out if the transdominant inhibition is related to the conformation of LFA-1, we studied the expression of activation epitopes with antibodies KIM127 (that recognises the extended integrin) and mAb24 (extended, open conformation). It was shown that the KIM127 epitope was constitutively expressed on cells regardless of phosphorylation or activation, whereas mAb24 only recognised wt LFA-1 (and not Ser1140Ala-mutant) and only when activated with SDF-1α, anti-CD3 or ligand binding (IV fig 4C-D). These results are in accordance with the previously reported α phosphorylation studies, where SDF-1α could not induce full activation of αL chain phosphorylation mutant LFA-1 but TCR ligation could. On the other hand, Fagerholm et al did not see mAb24 epitope expression following soluble ICAM-2 activation in the Ser1140Ala mutants, whereas our results show that the Ser1140Ala mutant is both expressing the mAb24 epitope and capable of ICAM-induced transdominant inhibition (Fagerholm et al 2005). The phosphorylation of the β2 chain is induced when the cells are activated with SDF-1α or through TCR ligation, indicating that β2 phosphorylation may be needed for this signalling (IV fig 4F).

Next, we went on to study the effects of β2 chain phosphorylation on transdominant inhibition. The Jβ2.7 cells lacking LFA-1 were transfected with β2-chain cytoplasmic peptide where Thr758 is phosphorylated (pβ2). This transfection was enough to induce transdominant inhibition, i.e. decrease in VCAM-1 binding (IV fig 5A). This clearly shows that, even if the α-chain is needed for the activation of the integrin with some activation methods, the phosphorylated β2 is needed for signalling to inhibit VLA-4/VCAM-1 interaction.

7.3.3 Signalling through 14-3-3, Tiam1 and Rac-1 leads to VLA-4 inhibition

An important signalling cascade, involving 14-3-3 protein binding to the phosphorylated β2 tail and subsequent activation of Tiam1 leading to Rac-1 activation and actin cytoskeleton rearrangements has been reported (Gronholm et al 2011). To test if this pathway was involved in transdominant inhibition of VLA4, we treated the cells with 14-3-3 blocking peptide or Tiam1-inhibitor prior to flow adhesion assays. Indeed, pβ2-induced transdominant inhibition was overridden with these inhibitors, suggesting that 14-3-3 and Tiam1 are essential for the transdominant inhibition to occur (IV fig 5B-C). The interaction of 14-3-3 with β2 tail upon cell activation was also studied and, in consensus with previous reports (Gronholm et al 2011) and results presented above, the 14-3-3 binding to β2 chain is attenuated when LFA-1 is not fully activated, as in the case of the α-chain phosphorylation mutant after activation with SDF-1α (IV fig 5D-F). As discussed in chapter 4.3, Rac-1 is an important GTPase regulating...
cytoskeletal rearrangements, which invites to hypothesize on the role of the actin cytoskeleton in the regulation of VLA-4 by LFA-1. Indeed Rac-1 inhibitor abolished cell adhesion and migration of all three cell lines (Jβ2.7 without LFA-1, with wt LFA-1 and with LFA-1 S/A), so the input of Rac-1, specifically downstream of LFA-1 in the transdominant inhibition could not be studied.

### 7.3.4 LFA signalling leads to changes in VLA4 phosphorylation and complex formation

To figure out what the consequences of these signalling events are on VLA-4, we elucidated the phosphorylation status of both α4 and β1 chains of VLA-4 after LFA-1 activation. Interestingly, phosphorylation of the α4 chain was similar in all three cell lines (Jβ2.7 lacking LFA-1, expressing wt LFA-1 and expressing LFA-1 Ser1140Ala), even though Ser988 phosphorylation has been shown to be essential in dynamic adhesion events through the regulation of paxillin binding (Han et al 2001). On the other hand, β1 phosphorylation was inhibited in the cells expressing wt LFA-1, whereas in Jβ2.7 or LFA-1 S/A cells, β1 was clearly phosphorylated on Thr788/789.

Filamin, a negative regulator of integrin activation (Takala et al 2008), only bound to VLA-4 when wt LFA-1 was present and activated, whereas 14-3-3ζ binding to the α4 chain was decreased in LFA-1 expressing cells (IV fig 6A). And, on the contrary, when LFA-1 was activated, it bound 14-3-3 whereas in the resting state it interacted with filamin (IV fig 6B). Based on these results, we propose that filamin and 14-3-3ζ alternate in binding to LFA-1 and VLA-4, and regulate the activation state of the integrins in this way. 14-3-3ζ is known to bind efficiently to the phosphorylated β2 tail and to regulate the subsequent signalling events through Tiam1 and Rac1 (Nurmi et al 2007, Gronholm et al 2011). 14-3-3ζ also binds to VLA-4 α4 chain when it is phosphorylated on Ser978. When bound, it participates in α4 – 14-3-3ζ –paxillin complex formation that activates Cdc42 at the leading edge of a migrating leukocyte (Deakin et al 2009). Recently the affinity of phosphorylated β2 towards 14-3-3ζ has been found to be higher than that of phospho-α4. The β1 chain was also reported to bind 14-3-3ζ, but this interaction does not require phosphorylation and is of lower affinity than phospho-α4 or phospho-β2 binding (Bonet et al 2013). With this information available, we propose that activation of LFA-1 and thus phosphorylation of the β2 chain, may sequester the available 14-3-3ζ from the surroundings (and from α4), allowing space for filamin to bind to VLA-4 and lead to the inhibition of VCAM-1 binding (figure 14).
7.3.5 **Transdominant inhibition could be utilised for efficient therapies**

In addition to mediating adhesion and signalling, integrins need to be able to release their ligands in order to fulfil their mission. This is evident in transendothelial migration, but also immune synapses need to unwind so that the cells can continue patrolling the circulation. In the context of extravasation, the fundamental steps are selectin-mediated rolling, slow integrin-mediated rolling, tight adhesion and migration along the endothelium and then transmigration towards the inflamed area (the whole process is explained in more detail in section 1.2.2). Different adhesion molecules are involved in these different steps, including selectins and their ligands, VLA-4 and VCAM-1 and β₂ integrins, especially LFA-1 and ICAMs. The roles of these (and many other) molecules have been attributed to distinct steps of the extravasation, and in order to continue to the next phase of extravasation, the previous receptor-ligand interactions need to be released. This is especially true for the migration, as the cell is constantly building up new adhesions at the lamellipodia, strengthening them in FAs and releasing them in the uropod area. The regulation of all these steps, adhesion and release in space and time, is of primary importance for many immune functions, but little has been known about the mutual control of the different adhesion molecules involved. Transdominant inhibition is probably one of the main mechanisms orchestrating the continuum of the steps of extravasation as it enables inactivation of previous adhesion steps simultaneously to the activation of the next adhesion molecule.

Another point of view related to transdominant control of different integrins is the therapeutical aspect, as integrins have been targets for drug development almost since they were discovered (Hilden et al 2006, Millard et al 2011). However, the anti-integrin therapies in the market are not numerous although a few successful drugs have been designed. One of the anti-integrin drugs on the market at the moment is Efalizumab or Raptiva®, an antibody against LFA-1 used in treatment of psoriasis (Lebwohl et al 2003). On the contrary, a promising therapy against multiple sclerosis and Crohn’s disease (Natalizumab or Tysabri®) was developed based on an antibody against VLA-4 but unfortunately, severe side effects causing progressive multifocal leukoencephalopathy (PML) led to withdrawal from the market (Linda et al 2009).
However, the withdrawal was only temporary, as Natalizumab is at the moment widely used in the treatment of severe MS. One possible explanation for unexpected side effects or inefficient function of a therapeutic agent may be the consequences controlled by transdominant inhibition (or activation) that may induce unwanted cellular events, since this crosstalk appears to be a fairly common form of integrin regulation. However, transdominant inhibition could also be used as a tool to a combined inhibition of for example LFA-1 and VLA-4, if a suitable antibody or other reagent activating the transdominant inhibition of VLA-4 and simultaneously inhibiting LFA-1 adhesion could be developed.

### 7.4 Regulation of leukocyte integrin binding to Ig-family ligands

In the experimental part I have characterized integrin activities from different angles in order to pinpoint some of the regulatory mechanisms involved in blood cell adhesion. On the basis of my research, and with help from the literature I have summarized the most relevant ways to regulate leukocyte integrin functions and signalling. They are as follows.

1. **The properties of the ligand are naturally the main factor in the specificity of the integrins.** Interesting observations were made when comparing the binding of ICAM-4 to the three members of leukocyte integrins that are close homologues, showing that distinct, although overlapping regions in both ICAM-4 and on β2 integrins are responsible for the binding.

2. **The environment of the adhesion, especially the presence of divalent cations Mg\(^{2+}\), Ca\(^{2+}\) and Mn\(^{2+}\) directly affects the ligand binding capacity of the integrins.**

3. **Phosphorylation of the integrin α and β chain cytoplasmic parts is thought to take part in the regulation of the integrins extracellular conformation and transmission of signals across the plasma membrane.** Phosphorylation is a specific and rapid way to control integrin activities in inside-out activation and outside-in signalling.

4. **Binding of intracellular signaling molecules to the cytoplasmic parts of integrins may precede or follow phosphorylation and cascades of signalling proteins may convey signals that are also spatially distant.** These often integrin-specific interactions are able to induce active conformation by separating the cytoplasmic tails, keep the integrin in the resting state, or initiate signalling cascades downstream of integrins.

5. **The conformation of the extracellular part of the integrin is the ultimate outcome of different activation steps, and it is the limiting factor in ligand binding.** However, the active conformation may also be induced from outside with an activating antibody or ligand binding. Strengthening of the conformation is vital for leukocyte adhesion in flow.

6. **Transdominant inhibition may regulate and coordinate many leukocyte functions where adhesion and release of different integrins are involved.**
Integrins, and the adhesion and signalling events they mediate, are essential for the proper function of probably all tissues. Especially in blood cells (red cells, leukocytes and platelets), the role of concerted regulation of the adhesion events is the cornerstone of all functions, including haematopoiesis, immune functions, haemostasis and the delivery of oxygen to the tissues. Leukocyte integrins control these adhesions by binding to ligands, of which the most important group is formed by Ig-superfamily of adhesion proteins. As important as adhesion is the ability to detach from the ligands, when the cells need to move on to the tissues, to the lymph nodes or into circulation. In the current work I have analysed the properties of leukocyte integrins and their ligands as well as the regulation of their interactions. I have observed that the red cell adhesion molecule ICAM-4 can bind to CR4, a leukocyte integrin expressed on monocytes and macrophages, and that the I domain is the ICAM-4 binding site on leukocyte integrins (LFA-1, Mac-1 and CR4). We have also characterised the phosphorylation of the cytoplasmic tail of CR4, and found that αx chain is phosphorylated on Ser1158, and that this phosphorylation is essential for CR4 inside-out activation, adhesion and phagocytosis but not for outside-in signalling initiated by CR4. Finally we analysed the regulation of VLA-4 mediated adhesion to VCAM-1 that is controlled by the β2 integrins. The findings of my studies show how leukocyte integrins are involved in numerous blood cell functions and that their functions are tightly regulated. Due to their multifocal roles, they also offer attractive targets for therapeutical use. The specificity of phosphorylations or ligands may serve as distinctive factors between different integrins, even members of the same family.

Even though the studies presented here may have revealed some of the regulation mechanisms of the leukocyte integrin activities, there remains certainly a large number of questions left unanswered or new ones coming up, which require more experiments and structural studies. One of them is the role of ICAM-4 in erythropagocytosis, as the results with anti-integrin and anti-ICAM-4 antibodies indicate an important role for these molecules. However, more in vivo studies in the context of spleen are required to answer this question. ICAM-4 also interacts with the platelet integrin α₅β₃, which suggests red cell adhesion involvement in haemostasis and/or thrombosis, but more work with platelets is needed to solve the role of this interaction. As for regulation of CR4 activities, several aspects may be envisioned. Could atherogenesis be inhibited by decreasing CR4-dependent monocyte adhesion to endothelium and could this inhibition be reinforced by transdominant inhibition between CR4 and VLA-4? Could it be possible to activate the uptake of antigen into DCs and hence strengthen DC-based vaccine efficiency by manipulating the CR4 activation? Another interesting application for transdominant inhibition between β2 integrins and VLA-4 would be targeted inhibition of autoimmune reactions in diseases like MS.
ACKNOWLEDGEMENTS

This study was carried out at the Division of Biochemistry and Biotechnology, Department of Biosciences, Faculty of Biological and Environmental Sciences, University of Helsinki, under the supervision of professor Carl Gahmberg. The work has been financially supported by the Magnus Ehrnrooth foundation and the Helsinki Graduate Program in Biotechnology and Molecular Biology.

I am deeply grateful to my supervisor Calle for patiently guiding me on my long and winding road towards being a scientist. Especially I appreciate the freedom you have given me to follow my own path and to independently carry out the weirdest experiments I came up with. You have succeeded in creating a welcoming atmosphere of scientific wonder in the lab, extending far beyond the world of integrins and adhesion, and shown that scientists should not lock themselves in the lab, but be active members of the society. I also want to thank professor Kari Keinänen, head of the division, for excellent research environment.

I want to express my gratitude to Eveliina Ihanus and Mikaela Grönholm for showing me the way. Both of you have taught me important lessons of science in practice. Eve told me all there is to know about cell adhesion in the very beginning of my PhD project, and Miku has shown, amongst other things, how fruitful a good teamwork can be. Thank you for your support and friendship!

I am privileged to have professor Francisco Sánchez-Madrid, who is one of the godfathers of the integrins, as my opponent. I am thankful to professor Jorma Keski-Oja and professor Manuel Patarroyo for the expert review of my thesis, and for providing me valuable feedback and suggestions. I want to thank professor Jukka Finne for serving as my custos. My follow-up group, Tiina Ohman and Pia Siljander did not gather that often (that was all my fault), but thank you anyway for your feedback and encouragement.

Calle’s lab has, besides excellent facilities, a superb atmosphere. I’m sure that the biochemistry coffee room is the place where all the problems, scientific or worldly, are solved in theory. I want to thank all of you Calle’s lab members for enduring the good and the bad days with me: Esa, Farhana, Lin, Sonja, Kate, Erkki and Miku. A special kiitos goes to Leena Kuoppasalmi for the complete care of the lab (and its members), for fulfilling our weirdest wishes, and for the patience. Kuoppis has been an important guide on my way, and especially a good friend. Many other people at the biochemistry division are also worth thanking, especially Pirjo Nikula-ljäs for the expert study counselling and Pia again for taking the immunochemistry course to the next level. Both are also thanked for always being supportive and encouraging.

As I have spent quite a few years in the lab, there is also a bunch of former members of the group and the division who I want to thank for sharing the joy of science with me: Anne, Suvi, Maria A., Maarit, Tiina, Suski, Susanna, Minna, Pauli, Suneeta, Emiliano, Kaj, Matti and others I forgot to mention. I have also been lucky enough to be able to dive into the sea of flow cytometry with Marias Semenova and Aatonen, thank you for sharing that world with me. Important people from the second floor are acknowledged for taking care of the facilities: Esa, Lefa, Paula, Tuukka and Olavi. I want to thank Yvonne, Katarina, Lea and Heidi for doing the paperwork and much more. All the other people at the Division of Biochemistry and Biotechnology are thanked for occasionally laughing at my bad jokes and keeping me scientific company in the coffee room.
Teaching has been and will hopefully remain an important part of my life and I am very grateful to all the students I have taught and supervised throughout the years. I’m sure I’ve learnt more than you!

I want to thank my dear friends Inka, Nelli, Hanna, Ella, Liisa K., Jussi, Lotta, Ankka, Sini and all of you others for the discussions, dinners, drinks, dreams – and giving me better things to think of than cells and adhesion. I am deeply grateful to my parents Helinä (& Heikki) and Tapsa (& Pirjo) for your endless support and belief in me, for not asking the “when is it ready?” too often and for your help in all the areas of life. I want to thank also my in-laws Katri and Voitto for their help and friendship.

Heikki, thank you for being there all the time and helping me through the hard times with this. I appreciate your support more than you can ever imagine. Kerttu, without you this or anything else wouldn’t make any sense. Never stop asking why!

Helsinki, October 2014
REFERENCES


Alon R & Dustin ML (2007) Force as a facilitator of integrin conformational changes during leukocyte arrest on blood vessels and antigen-presenting cells. *Immunity* 26: 17-27


Dustin ML & Springer TA (1988) Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol* **107**: 321-331


References


Hanspal M & Hanspal JS (1994) The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact. *Blood* 84: 3494-3504


References


References


References


Lim J, Dupuy AG, Critchley DR, & Caron E (2010) Rap1 controls activation of the alpha(M)beta(2) integrin in a talin-dependent manner. J Cell Biochem 111: 999-1009


of Kindlin-3 in LAD-III eliminates LFA-1 but not VLA-4 adhesiveness developed under shear flow conditions. *Blood* **114**: 2344-2353


O’Toole TE, Bialkowska K, Li X, & Fox JE (2011) Tiama1 is recruited to beta1-integrin complexes by 14-3-3zeta where it mediates integrin-induced Rac1 activation and motility. *J Cell Physiol* **226**: 2965-2978


Pavalko FM & LaRoche SM (1993) Activation of human neutrophils induces an interaction between the integrin beta 2-subunit (CD18) and the actin binding protein alpha-actinin. *J Immunol* **151**: 3795-3807


References


Tian L, Kilgannon P, Yoshihara Y, Mori K, Gallatin WM, Carpen O, & Gahmberg CG (2000a) Binding of T lymphocytes to hippocampal neurons through ICAM-5 (telencephalin) and characterization of its interaction with the leukocyte integrin CD11a/CD18. *Eur J Immunol* **30**: 810-818


References


References


References