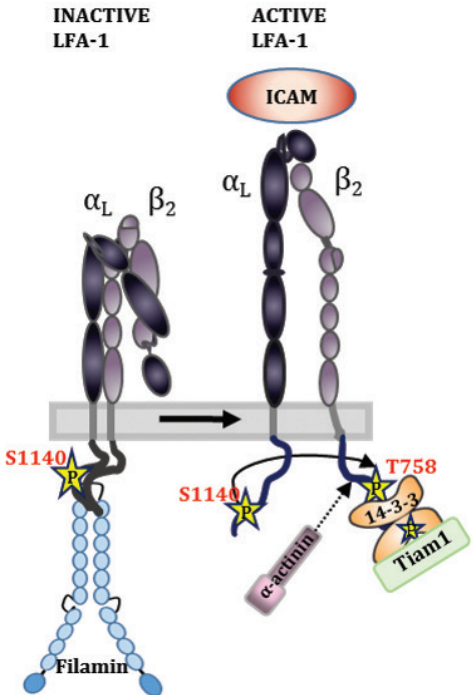
DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

86/2018

FARHANA JAHAN

Phosphorylation of the α- and β-Chains of LFA-1 Regulates its Interaction with Cytoplasmic Proteins and Crosstalk to VLA-4 Integrin



MOLECULAR AND INTEGRATIVE BIOSCIENCES RESEARCH PROGRAM
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

Phosphorylation of the α- and β-chains of LFA-1 regulates its interaction with cytoplasmic proteins and crosstalk to VLA-4 integrin

Farhana Jahan

Molecular and Integrative Biosciences Research Program
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 an Auditorium 1041, Bio-center 2, Viikinkaari 5, 00790, on 20th of December at 12 noon

Helsinki 2018

Supervisors: Professor Carl Gahmberg, M.D., Dr. Med. Sci.
Adjunct Professor Mikaela Grönholm, Ph.D.
Molecular & Integrative Biosciences Research Program
Faculty of Biological & Environmental Sciences
University of Helsinki

Advisory committee members: Professor Kari Keinänen, Ph.D.
Molecular & Integrative Biosciences Research Program
Faculty of Biological and Environmental Sciences
University of Helsinki

Adjunct Professor Tiina Öhman, Ph.D.
Institute of Biotechnology
University of Helsinki

Reviewers: Professor Jyrki Heino, M.D., Dr. Med. Sci.
Department of Biochemistry
University of Turku

Professor Jyrki Kukkonen, Ph.D.
Veterinary Biochemistry and Cell Biology
Veterinary Biosciences
University of Helsinki

Opponent: Olli Carpén, M.D., Dr. Med. Sci.
Medicum
Department of Pathology
University of Helsinki

Custos: Professor Kari Kenänen, Ph.D.

Dissertationes Scholae Doctoralis Ad Sanitatem Investigandam Universitatis Helsinkiensis

Layout: Tinde Paivarinta/PSWFolders Oy

ISBN 978-951-51-4670-0 (paperback)
ISSN 2342-3161 (print)
ISBN 978-951-51-4671-7 (PDF)
ISSN 2342-317X (Online)
Painosalama Oy, Turku, Finland 2018

"রাতের সব তারাই আছে দিনের আলোর গভিরে"

"The starts still twinkle, beneath the light of the sun"

-Rabindranath Tagore

Contents

I. List of original publications

I. Grönholm M*., Jahan F*., Marchesan S., Karvonen U., Aatonen M., Narumanchi S., Gahmberg CG. (2011). TCR-induced activation of LFA-1 involves signaling through Tiam1. Journal of Immunology. 187:3613–3619. (Shared first author)

II. Uotila, LM. Jahan F., Soto Hinojosa L., Melandri E., Grönholm M*., and Gahmberg CG*. (2014). Specific phosphorylations transmit signals from leukocyte beta2- to beta1integrins and regulate adhesion. Journal of Biological Chemistry. 289(46):32230–33242.

III. Grönholm M*., Jahan F*., Bryushkova E., Madhavan S., Aglialoro F., Soto Hinojosa L., Uotila LM., Gahmberg CG. (2016). LFA-1 antibodies inhibit leukocyte α4β1-mediated adhesion by intracellular signaling. Blood. 128(9):1270–1281. (Shared first author)

IV. Jahan F., Madhavan S., Taisia R., Viazmina L., Grönholm M*., Gahmberg CG*. Phosphorylation of the α-chain in the integrin LFA-1 enables β2-chain phosphorylation and α-actinin binding required for cell adhesion (2018). Journal of Biological Chemistry. 293(32):12318–12330.

*These authors have contributed equally to the work
The articles have been reproduced with the permission of the copyright holders and are referred to in the text by their Roman numerals.

Contributions

I. FJ performed protein purification, cell cultures, immunoprecipitation, affinity chromatography, immunofluorescence, static cell adhesion assays with MG, SM, UK and participated in planning the experiments, analyzing the results and writing the manuscript with the other authors.

II. FJ performed cell cultures, immunoprecipitation, immunofluorescence, cell adhesion assays both static and flow, cell migration with LU, LSH, EM, MG and participated in planning the experiments, analyzing the results.

III. FJ planned the experiments with MG and CG, performed most experiments, analyzed the data with MG, supervised SM's and FA's work and participated in writing the manuscript with MG and CG.

IV. FJ planned the experiments together with MG and CG, performed most experiments, supervised SM's work and wrote the article with MG and CG.

II. Abbreviations

aa	amino acid
ADAP	adhesion- and degranulation-promoting adapter protein
AIA	antigen-induced arthritis
AP-1	activator protein-1
APCs	antigen-presenting cells
BCR	B-cell receptor
CFDA-SE	carboxyfluorescein diacetate succinimidyl ester
CNS	central nervous system
CR4	complement receptor 4
cSMAC	central supramolecular activation cluster
DAG	diacylglycerol
DCs	dendritic cells
dSMAC	distal supramolecular activation cluster
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
FA	focal adhesion
FAK	focal adhesion kinase
FDCs	follicular dendritic cells
FERM	band 4.1, ezrin, radixin, moesin
FN	fibronectin
FRET	fluorescence resonance energy transfer
GDP	guanosine di-phosphate
GEF	guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
GTP	guanosine tri-phosphate
HEV	high endothelial venule
ICAM	intercellular adhesion molecule
DM	diabetes mellitus
EGF	epidermal growth factor
Ig	immunoglobulin
IgFLNa21	filamin immunoglobulin-like domain 21
IL	interleukin
ILK	integrin-linked kinase
IP_3	inositol trisphosphate

IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
JAB-1	Jun activation domain-binding protein-1
JNK	c Jun N terminal kinase
LAD	leukocyte adhesion deficiency
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LFA-1	lymphocyte function-associated antigen-1
LNs	lymph nodes
LPA	lysophosphatidic acid
Mac-1	macrophage-1 antigen
MAdCAM-1	mucosal vascular addressin cell-adhesion molecule-1
MAP	mitogen-activated protein kinase
MHC	major histocompatibility complex
MIDAS	metal ion-dependent adhesion site
MS	multiple sclerosis
NK	natural killer
PAK	p21-activated kinase
PAK-PBD	PAK-p21-binding domain
PDBu	phorbol-12, 13-dibutyrate
PH	pleckstrin homology
$PI(4,5)P_2$	phosphaditylinositol (4,5)–bisphosphate
PI3K	phosphoinisitide 3–kinase
$PI(3,4,5)P_3$	phosphatidylinositol (3,4,5)-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PSGL-1	P-selectin glycoprotein ligand-1
pSMAC	peripheral supramolecular activation cluster
PTB	phosphotyrosine-binding
Rac1	Ras-related C3 botulinum toxin substrate 1
RACK1	receptor for activated C kinase-1
RapL	regulator for cell adhesion and polarization protein L
RGD	Arg-Gly-Asp (peptide)

RIAM	Rap1 guanosine triphosphate interacting adapter molecule
RIPA	radio immunoprecipitation assay
ROS	reactive oxygen species
S1P	sphingosine-1-phosphate
SDF-1α	stromal cell-derived factor-1α
siRNA	small interfering RNA
SKAP55	Src kinase-associated phosphoprotein-55 kD
SLP-76	SH2 domain-containing leukocyte protein-76 kD
SMAC	supramolecular activation cluster
Tc	cytotoxic T-cell
TCR	T-cell receptor
T_H	helper T-cell
Tiam1	T cell lymphoma invasion and metastasis 1
TM	transmembrane
TMD	transmembrane domain
TNFα	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
VS	virological synapse
ZAP-70	zeta chain-associated protein kinase-70

Three-letter amino acids coding is used in the thesis

Amino acid	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

III. Summary

Inflammation is a self-protective response in which the body tries to get rid of harmful stimuli including damaged cells or pathogens. Leukocytes circulating in the blood stream are stimulated by chemokines released by endothelial cells that enable them to adhere and transmigrate to the site of infection. Leukocyte extravasation from the blood vessels towards the site of inflammation is a sequential and overlapping process involving leukocyte rolling, adhesion and transendothelial migration. During inflammation, antigen-presenting cells (APCs) present antigen to the T cells by binding to the T cell receptor (TCR). Adhesion is critical for T-cell trafficking and antigen recognition and is mediated in part by integrins, a large family of αβ heterodimeric cell surface proteins. Integrins exist in at least three different conformations: bent, extended-closed and extended-opened. The β2-integrin lymphocyte function-associated antigen-1 (LFA-1 /αLβ2) and the β1-integrin very late antigen-4 (VLA-4/α4β1) promote T-cell interactions with their ligands intercellular and vascular cell adhesion molecules (ICAMs and VCAMs) respectively which are expressed on endothelial cells. VLA-4 and LFA-1 directly participate in cell arrest under flow, whereas firm adhesion is mediated by LFA-1.

The cytoplasmic tails of integrins are short and lack catalytic activity. Several proteins, such as filamin, talin, 14-3-3, α-actinin and kindlin interact with the cytoplasmic part of the β2-chain. Some of these proteins–integrin interactions are in turn regulated by phosphorylation and dephosphorylation states of the β2-chain. As an adaptor protein, 14-3-3 is shown to interact with the phospho (p) Thr-758 of the β2-tail and activates Rac1, an actin–regulating protein. The first finding in this thesis was that in T cells, Tiam1 binds with 14-3-3 and is essential for Rac1 activation and cell migration. Tiam1 is a Rac guanine nucleotide exchange factor (GEF). Upon LFA-1 activation via anti-CD3 against the TCR or via stromal cell-derived factor 1α (SDF-1α), Tiam1 forms a complex with β2 phosphorylated on Thr-758 and the scaffolding protein 14-3-3, which activates Rac1. Downregulation of Tiam1 inhibits Rac1 activation and furthermore, it impairs cell adhesion to the LFA-1 ligand ICAM-1.

Integrins have unique characteristics of signaling at both directions across the membranes. Inside-out signaling occurs by ligand binding to non-integrin receptors, which can activate integrins through intracellular signal transduction whereas outside-in signaling takes place by binding of ligands to the integrin itself. The next finding was that the activation of LFA-1 via inside-out or outside-in

signaling downregulates VLA-4 binding to its ligand VCAM-1. When LFA-1 is activated upon SDF-1α, it causes the phosphorylation of Thr-758 on the β2-chain, which recruits 14-3-3 and Tiam1 and results in crosstalk to VLA-4. It leads to the dephosphorylation of Thr-788/789 on the β1-chain, which results in recruiting more filamin but less 14-3-3 to the β1-chain.

Another finding of this thesis was that the activating and some inhibiting antibodies against LFA-1 binding to the extracellular part, block VLA-4 binding to its ligand VCAM-1. We identified that LFA-1-specific monoclonal antibodies that activate LFA-1 but inhibit VLA-4, inhibit both LFA-1 and VLA-4, inhibit LFA-1 but not VLA-4, and neither affect the activity of LFA-1 nor VLA-4. We found that 7E4, an antibody that blocks LFA-1 binding to ICAM-1, concurrently activates intracellular signaling resulting in the phosphorylation of Thr-758 of the β2-chain, which leads to crosstalk to the VLA-4, inhibiting its activity. The activation of LFA-1 was mediated through phospholipase C (PLC) and protein kinase C (PKC) activation, which causes the phosphorylation of the Thr-758 on the β2-chain. These findings are significant for understanding the effects of antibody treatment on integrins, i.e. how they regulate integrin functions, and thus also in the use of LFA-1-specific antibodies in clinical applications.

In my fourth publication, I have also shown that the phosphorylation of the LFA-1 α-chain at Ser-1140 is essential for the functionally significant phosphorylation of the β-chain Thr-758 and protein interaction with the β2-chain. Mutation of Ser-1140 to alanine in the αL-chain significantly decreased Thr-758 phosphorylation of β2-chain after SDF-1α or anti-CD3 activation. This αL mutation also affects the exposure of the LFA-1 activation epitope for ligand interaction. α-Actinin is an actin-binding protein, which links integrins to the actin cytoskeleton and it is crucial for cell migration. We showed that mutation of Ser-1140 to alanine in the αL-chain affected the α-actinin interaction with the β2-chain and chemotactic directional migration. Phosphorylation and de-phosphorylation of αL- and β2-chains may be a way to regulate adhesion and deadhesion turnover of cells during migration.

To summarize, the thesis has elucidated the importance of the phosphorylation of Thr-758 on the β2-chain and Ser-1140 on the αL-chain of LFA-1. We have identified a new key molecule, Tiam1 in intracellular signaling downstream of LFA-1. We also have elucidated the crosstalk mechanisms of the activation of LFA-1 to VLA-4. We have revealed that the anti-LFA-1 activating or inhibiting antibodies crosstalk to VLA-4 as well. Finally, we have demonstrated that

phosphorylation of the αL-chain on Ser-1140 is vital for β2-chain phosphorylation and protein-protein interactions.

De-regulation of integrins' function may lead to auto-immune and chronic inflammatory diseases. Here in this thesis, I have now revealed some novel, important mechanisms of the regulation of the leukocyte integrins. The knowledge of how integrins are regulated could be beneficial in clinical applications as well as for developing drugs with high specificity in future.

IV. Review of the literature

1. The immune response

When the body is invaded by foreign particles, it reacts through a defense mechanism known as the immune system. In an immune reaction, several types of cells are activated to destroy foreign substances. Two fundamental immune response mechanisms have been described: the innate immune system in which cells encounter invaders immediately or within hours, and the adaptive immune system in which the initial interaction with pathogens result in an immunological memory that is specific to precise antigen.

2. Leukocytes

The immune mechanism is the body's defense mechanism involving a synchronized network of different organs, tissues, and cells. White blood cells come in two major types: myeloid and lymphocytes. These cells work together to seek and destroy any foreign molecules entered the body. Phagocytic cells in the innate immune system, such as macrophages, monocytes, and neutrophils, kill pathogens rapidly. The lymphocytes, T and B cells are the most critical cells in the adaptive immune responses.

During inflammation, T cells recognize the antigens, which are presented by the antigen-presenting cells (APCs) and kill pathogens or impaired the cells. Pluripotent hematopoietic stem cells in the bone marrow develop into immune cells like T cells and B cells. T cells mature and differentiate in the thymus whereas B cells mature in the bone marrow (Ross, 1994). Before entering the thymic cortex, T cells go through a positive selection to check that the T cells can interact with major histocompatibility complex (MHC) molecules. If T cells can interact with the MHC molecules, they will receive a survival signal; however if they are unable to interact, they will get a non-survival signal, and will eventually die. The survived T cells that interacted with MHC I, differentiate into CD8 T cells, also called cytotoxic T (T_c) cells, while those that interacted with MHC II, become CD4 T cells, also called helper T (T_H) cells. After T cells differentiate into CD8 or CD4 cells, they move to the thymic medulla where they undergo a negative selection. This selection examines how the

newly produced T cells will interact with the self -MHC molecules. If the interaction is strong, the cells will receive apoptotic signals. And if they somehow survive, T cells will induce autoimmune diseases. After the positive and negative selection stages, T cells will be released from the thymus as mature CD4/T_H cells or mature CD8/T_C single positive cells and circulate in the periphery. The function of the T_C cells is to remove virus-infected cells and neoplastic cells. T_H cells are capable of influencing a variety of immune cells and provide immediate response to cytokines, which is essential for an effective outcome against infection. Proliferating T_H cells referred to as effector T_H cells are divided into T_H1 and T_H2 cells; helper T cells which encounter interleukin-12 (IL-12) become T_H1 and those influenced by IL-4 become T_H2 cells (Cota & Midwinter 2015).

A successful defense mechanism depends on the ability of the leukocytes to travel in the blood and lymph as non-adherent cells and to migrate as adherent cells into the lymph node and tissue in response to invaders. Lymphocytes, which are activated by dendritic cells (DCs) or APCs must be able to assemble in the lymphoid organs, cross endothelial membrane barriers and aggregate at the site of infection. The transition between the nonadherent and adherent phenotype is crucial for immune reaction and surveillance.

Three main groups of adhesion receptors are involved in the processes of lymphocyte homing to different lymphoid organs and lymphocyte filtration to the site of inflammation. First, the immunoglobulin (Ig) superfamily, which contains among others the antigen-specific receptors for T and B lymphocytes; secondly the integrins, which are essential for the maintenance of cell adhesion and migration; and finally the selectins, which are important for lymphocyte interactions with the endothelium (Springer 1990). Too tight or continuous adhesion of cells with different receptors can cause chronic inflammation. Stringent regulation of lymphocyte adhesion from circulation to the site of inflammation is important for a fine-tuned immune system.

2.1 The immune synapse

Immune cells like T cells or natural killer (NK) cells communicate with the APCs through a close cell–cell interaction, which is known as the immune synapses (IS). Additionally, cytotoxic T cells or killer cells use the immune synapse to target the infected or malignant cells more specifically and eliminate them from the system, leaving the healthy cells unaffected (Dieckmann et al. 2016). Formation of the

immune synapse is vital for T-cell activation. Necessary physiognomies of a matured immune synapse include the transformation of peptide–MHC complexes from APCs to T cells and the control of secreted cytokine lytic granules (Davis & Dustin 2004).

Successful formation of the immunological synapse requires TCR reorganization to recognize MHC peptides and adhesion via LFA-1 to form a functional domain at the edge of the two cells. The structure of the immunological synapse refers to the 'bullseye' model, which makes concentric rings of different clusters of proteins in various layers (Figure 1). The formation of the immunological synapse was first presented by Kupfer in 1995 in a 'Keystone Symposium' where he showed three-dimensional images of CD4 T cells targeting another cell (Monks et al. 1998). Another name of the immune synapse is supramolecular activation cluster or SMAC. SMAC contains three unique rings: the central SMAC (cSMAC), peripheral SMAC (pSMAC) and distal SMAC (dSMAC). In the developed IS, TCR cluster in the central ring of the IS (cSMAC) with its co-receptor CD28, protein kinase C-θ (PKC) and lymphocyte-specific protein tyrosine kinase (Lck). Adhesion molecule LFA-1 with its adaptor protein talin is usually found at the periphery of cSMAC called pSMAC, and actin, CD43, CD44 and CD45 in the dSMAC (Dieckmann et al. 2016).

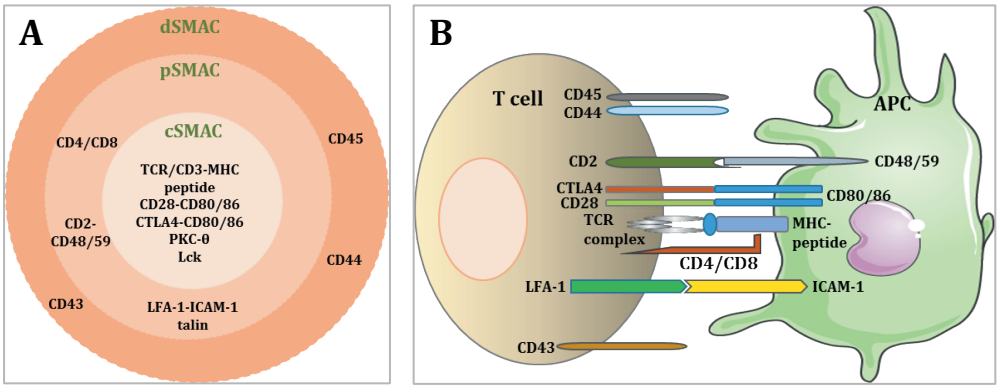


Figure 1: A. The molecular distribution of the immune synapse with the characteristic 'bullseye' view cSMAC (central supramolecular activation cluster), pSMAC (peripheral SMAC), dSMAC (distal SMAC). **B.** A schematic drawing of the immunological synapse and the molecular dissemination between the T cells and the APC (antigen presenting cell). Modified from Huppa and Davis 2003.

The TCR is a complex of antigen specific α and β chains associated with the CD3 γ, CD3 δ, CD3 ε and CD3 ζ chains. Each of CD3 chains has the common signaling motif called immunoreceptor tyrosine-based activation motif (ITAM) in the

cytoplasmic domain. T cell activation requires the interaction with other molecules such as CD45, a tyrosine phosphatase enzyme, and the T cell co-receptors, either CD4 or CD8. In the activation of CD4 co-receptor, quite a number of events take place after the MHCII complex with peptides binds to the TCR. CD45 dephosphorylates the terminal tyrosine of Lck and initiate its kinase activity. When CD4 interacts with the MHCII molecules, Lck phosphorylates the ITAM domains, and at this point, tyrosine kinase ZAP-70 (Chan et al. 1992) is recruited to the ITAM domain and binds through the SH2 domain. Then ZAP-70 is phosphorylated by Lck and able to phosphorylate transmembrane proteins with long intracytoplasmic domain LAT (linker activated of T cells). It binds to the adaptor protein Gads and recruits the cytoplasmic protein SLP-76 and forms a large scaffold (Chakraborty & Weiss 2014) (Figure 2). This scaffold then is used as a subsequent signaling pathway activator. Following events, including the activation of PLCγ, requires the contribution of another co-stimulatory pathway involving CD28. Furthermore, PLCγ generates second messenger inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Dieckmann et al. 2016). The cascades downstream of TCR activation ultimately result in cellular proliferation, differentiation and cytokine production.

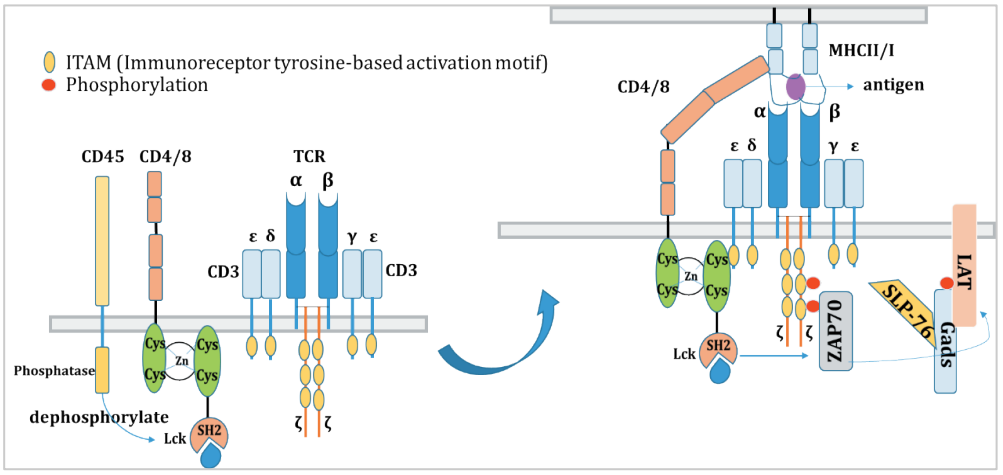


Figure 2: A simplified schematic picture of TCR (T cell receptor) activation. Description of the TRC activation is mentioned in the earlier paragraph.

Recently, it has been found that the formation of the immune synapse helps to localize the cytotoxic substances in the T cells for killing the tumor or virally infected cells more specifically (Dieckmann et al. 2016). Another fascinating feature of the immunological synapse is to function as a platform to transfer cellular or

genetic molecules via extracellular vesicles between T cells and the APC (Finetti et al. 2017). It has been described that human lymphotropic viruses such as HIV-1 ensures its spread and escaping from the host immune response by formation of the synapse. HIV-1 hijacks the diverged vesicular machinery from the host cells for assembly and focuses on producing new virions at the virological synapse (VS), which is a contact zone between the infected and uninfected CD4 T cells (Soares 2014). There are structural similarities between IS and VS. Same regulators like EW1-2, α-actinin and TCR signaling components have been found in both VS and IS. Interestingly, engagement of TCR by pMHC (peptide-MHC) leads to the enrollment and the accumulation of gag (a genetic code for group specific antigen) for HIV-1 at the IS, and will result in budding of gag microvesicles. Afterward, vesicles secreted by HIV-1-infected cells will carry chemokine receptors (CXCR4) that facilitate entering the non-permissive cells (Finetti et al. 2017). HIV-1 then takes control of the assembly of the IS by impairing both the interaction of Lck with the TCR and also the association of LAT with the actin cytoskeleton (Silva et al. 2016).

2.2 Leukocyte adhesion and migration

In response to the infections, leukocytes must leave the blood vessels to get into the inflamed tissue. The journey of a leukocyte from the bloodstream into tissues is a highly organized and well-maintained process, which involves the multifaceted interaction between the leukocyte and the endothelium. In immunology, the process of the outward way of the immune cells through the integral vessel is known as leukocyte extravasation or diapedesis. The leukocyte interacts with the endothelial cells via selectins, integrins, intercellular adhesion molecules (ICAMs), vascular adhesion molecules (VCAM-1), junctional adhesion molecules (JAMs), and platelet endothelial cell adhesion molecules (PECAMs). In the human liver, VAP-1 (vascular adhesion protein-1) plays an important role as an adhesion receptor expressed by endothelium. Since sinusoidal endothelial cells lack selectin expression, VAP-1 is critical for lymphocyte adhesion and transmigration through the endothelial to the recruitment in the liver (Lalor et al. 2002). Extravasation is based on a sequential and overlapping process including leukocyte rolling, arrest, firm adhesion and transendothelial migration (Figure 3). This phenomenon was already described in the nineteenth century by Rudolf Wagner and Rudolf Virchow (Ley 2008).

The first step of the leukocyte extravasation is the release of inflammatory cytokines and bacteria-derived peptides, which induce the upregulation of adhesion

molecules on the endothelial cells, further promoting labile and transient adhesion interactions (rolling) between leukocytes and the endothelial cells. Chemokine deposition on the endothelial luminal surface then triggers the integrin-mediated arrest via their ligands VCAM-1 and ICAM-1, and promote strong adhesion. After this firm adhesion, the cells start to transmigrate between or through the endothelial cells and reach the inflamed tissue in a process called diapedesis. The more detailed mechanisms are shown below.

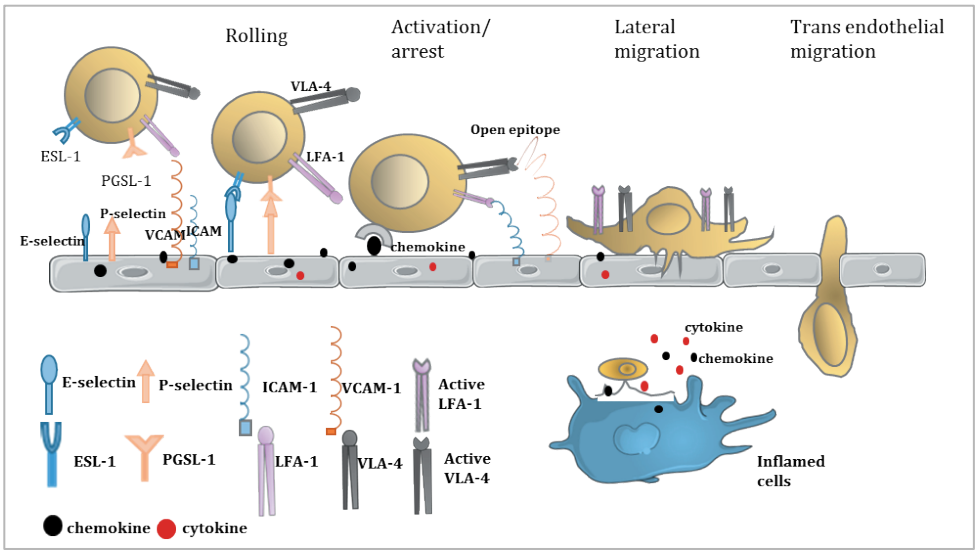


Figure 3: Essential steps of leukocyte extravasation and molecules involved in this process. Tethering of the leukocytes on the surface of activated endothelium occurrs through the interactions between E-selectin with its ligand ESL-1 and P-selectin with the ligand PGSL-1. Tethering and rolling precede firm adhesion, which is mediated by the interaction of VLA-4 and LFA-1 with VCAM-1 and ICAM-1, respectively.

2.2.1 Selectin-mediated adhesion

The first step of extravasation is rolling of the leukocytes on the endothelial cells, which is mediated by selectins. Selectins have lectin N-terminal domains which initiate the transient adhesion of cells to their transmembrane glycoprotein and glycolipid ligands containing the tetrasaccharide sialyl-Lewis. This results in rolling on the endothelial cells (Homeister et al. 2001). Three selectins have been described: L-selectin, P-selectin, and E-selectin. L-selectins are mostly expressed on the leukocytes, while E-selectins are expressed on the activated endothelial cells and are induced by cytokines, like tumor necrosis factor (TNF) and interleukin-1 (IL-1)

(Bevilacqua et al. 1987). On the other hand, P-selectins are expressed both on the platelets and the endothelial cells by the influence of released histamine and thrombin (Homeister et al. 2013). The interaction between the selectins and the leukocytes is transient and it initiates the rolling of leukocytes along the endothelial in the direction of blood flow (Lawrence & Springer 1991). During the rolling steps, bonds are formed at the leading edge and released at the trailing edge of the cells. Initially, leukocyte integrins stay in a resting state, and immunoglobulins on the endothelial cells remain at basal levels. P-selectin is the essential selectin for rolling processes, but it can also support capture (McEver 2015). P-selectin glycoprotein ligand-1 (PSGL-1) is constitutively expressed in all lymphocytes and other leukocytes as well. L- and E–selectins also take part in the rolling steps. It was shown that in the absence of P-selectin, rolling became L-selectin dependent, but the rolling was much slower compared to the P-selectin-dependent rolling. L-selecting-dependent rolling also requires the expression of E-selectin on the endothelial cells and CD18 integrins expression on the rolling leukocytes (Jung et al. 1998, Jung & Ley 1997). Even though E-selectin is less efficient for leukocyte rolling, it is necessary for the typical inflammatory response in capturing leukocytes and initiating rolling (McEver 2015).

2.2.2 Integrin mediated adhesion and transmigration

Integrins are as well involved in rolling and mediate firm leukocyte adhesion on endothelial cells. In the gut, α4β7 mediates rolling of the cells on endothelial via the interaction of mucosal vascular addressin cell-adhesion molecule-1 (MAdCAM-1) (Erle et al. 1994), and α4β1 (VLA-4) mediates rolling of the cells through the interaction with VCAM-1 (Berlin et al. 1995). The integrin is thought to be essential for monocytes and T cells is VLA-4, and VLA-4 interacts with its ligand VCAM-1, which is expressed widely on activated endothelial cells (Imai et al. 2010). The upregulation of integrin expression on the leukocytes and their ligand expression on the endothelial cells result in adhesion (Springer 1995, Zhou et al. 2005). It has been shown that lymphocyte rolling and adhesion in the venules of the central nervous system (CNS) is VLA-4 mediated independently of P-selectin engagement (Vajkoczy et al. 2001). In neutrophils, the rolling and capturing are mediated by the selectins and the interaction between LFA-1 and its ligands ICAM-1 and -2 on the endothelial cells (Springer 1990, Gahmberg et al. 1997).

Chemokines that are secreted by the endothelial cells activate leukocyte adhesion. Chemokines bind to G-protein coupled receptors (GPCRs) on leukocytes,

facilitating the rapid intracellular signaling events that activate the β2 integrin for their interaction with the ICAM counter-receptors expressed on the endothelium. The interaction between the integrin and the ligands initiate a signaling cascade in the leukocytes, which cause further strengthening of the leukocyte–endothelium adhesion by changes in the actin cytoskeleton (Gahmberg et al. 1997, Hogg et al. 2011b, Ley et al. 2007, Toivanen et al. 2008). In contrast to naive or memory lymphocytes, the effector cells do not need chemokine activation to be arrested on the endothelium. Instead, the effector lymphocytes express a vast number of activated leukocyte integrins on the surface that can be available for ligand interaction (Shulman et al. 2011, Lek et al. 2013).

During crawling, leukocytes change their morphology drastically from round to flat and highly polarized, defining a cell 'front' and a 'rear' or 'uropod (Filippi 2016). This highly polarized leukocyte creates lateral movement through the formation of cell protrusions, lamellipodia, filopodia, and the invadosome to exit the endothelial cell layer. At the leading edge, lamellipodia go through an intensive dynamic actin cytoskeleton reorganization. Filopodia even extend further towards the direction of migration from the lamellipodia (Ley et al. 2007). In the lamellipodia, consistent formation of strong adhesions takes place that comprises integrins, talin, and other adhesion-associated molecules. The cell exploits stable adhesion sites while dragging the cell body along the surface. In the rear, adhesion sites are interrupted, and there is a turn-over of molecules that are then recycled to the leading edge of the cell (Ley et al. 2007).

The leukocytes migrate through the endothelial layers by two separate routes: the paracellular (between the cells) and the transcellular (through the cells) route (Carman et al. 2007, Mamdouh et al. 2009). In the paracellular route of migration, the interaction between the activated endothelium and the leukocytes opens the inter-endothelial cell junctions and stimulate leukocyte migration through cell–cell junctions. The transcellular migration takes place by the secretion of chemokines from the intracellular endothelial stores, which facilitates the leukocyte to migrate through the endothelium (Shulman et al. 2012). In the migration process, cells use different kinds of adhesion molecules including integrins and proteases compared to the rolling or adhesion process on endothelium.

3. Leukocyte integrins

Integrins are transmembrane cell adhesion proteins that link cell surface molecules of bound cells or the ECM to the cell cytoskeleton. They are composed of different α- and β- subunits which are type I membrane glycoproteins. They contain an extended extracellular part and usually a short cytoplasmic tail (Alberts et al. 1994). There are 18α, and 8β subunits identified in vertebrates, and these form 24 distinct α–β heterodimer receptors (Figure 4). The diversity of these subunits enables a large variety of ligand recognition, in binding to cytoskeletal components and coupling to downstream signaling pathways. In leukocytes, integrins play a crucial role in trafficking, migration, immunological synapses formation and phagocytosis (Luo et al. 2007).

3.1 Leukocyte integrin classification

In leukocytes, several integrins are expressed depending on the activation and maturation status and on the cells types. LFA-1, also known as αLβ2 or CD11a/CD18, macrophage antigen-1 (Mac-1, αMβ2 or CD11b/CD18), αXβ2 (CD11c/CD18) and very late antigen-4 (VLA-4, α4β1 or CD49d/CD29) are integrins expressed on leukocytes (Chigaev and Sklar 2012, Abram and Lowell 2009). α4β7 (Ruegg et al. 1992) and αEβ7 (Andrew et al. 1996) are also expressed exclusively in leukocytes.

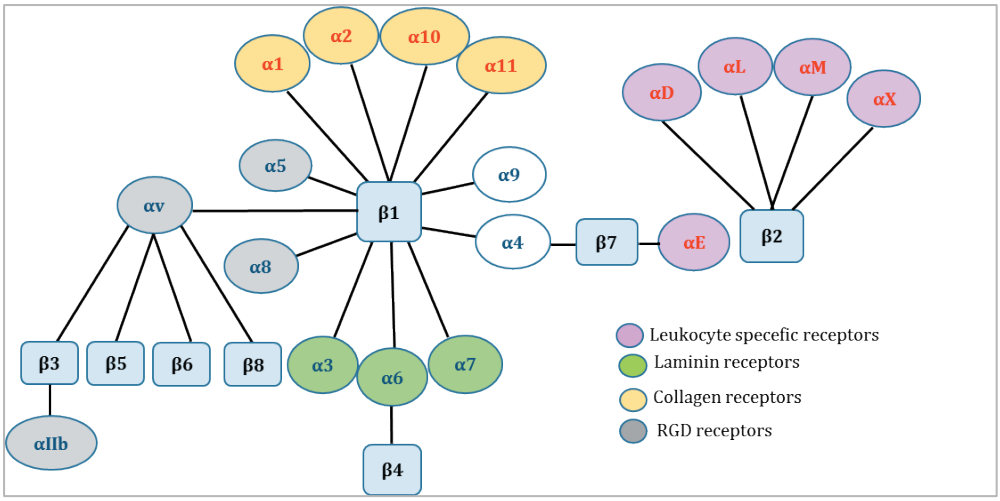


Figure 4: The classification of integrins and their heterodimers. Integrins containing the inserted I domains for α-chains are indicated in red (α1, α2, α10, α11, αE, αD, αL, αM and αX).

3.1.1 LFA-1 (αLβ2, CD11a/CD18)

LFA-1 is expressed on most leukocytes (Cornwell et al. 1993). In 1979, a child was diagnosed with defects in leukocyte migration and phagocytosis with recurrent infections. The reason behind these defects was found to be the lack of a gp150 surface membrane glycoprotein complex (LeBien & Kersey 1980, Arnaout et al. 1984). The glycoprotein was shown to make a heterodimer with to a 95kd glycoprotein (Beatty et al. 1984), and it was later named LFA-1. The LFA-1 belongs to the β2/ CD18 leukocyte differentiation antigen family that consists of p150/95 glycoproteins (Davignon et al. 1981, Springer & Anderson 1986). In 1985, LFA-1 was identified as an adhesion molecule using phorbol ester activation of T cells and adhesion to ICAM-1 using blocking antibodies (Patarroyo et al. 1985). LFA-1 binds to the members of the ICAM protein family (Patarroyo et al. 1987, Marlin & Springer 1987, Staunton et al. 1989, de Fougerolles & Springer 1992, Tian et al. 1997). LFA-1 also binds to JCAM (Ostermann et al. 2002), E-selectin (Kotovuori et al. 1993) and collagen (Garnotel et al. 1995a, Lahti et al. 2013).

LFA-1 plays a crucial role in cell–cell interactions, in leukocyte adhesion to the endothelial cells during the extravasation, for T cell interaction with the APCs in the lymph node (Hogg et al. 2011), and cytotoxic T cell-mediated killing of infected cells (Davignon et al. 1981). In the immunological synapse, LFA-1 is necessary for the formation of sMAC strengthening (Monks et al. 1998). It has been shown that either an absolute or a functional loss of β2 integrins leads to the rare immune deficiency disease called leukocyte adhesion deficiency (LAD) type I (Anderson 1987).

3.1.2 VLA-4 (α4β1, CD49d/CD29)

The VLA-4 is the CD49d (α4) and CD29 (β1) heterodimer and belongs to the β1 integrin family, first discovered in 1987 in multiple T-lymphoblastoid cell lines (Hemler et al. 1987). VLA-4 is expressed in many cells, stem and progenitor cells, T and B cells, monocytes, NK cells, and eosinophils. VCAM -1 and fibronectin are ligands for VLA-4 (Elices et al. 1990, Guan & Hynes 1990). However, without activation, VLA-4 cannot interact with its ligands. Activation of VLA-4 goes through the different conformational changes to bind to the ligands. The α4-chain lacks an I–domain and easily become activated upon stimulation (Chigaev & Sklar 2012).

VLA-4 is considered a critical adhesion molecule for maintaining hematopoiesis in the adult. It is reported that VLA-4 is not involved in the differentiation or proliferation of the hematopoietic cells, but instead, it is essential

for homing of hematopoietic cells in the bone marrow and helps for releasing the mature lymphocyte in the circulation (Imai et al. 2010). Antagonists of VLA-4 are shown to be clinically important in many inflammatory diseases (Chigaev et al. 2011). Moreover, it is also shown that in multiple sclerosis, VLA-4 enables T cells to cross the blood-brain barrier (Lin & Castro 1998).

3.2 Integrin structure and conformation

The α and the β subunits of integrins comprise several domains in which each subunit contains a single membrane-spanning helix and a cytoplasmic part (Figure 5A for the general structure of the integrins). Typically, the α- and β-subunits contain around 1000 and 750 amino acids respectively (Luo et al. 2007, Campbell & Humphries 2011).

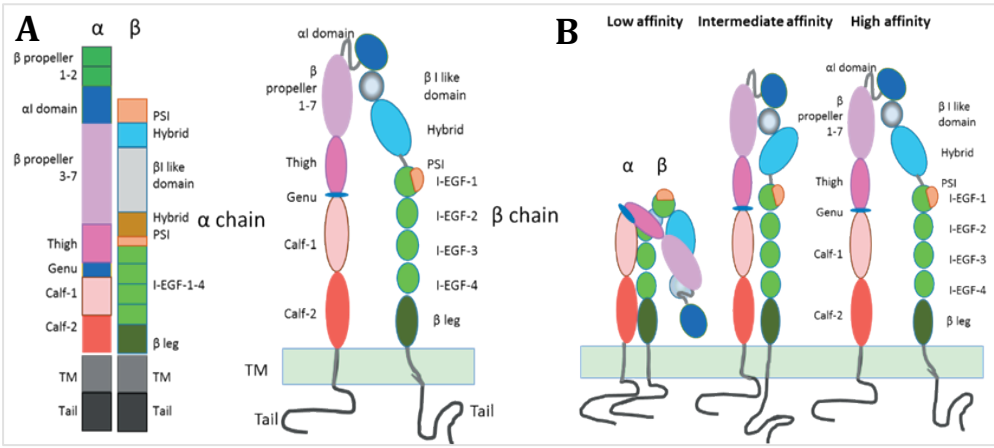


Figure 5: A. A schematic drawing of the integrin α- and β- chains. **B.** Three different conformational stages of integrins. Bent (low affinity), extended (intermediate affinity), extended and open (high affinity).

There are three different conformations of the extracellular part of the integrins: bent, extended but closed headpiece, extended and opened headpiece (Figure 5B). In the low-affinity state, the head of the integrins bends towards the plasma membrane. In the extended conformation, the two subunits closely associate with each other. This intermediate state of the integrins can have either low or intermediate affinity to the ligands. In the fully-active conformational stage of integrin, cytoplasmic domains of the α and β subunits are separated, and the ligand-binding site is entirely open (Lefort & Ley 2012).

3.2.1 The structure of integrin α-chains and the role of conformational change

The α-chain of integrin contains 4–5 extracellular domains. All integrins have a seven-bladed β-propeller, a thigh, and two calf domains. Among all the integrins nine α-chain integrins contain inserted I-domains: α1, α2, α10, α11, αE, αD, αL, αM and αX. An inserted (I) αI domain or a von Willebrand factor A domain is composed of about 200 amino acids and the ligand-binding site resides in the αI-domain. The αI-domain is inserted between blades 2 and 3 of the β-propeller, and it has five β-sheets surrounded by seven α-helices (Larson et al. 1989). On the other hand, in integrins lacking the I-domain, the interface (integrin head) between the α-subunit β-propeller domain and β-subunit βI-domain serves as the ligand-binding site (Springer & Dustin 2012).

The αI-domain plays a vital role in ligand interaction and mutations in the αI-domain alter the structure of the metal-ion-dependent adhesion site (MIDAS). The crystal structures of the I-domain of many integrins have been determined: αM (Bierema et al. 2017), αL (QU & Leahy 1995), α2 (Emsley et al. 1997), α1 (Salminen et al. 1999) and αX (Vorup-Jensen et al. 2003). MIDAS binds divalent metal cations (predominantly Mg^{2+}) and plays critical roles in ligand binding. The switch from a closed, resting state to an active conformation in integrins leads to increased ligand affinity. The increased affinity for ICAM-1 due to a conformational change of integrins shows that the αI-domain is efficient in changing the conformation to change its affinity for ligand (Liddington & Ginsberg 2002, Luo et al. 2007).

The C-terminal part of the α-subunit contains an immunoglobulin-like (Ig) "thigh," two β-sandwich "calves," a transmembrane domain and a cytoplasmic tail. The thigh and calf domains are rich in β-strands as compared to the typical Ig-like domains. One linker, between the β-propeller, the thigh and another linker, the "genu" or knee, is at the bend between the thigh and the calf-1 domain and provides inter-domain flexibility for the α-subunit (Figure 5A) (Xiong et al. 2001, Gahmberg et al. 2009).

3.2.2 The structure of the β-chains and the role of conformational change

The β-chain contains a plexin–semaphorin–integrin (PSI) domain, a βI-like domain, an Ig-like hybrid domain, four epidermal growth factor-like (EGF) domains, a β-tail, a transmembrane domain (TM) and a cytoplasmic tail. The βI-domain, homologous to αI, is inserted in the hybrid domain, which in turn is inserted in the PSI domain to

form the upper portion of the β-leg. The rest of the β-leg of the ectodomain is composed of four cysteine-rich regions of I-EGF domains and an α+β fold containing the β-tail domain (Campbell & Humphries 2011, Springer & Dustin 2012, Xiong et al. 2001).

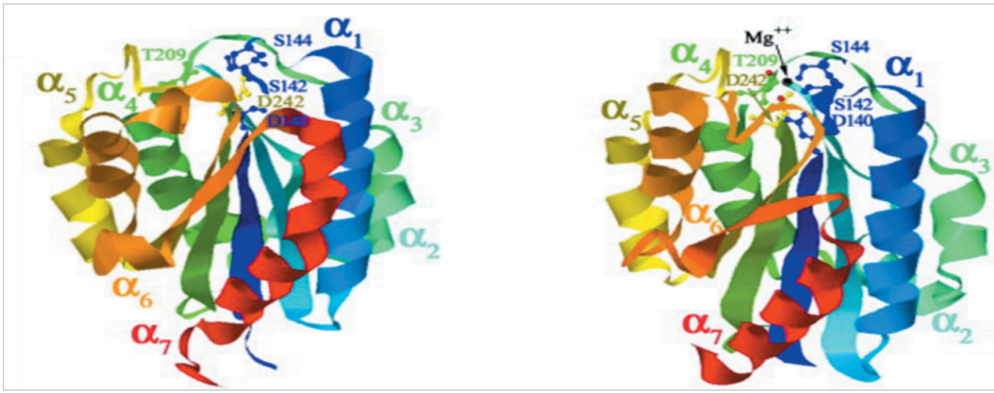


Figure 6: Inactive structure of Mac-1 I-domain (left). Active structure of the Mac-1 I-domain (right). When magnesium ions (black dots) activate Mac-1, the α7 helix (red) shifts position (Gahmberg et al. 2009). (Published with permission)

The flexibility of the β-leg reported is based on the different crystallographic structures of the αXβ2 integrin (Xie et al. 2010). Plasticity of the EGF domains is seen between EGF-1 and EGF-2, the β- knee, and at the PSI/hybrid and hybrid/I-EGF1 junctions. When the α7-helix of the βI domain moves toward the hybrid domain, a transition from a "closed" to an "open" conformation has been observed (Xia et al. 2004). Inhibition of the allosteric communication between I-domains by small adhesion antagonist molecules that bind βI-domains demonstrates the importance of the close interaction between the αI- and the βI-like domain (Nishida et al. 2006). Allosteric regulation of the α-chain I-domain by the β-chain I-like domain is essential for regulating integrin conformation. βI-MIDAS activates the integrin αI-domain, dragging the α7 helix by the interaction with the aspartic acid which is linked to the βI-domain from its resting position and the tail of the α and β subunit apart from each other. βI-MIDAS also utilizes glutamate (E310) as a ligand which is present in the linker formed by the α7 helix and the β-sheet 3 of the β2- propeller (Gahmberg et al. 2009) (Figure 6). It has also been shown that the Mg^{2+} ion at MIDAS interacts directly with the aspartic residue of RGD, which is the major binding site of many integrins (Gahmberg et al. 2009). The whole extracellular crystallographic

structures of αVβ3 (Xiong et al. 2001), αIIbβ3 (Zhu et al. 2008), αXβ2 (Xie et al. 2010) and α5β1 (Nagae et al. 2012) have been reported.

3.2.3 The transmembrane domains

Relatively little is known about the transmembrane regions of the α- and β-chains. The transmembrane domains (TMDs) of integrins are single spanning structures composed of approximately 24–30 amino acids, which form an α-helical coiled structure (Adair & Yeager 2002). The structures of the TM domains of αIIb and β3 in phospholipid bicelle membranes have been solved by NMR separately (Lau, Dua, et al. 2008, Lau, Partridge, et al. 2008) and in a complex (Lau et al. 2009). It was shown that the αIIb-TM is a 24 residue α-helix that is perpendicular to the membrane whereas the β3-TM is a 30 residue linear α helix, The β3-TM domain is somehow longer than the width of a typical lipid bilayer suggesting that a helix tilt is present within the plasma membrane (Figure 7) (Campbell & Humphries 2011). The NMR structure shows that there are glycines (Gly) at the helix–helix interface of the αIIb in the membrane that makes a pair of phenylalanine residues (Phe) on the β3-TM part, which promotes electrostatic interactions between the aspartic acid (Asp-723) on β3 and arginine (Arg-995) on αIIb.

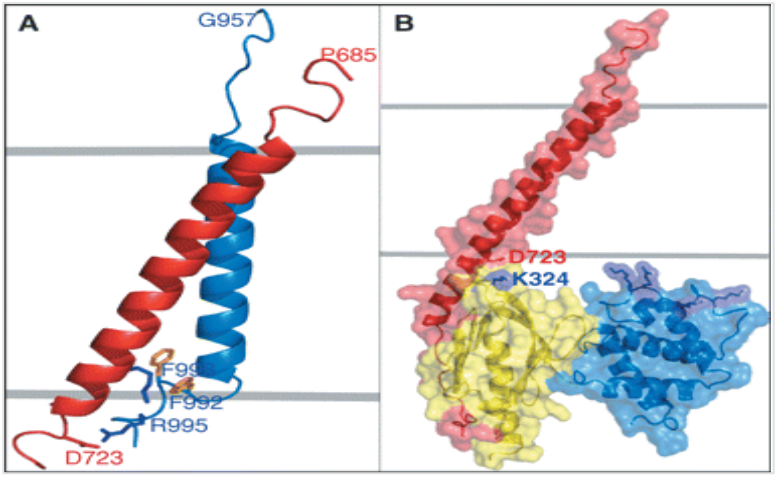


Figure 7: A) NMR structure of the complex of the TMs (transmembrane domains) of the αIIb (blue) and β3 (red). Gray outlines show the membrane glycerol backbone. **(B)** Talin domains F2 in blue and F3 in yellow make a pair with a β-chain (red). The salt bridge forms between Lys-324 on F3 of talin and Asp-723 of β3-tail (Campbell & Humphries 2011). (Published with permission)

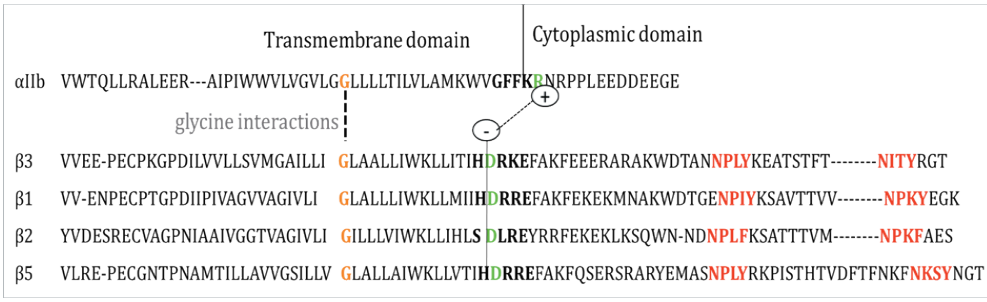


Figure 8: Sequence homologies of integrin TM (transmembrane) domains. The α and β-subunits are packed through glucine–glycine (orange) interactions in the TM domain. The salt bridge is shown in green in the membrane-proximal site of the cytoplasmic region. The functionally important amino acid residues NPXY and NXXY are shown in red.

Moreover, both the α and the β integrin TMDs possess 4–5 hydrophobic amino acids towards the C-terminal Lys residue in the transmembrane domains (Figure 8). The membrane-proximal region of α- subunits have a highly conserved Gly-Phe-Phe-Lys-Arg (GFFKR) motif, in which the two Phe residues are observed in all α-subunits regardless of species or integrin subtype. The conserved GFFKR sequence in the α-chains is significant for the non-adhesive state of integrins, while deletion or mutation of this motif results in integrin activation (O'Toole et al. 1994).

3.2.4 The cytoplasmic tails

The integrin cytoplasmic domains are short, mostly unstructured and comprised of 10–70 amino acid residues except for the β4- subunit, which contains more than 1,000 residues. The β-chain cytoplasmic tails are highly conserved while the α subunit tails are divergent (Figure 9). By studying the cytoplasmic peptides including the transmembrane/cytoplasmic interphase regions, it has been reported that the proximal portions of the α- and the β- cytoplasmic parts are α-helical and associate with each other (Iroumé et al. 2005). There are differences in published NMR studies of cytoplasmic tails. To date, only the structures of the αIIb/β3 cytoplasmic tails have been characterized under heterodimeric conditions. X-ray crystallography, electron microscopy (EM) and cryo-EM structures reveal that in the inactive state of integrins, the cytoplasmic domains of α and β chains stay close. Association of either the α and β cytoplasmic domains or the α- and β- subunit membrane-proximal domain stalks results in integrin inactivation (Adair & Yeager 2002, Lu et al. 2001). Also, Förster resonance energy transfer (FRET) -based studies provide evidence that separation of the cytoplasmic domains takes place after TCR activation of the integrins but not by ligand interaction (Wegener & Campbell 2008).

β1 KLLMIIHDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK

β2 KALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES

αL KVGFFKRNLKEKMEAGRGVPNGIPAEDSEQLASGQEAGDPGCLKPLHEKDSESGGGKD

αX KVGFFKRQYKEMMEEANGQIAPENGTQTPSPPSEK

αM KLGFFKRQYKDMMSEGGPPGAEPQ

αD KLGFFKRHYKEMLEDKPEDTATFGSGDDFSCVAPNVPLS

α4 KAGFFKRQYKSILQEENRRDBSWSYINSKSNDD

Figure 9: The cytoplasmic amino acids sequence of leukocyte integrin chains β1, β2, αL, αX, αM, αD, and α4. The amino acids which are important for phosphorylations are marked in red.

4. Leukocyte integrin signaling

Integrins transduce signals bidirectionally across the plasma membrane, referred to as "inside-out" and "outside-in" signaling. Each of these pathways is coupled to conformational changes in the integrin structures. Inside-out signaling is stimulated by chemokines or TCR activation that elicits the ligand-binding epitope, maintaining a higher-affinity state of the integrin ectodomain for its ligands. In contrast, outside-in signaling triggers cellular responses upon ligand binding to integrins and is involved in integrin clustering, cell spreading, retraction, migration, and proliferation of the cells (Abram & Lowell 2009, Davis & Dustin 2004, Shen et al. 2012).

4.1 Inside-out signaling

Binding of ligands to non-integrin receptors on the plasma membrane initiate a series of intracellular signals, which leads to integrin conformational change and activation. Activation of the TCR, B cell receptor (BCR), chemokine receptor, and selectins may lead to inside-out activation of LFA-1 (Kinashi 2005). In T cells, LFA-1-mediated adhesion can also be induced by CD3, CD2, CD44, CD45, CD73, CD98 and IL-2 receptors (Dustin & Springer 1989).

The intracellular signaling cascades of leukocytes have already been discussed in connection with the formation of the immunological synapse and extravasation part in chapters 2.1 and 2.2. Even though the activation mechanisms may be similar among all leukocytes, variations also occur in different cell types or the status and maturation and expression of the integrins on the cells. As an example, during adhesion and extravasation, it has also been shown that the effector T cells are not

dependent on chemokine signaling, but in contrast, naive T cells are solely reliant on the chemokine activation for adhesion and transmigration (Shulman et al. 2012).

In brief, ligation of TCR by anti-CD3 activates Lck, which in turn phosphorylates tyrosines in the ITAM motif of the CD3ζ chain. Activation of ITAM by phosphorylation further phosphorylates ZAP-70, and consequently, SLP-76 will be recruited to the phosphorylated LAT. The LAT/SLP-76 complex recruits and activates PLCγ. PLCγ hydrolyzes PIP_2 into IP_3 and DAG. IP_3 induces the release of Ca^{2+} from intracellular stores into the cytoplasm by binding to IP3 receptors in the endoplasmic reticulum (Lewis 2001). DAG, in turn, activates PKC enzymes that phosphorylate Thr-758 in the β2-chain of the LFA-1 (Figure 10) (Fagerholm et al. 2005).

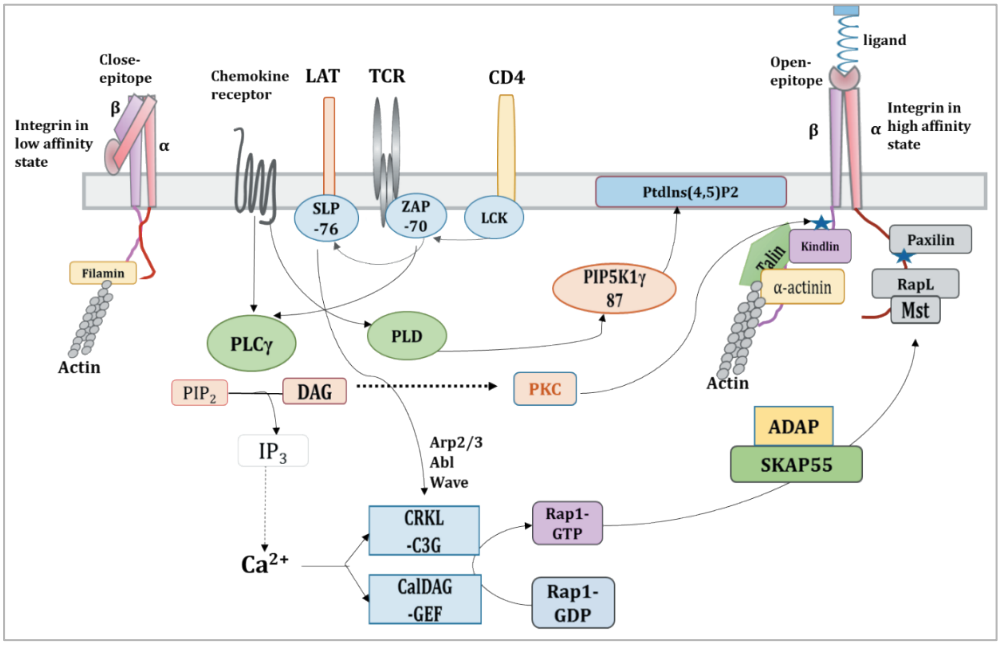


Figure 10: A simplified scheme of inside-out signaling of integrins. After activation of TCR (T cell receptor), the protein tyrosine kinase Lck is phosphorylated and activated, leading to the phosphorylation of TCR. After that ZAP-70 is recruited to the TCR. ZAP-70 then phosphorylates LAT (linker for activation of T cells) and SLP-76 (leukocyte protein of 76 Kd). SLP-76 is a scaffold protein, associated with GEF (guanine-nucleotide exchange factor) like Vav1 and activates Rac1, and Arp2/3 (actin-related protein-2/3) complex. SLP-76 and some GTPases also interact with ADAP (adhesion and degranulation-promoting adaptor protein), and ADAP directly makes a complex with SKAP-55 (Src kinase-associated protein of 55Kd.). SKAP-55 binds and brings RapL to the membrane, resulting in interaction with LFA-1. The ZAP-70 and LAT/SLP-76 complex activates PLCγ and PLCγ hydrolyzes PIP2 to IP3 and DAG. DAG activates PKC, which phosphorylates β2-chain and activates integrins from bent to extended conformation to interact with the ligand and facilitates interaction with talin, kindlin and α-actinin.

The initial result from the inside-out activation of the integrin is the separation of the α- and the β-chains, which increase the ligand binding and promote strong adhesion. The conformation and activation of the integrin affect the interactions of the cytoplasmic proteins with the integrins. There are many studies using mutations in the integrins, which cause either constant activation of the integrin or inhibit ligand binding. X-ray crystallography and EM studies reveal regions or sites, which are necessary for β2 and β3 integrins' activation (Luo et al. 2007). The regulation of the inside-out signaling can be typical for the most of the integrins or specific for some integrins depending on the cellular situation. For example, αIIβ3-expressing CHO cells interact with an activation- recognizing antibody after treatment with phorbol 12-myristate 13-acetate (PMA) only if the cells express equal amounts of talin and PKC_{α} (Han et al. 2006). The authors also found that Rap1 can activate αIIβ3 independent of PMA and PKC_{α} stimulation (Han et al. 2006). Ligation of TCR activates PLC, which in turn triggers DAG and Ca^{2+} release and excite PKD. PKD relocalizes Rap1 to PKD1 (PKC effector protein D1) in the cell membrane and association with the β1 integrins (Medeiros et al. 2005). In CalDAG-GEFI knockout mouse, impairment of αIIβ3 activation and aggregation is seen (Crittenden et al. 2004). Neutrophils from such mice show defects in Rap1 localization and consequently activation of β1 and β3 integrins. CalDAG-GEFI–null-mouse resembles the defects of leukocyte adhesion deficiency type III (LAD III) disease where mutations are found in CalDAG-GEFI (Bergmeier et al. 2007). There is a signaling cascade describing the complex of talin and Rap1 effector protein, RIAM, which binds to the α-chain and activates the integrins αIIbβ3 and LFA-1 (Watanabe et al. 2008, Zhang & Wang 2012). RIAM again links Rap1 to ADAP and Skap55, which are essential for αIIbβ3 and LFA-1 activation. In T cells, it was shown that the ADAP–Skap55 module makes a complex with Rap1–RapL after TCR activation. The interaction between RapL and the activated Rap1 brings RapL close to the cell membrane and allows RapL binding to the αL cytoplasmic chain of LFA-1 (Zhang & Wang 2012). It has also been shown that the N-terminal domain of Skap55 interacts with the C-terminal SARAH domain of RapL and forms a complex of Skap55-RapL-Rap1, which binds LFA-1 and increases binding to ICAM-1. In T cells, silencing of Skap55 impairs TCR-facilitated LFA-1 clustering and T cell–APC interaction (Jo et al. 2005). On the other hand, SDF-1α- or CCL21-induced regular migration is seen in Skap55-deficient T cells. It means that Skap55 is a specific adaptor protein that couples the TCR with the activation of Rap1–RapL component for LFA-1 adhesion. Skap55 knockout mice show deficiencies in β2 integrin

adhesion, clustering and downstream signaling (Wang et al. 2007). Thus, in T cells the Rap1/RIAM/ADAP/Skap55 complex formation at the membrane leads to talin activation and triggers integrin tail separation (Zhang & Wang 2012).

4.2 Outside-in signaling

Integrin outside-in signaling occurs through ligand interaction or integrin antibody binding (Campbell & Humphries 2011), or cation activation (Dransfield et al. 1992). Outside-in signaling may lead to firm cell adhesion, cell spreading, migration, and actin cytoskeletal rearrangement, even though all of these events depend on the types and conditions of the cell. For instance, in T cells, the outside-in cascade is connected to cell proliferation, interleukin-2 (IL-2) production and establishment of the IS formation between the T cell and the APC. Alternatively, in neutrophils, the signal enhances the degranulation and activation of the NADPH oxidase enzyme and reactive oxygen species (ROS) production, which increase the cytotoxic efficacy of the cells. The outcomes of the outside-in signal in macrophages are the cell differentiation and control of the IL-1β mRNA production (Abram & Lowell 2009).

There are two significant downstream consequences of outside-in signaling described (Figure 11). First, the formation of the focal adhesion (FA), a significant dynamic large protein assembly complex that spreads mechanical forces from sites of cell adhesion, and the reorganization of the actin cytoskeleton and integrin clustering . αIIbβ3 outside-in signaling occurs through tyrosine kinases particularly Src and Syk (Durrant et al. 2017). A lack of these kinases might affect both the signaling mechanisms of outside-in and inside-out of the integrins. In Jurkat cells, ligation of monoclonal antibodies to the β2 integrins leads to the clustering of integrins which then changes the focal zone of actin polymerization associated with other adaptor proteins and other downstream effector molecules in the outside-in signaling. Syk is capable of interacting directly both with the β3 cytoplasmic tail and ITAM via an SH2 domain although the interaction with the ITAM motif is independent of the tyrosine phosphorylation of the β3 integrin (Durrant et al. 2017). The lack of Syk kinase blocks entirely the signaling events of β1, β2 and β3 integrins in neutrophils and macrophages (Mócsai et al. 2002). On the contrary, the absence of this kinase does not inhibit the GPCR signaling events in neutrophils or mast cells.

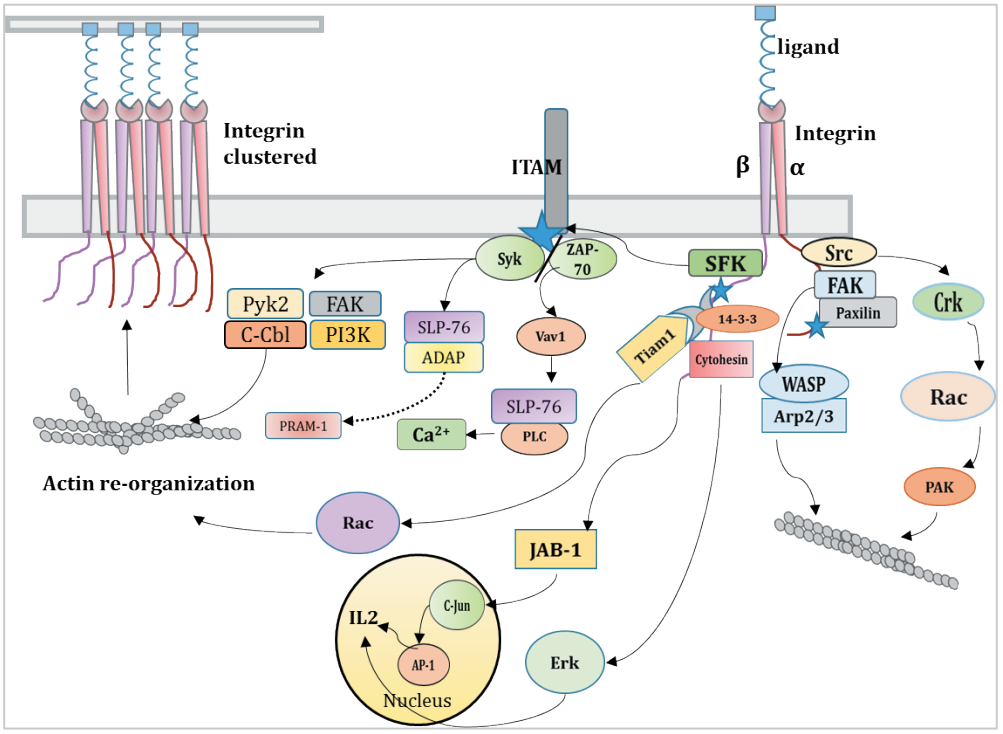


Figure 11: A simplified schematic path of outside-in signaling of integrins. Ligand binding to the integrin activates several kinases, including Src, FAK, and integrin-linked kinase (ILK). FAK binds to talin or paxillin, which interact with integrins. Phosphorylation of FAK, paxillin, and Src activates GTPase like WASP, also leading to Arp2/3 activation and actin polymerization. Ligand binding initiates the phosphorylation of SFK (Src family kinases) that phosphorylates ITAM, and activation of ITAM offers docking site for Syk (Syk family kinases) and ZAP-70. Downstream of signaling of SFK and Syk activates SLP-76 and ADAP. Ligand binding to integrins also phosphorylates cytohesin-1, and it is involved in ERK activation and IL-2 production. Ligand binding to LFA-1 releases JAB-1 (Jun-activating transcription factor) from its tail and transfer to the nucleus. In the nucleus, JAB-1 co-activates AP-1 (activator protein-1) resulting in IL-2 production.

Some adaptor proteins also function as scaffold proteins involved in the outside-in signaling of integrins. Among them, SLP-76 is the best-studied one. Inhibition of SLP-76 in neutrophils, dendritic cells and platelets block outside-in signaling of β2 and β3 integrins (Bezman & Koretzky 2007). Another interesting finding of SLP-76 is that the mutations of Tyr to Phe in the N-terminal or mutated the Gads-binding regions interupt the SLP-76 interaction with Gads and subsequently impair the TCR signaling pathway (Abram & Lowell 2009). Furthermore, another adapter protein ADAP and its myeloid-specific homolog PRAM1 are also involved in outside-in and inside-out signaling.

Another protein named cytohesin-1 has been reported both in outside-in and inside-out signaling (Verma & Kelleher 2014). Blocking of this protein affects the LFA-1 mediated ERK activation and IL-2 production. The transcription factor, JAB-1 (Jun-activating binding protein-1) is also involved in the outside-in signaling of LFA-1. When ligand binding activates LFA-1, JAB-1 is dissociated from the LFA-1's cytoplasmic part and migrates to the nucleus and coactivates AP-1 and consequently facilitates IL-2 production (Perez et al. 2003).

In outside-in signaling, the cytoskeleton is regulated through the modulation of RhoGTPases, which are governed by the Vav family of Rho-GEFs. Silencing or absence of Vav family proteins in neutrophils impairs β2-mediated adhesion, spreading of the cells and ROS production (Graham et al. 2007). In Vav1/3 knockout neutrophils, defects are also seen in β2-integrin mediated activation of Cdc42, Rac1, and RhoA. Vav proteins play significant roles in the regulation of PLCγ and Ca^{2+} signaling, predominantly in immunoreceptor (through TCR and BCR) pathways of T and B cells (Swat & Fujikawa 2005). Defects of PLCγ signaling and a poor Ca^{2+} response are seen in Vav knockout mice, and Vav signaling is also impaired in PLCγ knockout mice, as expected (Pearce et al. 2007). Other prime effectors of the cytoskeleton rearrangements in the integrin outside-in signaling pathways are RhoGTPases family members Rac, Rho, and Cdc42, which are vital for cell polarization and cell migration following adhesion (Ivetic & Ridley 2004).

5. Regulation of leukocyte integrins

Adhesion of leukocytes via integrins are pivotal mechanisms that convert biochemical signals to mechanical ones and vice versa or convey signals between cells. Regulation of integrins occurs at many levels. Integrins can be regulated through conformational changes, by integrin clustering and adhesion, and the timing of the interacting partners regulated by phosphorylation and adhesion maturation.

5.1 Affinity and avidity regulation

Integrin heterodimers undergo a process by which they change their conformation to increase the affinity of the ectodomains for extracellular ligand interaction (Iwamoto & Calderwood 2015). Crystallography and electron microscopic studies have described molecular mechanisms of integrin–ligand interactions and integrin

activation, and shown ectodomain extensions from bent to straight (Campbell & Humphries 2011, Gahmberg et al. 2009). However, the ectodomain rearrangements may vary between the heterodimers of different integrins (Xiong et al. 2009). Even so, most of the studies agree that the activation involves separation of the α- and β-cytoplasmic tails and TMDs which can be elicited by talin and kindlin on the β-chain (Harburger & Calderwood 2009, Shattil et al. 2010).

Inside-out activation of integrins transmitted from nonintegrin-mediated receptor would rapidly changes the conformation of the integrins and their affinity to bind the ligands. It has been shown by in vitro assays that this signal can be mimicked by adding divalent cations like Mn^{2+} (Smith et al. 1994), and Mg^{2+}(Dransfield et al. 1992) or activating monoclonal antibodies (Luque et al. 1996).

Inside-out signaling pathways can also stimulate integrin clustering, which contributes to increased integrin avidity. It is unclear whether the same signaling pathways stimulate both the affinity and clustering of the integrins at the same time, although it is evident that specific stimuli like Mn^{2+} activate the adhesion of LFA-1 but are unable to alter LFA-1 clustering (Kim et al. 2004). Research using T cells shows that upon PMA activation LFA-1 becomes more mobile in the lipid bilayer and clusters in the rafts (Krauss & Altevogt 1999). Interruption of lipid rafts inhibits leukocyte adhesion (Shen et al. 2012). However, in platelets lipid rafts do not influence the αIIβ3-mediated adhesion in outside-in signaling, but treatment of neutrophils with methyl-β-cyclodextrin (a chelating agent that disrupts the lipid rafts) induces integrin-mediated cell adhesion (Solomkin et al. 2007). Furthermore, crosslinking antibodies to the raft-associated CD24 increase LFA-1 clustering on the lipid rafts and also increase adhesion for ICAM-1, and this signaling is phosphatidylinositol-3-kinase (PI3K)-dependent. Additionally, exhaustion of cholesterol in the rafts prevents antibody- or PMA-mediated LFA-1 ligand binding, but will not affect the extracellular cation (Mg^{2+}, Mn^{2+})-mediated ligand communication (Marwali et al. 2003).

Leukocytes in the blood flow encounter shear force, which activates integrins; mechanical force can alter the conformation from bent to extended (Katsumi et al. 2005) as well as the integrin clustering (Katagiri et al. 2006). Neither LFA- nor VLA-4-mediated adhesion is seen in the absence of shear-pressure in the lymph node (Woolf et al. 2007).

It has also been suggested that the conformational change of the integrins by inside-out activation is transduced by the β-chain, whereas the outside-in signaling

initiates the integrin clustering carried through the α-chain (Kliche et al. 2012). However, a mutation in the β-chain of triple Thr-(758–760) to Ala-(758–760) did not change the initial interaction with the ligand, but on the other hand there was no adhesion between the integrin and the ligand ICAM-1 (Morrison et al. 2013).

5.2 Regulation through phosphorylation

Phosphorylation reported for integrins is an important mechanism for the regulation of protein-protein interactions. Both inside-out and outside-in activation of integrins can be regulated by the phosphorylation of integrins (Gahmberg et al. 2009). The cytoplasmic tails of the integrin are short but contain many potential protein binding sites. However, all proteins are not able to bind at the same time, so phosphorylation is one of the factors that regulate protein-protein interactions. It was reported already over two decades ago that the α-chains (αL,αM, and αX) of the β2 integrins are phosphorylated constitutively and the β2-chain upon cell activation (Gahmberg et al. 2009).

5.2.1 Phosphorylation sites in β2 integrins

There are several phosphorylations sites in the β2-chain. Tyr-735 in the β2-chain is found to be phosphorylated after IL-2 treatment of NK cells (Umehara et al. 1993) and during adhesion to type I collagen in neutrophils (Garnotel et al. 1995). This phosphorylation is vital for the LFA-1 internalization in the migration of T cells (Tohyama et al. 2003). The triple threonine on the β-chain (Thr-758–760) has been reported to be essential for the function of the leukocyte integrins (Hibbs et al. 1991). Later studies have shown that phosphorylation is vital for LFA-1-mediated ICAM-1 adhesion. However, these phosphorylations are very transient and without okadaic acid (phosphatase inhibitor) easily missed (Valmu & Gahmberg 1995). It has been shown that after the treatment of T cells with phorbol ester, the Thr-758–760 tripeptide on the β2-chain becomes phosphorylated and more detailed studies showed that Thr-758 is the major phosphorylated residue after TCR activation (Valmu et al. 1999, Hilden et al. 2003).

Two serine phosphorylations sites Ser-756 and Ser-745 are known in β2-chain (Fagerholm, Morrice, et al. 2002b, Hibbs et al. 1991). In T cells, Ser-756 becomes phosphorylated after PMA treatment but not upon CD3 ligation, and it is not significant for ICAM-1-mediated integrin adhesion (Fagerholm et al. 2002). However, phosphorylation of Ser-756 is vital for Mac-1-dependent phagocytosis through Rap1 and talin interaction with the β2 integrins (Lim et al. 2011). In T cells,

Ser-745 becomes phosphorylated after the PMA treatment. The function for this phosphorylation is that it releases JAB-1 from LFA-1 and transfers it to the nucleus where it co-activates the c-Jun transcription factor, AP-1 resulting in ERK1/2 signaling (Perez et al. 2003).

There are many PKC members involved in the phosphorylation of the Ser and Thr amino acids. Mainly PKCδ and PKCβI/II phosphorylate Ser-745 and Thr-758 but also PKCα, and PKCη may phosphorylate these amino acids. The PKCα isoform is also able to phosphorylate the Thr-760. PKC families cannot directly phosphorylate the Ser-756. Nevertheless, Ser-756 gets phosphorylated upon phorbol ester activation, demonstrating that PKC may be indirectly involved (Fagerholm et al. 2002).

The phosphorylation site on the αL-chain is Ser-1140, and this phosphorylation is needed for the activation epitope expression and as well as for ligand interaction (Fagerholm et al. 2005). In Jurkat cells, the mutation of Ser-1140 to Ala on the αL-chain of LFA-1 was unable to be activated by treatment with SDF-1α-, the ligand ICAM-2 or Mg^{2+} (Fagerholm et al. 2005). However, phorbol esters and anti-CD3 could activate Jurkat cells containing the mutated LFA-1 for ligand binding (Gahmberg et al. 2014). It has also been shown that SDF-1α- or ICAM-2-activated Jurkat cells containing the Ser mutation of LFA-1 cannot adopt the high-affinity conformation detected with a mab24 antibody, which recognizes the openly extended conformation (Fagerholm et al. 2005).

In Mac-1, the phosphorylation site for αM is Ser-1126, and it is constitutively phosphorylated like αL Ser-1140. Mutation of Ser-1126 to Ala affected ICAM-1/ICAM-2 binding after phorbol esters or after extracellular Mg^{2+} treatment, but on the other hand binding to other ligands such as iC3b and denatured serum albumin (BSA) was not reduced (Fagerholm et al. 2006). Importantly, i*n* vivo, homing of lymphocytes are also affected in the cells where αM contains the mutation. Attenuation of the phosphorylation of αM made cells stay in the circulation whereas WT cells accumulated in the spleen and lungs (Fagerholm et al. 2006). In αX, the phosphorylation site is Ser-1158. This phosphorylation is essential for adhesion and phagocytosis (Uotila et al. 2013). Phosphorylation sites for the αD-chain have not been reported yet.

As mentioned earlier, phosphorylations of the amino acid residues in the integrin directly or indirectly influence the protein–protein interactions. Phosphorylation of Thr-758 directly guides the 14-3-3 and filamin interaction with the β2-cytoplasmic tail. It is shown that upon phosphorylation of Thr-758 filamin

detaches and simultaneously more 14-3-3 binds to the β2-chain (Takala et al. 2008). The phosphorylations of αL, αM, and αX are functionally important but which kinases phosphorylate them or how these phosphorylation affect the interaction of the protein are not fully elucidated.

5.2.2 Phosphorylation sites in α4β1 and other integrins

The phosphorylation of amino acid residues Tyr-783, Tyr-795, Ser-785, Thr-788 and Thr-789 in the β1-chain have been described. Most of the studies use point mutations to clarify the importance of tyrosine for the cellular function. The tyrosine amino acids in the chain may be phosphorylated, but mutation of tyrosine to phenylalanine does not seem to affect integrin functions (Wennerberg et al. 1998). In contrast, phosphorylation of serine in the β1 shows the importance for integrins' localization in the focal adhesion sites. Phosphorylation of Ser thus increased cell adhesion and concurrently inhibited cell spreading and migration (Mulrooney et al. 2001). Threonine 788/789 residues of the β1-chain correspond to the phosphorylation site of Thr-758–760 of the β2-chain. The phosphorylation of these threonines in the β1 integrins is necessary for the activation epitope expression and fibronectin (Fn) binding (Wennerberg et al. 1998). Mutation of Thr to Asp acid on 788, which mimics the negative charge of a phosphate group, makes the β1 integrin constitutively active and increased cell adhesion of GD25 (fibroblast) cells (Nilsson et al. 2006). Furthermore, it has been shown that extracellular pressure induces phosphorylation of Thr-788/789, which increases β1 affinity towards collagen, laminin, and Fn, although mutation of threonine to aspartic acid in these sites did not increase the adhesion over the basal level (Craig et al. 2009).

Two phosphorylation sites of the α4 of VLA-4, Ser-988 and Ser-978, have been reported. It was shown that PKA phosphorylates Ser-988, and this is vital for paxillin interaction with the α-chain and VLA-4 function (Lim et al. 2008). Mutation of Ser-988 to Ala of α4 integrin reduced cell spreading, migration, and mutation to Asp induced cell spreading but inhibited migration. In contrast, both mutations can mediate cell adhesion to VCAM-1. Thus both phosphorylation and de-phosphorylation of Ser-988 are functionally important (Han et al. 2003). So, it seems that both wt and Ser-988 to Ala mutations can undergo outside-in signaling via VCAM-1 binding, while Ser-988 to Asp is not activated by ligand binding (Han et al. 2001, Han et al. 2003).

For leukocyte recruitment to the site of inflammation, paxillin interaction with the α4 integrin is needed (Alon et al. 1995). In the migratory cells,

phosphorylation of α4 enables integrin to be localized to the leading edge and mutation of Ser to Ala in the α4-chain destroyed lamellipodia formation and integrin localization. In contrast, there was no change in ligand affinity. Another interesting observation was that Ser-988 mutation to Asp acid in the α4-chain changed its localization; it was no longer enriched in lamellipodia (Goldfinger et al. 2003). Phosphorylation of Ser-978 was essential for 14-3-3 ζ interaction with VLA-4 and formed a complex of α4/14-3-3/paxillin that was needed for the activation of Rho-GTPase and cell migration (Deakin et al. 2009).

Phosphorylation of Tyr-759 of β3 integrins showed a vital role in signaling and platelet aggregation. Phosphorylation of Tyr-759 in β3 made the polypeptides resistant to calpain cleavage and inhibited integrin signaling, which is essential for cell spreading and focal adhesion formation (Phillips et al. 1999, Stojanovic et al. 2006).

6. Proteins involved in the signaling of β2 integrins

Integrins inside-out and outside-in signaling are regulated by phosphorylation of the α- and β-chains and by cytoplasmic protein interactions with the tail of the integrins. Many studies show that the competition and the phosphorylations are important mechanisms to regulate the interactions of cytoplasmic proteins with the integrin tails (Gahmberg et al. 2009).

6.1 Talin

Talin is a cytoskeletal protein and one of the interacting proteins that links the cytoplasmic domains of integrins to the actin cytoskeleton (Hemmings et al. 1996). In vertebrates there are two talin genes encoding isoforms talin 1 and talin 2. Talin 1 is ubiquitously expressed in mammals, on the other hand, talin 2 expression is limited with high levels in the heart, brain, and skeletal muscle (Monkley et al. 2001). Talin is 270 kDa in size and contains a globular head domain around 50 kDa in the N-terminal and a more extended C-terminal tail domain, which is around 220 kDa. The globular head domain has four FERM (FERM is protein 4.1, ezrin, radixin and moesin domain) subdomains and a C-terminal rod part that contains actin and vinculin binding sites (Hogg et al. 2011a). Talin is a crucial player in the activation of β1, β2, and β3 integrins and is pivotal for inside-out integrin signaling (Shattil et al. 2010). The FERM domain has three subdomains: F1, F2, and F3. The F3

subdomain interacts with the integrin membrane proximal NPXY/F region of the β-chain via the phosphotyrosine binding (PTB) site (Madejón et al. 2012).

An NMR study has shown that the K_d value of the talin head for the β2-tail is 0.1–0.5 nM while the K_d αL for β2 is 2.6 nM meaning that talin head can disrupt the αβ heterodimer (Bhunia et al. 2009). Additionally, it has also been shown that the head domain of talin is involved in the separation of the αβ tails and the stabilization of the high-affinity conformation of αIIbβ3 integrin (Iroumé et al. 2005). It is known that talin binds to the integrin tail and triggers integrin activation but how talin binding to the tail is regulated is still not clear (Calderwood et al. 2013). A study shows the involvement of the Rap1 effector RIAM in talin-mediated αIIbβ3 activation (Han et al. 2006). RIAM bound to the talin rod, and the RIAM–talin complex was recruited to the membrane with the active Rap1 (Lee et al. 2013). Later it has been shown that the RIAM-talin complex is needed for the activation of LFA-1 but not necessarily for β1 and β3 integrin activation (Calderwood 2015). In the studies where researchers show that RIAM knockout mice are fertile and viable, platelets also aggregated normally, but there were severe defects in β2 integrin-mediated leukocyte adhesion and trafficking (Su et al. 2015, Klapproth et al. 2015). Talin plays a vital role also in leukocyte regulation. Knockdown of talin by siRNA in both Jurkat and primary T cells showed a defect in adhesion to ICAM-1, decrease of LFA-1 clustering at the synapse, and impaired T cell-APC interactions (Verma & Kelleher 2014).

6.2 Kindlin

Many studies show that kindlin family proteins cooperate with talin (Calderwood et al. 2013). Kindlin contains FERM domains that are shown to interact with the distal NPXY motif, different from the talin binding site of the β1 and β3 integrin (Abram & Lowell 2009). Three kindlins are known: kindlin-1, -2 and -3 (Ussar et al. 2006). In Kindler syndrome, there is a mutation found in the gene encoding kindlin-1 protein (Karakose et al. 2010). The syndrome causes skin blistering and occurs due to the failure of actin function in keratinocytes. Kindlin-2 is ubiquitously expressed and interacts with ILK and migfilin (Montanez et al. 2008). Inhibition of kindlin-2 prevents integrin activation, whereas co-expression of kindlin-2 and talin activates integrins. Overexpression of the talin FERM domain is sufficient to activate the αIIbβ3 integrin, whereas overexpression of kindlin-2 does not change integrin activation (Montanez et al. 2008). Interaction of LFA-1 with ICAM-1 induces integrin

clustering on T cells which in its turn induces association of kindlin-3 with the scaffold protein RACK1 (receptor for activated C kinase 1). This association activates the PKC enzymes and translocates the PKCs to different sites in the cells (Feng et al. 2012). ICAM-1 ligation to LFA-1 activates T cells and makes a ternary complex of β2-tail/RACK1/kindlin-3, which further activates LFA-1 (Verma & Kelleher 2014).

6.3 Filamin

Filamin is a large cytoskeletal protein that binds to the integrin β-chain. Filamin mediates the interaction between integrin cytoplasmic tails to F-actin. The binding site for filamin is localized in the C-terminal portion of the β- tail and partially overlaps with that of talin. Filamin appears to be a negative regulator of at least the β7 and β2 integrins by competing with talin for the β-integrin tail (Calderwood et al. 2001). Filamin has an N-terminal domain that binds to actin and a C-terminal domain composed of 24 tandem immunoglobulin-like domains, responsible for protein-protein interactions (Himmel et al. 2003). Filamin also binds to other receptors and molecules that affect the actin cytoskeleton, such as the small GTPases RhoA, Rac1, and Cdc42 (Ohta et al. 1999). The structure of the integrin-IgFLN21 (filamin immuno- globulin-like domain (IgFLN) 21) complex has been resolved, and the crystal structure shows a general model for the filamin–integrin β7 interaction (Kiema et al. 2006). Phosphorylation of Thr-758 in the β2-tail inhibited filamin binding and at the same time recruited more 14-3-3 (Takala et al. 2008). Filamin plays a significant role in the regulation of cell motility, migration, and integrin signaling. It has been suggested that mutation or lack of filamin could be the cause of disorders in human. Mutation in the filamin A gene causes X-chromosome-linked brain disorders, in which neuronal migration is impaired during fetal development (Zhou et al. 2010). In melanocytes, loss of filamin causes impairment in migration and changes cell morphology (Liu et al. 2000).

Figure 12: Amino acid sequence of the β2-chain with the known phosphorylation sites in red and the binding site for talin, α-actinin, 14-3-3,kindlin and filamin in blue.

6.4 α-actinin

α-actinin is an antiparallel homodimeric rod-like and actin-cross-linking protein, which is expressed in all cells. In leukocytes, α-actinin plays a significant role in crosslinking the actin cytoskeleton with integrins (Gitlin & Colonna 2007). There are four isoforms of α-actinin called α-actinin 1-4. α-actinin 2 and α-actinin 3 are found in muscle cells whereas α-actinin 1 and α-actinin 4 are the non-muscle isoforms that are expressed in leukocytes. In leukocytes, α-actinins play a significant role in crosslinking integrins with the actin cytoskeleton (Otey & Carpen 2004, Foley & Young 2014). α-actinin is approximately 100 kDa and it contains three main parts: an N-terminal actin-binding domain, a central region of four spectrin-like repeats and a C-terminal domain containing two calcium-binding EF-hands (Virel & Backman 2004). The membrane-proximal region (aa 736–746) of the β2 integrin polypeptide contains the binding site for α-actinin, whereas the membrane-distal portion of β2-integrins has an inhibitory effect on binding to α-actinin (Sampath et al. 1998). It has been demonstrated that upon talin binding to the integrin, the α-actinin binding site is exposed due to tail displacement in a membrane-proximal region of the tail, which may stabilize the integrin open conformation and allow firm adhesion (Hyduk et al. 2011). Phospholipids and calcium can also regulate α-actinin interactions. α-Actinin may be involved in the regulation of cell–matrix adhesion disassembly in motile cells. Furthermore, α-actinin interacts with intermediate affinity with LFA-1 at the leading edge, and thus controls T cell migration (Stanley et al. 2008).

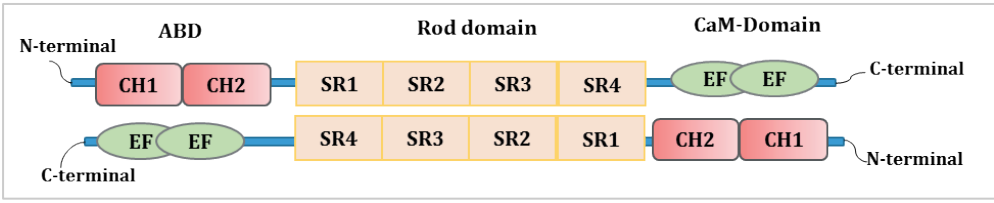


Figure 13: Schematic picture of the α-actinin protein. Each monomer contains an N-terminal actin-binding domain (ABD), a rod domain and a calmodulin (CaM)-like domain. ABD contains two calpain homology domains (CH1 and CH2), the rod domain comprise four spectrin repeats (SRs), and the CaM-like domain has two EF motifs.

Using light scattering, it was shown that filamin and α-actinin bind weakly to the β-chain compared to the talin interaction (Goldmann 2000). α-actinin localizes with the integrin in the focal adhesions similar to talin in fibroblasts (Otey et al.

1990). α-actinin is also present along stress fibers. In fibroblasts and osteoblasts, integrin-binding fragments of α-actinin interrupt stress fibers, the formation of focal adhesions and shear-induced mechanical force signaling of cells (Pavalko & Burridge 1991, Pavalko et al. 1998). Mutations in the membrane-proximal site of the integrin tails also change focal adhesion formation and alter stress fibers (Liu et al. 2000).

6.5 14-3-3

The 14-3-3 proteins are small dimeric adapter proteins of 28–33 kDa that bind to phosphorylated Ser/Thr sequences in proteins altering their localization and activity. The association of 14-3-3 with the Thr-758-phosphorylated β2 chain is crucial for actin cytoskeleton rearrangements, cell spreading and adhesion to ICAM-1 (Gahmberg et al. 2009). The 14-3-3 proteins are highly conserved from plants to mammals, and seven known mammalian isoforms of 14-3-3 have been identified. Although it has been shown that 14-3-3 can bind to the non-phosphorylated β2 chain (Grönholm et al. 2011), 14-3-3 binds stronger to the phosphorylated chain (Fagerholm et al. 2005). Most of the ligands of 14-3-3 contain a specific sequence (RSXpS/TXP) which allows optimal binding. The interaction between 14-3-3 and integrin β2 is functionally significant since a mutation in Thr-758 to Ala of β2 chain abrogates actin cytoskeleton rearrangements, cell spreading, and adhesion to ICAM ligands (Fagerholm et al., 2005; Takala et al., 2008).

6.6 T-lymphoma invasion and metastasis (Tiam1)

Tiam1 is a Rac GEF (Guanine nucleotide exchange factor) protein and GEF proteins activate monomeric GTPases by stimulating the release of GDP (guanosine diphosphate) to allow binding of GTP (guanosine triphosphate) (Cherfils & Zeghouf 2013). Tiam1 is mainly present in the cytoplasm of cells; however, upon activation, Tiam1 translocates to the plasma membrane. Under normal cellular conditions, the concentration of GTP is higher than that of GDP, favoring the replacement of GDP by GTPases. As a GEF for Rac, Tiam1 is impotant for cell adhesion, migration, invasion, metastasis, and carcinogenesis (Minard et al. 2004). Initially, Tiam1 was identified as a gene that confers an invasive and metastatic phenotype to the murine T-lymphoma cells and activates Rho-like GTPase (Mohammed et al. 2014).

Tiam1 is significant for cell migration. Overexpression of Tiam1 abrogates migration and invasion of epithelial cells by stimulating E-cadherin-mediated cell-cell adhesion between renal carsinoma cells. In contrast to this finding, Tiam1 may also stimulate cell migration and invasion of epithelial cells. Thus, it seems that the effects of Tiam1 on invasion to some extent is dependent on cell type and the levels of Rho and Rac (Ellenbroek & Collard 2007). Tiam1 has also been found in the progression of malignant tumor either as a mediator of Ras signaling in skin tumors or as a suppressor of the Wnt-responsive genes (Engers et al. 2006). Furthermore, overexpression of Tiam1 changes neuroblastoma and breast cancer cell morphology and cells become flattened epithelioid or sickle-shaped with extensive membrane ruffled (Minard et al. 2004).

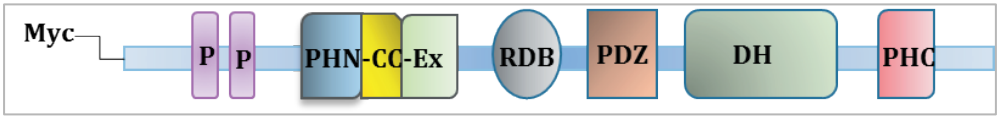


Figure 14: Tiam1 protein domain structure. Myc, myristoylation site; P, PEST sequence; PHN, N-terminal pleckstrin homology; CC, coiled-coil area; Ex, extended assembly; RBD, Ras-binding area; PDZ- PSD-95/DlgA/ZO-1 domain; DH: Dbl homology domain; PHC: C-terminal Pleckstrin homology domain.

Tiam1 interacts through its N terminus with 14-3-3 in HeLa cells and transfected COS7 cells (Woodcock et al. 2009, Pozuelo Rubio et al. 2004). It has also been shown that β1 recruits Tiam1 by 14-3-3ζ and subsequently facilitates Rac1 activation (O'Toole et al. 2011). Tiam1 is also involved in T cell trafficking and transendothelial migration. It was shown that although PKCζ/Tiam1/Rac signaling is not essential for T cell arrest, this signaling is significant for the stabilization of polarization and efficient crawling of T cells on endothelium (Gérard et al. 2009). Tiam1 plays an essential role for chemokine receptor (CXCR4) and Rac activation and cell migration. Knockout of Tiam1 in T cells reduces chemotactic migration (Gérard et al. 2009).

6.7 RapL

RapL was found in a yeast two-hybrid screen as a Rap1 binding protein (Katagiri et al. 2003). Rap1 is also known as Ras-related protein 1. It is a small cytosolic GTPase. RapL consists of an N-terminal Ras–Rap binding domain and a C-terminal coiled-coil domain, which are required for the direct or indirect interaction

with αL subunits (Cantor et al. 2008). Upon TCR or chemokine stimulation, RAPL binds to GTP-bound Rap1 and is essential for Rap1-mediated redistribution and clustering of LFA-1 (Hogg 2003). RAPL co-localizes with LFA-1 at the leading edge of migrating cells. Due to the proximity of a site consisting of two lysine residues following the GFFKR sequence in the αL cytoplasmic domain, it is proposed that, like talin, RAPL functions by destabilizing the αβ-cytoplasmic tail (Katagiri et al. 2003). RIAM-mediated binding of talin to the integrin β-tail and RAPL's interaction with the kinase Mst1 promote LFA-1 clustering and integrin activation (Tadokoro et al. 2003). Rap1/RAPL-mediated LFA-1 activation is regulated through the lysines-1097 and 1099 (Cantor et al. 2008). It has also been reported that for VLA-4-mediated T cell adhesion to VCAM-1, RapL interaction is essential for potent CCL25 chemokine triggering (Parmo-Cabañas et al. 2007). Lymphocytes and dendritic cells of RAPL-deficient mice show reduced adhesion in response to chemokines, and homing and migration defects in vivo (Katagiri et al. 2004).

7. Crosstalk between leukocyte integrins

7.1 Crosstalk between β2 and β1 integrins

A crosstalk between integrins means that the activation of one integrin regulates the function of other integrins. More than two decades ago, van Kooyk first noticed that in naive T cells and different T cell lines there are variations in LFA-1- and VLA-4-mediated cell adhesion on activated endothelium. She found that naive T cells use both LFA-1 and VLA-4 for adhesion on endothelial cells, and as the cells further proliferate, the adhesion becomes dependent on LFA-1 rather than on VLA-4. She also explained that the cells adhere via VLA-4 only when LFA-1 is not activated (van Kooyk et al. 1993). Later on, it was shown that the activation of LFA-1 inhibits most VLA-4 molecules and to some extent VLA-5 binding to their ligands VCAM-1 and fibronectin (Porter & Hogg 1997). However, elimination of the I-domain from the αL-chain of LFA-1 shows a different outcome of increased VLA-4/VLA-5-mediated adhesion to VCAM-1 in Jurkat cells. It shows that the lack of the I-domain in LFA-1 induces LFA-1 to be constitutively active and also activates VLA-4 and VLA-5, which consequently increases the VCAM-1 ligand binding. The increase of the VCAM-1 ligand binding may be due to the VLA-4 clustering on the membrane that undergoes actin reorganization (Leitinger & Hogg 2000).

Reciprocally, the ligation of VLA-4 with VCAM-1 stimulates the LFA-1 to bind ICAM-1. LFA-1 clustering on the cell membrane induced and enhanced adhesion of LFA-1 to ICAM-1 ligand. In increased adhesion due to avidity change of LFA-1, there is no change in the open activation epitope of LFA-1 as measured with the mab24 antibody (Chan et al. 2000). Importantly, it has been shown that phosphorylation of α4 on Ser-988 is needed for the trans-dominant regulation of the LFA-1. If the α4-chain becomes constitutively active by mutating the aa Ser988 to Asp, the interaction of paxillin with the integrin is inhibited and it is unable to convey signals to LFA-1 (Han et al. 2003).

Other β2 integrins have also been shown to exhibit crosstalk. In neutrophils, activation of β1 integrins via an activating antibody increased the Mac-1-dependent adhesion to fibronectin (Poloni et al. 2001). Some pathogens change the adhesion signaling in host cells via crosstalk mechanisms. For example, in whooping cough (*Bordetella pertussis* infection), activation of the β1 signaling pathway increases the bacterial adherence to monocytes or macrophages through Mac-1 integrin (Ishibashi et al. 1994). Moreover, CD11c is also reported to be involved in crosstalk in monocytes. After a high-fat meal, the expression of CR4 is increased together with VLA-4 which increases the adhesion of monocytes to aortic endothelium VCAM-1. This research suggested that in monocytes expression of CR4 not only facilitates the VLA-4 binding but also supports the VLA-4/VCAM-1 interaction (Gower et al. 2011).

7.2 Crosstalk between other integrins

Glazmann's thrombasthenia is a disease where platelet aggregation is defective, and patients suffer from severe bleeding. The mechanism behind this disease is integrin crosstalk. It was found that mutation of Ser-752 to Pro on the β3 integrin inhibited the inside-out activation of αIIbβ3 in platelets, which consequently inhibited the α2β1-mediated collagen binding (Gonzalez et al. 2010). Another crosstalk mechanism is also seen in this disease like that the binding of fibrinogen to β1 inhibits the collagen binding to β1 (Van de Walle et al. 2007, Riederer et al. 2002). Additional crosstalks have been reported between αvβ3 and α5β1integrins. Ligation of αvβ3 also speeds up the VLA-4-dependent migration on VCAM-1 as well as extravasation of the cells (Imhof et al. 1997). It has as well been shown that activation of the αvβ3 integrin inhibits ICAM binding of LFA-1 in monocytes (Weerasinghe et al. 1998). In phagocytosis, crosstalk of integrins has been reported. Blocking phagocytosis by αVβ3 integrin also inhibits the α5β1-mediated

phagocytosis. One study suggests that αVβ3 ligation inhibits α5β1-dependent phagocytosis without an effect on α5β1-mediated adhesion to fibronectin (Blystone et al. 1994). Later on, it was found that monocyte α5β1 is activated by Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), which mediates phagocytosis and migration, and α5β1 can be inhibited by the interaction of integrin-asociated protein (IAP) with the β3 cytoplasmic tail (Blystone et al. 1995, Blystone et al. 1999). There is a report of crosstalk of αIIbβ3 and α5β1 integrins in platelets. Usually, cells bind to Fn via α5β1 integrin; however, upon the activation of αIIbβ3, binding and spreading of FN is reduced (Díaz-González et al. 1996). αVβ5-mediated phagocytosis is vital for maintaining the physiological condition in the eye, and in glaucoma, it was found that activation of αVβ3 inhibited the αVβ5-mediated phagocytosis. Upon steroid treatment, αVβ3 was activated and inhibited αVβ5-mediated phagocytosis, leading to glaucoma (Gagen et al. 2013).

8. Clinical significance of leukocyte integrins

The leukocyte adhesion cascade firmly controls leukocyte trafficking to the site of infection. The inflammatory response of endothelial and many other cells produces cytokines and chemokines, which promote leukocyte recruitment by inside-out activation of integrins and upregulation of the adhesion molecules on the surface of endothelial cells which function as counter-receptors of integrins (Gahmberg et al. 2009). Too much or insufficient leukocyte recruitment to the inflamed site by integrins is a hallmark of several inflammatory diseases like multiple sclerosis (MS), psoriasis, inflammatory bone loss, inflammatory bowel disease and many more (Yonekawa & Harlan 2005).

MS is an autoimmune disease that is characterized by demyelination in the central nervous system (CNS). In this disease, leukocytes are capable of penetrating the blood brain barrier, start attacking the neuron-protecting myelin sheath, and leave a lesion in the CNS (Lassmann et al. 2012). The involvement of VLA-4 in leukocyte recruitment to the inflamed CNS sites has been extensively studied (Sheremata et al. 2005). Natalizumab, a monoclonal antibody to α4 integrin has been approved for the treatment of MS (Weinstock-Guttman 2013). Initially it was shown in a rat model of MS that continuous blocking of VLA-4 reduces the disease lesions in the CNS (Yednock et al. 1992, Baron et al. 1993). Furthermore, several studies also elucidated the significance of β2 in this disease model. Gordon and others have used blocking antibodies against CD11a and CD11b and have shown

that targeting the β2 integrins also blocks or delays the onset of the autoimmune encephalomyelitis (Gordon et al. 1995). Blocking the adhesion of Mac-1 to the platelet GPIba (platelet specific receptor glycoprotein Iba) also blocks the leukocyte-platelet interaction, which is significant for the amelioration of the disease severity (Langer et al. 2012).

An inflammatory disease of the skin is psoriasis. Usage of efalizumab, a monoclonal antibody against the αL chain in the treatment of psoriasis showed significant reduction of TNF, nitric oxide synthase production and the number of DCs in the lesion area (Lowes et al. 2005). It has as well been shown *in vivo* that blocking of Mac-1 together with LFA-1 improves the skin manifestations (Schön et al. 2000).

Blocking of VLA-1 integrin, a collagen receptor, reduces the infiltration of T cells into dermis, in a in vitro transplantation psoriasis model. The presence of VLA-1 in the epidermis activated effector T cells that increased the expression of interferon-γ (Conrad et al. 2007).

One more inflammatory disease is rheumatoid arthritis (RA), where synovial inflammation occurs by infiltrating neutrophils, T cells, B cells, DCs, and macrophages (Choy 2012). The role of leukocyte integrins has been reported in RA in many animal models. For example, concomitant inhibition of LFA-1 and Mac-1 completely blocks the neutrophil recruitment to the synovial inflammation (Issekutz & Issekutz 1993). Later on, the same group showed that blockage of LFA-1 and VLA-4 are required for the total inhibition of monocytes (Issekutz & Issekutz 1995).

Additionally, VLA-2 was shown to stimulate arthritis in an antigen-induced arthritis (AIA) model. VLA-2-deficient mice show less cartilage loss and joint pathology compared with the wild-type (Peters et al. 2006).

The central role of β7-integrins has been described in the formation of gut-associated lymphoid tissue (Koboziev et al. 2010). The humanized monoclonal antibody etrolizumab specifically binds to the β7 integrin in both α4β7 and αEβ7 heterodimers. This antibody provoked both α4β7-MAdCAM-1-mediated lymphocyte recruitment and αEβ7–E-cadherin interaction (Lin & Mahadevan 2014), and at the same time promoted adhesion of αEβ7 T cells to epithelial cells (Hadley & Higgins 2014). Several studies suggested a significant role of β2-integrins in a rodent experimental model (Palmen et al. 1995, Ostanin et al. 2007).

V. Aims of the study

The aims of this study were

- To discover signaling molecules downstream of LFA-1/14-3-3
- To investigate how the inside-out and outside-in activations of the LFA-1 regulate VLA-4 binding to its ligand VCAM-1
- To investigate how the treatment with LFA-1-specific antibodies via outside-in signaling activates LFA-1's intracellular signaling and affects the VLA-4 mediated adhesion to VCAM-1
- To study how the phosphorylation of the LFA-1 α-chain regulates the function of the β2-chain

VI. Methods and experimental procedures

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

Experimental procedure	Original publications			
Reagents	I	II	III	IV
Cell Culture	I	II	III	IV
Antibodies	I	II	III	IV
Immunoprecipitation	I	II	III	IV
Co-immunoprecipitation	I	II	III	IV
SDS-PAGE	I	II	III	IV
Immunoblot	I	II	III	IV
Affinity chromatography	I			
siRNA	I			
Rac-1 activity assay	I	II		
Immunofluorescence	I	II	III	IV
Cell adhesion assays (static)		II	III	IV
Cell adhesion assays (shear flow)		II	III	
Cell migration assays (Transwell)	I	II	III	
Cell migration assays (chemotactic directional)				IV
Flow cytometry		II	III	IV
T cells isolation from buffy coat cells	I	II	III	
Stable cell line production		II		
Pep-spot assay				IV
Peptide transfection		II		IV
DNA transfection				IV
Adhesion complex isolation				IV

VII. Results and Discussion

1. Tiam1 is found as a novel interacting protein in the complex of 14-3-3 and β2–chain in the intracellular signaling from LFA-1

1.1 Tiam1 links the phosphorylated LFA-1 to Rac activation

The cytoplasmic proteins 14-3-3, filamin, talin, kindlin, and α-actinin are involved in the intracellular signaling of LFA-1. Phosphorylations and dephosphorylation of the β2-chain regulate the interaction of these proteins with LFA-1, but the complete mechanisms of the regulation are not entirely understood (Gahmberg et al. 2009). Phosphorylation of the β2-chain on Thr-758 causes filamin release from the β2-tail and simultaneously promotes 14-3-3 binding to the phosphorylated β2 integrin, which activates Rac-1 (Nurmi et al. 2007). However, how phosphorylated β2 integrins via 14-3-3 binding activate Rac and finally actin cytoskeletal reorganization was not known. We were interested in Tiam1 as the potential Rac-GEF for a 14-3-3 binding partner. Tiam1 is a Rac-specific GEF and interacts with 14-3-3 in HeLa cells and transfected Cos-7 cells (Pozuelo Rubio et al. 2004; Woodcock et al. 2009). 14-3-3 proteins do not bind directly to G proteins but are instead involved in protein regulation interacting with other proteins (Jin & Li 2002). To investigate if Tiam1 delivers signals from LFA-1 and 14-3-3 to Rac1, co-immunoprecipitation experiments were done with JE6.1 Jurkat cells. These showed that Tiam1 was pulled down with 14-3-3 and vice versa and the 14-3-3–Tiam1 complex was also found in β2 precipitation experiment. Thus, Tiam1 formed a complex with β2 and 14-3-3 (I, fig. 1A & B).

The β2–14-3-3–Tiam1 complex was seen in both non-stimulated and anti-CD3 stimulated T cells but more protein was seen in the complex in the stimulated cells as compared to non-stimulated cells. We knew from before that TCR activation by anti-CD3 leads to the phosphorylation of Thr-758 in the β2-chain of LFA-1 (Pazos et al. 2017) and more 14-3-3 binds to pThr-758 (Fagerholm et al. 2002). Next, our quest to answer whether the activation of T cells affects the Tiam1 interaction with 14-3-3. Therefore, unstimulated and stimulated T cells with anti-CD3 were put over 14-3-3-conjugated Sepharose columns, and found that more Tiam1 bound with 14-3-3 from activated cells (I, fig. 1D and 2A). Next, we determined whether Tiam1

directly binds to the β2-chain or interact to the β2 via 14-3-3. For this, we blocked 14-3-3 interaction with the β2 integrins with the peptide R18 (PHCVPRDLSWLDLEANMCLP). We conjugated two short peptides corresponding to the β2's cytoplasmic tail containing phosphorylated (CLFKSApTTTVMN) and non-phosphorylated (CLFKSATTTVMN) Thr-758 with Sepharose beads. Afterward, we blocked all the 14-3-3 binding sites using the peptide R18 or– as a control – P621 peptide (VDVDSDGSTDLVIGA) with the pβ2/β2 bound Sepharose column and incubated with unstimulated JE6.1 Jurkat cell lysates. Significantly more 14-3-3 and Tiam1 interacted with pβ2 compared to the β2 column, and when 14-3-3 was blocked, no or rare interaction of 14-3-3 and Tiam1 was visible irrespective of the phosphorylation of β2 (I, fig2B). Hence, 14-3-3 is required for LFA-1/Tiam1complex formation.

Very little is known about the signaling pathway by which Tiam1 is controlled in T cells. Tiam1 has been reported as a specific activator for the Rho-like GTPase Rac (Mertens et al. 2003). It was shown before that chemokine-activated Rap1 triggered Par polarity complex with PKCζ and consequently activates Tiam1 and then Rac1, which is essential for T cell polarization (Gérard et al. 2007). We also showed that Tiam1 activates Rac1 and actin cytoskeletal reorganization. For that, we knocked down Tiam1 by siRNA in JE6.1 Jurkat cells and looked for active Rac1 which was determined by the PAK-PBD (p21 activated kinase–p21 binding domain) agarose. We found that introduction of Tiam1 targeting siRNA reduced active Rac1 whereas control siRNA had no effect. Inhibition of Tiam1 also decreased the total active Rac-1 both in JE6.1 Jurkat and T cells (I, fig 3A & B). Thus, we found that in Jurkat cells the Tiam1 complex with activated β2 is a prerequisite for Rac-1 activation after TCR activation. Tiam1 is also a crucial player in α3β1 integrin-facilitated activation of Rac, which is vital for the production and the secretion of the laminin-5 protein (to the new nomenclature it is called as α3β3γ2, laminin-332 (Aumailley et al. 2005)). Laminin-5 is required for spreading and migration of keratinocytes (Hamelers et al. 2005). Recently, it was shown that Tiam1 activates Rac through an association with the transmembrane cysteine-rich protein Semaphorin4D, which promotes cell proliferation, invasion and metastasis in oral squamous cell carcinoma (OSCC) cells (Zhou et al. 2017).

1.2 Tiam1 is required for LFA-1-mediated cell adhesion and migration

Tiam1 is reported to increase or decrease cellular adhesions and migrations depending on the cell lines (Minard et al. 2004). Malliri et al. 2004 found that Tiam1 was involved in the formation and the maintenance of cadherin-dependent adhesions in fibroblasts. We were interested in observing the effect of Tiam1 in T cell adhesion since it has been shown before that Rac1 regulates integrin-mediated adhesion (D'Souza-Schorey et al. 1998). The Tiam1 inhibitor (*N*6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine trihydrochloride) inhibited 60% of the anti-CD3-activated T cell adhesion to its ligand ICAM-1 (I, fig. 3C). So it seems that Tiam1 is involved in Rac dependent cell adhesion in T lymphocytes. Chemokine signaling regulates several signaling events in lymphocytes, including the Rac activation (Kinashi 2005, Zhou et al. 2017). T cells migrate towards the chemotactic attractants (Springer 1994). We showed here that chemokine activation phosphorylated the β2 integrin on Thr-758 and increased cell migration. This migration was LFA-1-dependent as cells migrated on ICAM-1 and this migration was reduced when LFA-1–ICAM-1 binding was blocked by the LFA-1-specific blocking antibody 7E4. We then studied whether Tiam1 was required for migration and we found that treatment of cells with the Tiam1 inhibitor reduced cell migration as well as the LFA-1 blocking 7E4 antibody. These results showed that Tiam1 is required for LFA-1-dependent cell migration towards the chemokine SDF-1α (I, fig 4 A, B, and C). Next, we aimed to elucidate if the migration was dependent on the signaling pathway from pβ2–14-3-3–Tiam1. Therefore, cells were transfected either with the β2 or pβ2 peptide to activate downstream signaling, and the migration assay was performed. Activation of the downstream signaling of LFA-1 by phosphorylation of Thr-758 on the β2-chain doubled the number of migrating cells and migration was significantly inhibited by incubating with the Tiam1 inhibitor and with R18 (14-3-3 blocking) peptide (I, fig 5A, B, & C). Thus, 14-3-3 and Tiam1 were required downstream of LFA-1 signaling for migration. Active Tiam1/Rac drives invasion of T-lymphoma cells on fibroblasts via activation of lysophosphatidic acid (LPA) and sphingosine one phosphate (S1P) receptor-mediated Rho A and PLC signaling (Stam et al. 1998). Activation of Tiam1 is a prerequisite for actin cytoskeleton reorganization during neuronal cell migration and neurite extensions (Ehler et al. 1997). These results correspond to our observations.

Tiam1 localize differently in cells depending on the morphology and differential phase of the cells. In non-motile fibroblast cells Tiam1 localizes to the adherens junctions, while Tiam1 is found in the lamellae of migrating cells (Sander et al. 1998). In this study, Tiam1 formed a capped like structure with LFA-1 in activated JE6.1 Jurkat cells. The inhibitor against Tiam1 inhibited the formation of capped like structure of Tiam1 in Jurkat cells (I, fig. 6A, B). LFA-1 can adopt in three conformational stages: bent, extended and open-extended as also mentioned earlier. In our hands (immune fluorescence experiments), we observed that activation of Jurkat cells by anti-CD3 and SDF-1α- increased the numbers of extended conformational state of LFA-1 as detected with KIM127 antibody. However, conversely, the open-extended conformation of LFA-1, recognized by mab24 was found only in anti CD3-activated cells (I, fig. 6 A–E). It may be due to the lack of the spreading and sheer force that this experimental setup was unable to open the mab24 epitope after SDF-1α- activation or the activation is too rapid to observe. It has recently been shown in neutrophils that β2 integrins can interact with ICAM-1 also in bent conformation which subsequently inhibits leukocyte adhesion (Fan et al. 2016). Tiam1 is reported as a vital player for Rac/Rho activation in many signaling pathways. Tiam1 may also be involved in cell division (Chen et al. 2012). PAK, a downstream effector of Rac1, promotes gene transcription and regulates cell division by MAP kinase pathway. For that reason, Rac activation via Tiam1 indorses cells division and cell migration. Importantly, Tiam1 is found to be overexpressed in many malignancies including T cell lymphomas. Tiam1 knockout mice show impaired carcinogen-induced HRAS (the gene for H-Ras enzyme) activation which prevented skin carcinoma formation (Vigil et al. 2010).

2. Intracellular activation of LFA-1 transmits signals to the leukocyte integrin VLA-4

2.1 LFA-1 inhibits adhesion of VLA-4 to its ligand VCAM-1

T cells express different kinds of integrins, of which LFA-1 and VLA-4 are mainly important for the adhesion to endothelial cells. These integrins play different functions in the cascade of transmigration. VLA-4 mediates cell tethering and rolling along with firm adhesion (Alon et al. 2005), while LFA-1 takes part in the formation of strong adhesions and regulates migration on the endothelial (Hogg et al. 2011b). Little known how integrins work together. They can be regulated seperately but also

regulate the function of each other. Crosstalk between LFA-1 and VLA-4 in leukocytes was first observed in 1993 (van Kooyk et al. 1993). Crosstalk among different integrin subclasses has also been reported in many other cells. It has been shown that when one integrin is activated, it affects the ligand binding of other integrins (Chan et al., 2000), but the mechanisms behind this are not well understood.

Jβ2.7 is an αL-chain lacking (Jβ2.7/LFA-1 negative) Jurkat cell, derived from the parental Jurkat T cell line (Weber et al. 1997), lacking LFA-1 expression on the cell surface. To study crosstalk between LFA-1 and VLA-4 we made Jβ2.7 Jurkat cells stably expressing LFA-1 (Jβ2.7/LFA-1 positive) by transduction (Uotila et al. 2014). We verified the expression of LFA-1 and VLA-4 on the surface of Jβ2.7/LFA-1 cells by flow cytometry. During the extravasation cascade of leukocytes, VLA-4 is reported to participate in cell tethering, rolling and arrest (Shamri et al. 2005), whereas LFA-1 is involved in tight adhesions on the endothelium (Hogg et al. 2011a). Jurkat cells are reported to adhere well to VCAM-1 through VLA-4 (Manevich et al. 2007). We showed that Jurkat cells which don't express LFA-1 on the surface, interacted with VCAM-1 only via VLA-4, as we could block VLA-4 binding using blocking antibody towards α4 or β1 which blocked VCAM-1 interaction. The binding of VLA-4 to VCAM-1 was also inhibited when LFA-1 was expressed, and activated by SDF-1α. Significantly more inhibition was observed both from the static and flow adhesions on purified VCAM-1 as well as on the activated endothelium. Jβ2.7/LFA-1-negative cells spread and made long extensions as well as bound strongly to VCAM-1 as compared to the Jβ2.7/LFA-1expressing cells (II, fig. 1A–E).

The firm adhesion of VLA-4 and VCAM-1 was also seen in the Transwell migration assays. Jβ2.7 cells lacking the LFA-1 on the surface were unable to transmigrate through VCAM-1 coated filters towards chemokines. These stable adhesions were released and cells were able to migrate when cells were treated either with α4- or β1-blocking antibodies or upon the expression of LFA-1. Furthermore, the long extensions from the Jβ2.7 on VCAM-1 also started retracting with time and disappeared almost completely after 120 minutes (II, Fig. 2 A–C).

2.2 LFA-1 signaling through pThr-758 β2, 14-3-3 and Tiam1 causes transdominant inhibition of VLA-4

Naive T cells adhere to ligands via LFA-1 and VLA-4, but blasts T cells adhere mainly via LFA-1, even though the expression of LFA-1 and VLA-4 stays the same (van Kooyk et al. 1993). In the activated T cells, VLA-4 is involved in slowing down the

rolling cells while LFA-1 captures the cells and promotes adhesion on endothelium (Porter & Hogg 1997). We saw that the occupancy of LFA-1 induces crosstalk to VLA-4. Transfection of the pThr-758 β2 peptide was enough to initiate the β2 signaling pathway (Grönholm et al. 2011). When we introduced the pβ2 peptide but not the β2 peptide to the Jβ2.7/LFA-1 negative Jurkat cells, it inhibited the adhesion of VLA-4 to its ligand VCAM-1. We were then interested in knowing whether the transdominant inhibition of VLA-4 happened via the pThr-758/β2–14-3-3–Tiam1 signaling pathway. Hence, we treated the cells with the 14-3-3- blocking peptide R18 or the Tiam1 inhibitor before performing flow adhesion experiments on VCAM-1, and indeed, pThr-758 β2-mediated transdominant inhibition was overruled with these inhibitors. These results suggest that 14-3-3 and Tiam1 are necessary for the crosstalk of VLA-4 inhibition to the ligand VCAM-1(II, fig.5A–C).

2.3 LFA-1 activation leads to changes in the phosphorylation status of VLA-4 and consequently affects protein-protein interactions

As we described that the activation of LFA-1 caused the phosphorylation of Thr-758 in the β2-chain and therefore inhibited the VLA-4 ligand binding, we were interested in studying the effect of the phosphorylation state of the β1 integrins and adaptor protein binding. The importance of phosphorylation of Thr-788/789 of the β1 integrins (Thr-788/789 sites of β1 integrin corresponds to the triple Thr-758–760 of the β2 integrin) have been described before. The phosphorylation of Thr-788/789 of β1 is necessary for fibronectin binding and for the expression of the activation epitope (Wennerberg et al. 1998). Mutation of the Thr-788 to Asp that mimics the phosphorylation of the β1 integrin is shown to induce active β1 and increase cell adhesion via inside-out activation (Nilsson et al. 2006). Suzuki and Takahashi reported that dephosphorylation of Thr-788/789 of β1 reduces β1-mediated adhesion to laminin in human mammary epithelial cells and that phosphorylation of β1 reduces the β1 association with the actin cytoskeleton (Suzuki & Takahashi 2003). It has also been shown in cancer cells that extracellular pressure induces phosphorylation of β1 in Thr-788/789 which increases integrin affinity (Craig et al. 2009). Phosphorylation of α4 regulates the dynamic adhesion events in Jurkat cells through the regulation of paxillin binding (Han et al. 2001). Ser-988 phosphorylation on α4 was found to be equally strong despite the active expression of LFA-1 on the cell surface. However, we observed that after SDF-1α activation, there was a drastic reduction of the phosphorylation of Thr-788/789 of

β1 in Jβ2.7/LFA-1 positive cells, but not in Jβ2.7/LFA-1 negative cells (II, fig. 6A). Next, we investigated how the adaptor proteins were involved in the regulation of integrin activity. 14-3-3ζ has been reported to bind more to the phosphorylated β2 integrin, which facilitated Tiam1 interaction with the β2–14-3-3 complex and further activated Rac-1 (Nurmi et al. 2007; Grönholm et al. 2011). The cytoplasmic tail of β1 also interacts with filamin, 14-3-3 and talin (Legate & Fassler 2009). Filamin is reportedly a negative regulator of integrin activation (Takala et al. 2008). Filamin was found to interact with VLA-4, only when LFA-1 was present and activated, whereas 14-3-3ζ binding to VLA-4 was decreased in cells expressing active LFA-1. In contrast, when LFA-1 was activated, it bound to14-3-3 and not to filamin (II, fig. 6A and B). LFA-1 interacts with 14-3-3 only through the β2-chain whereas VLA-4 binds with 14-3-3 via both the α and β chains. Phosphorylation of α4 on Ser-978 integrin engages 14-3-3ζ binding and further forms a complex with paxillin, which activates the Rho GTPase Cdc42 at the leading edge of migratory leukocytes. In contrast, the interaction between α4 and paxillin is enough to regulate the function of another Rho GTPase, Rac1 (Deakin et al. 2009). Mutation of the Ser-978 to Ala of the α4 integrin blocked migration as well as reduced lamellipodia stability, but the affinity of the ligand binding remained constant (Goldfinger et al. 2003). The affinity of 14-3-3ζ to the phosphorylate β2 was higher as compared to the phosphorylated α4 (Bonet et al. 2013). The cytoplasmic tail of the β1 interacted with 14-3-3ζ regardless of the phosphorylation of the β1, but the 14-3-3 interactions with β1 was much weaker than with the phosphorylated α4 and β2 polypeptides (Bonet et al. 2013). Thus activation of LFA-1 leads to the phosphorylation of β2 integrin that sequestered the 14-3-3 proteins and facilitated filamin binding to the β1 chain and inhibited VLA-4–VCAM-1 interaction.

We also studied the distribution of α4, talin, and phalloidin in Jβ2.7/LFA-1 negative and Jβ2.7/LFA-1 positive Jurkat cells. We found that more α4 and talin were co-localized with phalloidin in the LFA-1 negative cells than in LFA-1 positive cells. Confocal microscopy data suggested that filamin stayed firmly on the adhesion sites whereas talin and 14-3-3 localized to the membrane of the non-migratory, LFA-1 negative cells (II, fig. 6D and E). Because of the absence of the LFA-1, adhesion with the ligand took place via VLA-4 and thus more α4, talin and 14-3-3 were found in the adhesion sites.

3. LFA-1-specific antibodies induce crosstalk from LFA-1 to VLA-4

3.1 LFA-1-specific antibodies differently regulate VLA-4–VCAM-1 binding

Integrins communicate bi-directionally across the plasma membrane. As also mentioned earlier outside-in signals happen by binding of ligands or antibodies to integrins, whereas inside-out signals are due to the ligand binding to non-integrin receptors, such as chemokine receptors or the T-cell receptor. After studying how inside-out activation of LFA-1 affects VLA-4 adhesion we wanted to study the crosstalk to VLA-4 after the activation of LFA-1 via outside-in signaling. Therefore, in this investigation, we used antibodies that target LFA-1. The use of neutral, activating or blocking antibodies for targeting the integrins is one of the consistent tools for the study of integrin regulation. Activating antibodies stimulate ligand engagements or conformation resulting in intracellular signaling, inhibiting antibodies are those that impede ligand engagement and neutral antibodies usually do not have any effect on integrin regulation (Byron et al. 2009). These three categories of antibodies can bind to the same or different domains (Schiffer et al. 1995) of LFA-1.

The binding epitopes and functionality (activating, inhibiting or neutral) of the antibodies towards LFA-1 used in our study were mapped before. However, we verified them by static adhesion assays on ICAM-1. Static adhesion showed that treatment of activating antibodies increased ICAM-1 binding, inhibiting antibodies blocked ligand binding, whereas neutral antibodies did not have any effect on integrin–igand interaction (III, fig.1A, and B). Transdominant inhibition of VLA-4 with its ligand VCAM-1 was observed in the flow adhesion assay of Jβ2.7 Jurkat cells and T blast cells with the LFA-1-specific activating antibodies CBR1/2, MEM48 and MEM148. Strong transdominant inhibition was observed with the inhibiting antibody 7E4 and, a slight inhibition with TS1/18, but not with MHM24, MHM23, and TS1/22. Neutral antibodies did not have any effect on the transdominant inhibition of VLA-4. The transdominant inhibition of VLA-4 by 7E4 was not dependent on the concentration nor Fab fragment (III, fig. 2A-D) of the antibody.

	CHAIN	BINDING SITE	LFA-1 ACTIVITY
CBR1/2	β2	I-EGF-3	ACTIVATING
MEM48	β2	I-EGF-3	ACTIVATING
MEM148	β2	HYBRID	ACTIVATING
7E4	β2	HYBRID	INHIBITING
TS1/18	β2	β I-LIKE	INHIBITING
MHM23	β2	β I-LIKE	INHIBITING
CBR1/7	β2	I-EGF-1	NO EFFECT
MEM83	αL	α I-DOMAIN	ACTIVATING
TS1/22	αL	α I-DOMAIN	INHIBITING
MHM24	αL	α I-DOMAIN	INHIBITING
TS2/4	αL	β PROPELLER	NO EFFECT

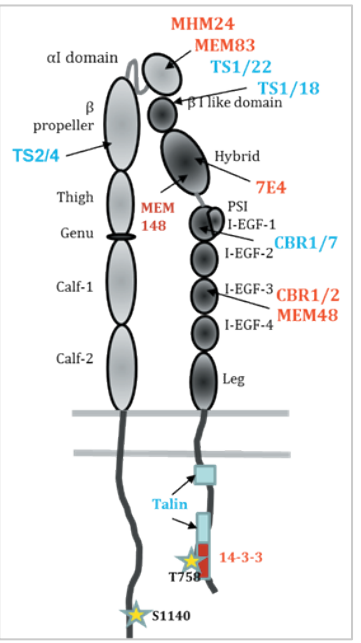


Figure 15: Binding sites and effects of antibodies against LFA-1. B. A schematic picture of LFA-1 αL- and β2-chains and the binding sites of the antibodies used. Yellow stars indicate the phosphorylation sites Ser-1140 at the α-chain and Thr-758 at the β-chain. The binding sites for talin and 14-3-3 are shown in blue and red, respectively.

In figure 15 is shown the regulation of integrin–ligand interaction by antibodies based on the antibody-binding sites on the extracellular domain of the integrin. Activating antibodies towards LFA-1 initiate inhibitory signals to VLA-4, but inhibitory antibodies towards LFA-1 show variable effects. Antibodies that bind more towards the C-terminal are activating or neutral, and antibodies that bind to the αI domain or the βI like domain are usually inhibitory (Huang et al. 2000).

3.1.1 Activating antibodies towards LFA-1

CBR1/2 and MEM48 are activating antibodies, which bind to the I-EGF-3 repeat region in the β2-chain (Petruzzelli et al. 1995; Bazil et al. 1990) of LFA-1. When CBR1/2 and MEM48 bind to the I-EGF-3 domain of the β2-chain, β2 moves away from the αL-chain and becomes activated for ligand binding (Nishida et al. 2006). This activation consequently phosphorylates Thr-758 of the β2 integrin and alters protein-protein interactions. Another β2-activating antibody is MEM148. It binds to the β2-hybrid domain facing towards the αL's β-propeller domain (Chen et al. 2010).

Bound MEM148 stabilizes the active conformation of LFA-1 and increases ligand binding affinities. MEM83 is reported to be an αL-specific activating antibody, and it binds to the I-domain of the αL chain. MEM83-binding residues are situated in the front of the αL-I that is opposite and far from the ligand binding site (Lu et al. 2004). MEM83 also causes inhibition of VLA-4 binding to its ligand, VCAM-1 but less efficiently than CBR1/2 and MEM48. MEM83 increases the ligand-binding affinity by changing the orientation of the I domain, which in turn makes it more accessible for ICAM-1.

3.1.2 Inhibitory antibodies towards LFA-1

7E4, TS1/18, and MHM23 are inhibitory antibodies specific for the β2-chain of LFA-1–ICAM-1 binding. MHM23 binds to the loop between the β1and β3 strands in the β-I like domain (Hildreth et al. 1983). We did not see any inhibition of VLA-4 with this antibody. On the other hand, TS1/18, another blocking antibody also binds the βI like domain (Sanchez-Madrid et al. 1982), and we observed mild inhibition of VLA-4 upon TS1/18 treatment. The 7E4 inhibitory antibody (NORTAMO et al. 1988) of β2 binds to the hybrid domain and inhibits ligand binding, and simultaneously caused the phosphorylation of the Thr-758 of the β2-chain. The epitopes for both 7E4 and TS1/18 alter their orientation when integrins shifts to the open conformation. Binding of these both antibodies stabilizes the closed conformation and block ligand binding (Chen et al. 2010), but simultaneously may cause a conformational change of the cytoplasmic domain of the LFA-1 that results in intracellular signaling, similar to the activating antibodies. TS1/22 and MHM24, two of the αL- specific blocking antibodies bind to the α-I domain (Hildreth et al. 1983; Lu et al. 2004b) and inhibit ICAM-1 ligand binding. Only MHM24 induced minimal crosstalk to VLA-4, like the αL-activating antibody MEM83. The reason behind this slight inhibition of VLA-4 by MHM24 might be the binding region of MHM24, which is very close to the binding site of MEM83. Both of these antibodies bind the αL-I domain. The epitope for MEM83 is the β3–α2 loop and the α4 helix residues 153–183 and 217–248, while MHM24 binds to the α4 loop residues 197–201. On the other hand, TS1/22 did not show a crosstalk effect. Its binding region is further from the MEM83 in the C-terminal end of the β5–α6 loop and the short α6 helix residues 266–270 (Lu et. 2004).

3.1.3 Neutral antibodies towards LFA-1

Two neutral antibodies CBR1/7 and TS2/4 were used as control antibodies in this study. CBR1/7 binds to the first EGF-I domain of the β2 chain (Lu et al. 2001) and TS2/4 to the β-propeller on the αL-chain (C. Huang 1997).

3.2 The crosstalk from LFA-1 to VLA-4 involves the phosphorylation of PLC

We have described that the transdominant inhibition of VLA-4 by LFA-1 facilitated intracellular signaling and requires phosphorylation of β2, which therefore alters the protein-protein interactions (Uotila et al. 2014). We showed that treatment with activating (CBR1/2, MEM48) and inhibiting (7E4) antibodies of both Jβ2.7/LFA-1-expressing Jurkat and T–blasts resulted in the phosphorylation of the β2- chain on Thr-758 (III, fig. 4A, and B). Moreover, we observed that the phosphorylation of β2 was the result of the phosphorylation of PLCγ1 on Tyr-783 and PLCβ3 on Ser-1105 after the antibody treatments (III, fig. 4A, and B). SDF-1α activation causes phosphorylation of PLC which in turn catalyzes the cleavage of PIP_2 into IP_3 and DAG. DAG activates PKC which then phosphorylates Thr-758 on the β2-chain (Gahmberg et al. 2002). Activation of LFA-1 via ICAM-1/Rg (soluble Ig C gamma1 fusion chimeras called receptor globulins) fusion protein causes the phosphorylation of Tyr on PLCγ. Monoclonal antibodies to both the αL and β2 chains induced different levels of Ca^{2+} mobilization in the cells, which suggests that activation of LFA-1 may depend on the epitope for antibody binding (Kanner et al. 1993). We also think that antibodies to LFA-1 may change the conformation of the integrin which can signal into the cells, cause the phosphorylation of PLC and thus activate PKC. By contrast to our results, Caiping Yue showed that the activation of G_q-coupled receptor leads the phosphorylation of Ser-1105 on PLCβ3 which inhibits PKC activity in Hela, COSM6, and RBL-2H3 cells, although by PMA activation PKC inhibition is not apparent (Yue et al. 2000). Thus it seems that upon ligand binding, specific signaling pathways were induced that lead to increase or decrease of cell adhesion or migration, although the final result depends on cell types and its characteristics.

We showed that the signal of transdominant inhibition of α4β1 by LFA-1 activating antibodies and 7E4 required Tiam1 involvement (III, fig. 5A, B, and C) and dephosphorylation of the β1-chain, which then altered protein-protein interactions (III, fig. 6A, B, E, and F). Another phenomenon is that VLA-4 mediates rolling of cells

and simultaneously conveys activation signals, which enhances the LFA-1 interaction with ICAM-1 and this interaction is required for firm adhesion (Chan et al. 2000). An intact cytoskeleton is required for the crosstalk of integrins (Leitinger & Hogg 2000). Treatment of cells with cytochalasin D decreased the stimulated interaction of LFA-1 and ICAM-1. However, the basal adhesion of LFA-1 to ICAM-1 and activation of LFA-1 (detected by mab24 positivity) were not changed. These observations strongly suggest that the increase in LFA-1 ligand binding via activation of VLA-4 is dependent on the cytoskeleton and LFA-1 avidity. We also saw that adding of cytochalasin D to MEM48 and 7E4 treated Jβ2.7/LFA-1-expressing cells reversed the VLA-4 binding to VCAM-1 in the flow adhesion experiments (III, fig., 5D). Treatment with cytochalasin D was also able to restore the phosphorylation of the β1 integrin in the activated Jβ2.7/LFA-1 expressing Jurkat and T cells (III, fig. 6C). Therefore we conclude that antibodies against LFA-1 can 1) activate LFA-1 and inhibit α4β1, 2) inhibit both LFA-1 and α4β1, 3) inhibit LFA-1 but not α4β1, or 4) not affect LFA-1 or α4β1.

4. α-chain phosphorylation regulates β2-integrin functions

The cytoplasmic tails of integrins are short and lack catalytic activity. Several proteins compete with each other for the interaction with the short β2-cytoplasmic domain. The cytoplasmic parts of the αL, αM, αX and αD chains of β2 integrins differ in length and sequence. Phosphorylation of the cytoplasmic tails of integrins is an important way of regulating adaptor protein interactions with integrins (Gahmberg et al. 2009). The phosphorylation of the α- and the β-chains is reported to be constitutive or to take place upon activation, respectively (Fagerholm et al. 2004; Buyon et al. 1990).

4.1 αL and αX phosphorylation is required for the transdominant inhibition of VLA-4 after chemokine activation

The αL, αM, and αX polypeptides have been reported to be phosphorylated on Ser-1140, Ser-1126 and Ser-1158, respectively (Fagerholm et al. 2005; Fagerholm et al. 2006; Uotila et al. 2013). Phosphorylation of αL on Ser-1140 affects LFA-1–ICAM-1 binding and the expression of the open conformation of LFA-1. Therefore, we made

a Jβ2.7 Jurkat cell line stably expressing the phosphorylation mutant of αL Ser1140Ala (Jβ2.7/LFA-1 αL mutant), and we wanted to know how the mutation in Ser-1140 to Ala on the αL-chain regulates the LFA-1 and VLA-4 adhesion to their ligands. We found that the Jβ2.7 Jurkat cells carrying the mutated αL-chain were unable to detach the uropod and instead left long extensions while migrating on VCAM-1, similar to Jβ2.7/LFA-1 negative cells (III, fig. 3A–C). Because of the mutation in the αL chain, cells adhered tightly to VCAM-1, which prevented migration. The same phenomena were also seen in K562-αXβ2-transfected cells. K562 cells were able to inhibit the VLA-4 mediated adhesion to VCAM-1 only when they express the wt αX (III, fig. 3D)

A significant fraction of the αL-chains is phosphorylated in Jurkat cells. Approximately 40% of total αL chains of LFA-1s are found in the phosphorylated form (Fagerholm et al. 2005). Interestingly, αL phosphorylation is partially dependent on the activation of LFA-1s. Cells expressing the mutated αL-chain are not affected by phorbol ester activation, as it does with activating antibody MEM83 or Mg^{2+}. Jβ2.7/LFA-1 αL mutant-expressing cells bind less efficiently to ICAM-1 (Fagerholm et al. 2005). We also found that when the cells were activated with SDF-1α, the phosphorylation of the αL-chain was essential for the transdominant inhibition of VLA-4. However, the transdominant inhibition of VLA-4 to its ligand VCAM-1 was independent of αL phosphorylation when LFA-1 was activated by anti-CD3 or ligand ligation (II, fig. 4A and B). As the phosphorylation of the αL-chain also affects the conformation of the integrin, we studied the activation stages of the LFA-1 for transdominant inhibition of VLA-4 using epitope-specific antibodies. We found that the KIM127-epitope (recognizing the extended conformation) was expressed irrespective of activation and mutation of the αL-chain. However, the mab24-epitope (recognizes open-extended) was expressed only in wt αL-expressing Jurkat cells when cells were activated by SDF-1α or anti-CD3 or via ligand interaction (II, fig. 4 C, and D). The NKI-L16 monoclonal antibody towards LFA-1 reacts with the unique epitope, called L16, on the α-chain in activated JS-136 T cells and is also capable of inducing cell adhesion by LFA-1–ICAM-1 (van Kooyk et al. 1989). By FACS assays, we found that the activation epitope defined by L16 is only expressed in the αL-wt-expressing T cells after SDF-1α activation but not in the αL-mutant-expressing T cells (IV, fig. 4 C). However, the L16 epitope expression was seen both in the αL-wt and αL-mutant-expressing T cells after anti-CD3 activation. The L16 epitope seems to appear only after stimulation of naive T lymphocytes with similar

kinetics as the conversion of LFA-1 from the low to the high avidity state (Larson et al. 1990). Previously we reported that the SDF-1α was incapable of activating Jurkat Jβ2.7/LFA-1 αL mutant-expressing cells (Fagerholm et al. 2005). Importantly, the αM chain with a mutation of Ser-1126 to Ala in was not able to be activated by phorbol esters or extracellular Mg^{2+}/EGTA treatment which consequently affects ICAM-1and ICAM-2 binding. However, this mutation in αM did not change the binding of the ligand iC3b or denatured BSA (bovine serum albumin) (Fagerholm et al. 2006). Mutation in the αM chain severely affected homing of lymphocytes and migration on endothelium in vivo (Fagerholm et al. 2006). Transdominant regulation of β1 integrins by Mac-1 has also been reported in neutrophils (Poloni et al. 2001). In whooping cough, activation of β1 integrins enhances the adhesion of bacterium to monocytes/macrophage via Mac-1 activation (Ishibashi et al. 1994).

4.2 Phosphorylation of the αL-chain is important for β2 phosphorylation and protein interactions with the β2-chain

There are many signaling, adaptor, and cytoskeletal linker proteins which can interact with the β-chain of LFA-1 (Legate & Fassler 2009). In contrast, very few proteins are known to bind specifically to the α-chain of LFA-1(Abram & Lowell 2009). The regulation of the integrins via interactions of these proteins with the cytoplasmic parts of the integrins is still poorly understood. Phosphorylations of the α- and β-chains are important in the regulation of integrin's function and protein-protein interactions (Grönholm et al. 2011). As mentioned earlier, a significant fraction of the α-chains of leukocyte integrins are phosphorylated (Fagerholm et al. 2005), while the β-chains become phosphorylated upon activation through SDF-1α, TCR or phorbol esters (Chatila et al. 1989, Valmu et al. 1999, Hilden et al. 2003, Fagerholm et al. 2005). We have now shown that the mutation of Ser to Ala at 1140 on the αL-chain, inhibits the phosphorylation of Thr-758 of the β2-chain and the conformational activation of LFA-1, which results in changes in protein interactions with the cytoplasmic tail of the β2-chain. We used Jurkat Jβ2.7/ LFA-1 αL wt and αL-expressing the Ser1140Ala mutation to elucidate the importance of the αL serine phosphorylation. There are three conserved regions, the two NPXY/F sequences and the Ser/Thr sequence between them in the β2-tail. GFFKR, a motif in the α-chain is also conserved. Initially, it was shown that mutations of Thr-758 to Ala in the β2-tail leads to the retraction of cell adhesion to ICAM-1 (Hibbs et al. 1991). Using

okadaic acid, a robust phosphorylation of Thr-758 was observed after activation of the cells by a phorbol ester, through TCR ligation or, SDF-1α treatments (Valmu & Gahmberg 1995, Hilden et al. 2003, Abram & Lowell 2009, Uotila et al. 2014). GFFKR in the αL-chain is essential to keep the integrins in an inactive conformation, and when this motif is deleted, the integrins become active (Hughes et al. 1995). Importantly, the Thr-758 β2 phosphorylation was not seen in SDF-1α- or anti-CD3-activated, αL mutant-expressing Jurkat cells as compared to the αL wild-type (IV, fig. 1B and F). The phosphorylations' level of Thr-758 of β2-chain varies between the SDF-1α and anti-CD3 activated T cells. The reasons behind this variation might be the difference among the key players involved in the signaling pathways. SDF-1α binds its receptors on the T cells and activates PI3K and PLCβ, and the latter activates CalDAG, Rap1, RapL and PKC (Kinashi 2005). On the other hand, the activation of integrins through TCR includes Lck (Fagerholm et al. 2002), which phosphorylates Zap-70, and PLCγ (Kinashi 2005). However, both chemokine and TCR pathways activate PKC which leads the phosphorylation of Thr-758 on β2-chain of LFA-1 (Gahmberg et al. 2014).

We showed that phosphorylation of the αL-chain played a significant role in the interaction of proteins with the β-chain. The phosphorylation of Thr-758 of the β2-chain lead to an increase in the 14-3-3 binding and filamin release from the β2-tail (Grönholm et al. 2011). We found a significantly lower amount of 14-3-3, α-actinin and at the same time also significantly more filamin protein was pulled down with LFA-1 after SDF-1α stimulation of αL mutant-expressing Jurkat cells as compared to the wt. Talin interaction was reduced but not significantly. There were also less 14-3-3 and α-actinin protein pulled down with LFA-1 from the anti-CD3 activated αL mutant-expressing Jurkat cells as compared to the wt cells (IV, fig. 2A and B). There were different amounts of protein binding was seen from the SDF-1α and anti-CD3 treated wt αL-expressing cells and the reason might be that the phosphorylation levels of Thr-758 also varies after the SDF-1α and anti-CD3 treatment of the the cells. Thus, the phosphorylation of Ser-1140 is important for the phosphorylation of Thr-758 and protein-protein interactions with the LFA-1. Furthermore, we showed that 14-3-3, talin and α-actinin interact only with the β2-chain and not at all with αL-chain, and phosphorylation of Thr-758 increases 14-3-3, talin and α-actinin binding to the β-chain in Jurkat cells (IV, Fig. 2 C and D). Ultimately, it is obvious that both α- and β-chain phosphorylations are important for the function of LFA-1. The importance of the α-tail in the regulation of integrins has previously been shown (O'Toole et al. 1994, Hughes et al. 1995). The proximal

membrane sites of both α and β-chains are α-helical and linked to each other. Rap1 is reported to be an activator of LFA-1 by TCR activation or CXCR4 activation (Lagarrigue et al. 2016, Calderwood 2015). RapL binds to a site consisting of two Lys residues following the GFFKR motif in the αL-chain (Tohyama et al. 2003). Mutations of these two Lys to Ala inhibited the RapL binding and resulted in inactivation of integrin and ICAM-1 interaction (Tohyama et al. 2003). Binding of talin to the β2-chain also activates the integrins (Lagarrigue et al. 2016). Binding of the talin head inhibited the association of the proximal membrane site and pushed cytoplasmic tails of the α and β-chains apart from each other and activated LFA-1 (Vinogradova et al. 2004). The distal membrane regions vary in sequences and length, and their functions are less clear in integrin activation. It has been shown that in the case of α-chains, mutation or deletion in the distal membrane part of some α-chains affect integrin activation and adhesion to their ligands. For example, mutation of Ser-1158 to Ala in the αX chain in myeloid cells affects β2-integrin mediated adhesion to the ligand iC3b (Uotila et al. 2013), and deletion of the α6's distal part of the cytoplasmic tail abolished α6β1 integrin mediated adhesion to laminin. However, mutation of Ser-1064 and -1071 to Ala in α6-tail did not affect adhesion (Shaw & Mercurio 1993). This indicates that there may be different regulatory mechanisms at the membrane distal-parts among different integrins. Another study showed that deletion of the αL-membrane distal region inhibited talin- and kindlin-mediated integrin activation and ligand binding (Liu et al. 2015). NMR studies (Bhunia et al. 2009) showed that the αL cytoplasmic tail forms a triple-helical structure, i.e. involving helices 1, 2 and 3. Helix 1 is shorter and helix 3 is longest. Helix 3 is connected both with helices 1 and 2, and also both helices 1 and 3 are in connection with the β2-tail. Bhunia and colleagues also showed that Ser-1140 is located in the negatively charged helix 3 position of the αL-tail, and phosphorylation of Ser-1140 further enhances the negative charge of this surface. They proposed that the phosphorylation of Thr-758 allowed 14-3-3 docking in the β2-chain in such a way the activation of LFA-1 is maintained (Bhunia et al. 2009) . We also showed that the negative charge in the αL 1140 position, which resembled the phosphorylation of Ser-1140, was enough to enhance α-actinin binding. COS-7 cells have been used for functional studies of the integrins before (Larson et al. 1990) and it has also been shown that the phosphorylation of the αL-chain of Ser-1140 in COS-7 takes place (Fagerholm et al. 2005). We tranfected COS-7 cells to express active LFA-1 on the surface. First, we activated COS-7 cells by PMA since COS cells lack chemokine and TCR receptors.

The activation by PMA increased the phosphorylation of Thr-758 and 14-3-3 and α-actinin interactions with the β2-chain. We transfected αL Ser1140Ala and Ser1140Asp in combination with the wt β2-chain in COS-7 cells. We found strong α-actinin binding and phosphorylation of both αL Ser-1140 and β2 Thr-758 of LFA-1 after PMA activation. Mutation of Thr-758 to Ala reduced the α-actinin interaction. Again, when the cells expressed Thr758Asp in β2 in combination with Ser1140Ala, there was no α-actinin binding. However α-actinin interaction with β2-chain was further increased when a negative charge was introduced in both the α- and β-chains (IV, fig. 3A, B, C, D and E). The results showed that phosphorylation or negative charges on both αL-Ser-1140 and β2-Thr-758 are vital for the regulation of the α-actinin interaction with the β2-chain of LFA-1.

Phosphorylation of both the α- and β-chains are important for functionally active LFA-1. In our study, we showed that of Ser1140Ala mutation on the α-chain abrogates cell adhesion as well as directional migration. This may be due to the lack of an adequate bridge between the integrin and the cytoskeleton because of the loss of α-actinin interaction with the β2-chain. We showed that the adhesion of Jβ2.7/LFA-1 αL mutant-expressing cells to the ligand ICAM-1 was significantly less as compared to the Jβ2.7/LFA-1 wt αL-expressing cells after both SDF-1α and anti-CD3 activation (IV, fig. 1 D). Furthermore, we found that SDF-1α activated Jβ2.7/LFA-1 wt-expressing cells made long extensions on ICAM-1 and formed clusters on SDF-1α-coated wells, and these features of the cells were not seen in Jβ2.7/LFA-1 mutant-expressing cells (IV, fig. 1E). We performed adhesion complex isolation experiment, where we let the Jβ2.7/LFA-1 wt, and Jβ2.7/LFA-1 mutant adhere to the ICAM-1 coated plate after SDF-1α and anti-CD3 activation and purified adhesion complexes according to the protocol (Jones et al. 2015). As expected, we found α-actinin, 14-3-3, talin, and actin only in Jβ2.7/LFA-1 wt-expressing cells from SDF-1α-activated cells. Additionally, none of these proteins were found in the Jβ2.7/LFA-1 mutant-expressing cells. Instead, they were in the fraction of the unbound cells. It seems that SDF-1α is unable to activate the αL mutant Jurkat cells, and there is no active LFA-1 to make firm adhesion complexes with ICAM-1. On the other hand, in anti-CD3 activated cells less α-actinin, 14-3-3, and talin were found in the Jβ2.7/LFA-1 mutant-mediated adhesion complex with ICAM-1 compared to the wild-type. After these observations we concluded that Jβ2.7/LFA-1 αL mutant cells could be partially activated by anti-CD3 to bind ICAM-1, which was not the case for SDF-1α activation (IV, fig. 2 E, F, and G). The binding site of α-actinin resides in the β2-chain residues 736–746 (Sampath et al. 1998). α-actinin plays a vital role in

linking of the integrins to the cytoskeleton, which is essential for cell adhesion and migration (Otey & Carpen 2004). In our study we also studied cell adhesion by transfecting a peptide containing the α-actinin binding site of β2 in the Jβ2.7/LFA-1 αL wt- expressing cells. This peptide blocked the interaction between α-actinin and the β2-chain. However, it did not affect talin's interaction with the β-chain (Stanley et al. 2008). Static cell adhesion to ICAM-1 was inhibited by β2-peptide in Jβ2.7/LFA-1 αL wt cells activated with SDF-1α, but not by the control peptide (IV, fig. 5A). Jβ2.7/LFA-1 αL mutant-expressing cells with decreased α-actinin interaction also showed impaired cell migration towards a chemokine gradient. For this migration assay, we let the cells (both Jβ2.7/LFA-1 αL wt and mutant-expressing, and both α-actinin binding site containing β2- and control peptide transfected in Jβ2.7/LFA-1 wt-expressing cells) migrate over an ICAM-1-coated chamber towards an SDF-1α gradient. We found that the directional migration towards the chemokine was impaired in the Jβ2.7/LFA-1 αL mutant cells and β2-peptide transfected Jβ2.7/LFA-1 αL wt-expressed cells, which was not the case for either wt LFA-1 or control peptide transfected in Jβ2.7/LFA-1 wt cells (IV, fig. 5B). Lost or reduced of α-actinin interaction between the integrin and the cytoskeleton, turnover of adhesion and de-adhesion of cells decreased and directional migration was impaired. α-Actinin plays a vital role in linking the β2-integrins to the cytoskeleton, which is known to be essential for cell migration (Otey & Carpen 2004, Stanley et al. 2008a). It was shown that a conserved region (residues 733–742) in the β2-tail is critical for the cytoskeletal association (Pardi et al. 1995) and this region overlaps with the α-actinin interaction site (residues 736–746). Besides, a regulatory domain (residues 748–762) in the β2-chain inhibits the constitutive association between the β2-tail and α-actinin (Sampath et al. 1998). This regulatory domain also contains the Thr-758 phosphorylation site of the β2 chain. Thus, the phosphorylation of Ser-1140 on αL- and Thr-758 on β2-tail may influence the separation of the α- and β-tails from each other and facilitate the α-actinin binding by releasing the inhibitory structure. This results in the activation of the ligand binding domain of the integrin, as shown with the active epitope NKI-L16 antibody, and migration of the cells. The importance of α-chain phosphorylation for migration is also observed with the α4 integrin. Phosphorylation of Ser-988 is required for α4-dependent migration and lamellipodia stability, and it blocks the binding of paxillin (Goldfinger et al. 2003, Han et al. 2003). The morphology of Jβ2.7/LFA-1 αL mutant cells was rounded and less polarized as compared to αL wt-expressing cells. In our migration assay, Jβ2.7/LFA-1 αL mutant cells were not able to move towards SDF-

1α gradient. Instead, they ruffled in different directions, and the cell body remained in place. Stanley et al. 2008a observed that the cells lacking α-actinin adhered on ICAM-1 but were unable to translocate on the membrane. Furthermore, both the accumulated and euclidean distances, as well as directionality, were reduced in cells with impaired α-actinin–integrin binding (IV, fig. 4C, D, and E). These observations suggest that α-actinin links the active integrins to the cytoskeleton to mediate directional migration.

VIII. Conclusions

Integrins not only function as adhesion receptors, but also convey signals either from inside-out or outside-in to the cells, and induce cell migration upon chemokine stimulation. We now have a deeper understanding of integrin structure, integrin–ligand interaction, cytoplasmic chain interacting proteins and their influence on cytoskeleton reorganization. Integrins are also vital for mechanotransduction and motility of the cells, and a knowledge of the role of integrins in the control of the tumor microenvironment is emerging (Longmate & DiPersio 2017). In my studies, I have elucidated the importance of the phosphorylation of Thr-758 on the β2-chain and Ser-1140 on the αL-chain of the LFA-1 concerning the regulation of the LFA-1 signaling. I have identified a new key player, Tiam-1, in intracellular signaling downstream of LFA-1. Tiam1 forms a complex with pThr-758 of the β2-chain and 14-3-3, which activates Rac1. I have explained the crosstalk mechanisms of the activation of LFA-1 to the VLA-4. The regulation of the VLA-4-mediated adhesion to VCAM-1 is controlled through the phosphorylation of the Thr-758 on the β2-chain of LFA-1(Figure 16). I have shown that the phosphorylation of the Ser-1140 of αL-chain is significant for the functionally important phosphorylation of Thr-758 on the β2-chain. Phosphorylation of Ser-1140 is important for the interaction of LFA-1 with cytoplasmic proteins although the binding sites for these proteins reside in the β-chain (Figure 17).

Because of the ability of the integrins to regulate cell adhesion, migration and protein-protein interactions, they are potential therapeutic targets. The specificity of phosphorylation or ligand interaction are distinctive features of specific integrins, even between members of the same family of integrin. Although we have revealed some of the important and novels mechanisms for the regulation of the leukocyte integrins, certainly a large number of questions remains unanswered and new ones are emerging, which require more experiments to answer. For example, we do not know which kinases are responsible for the α-chain phosphorylation of β2-integrins. Another exciting phenomenon is that the transdominant inhibition of VLA-4 by activated LFA-1. The transdominant inhibition between VLA-4 and β2-integrins could be targeted for the inhibition of autoimmune diseases, such as MS.

Schematic conclusions of the research

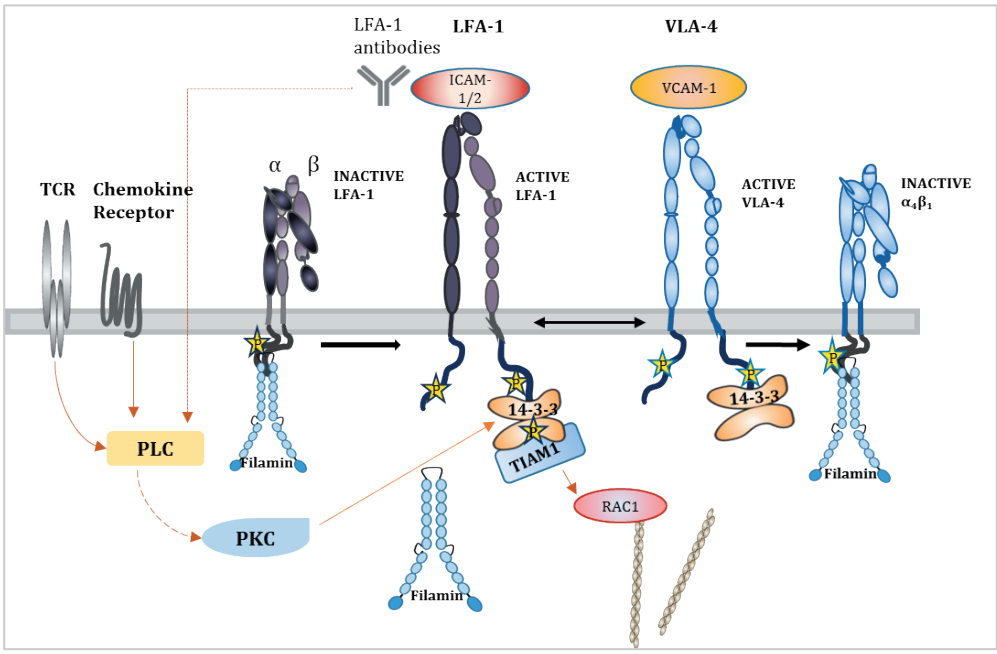


Figure 16: Activation of LFA-1 through TCR or CXCR4 or by antibodies against LFA-1, causes the phosphorylation of PLC, which activates PKC to phosphorylate Thr-758 of the β2-chain and turns LFA-1 into the active conformation. Active LFA-1 releases filamin from the β2-chain, 14-3-3 binds to the phosphorylated β2, and Tiam1 makes a complex with 14-3-3, activating Rac1. Activate LFA-1 also induced dephosphorylation of Thr-788/789 of the β1-chain of VLA-4 inactivating VLA-4. 14-3-3 is released from the cytoplasmic tail of the VLA-4, and filamin binds to the β1-chain of VLA-4.

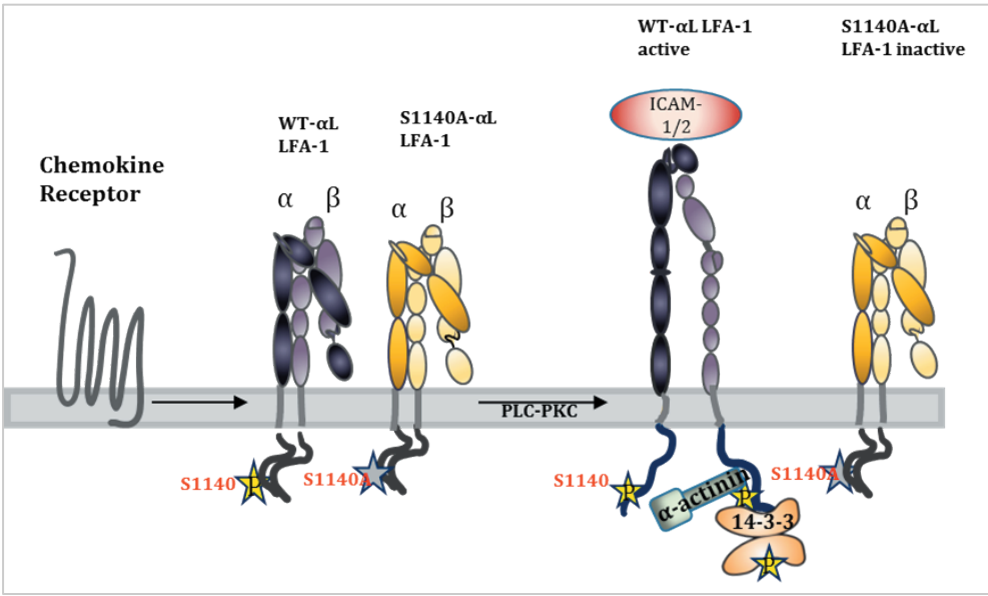


Figure 17: The chemokine SDF-1α cannot activate the LFA-1 when there is a mutation in the αL-chain at Ser-1140. When wt LFA-1 is activated by SDF-1α, it phosphorylates Thr-758 of the β2-chain, which facilitates the binding of α-actinin and 14-3-3. On the other hand, a mutation in Ser-1140 to Ala in the αL chain inhibits Thr-758 β2 phosphorylation and further α-actinin and 14-3-3 binding to LFA-1, which as a result cannot open the activation epitope for ligand binding.

IX. Acknowledgments

This study was carried out at the Molecular and Integrative Biosciences Research Program, Faculty of Biological and Environmental Sciences, University of Helsinki, under the supervision of Professor Carl Gahmberg and Adjunct Professor Mikaela Grönholm. The work has been mostly financially supported by the Magnus Ehrnrooth foundation.

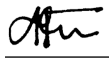
First and foremost I want to thank my supervisor, Professor Carl Gahmberg. It has been an honor to be a Ph.D. student of a leading scientist in the field. I am deeply grateful to my other supervisor Mikaela Grönholm for patiently guiding me on my long and winding road towards being a scientist. Without all her support, time, and above all friendship, I would not be where I am now. She has shown me, by example, what a good scientist (and person) should be. Thank You!

My sincerest gratitude goes to Professor Olli Carpén for accepting to be my opponent for my big day. I am thankful to Professor Jyrki Heino and Professor Jyrki Kukkonen for their expert review and constructive criticism on my thesis. I want to thank Professor Kari Keinänen for serving as custos on the day of my defense. I also wish to acknowledge my Ph.D. follow-up group members, Professor Kari Keinänen and Tiina Öhman for all the feedback and encouragement. My gratitude also goes towards Professor Susanna Fagerholm and Assistant Professor Pia Siljander who have agreed to be the Faculty representatives for my defense.

Liisa, special thanks to you for all the help and friendship inside and outside the lab. I like to thank Tahira and Maria for their friendship and being there always for me. Lin, Sonja and Taisia, thank you for everything from the scientific discussion to the chit-chat. My thanks go to all the present members in the group: Esa, Erkki, Sudarrshan, Larisa and Edward. My deepest thanks go to Katarina for all the affection, encouragement and assistance. As I have spent quite a few years in this lab, I would like to acknowledge the work and friendship of all the former members: Silvia, Ulla, Emiliano, Suneeta, Leena, Yvonne, Hetti, Francesca and others I forgot to mention. Important people from the program unit are acknowledged for taking care of the facilities: Esa, Aari, Tuukka, Taru and others.

Lastly, I would like to thank my family and friends for all their love and encouragement. Aunty and Uncle special thanks to you both for adoring me like your child. Life gets easier and happier in Finland knowing that I have Your supports always with me at any circumstance. Also thanks to my sweet loving sis, Moutushi for your help and most importantly for the precious time you spare for me.

And most of all, my encouraging and patient husband Hassan, who has been by my side throughout this challenging period, living every single minute of it, and without whom, I would not have had the courage to embark on this journey in the first place. The last words go to my darling son Ryan, for being such an inspiration in this journey and making it possible for me to complete what I started. Above all, Thanks to Almighty for every second I get a chance to spend upon His blessings!



Farhana Jahan

X. References

Abram, C.L. & Lowell, C.A., 2009. The Ins and Outs of Leukocyte Integrin Signaling. *Annual Review of Immunology*, 27(1), pp.339–362.

Adair, B.D. & Yeager, M., 2002. Three-dimensional model of the human platelet integrin IIb 3 based on electron cryomicroscopy and x-ray crystallography. *Proceedings of the National Academy of Sciences*, 99(22), pp.14059–14064.

Alberts, B. et al., 1994. *Molecular biology of the cell*, Garland Science.

Alon, R. et al., 1995. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *Journal of Cell Biology*, 128(6), pp.1243–1253.

Alon, R. et al., 2005. A4B1-Dependent Adhesion Strengthening Under Mechanical Strain Is Regulated By Paxillin Association With the A4-Cytoplasmic Domain. *Journal of Cell Biology*, 171(6), pp.1073–1084.

Anderson, D., 1987. Leukocyte Adhesion Deficiency: An Inherited Defect In The Mac-1, LFA-1, And p150,95 Glycoproteins. *Annual Review of Medicine*, 38(1), pp.175–194.

Andrew, D.P. et al., 1996. Distribution of α4β7and αEβ7integrins on thymocytes, intestinal epithelial lymphocytes and peripheral lymphocytes. *European Journal of Immunology*, 26(4), pp.897–905.

Arnaout, M.A. et al., 1984. Deficiency of a leukocyte surface glycoprotein (LFA-1) in two patients with Mo1 deficiency. Effects of cell activation on Mo1/LFA-1 surface expression in normal and deficient leukocytes. *Journal of Clinical Investigation*, 74(4), pp.1291–1300.

Aumailley, M. et al., 2005. A simplified laminin nomenclature. *Matrix Biology*, 24(5), pp.326–332.

Baron, J.L., 1993. Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. *Journal of Experimental Medicine*, 177(1), pp.57–68.

Bazil, V. et al., 1990. Monoclonal antibodies against human leucocyte antigens. IV. Antibodies against subunits of the LFA-1 (CD11a/CD18) leucocyte-adhesion glycoprotein. *Folia Biologica*, 36(1), pp.41–50.

Beatty, P.G. et al., 1984. Absence of Monoclonal-Antibody-Defined Protein Complex in Boy With Abnormal Leucocyte Function. *The Lancet*, 323(8376), pp.535–537.

Bergmeier, W. et al., 2007. Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III. *Journal of Clinical Investigation*, 117(6), pp.1699–1707.

Berlin, C. et al., 1995. A4 Integrins Mediate Lymphocyte Attachment and Rolling Under Physiologic Flow. *Cell*, 80(3), pp.413–422.

Bevilacqua, M.P. et al., 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proceedings of the National Academy of Sciences*, 84(24), pp.9238–9242.

Bezman, N. & Koretzky, G.A., 2007. Compartmentalization of ITAM and integrin signaling by adapter molecules. *Immunological Reviews*, 218(1), pp.9–28.

Bhunia, A. et al., 2009. NMR solution conformations and interactions of integrin aLb2 cytoplasmic tails. *Journal of Biological Chemistry*, 284(6), pp.3873–3884.

Bierema, A.M.K., Schwartz, R.S. & Gill, S.A., 2017. To what extent does current scientific research and textbook content align? A methodology and case study. *Journal of Research in Science Teaching*, 54(8), pp.1097–1118.

Blystone, S.D. et al., 1999. A molecular mechanism of integrin crosstalk: α(v)β3Suppression of calcium/calmodulin-dependent protein kinase II regulates α5β1function. *Journal of Cell Biology*, 145(4), pp.889–897.

Blystone, S.D. et al., 1994. Integrin α(v)β3differentially regulates adhesive and phagocytic functions of the fibronectin receptor α5β1. *Journal of Cell Biology*, 127(4), pp.1129–1137.

Blystone, S.D. et al., 1995. Integrin β3cytoplasmic tail is necessary and sufficient for regulation of α5β1phagocytosis by α(v)β3and integrin-associated protein. *Journal of Cell Biology*, 130(3), pp.745–754.

Bonet, R., Vakonakis, I. & Campbell, I.D., 2013. Characterization of 14-3-3-ζ interactions with integrin tails. *Journal of Molecular Biology*, 425(17), pp.3060–3072.

Bridgewater, R.E., Norman, J.C. & Caswell, P.T., 2012. Integrin trafficking at a glance. *Journal of Cell Science*, 125(16), pp.3695–3701.

Buyon, J.P. et al., 1990. Constitutive and induced phosphorylation of the a- and b-chains of the CD11/CD18 leukocyte integrin family. *The Journal of Immunology*, 144(1), pp.191–197.

Byron, A. et al., 2009. Anti-integrin monoclonal antibodies. *Journal of Cell Science*, 122(22), pp.4009–4011.

C. Huang, T.A.S., 1997. Folding of the B-propeller domain of the integrin aL subunit is independent of the I domain and dependent on the B2 subunit. *Proceedings of the National Academy of Sciences USA*, 94(7), pp.3162–3167.

Calderwood, D.A., 2015. The Rap1-RIAM pathway prefers b2 integrins. *Blood*, 126(25), pp.2658–2659.

Calderwood, D.A., Campbell, I.D. & Critchley, D.R., 2013. Talins and kindlins: Partners in integrin-mediated adhesion. *Nature Reviews Molecular Cell Biology*, 14(8), pp.503–517.

Campbell, I.D. & Humphries, M.J., 2011. Integrin structure, activation, and interactions. *Cold Spring Harbor Perspectives in Biology*, 3(3), pp.1–14.

Cantor, J.M., Ginsberg, M.H. & Rose, D.M., 2008. Integrin-associated proteins as potential therapeutic targets. *Immunological Reviews*, 223(1), pp.236–251.

Carman, C. V. et al., 2007. Transcellular Diapedesis Is Initiated by Invasive Podosomes. *Immunity*, 26(6), pp.784–797.

Chakraborty, A.K. & Weiss, A., 2014. Insights into the initiation of TCR signaling. *Nature Immunology*, 15(9), pp.798–807.

Chan, A.C. et al., 1992. ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell*, 71(4), pp.649–662.

Chan, J.R., Hyduk, S.J. & Cybulsky, M.I., 2000. 4 1 Integrin/VCAM-1 Interaction Activates L 2 Integrin-Mediated Adhesion to ICAM-1 in Human T Cells. *The Journal of Immunology*, 164(2), pp.746–753.

Chatila, T.A., Geha, R.S. & Arnaout, M.A., 1989. Constitutive and stimulus-induced phosphorylation of CD11/CD18 leukocyte adhesion molecules. *Journal of Cell Biology*, 109(6 II), pp.3435–3444.

Chen, B. et al., 2012. Tiam1, overexpressed in most malignancies, is a novel tumor biomarker. *Molecular Medicine Reports*, 5(1), pp.48–53.

Chen, X. et al., 2010. Requirement of open headpiece conformation for activation of leukocyte integrin X 2. Proceedings of the National Academy of Sciences, 107(33), pp.14727–14732.

Cherfils, J. & Zeghouf, M., 2013. Regulation of Small GTPases by GEFs, GAPs, and GDIs. Physiological Reviews, 93(1), pp.269–309.

Chigaev, A. et al., 2011. Discovery of Very Late Antigen-4 (VLA-4, α4β 1 integrin) allosteric antagonists. Journal of Biological Chemistry, 286(7), pp.5455–5463.

Chigaev, A. & Sklar, L.A., 2012. Aspects of VLA-4 and LFA-1 regulation that may contribute to rolling and firm adhesion. Frontiers in Immunology, 3(AUG), p.242.

Choy, E., 2012. Understanding the dynamics: Pathways involved in the pathogenesis of rheumatoid arthritis. Rheumatology (United Kingdom), 51(SUPPL.5), pp.v3–v11.

Conrad, C. et al., 2007. α1β1integrin is crucial for accumulation of epidermal T cells and the development of psoriasis. Nature Medicine, 13(7), pp.836–842.

Cornwell, R.D., Gollahon, K.A. & Hickstein, D.D., 1993. Description of the leukocyte function-associated antigen 1 (LFA-1 or CD11a) promoter. Immunology, 90, pp.4221–4225.

Cota, A.M. & Midwinter, M.J., 2015. The immune system I. R. Mackay & F. S. Rosen, eds. Anaesthesia and Intensive Care Medicine, 16(7), pp.353–355.

Craig, D.H. et al., 2009. Increased extracellular pressure enhances cancer cell integrin-binding affinity through phosphorylation of beta1-integrin at threonine 788/789. American journal of physiology. Cell physiology, 296(1), pp.C193-204.

Crittenden, J.R. et al., 2004. CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nature Medicine, 10(9), pp.982–986.

D'Souza-Schorey, C., Boettner, B. & Van Aelst, L., 1998. Rac regulates integrin-mediated spreading and increased adhesion of T lymphocytes. Molecular and Cellular Biology, 18(7), pp.3936–3946.

Davignon, D. et al., 1981. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2,3 that participates in T lymphocyte-mediated killing. Proceedings of the National Academy of Sciences, 78(7), pp.4535–4539.

Davis, D.M. & Dustin, M.L., 2004. What is the importance of the immunological synapse? Trends in Immunology, 25(6), pp.323–327.

Deakin, N.O. et al., 2009. An integrin- 4-14-3-3 -paxillin ternary complex mediates localised Cdc42 activity and accelerates cell migration. Journal of Cell Science, 122(10), pp.1654–1664.

Díaz-González, F. et al., 1996. Trans-dominant inhibition of integrin function. Molecular biology of the cell, 7(12), pp.1939–51.

Dieckmann, N.M.G. et al., 2016. The cytotoxic T lymphocyte immune synapse at a glance. Journal of Cell Science, 129(15), pp.2881–2886.

Dransfield, I. et al., 1992. Divalent cation regulation of the function of the leukocyte integrin LFA-1. Journal of Cell Biology, 116(1), pp.219–226.

Durrant, T.N., Van Den Bosch, M.T. & Hers, I., 2017. Integrin αIIbβ3outside-in signaling. Blood, 130(14), pp.1607–1619.

Dustin, M.L. & Springer, T.A., 1989. T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature, 341(6243), pp.619–624.

Ehler, E. et al., 1997. Expression of Tiam-1 in the developing brain suggests a role for the Tiam-1-Rac signaling pathway in cell migration and neurite outgrowth. Molecular and Cellular Neurosciences, 9(1), pp.1–12.

Elices, M.J. et al., 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/Fibronectin binding site. Cell, 60(4), pp.577–584.

Ellenbroek, S.I.J. & Collard, J.G., 2007. Rho GTPases: Functions and association with cancer. Clinical and Experimental Metastasis, 24(8), pp.657–672.

Emsley, J. et al., 1997. Crystal structure of the I domain from integrin α2β1. Journal of Biological Chemistry, 272(45), pp.28512–28517.

Engers, R. et al., 2006. Prognostic relevance of Tiam1 protein expression in prostate carcinomas. British journal of cancer, 95(8), pp.1081–6.

Erle, D.J. et al., 1994. Expression and function of the MAdCAM-1 receptor, integrin alpha 4 beta 7, on human leukocytes. Journal of Immunology, 153(2), pp.517–528.

Fagerholm, S. et al., 2002. Phosphorylation of the cytoplasmic domain of the integrin CD18 chain by protein kinase C isoforms in leukocytes. Journal of Biological Chemistry, 277(3), pp.1728–1738.

Fagerholm, S.C. et al., 2006. alpha-Chain phosphorylation of the human leukocyte CD11b/CD18 (Mac-1) integrin is pivotal for integrin activation to bind ICAMs and leukocyte extravasation. Blood, 108(10), pp.3379–3386.

Fagerholm, S.C. et al., 2005. Specific integrin α and β chain phosphorylations regulate LFA-1 activation through affinity-dependent and -independent mechanisms. Journal of Cell Biology, 171(4), pp.705–715.

Fagerholm, S.C., Hilden, T.J. & Gahmberg, C.G., 2004. P marks the spot: Site-specific integrin phosphorylation regulates molecular interactions. Trends in Biochemical Sciences, 29(9), pp.504–512.

Fan, Z. et al., 2016. Neutrophil recruitment limited by high-affinity bent β2integrin binding ligand in cis. Nature Communications, 7, p.12658.

Feng, C. et al., 2012. Kindlin-3 mediates integrin αLβ2 outside-in signaling, and it interacts with scaffold protein receptor for activated-C kinase 1 (RACK1). Journal of Biological Chemistry, 287(14), pp.10714–10726.

Filippi, M.D., 2016. Mechanism of Diapedesis: Importance of the Transcellular Route. Advances in Immunology, 129, pp.25–53.

Finetti, F., Cassioli, C. & Baldari, C.T., 2017. Transcellular communication at the immunological synapse: a vesicular traffic-mediated mutual exchange. F1000Research, 6, p.1880.

Foley, K.S. & Young, P.W., 2014. The non-muscle functions of actinins: an update. Biochemical Journal, 459(1), pp.1–13.

de Fougerolles, A.R., 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. Journal of Experimental Medicine, 175(1), pp.185–190.

Gagen, D. et al., 2013. Activated αvβ3 integrin regulates αvβ5 integrin-mediated phagocytosis in trabecular meshwork cells. Investigative Ophthalmology and Visual Science, 54(7), pp.5000–5011.

Gahmberg, C.G. et al., 2009. Regulation of integrin activity and signalling. Biochimica et Biophysica Acta - General Subjects, 1790(6), pp.431–444.

Gahmberg, C.G., Grönholm, M. & Uotila, L.M., 2014. Regulation of integrin activity by phosphorylation. In Advances in Experimental Medicine and Biology. Springer, Dordrecht, pp. 85–96.

Gahmberg, C.G., Tolvanen, M. & Kotovuori, P., 1997. Leukocyte adhesion - Structure and function of human leukocyte β2-integrins and their cellular ligands. European Journal of Biochemistry, 245(2), pp.215–232.

Garnotel, R. et al., 1995. The binding of type I collagen to lymphocyte function-associated antigen (LFA) 1 integrin triggers the respiratory burst of human polymorphonuclear neutrophils. Role of calcium signaling and tyrosine phosphorylation of LFA 1. Journal of Biological Chemistry, 270(46), pp.27495–27503.

Gérard, A. et al., 2007. The Par polarity complex regulates Rap1- and chemokine-induced T cell polarization. Journal of Cell Biology, 176(6), pp.863–875.

Gérard, A. et al., 2009. The Rac activator Tiam1 controls efficient T-cell trafficking and route of transendothelial migration. Blood, 113(24), pp.6138–6147.

Gitlin, L. & Colonna, M., 2007. A fresh look at an antiviral helicase. Cell Research, 17(11), pp.898–900.

Goldfinger, L.E. et al., 2003. Spatial restriction of ??4 integrin phosphorylation regulates lamellipodial stability and ??4??1-dependent cell migration. Journal of Cell Biology, 162(4), pp.731–741.

Goldmann, W.H., 2000. Kinetic determination of focal adhesion protein formation. Biochemical and Biophysical Research Communications, 271(2), pp.553–557.

Gonzalez, A.M. et al., 2010. Transdominant regulation of integrin function: Mechanisms of crosstalk. Cellular Signalling, 22(4), pp.578–583.

Gordon, E.J. et al., 1995. Bothanti-CD11a(LFA-l) and anti-CD11b (MAC-1) therapy delay the onset and diminish the severity of experimental autoimmune encephalomyelitis. Journal of Neuroimmunology, 62(2), pp.153–160.

Gower, R.M. et al., 2011. CD11c/CD18 Expression is upregulated on blood monocytes during hypertriglyceridemia and enhances adhesion to vascular cell adhesion molecule-1. Arteriosclerosis, Thrombosis, and Vascular Biology, 31(1), pp.160–166.

Graham, D.B. et al., 2007. Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLC??2 signaling axis in mice. Journal of Clinical Investigation, 117(11), pp.3445–3452.

Grönholm, M. et al., 2011. TCR-Induced Activation of LFA-1 Involves Signaling through Tiam1. Journal of Immunology, 187(7), pp.3613–3619.

Guan, J.L. & Hynes, R.O., 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor α4β1. Cell, 60(1), pp.53–61.

Hadley, G.A. & Higgins, J.M.G., 2014. Integrin αEβ7: Molecular features and functional significance in the immune system. In Advances in Experimental Medicine and Biology. pp. 97–110.

Hamelers, I.H.L. et al., 2005. The Rac activator Tiam1 is required for α3β1-mediated laminin-5 deposition, cell spreading, and cell migration. Journal of Cell Biology, 171(5), pp.871–881.

Han, J. et al., 2003. Integrin α4β1-dependent T cell migration requires both phosphorylation and dephosphorylation of the α4cytoplasmic domain to regulate the reversible binding of paxillin. Journal of Biological Chemistry, 278(37), pp.34845–34853.

Han, J. et al., 2001. Phosphorylation of the Integrin α4Cytoplasmic Domain Regulates Paxillin Binding. Journal of Biological Chemistry, 276(44), pp.40903–40909.

Han, J. et al., 2006. Reconstructing and Deconstructing Agonist-Induced Activation of Integrin αIIbβ3. Current Biology, 16(18), pp.1796–1806.

Hemler, M.E., Huang, C. & Schwarz, L., 1987. The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight β subunit. Journal of Biological Chemistry, 262(7), pp.3300–3309.

Hemmings, L. et al., 1996. Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. Journal of Cell Science, 109 (Pt 1, pp.2715–2726.

Hibbs, B.M.L. et al., 1991. The Cytoplasmic Domain of the Integrin Lymphocyte Function-associated Antigen 1 / 3 Subunit : Sites Required for Binding to Intercellular Adhesion Molecule 1 and the Phorbol Ester-stimulated Phosphorylation Site. The Journal of experimental medicine, 174, pp.1227–38.

Hildreth, J.E.K. et al., 1983. A human lymphocyte-associated antigen involved in cell-mediated lympholysis. European Journal of Immunology, 13(3), pp.202–208.

Himmel, M. et al., 2003. The limits of promiscuity: Isoform-specific dimerization of filamins. Biochemistry, 42(2), pp.430–439.

Hogg, N., 2003. T-cell integrins: more than just sticking points. Journal of Cell Science, 116(23), pp.4695–4705.

Hogg, N., Patzak, I. & Willenbrock, F., 2011a. The insider's guide to leukocyte integrin signalling and function. Nature Reviews Immunology, 11(6), pp.416–426.

Hogg, N., Patzak, I. & Willenbrock, F., 2011b. The insider's guide to leukocyte integrin signalling and function. Nature Reviews Immunology, 11(6), pp.416–426.

Homeister, J.W. et al., 2001. The α(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity, 15(1), pp.115–126.

Homeister, J.W., Jennette, J.C. & Falk, R.J., 2013. Immunologic Mechanisms of Vasculitis. In Seldin and Geibisch's The Kidney. Elsevier, pp. 2817–2846.

Huang, C. et al., 2000. Structural and functional studies with antibodies to the integrin β2 subunit. Journal of Biological Chemistry, 275(28), pp.21514–21524.

Hughes, P.E. et al., 1995. The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. Journal of Biological Chemistry, 270(21), pp.12411–12417.

Hyduk, S.J. et al., 2011. Talin-1 and Kindlin-3 Regulate α 4 β 1 Integrin-Mediated Adhesion Stabilization, but Not G Protein-Coupled Receptor-Induced Affinity Upregulation. Journal of Immunology, 187(8), pp.4360–4368.

Imai, Y., Shimaoka, M. & Kurokawa, M., 2010. Essential roles of VLA-4 in the hematopoietic system. International Journal of Hematology, 91(4), pp.569–575.

Imhof, B.A. et al., 1997. Cross talk between αvβ3 and α4β1 integrins regulates lymphocyte migration on vascular cell adhesion molecule 1. *European Journal of Immunology*, 27(12), pp.3242–3252.

Iroumé, A., Huber, A. & Schulz, K., 2005. Summer flows in experimental catchments with different forest covers, Chile. *Journal of Hydrology*, 300(1–4), pp.300–313.

Ishibashi, Y., Claus, S. & Relman, D.A., 1994. Bordetella pertussis filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *Journal of Experimental Medicine*, 180(4), pp.1225–1233.

Issekutz, A.C. & Issekutz, T.B., 1993. A major portion of polymorphonuclear leukocyte and T lymphocyte migration to arthritic joints in the rat is via LFA-1/MAC-1-independent mechanisms. *Clinical Immunology and Immunopathology*, 67(3 I), pp.257–263.

Issekutz, A.C. & Issekutz, T.B., 1995. Monocyte migration to arthritis in the rat utilizes both CD11/CD18 and very late activation antigen 4 integrin mechanisms. *Journal of Experimental Medicine*, 181(3), pp.1197–1203.

Ivetic, A. & Ridley, A.J., 2004. Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology*, 112(2), pp.165–176.

Iwamoto, D. V. & Calderwood, D.A., 2015. Regulation of integrin-mediated adhesions. *Current Opinion in Cell Biology*, 36, pp.41–47.

Jin, T. & Li, J., 2002. Dynamitin controls β2 integrin avidity by modulating cytoskeletal constraint on integrin molecules. *Journal of Biological Chemistry*, 277(36), pp.32963–32969.

Jo, E.-K., Wang, H. & Rudd, C.E., 2005. An essential role for SKAP-55 in LFA-1 clustering on T cells that cannot be substituted by SKAP-55R. *The Journal of Experimental Medicine*, 201(11), pp.1733–1739.

Jones, M.C. et al., 2015. Isolation of integrin-based adhesion complexes. In *Current Protocols in Cell Biology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., p. 9.8.1-9.8.15.

Jung, U. et al., 1998. Transit time of leukocytes rolling through venules controls cytokine- induced inflammatory cell recruitment in vivo. *Journal of Clinical Investigation*, 102(8), pp.1526–1533.

Jung, U. & Ley, K., 1997. Regulation of E-selectin, P-selectin, and intercellular adhesion molecule 1 expression in mouse cremaster muscle vasculature. *Microcirculation*, 4(2), pp.311–319.

Kanner, S.B. et al., 1993. Beta 2-integrin LFA-1 signaling through phospholipase C-gamma 1 activation. *Proc Natl Acad Sci U S A*, 90(15), pp.7099–7103.

Karakose, E., Schiller, H.B. & Fassler, R., 2010. The kindlins at a glance. *Journal of Cell Science*, 123(14), pp.2353–2356.

Katagiri, K. et al., 2004. Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. *Nature Immunology*, 5(10), pp.1045–1051.

Katagiri, K. et al., 2003. RAPL, a Rap1-binding molecule that mediates Rap1-induced adhesion through spatial regulation of LFA-1. *Nature Immunology*, 4(8), pp.741–748.

Katagiri, K., Imamura, M. & Kinashi, T., 2006. Spatiotemporal regulation of the kinase Mst1 by binding protein RAPL is critical for lymphocyte polarity and adhesion. *Nature Immunology*, 7(9), pp.919–928.

Katsumi, A. et al., 2005. Integrin activation and matrix binding mediate cellular responses to mechanical stretch. *Journal of Biological Chemistry*, 280(17), pp.16546–16549.

Kiema, T. et al., 2006. The molecular basis of filamin binding to integrins and competition with talin. *Molecular Cell*, 21(3), pp.337–347.

Kim, M. et al., 2004. The primacy of affinity over clustering in regulation of adhesiveness of the integrin αLβ2. *Journal of Cell Biology*, 167(6), pp.1241–1253.

Kinashi, T., 2005. Intracellular signalling controlling integrin activation in lymphocytes. *Nature Reviews Immunology*, 5(7), pp.546–559.

Klapproth, S. et al., 2015. Loss of the Rap1 effector RIAM results in leukocyte adhesion deficiency due to impaired β2 integrin function in mice. *Blood*, 126(25), pp.2704–2712.

Kliche, S. et al., 2012. CCR7-mediated LFA-1 functions in T cells are regulated by 2 independent ADAP/SKAP55 modules. *Blood*, 119(3), pp.777–785.

Koboziev, I., Karlsson, F. & Grisham, M.B., 2010. Gut-associated lymphoid tissue, T cell trafficking, and chronic intestinal inflammation. *Annals of the New York Academy of Sciences*, 1207, pp.E86–E93.

van Kooyk, Y. et al., 1993. Lymphocyte function-associated antigen 1 dominates very late antigen 4 in binding of activated T cells to endothelium. *J Exp Med*, 177(1), pp.185–190.

Kotovuori, P. et al., 1993. The vascular E-selectin binds to the leukocyte integrins CD11/CD18. *Glycobiology*, 3(2), pp.131–136.

Krauss, K. & Altevogt, P., 1999. Integrin leukocyte function-associated antigen-1-mediated cell binding can be activated by clustering of membrane rafts. *Journal of Biological Chemistry*, 274(52), pp.36921–36927.

Lagarrigue, F., Kim, C. & Ginsberg, M.H., 2016. The Rap1-RIAM-talin axis of integrin activation and blood cell function. *Blood*, 128(4), pp.479–487.

Lahti, M., Heino, J. & Käpylä, J., 2013. Leukocyte integrins αlβ2, αmβ2 and αxβ2 as collagen receptors - Receptor activation and recognition of GFOGER motif. *International Journal of Biochemistry and Cell Biology*, 45(7), pp.1204–1211.

Lalor, P.F. et al., 2002. Vascular Adhesion Protein-1 Mediates Adhesion and Transmigration of Lymphocytes on Human Hepatic Endothelial Cells. *Journal of Immunology*, 169(2), pp.983–992.

Langer, H.F. et al., 2012. Platelets contribute to the pathogenesis of experimental autoimmune encephalomyelitis. *Circulation Research*, 110(9), pp.1202–1210.

Larson, R.S. et al., 1989. Primary structure of the leukocyte function-associated molecule-1α subunit: An integrin with an embedded domain defining a protein superfamily. *Journal of Cell Biology*, 108(2), pp.703–712.

Larson, R.S., Hibbs, M.L. & Springer, T.A., 1990. The leukocyte integrin LFA-1 reconstituted by cDNA transfection in a nonhematopoietic cell line is functionally active and not transiently regulated. *Cell Regulation*, 1, pp.359–367.

Lassmann, H., Van Horssen, J. & Mahad, D., 2012. Progressive multiple sclerosis: Pathology and pathogenesis. *Nature Reviews Neurology*, 8(11), pp.647–656.

Lau, T.L., Partridge, A.W., et al., 2008. Structure of the integrin β3 transmembrane segment in phospholipid bicelles and detergent micelles. *Biochemistry*, 47(13), pp.4008–4016.

Lau, T.L. et al., 2009. The structure of the integrin αiIbB3 transmembrane complex explains integrin transmembrane signalling. *EMBO Journal*, 28(9), pp.1351–1361.

Lau, T.L., Dua, V. & Ulmer, T.S., 2008. Structure of the integrin αIIb transmembrane segment. *Journal of Biological Chemistry*, 283(23), pp.16162–16168.

Law, D.A. et al., 1999. Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. *Nature*, 401(6755), pp.808–811.

Lawrence, M.B. & Springer, T.A., 1991. Leukocytes roll on a selectin at physiologic flow rates: Distinction from and prerequisite for adhesion through integrins. *Cell*, 65(5), pp.859–873.

LeBien, T.W. & Kersey, J.H., 1980. A monoclonal antibody (TA-1) reactive with human T lymphocytes and monocytes. *Journal of immunology*, 125(5), pp.2208–14.

Lee, H.-S. et al., 2013. Two modes of integrin activation form a binary molecular switch in adhesion maturation. *Molecular Biology of the Cell*, 24(9), pp.1354–1362.

Lefort, C.T. & Ley, K., 2012. Neutrophil arrest by LFA-1 activation. *Frontiers in Immunology*, 3, p.157.

Legate, K.R. & Fassler, R., 2009. Mechanisms that regulate adaptor binding to -integrin cytoplasmic tails. *Journal of Cell Science*, 122(2), pp.187–198.

Leitinger, B. & Hogg, N., 2000. Effects of I domain deletion on the function of the beta2 integrin lymphocyte function-associated antigen-1. *Molecular biology of the cell*, 11(2), pp.677–90.

Lewis, R.S., 2001. Calcium signaling mechanisms in T lymphocytes. *Annual Review of Immunology*, 19(1), pp.497–521.

Ley, K. et al., 2007. Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nature Reviews Immunology*, 7(9), pp.678–689.

Ley, K., 2008. The Microcirculation in Inflammation. In *Microcirculation*. Elsevier, pp. 387–448.

Liddington, R.C. & Ginsberg, M.H., 2002. Integrin activation takes shape. *Journal of Cell Biology*, 158(5), pp.833–839.

Lim, C.J. et al., 2008. Integrin-mediated Protein Kinase A Activation at the Leading Edge of Migrating Cells. *Molecular Biology of the Cell*, 19(11), pp.4930–4941.

Lim, J., Hotchin, N.A. & Caron, E., 2011. Ser 756 of β2 integrin controls Rap1 activity during inside-out activation of αMβ2. *Biochemical Journal*, 437(3), pp.461–467.

Lin, K.C. & Castro, A.C., 1998. Very late antigen 4 (VLA4) antagonists as anti-inflammatory agents. *Current Opinion in Chemical Biology*, 2(4), pp.453–457.

Lin, K.K. & Mahadevan, U., 2014. Etrolizumab: Anti-β7—A Novel Therapy for Ulcerative Colitis. *Gastroenterology*, 146(1), pp.307–309.

Liu, J. et al., 2015. The dual structural roles of the membrane distal region of the -integrin cytoplasmic tail during integrin inside-out activation. *Journal of Cell Science*, 128(9), pp.1718–1731.

Liu, S., Calderwood, D. a & Ginsberg, M.H., 2000. Integrin cytoplasmic domain-binding proteins. *Journal of cell science*, 113 (Pt 2, pp.3563–71.

Longmate, W. & DiPersio, C.M., 2017. Beyond adhesion: emerging roles for integrins in control of the tumor microenvironment. *F1000Research*, 6, p.1612.

Lowes, M.A. et al., 2005. Increase in TNF-alpha and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a). *Proc. Natl. Acad. Sci. USA*, 102(52), pp.19057–19062.

Lu, C. et al., 2001. Epitope Mapping of Antibodies to the C-Terminal Region of the Integrin 2 Subunit Reveals Regions that Become Exposed Upon Receptor Activation. *The Journal of Immunology*, 166(9), pp.5629–5637.

Lu, C. et al., 2004. The binding sites for competitive antagonistic, allosteric antagonistic, and agonistic antibodies to the I domain of integrin LFA-1. *Journal of Immunology*, 173(6), pp.3972–3978.

Luo, B.-H., Carman, C. V. & Springer, T.A., 2007. Structural Basis of Integrin Regulation and Signaling. *Annual Review of Immunology*, 25(1), pp.619–647.

Luque, A. et al., 1996. Activated conformations of very late activation integrins detected by a group of antibodies (HUTS) specific for a novel regulatory region (355-425) of the common β1 chain. *Journal of Biological Chemistry*, 271(19), pp.11067–11075.

Madejón, E. et al., 2012. Biosolids, mycorrhizal fungi and eucalypts for phytostabilization of arsenical sulphidic mine tailings. *Agroforestry Systems*, 84(3), pp.389–399.

Malliri, A. et al., 2004. The Rac exchange factor Tiam1 is required for the establishment and maintenance of cadherin-based adhesions. *Journal of Biological Chemistry*, 279(29), pp.30092–30098.

Mamdouh, Z., Mikhailov, A. & Muller, W.A., 2009. Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. *The Journal of Experimental Medicine*, 206(12), pp.2795–2808.

Manevich, E. et al., 2007. Talin 1 and paxillin facilitate distinct steps in rapid VLA-4-mediated adhesion strengthening to vascular cell adhesion molecule. *Journal of Biological Chemistry*, 282(35), pp.25338–25348.

Marlin, S.D. & Springer, T.A., 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell*, 51(5), pp.813–819.

Marwali, M.R. et al., 2003. Membrane cholesterol regulates LFA-1 function and lipid raft heterogeneity. *Blood*, 102(1), pp.215–222.

McEver, R.P., 2015. Selectins: Initiators of leucocyte adhesion and signalling at the vascular wall. *Cardiovascular Research*, 107(3), pp.331–339.

Medeiros, R.B. et al., 2005. Protein kinase D1 and the β1 integrin cytoplasmic domain control β1 integrin function via regulation of Rap1 activation. *Immunity*, 23(2), pp.213–226.

Menon, V. V., Sharma, V.D. & Jeffrey, A., 1983. On the genera! behavior of acceleration waves. *Applicable Analysis*, 16(2), pp.101–120.

Mertens, A.E., Roovers, R.C. & Collard, J.G., 2003. Regulation of Tiam1-Rac signalling. *FEBS Letters*, 546(1), pp.11–16.

Minard, M.E. et al., 2004. The role of the guanine nucleotide exchange factor Tiam1 in cellular migration, invasion, adhesion and tumor progression. *Breast Cancer Research and Treatment*, 84(1), pp.21–32.

Mócsai, A. et al., 2002. Syk is required for integrin signaling in neutrophils. *Immunity*, 16(4), pp.547–558.

Mohammed, H.S. et al., 2014. Identification and phylogenetic analysis of common pumpkin viruses in Sudan. *Journal of Plant Pathology*, 96(1), pp.77–84.

Monkley, S.J., Pritchard, C.A. & Critchley, D.R., 2001. Analysis of the mammalian talin2 gene TLN2. *Biochemical and Biophysical Research Communications*, 286(5), pp.880–885.

Monks, C.R.F. et al., 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature*, 395(6697), pp.82–86.

Montanez, E. et al., 2008. Kindlin-2 controls bidirectional signaling of integrins. *Genes and Development*, 22(10), pp.1325–1330.

Morrison, V.L. et al., 2013. The b2 integrin-kindlin-3 interaction is essential for T-cell homing but dispensable for T-cell activation in vivo. *Blood*, 122(8), pp.1428–1436.

Mulrooney, J.P., Hong, T. & Grabel, L.B., 2001. Serine 785 phosphorylation of the {beta}1 cytoplasmic domain modulates {beta}1A-integrin-dependent functions. *J. Cell Sci.*, 114(13), pp.2525–2533.

Nagae, M. et al., 2012. Crystal structure of α5β1 integrin ectodomain: Atomic details of the fibronectin receptor. *Journal of Cell Biology*, 197(1), pp.131–140.

Nilsson, S. et al., 2006. Threonine 788 in integrin subunit β1 regulates integrin activation. *Experimental Cell Research*, 312(6), pp.844–853.

Nishida, N. et al., 2006. Activation of Leukocyte β2Integrins by Conversion from Bent to Extended Conformations. *Immunity*, 25(4), pp.583–594.

Normato, P. et al., 1988. Immunological Mapping of the Human Leucocyte Adhesion Glycoprotein gp90 (CD 18) by Monoclonal Antibodies. *Scandinavian Journal of Immunology*, 28(5), pp.537–546.

Nurmi, S.M. et al., 2007. Phosphorylation of the LFA-1 integrin ??2-chain on Thr-758 leads to adhesion, Rac-1/Cdc42 activation, and stimulation of CD69 expression in human T cells. *Journal of Biological Chemistry*, 282(2), pp.968–975.

O'Toole, T.E. et al., 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. *Journal of Cell Biology*, 124(6), pp.1047–1059.

O'Toole, T.E. et al., 2011. Tiam1 is recruited to β1-integrin complexes by 14-3-3ζ where it mediates integrin-induced Rac1 activation and motility. *Journal of Cellular Physiology*, 226(11), pp.2965–2978.

Ohta, Y. et al., 1999. The small GTPase RalA targets filamin to induce filopodia. *Proceedings of the National Academy of Sciences*, 96(5), pp.2122–2128.

Ostanin, D. V et al., 2007. T cell-associated CD18 but not CD62L, ICAM-1, or PSGL-1 is required for the induction of chronic colitis. *American journal of physiology Gastrointestinal and liver physiology*, 292(6), pp.G1706–G1714.

Ostermann, G. et al., 2002. JAM-I is a ligand of the β2integrin LFA-I involved in transendothelial migration of leukocytes. *Nature Immunology*, 3(2), pp.151–158.

Otey, C.A. & Carpen, O., 2004. α-actinin revisited: A fresh look at an old player. *Cell Motility and the Cytoskeleton*, 58(2), pp.104–111.

Otey, C.A., Pavalko, F.M. & Burridge, K., 1990. An interaction between α-actinin land the β1integrin subunit in vitro. *Journal of Cell Biology*, 111(2), pp.721–729.

Palmen, M.J.H.J. et al., 1995. Anti-CD11b/CD18 antibodies reduce inflammation in acute colitis in rats. *Clinical and Experimental Immunology*, 101(2), pp.351–356.

Pardi, R. et al., 1995. Conserved Regions in the Cytoplasmic Domains of the Leukocyte Integrin Alpha(L)Beta(2) Are Involved in Endoplasmic-Reticulum Retention, Dimerization, and Cytoskeletal Association. *Journal of Immunology*, 155(3), pp.1252–1263.

Parmo-Cabañas, M. et al., 2007. Intracellular signaling required for CCL25-stimulated T cell adhesion mediated by the integrin α4β1. *Journal of Leukocyte Biology*, 82(2), pp.380–391.

Patarroyo, M. et al., 1987. Identification of a novel adhesion molecule in human leukocytes by monoclonal antibody LB-2. *FEBS Letters*, 210(2), pp.127–131.

Patarroyo, M. et al., 1985. Identification of a Cell Surface Protein Complex Mediating Phorbol Ester-Induced Adhesion (Binding) among Human Mononuclear Leukocytes. *Scandinavian Journal of Immunology*, 22(2), pp.171–182.

Pazos, M. et al., 2017. Neoadjuvant radiotherapy followed by mastectomy and immediate breast reconstruction. *Strahlentherapie und Onkologie*, 193(4), pp.324–331.

Pearce, A.C. et al., 2007. Vav family proteins are required for optimal regulation of PLCγ2 by integrin αIIbβ3. *Biochemical Journal*, 401(3), pp.753–761.

Perez, O.D. et al., 2003. Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nature Immunology*, 4(11), pp.1083–1092.

Peters, T. et al., 2006. CD18 in monogenic and polygenic inflammatory processes of the skin. *Journal of Investigative Dermatology Symposium Proceedings*, 11(1), pp.7–15.

Petruzzelli, L., Maduzia, L. & Springer, T.A., 1995. Lymphocyte Function-Associated Molecule4 (CDlla/CD18) and Mac-1 (CD11 b/CD18) Mimicked. , 4.

Poloni, F. et al., 2001. B1Integrin Activation on Human Neutrophils Promotes B2Integrin-Mediated Adhesion To Fibronectin. *European Journal of Immunology*, 31(1), pp.276–284.

Porter, J.C. & Hogg, N., 1997. Integrin cross talk: Activation of lymphocyte function-associated antigen-1 on human T cells alters α4β1- and α5β1-mediated function. *Journal of Cell Biology*, 138(6), pp.1437–1447.

QU, A.D. & Leahy, D.J., 1995. Crystal-Structure of the I-Domain From the Cd11a/Cd18 (Lfa-1, Alpha(L)Beta-2) Integrin. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 92(22), pp.10277–10281.

Riederer, M.A., Ginsberg, M.H. & Steiner, B., 2002. Blockade of platelet GPIIB-IIIA (integrin αIIbβ3) in flowing human blood leads to passivation of prothrombotic surfaces. *Thrombosis and Haemostasis*, 88(5), pp.858–864.

Rubio, M.P. et al., 2004. 14-3-3-Affinity Purification of Over 200 Human Phosphoproteins Reveals New Links To Regulation of Cellular Metabolism, Proliferation and Trafficking. *Biochemical Journal*, 379(2), pp.395–408.

Ruegg, C. et al., 1992. Role of integrin α4β7/α4βP in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *Journal of Cell Biology*, 117(1), pp.179–189.

Salminen, T.A. et al., 1999. Production, crystallization and preliminary X-ray analysis of the human integrin α1 I domain. *Acta Crystallographica Section D: Biological Crystallography*, 55(7), pp.1365–1367.

Sampath, R., Gallagher, P.J. & Pavalko, F.M., 1998. Cytoskeletal interactions with the leukocyte integrin cytoplasmic tail: Activation-dependent regulation of associations with talin and α-actinin. *Journal of Biological Chemistry*, 273(50), pp.33588–33594.

Sanchez-Madrid, F. et al., 1982. Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. *Proceedings of the National Academy of Sciences*, 79(23), pp.7489–7493.

Sander, E.E. et al., 1998. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *Journal of Cell Biology*, 143(5), pp.1385–1398.

Schön, M. et al., 2000. Critical role of neutrophils for the generation of psoriasiform skin lesions in flaky skin mice. *Journal of Investigative Dermatology*, 114(5), pp.976–983.

Shamri, R. et al., 2005. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nature Immunology*, 6(5), pp.497–506.

Shattil, S.J., Kim, C. & Ginsberg, M.H., 2010. The final steps of integrin activation: The end game. *Nature Reviews Molecular Cell Biology*, 11(4), pp.288–300.

Shaw, L.M. & Mercurio, A.M., 1993. Regulation of α6β1 integrin laminin receptor function by the cytoplasmic domain of the α6 subunit. *Journal of Cell Biology*, 123(4), pp.1017–1025.

Shen, B., Delaney, M.K. & Du, X., 2012. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Current Opinion in Cell Biology*, 24(5), pp.600–606.

Sheremata, W.A. et al., 2005. The role of alpha-4 integrin in the aetiology of multiple sclerosis: Current knowledge and therapeutic implications. *CNS Drugs*, 19(11), pp.909–922.

Shulman, Z. et al., 2012. Transendothelial migration of lymphocytes mediated by intraendothelial vesicle stores rather than by extracellular chemokine depots. *Nature Immunology*, 13(1), pp.67–76.

Silva, J.G. et al., 2016. HIV-1 Nef Impairs the Formation of Calcium Membrane Territories Controlling the Signaling Nanoarchitecture at the Immunological Synapse. *The Journal of Immunology*, 197(10), pp.4042–4052.

Smith, J.W., Piotrowicz, R.S. & Mathis, D., 1994. A mechanism for divalent cation regulation of b3-integrins. *Journal of Biological Chemistry*, 269(2), pp.960–967.

Soares, H., 2014. HIV-1 intersection with CD4 T cell vesicle exocytosis: Intercellular communication goes viral. *Frontiers in Immunology*, 5(SEP), p.454.

Solomkin, J.S. et al., 2007. Alterations in membrane cholesterol cause mobilization of lipid rafts from specific granules and prime human neutrophils for enhanced adherence-dependent oxidant production. *Shock*, 28(3), pp.334–338.

Springer, T.A., 1990. Adhesion receptors of the immune system. *Nature*, 346(6283), pp.425–434.

Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell*, 76(2), pp.301–314.

Springer, T.A. & Anderson, D.C., 1986. The importance of the Mac-1, LFA-1 glycoprotein family in monocyte and granulocyte adherence, chemotaxis, and migration into inflammatory sites: insights from an experiment of nature. *Ciba Foundation symposium*, 118, pp.102–26.

Springer, T.A. & Dustin, M.L., 2012. Integrin inside-out signaling and the immunological synapse. *Current Opinion in Cell Biology*, 24(1), pp.107–115.

Stam, J.C. et al., 1998. Invasion of T-lymphoma cells: Cooperation between Rho family GTPases and lysophospholipid receptor signaling. *EMBO Journal*, 17(14), pp.4066–4074.

Stanley, P. et al., 2008. Intermediate-affinity LFA-1 binds α-actinin-1 to control migration at the leading edge of the T cell. *EMBO Journal*, 27(1), pp.62–75.

Staunton, D.E., Dustin, M.L. & Springer, T.A., 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature*, 339(6219), pp.61–64.

Su, W. et al., 2015. Rap1 and its effector RIAM are required for lymphocyte trafficking. *Blood*, 126(25), pp.2695–2703.

Swat, W. & Fujikawa, K., 2005. The Vav family: At the crossroads of signaling pathways. *Immunologic Research*, 32(1–3), pp.259–265.

Tadokoro, S. et al., 2003. Talin binding to integrin β tails: A final common step in integrin activation. *Science*, 302(5642), pp.103–106.

Takala, H. et al., 2008. β2 integrin phosphorylation on Thr758 acts as a molecular switch to regulate 14-3-3 and filamin binding. *Blood*, 112(5), pp.1853–1862.

Tian, L. et al., 1997. The neuronal glycoprotein telencephalin is a cellular ligand for the CD11a/CD18 leukocyte integrin. *Journal of immunology (Baltimore, Md.: 1950)*, 158(2), pp.928–936.

Tohyama, Y. et al., 2003. The critical cytoplasmic regions of the αL/β2 integrin in Rap1-induced adhesion and migration. *Molecular biology of the cell*, 14(6), pp.2570–82.

Umehara, H. et al., 1993. Signal transduction via phosphorylated adhesion molecule, LFA-1β (CD18), is increased by culture of natural killer cells with IL-2 in the generation of lymphokine-activated killer cells. *International Immunology*, 5(1), pp.19–27.

Uotila, L.M. et al., 2014. Specific phosphorylations transmit signals from leukocyte β2 to β1 integrins and regulate adhesion. *Journal of Biological Chemistry*, 289(46), pp.32230–32242.

Uotila, L.M., Aatonen, M. & Gahmberg, C.G., 2013. Integrin CD11c/CD18 alpha-chain phosphorylation is functionally important. *Journal of Biological Chemistry*, 288(46), pp.33494–33499.

Ussar, S. et al., 2006. The Kindlins: Subcellular localization and expression during murine development. *Experimental Cell Research*, 312(16), pp.3142–3151.

Vajkoczy, P., Laschinger, M. & Engelhardt, B., 2001. α4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *Journal of Clinical Investigation*, 108(4), pp.557–565.

Valmu, L. et al., 1999. Characterization of beta2 (CD18) integrin phosphorylation in phorbol ester-activated T lymphocytes. *The Biochemical journal*, 339(Pt 1), pp.119–25.

Valmu, L. & Gahmberg, C.G., 1995. Treatment with okadaic acid reveals strong threonine phosphorylation of CD18 after activation of CD11/CD18 leukocyte integrins with phorbol esters or CD3 antibodies. *Journal of immunology (Baltimore, Md.: 1950)*, 155(3), pp.1175–83.

Verma, N.K. & Kelleher, D., 2014. Adaptor regulation of LFA-1 signaling in T lymphocyte migration: Potential druggable targets for immunotherapies? *European Journal of Immunology*, 44(12), pp.3484–3499.

Vigil, D. et al., 2010. Ras superfamily GEFs and GAPs: Validated and tractable targets for cancer therapy? *Nature Reviews Cancer*, 10(12), pp.842–857.

Vinogradova, O. et al., 2004. Membrane-mediated structural transitions at the cytoplasmic face during integrin activation. *Proceedings of the National Academy of Sciences*, 101(12), pp.4094–4099.

Virel, A. & Backman, L., 2004. Molecular evolution and structure of alpha-actinin. *Molecular Biology and Evolution*, 21(6), pp.1024–1031.

Vorup-Jensen, T. et al., 2003. Structure and allosteric regulation of the αXβ2 integrin I domain. *Proceedings of the National Academy of Sciences of the United States of America*, 100(4), pp.1873–1878.

Van De Walle, G.R. et al., 2007. Activation of αIIbβ3is a sufficient but also an imperative prerequisite for activation of α2β1on platelets. *Blood*, 109(2), pp.595–602.

Wang, H. et al., 2007. Functional Defects of SKAP-55-Deficient T Cells Identify a Regulatory Role for the Adaptor in LFA-1 Adhesion. *Molecular and Cellular Biology*, 27(19), pp.6863–6875.

Watanabe, N. et al., 2008. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin αIIbβ3. *Journal of Cell Biology*, 181(7), pp.1211–1222.

Weber, K.S.C. et al., 1997. Characterization of lymphocyte function-associated antigen 1 (LFA-1)-deficient T cell lines: The αL and β2 subunits are interdependent for cell surface expression. *Journal of Immunology*, 158(1), pp.273–279.

Weerasinghe, D. et al., 1998. A role for the αvβ3 integrin in the transmigration of monocytes. *Journal of Cell Biology*, 142(2), pp.595–607.

Wegener, K.L. & Campbell, I.D., 2008. Transmembrane and cytoplasmic domains in integrin activation and protein-protein interactions (Review). *Molecular Membrane Biology*, 25(5), pp.376–387.

Weinstock-Guttman, B., 2013. An update on new and emerging therapies for relapsing-remitting multiple sclerosis. *American Journal of Managed Care*, 19(17), pp.s343-54.

Wennerberg, K. et al., 1998. Mutational analysis of the potential phosphorylation sites in the cytoplasmic domain of integrin beta1A. Requirement for threonines 788-789 in receptor activation. *Journal of cell science*, 111(8), pp.1117–1126.

Woodcock, S.A. et al., 2009. A modified tandem affinity purification technique identifies that 14-3-3 proteins interact with Tiam1, an interaction which controls Tiam1 stability. *Journal of Proteome Research*, 8(12), pp.5629–5641.

Woolf, E. et al., 2007. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nature Immunology*, 8(10), pp.1076–1085.

Wörheide, G., 2006. Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge Astrosclera willeyana across the Indo-Pacific. *Marine Biology*, 148(5), pp.907–912.

Xi, A. et al., 2006. Tyrosine phosphorylation of the integrin β3 subunit regulates β3 cleavage by calpain. *Journal of Biological Chemistry*, 281(40), pp.29426–29430.

Xia, T. et al., 2004. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature*, 432(7013), pp.59–67.

Xie, C. et al., 2010. Structure of an integrin with an alpha i domain, complement receptor type 4. *EMBO Journal*, 29(3), pp.666–679.

Xiong, J.P. et al., 2009. Crystal structure of the complete integrin αVβ3 ectodomain plus an α/β transmembrane fragment. *Journal of Cell Biology*, 186(4), pp.589–600.

Xiong, J.P. et al., 2001. Crystal structure of the extracettutar segment of integrin αVβ3. *Science*, 294(5541), pp.339–345.

Yednock, T.A. et al., 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against α4βl integrin. *Nature*, 356(6364), pp.63–66.

Yonekawa, K. & Harlan, J.M., 2005. Targeting leukocyte integrins in human diseases. *Journal of Leukocyte Biology*, 77(2), pp.129–140.

Yue, C. et al., 2000. Molecular mechanism of the inhibition of phospholipase C β3by protein kinase C. *Journal of Biological Chemistry*, 275(39), pp.30220–30225.

Zhang, Y. & Wang, H., 2012. Integrin signalling and function in immune cells. *Immunology*, 135(4), pp.268–275.

Zhou, A.X., Hartwig, J.H. & Akyürek, L.M., 2010. Filamins in cell signaling, transcription and organ development. *Trends in Cell Biology*, 20(2), pp.113–123.

Zhou, H. et al., 2017. Recruitment of Tiam1 to Semaphorin 4D Activates Rac and Enhances Proliferation, Invasion, and Metastasis in Oral Squamous Cell Carcinoma. *Neoplasia*, 19(2), pp.65–74.

Zhu, J. et al., 2008. Structure of a Complete Integrin Ectodomain in a Physiologic Resting State and Activation and Deactivation by Applied Forces. *Molecular Cell*, 32(6), pp.849–861.

Recent Publications in this Series

66/2018 Muntasir Mamun Majumder
Improving Precision in Therapies for Hematological Malignancies
67/2018 Lasse Karhu
Computational Analysis of Orexin Receptors and Their Interactions with Natural and Synthetic Ligands
68/2018 Hanna Pitkänen
Alterations and Impact of Thrombin Generation and Clot Formation in Solvent/Detergent Plasma, FXIII Deficiency and Lysinuric Protein Intolerance
69/2018 Riikka Havunen
Enhancing Adoptive Cell Therapy of Solid Tumours with Armed Oncolytic Adenoviruses
70/2018 Patrick Penttilä
Novel Biomarkers in Metastatic Renal Cell Carcinoma
71/2018 Jaakko Keinänen
Metabolic Changes, Inflammation and Mortality in Psychotic Disorders
72/2018 Heidi Marjonen
Effects of Prenatal Alcohol Exposure on the Epigenome, Gene Expression and Development
73/2018 Dyah Listyarifah
The Role of the Oral Spirochete *Treponema denticola* in Periodontitis and Orodigestive Carcinogenesis
74/2018 Teija-Kaisa Aholaakko
Intraoperative Aseptic Practices and Surgical Site Infections in Breast Surgery
75/2018 Cecilia Anna Brunello
Tau Pathology: Secretion and Internalization as the Key for Understanding Protein Propagation
76/2018 Niko M. Perttilä
Exercise and Falls among Frail Older People – Special Focus on People with Dementia
77/2018 Hanna Knihtilä
Assessment of Small Airway Function – Application of Impulse Oscillometry in Young Children with Asthmatic Symptoms
78/2018 Mikko Jalanko
Alterations in Myocardial Function and Electrocardiology in Hypertrophic Cardiomyopathy
79/2018 Jenni Lehtisalo
Diet, Diabetes, and Prevention of Cognitive Decline — Focus on Lifestyle Intervention
80/2018 Maria Sanz Navarro
Sox2 and Meis1 in Tooth Development and Murine Incisor Renewal
81/2018 Anna Boström
Evaluation of Epaxial Muscle Structure in Dogs with Spinal Disease
82/2018 Anna-Liina Rahikainen
Post-mortem Pharmacogenetics — Citalopram-positive Suicides as a Model Population
83/2018 Santeri Rouhinen
Role of Oscillations in Visual Perception: Attention and Working Memory
84/2018 Omar Youssef
Analysis of Gene Mutations in Exhaled Breath Condensate from Healthy and Lung Cancer Individuals and Profiling of Mutations and Gut Microbiota in Stools from Patients with Gastrointestinal Neoplasms
85/2018 Teija Kasteenpohja
Treatment Seeking, Treatment Adequacy and Outcome of Depressive and Anxiety Disorders among Young Adults in Finland — Findings from a Population-Based Sample

Helsinki 2018 ISSN 2342-317X ISBN 978-951-51-4671-7