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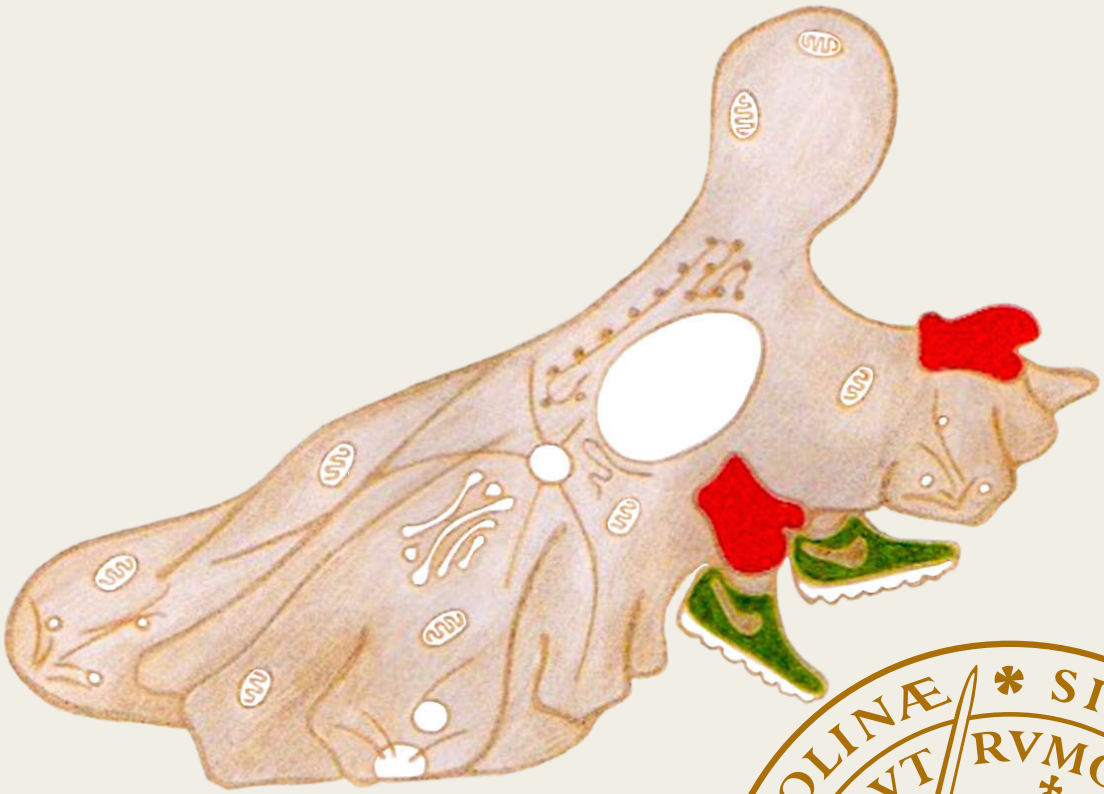

Dynamics of LFA-1 in T lymphocytes

Malin Samuelsson

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Abstract

Integrins are heterodimeric transmembrane proteins that play an essential role during leukocyte migration to sites of infection or injury as well as during immunological synapse formation. The regulation of cell adhesion and motility is very complex and requires intracellular integrin trafficking to and from the adhesion sites especially in fast moving cells like leukocytes.

LFA-1 is the main integrin expressed on T lymphocytes. In my thesis, we propose a model that identifies vesicle-associated RhoB as a novel regulator of Rab11-mediated recycling of surface-internalized LFA-1 along microtubules in T lymphocytes. Dysfunctional RhoB cause accumulation of both LFA-1 and Rab11 in the rear of migrating T lymphocytes. Rab4 does not compensate for this loss in RhoB-mediated Rab11-dependent LFA-1 recycling and T lymphocyte migration is consequently reduced.

We also identify the phosphatase PTPN22 as a novel negative regulator of LFA-1 in migrating T lymphocytes. PTPN22 exists in large clusters that disperse upon LFA-1 engagement with ICAM-1. Declustered PTPN22 then associates with both its substrates and LFA-1 at the leading edge of migrating T lymphocytes and subsequently inhibits LFA-1 clustering and signaling. We also show that PTPN22 is an important regulator of LFA-1-mediated adhesion when T lymphocytes form immunological synapses with antigen-presenting cells (APCs). PTPN22 polarizes towards the immunological synapse and regulate LFA-1 affinity through its substrates.

We additionally show that PTPN22W620, the SNP variant of PTPN22 that is associated with numerous autoimmune diseases, perturbs integrin function and is a loss-of-function variant, at least in the context of LFA-1 signaling. The failure of PTPN22W620 to be retained at the leading edge consequently limits its association potential with its substrates. It also enhances LFA-1 clustering at the leading edge, which further amplifies and sustains LFA-1 signal intensity and subsequently increases LFA-1-dependent adhesion.

Leukocyte recruitment into an inflamed tissue is a central hallmark for a proper immune response. However, it is also a central hallmark for malignancy and for several inflammatory and autoimmune diseases where basic regulation of the immune response is lost. Targeting these processes is an attractive model to either enhance the immune system or suppress inflammation-induced tissue destruction. However, the relatively frequent occurrence of severe side effects is a big complication in integrin-targeted therapies and future targeting strategies need to be more specific. Studies on cell type-specific integrin signaling pathways and studies on specific molecular interactions within these pathways are therefor required before therapies can be developed.

Key words

T lymphocytes, adhesion, migration, integrins, LFA-1, regulation, recycling, Rab, RhoB, PTPN22

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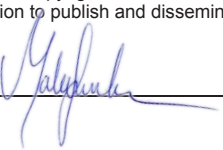
Signature  Date 2017-10-02

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Till mina stjärnor

Preface

How did I end up here? Well, I guess this is the question my twin sister asks herself every time I drag her into all of my ideas and schemes. However, and believe me when I write this, I have a tendency to ask myself this question on a regular basis as well since it is not always clear how I end up in the most unlikely, sometimes very odd and confusing situations. When it concerns my PhD studies, I have been asking myself this question multiple times the last six years. However, the answer appears to be quite straightforward, at least to some extent…

I did not really think about the future when I was a kid. Honestly, I just enjoyed my time spent together with my family and my friends, while also playing a huge amount of sports. It is no secret that my parents had a huge impact on the person I am today. My mom and dad always taught me that I could be anything and do everything. And in contrast to my sisters (they are amazing, by the way) who always wanted to go into the medical (Hanna) or publishing (Emma) professions, I had many callings during my childhood including but not limited to being a firefighter, ambulance driver, chef, physiotherapist, gym teacher and veterinarian. My inner flame for science and medicine grew stronger during junior high and high school. With my love for sports, my interest in physiology and medicine came along. My dad also liked to watch crime TV shows and this transferred onto me. I remembered thinking that I wanted to work with forensic science one day. So once all these ideas and interests of mine were mixed, I decided that I was going to study either medicine or medical science after high school. However, during high school I realized that I am allergic to hospitals, which consequently ruled out medicine. I then came to think about the fact that I have always been very curious and questioning - I was heading for medical science.

The night before the final call for university applications during the spring of 2006, I woke up and decided that I was going to change my biomedical program application, from Uppsala to Lund. Lund was just closer to home. What had I been thinking when I first choose Uppsala? Anyhow, my first week in Lund was quite chaotic. I injured the palm of my hand while removing the plastic cover on a cucumber with a sharp kitchen knife that was newly purchased from IKEA. My friend, Annie, drove me to the Emergency Room and after one hour, two extremely giggling girls where leaving the ER, one with her right hand closed with stitches and wrapped in a huge bandage. This actually made me miss out on my

first practical laboratory class on the biomedical program. I could not use gloves. However, I was still attending the days in the laboratory and it was during one of those coffee breaks my first encounter with PhD studies came along. I was amazed (but also slightly troubled) when some of my classmates had their next eight to ten years planned; first graduate studies and then continue on with doctoral studies. I remember thinking, what is a PhD student? What does a PhD student do? For your information, in contrast to Lund University that was established back in 1666, Växjö University was established way later, actually in 1999. The only thing I knew when I was starting my academic studies in 2006 was that I really wanted to study abroad in the United States at some point during my undergraduate studies. I did accomplish this wish of mine, and also my undergraduate studies together with my buddy and sidekick, Sara. Eventually, I also did become a PhD student and that is how this book came about and also why you currently are reading this preface.

My journey as a PhD student has been a true rollercoaster ride and an enormous mental challenge. I still have a hard time to facing the fact that hard work does not necessarily mean results. It has been a struggle and yes, I have considered giving in. However, I am a fighter and I am also as stubborn as my grandfather was (and my mom is). I kept going and going. I not only identify problems, I deal with them and find solutions. In fact, I am very proud of myself for accomplishing this journey. Now when I look back at my PhD studies I see that I have grown and learned a lot about myself. I am so excited about what to come next. To relate back to the rollercoaster ride: I'm now at the first height and totally ready for the journey.

The purpose of a preface is to persuade the reader, why he or she should continue on and read the rest of the book. The reader is in this case you. If you do continue on reading, I really think you will find my work very interesting, and also important. However, I honestly do not expect all of you to read my entire book, but I do expect many of you fabulous colleagues, friends and family to read my acknowledgements at page 111. This book would never have made it to print, if not all of you gorgeous people have helped and supported me during my PhD studies.

All the best,

Malin, scientist and very soon PhD in medical research, just as the original plan back in high school – almost.

Content

List of papers

Paper 1:

RhoB controls Rab11-mediated recycling and reexpression of LFA-1 in migrating T lymphocytes

Science Signaling, in press

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Paper 2:

Superresolution imaging of the cytoplasmic phosphatase PTPN22 links integrin-mediated T cell adhesion with autoimmunity

Science Signaling 04 Oct 2016:Vol. 9, Issue 448, pp. ra99

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Paper 3:

PTPN22 negatively regulates high affinity LFA-1 in the immunological synapse

Manuscript

Abbreviations

ABL	Abelson tyrosine protein kinase
ADAP	Adhesion and degranulation-promoting adapter protein
Akt	Stock “Ak”-transforming
AP	Adaptor protein
APC	Antigen-presenting cell
ARP-2/3	Actin-related protein homolog 2 or 3
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid
ATPase	Adenosine triphosphatase
BBB	Blood-brain barrier
Ca	Calcium
CalDAG-GEF-1	Calcium- and DAG-regulated GEF 1
Cbl-b	Casitas B-lineage lymphoma b
CCL	C-C motif ligand
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CD	Cluster of differentiation
CDC-42	Cell division cycle 42
cDNA	Complementary deoxyribonucleic acid
CLASP	Clathrin-associated sorting protein
CLIC	Chloride intracellular channel protein

CNS	Central nervous system
CrD	Crohn's disease
CRKL	CT10 regulator of kinase (Crk)-like protein
Csk	C-terminal Src kinase
cSMAC	Central SMAC
CTL	Cytotoxic T lymphocyte
Cys, C	Cysteine
CXCR-2	C-X-C chemokine receptor type 2
CXCL	C-X-C motif ligand
DAG	Diacylglycerol
DAMP	Damage-associated molecular pattern
Dia-1	Diaphanous-related formin 1
DOK-1	Docking protein 1
dSMAC	Distal SMAC
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EE	Early endosome
EEA-1	Early endosome-associated protein 1
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ERM proteins	Ezrin, radixin, moesin
E-selectin	Endothelial selectin
FERM	4.1 protein, ezrin, radixin, moesin
FRC	Fibroblastic reticular cell
GAP	GTPase-activating proteins
GDI	GDP-dissociation inhibitor
GDP	Guanosine diphosphate
GDF	GDI-displacement factor
GEF	Guanine nucleotide exchange factor

GEM	Glycolipid-enriched membrane domain
Glu, E	Glutamic acid
Gln, Q	Glutamine
Gly, G	Glycine
Grb-2	Growth factor receptor-bound protein 2
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HEK cell	Human embryonic kidney cell
His, H	Histidine
HSCT	Hematopoietic stem cell transplantation
IBD	Inflammatory bowel disease
iC3b	Inactive complement component 3
ICAM	Intercellular adhesion molecule
ICAP-1	Integrin cytoplasmic domain-associated protein 1
IFN-γ	Interferon γ
Ig-CAM	Immunoglobulin-like cell adhesion molecule
IL	Interleukin
ILT	Innate-like T lymphocytes
ILV	Intraluminal vesicle
IP_3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
JAB-1	Jun activation domain-binding protein 1
JAM-A/C	Junctional adhesion molecule A/C
LAD-I/II/III	Leukocyte adhesion deficiency type I/II/III
LAMP-1/2	Lysosomal-associated membrane protein 1 or 2
LAT	Linker of activated T cells
LBRC	Lateral border recycling compartment
LC-2	Light chain 2

Lck	Leukocyte C-terminal Src kinase
LE	Late endosomes
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
L-selectin	Leukocyte selectin
Mac-1	Macrophage-1 antigen
MadCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MAP-1A	Microtubule-associated protein 1 A
Mg	Magnesium
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
Mn	Manganese
Mst-1	Macrophage-stimulating protein 1
MS	Multiple sclerosis
MTOC	Microtubule-organizing center
MVB	Multivesicular body
NK cell	Natural killer cell
NOD	Non-obese diabetic
PAG	Protein associated with GEMs
PAMP	Pathogen-associated molecular pattern
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PECAM-1	Platelet/endothelial cell adhesion molecule 1
Pep	Proline-enriched phosphatase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP5K-1	Phosphatidylinositol 4-phosphate 5-kinase 1
PKB/C/D-1	Protein kinase B, C or D 1
PLC/D-1	Phospholipase C or D 1

PM	Plasma membrane
PML	Progressive multifocal leukoencephalopathy
PP	Peyer's patch
Pro, P	Proline
PRR	Pattern recognition receptor
P-selectin	Platelet selectin
PSGL-1	P-selectin glycoprotein ligand 1
pSMAC	Peripheral SMAC
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTPN	Tyrosine-protein phosphatase non-receptor
RA	Rheumatic arthritis
Rab	Ras-related in brain
Rac-1	Ras-related C3 botulinum toxin substrate 1
Rap-1	Ras-related protein 1
RapL	Rap ligand
R-G-D sequence	Arg-Gly-Asp sequence
RhoA/B/C/H	Ras homolog gene family member A, B, C or H
RIAM	Rap1-GTP-interacting adapter molecule
RLC	Regulatory Light Chain
ROCK	Rho associated coiled-coil containing protein kinase
Ser, S	Serine
SH2/3	Src Homology 2 or 3
SHANK	SH3 and multiple ankyrin repeat domains
SHARPIN	SHANK-associated RH domain-interacting protein
SHP-1/2	Src homology region 2 domain-containing phosphatase 1 or 2
siRNA	Small interfering ribonucleic acid
SKAP-55	Src kinase-associated phosphoprotein of 55 kDa
SLE	Systemic lupus erythematous

SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
SMAC	Supramolecular activation clusters
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor
SNP	Single-nucleotide polymorphism
Src	Sarcoma
Syk	Spleen tyrosine kinase
T1D	Type 1 diabetes
TGN	Trans-Golgi network
TNF-α	Tumor necrosis factor α
Th	T helper
Thr, T	Threonine
Tyr, Y	Tyrosine
UC	Ulcerative colitis
UV	Ultraviolet
Vav-1	Vav (the sixth letter of the Hebrew alphabet) type 1
VCAM-1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR-2	VEGF receptor 2
VLA-4	Very late antigen 4
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
WT	Wild-type
X	Any amino acid
Zap-70	Zeta chain of T-cell receptor associated protein kinase 70

List of figures

Populärvetenskaplig sammanfattning

Vi människor är uppbyggda av celler. En cell är en mer eller mindre självständig enhet, som kan föröka sig själv och ta emot olika typer av information från omgivningen. En del celltyper ingår i kroppens immunsystem, det vill säga kroppens eget försvar mot främmande och skadliga partiklar. Immunförsvaret är ett komplext system som reagerar mot virus och bakterier, men även mot tumörceller, skador mot kroppen och celler från andra människor. Många av kroppens immunceller kan liknas vid spanare som ständigt letar efter nya inkräktare, medan andra mer kan beskrivas som soldater som angriper dessa inkräktare.

I min värld, även om jag älskar mat, så är protein så mycket mer än kött, fisk, kyckling, ägg, bönor och linser. Det är inte bara muskler som byggs upp av proteiner, utan även kroppens celler. De är kroppens livsnödvändiga byggstenar. Proteiner kan byggas ihop på många olika sätt med hjälp av ärvda ritningar, gener, som finns lagrade i cellen. Cellens proteiner kan ha en mängd olika funktioner och se olika ut och organiseras på olika sätt beroende på vilken typ av funktion de fyller. Integriner är proteiner som byggs ihop på ett sätt medan exempelvis cytoskelettet, som är cellens egna skelett, byggs ihop på ett annat sätt.

Integriner kan liknas vid cellens fötter, som hjälper cellen att hålla sig fast på ett visst ställe i kroppen eller förflytta sig till ett annat. De hjälper även till med kommunikation mellan cellen och dess omgivning samt mellan celler genom att ta emot och skicka ut olika typer av information. Celler kan själv bestämma om integrinerna ska vara ”på” eller ”av” eller plockas in i cellen, transporteras till en bättre behövande plats och därmed återanvändas. Att ständigt tillverka nya integriner skulle kosta alldeles för mycket energi. Återvinning är viktigt och absolut en global angelägenhet – ända ner på molekylär nivå!

Målet med min avhandling har varit att studera hur celler i detalj kopplar ”av” samt återanvänder sina integriner. Till min hjälp har jag använt levande vita blodkroppar från friska donatorer. Jag har till största del använt olika mikroskopieringstekniker samt en vanlig biokemisk metod som kallas western blot, och som används inom proteinkemin för att detektera och identifiera specifika proteiner med hjälp av antikroppar.

För att celler ska kunna röra på sig måste de kunna återanvända sina integriner genom att förflytta dem mellan olika platser, fästpunkter eller förankringar. På så sätt pågår ett ständigt utbyte av integriner mellan insidan och utsidan av cellen. Inuti cellen är integrinerna förankrade i transportvesiklar, så kallade endosomer. Endosomerna är liksom själva cellen omgärdade av membran och bildar stora transportnätverk längs den del av cellens cytoskelett som kallas mikrotubuli. Denna struktur skulle kunna liknas vid ett gigantiskt, sammanflätat liftsystem som drivs av vajrar. Mikrotubuli utgör själva vajrarna och endosomerna är kabinerna som transporteras längs med. Transporten i mikrotubuli sker genom att särskilda proteiner, motorproteiner, binder sig vid det som ska transporteras och till mikrotubuli. Precis som alla skidliftar är cellens transporter också energikrävande och drivs av cellens energivaluta (ATP).

Det pågår en ständig transport av endosomer inuti cellen och för att alla transporter ska fungera och hamna på rätt ställe behövs proteiner, pistvakter, som signalerar och kommunicerar med varandra inuti cellen. Pistvakterna som ansvarar för koordineringen av cellens många olika endosomer och deras rörelser består av en grupp proteiner som kallas för Rab-proteiner. Men Rab-proteinerna behöver hjälp och till sin hjälp har de andra proteiner, liftvärdar, som hjälper både stor som liten vid på- och avstigningen. I nuläget vet vi inte i detalj vilka proteiner som jobbar som liftvärdar. Vi vet heller inte hur dessa transportvesiklar och endosomer kan styras till exakt rätt ställe och hur deras innehåll kan frisättas vid exakt rätt tidpunkt. I det första arbetet av min avhandling har jag försökt förstå just hur denna transport av integriner går till inuti vita blodkroppar.

Våra tekniker, där vi bland annat märker in specifika proteiner med fluorescense, möjliggör för oss att i detalj studera snabba förändringar som sker när integriner kopplas på och av samt rör sig inuti cellen. Hittills har vi resultat som visar att proteinet RhoB, en släkting till Rab-proteinerna, också finns med och dirigerar transporten av integriner inuti cellen. I det första arbetet av min avhandling fastställer vi att vita blodkroppar inte kan transportera sina integriner som de ska om inte RhoB finns på plast och hjälper till. Utan RhoB ansamlas integrinerna inuti cellen och bildar långa liftköer, likt dem i fjällen under sportlovet. Detta resulterar också i att cellen inte kan röra sig som den ska då inga integriner transporteras till nya förankringspunkter.

Det är viktigt att förstå hur cellens integriner regleras, dels för att störningar i dessa signalkaskader hos de vita blodkropparna påverkar vårt immunförsvar negativt, och dels för att cancerceller använder sig av dessa integriner för att sprida sig och bilda metastaser. Integrinerna ska dock inte bara kunna aktiveras och därmed kopplas ”på”, utan även inaktiveras och därmed kopplas ”av”. Fram till inte för så länge sedan trodde man att själva inaktiveringen bara var en passiv händelse som skedde förr eller senare efter det att integrinerna hade aktiverats.

Idag vet vi att det inte är så, då inaktiveringen är en aktiv process som också den regleras hårt och även om mycket forskning kvarstår inom detta område, så har några proteiner på senare tid identifierats som aktiva hämmare av just integrinernas aktivitet.

Målet med det andra och det tredje arbetet av min avhandling var att just studera hur cellen i detalj kopplar ”av” sina integriner samt om detta sker med hjälp av proteinet PTPN22. Det är också ett protein och ett fosfatas, vilket betyder att det kopplar bort fosfatgrupper på andra proteiner och därmed reglerar om dessa ska vara ”på” eller ”av”. Genom att studera friska individer har vi fått resultat som visar att PTPN22 reglerar LFA-1, den integrin som uttrycks mest hos de vita blodkropparna på just detta sätt. När LFA-1 ska kopplas på sprids en signal inuti den vita blodkroppen, vilket kan liknas vid när dominobrickor ställda på högkant faller. När en bricka knuffas till välter den på nästa, som välter på nästa och så vidare. Varje dominobricka är ett signalprotein som överför information om att LFA-1 ska kopplas på till nästa bricka. Varje ”knuff” innebär också att en fosfatgrupp kopplas på och får brickan att börja falla. PTPN22 kopplar bort fosfatgrupper på dessa signalproteiner (dominobrickorna) som då slutar falla. Signalen om att LFA-1 ska kopplas på kommer därmed inte fram som den ska. I det andra arbetet av min avhandling visar vi att PTPN22 reglerar LFA-1 när den typ av vita blodkroppar som kallas T-celler rör på sig, och i tredje arbetet att samma reglering sker när T-celler binder in och kommunicerar med B-celler som också är en slags vita blodkroppar.

Kopplingarna som bildas när celler i immunförsvaret (bland annat vita blodkroppar) binder in och kommunicerar med varandra kallas för immunologiska synapser. Dessa kopplingar används bland annat för att aktivera T-celler när immunförsvaret har upptäckt främmande eller skadliga partiklar som exempelvis invaderande virus, bakterier eller skadade celler. Forskning på integriner och immunologiska synapser är viktigt av många anledningar. Även om dessa regleras noggrant av cellerna själva så händer det ibland att de immunologiska synapserna inte fungerar som de ska, och att immunförsvaret inte aktiveras alls eller felaktigt. Immunförsvaret kan exempelvis felaktigt programmeras och därmed attackera kroppens egna celler och det är då som autoimmuna sjukdomar och allergier kan uppstå. Immunförsvaret kan dessutom missa att upptäcka cancerceller om inte de immunologiska synapserna fungerar som de ska. Upptäcks inte en cancercell i tid och därefter inte förstörs av immunförsvaret kan denna börja dela sig ohämmat och bilda en tumör.

När väl en tumör har bildats har den som mål att sprida sig till andra ställen av kroppen och bilda metastaser. Även här är integrinerna viktiga då dessa också är cancercellernas fötter och därmed används av enstaka cancerceller från tumören för att smita iväg mot ett blodkärl. Väl i ett blodkärl transporteras de mot andra

organ och fäster sedan sig fast med hjälp av sina integriner på outforskad mark där metastaser därefter eventuellt bildas. Spridd cancer, metastaser, är svår att behandla och i många fall detsamma som en dödsdom. Om inga metastaser fanns skulle de flesta cancerpatienter överleva. Men hur får vi stopp på cancercellerna? För att förebygga eller minska uppkomsten av metastaser är forskningen på integriner enormt viktig. Om kunskapen om hur både friska celler och cancerceller använder sina integriner för att hålla fast, fästa och röra på sig ökar, skulle det eventuellt kunna gå att utveckla mediciner mot olika proteiner i dessa processer och därmed stoppa cancercellernas spridning. Man skulle rädda liv. Många liv.

Slutligen en kort sammanfattning: Målet med min avhandling har varit att studera hur den typ av vita blodkroppar som kallas T-celler i detalj reglerar samt återanvänder sina integriner. I det första arbetet av min avhandling fastställer vi att T-celler inte kan återanvända sina integriner som de ska om inte proteinet RhoB finns på plast och hjälper till. I det andra arbetet visar vi att proteinet PTPN22 hjälper till att koppla ”av” integriner när T-celler rör på sig och att denna mekanism inte fungerar hos personer som bär på den sjukdomsrelaterade genvarianten av just PTPN22. Slutligen, i det tredje arbetet, bekräftar vi att samma reglering som i det andra arbetet sker när T-celler binder in och kommunicerar med en annan typ av vita blodkroppar som kallas för B-celler. På sikt hoppas vi att vår forskning ska leda till ny kunskap om cellers rörelse och därmed kunna vara behjälplig i nya behandlingsstrategier och utvecklandet av mer specifika läkemedel inom bland annat cancersjukdomar och autoimmuna sjukdomar.

Aims of thesis

My thesis aimed to investigate the dynamics of LFA-1 in T lymphocytes. My specific aims and research questions were:

- What signaling pathways and intermediates are involved in intracellular transport and recycling of LFA-1?
- If and how PTPN22 is regulating LFA-1 during both T lymphocyte migration as well as synapse formation between a T lymphocyte and an antigen-presenting cell?
- What is the consequence of the SNP in PTPN22 that subsequently leads to an Arginine to a Tryptophan substitution at amino acid position 620 within the protein? Is it a gain-of-function variant or a loss-of-function variant when it comes to regulation of LFA-1?

By answering these questions, we aimed to provide a better understanding of migration when it comes to rapidly moving cells such as leukocytes and cancer cells. Reusing and consequently recycling its integrins means efficient energy use for any given cell type. Investigating how these recycling mechanisms and also overall regulatory mechanisms occur for LFA-1 in T lymphocytes when these cells move forward and form immunological synapses, could aid in the development of new therapeutic strategies in both cancer and autoimmune diseases. Many a little makes hopefully a mickle…

Background

Inflammation is extremely complex and equally fascinating. It plays a crucial role in mammalian physiology and is indispensible for survival. Regardless of the cause, the purpose of the inflammatory response is to remove the source of disturbance and restore homeostasis (on all levels, from cellular to systemic state). The origin of inflammation is best understood in the context of fighting an infection or an injury, but these triggers are at the extreme end of all the conditions that can trigger inflammation. Indeed, the magnitude of the inflammatory response can differ greatly. Whenever possible, smaller disruptive threats will be monitored and generally taken care of by tissue-resident macrophages and the inflammatory response is consequently only engaged when the local tissue-defense is insufficient(1).

The protective capacity of inflammation is great but comes with a price. It has both favorable and unfavorable consequences. With such a complex and multistep regulatory system much can go wrong and the pathological potential of inflammation can cause devastating consequences. An inflammation that persists too long, is self-directed and/or is at the wrong place at the wrong time can cause various inflammatory pathologies that are associated with many modern human diseases. There is currently no clear understanding of what the physiologically cause behind chronic inflammation is, or whether there even is one.

Despite the potentially negative consequences, the inflammatory response is heroic and comes to our rescue like a true soldier over and over again during our lifetime. One of the main principles during this inflammatory response is leukocyte migration – the actual subject of my thesis. There wouldn't be a response unless leukocytes migrate and cross blood and/or lymphatic vessels(1-3).

Migration – making and breaking of adhesions

Cell migration to specific locations is an essential process for the maintenance of multicellular organisms during embryonic development, immune responses, tissue repair and regeneration. The orchestrated movement of cells is also crucial in various pathologies including cancer, arthritis, atherosclerosis, osteoporosis and

during congenital development brain defects(4). Cell migration can be viewed as a multistep cycle of making adhesions and breaking adhesions over and over again. In this chapter I will briefly summarize the main adhesion molecules used by human cells and also the main principles of cell migration.

Adhesion molecules

Adhesions can be made between cells and between cells and extracellular matrix (ECM). Mammalian cells express many different types of adhesion molecules and many of these proteins are also signaling receptors and propagate intracellular signals due to their connections with various signaling networks that further control most cellular responses. The major adhesion families are cadherins, selectins, integrins and Immunoglobulin-like cell adhesion molecules (Ig-CAMs)(5).

Stabile adhesion junctions

There are two main ways in which cells are tightly anchored together. Cells may be linked by direct interactions or they may be held together within the ECM. Adherence junctions (or anchoring junctions) are anchorage sites for actin filaments. This type of adhesion is mostly mediated by cadherins in a calcium^{2+} (Ca^{2+})-dependent feature(5). Most cadherins form homophilic interactions but they can also interact with other cell adhesion families although this is less common(6). Homophilic cadherin interactions create a zipper-like structure along the cell periphery that promotes tight adhesion between cells. Indeed, their primary function is to resist external force that can pull cells apart. However, unlike many other signaling receptors, the cadherins bind their ligands with weak affinity and strong attachments are first generated when multiple cadherins cluster together(5).

Desmosomes are anchorage sites for intermediate filaments and are generally present in tissues that are subjected to high level of mechanical stress such as the myocardium, the gastrointestinal mucosa and the skin. The major desmosome building blocks comprise desmosomal (non-classical) cadherins. In contrast to classical cadherins, non-classical cadherins can vary in both size and shape. It also remains unclear whether homophilic or heterophilic interactions are primarily responsible for desmosome adhesion(7).

Cells may also be held together within the ECM and there are two main types of junctions in which cells are held together within the ECM. Actin-linked cell-matrix junctions anchor cellular actin filaments to ECM while hemidesmosomes anchor cellular intermediate filaments to the same ECM structure. Whereas cadherins chiefly mediate cell-cell attachments, integrins mediate both cell-ECM and cell-cell attachments(5).

Tight junctions act in concert with anchoring junctions to establish cell polarity by acting as diffusion barriers(5). Claudins are the main transmembrane protein that seal adjacent epithelial or endothelial cells together in tight junctions. The opposite of tight junctions is gap junctions. Whereas tight junctions block the passageways through adjacent cells, gap junctions create direct channels from the cytoplasm of one cell to that of another. The connexins are the main transmembrane proteins within these structures that allow for both electrically and metabolically cell exchanges(8).

Transient adhesion junctions

Beside the above-described junctions, there are also more specialized adhesions that are used in specific tissues. These junctional adhesions are generally not as strong as those mediated by cadherins, but comprise fine-tuning adhesions that are as crucial as other adhesion junctions(5). The Ig-CAMs are cell surface glycoproteins that bind either homophilic or heterophilic to integrins or different Ig-CAMs(9). In contrast to both cadherins and claudins they are independent of Ca^{2+}. They are also generally distributed along intracellular boundaries without being associated with specific adhesive structures. In similarity to cadherins, Ig-CAMs form zipper-like structures that are supported by the actin cytoskeleton(5). Members of the Ig-CAM family function in a wide variety of cell types and contain many members including but not limited to Intercellular adhesion molecule 1 (ICAM-1), Vascular cell adhesion molecule 1 (VCAM-1), Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and Platelet/endothelial cell adhesion molecule 1 (PECAM-1)(10).

The selectins are cell-surface carbohydrate-binding proteins that also mediate various transient cell-cell adhesions in the blood stream(11). The three members of the selectin family include leukocyte selectin (L-selectin), endothelial cell selectin (E-selectin) and platelet and endothelial cell selectin (P-selectin)(12-14). Their main role is to guide trafficking leukocytes including T lymphocytes into lymphoid organs and into inflamed tissue(15). This is also one of the main roles for integrins and they will be discussed thoroughly in a later chapter.

Cells on the run

Cell adhesion is the main principle of cell migration. Almost all cell locomotion in human cells occurs by crawling. This is a complex multistep process that can be created by individual cells alone or in concert by multiple cells in sheets. Cell migration can generally be divided into three distinct activities: protrusion, attachment and traction. The crawling begins with membrane protrusion at the leading edge. This step is followed by formation of stable protrusive attachments

in which the actin cytoskeleton connects. The bulk of the trailing cytoplasm is then moved forward through traction forces, and in order for the cell not to tear adhesions are broken in the back of the cell(16).

Protrusion

Cells use membrane protrusions to scan the surrounding. They can extend their protrusions randomly or toward a directional cue such as a gradient of growth factors or chemokines, mechanical force, distinct ECM proteins or electrochemical gradients(16). The protrusions rely on the force of actin polymerization that pushes the front cell membrane forward. For migration to occur, the protrusions have to be stabilized by attachment to the substratum(17).

There are different protrusive structures dependent on cell type. Spike-like filopodia and broader lamellipodia are filled with dense actin(16). Podosomes are small and highly dynamic actin-rich protrusions that are characterized by its ring-shaped adhesion clusters. Invadopodia are also actin-rich protrusions and are used by cancer cells that cross tissue barriers. Both podosomes and invadopodia contain dense vesicles with ECM-degrading matrix metalloproteinases (MMPs). These protrusions are therefore location sites for ECM degradation and are thought to contribute to the invasiveness of both extravasating leukocytes and cancer cells(18).

Attachment

In order for the migrating cell to move forward, the leading edge must be attached to the substrate. These attachments are mediated through interactions between the integrins and the ECM. These small nascent adhesions are stabilized by the recruitment of both signaling and cytoskeletal proteins and can either rapidly turn over or mature to larger adhesions termed focal complexes. These dot-like adhesions locate slightly further back from the leading edge and serve as traction points when the cell is pushing its cell body forward. Focal complexes can continue to expand and turn into large and organized adhesion structures called focal adhesions. They reside in both central and peripheral regions of the cell. Focal adhesions are characterized by long lifetimes. They are composed of multiple proteins, which connect integrins with networks of large actin bundles or stress fibers. These adhesion structures are therefore generally not seen in rapidly moving cells like leukocytes since they are very stable and consequently tend to constrain cell migration(19).

The precise molecular linkage that forms between the integrin and the actin cytoskeleton during adhesion remains unclear and the scale of the cytoskeletal linkage also depends on the magnitude of the adhesion. In this thesis I will generally focus on the signaling molecules participating in nascent adhesions and in focal complexes since these are typically present in migrating cells. These

adhesions are small and dynamic and many of their key components will be discussed later in this thesis.

Traction

Adhesion disassembly occurs both at the front and at the back of the cell. During migration cells can extend and retract protrusions for long period of time. Adhesion disassembly in the back of migrating cells results in tail retraction and net translocation of the cell body forward. It is a quite complex process and does not appear to simply be the reverse of adhesion formation. It is driven, at least in part, by contractile forces. As the cell moves forward and the integrins remain bound to the substratum, a tension is created within the intracellular adhesive components. When the tension is too high the adhesion complex with its cytoskeletal linkage disperses from the integrin attachment and move forward by force along with the entire cell body. The integrins then face two destinies. They either remain in the cell membrane (more commonly) or become left behind in small units as "foot prints"(17).

Individual cell migration

The movement of individual cells is commonly described as either mesenchymal or amoeboid migration. However, these terms can be confusing and are not as straightforward as it may seem at one extreme. Mesenchymal migration applied by mesenchymal and epithelial cells, typically displays a flat and spread morphology due to many and strong focal adhesion anchorages. The migratory speed is slow, typically 0.1-0.5 μm/min. In contrast, a rounded cell shape, weak cell-substrate adhesions and constant cell shape changes characterize amoeboid migration that is applied by leukocytes(18). The migratory speed is much faster compared to mesenchymal migration with typically 5-40 μm/min(20).

Collective migration

Collective migration, in which large sheets of cells migrate in a coordinated manner toward the same direction and at similar speed, is present both during the development but also later in life during for example wound healing, tissue renewal and angiogenesis. During later decades it has also been giving a great role during tumor spreading, especially in carcinomas. Cells that are migrating together generally move with lower velocity but tend to migrate more persistent compared to cells migrating individually and they coordinate their responses from the environment through physical and chemical crosstalk(21).

Cell polarity

Individual cell migration requires communication and coordination between one cell end and to another. The back and the front (as well as the top and the bottom)

of the cell are all structurally and functionally different and it is crucial for the cell to maintain these differences during migration. The mechanisms that generate cell polarity in human cells are not fully understood but rely on cytoskeletal remodeling and members of the Ras homolog gene family (Rho) protein family of small Guanosine triphosphatases (GTPases)(21).

The Rho GTPases ras-related C3 botulinum toxin substrate 1 (Rac-1), Rho member A (RhoA) and cell division cycle 42 (CDC-42) regulate adhesion during migration by directly controlling the balance between actin-mediated protrusion and myosin-dependent contraction(19). Only a small fraction of these GTPases is active at the same time inside the cell. It has been shown that even with robust stimuli only 5% of Rac-1, RhoA and CDC-42 are in their active state at a given time in a neutrophil(22). Indeed, one should remember that where and when these GTPases are active is a different entity from where they are located.

Rac-1 and CDC-42 mediate rapid membrane protrusion in the front of a migrating cell by promoting actin monomer formation, branching and elongation. Rac-1 and CDC-42 are commonly known to stimulate lamellipodia and filopodia formation respectively, however, leading edge protrusions in most cell types probably involves both proteins in synergy(23). Both GTPases activate downstream effectors that belong to the Wiskott–Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) family of proteins that subsequently activate actin-related protein homolog 2 and 3 (ARP2/3) along with cofilins and formins, which finally catalyze actin polymerization(19, 24).

RhoA is generally involved in the adhesion disassembly at the rear of the cell. Actin filaments in the central and rear regions of a migrating cell are often organized into thick bundles called stress fibers. When these bundles contract, antiparallel actin filaments pass each other and thereby provide the force that rearranges the actin cytoskeleton. Myosin II mediates this contraction, and RhoA indirectly activates myosin II by activating Rho associated coiled-coil containing protein kinase 1 (ROCK-1) and ROCK-2. They subsequently phosphorylate and activate myosin light chain (MLC) as well as phosphorylate and inhibit myosin II phosphatases(19, 25).

Rho GTPase signaling and function is complex and RhoA is also activated at the leading edge and promote actin polymerization through mouse diaphanous-related formin 1 (mDia-1)(19). Rac-1 and CDC-42 may also regulate myosin activity by inhibiting myosin light chain kinase (MLCK) or the regulatory light chain (RLC)(26). Rho GTPases also promote the polarization of the microtubule network and its associated vesicular traffic machinery, thereby providing the cell front with additional membrane and membrane receptors(21). In addition, the complex feedback loops initiated by external cues that control Rho GTPase activity are currently poorly understood. Integrin signaling networks can regulate

the Rho GTPase activity by recruiting Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) to the adhesion complex. Some GEFs are specific for an individual Rho family member whereas others act on multiple family members. Various scaffolding proteins of the protein tyrosine kinase (PTK) family are also involved in the organization of signaling complexes that regulate Rho GTPases(19).

Collective cell migration also requires communication and coordination between one cell end and another. However, in contrast to individual cell polarization, the cohesive cell group is forming one large polarized unit. The cells that are located in the front line are called leader cells. Due to their position, they are exposed to high levels of external signals and dictate the direction and migratory speed of the entire cell cluster(27). The leader cells also play a major part in the ECM remodeling, and in similarity to single-migrating cells, leader cells are also polarized and display actin-based protrusions(28). The following cells (called followers) must rely on strong cellular interactions to collectively polarize and both the leader cells and the followers are consequently linked tightly together by lateral adhesive structures and transverse acto-myosin cables that move in concert in multiple cells. The followers communicate with the leader cells and can if necessary also influence migratory behavior(27). The overall understanding of collective migration is somewhat limited. Various β1 integrins are generally involved in collective migration but this also depends on cell type and cell substrate(21).

Integrin-independent migration

Basically every mammalian cell type has integrins on its surface and the only exception is erythrocytes and they lack migratory capacity. However, adhesion-independent migration has recently emerged as a possible although still poorly understood mechanism(18). One of the motility mechanisms that appear to require less, or no, specific adhesive interactions with the environment is called blebbing(29, 30). Bleb-based migration is more commonly found in 3D environments and appear to be traction-dependent and a direct consequence of intracellular pressure that is pushing against the plasma membrane (PM)(29, 31). Its role and its regulation in cell migration is not fully understood but it is studies like these that challenge the common view that migration universally depends on integrin-based attachments(18, 31). Still, the detrimental consequences of integrin loss for most cell types additionally speak for their importance in force-generated migration(32).

Measuring velocities as phenotypic outcome can also be misleading. For example, integrin-deficient cells can compensate for inefficient force transduction by significantly increase the rate of actin polymerization at the leading edge. For sure, such compensation mechanisms make it almost impossible to allow for clear

conclusions. Most cells also migrate in a 3D confinement and to monitor actomyosin dynamics in 3D (and *in vivo*) is challenging. In addition, the use of fluorescently tagged adhesion proteins, cell-shape measurements and cell-substrate contact angles are also great migratory read outs, but can as well be a challenge, as well as more complex in vivo and in 3D matrices(18). Consequently, the extent of cells' ability to migrate independently of integrins is unclear and currently an unsettled question. In order for the answer to be conclusive one must use a combination of approaches and also study both physiological and pathological processes.

Some of the first cell types that was studied during integrin-independent migration were leukocytes since they also posses the ability to polarize along the front-back axis even in suspension(18). Some of these first studies reported dysfunctional migration when specific integrin expression was reduced, however the migratory capacity was not completely abolished(33). Lämmermann *et al.* further argued the importance of integrins during migration when they showed that *in vivo* interstitial migration of Dendritic cells (DCs; amoeboid migration) could occur in the absence of integrins or its cofactor talin(34). Still, integrins are required for leukocyte extravasation under shear, an essential process that will be thoroughly discussed in the next chapter of this thesis(35).

Getting leukocytes to the site of inflammation

Innate control and recognition

The insulting challenge can be of various kind an include pathogens, toxins, irritants, unscheduled cell death, ECM degradation products and also tissue damage(36). These challenges can be sensed either directly or indirectly. A direct sensing strategy includes pattern recognition receptors (PRRs) that recognize conserved pathogen-associated molecular patterns (PAMPs) on the disruptive agent such as allergens, toxins, poisons and viral nucleic acids. However, both beneficial commensal microorganism and pathogenic microorganism express PAMPs and consequently the innate control must use additional characteristics to be able to distinguish beneficial microbes from harmful ones. Indeed, indirect sensing mechanisms include sensing the functional harm or features that are caused by disruptive agents such as membrane pore formation, tissue damage (DAMPs) or enzymatic activity that alters homeostasis(1, 2).

The first surveillance system the pathogen encounter in the host is generally composed of macrophages that are located in the mucosal layer above the

epithelium, the epithelium itself with tissue-resident macrophages, DCs and mast cells that are located underneath the endothelium. This local defense has a great sensing capability and is mostly able to recognize the harmful source and eliminate it before it has the chance to cause any damage. However, when the local defense is insufficient to eliminate the harmful source an immune response is initiated. A combination of direct and indirect recognition during a viral, bacterial or fungal infection leads to a type 1 immune response. Indirect recognition alone during for example a parasitic infection leads to a type 2 immune response(1).

The leukocyte adhesion cascade – crossing the vascular wall

Correct cells, correct molecules and correct order

Once the local defense has been conquered the magnitude of the immune response becomes greater and includes the recruitment and the amplification of various leukocytes at the site of inflammation to combat the spreading pathogen. Leukocyte extravasation is one of the first steps during inflammation. During this step the magnitude of the immune response become prominent and will start to cost and show necessary local and systemic damage (immunopathology). The inflammatory response is capable of causing great tissue damage and that is why it is in the host's best interest to have an extended series of checkpoints in place before leukocytes enter the tissue where they perform their effector functions that ultimately kill and expulse the pathogen(1, 35).

In response to inflammatory stimulus, neutrophils are generally the first leukocytes to exit the blood stream. Monocytes are next in line and finally lymphocytes. Extravasation of all these leukocytes is compulsory for a proper immune response since they fulfill different functions once they are recruited to the inflamed tissue. For example, besides being involved in the initiation of pathogen destruction, an additional role of neutrophils and monocytes is to prepare the site for following lymphocyte arrival(37). The precise molecular extravasation mechanism is still under discovery, but the overall strategy for all leukocytes is similar in term of receptor-ligand interactions and signaling pathways(38).

Capture

In a normal state, circulating leukocytes move passively in the center of the blood stream by laminar blood flow. To increase the chances of leukocytes to be exposed to local inflammatory signals and come in direct contact with the vascular endothelium that lines the inflammatory site, local changes in hemodynamics results in a greatly reduced blood flow(35).

Rolling

Rolling of leukocytes on the vascular endothelium is mediated by selectins. Under normal conditions, the endothelium expresses low levels of adhesion molecules and is accordingly undergoing limited immune surveillance. Upon stimulation of histamine or other acute inflammatory mediators such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), preformed P-selectin that is stored in intracellular endothelial vesicles (Weibel-Palade bodies) become translocated toward the endothelial cell surface and consequently interact with selectin ligands that are expressed on circulatory leukocytes(35). The best-characterized P-selectin ligand is P-selectin glycoprotein ligand 1 (PSGL-1) that is expressed on leukocytes. However, many surface proteins on leukocytes can become glycosylated by glycosyltransferases and many of these formed glycoproteins bind to the endothelium-expressed selectins(38).

A complementary selectin is also expressed on leukocytes. L-selectin interacts with cluster of differentiation 34 (CD34) expressed on endothelial cells and promotes further binding. Both P- and L-selectin have very fast on and of rates that allows the leukocytes, in combination with the blood flow, to bind tentatively (called rolling) to the endothelial cell layer(35).

T lymphocytes differ slightly from other leukocytes when establishing vascular endothelial cell contact. In contrast to neutrophils and monocytes, T lymphocytes generally do not constitutively express all those glycosyltransferases required for proper selectin ligand glycosylation. T lymphocytes consequently need additional inflammatory stimulus that initiates proper glycosylation of selectin ligands(39). T lymphocytes also randomly adhere and role on endothelial cell lining in order for their T cell receptors (TCRs) to scan and recognize potential antigens presented by the vascular endothelium. TCR signaling itself can also initiate transmigration(40).

Slow rolling

When endothelial cells have been exposed to pro-inflammatory cytokines for several hours, they begin to express an additional selectin called E-selectin. This step brings the leukocytes in closer contact to the vascular endothelium. It also promotes further activation of the leukocytes since the endothelial cells also presents chemokines and lipid chemoattractants that are produced by either the endothelial cells themselves or by underlying interstitial inflammatory cells(35).

Sundds *et al*. have also showed that neutrophils produce extensions of their PMs, called slings that help tether the neutrophils to the endothelium(41). The neutrophils cast their slings downstream, in the direction of flow, where they adhere to the endothelium. As the neutrophils roll, the slings wrap around the cells and provides additional adherence(41). Fascinating.

Arrest – nearly exclusively mediated by integrins

The loosely attached leukocytes roll along the endothelial surface until chemokines, which are induced in the injured tissue and tethered to proteoglycans on the vascular wall, initiate the next step of the adhesion cascade. Contact between chemokines and G protein–coupled receptors (GPCRs) on the leukocytes initiates intracellular signaling cascades that activate integrins on the surface of leukocytes. Leukocytes express integrins of the β1 and β2 family that are called very late antigen 4 (VLA-4) and lymphocyte function-associated antigen 1 (LFA-1), respectively, and their ligands are expressed on endothelial cells (VCAM-1 for VLA-4 and ICAM-1 and ICAM-2 for LFA-1)(35). Rolling integrins can be partly activated but once fully activated through GPCR signaling, integrins bind tightly to their ligands, which further allow the rolling leukocytes to arrest on the vascular endothelium(42, 43). The primary integrin-inducing arrest and further adhesion varies among leukocyte subsets and is believed to be dependent on the stimulus(37).

Adhesion strengthening and spreading

Once arrested, leukocytes begin to strengthen their adhesions and spread on to the vascular endothelium(35). During this step (and the proceeding ones), integrin ligands become enriched under the leukocytes in a process that is dependent on the actin cytoskeleton(44). The clustering of preformed membrane ICAM-1 and VCAM-1 nanodomains on the endothelium initiates activation of several intracellular signaling cascades that includes intracellular Ca^{2+}, several kinase cascades and also Rho-GTPase activation(45). These signaling cascades prepare for leukocyte transmigration by loosening the endothelial junctions and help the endothelial cells to contract and separate(37).

Crawling

After some time and in order to find a good extravasation site, adherent leukocytes begin to crawl on the vascular endothelium. Intravascular crawling, generally upstream against the blood flow, is partly mediated by LFA-1 but mostly by a third integrin expressed on leukocytes called macrophage-1 antigen (Mac1), which interact with its ligand ICAM-1 on endothelial cells(38).

Transmigration

Transmigration (or diapedesis) can be achieved by either a paracellular or a transcellular fashion. Transmigration could previously be described by using some famous song lyrics written by Andrew Lloyd Webber, the man behind the best musical that has ever been written. It was believed to be the point of no return in the initiation of an inflammatory response. Once past this final threshold there were generally no backward glances. The leukocytes had committed to

transmigration and would generally not come back to the circulation. At least not as the same cell type(35). However, the current view is slightly less dramatic and leukocytes have been found to return to the circulation after transmigration. For example, Woodfin *et al.* showed that some neutrophils do migrate back into the circulation after crossing the vascular endothelium during ischemia-reperfusion injury(46).

During transmigration, when leukocytes move across the endothelial cells, the leukocyte-endothelial cell interaction changes, from a 2D state to a 3D state. The leukocytes can choose to migrate in a paracellular (most common) or transcellular manner. Why they chose one path over the other is not known but it is believed to be dependent on tightness of the endothelial junctions and consequently the ability of the leukocytes to break through them(47). During paracellular transmigration some junctional proteins such as vascular endothelium cadherin (VE-cadherin) becomes locally dispersed whereas in contrast both platelet/endothelial cell adhesion molecule 1 (PECAM-1) and CD99 become concentrated at the junction border (48-51). Various integrins also participate during transmigration. For example, junctional adhesion molecule (JAM-A) binds to LFA-1 and JAM-C binds to Mac-1 on transmigrating leukocytes(52, 53). Transmigration might also be supported by endothelial lateral border recycling compartments (LBRCs) that are located in subcellular vesicular compartments. Rather than having to unzip high-density homophilic adhesions of VE-cadherins, catenins and JAMs, LBRCs and their membranes push aside present junctional adhesion structures and provide the junction with unligated CD99, PECAM-1 and JAM molecules(35).

Once past the epithelial cell lining leukocytes face additional challenges. First they have to cross the endothelial cell basement membrane that also contains pericytes, and then also the surrounding interstitial ECM(37). Transmigration trough the vascular endothelium can be a rapid process whereas penetrating the following layers can take much longer time (<2-5 minutes and >5-15 minutes, respectively)(37). Due to the difficulty in reproducing this complete structure *in vitro* or to visualize it *in vivo*, relatively little is known about the molecular interactions involved in the transmigration process. It has been shown that neutrophils and monocytes tend to migrate across the least dense basal membrane where collagen IV and laminins are expressed in relatively low density(54, 55). Neutrophils also seem to be guided toward a less dense area by communicating with pericytes trough LFA-1- or Mac1-interactions with ICAM-1(56). Real-time leukocyte recruitment into inflamed tissues is not well characterized but Hyun *et al*. have shown that extravasating leukocytes (neutrophils, monocytes, and T lymphocytes) show delayed detachment of LFA-1 in the uropod and become extremely elongated before complete transmigration across the vascular endothelium occur(57). Ligation of PECAM-1 and other similar molecules can

also recruit and activate ECM-binding β1 integrins during transmigration. This includes VLA-2, VLA-3 and VLA-6(57-59).

In summary, leukocyte recruitment to inflamed tissue forms the basis of all local immune responses. It is a complex and multistep regulatory system and many steps can go wrong and the pathological potential of inflammation can cause devastating consequences. However, the protective capacity of inflammation is great and comes to our rescue multiple times during our lifetime. Next time your skin cut on your finger gets infected and you experience redness, swelling, heat, immobility and pulsating pain, think about it as a positive and beneficial process. Look down on your finger and say, "Good job guys. Just get those invading crooks."

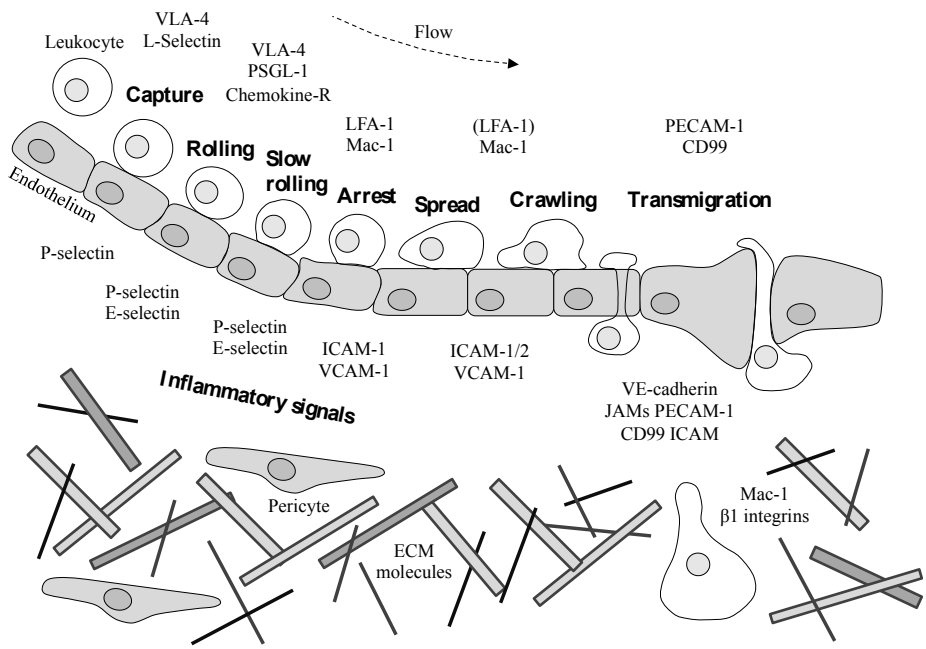

Figure 1. General steps of the leukocyte extravasation cascade.

The integrin LFA-1

The basics of integrins

The integrins are implicated in cell migration in many contexts, which include embryonic morphogenesis, immune responses, tissue repair and regeneration as well as stem cell homing(24). Integrins are transmembrane heterodimers composed of two non-covalently associated glycoprotein subunits called α and β. Both subunits contain a long N-terminal extracellular domain and a short C-terminal cytoplasmic domain(60). The extracellular domain determines binding specificity and it can recognize a variety of ligands on cells (Ig-CAMs) or in the ECM (fibronectins, collagens and laminins). Integrins can roughly be classified into four families based on ligand preference. These families include arginine-glycine-aspartic acid (Arg-Gly-Asp, R-G-D)-binding integrins, which bind the peptide motif Arg-Gly-Asp on various proteins such as fibronectins, as well as collagen-binding integrins, laminin-binding integrins and leukocyte-specific integrins(61).

Similar to the author of this thesis, integrins made an entrance back in 1986(62). Tamkun and Hynes *et al.* named the protein integrin because it linked the ECM to the actin cytoskeleton. The cytoplasmic integrin domain assembles several different proteins that together form the linkage to the cytoskeleton. The size of the cytoskeletal linkage depends on the adhesion magnitude, with larger linkages being formed under stronger adhesions(24).

Different stages of activation

Ligand binding can be regulated through integrin clustering and through modulation of the activity of individual integrins. Most integrins are not constitutively active. Electron microscopic and crystallographic studies have identified three conformations that reflect three different stages of activation. The conformations include the bent and low-affinity form, the extended and intermediate-affinity form with a closed ligand-binding head domain and finally the high-affinity form. Here, the α- and β-subunits have separated and the ligand-binding head domain has become more accessible(63). The exact dynamic equilibrium among different conformations remains controversial. Various studies suggest that integrins are bent with various degrees and not all inactive integrins are bent with an obtuse angle. A fully extended conformation with fully separated cytoplasmic tails may also not be required for all integrins in order to bind ligands(64).

Different integrins use different regulatory mechanisms that have evolved to meet varying biological requirements. Integrins on adherent cells are generally found in

their active and extended conformation whereas integrins on fast moving cells like leukocytes generally are found in their inactive and bent conformation. Integrin activation on adherent cells mainly occurs by integrin clustering through high concentration of available ligands in the ECM. It can also occur by force. Although activation of integrins on rapidly migrating cells also can occur by force when exposed to shear flow in the circulation, conformation changes through complex signaling systems dominate(61, 65, 66).

Importance of force

Apart from intracellular and extracellular stimuli, also tensile force can induce conformational changes and consequently stabilize the interaction between integrins and their ligands. In the vascular system, force is generated by fluid flow and when not present, migrating cells rely on traction force. This is dependent on the rigidity of the extracellular substrate and the tensile forces it applies on the extended integrin's headpiece. Traction force also relies on the cytoskeletal network that is linked to the integrins and the lateral pulling force it applies on the β tail that consequently generates the separated integrin tail and also the open headpiece conformation(67, 68). Most integrins connect with the actin cytoskeleton to generate this type of force but some integrins also connect with the intermediate filament system (α6β4)(69, 70).

Leukocyte integrins

Leukocytes can express at least 12 of the 24 known integrin heterodimers, and the expression pattern depends on the subset and the maturation state of the cell(60). LFA-1 (αLβ2), Mac-1 (αMβ2) and VLA-4 (α4β1) are common integrins expressed on leukocytes(71). LFA-1 recognizes ICAMs (1-5) as well as JAM-1. VLA-4 recognizes VCAM-1 but also fibronectin(64, 71, 72). Mac-1 recognizes various ligands including different ECM proteins such as fibrinogen and fibronectin as well as activated complement proteins such as inactive complement component 3 (iC3b)(71).

β2 integrins are only expressed on cells of the hematopoietic lineage and besides LFA-1 and Mac-1, leukocytes also express the remaining leucocyte-restricted β2 integrins αXβ2 and αDβ2, that also are required for a functional immune system(73). LFA-1 is expressed by neutrophils, monocytes and lymphocytes and Mac-1 is mainly expressed on neutrophils and monocytes whereas VLA-4 is expressed on monocytes and T lymphocytes. The less common leukocyte integrins αXβ2 and αDβ2 are expressed on macrophages and DCs and monocytes and macrophages, respectively(74). Leukocytes also express the β7 integrins (α4β7

and αEβ7) that are essential for homing and retention of lymphocytes to the gut(75, 76).

The integrin LFA-1

LFA-1 is the main integrin that is expressed on T lymphocytes. Since the development of specific monoclonal antibodies that detect either the two extended states of LFA-1 (KIM-127), or the extended and fully active state of LFA-1 (m24) unique features of LFA-1 activity and its localizations have been discovered(77-80). Circulating leukocytes generally display their LFA-1 integrins in a bent and non-adhesive state that restrict their ability to bind ICAMs. Their LFA-1 integrins are also randomly dispersed over the cell surface, but this distribution is rapidly changed upon ligand contact. On migrating T lymphocytes, LFA-1 levels varies from lower levels at the leading edge to higher levels in the non-attached uropod(81). Although discrete focal adhesions are lacking in migrating T lymphocytes, different cellular zones contain populations of LFA-1 with different ligand-binding affinities. During migration intermediate-affinity LFA-1 is localized to the more dynamic leading edge (lamellipodia) where membranes rapidly protrude and retract (81, 82). These highly dynamic changes rely on fast make-and-break binding of LFA-1 to ICAM-1 and consequently favors intermediate-affinity LFA-1 with quicker on-off kinetics compared to high-affinity LFA-1(20). Intermediate-affinity LFA-1 at the leading edge is associated with the actin cytoskeleton through α-actinin(82). High-affinity LFA-1 is generally localized to a distinct adhesion zone in the mid cell (focal zone), which provides firm attachment to ICAM-1. The high-affinity LFA-1 is associated with the actin cytoskeleton through talin(81, 82).

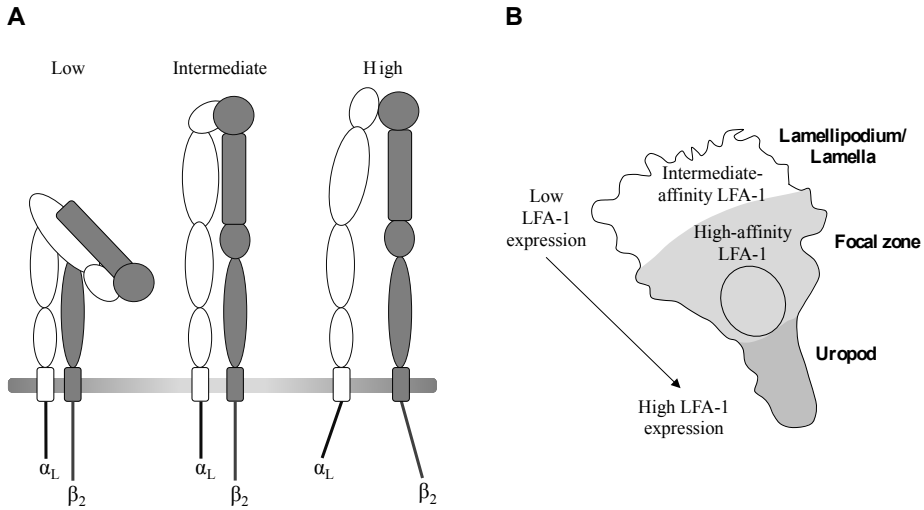

Figure 2. (A) LFA-1 conformations. (B) LFA-1 conformation zones and expression pattern in a migrating T lymphocyte.

Integrins – more than just adhesion molecules

Integrins do more than just create attachments. They are also signaling receptors and activate intracellular signaling pathways that control a broad range of the cell's behavior including proliferation, gene expression and cell survival(24).

Integrin receptor signaling

Integrin receptor signaling is bidirectional through the PM. The intracellular signaling cascades that are responsible for the transition from a low-affinity state to an intermediate-affinity state are referred to as inside-out signaling. These signals transfer from the cytoplasm, through the intracellular cytoplasmic integrin tail and further toward the extracellular integrin domain(83). Integrins can also activate intracellular signaling cascades by transferring signals from the extracellular integrin domain through the intracellular cytoplasmic tail and further towards the cytoplasm. These signals are referred to as integrin outside-in signaling and promote the transition from the intermediate-affinity state to the high-affinity state(71).

The key signaling events during integrin signaling have been identified by using various methods and approaches including but not limited to knockout mouse models, small interfering ribonucleic acid (siRNA) silencing, over/dysfunctional complementary deoxyribonucleic acid (cDNA) expression, co-

immunoprecipitation, fluorescence imaging and pharmacological inhibitors. Many of these studies have shown that multiple proteins assemble with the integrin cytoplasmic tail, however the binding kinetics and also the binding order that leads to integrin conformational changes are still poorly understood(71).

Integrin activation in T lymphocytes is accomplished through similar intracellular signaling pathways that are independent on initiating stimulus (selectin, GPCR or TCR signaling). GPCR signaling generally promotes cell migration whereas TCR signaling generally promotes stable cell-cell contacts(71). In the following sections, I will try to explain the main known signaling events in both LFA-1 inside-out and outside-in signaling.

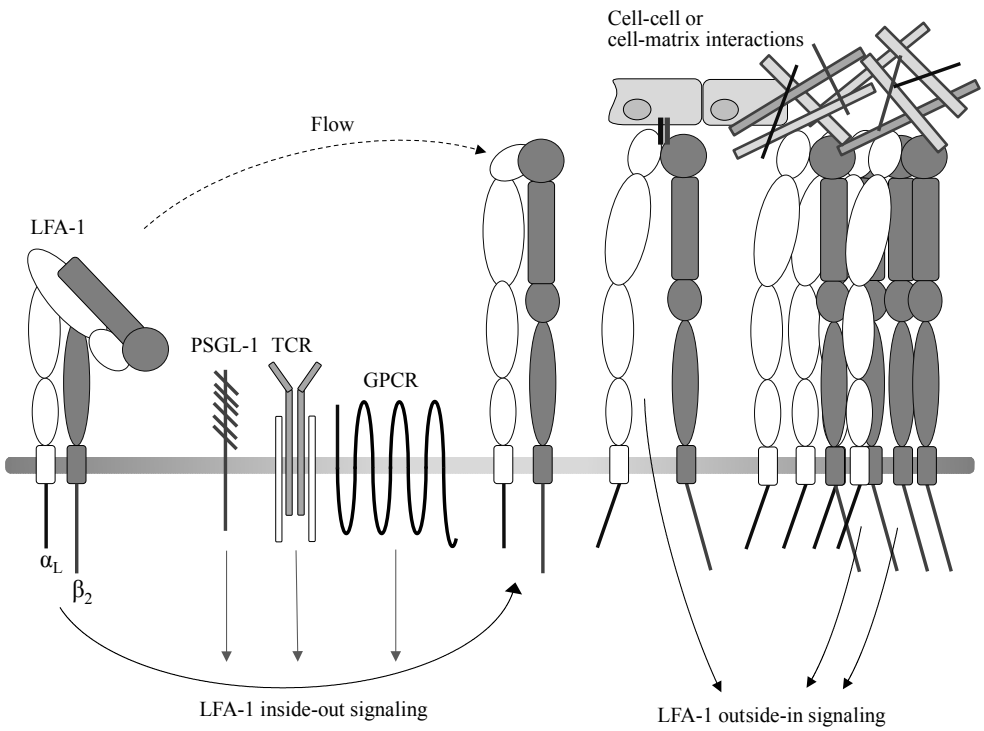

Figure 3. LFA-1 inside-out versus LFA-1 outside-in signaling.

LFA-1 inside-out signaling

Signal initiation

Circulating leukocytes generally display their LFA-1 integrins in a bent and non-adhesive state that restrict their ability to bind ICAMs. LFA-1 inside-out signaling and subsequently transition from the bent to the extended state can be initiated by

various stimuli that differ in their mechanism of action. E-selectin triggered signals require seconds of rolling and during this time, LFA-1 is globally triggered over the entire surface of the rolling leukocyte. In contrast, GPCR-mediated LFA-1 activation is a rapid and highly localized event that simply involves a small subset of the entire surface-expressed LFA-1 molecules(84). Selectins and GPCRs work toward the common goal of integrin activation during leukocyte migration across the vascular endothelium. When leukocytes establish cell contact, selectin-mediated rolling facilitates the rapid GPCR-mediated LFA-1 activation by promoting a close contact between the leukocytes and the endothelium. Similar to selectins and GPCRs on T lymphocytes, TCRs can also initiates inside-out signaling(38)

Action at the αL integrin tail

A crucial step during LFA-1 activation is the delivery and subsequent activation of ras-related protein 1 (Rap-1) and its guanine nucleotide exchange factor (GEF) at the cell membrane but how this precisely occurs is currently unknown(85). Various pathways initiated by either GPCR or TCR signaling can converge on Rap-1 activation by activating its GEFs including calcium- and diacylglycerol (DAG)-regulated GEF 1 (CalDAG-GEF-1) and RapGEF-1(86). Rap-1 activation also occurs through the routes of either phospholipase C (PLC) or protein kinase C (PKC)(85, 87). PKC is in similarity to CalDAG-GEF-1 also responsive to Ca^{2+} and DAG. DAG is believed to activate PKC by recruiting and attaching it to the PM where Ca^{2+} binds(88). Another downstream effector of PKC that is also involved in Rap-1 activation is protein kinase D 1 (PKD-1)(87).

Activated Rap-1 acts on its effector Rap ligand (RapL), which interact with the intracellular αL integrin tail(89). RapL next form a complex together with macrophage-stimulating protein 1 (Mst-1) and consequently mediates its activation(90). Both RapL and Mst-1 have been found in the same recycling compartment as Rap-1 and this also includes LFA-1 since RapL binds LFA-1 and acts as its transporter(90, 91). RapL also bind Sarcoma (Src) kinase-associated phosphoprotein of 55 kDa (SKAP-55) and both these proteins have been suggested to be responsible for correctly positioning Rap-1 and also LFA-1 at the cell membrane(92). In addition, RapGEF-1 transport appears to be mediated in a complex together with CT10 regulator of kinase (Crk)-like protein (CRKL) in a mechanism that additionally depends on Abelson tyrosine protein kinase (ABL) as well as WASP, WASP-family verprolin-homologous protein (WAVE) and actin-related protein homolog 2/3 (ARP2/3)(93).

Action at the β2 integrin subunit

Inside-out signaling culminates in delivery and subsequent binding of talin to LFA-1 at the β2 integrin tail. The interaction between LFA-1 and talin is complex

and requires calpain-mediated cleavage, binding of phosphoinositol phosphatase and phosphorylation(94, 95). Talin contains various 4.1 protein, ezrin, radixin, moesin (FERM) domains and one such FERM domain binds and responds to local changes in phosphatidylinositol 4,5-bisphosphate (PIP_2) that is found at the PM. Both GPCR and TCR signaling can activate PLD-1, which subsequently generates phosphatidic acid that stimulates phosphatidylinositol 4-phosphate 5-kinase 1 (PIP5K-1) that is responsible for PIP_2 production(96). This is another crucial step during LFA-1 activations since PIP_2 binding to talin cause some unfolding that potentially could unmask additional binding domains(97). One such domain that potentially could become accessible after PIP_2 binding (and calpain-mediated talin cleavage) is also another FERM domain on talin that interacts with the β2 integrin tail(98). PIP_2 production can also be driven by GPCR-activated RhoA, which may locally elevate PIP_2 near LFA-1 and thereby activate talin through both direct binding and subsequent recruitment of the CalDAG-GEF-1/Rap-1 complex that also includes Rap1-GTP-interacting adapter molecule (RIAM)(88). In addition, talin also contain multiple binding sites for the F-actin-binding protein vinculin(99).

Kindlin-3 is another crucial signaling intermediate for LFA-1 activation that works as a co-activator together with talin. Kindlin-3 also contains a FERM domain that interacts with the β2 integrin tail at a site distal of talin(100). Rap-1 is also a player at the β2 integrin tail and Rap-1-mediated recruitment of RIAM links Rap-1 to adhesion and degranulation-promoting adapter protein (ADAP) and SKAP-55. Recruitment of the ADAP/Skap-55 complex to the PM is further mediated by Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76kDa (SLP-76), at least during TCR signaling(101). The complex of Rap-1/RIAM/ADAP/SKAP-55 at the membrane is also an early step during talin activation(102). Rap-1 links RIAM to talin and binding of RIAM to talin unmasks the β3 integrin-binding site in talin, at least in platelets(103).

The actin-binding protein α-actinin also binds the β2 integrin tail of intermediate-affinity LFA-1 and consequently link LFA-1 to the cytoskeleton. α-actinin is part of the F-actin network that is responsible for propelling the cell forward. α-actinin loses its attachment in favor to talin during transition to high-affinity LFA-1(81, 82). This is also true for filamin, an actin filament cross-linking protein, which interacts with migfilin and together they compete with talin for binding to β2 integrin tail(104).

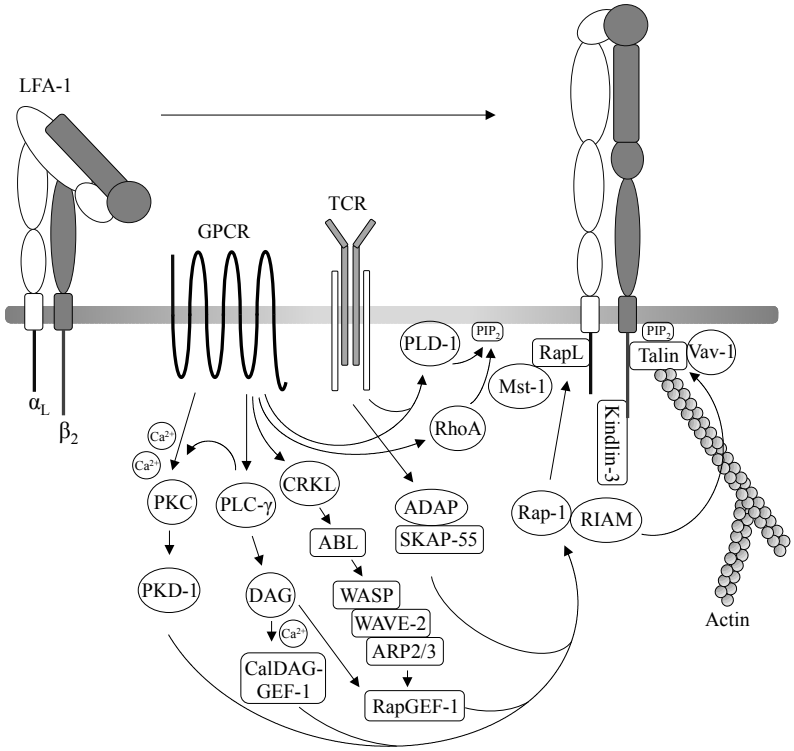

Figure 4. Schematic view of LFA-1 inside-out signaling.

LFA-1 outside-in signaling

LFA-1 outside-in signaling mediates the transition from the intermediate-affinity state to the high-affinity state, which consequently brings LFA-1 in close contact with ICAM-1 and promotes leukocyte function and activation. Besides firm adhesion, cell spreading and migration, outside-in signaling responses in T lymphocytes also include proliferation, IL-2 production and stronger T lymphocyte binding to an antigen-presenting cell (APC)(71).

Since signaling from the integrins themselves often follows inside-out signaling, identifying unique signaling events downstream of the integrins have been a challenge. Many of these signaling intermediates also hold dual roles and are key-signaling mediators during both inside-out and outside-in signaling(71). However, by for example using stimulators that bypass inside-out signaling such as phorbol myristate acetate (PMA) and manganese^{2+} (Mn^{2+}), outside-in signaling has been able to be studied. However, the signaling pathways downstream of ligand-bound

LFA-1 that participate in outside-in signaling have not been well characterized in T lymphocytes. The next section will consequently describe details that generally have been obtained in other lymphocytes including macrophages and neutrophils.

Src and Syk family of kinases

Two kinase families are main players in outside-in signaling. In T lymphocytes they include the Src kinase leukocyte C-terminal Src kinase (Lck) and the spleen tyrosine kinase (Syk) zeta chain of T-cell receptor associated protein kinase 70 (Zap-70)(71). As in other signaling cascades (including TCR signaling) they are believed to phosphorylate each other and consequently initiate downstream signaling(105, 106). Since most integrins do not possess an obvious interaction motif (such as SH2 or SH3 domains) for these kinases, a previous unanswered question was if and how these kinases become coupled to integrins. However, recent studies have shown that active LFA-1 directly interacts with Zap-70 and both inactive and active LFA-1 interacts with Lck in T lymphocytes(107, 108). We have also recently shown that phosphorylation of Zap-70 and Lck increases upon LFA-1 engagement with ICAM-1(109). Additional studies have also specifically shown that various immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor proteins such as SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) can couple these kinases to integrins(110, 111).

Connecting LFA-1 outside-in signaling to the actin cytoskeleton

Cytoskeletal reorganization is a key downstream effect of outside-in signaling. It is only after external and opposing forces supplied by ICAM-1 and the cytoskeleton that full LFA-1 activation takes place(68). The Rho-GEF vav type 1 (Vav-1) is a main signaling intermediate in this signaling pathway and activates the Rho GTPases CDC-42, Rac-1 and RhoA (112, 113). A pool of Vav-1 also binds to talin in response to PIP_2. Although not yet confirmed in β2 signaling, it is predicted that Zap-70-medaited phosphorylation of Vav-1 leads to its dissociation from talin. Free talin with its full LFA-1 binding potential promotes the final transition to high-affinity LFA-1 by separating the αL and β2 tails from one another(114). RAPL might contribute to a similar allosteric effect as talin, but on the opposing αL tail side(86). In addition, kindlin-3 also appears to be involved in outside-in signaling and consequently provide additional cytoskeletal linkage(115).

Another main function of Vav-1 is also to regulate phospholipase C γ (PLC-γ) and downstream Ca^{2+}-signaling(112). The mechanism behind this regulation is not fully understood, but does likely involve a complex of SLP-76/Vav-1/PLC-γ similar to inside-out signaling. PLC-γ also likely plays a role in inositol 1,4,5-trisphosphate (IP_3) production during outside-in signaling, which further activates

CDC-42, Rac-1 and RhoA(71). Integrin activation is additionally also dependent on the presence of cations, especially Mn^{2+} and magnesium^{2+} (Mg^{2+})(116).

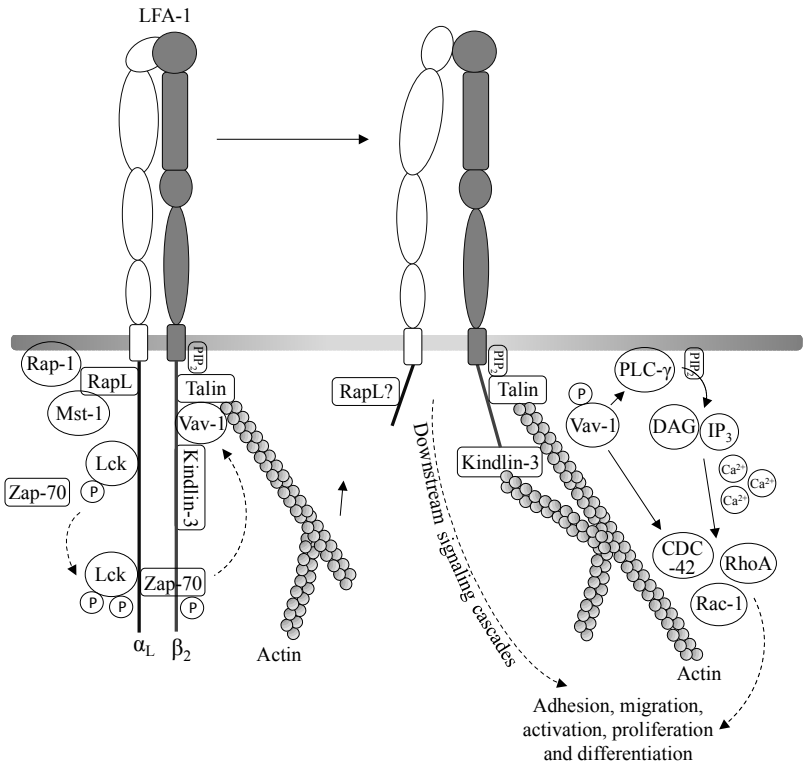

Figure 5. Schematic view of LFA-1 outside-in signaling.

Integrin recycling

Going inside

Endocytosis is a dynamic process that is essential for maintaining PM composition as well as regulating signaling pathways. It is a complex process and surface molecules can be endocytosed by several different routes. The two primary internalization routes are clathrin- and caveolae-mediated endocytosis(117). The coat proteins clathrin and caveolin are responsible for invagination and vesicle

formation during cargo internalization by mechanisms that are incompletely understood. Whether preformed invaginations (called clathrin-coated pits (CCPs) and caveolae) trap and internalize cargo, or if the presence of cargo molecules stimulates vesicle formation is one of the key questions waiting to be answered. One of the reasons for the lack of understanding is the complexity and diversity of vesicle formation in different species and in different cell types(117).

Although clathrin is the mechanical backbone in CCPs it cannot directly adhere to the membrane components that form the vesicle. It needs a linker that provides multiple binding sites for the vesicular backbone, the accessory proteins, the lipids and finally the vesicle cargo(117). Adaptor protein 1 (AP-1), AP-2 and various clathrin-associated sorting proteins (CLASPs) are the most widely recognized adaptor proteins in clathrin-mediated endocytosis. They select transmembrane cargoes based on sorting signals and also assemble the clathrin coat and other components necessary to shape the CCP(117, 118). The CCP is stabilized by accessory and regulatory proteins and becomes subsequently pinched off by scission factors such as dynamins. The internalized clathrin-coated vesicle (CCV) is then propelled into the cytoplasm with an incorporating signal that directs it towards a desired intracellular compartment where it ultimately docks and fuses with target membranes(118).

Alternative endocytic pathways besides clathrin- and caveolae-mediated endocytosis also exist. Although the understanding of these pathways is incomplete, they share similar mechanism as clathrin- and caveolae-mediated endocytosis in terms of cargo recruitment, invagination and scission. Flotillin-regulated, chloride intracellular channel protein (CLIC)-dependent, CDC-42-dependent, lipid raft-dependent and macropinosome-mediated endocytosis are some of these less understood endocytic pathways(117).

Endosomal traffic - more than just a social network

The molecular machinery of endosomal traffic is complex and not fully understood. The distinction between different endosomal compartments is somewhat blurred at the molecular level but unique variations in both protein and lipid compositions have been identified. Despite the existence of numerous internalization routes, the early endosome (EE) serves as a key sorting station within most endocytic routes and most internalized cargo is delivered to this membrane machinery. The GTPase ras-related in brain (Rab) 5 (Rab5) and its effectors such as early endosome-associated protein 1 (EEA-1) function as regulatory factors at the EE and from here cargo can become assigned to various faiths. Many receptors are returned back to the PM via either a rapid recycling route in a Rab4-dependent manner or by a longer recycling route in a Rab11-

dependent manner(119). Internalized cargo can also be routed towards the trans-Golgi network (TGN) and some newly synthesized membrane proteins can also transit from the TGN via the EE on their way to PM(119).

Internalized cargo can also be directed towards a second trafficking station within the endosomal system that is regulated by Rab7. Transport from the EE to the late endosomes (LE) is mediated by endosomal intraluminal vesicles (ILVs), which travel along the microtubule network. The ILVs are pinched off as free cargo-containing vesicles and mature into free endosomal-carrier vesicles that are called multivesicular bodies (MVBs), which eventually fuse with LE. EE-LE transport is characterized by concomitant changes in the endosomal luminal milieu. The slightly acidic milieu of the EE promotes uncoupling between receptor and ligand and during the EE-LE transport the pH value inside the endosome becomes even more acidic, which further aids in the uncoupling of receptor with its ligand. The LEs are also a signaling station and is involved in the sensing of nutrient availability and other environmental cues that subsequently control cell growth/differentiation and metabolism(119).

Most cargo that ends up in the LE is domed for degradation and consequently further transport to the lysosome. This transport involves further acidification of the LE that subsequently activates lysosomal enzyme activity. The main transmembrane component of both LEs and lysosomes are lysosomal-associated membrane protein (LAMP) 1 (LAMP-1) and LAMP-2. These proteins are responsible for maintaining lysosomal integrity by forming a layer around the acidic vesicle that consequently limits the risk of unwanted leakage throughout the cell. Cargo that ends up in the LE can however also be routed toward other destinations than the lysosome, and can for example become recycled back to the PM. In addition, the LE can also merge with an autophagosome in order to acquire degradative capacity(119).

The microtubular network

The cytoskeleton is mainly composed of actin filaments, intermediate filaments and microtubules. Both actin and intermediate filaments have been mentioned in previous chapters of this thesis due to their involvement in adhesion, protrusion and migration. The third part of the cytoskeleton, the microtubules, also contributes to protrusion and migration but has a slight different cellular location as well as method or manner of working. The microtubules provide cell polarity through its mechanical properties, its signaling properties and also via its role in intracellular traffic. Microtubule-based endocytic transport facilitates vesicle fusion and fission as well as sorting and delivery to various intracellular compartments(120). Target of adhesion complexes (focal adhesions in particular)

by microtubules is also an important step in adhesion turnover and cell migration since they deliver both MMPs that promote ECM-cell destabilization, as well as the molecular machinery that promotes integrins endocytosis(121).

Microtubule dynamics is largely regulated at fast growing plus ends that undergoes subsequent growth and shrinkage. The slow growing minus ends are generally located close toward the microtubule-organizing center (MTOC) that organizes the microtubular network. The microtubules expand from the uropod throughout the lamella but only to some extent in the actin-dense lamellipodium. However, even though present in a limited number, the presence of microtubules at the leading edge in migrating cells is crucial for the delivery of post-Golgi and recycling vesicles that includes membrane-associated signaling molecules as well as proteins that is crucial for actin polymerization and adhesion. The microtubule-network is in similar to the actin cytoskeleton regulated by the small GTPases Rac-1, CDC-42 and RhoA(120, 122). Likewise, the microtubular network also regulates the activity of Rac-1, CDC-42 and RhoA(120).

Motor proteins

Molecular motor proteins transport vesicles, proteins and mRNA from or to the cell periphery along the microtubule tracks(123). Since they are adenosine triphosphatases (ATPases), motor proteins utilize ATP hydrolysis to drive conformational changes within the protein that subsequently generates motile force(123). The motor protein dynein generally moves toward the more proximal minus-ends of microtubules and the motor protein kinesin generally moves toward the more distal plus-ends of microtubules. Consequently, traffic destined for central or apical compartments move with help of dyneins and traffic destined for the cell surface move with help of kinesins. Organelles such as Golgi, endoplasmic reticulum (ER) and mitochondria as well as endosomes move bidirectionally and accordingly use both dyneins and kinesins depending on destination, inward or outward. How a vesicle or an organelle decide to move one way instead of the other is currently unknown but the motor proteins appear to have receptors that link them to their cargo. The receptors in addition of finding specific cargo, can potentially also provide the mechanism by which competing motor proteins coordinates their activities(124). One such family of receptors that regulate both kinesin and dynein motor complexes and attach them to membranous cargo is the Rab family of small GTPases(123).

Rab proteins

Vesicles do not move randomly within the cell and the Rab proteins are master regulators of all stages of intracellular traffic along the microtubule and/or actin

cytoskeleton. Rab proteins belong to the same Ras GTPase superfamily as Rac-1, CDC-42 and RhoA. The localization of each Rab is unique. One Rab protein generally specifies (and regulates) a specific transport step along the exocytic, endocytic or recycling pathway(125). So far more than 70 different Rab proteins have been identified in human cells and the large number of Rab proteins and their multiple effectors reflect the complexity of the intracellular transport system(126).

The Rab GTPase cycle

When guanosine diphosphate (GDP)-bound and consequently inactive, Rab proteins are usually distributed in the cytosol where they form a complex with a GDP-dissociation inhibitor (GDI). Although not fully understood, it appears that a membrane-bound GDI-displacement factor (GDF) recognizes a specific Rab-GDP–GDI complex and promotes GDI release that subsequently facilitates Rab insertion into the membrane. Once in a membrane, a GEF facilitates the exchange of GDP for guanosine triphosphate (GTP). When GTP-bound and consequently active, the Rab protein recruits downstream effector proteins to facilitate selection of cargo into vesicles, vesicle movement on actin/microtubules through motor proteins and finally tethering of vesicle cargo to the target compartment(127). Rab proteins have also been implicated in vesicle fusion together with the SNARE complex(126). Rab proteins have an intrinsic GTPase activity but it is weak. The exchange back to GDP (hydrolyses of the terminal phosphate of the GTP molecule) is consequently stimulated by a GTPase-activating protein (GAP). Once again in an inactivate state, the GDP-bound Rab protein uncouples from its downstream effectors and becomes removed from the membrane and transported back to its donor compartment or to the cytosol by a GDI(127). The main pool of each Rab protein accumulates at its donor (or target) compartment and only a minor pool of each Rab protein localizes with GDI in the cytosol(126).

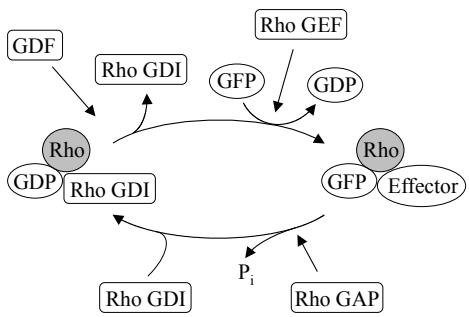

Figure 6. The Rho GTPase cycle.

Forming a Rab cascade

An upstream Rab protein can generally recruit a GEF for a downstream Rab protein and a downstream Rab protein can generally recruit a GAP for an upstream Rab protein. The combinational recruitment of a GEF and a GAP by upstream and downstream Rab proteins makes the transition from an early to a later endosomal compartment both effective and smooth(125). However, what mechanism that ensures properly programmed transition between Rab proteins and their specific effector cascade is a million-dollar question for the future.

Where to go and what to bind?

Targeting Rab proteins to the cytoplasmic face of exocytic and endocytic organelles and also to the membranes vesicles that couple these compartments requires posttranslational isoprenylation. The C-terminus of Rab proteins contains a conserved motif in which two cysteines are substrates for prenylation. This modification is crucial for Rab function since it adds a hydrophobic lipid anchor to the Rab protein that is essential for its membrane association(126).

Also another hypervariable region at the C-terminus of Rab proteins appears to be of great importance. This region of approximately 35-40 amino acids plays a role in Rab target to a specific membrane. There are also other Rab domains that are believed to direct the Rab protein to its proper localization but the overall target mechanism is incompletely understood(128).

Rab proteins are generally strikingly similar in their overall structure but their effectors are not. The specific binding between one Rab and its effector occurs when the Rab protein adopts its active GTP-bound state. Although there is very little change in the structure elsewhere, there is one important Rab region that undergoes major structural changes upon GTP-binding. This region shows great structural heterogeneity between Rab proteins and explains why different Rab proteins can recruit specific effectors to regulate their precise transport step along the exocytic, endocytic or recycling pathways(128).

Integrin traffic

The degradative turnover of integrins is slow and once internalized, most integrins are recycled back to the cell surface(121). Several studies have shown that integrins cycle from the rear to the front of the cell and this was previously assumed to be the common dogma for integrin recycling(17). However, since integrin endocytosis is found in the front of the cell and also since the fact that both clathrin-dependent endocytosis and a subsequent large pool of sorting endosomes are located near the front of a migrating cell, the idea about this long-range integrin movement has changed. Consequently, it is now clear that integrins

can follow a more locally displayed recycling route with both endocytosis and recycling near the lamellipodium. Integrins can also be endocytosed from either the cell front or the nuclear periphery and be recycled backwards to the cell rear(129). The long back-to-front transport is still believed to take place to some extent, at least in quickly migrating cells(130, 131).

Internalization

Integrin endocytosis can be mediated through more than one internalization route and has been shown to include clathrin-dependent endocytosis, caveolae-dependent endocytosis, CLIC-dependent endocytosis, lipid raft-mediated endocytosis and also macropinocytosis(121). A specific integrin can either follow one sole route or multiple internalization routes. For example, αvβ6 seems to use only a clathrin-dependent mechanism whereas α5β1 and αvβ3 can follow both clathrin- and caveolae-dependent endocytosis(129). This is also true for other β1 and β3 integrins. The choice of internalization route can depend on adhesion type, its location and on cell type(121). A parameter that also could affect which internalization route to use is the stiffness of the substrate to which the cells are attached. For example, β1 integrins on cells plated on a more rigid substrate were found to prefer caveolae-dependent endocytosis to clathrin-dependent endocytosis(132).

Endocytosis of β2 integrins are less studied compared to endocytosis of β1 and β3 integrins. Consequently, the way/s in which LFA-1 is recruited from the cell surface and into the cell are not fully defined but it has been shown to use a lipid raft- and cholesterol-dependent endocytic route in neutrophils(133). Endocytosis of LFA-1 in T lymphocytes is also dependent on Rap-2 and Gαq/11-mediated GPCR-signaling(134, 135). Endocytosis of β1 integrins in neutrophils is additionally dependent on JAM-A and a similar JAM-A-dependent mechanism is believed to take place during endocytosis of β2 integrins in the same leukocyte type(136). Also caveolin proteins, the major structural component of caveolae, have been linked to integrin signaling in multiple cell lineages(137). Its presence in T lymphocytes was identified only recently and caveolin-1-deficient murine CD8 T lymphocytes have interestingly been shown to have defective LFA-1-mediated adhesion under shear flow as well as defective LFA-recruitment during synapse formation(138, 139). These findings on caveolin-1-mediated membrane organization of LFA-1 could potentially indicate, that caveolin-1 control LFA-1 endocytosis and maybe also further LFA-1 recycling in T lymphocytes.

Endosomal trafficking – the first port of call

Like many other cargoes, endocytosed integrins can enter the EEs in mechanism regulated by Rab5 and Rab21 along with their multiple effectors (and also other regulators)(121). This is also true for β2 integrins and LFA-1 has been shown to

be associated with both Rab5 and the Rab5 effector EEA-1 in migrating T lymphocytes as well as with Rab5 in migrating neutrophils(133, 135, 140).

Onward trafficking depends on sorting decisions at the EE. It is generally taught that the majority of endocytosed integrins are routed to the recycling pathways rather than sent to the lysosomes for degradation. This is especially true for leukocytes. To migrate at speeds up to 40 μm/min and avoid static adhesion, their integrins must be rapidly recycled back to the surface(121). Various regulators have been found to be crucial for sorting decisions for β1, β3 and β5 integrins at the EE, but the overall mechanism through which specific integrin heterodimers are trafficked is incompletely understood. This is also true for β2 integrin trafficking and consequently the main question and the backbone of my thesis.

Endosomal trafficking – moving forward

Rab proteins and their effectors are key regulators during integrin trafficking. In regard of αvβ3 trafficking in fibroblasts that are stimulated with platelet-derived growth factor (PDGF), Rabaptin-5 binds and activates Rabex-5 that further promotes Rab5 activation within the EE. Phosphorylated Rabaptin-5 by PKD, that is activated through the PGDF-stimulated PLC-γ/IP3/DAG/PKC signaling cascade, also link Rab5 to Rab4 and subsequently promote fast recycling of αvβ3. Phosphorylated Rabaptin-5 only interacts with Rab4 but not with Rab5, and consequently αvβ3 is recycled to the leading edge where it drives persistent cell motility. Phosphorylation of Rab4 by PKD also drives recycling of α2β1 and α3β1, but not α5β1, although the specific PKD family member may vary. Reviewed in Caswell 2015(121).

Rab11 mainly drives the long recycling loop but Rab8, Rab22a and Arf66 can also drive integrin recycling through the long recycling loop. One of the challenges behind Rab11-directed traffic is the fact that Rab11 direct traffic both within the endocytic and exocytic pathways. Consequently it can be hard to distinguish internalized and recycling integrins from newly synthesized integrins on their way to the PM. However, despite the difficulties, several specific heterodimers of β1, β2, β3 and β4 integrins have been identified to use this pathway. LFA-1 has also been shown to use Rab11-medaited recycling in neutrophils(133) and we have recently shown a similar mechanism for LFA-1 in T lymphocytes (Samuelsson M *et al*. 2017, in press Science Signaling).

Some of the machinery components of recycling seem to be common between integrins, but also unique key regulators of each specific integrin have been found(121). For example, active Rab13 associates with Mst-1 and facilitates the delivery of LFA-1 to the leading edge of migrating lymphocytes in a mechanism that potentially also include RAPL(90, 141).

Integrins can also face the lysosomal route. Rab25 has been shown to localize at LEs and direct α5β1 to lysosomes. However, Rab25-directed and lysosomally routed α5β1 can still become rescued via a recycling pathway that requires CLIC-3(142). Integrins routed to the lysosomes for degradation but do not become rescued are probably influenced by ubiquitinylation similar to most degrading intracellular biomolecules(61).

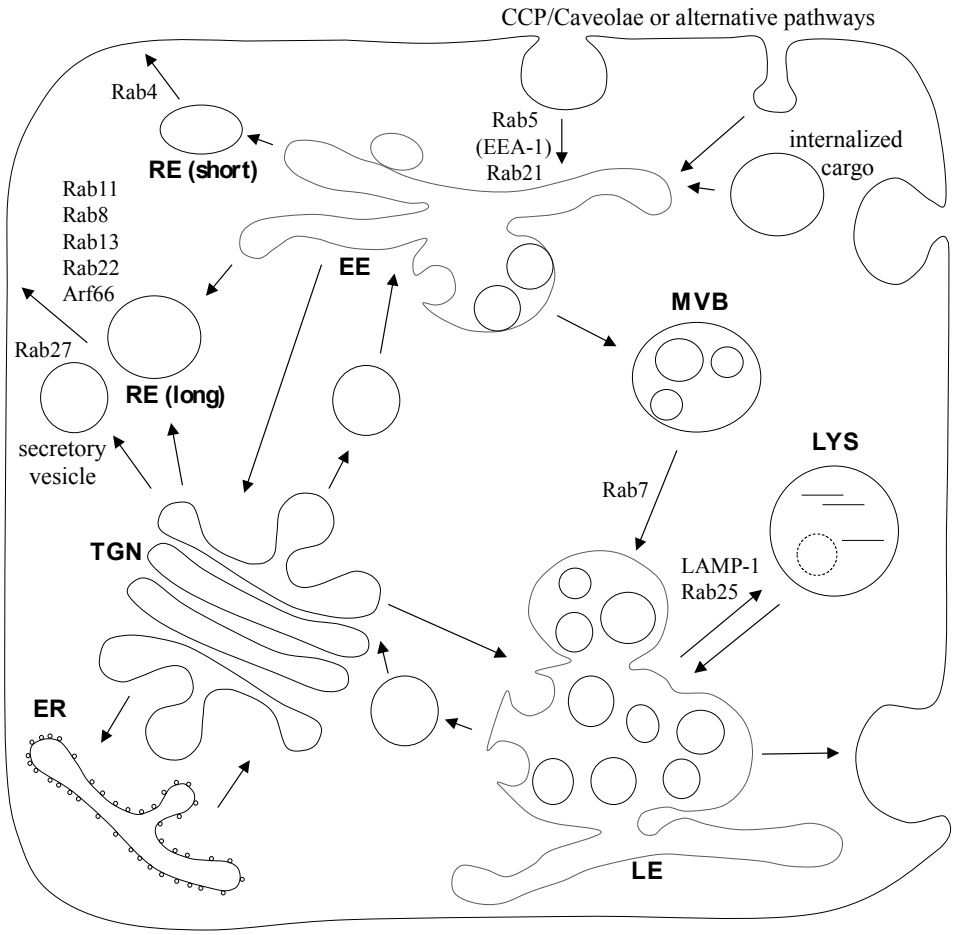

Figure 7. Intracellular transport, modified from originals made by Scott *et al.* and Hutagalung *et al.(119, 128)*.

Integrin activation status

The activation status of internalized integrins (as well as trafficking integrins) is somewhat controversial but a consensus that both inactive and inactive integrins can be internalized is emerging. For example, active α5β1 in epithelial cells are internalized in the front of the cell and also, while still in an active conformation,

recycled back to the cell surface(142). This is also true for active α5β1 in endothelial cells that spread on fibrinogen(143) and for LFA-1 (the intermediate-affinity conformation) in T lymphocytes that migrate on ICAM-1(134).

It has also been suggested that the integrin activity state influences what recycling route to use. For example, active β1 integrins are recycled through the long Rab11-dependent recycling route whereas inactive β1 integrins in the same epithelial cancer cell type are recycled through the short Rab4-dependent recycling route. Both conformations are endocytosed in a clathrin- and dynamin-dependent manner(144).

Endocytosed active β1 integrins are likely to be ligand-bound. A possible scenario for β1 integrins during focal adhesion disassembly is that ECM proteolysis is incomplete and active β1 integrins are consequently still bound to fibronectin fragments during endocytosis. The endocytosed β1/fibronectin complexes are likely routed toward the LE/lysosomal pathway where the proteins become disconnected from one another as the endosomal milieu becomes more acidic. The faith of the fibronectin fragments is likely lysosomal degradation whereas the faith of the β1 integrins is more likely recycling towards newly formed adhesion sites(121). In line whit this, inactive integrins appears to recycle faster than active integrins possibly owing to the requirement of ligand disassociation from the active integrin prior to recycling(145).

Not just endosomal passengers

The balance between the activities of different GTPases such as Rac-1, CDC-42 and RhoA determines the way in which a cell migrates and Rho GTPase signaling is closely linked to endosomal transport and integrin expression. For example, Rho GTPases control an antagonistic relationship between recycling of α5β1 and αvβ3. High levels of active Rac1 supports Rab4-mediated recycling of αvβ3 and directed migration in epithelial cells whereas high levels of active RhoA supports Rab11-medated recycling of α5β1 and random migration in epithelial cells. In addition, integrins are not just endosomal passengers and integrin traffic along with its signaling can also influence the balance of GTPase activity. For example, high expression level of α5β1 during directed migration in epithelial cells supports activation of RhoA in order to drive and favor random migration(121, 129).

Trafficking integrins can also direct or suppress traffic of various cargo and receptors. Going back to the antagonistic relationship between recycling of α5β1 and αvβ3, it has also been shown that inhibition of αvβ3 not only leads to increased recycling of α5β1 but also to increased recycling of epidermal growth factor receptor (EGFR). α5β1 and EGFR travel in a complex through the endosomal pathway and will consequently influence one another's transport(146). αvβ3 can additionally suppress Rab4-mediated recycling of vascular endothelial

growth factor (VEGF) receptor 2 (VEGFR-2) and consequently inhibit VEGF-signaling that promotes endothelial cell proliferation and migration (vascularization)(129). This also raises the question whether integrins are able to signal when they are transported through the endosomal system. Such a condition would then differ from the classical view of endocytosis as a means to terminate receptor signaling. The presence of active integrins in intracellular compartments does suggest a possibility for the integrins to transmit signals, however in similar to many other questions within the field of intracellular transport, a complete answer lies ahead.

The small GTPase RhoB

The Rho GTPase subfamily members Rac-1, CDC-42 and RhoA indirectly regulate endocytosis and vesicle traffic by controlling cytoskeletal reorganization as described in previous chapters of this thesis. The focus in the following section will be on Rho member B (RhoB), yet another member of Rho GTPase subfamily.

Mammals have three closely related Rho proteins, RhoA, RhoB and Rho member C (RhoC), which are highly homologous. Although their effector domains are identical, they differ in their hypervariable domains near the C-terminus. These alterations result in differences in subcellular localization and ultimately also function by distinct effector interactions(147, 148). RhoB also has additional features that make it distinct among the Rho proteins. Unlike most small GTPases, which are relatively stable, RhoB has a rapid turnover with a half-life of approximately 2 hours(149). RhoB expression in cultured cells can also rapidly become upregulated by various growth and stress stimuli such as ultraviolet (UV) irradiation, cytokines or growth factors(148). RhoB levels also vary through the cell cycle, which indicates that RhoB's function requires its expression to be highly regulated(150). Along with its close relatives and most Rho GTPases, RhoB is regulated through its GDP/GTP-bound status that is regulated by GEFs and GAPs(151).

Prenylation

In similar to Rab proteins, targeting of RhoB to membrane vesicles requires posttranslational prenylation at the C-terminus, which adds a hydrophobic lipid anchor to the protein(126, 152). RhoB exists as two different prenylated forms in cells, geranylgeranylated RhoB and farnesylated RhoB. These modifications make RhoB unique, and distinguish it from the highly homologous RhoA and RhoC isoforms, which are solely geranylgeranylated(152). The cellular location of the prenylated forms or RhoB differs in location at least in epithelial cells(153, 154). Farnesylated RhoB is localized in the PM and geranylgeranylated RhoB is

localized to various endocytic compartments(154). RhoB can also become posttranslationally modified through palmitoylation. This modification prevents GDI from binding to membrane-bound RhoB and consequently makes it insensitive to GDI-mediated removal into the cytoplasm(155).

Cytoskeletal regulation

RhoB was the first member of the Rho family that was found to localize to intracellular membrane vesicles. RhoB localizes to the cytoplasmic face of endosomal membranes from where it regulates endosomal traffic(156). For example, RhoB is involved in the transition of endosomal vesicles from the peripheral actin cytoskeleton to the more proximal microtubular network in epithelial cells. Activated RhoB recruits the Rho effector Dia-1 to the endosomes. By interacting with fast growing ends of the actin filaments and catalyzing actin polymerization, Dia-1 mediates the formation of an actin coat around endosomes downstream of RhoB(157).

Besides participating in the transition of endosomal vesicles from actin cables to microtubules, RhoB also provides a transport mechanism on the microtubules and may consequently have a role as a microtubule adaptor protein. RhoB directly interacts with light chain 2 (LC-2) of the microtubule-associated protein 1 A (MAP-1A) and regulates traffic of EGFR in epithelial cells(158). MAP-1A/LC-2 functions as an adaptor between microtubules and other molecules and the MAP-1A/LC-2/RhoB complex may regulate traffic of EGFR (and also other receptors) between various endosomal compartments(154, 158, 159).

RhoB has also an additional regulatory role in epithelial cells by controlling endothelial barrier function, mainly by regulating ROCKs, which drive actomyosin-mediated contractile force generation and modulate cell–cell junctions. RhoB is upregulated in response to inflammatory cytokines. Increased RhoB signaling during inflammation promotes endothelial contraction that leads to the disruption of vascular integrity. RhoB also negatively regulates Rac-1 activity and Rac-1-medaited endosomal traffic back to the PM during inflammation and barrier recovery. Active RhoB accumulates with Rac-1 in Rab7-positive LEs and delay Rac-1 recycling back to the PM. Intracellular Rac-1 accumulation and consequently reduced Rac-1 presence at the cell border impairs the formation of PM extensions and maturation of cell–cell junctions(160).

Endosomal kinase traffic

RhoB regulates endosomal traffic of various kinases. For example, it regulates cell survival by promoting stock “Ak”-transforming (Akt; also known as protein kinase B; PKB) traffic to the nucleus in vascular endothelial cells(161). RhoB is also associated with the multi-signaling kinase Src in perinuclear recycling compartments and regulates both its activation and its traffic back to the PM in

fibroblasts. Peripheral membrane target and activation of Src by RhoB is also dependent on Rab11(162).

The importance of RhoB in endosomal traffic has also been extended to include the traffic of various receptor tyrosine kinases (RTKs). As for most RTKs, one of the earliest signaling responses of both the EGFR and the PDGF receptor (PDGFR) are to stimulate their own internalization. Internalized receptors can recycle back to the cell surface or become sorted to the lysosome for degradation. RhoB regulates EGFR traffic from the EE to the LE/lysosome and associates with LAMP-1 in LE/lysosomes in endothelial cells(154, 159). RhoB also regulates PDGFR traffic from the cytoplasm to the nucleus in vascular smooth muscle cells(163). PDGF is an essential survival factor for vascular smooth muscle cells. Interestingly the important positive function for RhoB in these cells as well as in vascular endothelial cells where it supports cell survival trough it role in Akt traffic, is contradicting to it suppressive function of growth and survival in epithelial cells and in transformed cells(148, 154, 159, 161, 163-165). Still, tumor growth and metastasis depend on angiogenesis and RhoB-stimulated growth of the vasculature's both cell types, vasculature smooth muscle cells and endothelial cells, argue for a positive role of RhoB during malignant transformation(161, 163).

RhoB can also regulate GPCR signaling and plays for example a key role in the recycling/degradation sorting decision of cysteine (Cys, C)-any amino acid (X)-C chemokine receptor type 2 (CXCR-2) traffic in embryonic kidney epithelial cells(166).

Endosomal integrin traffic

The role of RhoB in intracellular integrin trafficking has not been thoroughly investigated. It has been shown that RhoB-deficient murine macrophages have an impaired attachment to ICAM-1 (LFA-1-mediated). These cells have normal β1 integrin expression, but express lower levels of β2 and β3 integrins on their surface. RhoB-deficient murine macrophages also have an increased migratory speed on fibronectin but no difference in attachment on fibronectin compared to control(167). Attachment and migration on fibronectin would be mediated through VLA-4 since this is the only fibronectin-binding integrin on macrophages(72). In addition, a human RhoB-deficient prostate cancer cell line also show a decrease in both total level of β1 integrins and β1 integrin activity at the PM, which correlates with an increase in cell motility(168).

Negative regulation of LFA-1

The adhesive activity of leukocyte integrins must be tightly regulated, ensuring that adhesive interactions with ligands occur only after leukocyte activation. The ability of the leukocyte to attach is regulated by its affinity conformations as well as through recycling and avidity modulation that modifies integrin diffusion and clustering in the membrane through cytoskeletal interactions. So far in this thesis all of the above regulatory modulations have been discussed except for affinity deactivation. The fine-tuned balance between activation and deactivation is crucial for appropriate integrin function. Most previous studies have focused on integrin activation and integrin deactivation is consequently a relatively unexplored field of research. However, the list of integrin-inactivating proteins is growing and our work has added yet another protein to the list. In the following chapter I will briefly discuss integrin inactivation and what is currently known.

Win or lose - competitive binding at the integrin tails

Until recently it was commonly thought that the inactive and bent integrin conformation was a passively adopted default state that shifted towards integrin activation upon stimulation(169). However, through the discovery of integrin-inactivating proteins (integrin inhibitors) such as sharpin, filamin and integrin cytoplasmic domain-associated protein 1 (ICAP-1) the view turned and it is now evident that both active and inactive integrins are actively regulated(170-174).

Integrin inhibitors include those that directly bind to integrins and interfere with recruitment of activators such as talin and kindlin (competitive binding) and those that function through more indirect mechanisms by interfering with integrin signaling intermediates or by regulating integrin surface levels(170). Filamin is an integrin inhibitor that regulates β1 and β7 integrins through both mechanisms. It directly inhibits integrins by competing with talin for the same binding site on the cytoplasmic β integrin tail(104). It indirectly inhibits integrins by recruiting GAPs, which locally inhibit Rac-1 activation and consequently cell spreading(104). Filamin also inhibits calpain-mediated proteolytic cleavage of talin and additionally suppresses metalloproteinase activity that result in inhibited cell adhesion and ECM degradation, respectively(175, 176).

Similar to filamin, docking protein 1 (DOK-1) also inhibits integrins by competing with talin for the same binding site on the cytoplasmic β3 integrin tail(177, 178). Also ICAP-1 competes, but instead of competing with talin it competes with kindlin on the cytoplasmic β1 integrin tail but not on cytoplasmic β3 and β5 integrin tails(174, 179). Sharpin is yet another integrin inhibitor but in contrast to

filamin, DOK-1 and ICAP-1, sharpin binds the cytoplasmic α integrin tail of β1 integrins. Albeit not confirmed, sharpin is believed to have a similar regulatory role in most integrins since it binds to a highly conserved motif on the α integrin tail(173). Even if the mechanisms behind these competitive integrin inhibitors are not fully understood, both sharpin and ICAP-1 have been suggested to maintain integrins uncoupled from the actin cytoskeleton and consequently sustain integrin movement in the plasma membrane(170).

The impact of the above described integrin inhibitors on β2 integrins have not been extensively studied and whether the same integrin inhibitors compete with integrin activation in leukocytes remain to be addressed. It has been shown that Rho member H (RhoH), jet another member of the Rho GTPase family negatively regulates LFA-1 activation and consequently maintain lymphocytes in their resting and non-adhesive state(180). In addition, also SH3 and multiple ankyrin repeat domains (SHANK)-associated RH domain-interacting protein (SHARPIN) has been found to directly interact and deactivate LFA-1 and control uropod detachment in lymphocytes migrating on ICAM-1(181). On the basis of existing knowledge it also appears that leukocyte β2 integrins can be kept in their inactive conformation by yet another mechanism. It has been shown that a salt-bridge between the cytoplasmic part of the α and β2 integrin tails forms a clasp that is crucial for keeping the integrin in its inactive state(169, 182).

The power of phosphorylation

Reversible phosphorylation is a key and maybe also the most common mechanism to regulate cellular activities. Compared to slower regulatory changes at the transcription and translation level, protein phosphorylation is a rapid event that directly alters enzymatic activity or binding affinity. There are many examples of how tyrosine (Tyr, Y), serine (Ser, S) and threonine (Thr, T) phosphorylation modify integrin interactions with adaptor and cytoskeletal proteins and consequently regulate integrin activation(170, 183). For example, phosphorylation of Ser745, Ser756 and Thr758 on the cytoplasmic β2 integrin tail as well as phosphorylation of Ser1140 and Ser1126 on the cytoplasmic αL and αM integrin tail, respectively, acts as molecular switches that promote integrin β2 activation(184-188). In the following section I will particularly focus on Tyr de/phosphorylation and especially those modifications that negatively regulate integrin signaling.

Reversible Tyr phosphorylation

Protein tyrosine receptors (PTKs) and protein tyrosine phosphatases (PTPs) are enzymes that catalyze the reversible addition or release of phosphate groups from

Tyr residues on signaling intermediates. With the possible exception of neurons, immune cells express more PTKs and PTPs than any other cell type. Previous studies have in general focused on PTKs and it is currently far from clear what substrates the majority of all PTPs that are expressed in immune cells have. Until recently it was also assumed that PTPs are secondary to PTKs and accordingly only sooner or later reverse the action of the PTKs. However, it is now established that PTPs possess active regulatory roles that is of similar importance to those of the PTKs(189).

There are several known PTPs that are restricted to lymphocytes, and many of these are found at or mainly near the PM where they participate in transmembrane signaling(189). T lymphocytes express as many as 60-70 different PTPs and the majority of these PTPs with known functions, regulate lymphocyte activation through TCR signaling and subsequently IL-2 production(189, 190). In contrast to CD45 and Src homology region 2 domain-containing phosphatase-2 (SHP-2) that mainly promote lymphocyte activation, most other PTPs in lymphocytes inhibit lymphocyte activation(189).

Tyr phosphorylation during deactivation of integrin signaling

Both PTKs and PTPs can promote integrin deactivation directly by acting on the integrins themselves or indirectly by acting on integrin activators and inhibitors. For example, direct inhibition includes Tyr phosphorylation within the two highly conserved asparagine (Asn, N)-X-X-Y β integrin motifs on the cytoplasmic integrin tail that consequently inhibit binding of talin and kindlin but promote binding of DOK-1(177, 191). Indirect inhibition includes phosphorylation of filamin and consequently promotes filamin interaction with the cytoplasmic β integrin tail that subsequently supports integrin deactivation(192).

Tyr dephosphorylation during deactivation of integrin signaling

The role of PTPs in integrin deactivation is fairly unexplored. The tyrosine-protein phosphatase non-receptor (PTPN) type 12 (PTPN12) has been shown to directly associate with the integrin adaptor protein paxillin in fibroblasts. Here, PTPN12 regulates migration by reciprocally modulating the activity of Rac-1 and RhoA through their upstream regulators Vav-2 and p190RhoGAP(193-195). Based on this very short section I would like to claim that our research and my thesis are needed.

The phosphatase PTPN22

PTPN22 expression, classification and structural aspects

PTPN22 belongs to the same PTP subfamily as PTPN12. It is exclusively expressed in hematopoietic cells where natural killer (NK) cell and neutrophils express the highest rates. CD8 T lymphocytes also express high PTPN22 levels whereas CD4 T lymphocytes and monocytes express lower PTPN22 levels(196). In addition, PTPN22 expression becomes upregulated following activation of naïve CD4 and CD8 T lymphocytes(197).

PTPN22 displays three major domains: the N-terminal PTP domain, the interdomain and the C-terminal domain that is rich in proline (Pro, P), glutamic acid (Glu, E), Ser (S) and Thr (T). This C-terminal amino acid sequence prompted this PTP subfamily its name PEST, but in contrast to other PEST-sequences on other proteins, this sequence on the PTP PEST subfamily does not act as a signal peptide for protein degradation. In fact it does not influence PTPN22 stability at all(198).

The ability of PTPN22 to dephosphorylate Tyr residues is critically dependent on the N-terminal catalytic domain since substitutions at Cys227 and Asp195 inactivate the enzyme(199). The C-terminal domain of PTPN22 includes four highly conserved Pro-rich regions called P1-P4(197). The P1 region is crucial for PTPN22's interaction with C-terminal Src kinase (Csk)(200). PTPN22 also exists in various isoforms but the functional relevance of these isoforms at the protein level remains incomplete(201).

PTPN22 substrates within TCR signaling

Initial TCR engagement with cognate antigen leads to mobilization and association of Lck with TCR co-receptors CD4 or CD8, as well as activation of Lck through autophosphorylation of Tyr394 in its activation loop(202). Phosphorylated and activated pLckY394 phosphorylates ITAMs on TCR-CD3ζ that subsequently promotes Zap-70 binding(203). Ones bound to TCR-CD3ζ, Zap-70 becomes released from its auto-inhibited state and exposes activatory Tyr residues. Both pLckY394 and Zap-70 itself phosphorylate Zap-70 at the newly exposed Tyr activatory residues, and in particularly Tyr493(204, 205). Next, phosphorylated and activated pZap-70Y493 subsequently phosphorylates another molecule in the signaling cascade called linker of activated T cells (LAT)(206). LAT serves as a docking site for a number of other proteins of which many also can become phosphorylated and activated by pLckY394 and pZap-70Y493(202, 207). LAT-assembling multi-signaling molecules includes growth factor receptor-bound protein 2 (Grb-2), SLP-76, Vav-1 and PLCγ-1, and the coordination between these signaling components regulates multiple downstream cellular responses(207, 208). Their signaling pathways culminates in intracellular Ca^{2+}

mobilization, cytoskeleton reorganization and activation of nuclear transcription factors within the T lymphocyte that subsequently promote its activation, proliferation and commitment into a fully functional and differentiated T lymphocyte. The T lymphocyte is now ready to do some action(202).

Since PTPN22 has been shown to dephosphorylate not only pLckY394 and pZap-70Y493 but also TCR-CD3ζ (at least *in vitro),* PTPN22 acts a negatively regulator of TCR signaling(199). PTPN22 deficiency or inhibition results in increased phosphorylation levels of pLckY394, pZap-70Y319 and pZap-70Y493 in both murine and human T lymphocytes and also in Jurkat T lymphocytes(209-211). PTPN22 also interacts with Grb-2 in Jurkat T lymphocytes and in human embryonic kidney (HEK) 293T cells when both proteins are overexpressed(212). In addition, PTPN22 immunoprecipitates from CD3-stimulated thymocytes and substrate trapping experiments in Jurkat T lymphocytes also show that PTPN22 interacts with the ubiquitin ligase casitas B-lineage lymphoma B (Cbl-b) that negatively regulates TCR signaling by accelerating TCR internalization and degradation. (189, 197, 199). Cbl-b also promotes dephosphorylation and subsequent inactivation of Zap-70, possible in concert with PTPN22.

Effector and memory T lymphocytes from mice deficient in the PTPN22 mouse orthologue proline-enriched phosphatase (Pep) show increased Ca^{2+} levels and enhanced proliferation and expansion after TCR triggering(210). Also regulatory T lymphocytes from Pep-deficient mice show greater proliferation after TCR triggering and also increased capacity to produce and secrete the immunosuppressive cytokine IL-10(209). To summarize, PTPN22 negatively regulates TCR signaling in both human and murine T lymphocyte subsets.

PTPN22 and Csk – coworkers or not?

Inactivation of Lck requires both dephosphorylation of activatory LckY394 and phosphorylation of inhibitory LckY505(108). The kinase Csk is found in the cytoplasm or in PM lipid rafts through interactions with protein associated with glycolipid-enriched membrane domains (GEMS; PAG)(213, 214). The interaction with PAG in resting T lymphocytes brings Csk in close proximity to Lck and subsequently enables Csk to keep Lck in its inactive state through phosphorylation of LckY505(213, 215, 216).

Csk has been shown to interact with the C-terminal P1 domain of PTPN22 and together synergistically inhibit Lck signaling. Here, PTPN22 dephosphorylates the activatory LckY394 whereas Csk phosphorylates the inhibitory LckY505(199, 200, 216). However, available studies also challenge this concept of a cooperative TCR inhibition between Csk and PTPN22. For example, active Csk has been shown to promote Lck-mediated phosphorylation of PTPN22Y536 in the interdomain, which subsequently decreases PTPN22 phosphatase activity and

results in enhanced TCR signaling(217). It was also recently shown that PTPN22 downmodulates TCR-induced signaling mainly when dissociated from Csk(211). Here, on the basis of co-immunoprecipitation experiments, Vang *et al*. showed that approximately half of the total cellular PTPN22 pool interacts with Csk in resting human T lymphocytes. After initiation of TCR signaling freed PTPN22 from Csk translocates to the PM and lipid rafts where it downmodulates TCR-signaling independent of Csk(211). A possible Csk-independent regulation of PTPN22 function has also been suggested. In this study, PKC-mediated phosphorylation of Ser35 within the C-terminal catalytic domain of PTPN22 impaired the ability of PTPN22 to inactivate Lck and consequently downregulate TCR signaling (218). In summary, only the future can explain the exact roll of the Csk-PTPN22 complex and its location. In addition, yet unknown PTPN22 regulatory mechanisms also likely exist.

PTPN22 substrates outside TCR signaling

In consistency with its established role as a potent suppressor of TCR signaling, PTPN22 also acts a negative regulator of LFA-1-signaling in migrating T lymphocytes(109). Lck, Zap-70 and also the cytoskeletal regulator Vav-1 all become phosphorylated following LFA-1 engagement in T lymphocytes and PTPN22 deficiency in the same cell type increase phosphorylation of all three signaling intermediates(107, 109, 219, 220). The increased phosphorylation of pLckY394, pZap-70Y493 and pVav-1Y174 results in increased LFA-1 adhesion to its ligand ICAM-1(109). Enhanced LFA-1 activation and subsequent increased LFA-1-mediated adhesive properties has also been shown in regulatory T lymphocytes from Pep-deficient mice(209).

PTPN22 location

The identification of PTPN22 substrates among signaling proteins in membrane-proximal and cytoplasmic regions suggests that PTPN22 may reside in these compartments. Indeed, PTPN22 is found both in the cytoplasm and in or close to the PM through interactions with other proteins(197, 221). Studies addressing the subcellular localization of both endogenous PTPN22 levels as well as overexpressed epitope-tagged PTPN22 in Jurkat T lymphocytes also show that PTPN22 can reside in the nucleus(201, 221). From our work, we conclude that LFA-1 signaling stimulates an association between PTPN22 and its phosphorylated substrates pLckY394, pZap-70Y493 and pVav-1Y174 at the leading edge in migrating T lymphocytes. We also show that PTPN22 associates with LFA-1 in a Lck-dependent manner and the interaction between PTPN22 and LFA-1 increase as a function of LFA-1 signaling(109).

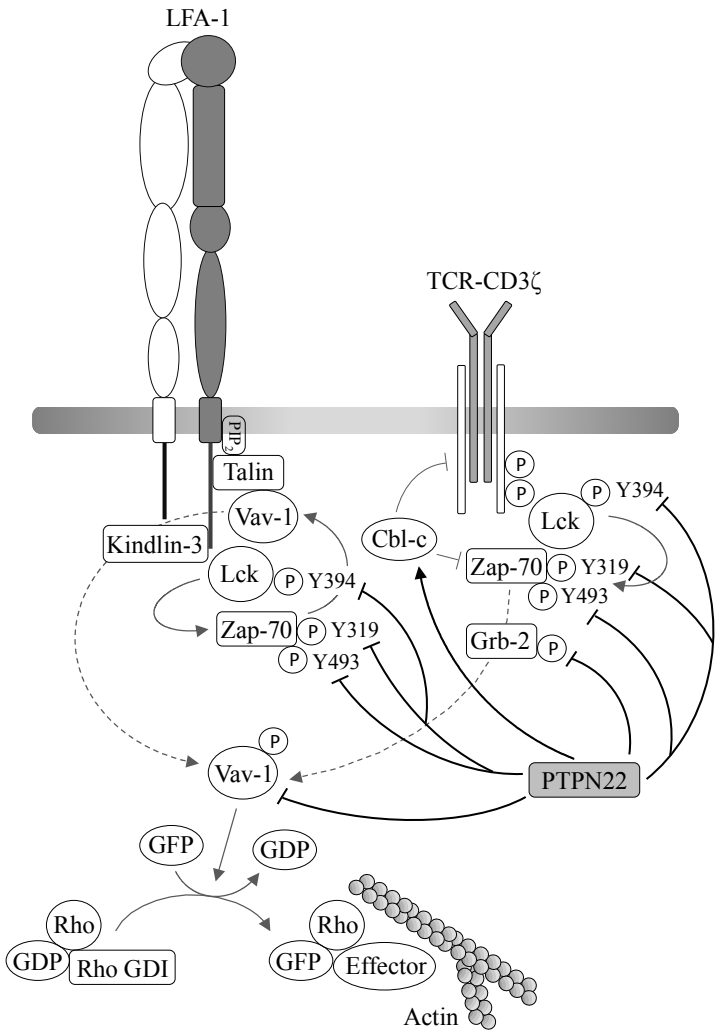

Figure 8. Phosphatase action of PTPN22.

Summary of the PTPN22-R620W story

The interest of PTPN22 in regulating immune cell function grew stronger when numerous studies back in 2004 confirmed a strong link between a variant of PTPN22 and the development of various autoimmune diseases. A missense single-nucleotide polymorphism (SNP) in PTPN22 exon 14 (*PTPN22-C1858T*) that subsequently lead to an Arg-to-Trp substitution within the P1 motif (position 620, R620W), was shown to increase the predisposition of multiple autoimmune diseases including type 1 diabetes (T1D), rheumatic arthritis (RA) and systemic lupus erythematous (SLE)(196, 222, 223). Since these initial findings, multiple

studies have been conducted and it is now evident that this PTPN22 variant is one of the strongest genetic risk factors for autoimmune disease outside of the major histocompatibility complex (MHC)(224).

The prevalence of the SNP varies notably in human populations. Northern Europeans have the strongest prevalence with the Swedish and Finnish populations having some of the highest. Here, the frequency of the variant allele is 12% and 15.5%, respectively(224). In contrast, the prevalence is almost non-existing in African, Middle Eastern, American, Indian, and Asian populations and the cause of these inter-population frequency differences is currently unknown(196, 222). The SNP could confer a protective advantage since studies have indicated that PTPN22W620 carriers may be protected from various infectious diseases including tuberculosis that has been known to mankind since ancient times(225, 226). However, another study indicates that PTPN22W620 carriers are at higher risk for developing certain invasive bacterial diseases(227). Consequently, why evolution has kept PTPN22W620 is unknown.

PTPN22R620 is crucial for the interaction between PTPN22 and Csk(200, 228, 229). Consequently, the exchange of PTPN22R620 with PTPN22W620 makes PTPN22 incapable of binding Csk(222, 230). However, in similar to the Csk-PTPN22 complex the effect of this SNP on PTPN22 function is currently controversial. Some studies claim that PTPN22W620 is a loss-of-function variant associated with increased T lymphocyte signaling whereas other studies suggest the opposite, that PTPN22W620 is a gain-of-function variant associated with decreased T lymphocyte signaling(217, 230-233).

It is clear that all data collected this far on the role of PTPN22W620 during TCR signaling is difficult to separate into a simple gain-of-function or loss-of-function model. When it comes to the role of PTPN22W620 during LFA-1-signaling, our data support a model of a loss-of-function variant. Both murine knockout and human knockdown of PTPN22 as well as overexpression PTPN22W620 resulted in the enhanced phosphorylation of signaling molecules downstream of integrins. Similar to unstimulated T lymphocytes, superresolution imaging also showed that PTPN22W620 remained as large clusters, following LFA-1 stimulation. Consequently, PTPN22W620 showed less association with its binding partners at the leading edge compared to PTPN22R620. Our data also confirm that although Csk is becomes declustered upon LFA-1 stimulation, its association with PTPN22-W620 is reduced in migrating T lymphocytes. The failure of PTPN22W620 to disaggregate upon stimulation of LFA-1 also resulted in increased LFA-1 clustering and integrin-mediated cell adhesion(109).

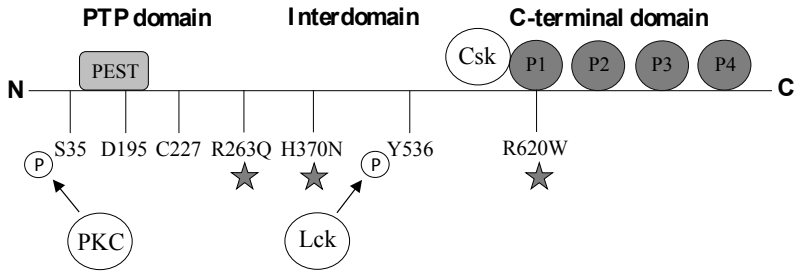

Figure 9. Schematic structure of PTPN22.

The immunological synapse

There are two distinct T lymphocyte functions in which LFA-1 is implicated. The first one is migration through making and breaking of adhesion and this has been the main focus in my theses – until now. In this chapter I will briefly discuss the second main LFA-1-mediated T lymphocyte function that involves making contact with an antigen-presenting cell (APC) and subsequent formation of an immunological synapse.

Leukocyte integrins must be able to rapidly create stable adhesions during for example leukocyte adhesion onto the endothelium in blood vessels that is subjected to flow. However, when it comes to immune surveillance and immunological synapse formation, such rapidly forming and strong adhesions are not needed. In fact, they are not wanted. Nonspecific short-lived contacts between T lymphocytes and APCs that lack cognate antigen recognition should solely remain just short-lived and not promote TCR signaling with following T lymphocyte commitment and activation.

Lymph nodes

The principle function of secondary lymphoid tissues is to serve as a meeting place where they bring APCs and lymphocytes together. Secondary lymphoid organs such as the spleen, lymph nodes (LNs) and Peyer's patches (PPs) are strategically positioned to sample antigens that enter through almost any body surface(234). LNs are small stations of the lymphatic system that are widely spread throughout the body but particularly more concentrated in the neck, the groins, the axillas and in various abdominal areas. Circulating naïve T lymphocytes continually enter LNs from the blood and migrate to the T cell cortex within the LNs where they survey for specific antigens(235). The T cell cortex comprises a network

composed of fibroblastic reticular cells (FRCs), collagen matrix and ECM molecules. The network also contains resident DCs within the T cell cortex, which takes up soluble antigens that enter via the afferent lymphatics before antigen-carrying DCs arrive from the periphery. The DCs are located in prime positions for making contact with newly recruited naïve T lymphocytes that prime for cognate antigens(236).

T lymphocyte migration within the LNs requires FRC-producing chemokines that include C-C motif ligand (CCL) 21 (CCL-21) and to smaller degree also CCL-19 and C-X-C motif ligand (CXCL) 12 (CXCL-12)(234). FRCs also produce the integrin ligands ICAM-1 and VCAM-1 although a surprising conclusion from a recent study showed that leukocytes migrate without using functional integrins once they have gained entry into the LNs(34). Instead, they migrate by the force of actin-network expansion, which promotes protrusive flowing of the leading edge(34). Integrins on T lymphocytes are consequently thought to be silenced and subsequently in there bent and low-affinity stage since this would make the scanning of antigen more effective within the LNs(34, 237). However, even though not essential for leukocytes during migration within the LNs, integrins are crucial for leukocytes during the actual entrance into the LNs(42, 43). In addition, LFA-1-deficient murine T lymphocytes pass through the LN more rapidly which consequently leads to ineffective priming of APCs and decreased likelihood of encounter a cognate antigen(238). Thus, even though the role of LFA-1 during the actual leukocyte trafficking within the LN is somewhat debated, LFA-1 is essential for LN entry and LN exit(42, 43, 238). In addition, and also described below, LFA-1 is needed to mechanically stabilize and prolong TCR signaling once a naïve T lymphocyte has recognized a cognate antigen that is displayed by an APC.

Stick, stop, activate and go!

Once a naïve T lymphocyte has engaged a cognate antigen, it starts to modulate its expression phenotype in order to extend its contact, become activated and remain within the LN. Whereas most CD4 T helper (Th) lymphocytes generally migrate to the boundary of the T cell cortex where they interact with antigen-engaged and activated B lymphocytes, most of the activated CD8 T lymphocytes and some of the CD4 T lymphocytes divide, expand and re-acquire an exit-capable effector phenotype. These changes equip the activated T lymphocytes with tools that allow them to migrate out of the LNs and home to inflamed tissue where they then carry out their effector functions(234).

T lymphocyte education - clearing out the bad guys from the repertoire

APCs present their antigens through major histocompatibility complexes (MHCs) on the cell surface. The antigens are peptides that can originate from both

endogenous self-molecules and exogenously pathogen molecules(239). In the case of T lymphocytes, TCRs are very specialized receptors that are very specific for a precise antigenic peptide.

T lymphocytes are educated in the thymus. The selection processes aim to select a functional and self-tolerant T cell repertoire. As reviewed by Starr *et al*., Anderson *et al*. and Palmer, peripheral and mature T lymphocytes arise from hematopoietic stem cells that migrate into the thymus. While in the thymus they undergo a multistep-selecting process that establishes the particular T lymphocyte repertoire of an individual(240-242). Upon thymic entry, the cells lack expression of both CD4 and CD8 and are consequently called double negative (DN) thymocytes. These cells will start to express TCRs, mature into one of the two T cell lineages (α/β and γ/δ) and develop into CD4 and CD8 double positive (DP) thymocytes(243). Most α/β TCR-expressing DP thymocytes will fail to engage peptide-expressing MHCs (pMHCs) and consequently die in a process called death by neglect(241). A small fraction (less than 5%) of these DP thymocytes succeed to engage pMHCs with moderate affinity, and dependent on what type of pMHC they recognize, they will either mature into a MHC class I-restricted CD8 or MHC class II-restricted CD4 single positive (SP) thymocyte. This selection process is termed positive selection(242). The third challenge that the thymocytes face is negative selection or clonal deletion. This is a process where most thymocytes that express TCRs with too strong affinity or show reactivity for self-peptides will undergo apoptosis because they present a risk of autoimmunity(240-242).

Positive and negative selection is not necessarily sequential events since studies suggest that thymocytes can be subjected to negative selection either before or after positive selection. Thymic selection is additionally still not fully understood and there is still relatively little consensus about what constitutes these pathways and how thymocytes distinguish between self and non-self and produce such outstanding naive T lymphocytes in the periphery(240-242).

Antigen presentation, TCR and the immunological synapse

T lymphocyte activation, proliferation and differentiation that ultimately generate effector T lymphocytes involve multiple cellular and molecular changes. They include cytoskeletal rearrangement and increased integrin affinity as well as coordinated production and mobilization of transcription factors that are crucial for T lymphocyte-activating gene expression. The signaling pathways that direct these events originate from TCR engagement but how precisely the TCR–pMHC ligation results in these changes is poorly understood(239).

The tight junction that is formed at the interface between a T lymphocyte and an APC is called the immunological synapse(244). The mature immunological

synapse is mostly organized into a distinct structure and composed of three distinct zones that are termed supramolecular activation clusters (SMACs)(245). The most central region is termed the central SMAC (cSMAC) and includes TCRs and their proximal signal intermediates. The peripheral SMAC (pSMAC) surrounds the cSMAC and includes LFA-1 and its adaptor protein talin as well as the highly conserved proteins ezrin, radixin, moesin (ERM proteins). The most distally located region is called the distal SMAC (dSMAC) and includes large and bulky molecules such as CD43 and CD45(245-247).

TCRs are engaged in two structures

Real time high-resolution imaging has recently shown that TCRs can signal through small clusters and that the large-scale SMAC rearrangements within the immune synapse are not required to drive T lymphocyte activation. The TCR microcluster forms within seconds after antigen binding, peak prior to cSMAC formation and consequently precedes SMAC maturation(248-250). It is from these TCR microclusters that proximal TCR signaling is initiated. The cSMAC is nowadays more thought to participate in TCR downregulation through TCR internalization and degradation(251). However, another model suggests that the role of cSMAC depends on antigen quality and that TCR signaling does occur in the cSMAC even at later time points. This data supports a model where cSMAC formation can enhance the stimulatory potency of weak agonists besides downregulating TCR signaling. Consequently, when TCRs engage peptides with long half-lives, its signaling can be transmitted without the requirement of cSMAC formation. However, when TCRs engage weaker peptides with shorter half-lives, its signaling is relatively inefficient without signal amplification from concentrating TCRs in the cSMAC(252, 253). As discussed previously, it is also here when TCRs engage weaker peptides that the phosphatase PTPN22 is believed to have a crucial regulatory but yet incompletely understood mechanistic role(254). In addition, the complexity is further increased by a conflicting study where immunological synapse formation between CD4 T lymphocytes and B lymphoma cells was analyzed and large-scale SMAC structures only were observed at high antigen concentration. Low antigen concentration, which might represent a more physiological situation, led to the formation of sole TCR microcluster structures(255). To conclude, immunological synapse formation is interesting but very complex.

Complexity of SMAC and synapse formation

The SMAC pattern was first described in Th lymphocytes but has later also been observed in cytotoxic T lymphocytes (CTLs), regulatory T lymphocytes, innate-like T lymphocytes (ILTs), B lymphocytes and Natural Killer (NK) cells(256-258). Synapse formation between DCs and T lymphocytes appears to be more complex and quite different from the prototypical mature immunological synapse

that is described above. For example, although the traditional SMAC organization has been observed between T lymphocytes and mature DCs, immunological synapses between these cell types seem to favor the existence of multiple cSMAC-like structures(259-261). In addition, naïve T lymphocytes can establish long-lasting contacts with mature DCs even in the absence of antigen(261). Thauland *et al*. further concluded that not only naïve T lymphocytes that are forming immunological synapses with mature DCs fail to form classical SMAC structures. DP thymocytes as well as peripheral Th type 2 (Th-2) lymphocytes can fail to form classical SMAC structures as well. These differentiated Th-2 lymphocytes formed solely TCR microclusters throughout the interface when conjugated with various B lymphocytes(262).

To summarize, well-defined and classic SMACs are a hallmark of immunological synapse formation when NK cells, CTLs and Th type 1 (Th-1) lymphocytes are involved. However, it is not the case when DP thymocytes, naïve T lymphocytes (when forming immunological synapses with mature DCs) and Th-2 lymphocytes are involved. Consequently, it appears to be the fact that well-defined SMAC structures are not required for thymocyte selection, T-cell priming and Th2 function. Why this diversity in immunological synapse structure? Thauland *et al.* suggest that the difference is linked to T lymphocyte function. For example, T lymphocyte delivery of certain effector molecules directly to the APC, such as cytolytic granules delivered by CTLs, requires the formation of a classical SMAC-structure in order to prevent leakage(262). In addition, it takes two to tango and the cell type that is conjugated with the T lymphocyte might also influence the formation of a certain immunological synapse structure.

Remodeling of the cytoskeleton during immunological synapse formation

Early studies of immunological synapse formation between APCs and CTLs or CD4 Th lymphocytes demonstrate that both the MTOC and the microtubules as well the actin cytoskeleton within the T lymphocyte becomes orientated towards the APC(263, 264). These cytoskeletal changes occur almost immediately after antigen recognition and result in a more rounded T lymphocyte morphology as well as a flattening of the T lymphocyte surface that is facing the APC(265).

The initial assembly of TCR microclusters and their subsequent migration towards cSMAC is dependent on dynamic remodeling of the actin cytoskeleton(266). Actin polymerization is accelerated in the peripheral border of the immunological synapse where the concentration of filament-inducing Arp2/3 and cofilin is high(267). Inward extension and depolymerization of the actin cytoskeleton near the cSMAC border, results in a retrograde flow that the TCR microclusters use for transport(268). Rearrangement of the actin cytoskeleton is also thought to govern additional aspects of immunological synapse formation by for example function as

a scaffold for further assembly and stabilization of signaling clusters including those of LFA-1(269).

The immunological synapse and integrins

Full T lymphocyte activation requires a stable contact between the T lymphocyte and the APC for several hours, but initial TCR signaling becomes decreased to undetectable levels after 10–20 minutes after TCR triggering(270). Consequently, signaling from other signaling intermediates in close proximity to the TCR microclusters as well as integrins are needed to form stable and long-lived immunological synapses that result in naïve T lymphocyte commitment and effector differentiation(248, 249). For example, integrins and VLA-4 in particularly, have been shown to favor the retention of mobile and effectively signaling TCR and SLP-76 microclusters in the periphery of the immunological synapse. VLA-4 integrins promote sustained SLP-76 microcluster signaling by attenuating cytoskeletal movements that drive the centralization and inactivation of SLP-76 microclusters by detaching SLP-76 from its upstream kinase ZAP-70(271).

LFA-1 has also been shown be critical for the exclusion of CD45 from the synapse, enhance engaged TCR accumulation within the cSMAC and consequently augment proximal TCR signaling. The large and bulky phosphatase CD45 is present in the center of the contact zone at early time-points but with the help of LFA-1 it gradually becomes cleared and constrained to regions outside the cSMAC during immunological synapse maturation(247, 272).

Moreover, binding of LFA-1 to ICAM-1 is not required for initial cSMAC formation, but it enhances the number and the extent of the interactions between T lymphocytes and APCs. Indeed, in the absence of interactions between LFA-1 and ICAM-1 only the cSMAC region is found between the T lymphocyte and the APC(273). However, there are many studies that confirm that LFA-1 engagement with ICAM-1 does enhance synapse formation and increase the area and density of accumulated complexes, which then increases the overall amplitude and duration of T cell signaling(274, 275). In addition, T lymphocytes have glycoproteins on their surfaces that more or less form a repulsing-cover around the surface(276). The interaction between LFA-1 and ICAM-1 supports the formation of an optimal interaction between the TCR and the pMHC by helping the cells to overcome the repulsive glycoprotein surface. The interaction also generates a starting point for actin polymerization that consequently can push on the membrane, rearrange surface proteins and generate optimal intermembrane space between the T lymphocyte and the APC(277). Presence of LFA-1 also results in an increased intracellular Ca^{2+} response that might reflect on the magnitude of proximal TCR signaling(273).

In summary, sole TCR signaling is not sufficient to form extensive interactions between T lymphocytes and APCs. Although stable contacts are not required for T lymphocyte proliferation and early cytokine release, the results that are described above show that LFA-1 is needed to sustain TCR signaling and organizing proteins within the immunological synapse and thereby regulate both T lymphocyte activation and differentiation into effector cells(272-275, 277, 278).

Cell migration in disease

When most people think about cell migration and disease they think about cancer and metastasis. Indeed the role of integrins in tumor biology is well described. Yet, integrins are not oncogenic themselves but since they regulate a diverse array of cellular functions, a lost regulation can cause much harm and contribute to malignancy. Beside the role of integrins in migration and invasion, integrins can also control proliferation, survival and angiogenesis based on their receptor signaling properties. The conversion from a normal cell to a cancer cell with an invasive phenotype requires the ability of a cancer cell to interact with its environment and transduce signals from the ECM into the cell. Indeed, integrins are appealing targets for the design of specific therapeutics, however, they are also important for the host cellular maintenance and its response to cancer. This is why both life and cancer therapeutics are complicated(279).

Some integrins are more crucial than others. Loss of some integrins (β1 subtypes) results in early embryonic lethality whereas loss of others results in tissue-specific abnormalities that range from milder to major malfunctions(170). Integrin-linked diseases often correlate with alterations in protein expression. For example, deletion of the α6β4 integrin leads to a skin blistering disease termed epidermolysis bullosa and deletion of the α7β1 integrin causes congenital muscular dystrophy (280, 281). Disease can also correlate with alterations in integrin-mediated functions and patients with Glanzmann's thrombasthenia have platelets that fail to aggregate due to dysfunctional defects of αIIbβ3(282). Integrin-mediated cell migration is also a key component in stem cell transplantation strategies and in wound repair. It is also important in chronic inflammation that I will describe after the following section.

Leukocyte adhesion deficiency

The leukocyte adhesion deficiency (LAD) syndromes are rare deficiency disorders that result from defective leukocyte adhesion to activated endothelium and consequently impaired leukocyte transmigration into a tissue subjected to injury or

bacterial infection. The clinical feature common to LAD syndromes is recurrent bacterial infections that are primarily localized to skin and mucosal surfaces. These diseases can be difficult to treat and even life-threatening(283).

LAD type I

LAD type 1 (LAD-I) is an autosomal recessive genetic disorder that is characterized by absent or reduced β2 integrin expression that is caused by a variety of mutations in the gene that encodes the β2 integrin subunit. Lymphoblasts of LAD-I patients synthesize normal levels of the α integrin subunit but in the absence of the β2 integrin subunit, it becomes degraded in the cytoplasm and consequently fails to reach the cell surface. The phenotype ranges from severe (< 2% β2 integrin expression) to moderate (2-30% β2 integrin expression). Rare occasions where β2 integrin expression is normal but still dysfunctional also occur. Patients with severe or moderate LAD-I mostly troubled by severe periodontitis, tooth loss and impaired wound healing at infected sites. They do survive with help from antibiotic treatment at infectious episodes, but they will always battle infections and even those infections that for most of us appear to be harmless(284).

LAD type II

LAD type II (LAD-II) is characterized by defects in fucosylation of selectin ligands that lead to impaired selectin signaling and consequently impaired leukocyte tethering and rolling during the leukocyte adhesion cascade on to the activated endothelium. A mutated human gene encoding a specific GDP-fucose transporter in the Golgi membrane is the cause behind dysfunctional fucosylation(283). Immunodeficiency also dominates the clinical picture of LAD-II but other severe symptoms also include severe mental retardation and various metabolic defects. The prognosis for LAD-II is poor (284).

LAD type III

LAD type III (LAD-III) patients show similar clinical symptoms as patients with LAD-I and Glanzmann's thrombasthenia. It is characterized by severe recurrent infections and bleeding disorder. Some LAD-III patients also exhibit an osteopetrosis-like bone defect (abnormal bone turnover). LAD-III leukocytes express normal levels of surface integrins and their rolling during the leukocyte adhesion cascade on to an activated endothelium is also intact. However activation of all major leukocyte integrins by chemokine receptor or TCR signaling is severely impaired due to their failure in integrin inside-out signaling. Consequently, LAD-III leukocytes fail to tether and arrest on an activated endothelium expressing integrin ligands. LAD-III platelets also fail to aggregate,

which is necessary for effective hemostasis that leads to the initiation and formation of a thrombus or hemostatic plug(284).

The primary genetic defect behind LAD-III was just recently revealed. Recent work on integrin activation defects in several knockout mice models as well as on leukocytes obtained from LAD-III patients ascribed the disease to a defect in kindlin-3(285-287). Kindlin-3 is exclusively expressed in hematopoietic cells and is crucial for LFA-1 activation by stabilizing the active integrin conformation together with talin(288). Transfection of lymphocytes from LAD-III patients with wild-type (WT) *KINDLIN-3* cDNA restored integrin-mediated adhesion and migration(285). Several different mutations in the gene that encodes kindlin-3 have been found to be the cause of LAD-III and the subsequent abnormal or absent kindlin-3 protein expression. These mutations have also been found in patients with various ethnic origins(285, 286, 289).

A mutation in the gene that encodes CalDAG-GEF-1 has also been found in some LAD-III patients of Turkish origin(290). However, the defect of CalDAG-GEF-1 is not fully understood since these patients generally express normal CalDAG-GEF-1 protein levels and transfection of lymphocytes from LDA-III patients with *CALDAG-GEF-1* cDNA fails to rescue the phenotype(285). Patients with LAD-III will die if not subjected to HSCT early in life(284).

Inflammatory diseases associated with leukocyte integrins

Leukocyte recruitment into an inflamed tissue is a central hallmark for a proper immune response. However, it is also a central hallmark for malignancy and for several inflammatory and autoimmune diseases where basic regulation of the immune response is lost(74, 279). Given the central involvement of leukocyte recruitment in both physiology and pathology, targeting this process is an attractive possibility to either enhance the immune system or suppress inflammation-induced tissue destruction. Indeed, the leukocyte adhesion cascade in general, and leukocyte integrins in particular, represent key therapeutic targets(74).

Uncontrolled and excessive leukocyte transmigration is characteristics for many pathologies such as various chronic inflammatory diseases including psoriasis, multiple sclerosis (MS), inflammatory bowel disease (IBD) and RA(291). Here, infiltrating leukocytes induce, promote and prolong inflammation without being subject for proper clearance. Although integrin-targeted therapies have been somewhat efficacious in animal models of inflammatory diseases, subsequent human studies haven't been as great as was hoped for. Only a few studies on integrin-targeted therapies have shown beneficial effect in the treatment of human inflammatory diseases(292-296).

The relatively frequent occurrence of severe adverse events is a big obstacle in integrin-targeted therapies(74). As for all treatments, the aim is always to effectively stop the pathology but at the same time of course also to limit the negative side effects to as few as possible. The issue with integrin-targeting therapies is the fact that it is difficult to develop treatments that only affect leukocyte trafficking in an inflammatory-site specific manner(292). If globally blocking integrin function by targeting major integrins in T lymphocytes such as LFA-1 and Mac-1 it is extremely difficult not to impair the normal immune response and compromise the health of the patient(74, 292). Alternative future strategies could potentially include those that either target integrin-signaling intermediates that are specific for a certain cell type or those that specifically target active integrin conformations(292). This is why studies on cell type-specific integrin signaling pathways and studies on specific molecular interactions within these pathways are required before therapies can be developed. In the following sections I will briefly describe some of the common inflammatory diseases associated with leukocyte integrins and also mention some of the different leukocyte integrin-targeting therapies that have reached clinical use.

Psoriasis

Psoriasis is a chronic inflammatory disease of the skin that is characterized by erythematous skin patches that are typically red, itchy, and scaly. The disease is also characterized by an abnormally excessive and rapid growth of the epidermal layer of the skin during wound repair(297). The protective capacity of inflammation is great but psoriasis is a good example of a pathologic consequence aroused from dysfunctional regulation of the inflammatory response. A typical psoriatic lesion contains infiltrating inflammatory cell populations (T lymphocytes and DCs in the dermis, and T lymphocytes and neutrophils in the epidermis) as well as epidermal keratinocytes that are subjected to hyperproliferation. Instead of usually being replaced every 28-30 days the keratinocytes are replaced every 3–5 days in psoriasis(297).

Various attempts to block integrin activity and consequently reduce trafficking and compartmentalization of leukocytes in the psoriatic lesions have been made(298). Inhibitory studies on LFA-1 resulted in the clinical use of Efalizumab, a monoclonal antibody that is directed against the αL integrin tail of LFA-1. When bound to LFA-1, Efalizumab prevents ICAM-1 binding via steric hindrance(299). Studies on Efalizumab showed reduced T lymphocyte activation, inhibited traffic and recruitment of T lymphocytes to the dermis/epidermis and subsequently decreased reactivation of T lymphocytes(299-301). However, Efalizumab was discontinued from the market in 2009 since cases of progressive multifocal leukoencephalopathy (PML) were observed after continuous and long-term use(298). PML is a viral-mediated demyelinating brain disease that develops when

leukocytes function and especially T lymphocyte function is heavily weakened(302). Indeed, inhibiting integrins that are multifunctional is always a risk since there is always a fine balance between increased clinical benefit and reduced probability for side effects like those seen for Efalizumab(74).

In similarity to the β2-integrin-blocking strategies of LFA-1 and also Mac-1 during psoriasis development, selectin-blocking strategies have also shown contradictory results(297). However, α1β1-integrin blocking (α1β1 is also known as VLA-1) has shown promising results in mice, but most faith is currently given to IL-17 blocking-strategies(303, 304).

Multiple sclerosis

MS is an autoimmune disease of the central nervous system (CNS) characterized by demyelination. The clinical course ranges from relapsing-remitting to progressive forms and can lead to severe disability and mortality. The pathogenesis leading to demyelination includes CNS-infiltrating autoreactive T lymphocytes that target myelin sheaths(74). Activated T lymphocytes can cross the blood-brain barrier (BBB) by using surface LFA-1 and VLA-4 that bind to ICAM-1 and VCAM-1, respectively, which are expressed on endothelial cells in the CNS(305). The role of both LFA-1 and VLA-4 in leukocyte recruitment to the inflamed CNS in the course of experimental autoimmune encephalomyelitis (EAE), the rodent model of MS, has been extensively investigated(74, 305). Inhibitory studies on β2-integrins have shown contradictory findings and have reported both effector and suppressor functions in EAE pathogenesis(74).

However, the identification of VLA-4 as a major player in EAE and MS resulted in the development of Natalizumab, a monoclonal antibody against α4, which has been approved for the treatment of patients with MS(293, 306). Natalizumab prevents the migration of leukocytes across the BBB into the CNS by blocking the interaction between VLA-4 and VCAM-1(305). Similar to Efalizumab that was previously used as psoriasis-therapy, Natalizumab is also associated with increased risk of PML development(307, 308). However, a recently published study confirmed the beneficial role of Natalizumab and additionally reported a diminished risk of PML side effects in patients with relapsing MS. It is consequently still on the market(309). In addition, blocking VLA-1 in both rats and mice has shown to prevent EAE disease development (310, 311).

IBD

IBD are a group of chronic disorders of the gastrointestinal tract that mainly include Crohn's disease (CrD) and Ulcerative colitis (UC). Studies with animal models of IBD have revealed a critical involvement of leukocyte integrins during intestinal inflammation. In various models, blocking β7 integrins has been shown to efficiently suppress inflammation, either alone or in combination with L-

selectin inhibition(74). Natalizumab, the monoclonal antibody used in MS therapy, block all α4-subunits independently of associating β-subunit. Consequently, Natalizumab not only block α4β1 integrins but also α4β7 integrins and has been used in Phase II and III studies on patients with CrD(74, 312-314). Blocking of α4β7 inhibit lymphocyte homing to gastrointestinal lymphoid tissue by preventing α4β7 interaction with endothelial MAdCAM-1(315). The clinical trials have shown efficient results, however, due to the risk for PML development it is only approved in the United States and under a restricted distribution program(316).

Vedolizumab is a selective antibody against α4β7 integrins and does not target α4β1 (VLA-4) integrin-mediated leukocyte recruitment to the CNS and consequently minimizes the risk of developing PML that is associated with VLA-4 inhibition(74). Vedolizumab is especially used as therapy for patients with CrD in whom TNF-α treatment failed(296).

Not only blocking of β7 integrins by administration of antibodies has been shown to have beneficial effect in the treatment of patients with moderate to severe IBD(74, 317). Several studies have also suggested a role of β2 integrins in experimental colitis and blocking β2 integrins by administration of antibodies result in significant inhibition of inflammatory cell recruitment and consequently decreased mucosal damage(74).

Association between PTPN22R620W and human autoimmunity

The SNP in PTPN22 that subsequently lead to an Arg-to-Trp substitution at amino acid position 620 (R620W) is currently one of the strongest genetic risk factors for autoimmune disease outside of genes that encodes either the MHC or the T lymphocyte inhibitory signal cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)(190). As reviewed by Bottini *et al.*, the association between PTPN22W620 and increased risk of developing various autoimmune diseases including but not limited to T1D, RA, SLE and Graves' disease is well established(201). These associations have been confirmed in multiple populations and carriage of the PTPN22W620 variant increases the risk of disease 1.3–2.56 times depending on the actual study and the disease type. The strongest effects have been observed in T1D, RA, SLE and vitiligo(201, 224). Interestingly, some other common autoimmune diseases including celiac disease, MS, UC and psoriasis are conclusively not associated with PTPN22W620 and a small but robust protective effect of PTPN22W620 is actually observed in CrD(318-322).

Although the association between PTPN22W620 and increased risk of developing various autoimmune diseases is established, mice deficient in the PTPN22 mouse orthologue Pep do not develop spontaneous autoimmunity(210). Still, positive selection is enhanced in thymocytes of Pep-deficient mice and consequently results

in increased numbers of both CD4 and CD8 memory and effector T lymphocytes in peripheral lymphoid compartments(210). In contrast, negative selection is not enhanced in thymocytes of Pep-deficient mice or in murine PTPN22W620-expressing T lymphocytes(254, 323). However, Pep-deficient T lymphocytes display an over-reactive phenotype with enhanced conjugate formation with APCs that are pulsed with weak peptides. This results in increased naïve T lymphocyte activation with subsequent production of inflammatory cytokines including TNF-α and Interferon γ (IFN-γ)(254). In summary, these findings suggest that Pep is a critical regulator for the ability of TCRs to discriminate among their ligands and that Pep regulates TCRs that signal through weak agonists and self-antigens but not through strong agonist. Moreover, Pep-deficient T lymphocytes also showed an enhanced ability to form stable LFA-1-dependent cell contacts with weak antigen-loaded APCs at later time points (< 2.5 hours). This could further indicate that the ability of PTPN22 to discriminate weak and potentially self peptide-mediated TCR signaling from strong peptide-mediated TCR signaling is LFA-1 dependent(254). Consequently, tolerance can potentially brake if dysfunctional PTPN22 is incapable of restraining TCR signaling engaged with weaker ligands that may include self-antigens. No good what so ever.

One finding that could explain why tolerance puzzlingly is maintained in Pep-deficient mice is that their regulatory T lymphocytes are intrinsically more effective than those of WT mice(209). When comparing with WT control, Pep-deficient regulatory T lymphocytes displayed a more activated phenotype, produced increased amounts of the immunosuppressive cytokines such as IL-10 and also had enhanced LFA-1-mediated adhesion(209).

To summarize, the role of PTPN22 in disease is complex but hopefully, as more detailed genetic analyses that cover the broad and increasing range of PTPN22-associated diseases become carried out, the more comprehensive understanding we will get. The complexity is also underpinned by the fact that there are additional disease-related SNPs identified in PTPN22. For example carriers of an additional missense SNP in PTPN22 that subsequently lead to an Arg-to-Glycine (Gln, Q) substitution within the PTPN22 catalytic domain (position 263, R263Q) have been shown to have a reduced risk of developing SLE, RA and UC but an increased risk of developing pulmonary TB(225, 320, 324, 325). In addition, another rare PTPN22 variant that subsequently lead to a Histidine (His, H)-to-Asn substitution within the PTPN22 interdomain (position 370, H370N) has been shown to slightly increase the risk of developing CrD whereas for example the more common PTPN22 R620W variant risk has a protective effect in the same disease as previously discussed(322, 326).

In addition, since PTPN22 and the mouse orthologue Pep are two of the most divergent example of phosphatase orthologous between human and murine

species, some caution should always be taken when it comes to extrapolating findings between them (327). For example, Pep deficiency has shown to both increase as well as reduce the severity of disease in different autoimmunity mouse models. To additionally complicate the role of PTPN22 in autoimmune disease development, discrepancies in the role of PTPN22 within the same autoimmune disease do occur(201). For example, both overabundance and deficiency of PTPN22 on a non-obese diabetic (NOD) mouse background have shown protection from diabetes (328, 329). However, some of the data from both human and mouse genotyped cells need to be interpreted with caution because several studies have been assessing small numbers of subjects and/or focused on narrow definitions of T lymphocyte signaling and function(201).

Results and Discussion

Relocating integrins to new locations within the cell membrane can control integrin activity. The degradative turnover of integrins is slow and once internalized, most integrins are recycled back to the cell surface(121). The mastermind behind this relocation is the cells' intracellular and very complex highway system, which facilitates the movement of essential molecules such as membrane-bound vesicles, organelles and proteins. The mechanisms behind this transport system are incompletely understood and recycling is consequently a global and hot topic all the way down to the cellular and molecular level.

With paper 1 of my thesis, we propose a model that identifies vesicle-associated RhoB as a novel regulator of Rab11-mediated recycling of surface-internalized LFA-1 in T lymphocytes. The main findings in paper 1 of my thesis are summarized as followed:

- Dysfunctional RhoB causes impaired migration by accumulation of LFA-1 in the rear of the T lymphocyte
- RhoB regulates vesicle transport of LFA-1 along the microtubules
- RhoB regulate Rab11-dependent intracellular transport and reentering of LFA-1, which is needed to support forward migration.
- Rab4 does not compensate for loss in RhoB-mediated Rab11-dependent LFA-1 recycling

Integrins play a crucial role in all cell adhesion and migration and not only their recycling but also their activity must be tightly regulated. Most previous studies have focused on integrin activation and the mechanistics behind integrin activation are fairly known. In contrast, integrin deactivation is less known and is in fact a relatively unexplored field of research. However, it is now established that integrin deactivation not just is a sole passively adopted default event that sooner or later follows after integrin activation(169). In both paper 2 and paper 3 of this thesis, we have identified the phosphatase PTPN22 as a novel negative regulator of LFA-1. The main findings in paper 2 is the following:

- PTPN22 associates with both its substrates and LFA-1 at the leading edge of migrating T lymphocytes upon LFA-1-stimulated migration

- PTPN22 exists in large clusters that disperse upon LFA-1 engagement with ICAM-1
- PTPN22 associates with LFA-1 signaling complex and inhibits LFA-1 clustering
- LFA-1-dependent adhesion during both static condition and shear flow is increased in T lymphocyte that express PTPN22-W620
- Expression of the PTPN22-W620 enhances LFA-1 clustering at the leading edge of migrating T lymphocytes

LFA-1 function is not only important for T lymphocytes' ability to move forward but also for their ability to form immunological synapses. Consequently, in addition to study PTPN22-mediated LFA-1 regulation in migrating T lymphocytes, one additional aim of this thesis was to study this regulation during cell-cell contacts that includes those that occur during immunological synapse formation. Our work in paper 3 concludes that PTPN22 is an important regulator of LFA-1-mediated adhesion also during immunological synapse formation. The main findings in paper 3 are summarized below:

- PTPN22 polarizes towards the immunological synapse
- Reduced PTPN22 expression in T lymphocytes increases immunological synapse formation efficiency
- Reduced PTPN22 expression in T lymphocytes induces phosphorylation of PTPN22 substrates and their associations with LFA-1 at the immunological synapse
- Reduced PTPN22 expression in T lymphocytes induces high affinity LFA-1 at the immunologic synapse

The previous dogma for integrin recycling that describes integrin recycling from the rear to the front of the cell is now under reconstruction(17). It is now evident (and generally assumed) that most integrins follow a more locally displayed recycling route with both endocytosis and recycling near the lamellipodium(129). However, the long back-to-front transport is still believed to take place to some extent, at least in quickly migrating cells(130, 131). For example, inhibiting LFA-1 internalization via endocytosis in mouse pro-B lymphocytes results in LFA-1 accumulation in the uropod, which severely impairs uropod detachment(89). Similar, our work in paper 1 also shows that impairing RhoB function leads to reduced LFA-1 recycling and accumulation of both LFA-1 and Rab11 at the rear of migrating T lymphocytes. All together, the fact that LFA-1 accumulates in the uropod during impaired intracellular transport suggests that LFA-1 can be recycled from the uropod in T lymphocytes.

The mechanism behind LFA-1 recycling is largely unknown and it is not clear which type of vesicles that are in use. Extensive work made by Katagiri *et al*. has found both Rap-1 and RapL as well as Mst-1 within the same recycling compartment as LFA-1 (90, 91). RapL also bind SKAP-55 and both these proteins have been suggested to be responsible for correctly positioning Rap-1 and also LFA-1 at the cell membrane(92). However, the vesicles do not move randomly within the cell. Indeed, Rab proteins are master regulators of all stages of intracellular traffic along the microtubule and/or actin cytoskeleton(125). Many receptors are internalized through a Rab5-mediated pathway and returned back to the PM via either a rapid recycling route in a Rab4-dependent manner or by a longer recycling route in a Rab11-dependent manner(119). LFA-1 has been shown to be associated with both Rab5 and the Rab5 effector EEA-1 in migrating T lymphocytes as well as with Rab5 in migrating neutrophils(133, 135, 140). We confirm this finding in paper 1 and additionally show that RhoB does not regulate Rab5-mediated internalization of LFA-1 in migrating T lymphocytes. Moreover, several specific heterodimers of β1, β2, β3 and β4 integrins have been identified to use Rab11-medaited recycling and LFA-1 has been shown to use Rab11-medaited recycling in neutrophils(133). With this work we show that LFA-1 has a preference for this longer Rab11-mediated recycling loop also in T lymphocytes. We also show that Rab4 does not compensate for the loss of Rab11-dependent LFA-1 recycling in RhoB-deficient T lymphocytes as we did hypothesize. It could be interesting to study weather Rab13 or Rab27 could compensate for the loss of Rab11-dependent recycling of LFA-1 in RhoB-deficient T lymphocytes. Rab27 was recently found to be crucial for chemotaxis of neutrophils and Rab13 to associate with Mst-1 and facilitates LFA-1 delivery to the leading edge of migrating pro-B BAF lymphocytes(90, 141, 330).

Another important issue that is remaining to be addressed is whether integrins can be endocytosed in different affinity conformations. For example, the fact that LFA-1 can be endocytosed both dependently and independently of Rab5 could point toward the possibility that LFA-1 use different internalization and intracellular transport pathways depending on its conformation state(133, 135, 140). So far, there have only been a few studies showing that integrins can be intracellular transported in their high affinity conformation(134, 144). However, one reason for this could be explained by the fact that many of the previous studies have used specific affinity antibodies that are proved to be functionally blocking. This could consequently have led to false conclusions. In addition to LFA-1 affinity conformations, one big unanswered question is also whether clustered LFA-1 is endocytosed and recycled back to the PM, or if these LFA-1 clusters undergo down-regulation by other mechanisms. Indeed, more theses on integrin recycling are warranted.

Phosphorylation and dephosphorylation can regulate protein function in many different ways including catalytic activity, stability, subcellular location and physical association between proteins(189). With paper 2 and 3, we show that the phosphatase PTPN22 is required for the regulation of LFA-1-mediated cell motility and immunological synapse formation. PTPN22 polarizes not only toward the leading edge in migrating T lymphocytes but also to the immunological synapse when T lymphocytes form synapses with APCs. The location of PTPN22 within the synapse is partly spread since it regulates both TCR signaling and LFA-1 signaling. PTPN22 can associate with more peripherally located LFA-1 and here regulate LFA-1 affinity conformations as well as associate with TCR and regulate it signaling, both in peripheral microclusters and in more centrally located TCR clusters in the cSMAC, although the latter is somewhat still debated. Overall, this could consequently explain why we find PTPN22 polarized towards the synapse in a slightly spread structure.

LFA-1 activation and its downstream signaling are very complex and include several cross-talking pathways. We found an increase in association between LFA-1 and the phosphorylated PTPN22 substrates LckY394 and Vav-1Y174 in T lymphocytes during both LFA-1-mediated cell motility and immunologic synapse formation. The Rho GTPase GEF Vav-1, together with its downstream Rho GTPase substrates promote cytoskeletal polarization, rearrangement and stabilization in T lymphocytes and subsequently determine both the direction of migration as well as the stability of the synapse(156, 255). Although not confirmed, the action of PTPN22 on Vav-1 could influence migratory direction by directly and negatively regulate Vav-1 and consequently be a part of regulating the balance of actin-mediated protrusions at the leading edge that subsequently drives cell migration(19). Moreover, the location of the PTPN22 substrate LckY394 is increased in both central and more peripheral locations of the synapse, which consequently suggest that PTPN22 dephosphorylates LckY394 during both TCR signaling and LFA-1 activation within the synapse. In addition, several other phosphatases besides PTPN22 are involved in the regulation of TCR signaling during synapse formation, but besides PTPN22 it is currently unknown which phosphatases, in more detail, that regulate LFA-1 activation and signaling during synapse formation(331).

Imbalance in integrin activity and in its signaling pathways may explain why leukocytes infiltrate into tissues in inflammatory and autoimmune diseases. The results from paper 2 and 3 of this thesis define a previously uncharacterized mechanism for fine-tuning LFA-1 signaling in T lymphocytes and aids in the understanding of autoimmunity in humans. Regulating LFA-1 signaling is important for many reasons. LFA-1 mediated leukocyte recruitment into an inflamed tissue is a central hallmark for a proper immune response, but also a central hallmark for malignancy and for several inflammatory and autoimmune

diseases(74, 279). Indeed, these common pathologies in which basic regulation of the immune response is lost can be a consequence of faulty LFA-1 regulation. For example, LFA-1 is needed to sustain TCR signaling, organize proteins within the immunological synapse and thereby regulate both T lymphocyte activation and differentiation into effector cells(272-275, 277, 278). Consequently, by providing this regulatory basis of T lymphocyte differentiation into effector cells, LFA-1 also regulates autoimmunity. Even in the absence of appropriate TCR signaling, that is when a TCR recognizes a peptide–MHC complex that is not the cognate one, LFA-1 may facilitate effector differentiation by increasing the contact between the T lymphocyte and the APC when PTPN22 is dysfunctional. No good. In addition, we show that PTPN22W620, the SNP variant of PTPN22 that is associated with numerous autoimmune diseases, perturbs integrin function and is a loss-of-function variant, at least in the context of LFA-1 signaling.

To summarize, this thesis aimed to investigate the dynamics of LFA-1 in T lymphocytes. We have identified vesicle-associated RhoB as a novel regulator of Rab11-mediated recycling of surface-internalized LFA-1 along microtubules in T lymphocytes. We have also identified the phosphatase PTPN22 as a novel negative regulator of LFA-1 in T lymphocytes during both migration and synapse formation. Targeting cell type-specific integrin signaling pathways is an attractive model when designing new therapies for various pathologies including cancer and autoimmune disease, since they potentially could either enhance the immune system or suppress inflammation-induced tissue destruction. By providing a better understanding of LFA-1 regulation and LFA-1 recycling, we consequently contribute the development of future integrin-targeting therapies.

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Put your faith in what you most believe in

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