**Univerzita Karlova v Praze**

**Přírodovědecká fakulta**

Studijní program: Biologie

Studijní obor: Imunologie


Bc Jan Valečka

# Identification of a new mechanism of Lck regulation via its C-terminal sequence

# Identifikace nového mechanismu regulace Lck zprostředkovanou její C-terminální sekvencí

Diplomová práce

Školitel: RNDr. Dominik Filipp, CSc.

Praha, 2014

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 21. 8 2014

……
Jan Valečka

## Poděkování

Chtěl bych nesmírně poděkovat svému školiteli Domiinikovi Filippovi za trpělivost a pomoc s prací na diplomové práci a za vedení v laboratři. Dále bych chtěl poděkovat Ondřeji Ballkovi za pomoc s diplomoou I laboratorní prací. A konečně bych rád velmi poděkoval rodině za podporu a pevné nervy.

# 1. Abstract

T-cell activation is a complex process crucial for a proper function of immune system. It has been extensively studied and its main features are well understood. However, some of the events involved in T-cell signalling are still unclear. After T-cell receptor stimulation, Src-family kinase Lck drives the initiation of signalling by tyrosine phosphorylation. Phosphorylation of several downstream targets is dependent on the redistribution of Lck to the different compartment of the plasma membrane, called lipid rafts. In lipid rafts, active Lck is juxtaposed and activates raft-resident substrates which then trigger downstream signalling. The critical in this process is the mechanism of Lck translocation to lipid rafts which has not been studied so far and represents the topic of great academic and clinical interests. Previously, we identified the adaptor protein RACK1 as a candidate protein mediating the redistribution of Lck to lipid rafts by linking it to the microtubular network. In this thesis, we analysed the structural features and functional role of RACK1 in its interaction with Lck. We show here, using the SYF cell lines expressing the wild type and various mutated forms of Lck, that intact SH3 or SH2 domains of Lck are required for an effective RACK1-Lck complex formation. We also documented a mild, but reproducible and sequence-specific effect of the C-terminal end of Lck in this process. This indicates that both SH2 and SH3 domains of Lck play a critical role in the complex formation with RACK1. The immunoprecipitation of SH3 and SH2 domains with RACK1 from lysates of transfected cells and cell-free *in vitro* translation system failed to detect their direct binding. This suggests that this interaction could be indirect and mediated by other proteins. Several such candidate proteins involved in the RACK1-Lck complex formation has been identified and one of them, the adaptor protein GADS, was tested as a possible linker between RACK1 and Lck. Adenovirus-induced overexpression of gene expression of RACK1 was used to assess its physiological role during the early onset of TCR signalling in primary T-cells. While these technically difficult experiments were completed, viral infection induced high levels of non-specific activation of T-cells and prevented us from a direct observation of influence of RACK1 overexpression on their activation. To circumvent these conditions, we prepared and successfully tested all reagents necessary for the downregulation of RACK1 by RNA interference. This alternative approach will be used in the near future for the functional characterisation of RACK1 in T-cell activation. Together, our data identified structural

elements of Lck involved in RACK1-Lck complex formation and provided insight into the composition of this complex. During this work, we also prepared all necessary molecular, cellular and organismal reagents to assess the role of RACK1 in the redistribution of Lck during early phases of T-cell activation.

Keywords: Lck, T-cell, lipid rafts, RACK1, GADS, T-cell activation

## 2. Abstrakt (CZ)

Aktivace T-lymfocytů je složitý proces zcela zásadní pro správnou funkci imunitního systému. Jako takový byl v minulosti důkladně zkoumán a jeho základní rysy jsou velmi dobře popsány. Neplatí to ovšem pro všechny události, které během T-buněčné signalizace nastávají. Po stimulaci T-buněčného receptoru kináza Lck z rodiny Src zajišťuje spuštění signalizace fosforylací tyrosinů. Fosforylace dalších proteinů je závislá na přesunutí Lck do jiných kompartmentů plazmatické membrány, takzvaných lipidových raftů. V lipidových raftech je Lck v blízkosti svých substrátů, které a aktivuje a které následně spouští další signalizaci. Kritickou částí tohoto procesu je translokace Lck do lipidových raftů, jejíž mechanismus zůstává dosud neprozkoumaný, a jako takový je objektem značného akademického i klinického zájmu. Již dříve jsme identifikovali adaptorový protein RACK1 jakožto kandidátní protein zprostředkovávající redistrubuci Lck do lipidových raftů pomocí propojení Lck s mikrotubulární sítí. V této práci jsme analyzovali strukturní znaky a funkci RACK1 v interakci s Lck. S využitím buněčných linií SYF exprimující buď přirozené nebo různě mutované formy Lck zde ukazujeme, že plně funkční SH3 a SH2 domény Lck jsou nezbytné pro efektivní vytvoření komplexu RACK1-Lck. Také jsme zjistili malý, ale reprodukovatelný a sekvenčně specifický vliv C-terminální domény Lck na tento proces. Tato zjištění naznačují, že SH2 i SH3 domény Lck hrají zásádní roli v tvorbě komplexu s RACK1. Nepodařilo se nám imunoprecipitací SH2 a SH3 domén s RACK1 z lyzátů transfekovaných buněk a z bezbuněčného in vitro translačního systému detekovat jejich přímou vazbu. To naznačuje, že interakce by mohla být nepřímá a zprostředkovaná dalšími proteiny. Identifikovali jsme několik kandidátních proteinů pro tuto roli, které se účastní formování RACK1-Lck komplexu. Jeden z nich, adaptorový protein GADS, byl testován jako možné pojítko mezi RACK1 a Lck. Overexprese genu RACK1 indukovaná adenovirem byla využita k objasnění fyziologické role tohoto proteinu během počínající signalizace přes TCR v primárních T-lymfocytech. Během dokončování těchto technicky náročných experimentů způsobovala virová infekce vysokou úroveň nespecifické aktivace T-buněk, což nám znemožnilo pozorovat vliv nadměrné exprese RACK1 na jejich aktivaci. Abychom obešli tyto problémy, připravili jsme a úspěšně otestovali reagencie nezbytné pro snížení množství RACK1 v buňkách pomocí RNA interference. Tento alternativní přístup bude v blízké budoucnosti použit ke zkoumání vlivu RACK1 na aktivaci T-lymfocytů. Společně naše data ukázaly strukturní části Lck využívané při utváření komplexu RACK1-Lck a poskytly nové informace o jeho dalších složkách. Během této práce jsme taktéž připravili veškeré

molekulární, buněčné a organismální reagencie potřebné ke zkoumání role RACK1 v redistribuci Lck během časné aktivace T-lymfocytů.

3.

4. Klíčová slova: Lck, T-lymfocyt, lipidové rafty, RACK1, GADS, aktivace T-lymfocytu

## 5. List of Abbreviations

| | |
|---|---|
| AP-1 | Activator protein 1 |
| APC | Antigen presenting cell |
| Arp2/3 | Actin related protein 2/3 |
| ATP | Adenosine triphosphate |
| Bcl-XL | B-cell lymphoma – extra large |
| Blk | B lymphocyte kinase |
| cAMP | Cyclic adenosine monophosphate |
| CAR | Coxsackie/adenovirus receptor |
| Cbp/PAG | Csk binding protein/protein associated with glycosphingolipid-enriched microdomains |
| CDC42 | Cell division control protein 42 |
| Csk | C-terminal Src kinase |
| CTLA4 | Cytotoxic T-lymphocyte-associated protein 4 |
| DAG | Diacyl glycerol |
| DPC | Distal pole complex |
| EBP50 | ERM binding protein 50 |
| EGF | Epidermal growth factor |
| EGFP | Enhanced green fluorescent protein |
| eIF6 | Eukaryotic translation initiation factor 6 |
| Erk | Extracellular signal-regulated kinase |
| ERM | Erzin, radixin, moesin protein family |
| FACS | Fluorescence-activated call sorting |
| F-actin | Filamentous actin |
| FAK | Focal adhesion kinase |
| Fgr | Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog |
| GABA | Gamma-aminobutyric acid |
| GADS | GRB2-related adapter protein 2 |
| GDP | Guanosine diphosphate |
| GEF | Guanine nucleotide exchange factor |

| | |
|---|---|
| GFP | Green fluorescent protein |
| GRB2 | Growth factor receptor-bound protein 2 |
| GST | Glutathione S-transferase |
| GTP | Guanosine triphosphate |
| HA | Hemagglutinin |
| ICOS | Inducible T-cell costimulator |
| ICOSL | Inducible T-cell costimulator ligand |
| IKK | IκB kinase |
| IL-2 | Interleukin 2 |
| IL-7 | Interleukin 7 |
| IP3 | Inositol-1,4,5-triphosphate |
| IRES | Internal ribosome entry site |
| IS | Immunological synapse |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| Itk | Interleukin-2-inducible T-cell kinase |
| IκB | Inhibitor on NFκB |
| JNK | c-Jun N-terminal kinase |
| LASP | LIM and SH3 domain protein 1 |
| LAT | Linker of activated T-cell |
| Lck | Lymphocyte-specific kinase |
| LFA-1 | Lymphocyte function-associated antigen 1 |
| MAP kinase | Mitogen-activated protein kinase |
| MEK | MAP/Erk kinase |
| MHC | Major histocompatibility complex |
| MOI | Multiplicity of infection |
| MS | Mass spectrometry |
| MTOC | Microtubule organizing centre |
| Nck | Non-catalytic region of tyrosine kinase adaptor protein 1 |
| NFAT | Nuclear factor of activated T-cells |

| | |
|---|---|
| NFκB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMDAR | N-methyl-D-aspartate receptor |
| PD1 | Programmed cell death protein 1 |
| PDK1 | 3-phosphoinositide dependent protein kinase-1 |
| PD-L1 | Programmed cell death protein ligand 1 |
| PH | Pleckstrin homology domain |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidylinositol-(3,4,5)-trisphosphate |
| PKC | Protein kinase C |
| PLCγ1 | Phospholipase C |
| pMHC | peptide-MHC complex |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| Rac1 | Ras-related C3 botulinum toxin substrate 1 |
| RACK1 | Receptor for activated C-kinase |
| Ras | Rat sarcoma protein |
| RasGRP | RAS guanyl nucleotide-releasing protein 1 |
| SFK | Src family kinase |
| SH1, SH2, SH3 | Src homology domain 1, 2, 3 |
| shRNA | Small hairpin RNA |
| SLP-76 | SH2 domain containing leukocyte protein of 76kDa |
| SMAC (cSMAC, pSMAC, dSMAC) | Supramolecular activation cluster (central, peripheral, distal) |
| Sos | Son of sevenless |
| SYK | Spleen tyrosine kinase |
| TCR | T-cell receptor |
| TLR | Toll-like receptor |
| WASP | Wiskott–Aldrich Syndrome Protein |
| WAVE2 | WASP-family verprolin-homologous protein 2 |
| WB | Western blotting |

| | |
|---|---|
| WT | Wild type |
| Yes | Yamaguchi sarcoma viral (v-yes) oncogene homolog 1 |
| ZAP-70 | zeta-chain associated protein kinase 70kDa |

# 6. Introduction

Immune system is a crucial mechanism that protects life of every organism, in one form or another. It protects us from pathogens as well as harmful processes of our own bodies. Immunity is traditionally divided into two parts, innate and adaptive. Innate is evolutionally older and is generally considered non-specific, while adaptive is induced by a specific molecule and is ready to recognise virtually endless range of antigens. These two parts are not independent, but tightly interconnected and even indispensable for proper function of each other.

Important part of adaptive immunity are T-cells. Their receptors are highly variable and T-cells are therefore able to recognise immense number of antigens. During their lives, they have to be able to react to various stimuli in a correct manner. For this purpose they employ numerous ways of signalling. Whether they mature, migrate, detect invaders, secret cytokine or kill pathogens, they always convey relevant signals to the nucleus to modulate their genetic program to adapt to a changing microenvironment and to communicate with other immune cells.

When T-cell recognises an antigen, it is activated and consequently orchestrates and performs immune response to danger. T-cell activation is a complex process engaging multiple signalling pathways. To achieve an unencumbered coordination, specificity and efficiency in signalling, the process requires physical segregation of signalling elements into appropriate confinement. The initial processes take place on the plasma membrane. T-cell receptor delivers a signal of recognition of antigen, which is further transmitted by protein tyrosine kinase Lck. Lck-induced phosphorylation leads to engagement of a cohort of other proteins, which leads to triggering of various signalling pathways.

In spite of the fact that the process of T-cell activation has been studied for decades and schematics of T-cell signalling are well understood, some crucial details are still obscure. The mechanism of Lck engagement in first moments after T-cell receptor stimulation is undescribed. It has been proposed that it is caused by change of localisation on the membrane, but its precise mechanism is still unknown. In this thesis, we tried to elucidate the translocation and machinery behind this process.

# 7. Literature review

This section summarises the information about the T-cell signalling. It describes pathways engaged during the initial stage of T-cell activation. It also introduces several currently considered models of T-cell activation and signal transduction from the TCR inside the cell. Furthermore, it highlights s the structure and mechanisms of regulation of Lck, the member of Src-family tyrosine kinases (SFKs), which generates the first detectable signal after T-cell activation and is absolutely critical for the initiation of TCR signalling. And finally, it describes structure and function of receptor for activated C-kinase, an adaptor protein which could play so far undescribed, but important role in the proximal T-cell signalling by regulating the subcellular redistribution of Lck.

## *7.1. T-cell signalling*

T-cells are crucial part of the adaptive immune system, highly efficient and extremely versatile. To ensure that they only response to potentially dangerous stimuli derived from pathogens, a complex mechanism of activation is employed. The engagement of TCR starts a rapid response leading to a lasting change in the behaviour of T-cell. To ensure the activation occurs under proper conditions and to avoid autoimmunity, T-cells require two separate signals from the antigen presenting cell (APC): (i) specific signal generated upon peptide-MHC-TCR interaction (Norcross, 1984) and (ii) costimulatory signal via CD28-CD80/86 interaction (Lenschow *et al.*, 1996). If both signals are delivered in appropriate spatio-temporal fashion, the process of T-cell activation is initiated. We can distinguish three stages of this process (Mempel *et al.*, 2004). The initial phase starts by forming very short and temporary associations of T-cells with APCs in secondary lymphoid organs, whereby T-cell scans the surface of APC for cognate agonist peptide-MHC complexes. If successful and such interaction is productive, the most proximal signalling event is triggered. When this initial phase is completed, in the second phase, the interacting cells form a stable conjugates and the close apposition of their membranes forms a visually distinguishable structure called the immunological synapse (IS) (Grakoui *et al.*, 1999). IS is a highly complex and dynamic arrangement of crucial signalling molecules and their complexes which are recruited and transiently harboured to this confined space in order to further drive the progression of T

cell activation process. The outcome of this early activation event is the expression of early activation markers CD69 and CD44 as well as the expression of CD25 (α-chain of IL-2 receptor). 24 hrs after, if the interaction persists, the third stage takes place during which conjugated cells dissociate, T-cells start to produce IL-2 and proliferate.

### 7.1.1. T-cell triggering

T-cell activation relies on the recognition of a peptide displayed in the context of MHC. There are two major subtypes of MHC, class I and II, both recognized simultaneously by TCR and a coreceptor, CD8 or CD4, respectively. MHC class I is expressed on all cell types and presents peptides from endogenous proteins. Its expression serves as a hallmark of healthy cells and their resident tissues. After infection, such as viral transmission, intracellular pathogen-derived peptides are copresented with normal endogenous proteins in the context of MHCI, enabling CD8+ T-cells to identify and eliminate infected cells. MHC class II proteins are expressed mainly on APCs and serve for the presentation of peptides derived from extracellular proteins, thus sampling the surrounding environment. APCs internalize and enzymatically process these antigens in the endolysosomal compartment and resulting peptides are loaded on MHC class II. Consequently, the peptide-MHCII complexes can be recognized by cognate CD4+ T-cells. (Vyas *et al.*, 2008)

The recognition is mediated by TCR complex, consisting of α and β chain of TCR and γ, δ, two ε and two ζ chains of CD3. Simultaneously, MHCII is recognised by CD4 coreceptor (or by CD8 in case of MHC class I) and signalling is triggered. CD3 γ, δ and ε chains possess one and CD3 ζ three ITAM motifs, summing their total number to ten per one TCR complex. ITAM motif is a specific aminoacid sequence containing two tyrosine residues susceptible to phosphorylation. Within seconds after TCR engagement, Lck tyrosine kinase phosphorylates the ITAM motifs, initiating one of the first experimentally detectable biochemical changes after T-cell activation. Phosphorylated tyrosine of ITAMs recruit ZAP-70, a SYK family tyrosine kinase, via its two SH2 domains (Wange *et al.*, 1993). ZAP-70 is then further activated by Lck phosphorylation and proceeds to phosphorylate the adaptor protein LAT, enabling engagement of multiple signalling pathways (Fig. 1.1b).

### 7.1.2. Downstream signalling

LAT is a transmembrane adaptor protein without any intrinsic catalytic activity. It contains the juxtamembrane CxxC motif for palmitoylation which targets the protein to the membrane microdomains, the event necessary for its proper function (Zhang *et al.*, 1998). It has a number of tyrosine residues, which serve as SH2 domain docking sites upon phosphorylation by ZAP-70 (Lin & Weiss, 2001). The association with these SH2 domain-containing proteins leads to the formation of a large multiprotein complex which serves as platform for downstream signalling.

The signalling complex built around LAT branches into several distinct pathways that are, however, tightly interconnected and interdependent. They ultimately lead to the activation of transcription factors which converge on numerous promoter sequences to initiate the expression of their genes, such as the expression of IL-2 and other cytokines (Fig. 1.1c).

When LAT is phosphorylated, it is bound by adaptor protein GADS, which in turn binds another adaptor protein SLP-76. SLP-76 serves as a docking platform for guanosinnucleotide exchange factor (GEF) Vav1, adaptor protein Nck, Tec family kinase Itk and PLCγ1, which also binds LAT directly. Itk is then phosphorylated and activated by Lck and in turn phosphorylates and thus activates PLCγ1. PLCγ1 then lyses phosphatidylinositol-4,5-bisphosphate ($PIP_2$) to inositol-1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG). Consequently, IP3 stimulates opening of Ca2+ channels on the endoplasmatic reticulum, which in turn leads to the opening of the Ca2+ channels on the cytoplasmatic membrane, providing a sustained influx of Ca2+. Increased calcium level activates calcineurin, which dephosphorylates transcription factor NFAT. NFAT is therefore translocated into the nucleus, where it alters gene expression in cooperation with other transcription factors. (Brownlie & Zamoyska, 2013)

Meanwhile, DAG in the membrane produced by PLCγ1 is recognized and bound by protein kinase Cθ (PKCθ). This enzyme then phosphorylates one subunit of IKK, causing it to phosphorylate inhibitor on NFκB (IκB), which serves as a signal for its polyubiquitinylation and subsequent degradation of IκB. Once free of the inhibitor, nuclear localization signal of NFκB is exposed, NFκB is translocated to the nucleus and triggers the transcription of genes relevant for the accomplishment of T cell activation process. (Vallabhapurapu & Karin, 2009)

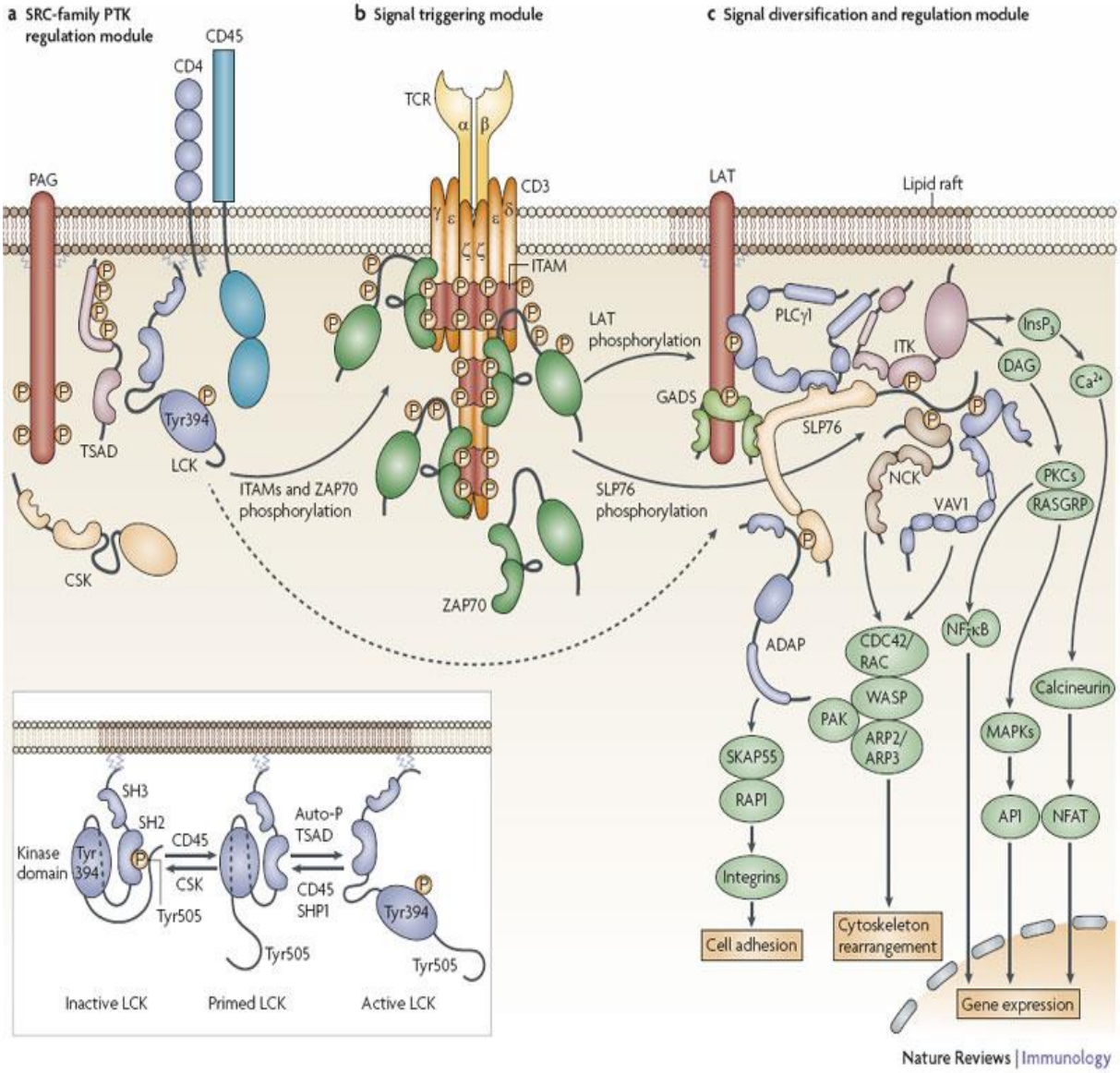



**Figure 1.1 T-cell signalling.** T-cell signalling pathway could be divided into three closely connected regulation and triggering modules. **(a)** Lck is negatively regulated via phosphorylation by Csk, which is recruited to the membrane by adaptor protein Cbp/PAG. **(b)** Once TCR is stimulated, CD45 phosphatase dephosphorylates the inhibitory tyrosine on Lck. Fully active Lck then phosphorylates ITAMs on the CD3 and activates ZAP-70. ZAP-70 consequently phosphorylates adaptor protein LAT **(c)**, which interacts with a number of other proteins. This leads to forming of a signalling complex that activates actin polymerisation and multiple downstream signalling pathways leading to altering the gene expression. Taken from (Acuto *et al.*, 2008).

DAG is also bound by another protein important for signalling, Ras guanylnucleotide-releasing protein (RasGRP). RasGRP is one of two GEFs for small GTPase Ras, the other being son of sevenless (Sos). Sos is bound to LAT indirectly by adaptor protein GRB2. Together, RasGRP and Sos activate Ras by causing exchange of GDP for GTP (Roose *et al.*, 2007). Ras then initiates MAP kinase cascade by activation of Raf1 kinase, which then phosphorylates MEK kinase, which in turn phosphorylates Erk, the effector MAP kinase. This leads to the engagement of transcription factor AP-1. (Smith-Garvin *et al.*, 2009)

LAT also stands at the beginning of actin remodelling. Aforementioned adaptor protein Nck associated with SLP-76 binds Wiskott-Aldrich syndrome protein (WASP). Vav1 then activates Rho family GTPases CDC42 and Rac1, which stimulate WASP and WAVE2 proteins responsible for actin remodelling. (Billadeau *et al.*, 2007)

### 7.1.3. Costimulatory signal

TCR/MHC complex formation alone is insufficient for T-cell activation. On the contrary, it leads to anergy, rendering T-cells unresponsive to physiological stimuli (Gimmi *et al.*, 1993). T-cell needs a second, so call costimulatory signal to promote T-cell survival and activation. This costimulatory signal is provided by molecules of B7 family, CD80 and CD86, which are upregulated on activated APCs (Inaba *et al.*, 1994). The absence of B7 molecules contributes to peripheral tolerance by inactivating T-cells recognizing antigens on non-activated APCs lacking costimulatory molecules. After the recognition of danger signal derived from microbes by pattern recognition receptors (e.g. TLRs), APCs upregulate the surface expression of costimulatory molecules, whose the presence is the prerequisite for activation of T-cells. The costimulatory signal is delivered into the T-cell by CD28, the receptor for CD80 and CD86. There are also other receptor-ligand pairs that contribute to stimulation or inhibition, such as ICOS-ICOSL, PD1-PD-L1, CD40-CD40L (Peggs & Allison, 2005).

Mechanistically, upon engagement, CD28 binds phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), protein capable of phosphorylation of phosphatidylinositol on the position 3, creating phosphatidylinositol-3,4,5-triphosphate ($PIP_3$) (Harada *et al.*, 2001). This phospholipid can be recognized and bound by pleckstrin homology (PH) domain. This domain is present in Akt protein, as well as its activatory kinase PDK1. After activation, Akt phosphorylates number of proteins which support the cell survival and the generation of antiapoptotic signals, including attenuation of NFκB, NFAT and Bcl-$X_L$ pathways (Smith-Garvin *et al.*, 2009). CD28 competes for CD80 and CD86 binding with a negative regulator of T-cells, CTLA4. This molecule does not provide the costimulatory signal. In contrast, it recruits protein tyrosine phosphatases which actively abolish T-cell signalling (Smith-Garvin *et al.*, 2009).

### 7.1.4. Role of cytoskeleton

During T-cell activation, the cell becomes polarized and MTOC is translocated underneath the contact site, the IS. These dynamic processes are fully dependent on the engagement of cytoskeleton which is therefore necessary structural component for proper T-cell activation.

The IS is a specialized area on the membrane on the contact site between T-cell and APC. It is formed after TCR stimulation by peptide-MHC and enables sustained activation necessary for full T-cell activation (Grakoui *et al.*, 1999). It aggregates proteins of the signalling cascades to enhance signal transduction, and also serves as an environment from which the negative regulators are sequestered.

The IS has a structure of three concentric circles. The inner one is called central supramolecular activation cluster (cSMAC), middle one is peripheral SMAC (pSMAC) and the outmost one is distal SMAC (dSMAC). cSMAC is the heart of the activation, containing TCR complex, CD4 or CD8, Lck, PKCθ and CD28. pSMAC harbours integrins (most importantly LFA-1) and is therefore responsible for maintaining the physical connection with APC. dSMAC contains phosphatases such as CD45, that are therefore spatially incapable of counteracting the tyrosine phosphorylation-propelled signalling in cSMAC. (Huppa & Davis, 2003)

The cytoskeleton plays a very important role in the establishment of IS as it is involved in the signalling leading to the formation of IS and T-cell polarisation. Crucial for the development of the IS are actin microfilaments since without the actin the synapse is not formed. After TCR engagement, there is an increase in actin polymerisation which results in the formation of lamellipodium that increases contact area between APC and T-cell (Fig. 1.2). The polymerisation is dependent on Vav1, which activates CDC42 and Rac1, which in turn activate WASP and WAVE2 respectively (Billadeau *et al.*, 2007). Both of these proteins associate with Arp2/3 complexes and are able to initiate actin polymerisation. Both WASP and WAVE2 are indispensable for normal signalling, but appear to play different roles, as suggested by their distinct spatiotemporal localizations. WAVE2 appears to be largely responsible for the actin polymerization (Nolz *et al.*, 2006), while WASP might be involved in endo- and exocytosis (Zhang *et al.*, 1999) and stabilization of the IS. During this process, actin also works as a scaffold, as a number of actin-related proteins are engaged in the regulation of activation.

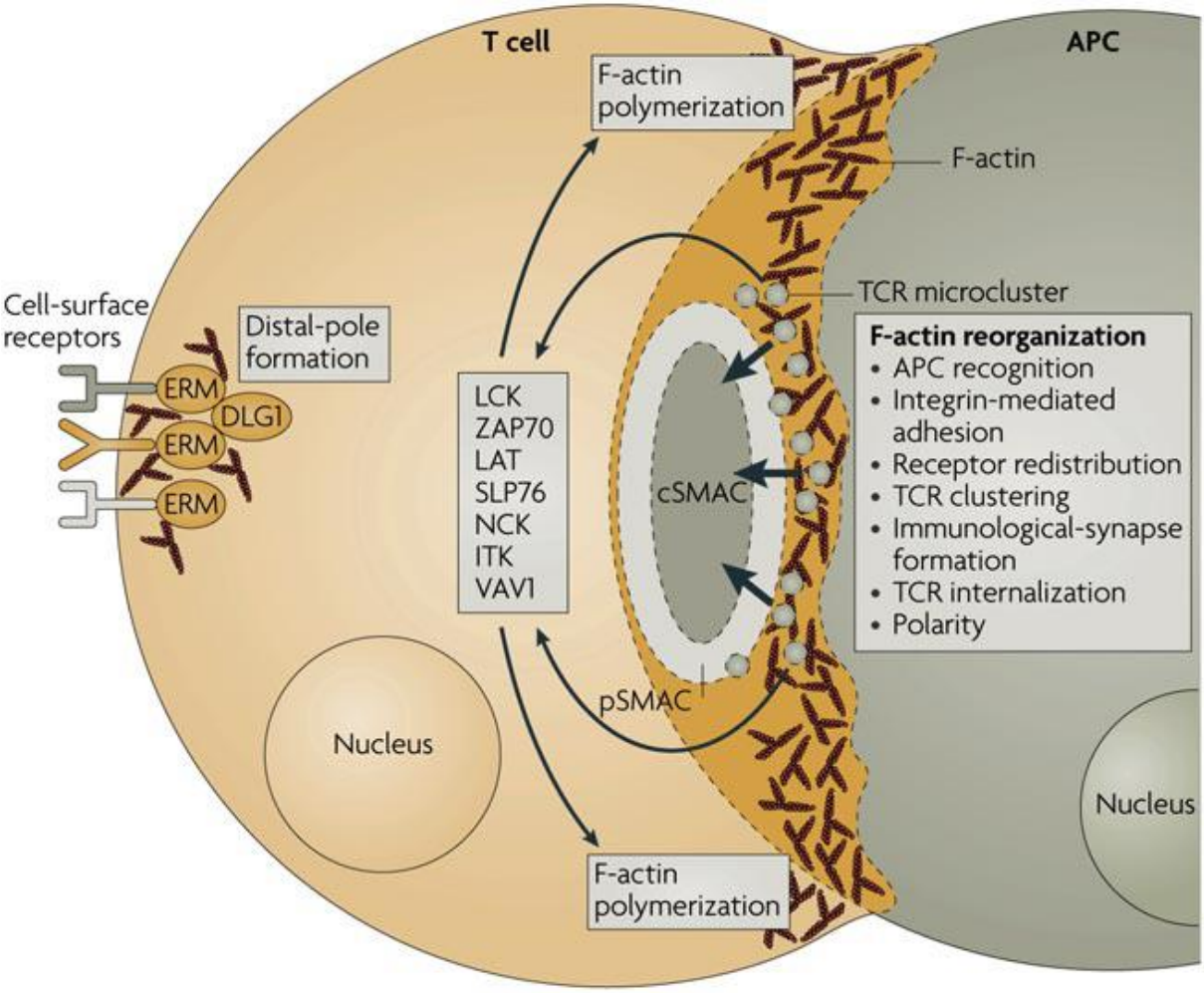



**Figure 1.2 Immunological synapse formation.** After TCR recognition of peptide-MHC, T-cell is polarised. On the contact site with APC, lamellipodium driven by F-actin extends the area of contact, and immunological synapse is formed. In the cSMAC are aggregated signalling molecules including TCR, CD4/8, Lck, ZAP-70, LAT and SLP-76. pSMAC harbours integrins, ensuring stable association with the APC. On the opposite end of the T-cell is formed distal pole complex, where are sequestered some of the proteins involved in the negative regulation of the signalling. Taken from (Billadeau *et al.*, 2007)

In the fully formed synapse, actin is restricted to the outer zones of the IS and is virtually excluded from the cSMAC, although some residual mircrofilaments has been observed. Other cytoskeletal network is placed in the centre of the IS, as the entire microtubule-organising centre (MTOC) moves and associates with the cSMAC through an Lck-dependent mechanism (Tsun *et al.*, 2011) and simultaneously brings Golgi apparatus to the vicinity of the IS. This facilitates secretion of cytokines and other protein through the IS directly towards the APC. Furthermore, during the formation of the cSMAC, TCR microclusters are formed on the periphery of the IS and are moved to its centre, first by actin-mediated mechanism (Kaizuka *et al.*, 2007), and then by mechanism depending on microtubules (Hashimoto-Tane *et al.*, 2011).

Important role in the formation of IS belongs to ERM proteins erzin and moesin. These proteins mediate interaction between actin cytoskeleton and cytoplasmic domains of transmembrane proteins. After TCR engagement, they are inactivated and the resulting detachment of the cortical actin network decreases the rigidity of the plasma membrane, enabling more efficient APC recognition (Faure *et al.*, 2004). ERM proteins are then translocated into a distal pole complex (DPC) (Fig. 1.2). DPC is a membrane structure on the opposite end of the cell than the IS and serves as a depository for proteins such as CD43, PSGL-1 and EBP50, which interacts with Cbp/PAG (Brdickova *et al.*, 2001), a negative regulator of Lck (Brdicka *et al.*, 2000). Moesin is consistently found in DPC, while erzin has been reported in the IS. Possible explanation for this is the regulation of translocation of other proteins.

## *7.2. T-cell activation models*

Despite the fact that the T-cell activation has been studied for several decades, the nature of its initial steps remains elusive. Many models have been suggested as to how is the signal from pMHC-TCR interaction transferred into the T-cell and to the downstream signalling (van der Merwe & Dushek, 2011). These models can roughly be divided into four groups, and are not necessarily mutually exclusive (Fig. 1.3).

### 7.2.1. Multimerisation of TCR

One problem with T-cell activation is low density of agonist pMHC on APC. Some theories suggest that the key to this problem is the multimerisation of surface molecules and their consequent activation. Besides, it is well established that it is possible to stimulate T-cells by artificial multimerisation by antibodies. In the most basic theory, it is heterodimerization of TCR with coreceptor CD4 or CD8 (Delon *et al.*, 1998), as they are associated with Lck (Rudd *et al.*, 1988; Veillette *et al.*, 1988). However, this cannot be the sole mechanism, as it is possible to elicit T-cell response even without the coreceptors (Schilham *et al.*, 1993). More complex theories has been therefore proposed, such as pseudodimer theory (Irvine *et al.*, 2002), which proposes that one pMHC is recognised by two complexes of TCR-coreceptor, one using TCR, the other coreceptor, and the latter amplifies the signal by interaction with

distinct self-peptide-MHC. This model has been discounted by the fact that MHC has a limited propensity to form dimers, even though, the formation of such complexes has been reported (Choudhuri & van der Merwe, 2007). Collectivelly, the receptor multimerization is not considered as the major driving mechanism of T cell proximal signaling, although it certainly propagates and enhances the signalling.

### 7.2.2. Change of conformation

This group of theories revolves around a very simple but strong premise that stimulated TCRs change their conformation or position with regards to neighbouring proteins. There is evidence suggesting that conformational change alone can induce at least some of the downstream signalling pathways (Gil *et al.*, 2002). Nevertheless, it seems unlikely that all the possible variety of the antigen recognition sites that are created largely by a random recombination would be able, upon the engagement, to undergo the same conformational in the cytoplasmic segments of TCR/CD3 chains. More complex theories

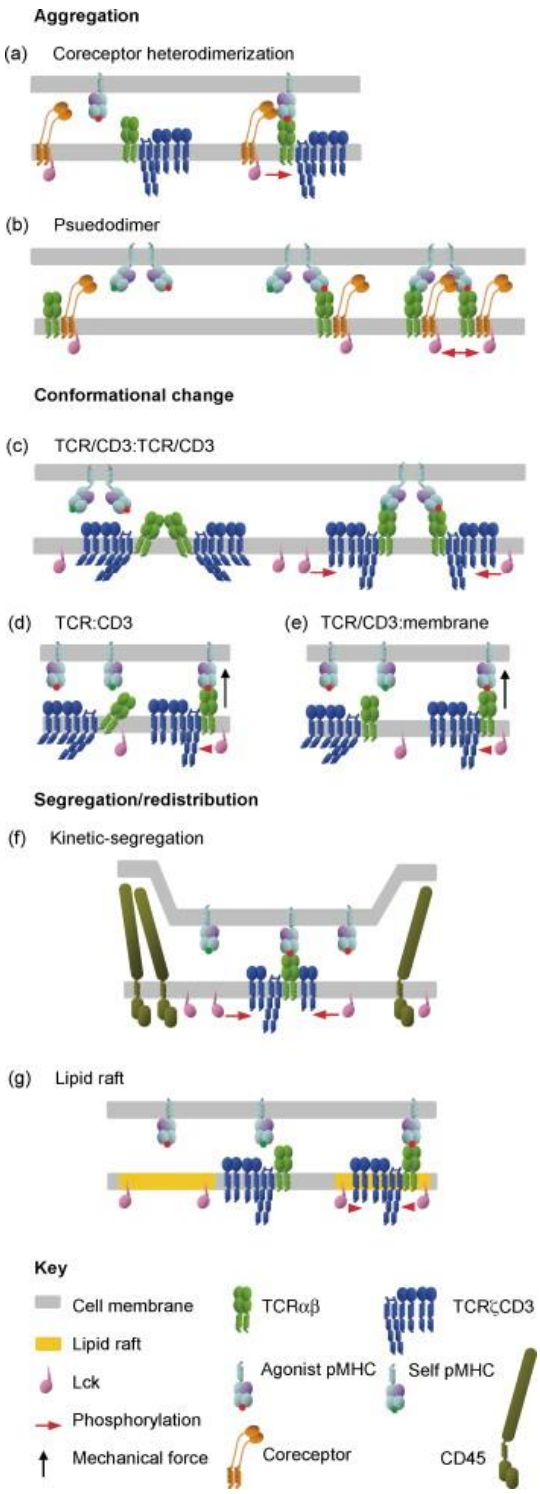



**Figure 1.3 T-cell activation models.** There are several theories trying to explain the mechanism of T-cell activation. Some of them see the key mechanism in clustering of TCR with CD4/8 (a) or distinct TCR complex (b). Next group expects that the activation is based on a conformational change between complexes (c), molecules within a single complex (d) or change in the relative position to the membrane by mechanical forces (e). In the kinetic segregation model, the activation is achieved by removing tyrosine phosphatases from signalling molecules due to their bulky extracellular domains (f), while lipid raft theory considers as crucial the translocation of the TCR complex to the lipid raft, where the rest of signalling machinery is located (g). Taken from (Choudhuri & van der Merwe, 2007)

circumvents this objection by moving from intramolecular change to intermolecular, suggesting positional changes of one TCR with respect to another (Ding *et al.*, 1999) or changes in mutual position of CD3 polypeptides to TCR complex or TCR complex itself within their multimers (Choudhuri & van der Merwe, 2007). This group also includes theories according to which the signal is transduced by a mechanical force exhorted on TCR upon engagement (Ma *et al.*, 2008). Such mechanical forces have indeed been determined on the T-cell-APC (Li *et al.*, 2010).

### 7.2.3. Kinetic segregation

The last model of T-cell activation is based on the mechanism of selective inclusion and exclusion of various proteins from the site of the forming IS based on the size of their extracellular domains (Davis & vanderMerwe, 1996). Specifically, many proteins required for activation of T-cells possess a relatively small extracellular domain; among those proteins are TCR, CD4, CD8 and CD28. The same applies to their binding partners on APCs, MHC and CD80/86. On the other hand, extracellular domains of phosphatases such as CD45 and CD148 are much larger (Fig. 1.4). These phosphatases are very potent and are capable of counteracting the tyrosine phosphorylation on ITAMs of CD3 and thus prevent the downstream signalling (Hui & Vale, 2014).

During the formation of the IS, a close membrane apposition limits the space for the interaction between MHC and TCR. Bulky extracellular domains of the phosphatases simply do not fit this confined space. They are therefore pushed out of this area and consequently the balance between tyrosine phosphorylation and dephosphorylation is nudged towards the former (Cordoba *et al.*, 2013).

This model is in agreement with the 'standby model' of TCR triggering proposed by Nika and colleagues (Nika *et al.*, 2010). Their and other's findings (Paster *et al.*, 2009) suggested that a substantial pool of Lck in naïve T-cells is activated prior to TCR engagement and upon T-cell activation remains constant. This is in a sharp contrast with previous dogma which dominated the field for previous 20 years that Lck is largely activated *de novo* after the TCR triggering. However, the persistence of the preactivated pool of Lck requires a mechanism preventing spontaneous triggering of T-cell activation. The authors suggested that the presence of CD45 phosphatase activity is sufficient to counteract SFK kinase activity through

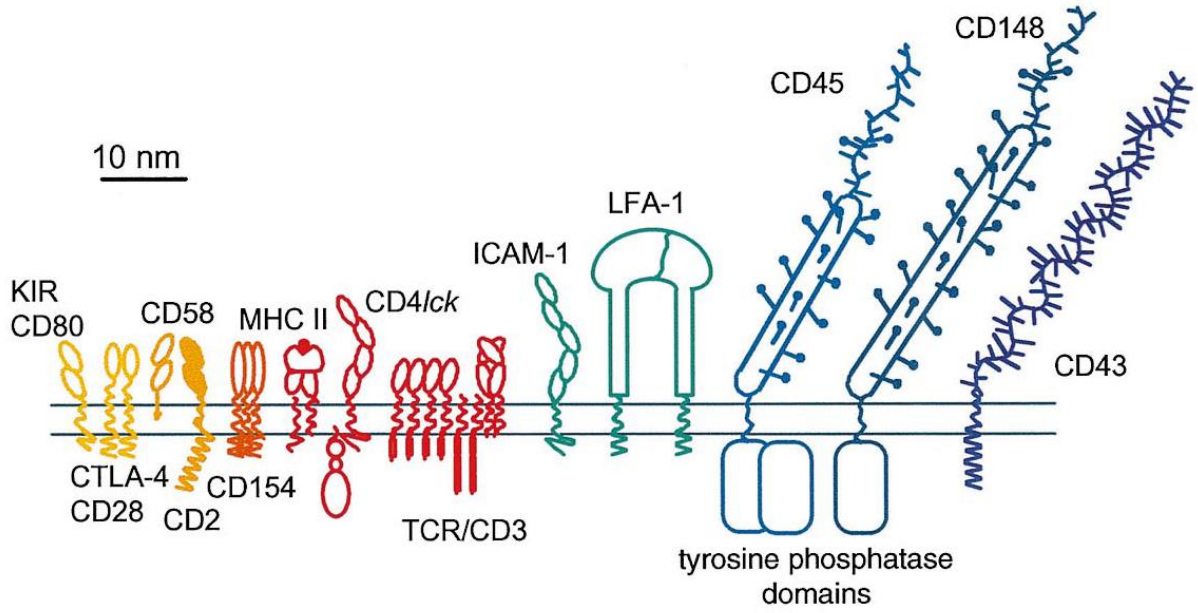



**Figure 1.4 Sizes of extracellular domains of selected signalling molecules.** Kinetic segregation theory expects exclusion of tyrosine phosphatases CD45 and CD148 from the immunological synapse because of their large extracellular domains that would not fit the space between membranes on the contact site between TCR and MHC, both of which have small extracellular domains. Integrin LFA-1, which has a medium-sized extracellular domain, can be found in the peripheral ring of the immunological synapse. Taken from (van der Merwe *et al.*, 2000)

highly effective dephosphorylation of CD3ζ chains, the proposition which finds its support in recently published experiments (Hui & Vale, 2014; Nika *et al.*, 2010).

### 7.2.4. Membrane microdomains

Another group of theories is based on the observed importance of membrane microdomains, or lipid rafts (LR), for TCR signalling (Horejsi, 2005). These models suggest that after the stimulation of the TCR, the entire TCR complex is translocated into the LR. LR represents a distinct membrane subcompartment, which concentrate molecules important for signal transduction, such as LAT. The translocation brings the TCR complex capable of initiation of the signalling to a specific environment conducive for sustained signal transduction into the interior of the cell.

An important role in this mechanism has been attributed to the translocation of Lck into LR and its subsequent interaction with Fyn, another member of SFKs (Filipp & Julius, 2004). In primary naive CD4+ T-cells Fyn is LR-resident protein and up to 98% is reproducibly detected in this membrane subcompartment. On the other hand, 75-95% of Lck is located

outside the lipid rafts prior to the T-cell activation. After TCR engagement by coaggregation of CD4 and TCR, Lck is activated and within seconds rapidly translocates alongside TCR and CD4 to the lipid rafts, where it activates Fyn (Filipp *et al.*, 2003). The translocation mechanism, however, remains obscure. It appears to be dependent on the kinase activity of Lck, since kinase inactive mutants fail interact with Fyn and activate it (Filipp *et al.*, 2008). It has also been suggested that the C-terminus of Lck may play role in this translocation. It has been shown that its sequential truncations lead to the loss of translocation capability and diminishment of kinase activity of Lck and subsequently decreased IL-2 production (Filipp *et al.*, 2008). We hypothesized that the C-terminus of Lck may be involved in a previously undescribed protein-protein interaction which allows its translocation to LR via linking to the elements of cytoskeletal network. Using the comparative 2D electrophoresis coupled with mass spectrometry analysis several candidate proteins were identified (Filipp, unpublished data). Using this screen, one of the most suitable Lck interaction candidate partner has been identified as adaptor protein RACK1 – Receptor of activated C kinase. The interaction of Lck with RACK1 and the role of the C-terminus of Lck in T-cell activation will be examined in this thesis.

## *7.3. Lck*

Lck is a prototypical member of SFKs, a group of essential nonreceptor tyrosine kinases involved in signal transduction. Three kinases of this family, Src, Yes and Fyn are ubiquitously expressed, while other members of the family (Fgr, Lck, Hck, Blk, Lyn and Frk) show more cell type specific expression pattern (Thomas & Brugge, 1997). Specifically, Lck expression is confined to leukocytes (Perlmutter *et al.*, 1988), with highest expression levels in T-cells and NK-cells and low expression in B-cells and dendritic cells.

### 7.3.1. Structure of Lck

The overall structure of Lck is the same as other SFKs. It consists of four domains, called a unique, SH3 (Src homology 3), SH2 and kinase, or SH1, domain. In addition, three functionally distinct segments can be also distinguished: (i) the N-terminal sequence responsible for the attachment of Lck to the membrane (see below), (ii) a proline-rich region

placed between SH2 and SH1 domain, and (iii) an unstructured C-terminal tail, which contains tyrosine residue Y505 that is crucial for the negative regulation of the kinase.

A relatively short N-terminal sequence of 10 aminoacids of Lck contains three potential sites for acylation, specifically myristoylation on glycine in position 2, and palmitoylation on cysteins in position 3 and 5 (Abraham & Veillette, 1990; Koegl *et al.*, 1994). These lipid modifications mediate Lck targeting into LR, which appears to be important for Lck function.

The following unique domain is the only sequence in which all SFKs differ significantly and is thus often used for the generation of a kinase specific antibody. Furthermore, this domain bears a dicystein motif, which via zinc ion coordinates the interaction between Lck and CD4 or CD8 coreceptor (Kim *et al.*, 2003). If this interaction is interrupted, the T-cell activation is prevented. Moreover, Lck binding to CD4 prevents the internalization and degradation of the latter by masking its dileucine internalisation motif.

SH3 domain is a protein domain fold widely used in various proteins. It is able to interact with proline-rich sequences in polyproline helix type II conformation. Such a helix displays a shape of a triangle on an intersection with proline residues on one side. The proline residues intercalate between the aromatic residues of the SH3 domain and form a stable complex (Feng *et al.*, 1994).

Similar to SH3 domain, SH2 domain is also very common in signalling proteins. It is capable of inducible binding to a phosphorylated tyrosine residue. It contains two pockets responsible for the recognition; one binds the phosphotyrosine, the other one binds a hydrophobic residue, preferably isoleucine (Waksman *et al.*, 1993). Between these two aminoacids are two other residues that are less important for binding, but, nevertheless, play a role in its binding specificity. The optimal consensus binding sequence of SH2 domain of Lck is pYEEI (Zhou *et al.*, 1993).

The only domain in Lck that exhibits a catalytic function is the kinase domain. It is analogous to other kinase domains and contains an N-lobe and a C-lobe with catalytic cleft in between. An important part of the C-lobe is an activation loop, whose tyrosine phosphorylation in position Y394 increases the activity of Lck by displacing it from a position preventing the kinase domain to acquire the optimal conformation for the catalysis (Xu *et al.*, 1999). The Y394F Lck mutant exhibits severely compromised kinase activity.

The C-terminus of Lck is an 11 aminoacids long unstructured tail. Despite its shortness, it is extremely important as it carries a negative regulatory tyrosine crucial for keeping Lck in inactivated or closed conformation. Other mechanism how the C-terminal tail sequence imposed its effect on the function of Lck has been proposed (Filipp *et al.*, 2008). This additional mode of action is described below and is the subject of investigation presented in this thesis.

### 7.3.2. Regulation of Lck

Activity of Lck is regulated by tyrosine phosphorylation. This phosphorylation is regulated by several additional proteins and their action results in important changes in the structure of Lck.

#### 7.3.2.1. Conformational forms

The regulation of Lck is based on phosphorylation of two regulatory tyrosines, activatory Tyr394 and inhibitory Tyr505. The activatory tyrosine 394 is located in the activation loop of the kinase domain and its phosphorylation enables more efficient phosphorylation of the substrate. In contrast, the regulation by phosphorylation of Tyr505 downregulates the kinase activity by causing the formation of intramolecular interactions between pY505 and its own SH2 domain (Xu *et al.*, 1999).

Although the SH2 and SH3 domains are on the opposite site of the kinase domain relative to its catalytic cleft, their interaction with pY505 and polyproline helix, respectively, change the angle between C- and N-lobes and decreases flexibility of the catalytic cleft, which results in decrease of the kinase activity (Cowan-Jacob *et al.*, 2005).

Three conformational states of Lck have been described with regard to its activity and phosphorylation state, termed closed, primed and open (Fig. 1.5). Closed and catalytically inactive conformation has phosphorylated Tyr505 bound to SH2 and both SH3 and SH2 domains are packed against the kinase domain. Primed conformation has neither of the regulatory tyrosines phosphorylated and SH3 and SH2 domain are thus unbound. The catalytic activity is approximately five times higher than that with the closed conformation with pTyr505 Lck (Hui & Vale, 2014). The phosphorylation on Tyr394 leads to additional two-

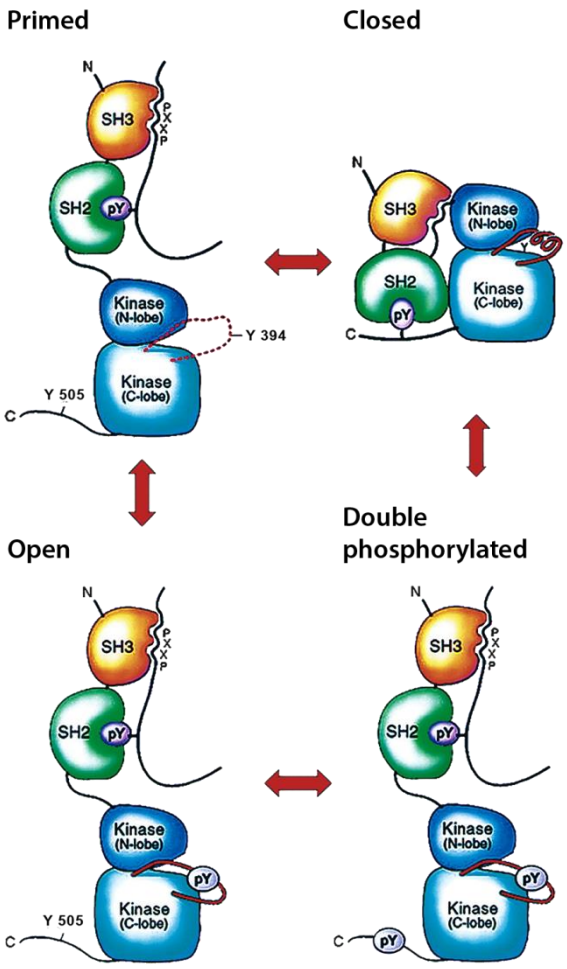



**Figure 1.5 Conformational forms of Lck.** Lck can be found in four conformational forms, based on the phosphorylation status of two regulatory tyrosines. Primed conformation carries no phosphorylated tyrosine and exhibits a basal level of kinase activity. The activity can be minimized by phosphorylation on negative regulatory Tyr505, which leads to the acquisition of closed conformation caused by intramolecular interaction of the SH domains. Phosphorylation of the activatory Tyr394 on the other hand leads to a full activation of Lck by fixing activation loop in a position favouring the phosphotransfer reaction. Double phosphorylated form has been described as mostly derived from an active conformation and its activity is comparable with that of primed form of Lck. Modified from (Harrison, 2003)

fold increase in Lck activity, making it approximately ten times more active than Lck with a closed conformation (Hui & Vale, 2014).

In 2010, Nika et al.(Nika *et al.*, 2010) described a previously observed but ignored phosphorylation state whereby Lck carries both Tyr394 and Tyr505 phosphorylated. Despite the phosphorylation of the inhibitory tyrosine, this form of Lck is active matching the kinase activity of primed Lck (Hui & Vale, 2014; Nika *et al.*, 2010). The conformational status of this form has not been addressed, but based on the kinase activity, SH3 and SH2 domains are unlikely to form intermolecular interactions.

#### 7.3.2.2. Regulation of phosphorylation

The activation on Tyr394 is mediated by trans-autophospharylation between two molecules of Lck (Hui & Vale, 2014). Lck has also been showed to cause trans-autophosphorylation on Tyr505, although the main protein performing this inhibitory phosphorylation is Csk (Bergman *et al.*, 1992). This kinase is considered to be a key negative regulator of Lck and its knockout leads to a spontaneous activation of T-cells (Schmedt *et al.*, 1998). However, Csk is a cytosolic protein, whereas most of Lck is membrane-bound. For the purpose of

translocation to the membrane, Csk binds to a membrane adaptor protein Cbp/PAG, thus enhancing the kinetics of Lck phosphorylation (Brdicka *et al.*, 2000). Furthermore, it associates with PEP phosphatases, capable of dephosphorylation of pTyr394, which strengthen the inhibitory effect of Csk (Cloutier & Veillette, 1996).

The main regulatory phosphatase of Lck is CD45, a very potent enzyme abundantly expressed in T-cells. It is able to dephosphorylate both activatory and inhibitory tyrosines, predisposing them for activation or inhibition, depending on the actual needs (Mustelin & Altman, 1990). Its overall effect on Lck is considered to be activatory, since the primed conformation exhibits kinase activity, although no full.

## *7.4. RACK1*

Receptor for activated C-kinase 1 (RACK1) belongs to an ancient and conserved family of WD40 proteins. Members of this family are present in all eukaryotes and even some prokaryotes (Li & Roberts, 2001). The family of these proteins contain four to sixteen WD40 repeats. The most typical and conformationaly favoured number of repeats is seven. These proteins acquire a doughnut-shaped conformation of seven-bladed β-propeller (Carrillo *et al.*, 2012). Each WD40 repeat consists of four β-sheets, first of which is part of the previous structural segment (Fig. 1.6). The loops between the β-sheets form variable surfaces capable of interaction with many other proteins. On the β-propeller structure, proteins can bind to its top, circumference or the bottom. The most commonly used is the top (Stirnimann *et al.*, 2010), and commonly used site is the central channel (Russell *et al.*, 1998). This extensive binding surface predicates WD40 proteins to work as scaffolding proteins and to regulate other proteins by specific interactions. WD40 domain also belongs to the most common folds, which indicates its importance (Stirnimann *et al.*, 2010). While WD40 domain itself never possesses the catalytic activity, they can be part of a protein whereby such activity is executed by other linked domains (Stirnimann *et al.*, 2010).

### 7.4.1. Roles of RACK1

RACK1 is a 36kDa seven-bladed β-propeller protein with no additional domains (Yatime *et al.*, 2011). It is able to interact with many proteins including itself (Yatime *et al.*, 2011).

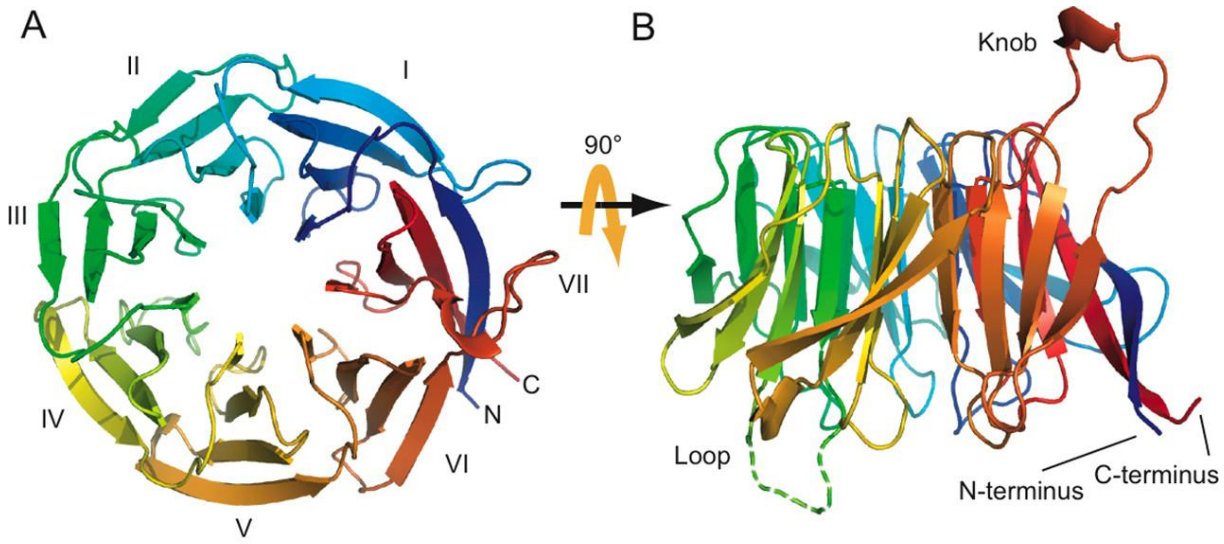



**Figure 1.6 Structure of RACK1.** RACK1 has a structure of seven-bladed β-propeller. It consists of seven WD40 repeats forming seven structural elements. Each element consists of four β-sheets, first of which belongs to a previous WD40 repeat. Loops between the sheets provide diverse binding sites and enable the interaction of RACK1 with a plethora of other proteins. Taken from (Coyle *et al.*, 2009).

RACK1 deficiency in mice is lethal during gastrulation, although heterozygotes compensate the gene dosage and their phenotype is very mild (Volta *et al.*, 2013). Although it has no catalytic activity, it is an important protein, as it is part of various protein complexes in signalling pathways, it is involved in the regulation of ubiquitinylation (Zhang *et al.*, 2012), transcription (Ceci *et al.*, 2003; Kouba *et al.*, 2012) and circadian rhythm (Robles *et al.*, 2010). Its propensity for homodimerisation expands its capacity to bind proteins which share the same docking site on RACK1 (Thornton *et al.*, 2004). Its importance is underlined by its high evolutionary conservation (Neer *et al.*, 1994). Some of the most important roles of RACK1 are briefly outlined below.

RACK1 was observed as a member of various signalling pathways, serving as an adaptor protein. To this day, almost 160 proteins have been reported to be associated with RACK1 (thebiogrid.org). This numerous binding capability predisposes RACK1 for mediating protein-protein interactions. Later another cellular pool of RACK1 was identified, associated with ribosomes, where it plays an indispensable role in activation of translation (Shor *et al.*, 2003).

RACK1 was first described as a protein binding activated PKCβII and translocating it within the cell to a distinct location (Ron *et al.*, 1994). RACK1 is able to associate also with other isoforms of PKC. Therefore it can recruit other proteins to the vicinity of PKC or move PKC to

their proximity. For example, it has been showed to mediate the crosstalk between PKC with MAP kinase pathway by associating with MAP kinase JNK (Lopez-Bergami *et al.*, 2005).

RACK1 has been detected in the crystal structure of the small 40S subunit of the ribosome (Sengupta *et al.*, 2004). Being position on the surface of the ribosome, it can serve as a platform for aggregation of signalling molecules. Among others, it has been shown to interact with the transcription initiation factor eIF3 which is essential for the regulation of transcription (Kouba *et al.*, 2012). Also, the recruitment of PKCβII to the ribosome by RACK1 is crucial for the initiation of translation (Ceci *et al.*, 2003). In this scenario, PKC phosphorylates eIF6, which consequently dissociates and allows the association of small and big subunit of the ribosome, thus initiating translation. On the other hand, RACK1 can also promote the repression of gene expression. This is mediated by the mechanism where RACK1 can enhance the recruitment of miRNA silencing complex directly to the ribosome (Jannot *et al.*, 2011). It has also been shown that RACK1 participates in the nascent peptide-dependent translation arrest (Kuroha *et al.*, 2010).

Upon the signal by cAMP, RACK1 can be translocated to the nucleus by 14-3-3ζ protein. Once there, it is capable of altering the expression by promoting histone acetylation what leads to the production of c-Fos, which in turn changes the expression pattern of numerous other proteins (He *et al.*, 2002).

RACK1 also plays a regulatory role in neurons. GABA receptor is modulated by phosphorylation caused by PKC, which is dependent on RACK1 (Brandon *et al.*, 2002). Glutamate receptor NMDAR is also regulated by RACK1. It brings Fyn kinase to its proximity by homodimerization (Thornton *et al.*, 2004), but Fyn cannot increase activity of the receptor by phosphorylation until both Fyn and NMDAR are released from RACK1 upon cAMP signalling (Yaka *et al.*, 2002).

Protein translocation by RACK1 is used also in the regulation of cytoskeleton. RACK1 interacts with plectin, protein interacting with all three elements of cytoskeleton (Osmanagic-Myers & Wiche, 2004). This study defines plectin as a protein sequestering RACK1 to the cytoskeleton, and plectin-RACK1-PKC complex is released after stimulation of the cell by EGF, leading to the formation of focal adhesions. RACK1 plays an important role in formation of focal adhesion. It binds β chains of integrins, Src and FAK kinases and integrates signals from integrins with those from growth factor receptors (Cox *et al.*, 2003). Src then phosphorylates FAK and hence increasing its activity (Kiely *et al.*, 2009).

Src family kinases Src, Fyn and Lck have been shown to bind RACK1 (Chang *et al.*, 1998). This interaction has an inhibitory effect on their kinase activity (Chang *et al.*, 1998). Src is bound to the phosphotyrosine 246 on the sixth WD40 domain (Chang *et al.*, 2001). Src itself, in turn, is capable of phosphorylation of Tyr228 and Tyr246 on RACK1 (Chang *et al.*, 2002). Other four tyrosines on RACK1 are phosphorylated by different kinases.

All these mechanisms, in which RACK1 plays an important role, indicate that this molecule is highly versatile and play a pleiotropic function in the translocation mechanisms of other proteins during activation events. Thus RACK1 was a well-suited candidate, identified in our functional screen, which could orchestrate the translocation of Lck to the lipid rafts during early phases of T-cell signalling. This potentially novel role in T cell activation was the main objective of inhere presented thesis.

# 8. Material and Methods

Cell lines

NIH3T3 and SYF fibroblast lines were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco); HEK293T, HEK293, AD 293 fibroblast cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies); J774.2 macrophage cell line and Jurkat T-cell line were maintained in RPMI 1640 (Life Technologies). All media were supplemented with 10% of inactivated fetal calf serum (FCS) and 100 Units of penicillin / 10µg streptomycin antibiotics (Sigma) per 1 ml of media.

Mice

Double transgenic mice tg D011.10 TCR / tg CARΔ (TgCAR) (Taconic) mouse strain expressing human coxsackie/adenovirus receptor lacking a cytosolic domain (Murphy *et al.*, 1990; Wan *et al.*, 2000a) six to eight weeks old, were used for experiments. Mice were housed in a specific pathogen-free animal facility at Institute of Molecular Genetics (Prague, Czech Republic).

Cloning

PCR constructs created using Pfu polymerase (Thermo Scientific) were first cloned into pJET using CloneJET PCR Cloning Kit (Thermo Scientific) according to manufacturer's instructions. Then they were excised by restriction endonucleases and ligated into definitive plasmid. Constructs were verified by sequencing.

Plasmids

Lck and RACK1 constructs were cloned into MIGR1 (Pear *et al.*, 1998) , plasmid with EGFP under control of IRES. Constructs for in-vitro translation were cloned into pET-42 (a gift from laboratory of Dr. Vladimír Kořínek; contains GST tag) or pET-15 (a gift from laboratory of Dr. Jan Černý) after it was provided with 3xFLAG tag derived from Sigma plasmids. HA-RACK for preparation of adenovirus was cloned into pSH-UP-UP (gift from laboratory of Dr. James DeGregory, University of Colorado) (Fig. 3.4a), plasmid with two ubiquitin promotors, one controlling inserted gene, the other EGFP.

Site-directed mutagenesis

ΔC7, ΔC9 and new ΔYQPQP mutants were created by GeneTailor site-directed mutagenesis system (Invitrogen) according to the manufacturer's instructions.

Bacteria transformation

50 µl of TOPO10 E. coli were thawed on ice, plasmid or ligation mixture was added, and kept 20 minutes on ice. Cells were then warmed to 42 °C for 90 seconds and cooled on ice for 1 minute. Then was added 200 µl of pre-warmed SOC medium (Invitrogen) and cells were shaking for 30 minutes at 37 °C. Cells were then seeded on plates with an antibiotic according to the plasmid (kanamycin or ampicillin).

Plasmid isolation

Plasmids from 3 ml (QIAprep Spin Miniprep Kit, Qiagen) or 100 ml (QIAGEN Plasmid Midi Kit, Qiagen) of overnight bacterial culture were harvested according to the manufacturer's instructions. Plasmid concentration was determined spectrophotometrically using Nanodrop ND-1000 (Thermo Scientific).

Transient transfection of the cells

Overnight, approx. 70% confluent culture of the cells was transfected using Lipofectamine LTX or Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Creation of stable cell lines (retroviral cell infection)

HEK 293T cells were transfected with equal amounts of GAG-POL, ENV and desired plasmid. Next day, medium of SYF cells was replaced by retrovirus-containing medium from HEK 293T cells; their medium was replenished. Same procedure was performed next day. After sufficient propagation of SYF cells, GFP-positive SYF cells were sorted.

Flow cytometry and cell sorting

FACS analysis was performed on LSRII or FACSCalibur cytometers (BD Biosciences). Data were analysed by FlowJo software. Cell sorting was performed by Zdeněk Cimburek on Influx cell sorted (BD biosciences).

Antibodies

*Primary antibodies*

| | |
|---|---|
| Anti-Lck | Gift from laboratory of prof. Václav Hořejší |
| Anti-pY394 Lck | Santa Cruz |
| Anti-pY505 Lck | Invitrogen |
| Anti-RACK1 | Santa Cruz |
| Anti-FLAG | Sigma |
| Anti-GST | Gift from laboratory of prof. Václav Hořejší |
| Anti-HA | Sigma |
| Anti-GFP | Abnova |
| Anti-Myc | Gift from laboratory of prof. Václav Hořejší |
| Anti-GADS | Santa Cruz |
| Anti-pY (4G10) | Gift from laboratory of prof. Václav Hořejší |
| Anti-GAPDH | Sigma |
| Anti-TCRβ (biotinylated) | eBioscience |
| Anti-CD4 (biotinylated) | eBioscience |
| Anti-CD69 – PE-Cy7 | eBioscience |
| Anti-CD62L – PE | eBioscience |
| Anti-CD25 – PE | eBioscience |

*Secondary antibodies*

| | |
|---|---|
| Goat-anti-Mouse HRP | BioRad |
| Goat-anti-Rabbit HRP | BioRad |
| IRDye 680 anti- Mouse | LI-COR |
| IRDye 800 ant- Rabbit | LI-COR |
| Goat-anti-Mouse light chain specific HRP | Jackson ImmunoResearch Laboratories |
| Mouse-anti-Rabbit light chain specific HRP | Jackson ImmunoResearch Laboratories |

SDS PAGE and western blotting

Samples were boiled for 10 minutes in 2x Laemmli sample buffer and then resolved on 8%, 10% or 12% polyacrylamide gels. Proteins were transferred onto PVDF membrane (Millipore) and blocked for 1 hour in 5% non-fat milk at RT. Blots were then incubated for 1 hour with

primary antibody diluted in blocking buffer, washed and incubated for 1 hour with secondary antibody conjugated to horseradish peroxidase (HRP), developed by incubation with ECL substrate (Thermo Scientific) and visualised by X-ray films. Blots for phosphotyrosine staining or analysis by fluorescence scanner were blocked for 1 hour in 3% gelatine in TBS-T at 37°C. Blots were then incubated for 1 hour with primary antibodies diluted in blocking buffer, washed and incubated for 1 hour with secondary antibodies conjugated fluorescent dye (IRDye) and scanned on Odyssey CLx Infrared Imaging System (LI-COR). Phosphotyrosine staining could be determined also by ECL substrate. Densitometry quantification was performed by AIDA image analyser software from the raw image data.

T-cell isolation and activation

The method was described in (Ballek *et al.*, 2012). Briefly, CD4+ T-cells were isolated from lymph nodes and purified by MACS CD4+ T-cell isolation kit (AutoMACS, Miltenyi Biotec) to ~95% purity. T/cells were precoated in biotinylated antibodies anti-TCR (1 µg/ml) and anti-CD4 (0,3 µg/ml), washed and activated by antibody-crosslinking by streptavidin (final concentration of 50 µg/ml in 20 µl). Activation was ended by addition of Laemmli sample

Adenovirus production and transduction of T-cells

HA-RACK was cloned into pSH-UP-UP plasmid. It was used for production of adenoviruses by AdEasy™ Adenoviral Vector System (Agilent Technologies) according to the manufacturer's instructions. Virus was created and amplified in AD-293 cells. Isolation of virus was performed when cytopathic effect of the virus was present (Fig. 2.1). Virus titer was determined by AdEasy™ Viral Titer Kit (Agilent Technologies) and MOI was calculated.

Transduction of naïve $CD4^+$ T-cells isolated from CAR mice was performed as described elsewhere (Zha et al., 2008). Briefly, T-cells were resuspended in DMEM with 2%. Viral particles are added at desired MOI and cells are incubated for 1 hour at 37°C. Cell with virus is then cultured at 37°C in complete RPMI 1640 medium supplemented with 2ng/ml IL-7 (PeproTech). Live T-cells were then sorted and left to rest for 4 hours. Then they are used for further experiments.

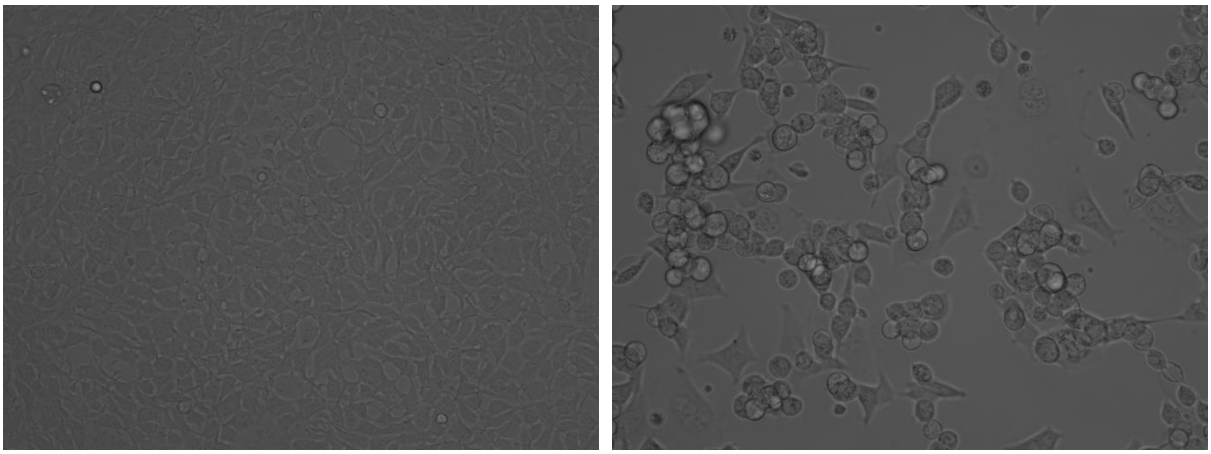


**Figure 2.1 Cythopatic effect of adenovirus.** Adenovirus amplification induces cytopathic efect. Compared to uninfected cells (left), infected cells round up and lose adhesion.

BMDC preparation and T-cell activation

Bone marrow was isolated from femurs and tibias of mice, erythrocytes were lysed by ACK buffer, remaining cells were washed and cultured in RPMI with GM-CSF for 7 days. Cultured cells were considered to be bone marrow-derived dendritic cells (BMDC).

BMDC were pulsed by $OVA_{323-339}$ peptide for 2 hours and mixed with $CD4^+$ T-cells at 1:4 ratio for T-cell activation. CD69 and CD25 production was analysed by FACS.

Proliferation assay

$CD4^+$ T-cells were stained by eFluo670 proliferation dye (eBioscience) for 10 min, washed and mixed with BMDC pulsed by $OVA_{323-339}$ peptide for 2 hours at 4:1 ratio. After 72 hours, cells were stained against CD4 and analysed by FACS.

In-vitro translation

TnT® T7 Quick Coupled Transcription/Translation System (Promega) was used. To 40 µl of mastermix was added 1µg of plasmid, 1 µl of methionine and water to 50 µl. The reaction was kept on 30°C for 90 minutes. 2 µl were used as control of successful transcription and 11,5 µl was used for immunoprecipitation experiments.

Immunoprecipitation

Cells were lysed in TNE buffer containing 1 % on NP-40 and protease and phosphatase inhibitor cocktails (Roche). Lysates were incubated on ice for 30 min; nuclei were then spun

down (800 RPM, 2 min, 4°C) and discarded. In vitro translation reactions were diluted in TNE buffer containing 1 % on NP-40 or RIPA buffer. Lysates were loaded on agarose anti-HA conjugated beads and incubated overnight 4°C. Alternatively, lysates were pre-cleared by unprecoated magnetic beads, and then loaded on magnetic beads precoated by antibody and incubated overnight at 4°C. Beads were washed 6 times by lysis buffer and boiled in 2x Laemmli sample buffer.

# 9. Results

## *9.1. The role of the C-terminus of Lck in the complex formation with RACK1*

RACK1 has been identified as the candidate Lck interacting partner based on its tyrosine phosphorylation which was dependent on the presence of the intact C-terminal tale of Lck (Filipp *et al.*, 2008). Based on our previous and already published results we predicted that the progressive truncations of the tail will have no or only a limited impact on the kinase activity of Lck, but will negatively affect its capacity to interact with RACK1 as well as to phosphorylate its multiple intracellular targets (Filipp *et al.*, 2008). In order to precisely map the sequence and the requirement for this Lck tail segment in this interaction, we created stable cell lines expressing various mutated forms of Lck. The cell lines were derived from SYF cells, mouse fibroblasts that lack the expression of the triad of ubiquitously expressed SFKs, Src, Yes and Fyn (Klinghoffer *et al.*, 1999). Therefore the only expressed SFK in these cells was the wild type and various mutant form of Lck introduced into these cells retrovirally.

### 9.1.1. Preparation of mutant cell lines

Mutated Lck forms were cloned into Migr1, a plasmid with EGFP under control of IRES downstream of the cloned protein, which enables retrovirus encapsulation. Migr1 empty (control), WT (wild type Lck), W97A (SH3 domain inactive), R154K (SH2 domain inactive), K273A, Y394F (both kinase domain inactive), Y505F (constitutionally active), ΔQP, ΔQPQP, ΔYQPQP and ΔC11 constructs (Fig. 3.1a, b) were prepared as described previously (Filipp *et al.*, 2008). Migr1 with ΔC7 and ΔC9 Lck mutants were prepared by the site-directed mutagenesis. All the truncation mutants were created on Y505F Lck template. Retroviral particles containing the constructs were created and used for SYF cells infection. SYF cells infected with retroviral particles carrying the Lck constructs were sorted out based on an equal expression of EGFP. During the sequencing analysis, we discovered a possible

mutation in a previously prepared plasmid with ΔYQPQP mutation and thus we generated its new version from ΔQPQP template.

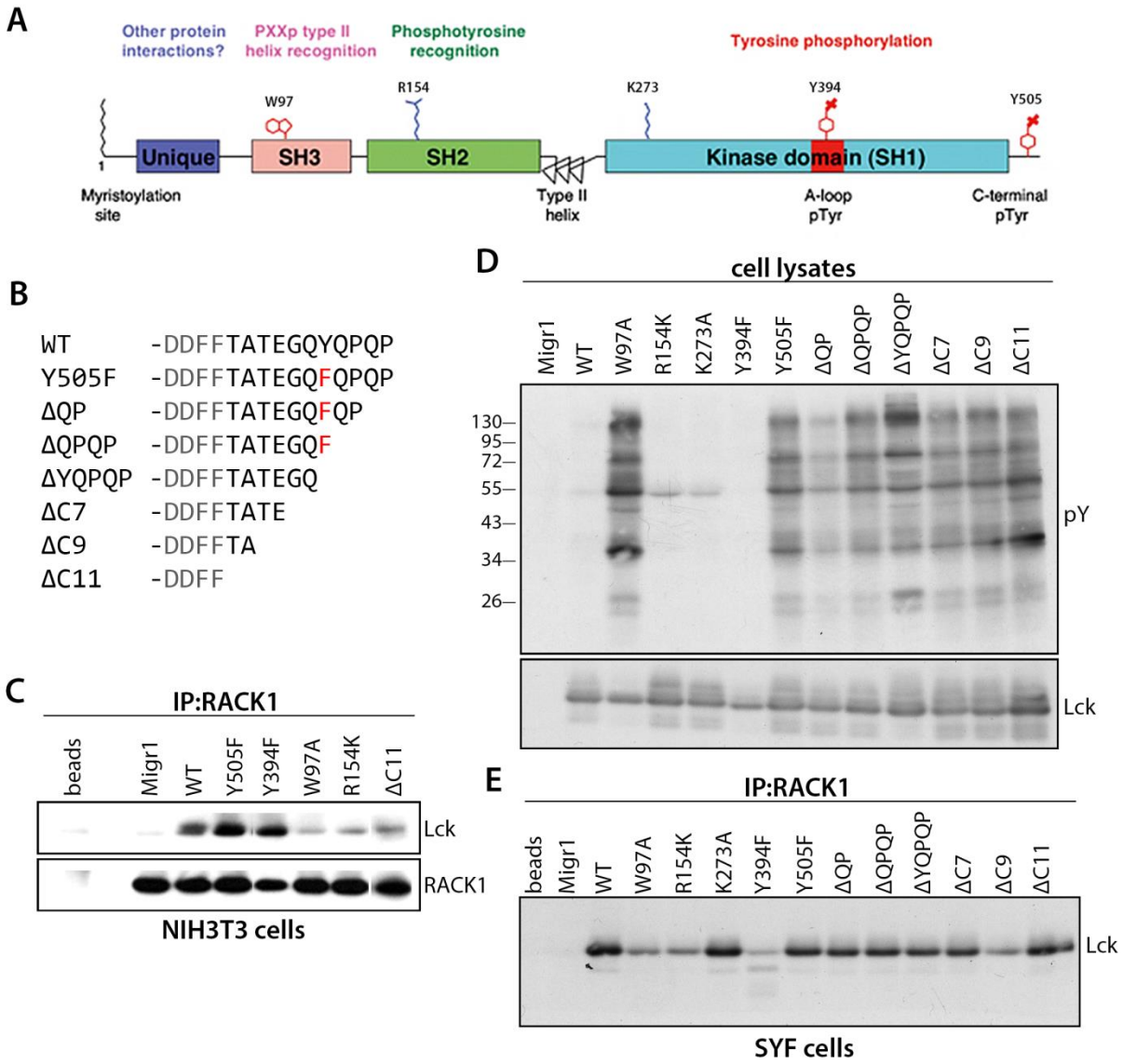



**Figure 3.1 Influence of C-terminus on activity of Lck and its interaction with RACK1.** Functional mutants of Lck were prepared, disabling SH3 (W97A), SH2 (R154K), kinase domain (K273A, Y394F) and negative regulatory segment (Y505F) (a), and cloned into Migr1 plasmid. A series of mutants containing progressive C-terminal truncations was prepared on the background of constitutively active (Y505F) Lck (b). It was previously shown that SH2, SH3 and the C-terminus of Lck are important for binding of RACK1 in NIH3T3 cells (c) (Ballek, 2009). We created cell lines with stable expression of our constructs in SYF cells which, unlike NIH3T3, lack the expression SFKs. These cells were then analysed for their global tyrosine phosphorylation (d). They were also used for experiments determining the influence of the mutations on the interaction of Lck and RACK1 by immunoprecipitation of RACK1 (e).

### 9.1.2. Tyrosine phosphorylation status

First, we assess the kinase activity of the wild type and mutated forms of Lck. The total lysates from infected SYF cells were stained by phosphotyrosine-specific antibody (4G10) (Fig. 3.1d). The observed tyrosine phosphorylation pattern can be attributed only to Lck since the background signals in mock cells infected with the control empty plasmid as well as those with the kinase inactive Lck, are negligible. As expected, the WT and kinase dead (K273A, Y394F) mutants showed limited or no phosphorylation. Somewhat unexpectedly, and as illustrated in Fig. 3.1d, phosho-pattern of some Lck mutant constructs differed from those previously published (Filipp *et al.*, 2008). Specifically, SH2 domain mutant (R154K) no longer possessed the expected kinase activity. In contrast, and in the context of the main purpose of this experiment, we did not see the expected pattern of kinase activity in the tail truncation mutants. While published data showed virtually no Lck activity of ΔYQPQP mutant towards its intracellular targets, the kinase activity of all truncation mutants in our experiments was comparable, and in case of ΔYQPQP even slightly elevated in comparison to that expressing the constitutively active Y505F. There was some decrease in the activity of ΔQP, but no clear and reproducible pattern could be identified.

### 9.1.3. RACK1-Lck interaction

Next, we tested whether the C-terminal sequential truncations of Lck impact its interaction with RACK1. It was previously shown that in NIH3T3 cell lines, SH3, SH2 and the C-terminal tail of Lck are involved in binding to RACK1 (Fig. 3.1c) (Ballek, 2009). We therefore performed the immunoprecipitation of RACK1 from freshly prepared SYF cells infected with Lck mutant constructs and compare the amount of Lck present in the complexes (Fig. 3.1e). Results confirmed that both SH3 and SH2 domains are involved in the interaction with RACK1. Specifically, as W97A and R154K mutants exhibited decreased binding, but neither mutant abolished this interaction completely. Y394F Lck mutant with removed activatory tyrosine 394 also caused severe diminishment of this interaction. The only apparent C-terminal truncation which exhibited influence over the Lck-RACK1 , interaction was ΔC9 mutant whose binding to RACK1 was decreased to the level comparable to those of SH2 and SH3 domain mutants.

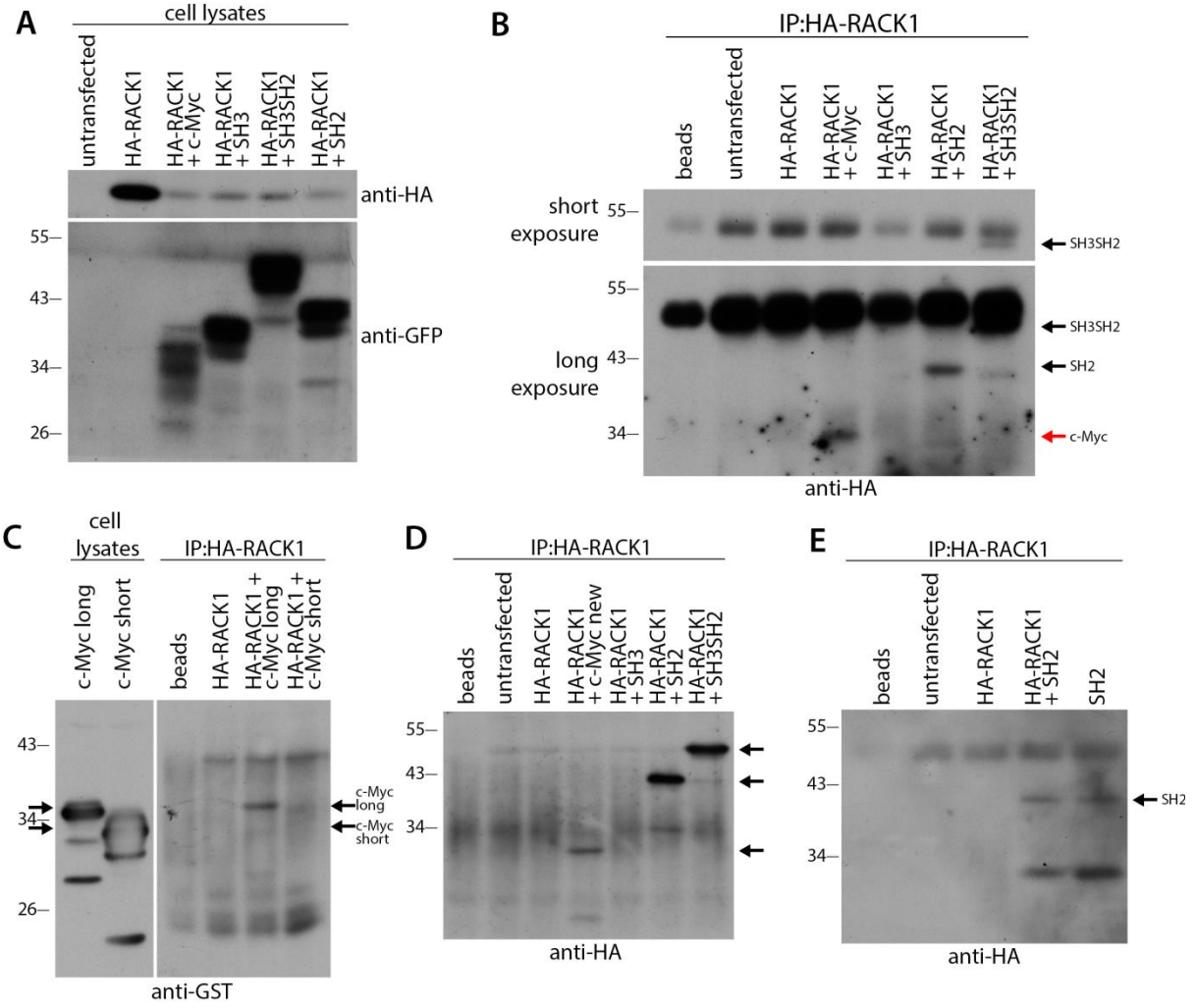



**Figure 3.2 Interaction of Lck and RACK1 in cell cultures.** Plasmids containing HA-RACK1, c-myc-EGFP as negative control, c-myc-EGFP-SH3, c-myc-EGFP-SH2 and c-myc-EGFP-SH3SH2 were created. HA-RACK1 was transfected to HEK293T cells alone or together with SH domains containing constructs (a). These cells were used for the immunoprecipitation of HA-RACK1 (IP: HA-RACK1) for the determination of its interaction with lck structural components (b). Two different times of exposure of the same membrane are displayed. Because the negative control showed a high background non-specific interaction (red arrow), an unnecessary sequence of multiple cloning site was removed, resulting in much reduced background signal (c). The IP:HA-RACK1 experiment was repeated, using shortened control (d); the interaction of control was still detectable, but lower than interaction of SH2 and SH3SH2 domains. SH2 domain was further analyzed for the specificity of binding, showing interaction with anti-HA beads even without presence of HA-RACK1 (e).

Thus, the immunoprecipitation of RACK1 in the SYF cell lines demonstrated that Lck interacts with RACK1. It further confirmed that this interaction is dependent on functional SH2, SH3 and to certain extend also the C-terminal tail of Lck. However, the limitation of this type of analysis is that it could not give us further insight into the specific mechanism of these interactions, i.e. whether the interaction between Lck and RACK1 is direct or mediated

through some intermediary products. We therefore decided to assess the capacity of SH2 and SH3 domains to associate directly with RACK1.

### 9.1.4. RACK1-SH2 and -SH3 Lck pull down assays

To address the Lck binding abilities to RACK1, several recombinant constructs from Lck and RACK1 were prepared. The full size molecule of RACK1 and separate sequence of SH2, SH3 as well as combined SH2-SH3 domains of Lck were tagged with HA oligopeptide and Myc-EGFP protein, respectively. HA-RACK1, Myc-EGFP-SH2, Myc-EGFP-SH3, Myc-EGFP-SH3SH2 and control Myc-EGFP constructs were prepared by Adela Brouckova, the former postdoc in our lab. After confirming that the proteins are well expressed (Fig. 3.2a), the constructs from Lck were cotransfected together with HA-RACK1 to HEK293T cells. Subsequently, pull-down assay was performed, using anti-HA antibody. We observed the interaction of HA-RACK1 with SH2 and SH3SH2 domain constructs, but we also got a signal in the negative control sample (Fig. 3.2b, red arrow). By analyzing the sequence of the negative control we found that it contains an additional sequence from multiple cloning site of the plasmid. We prepared its shorter version lacking most of this extra sequence. As illustrated in Fig. 3.2c and d, this modification decreased the non-specific interaction with Myc-EGFP-SH2 construct, although it did not eliminated it entirely. In addition, we found out that SH2 construct was able to bind to beads in the absence or presence of HA-RACK1 construct (Fig. 3.2e), suggesting this binding is non-specific.

### 9.1.5. In in-vitro translation and interaction reconstituting system

Due to our inability to eliminate the non-specific interactions of SH2 construct with the beads, we decided to study the RACK1-Lck interaction in more controlled environment of in-vitro translation procedure. We created a modular system for cloning any construct into a plasmid with 3xFLAG tag or GST tag under T7 promotor. We prepared 3xFLAG-SH3SH2, GST-SH3SH2, GST-SH3, GST-SH2, 3xFLAG-RACK1, GST-RACK1, 3xFLAG-WD1-3 (first three WD40 domains of RACK1) and 3xFLAG-WD4-7 (last four WD40 domains of RACK1). These constructs were then translated "*in vitro*" and the product of two or three translation reactions were mixed, incubated and then used for the immunoprecipitation.

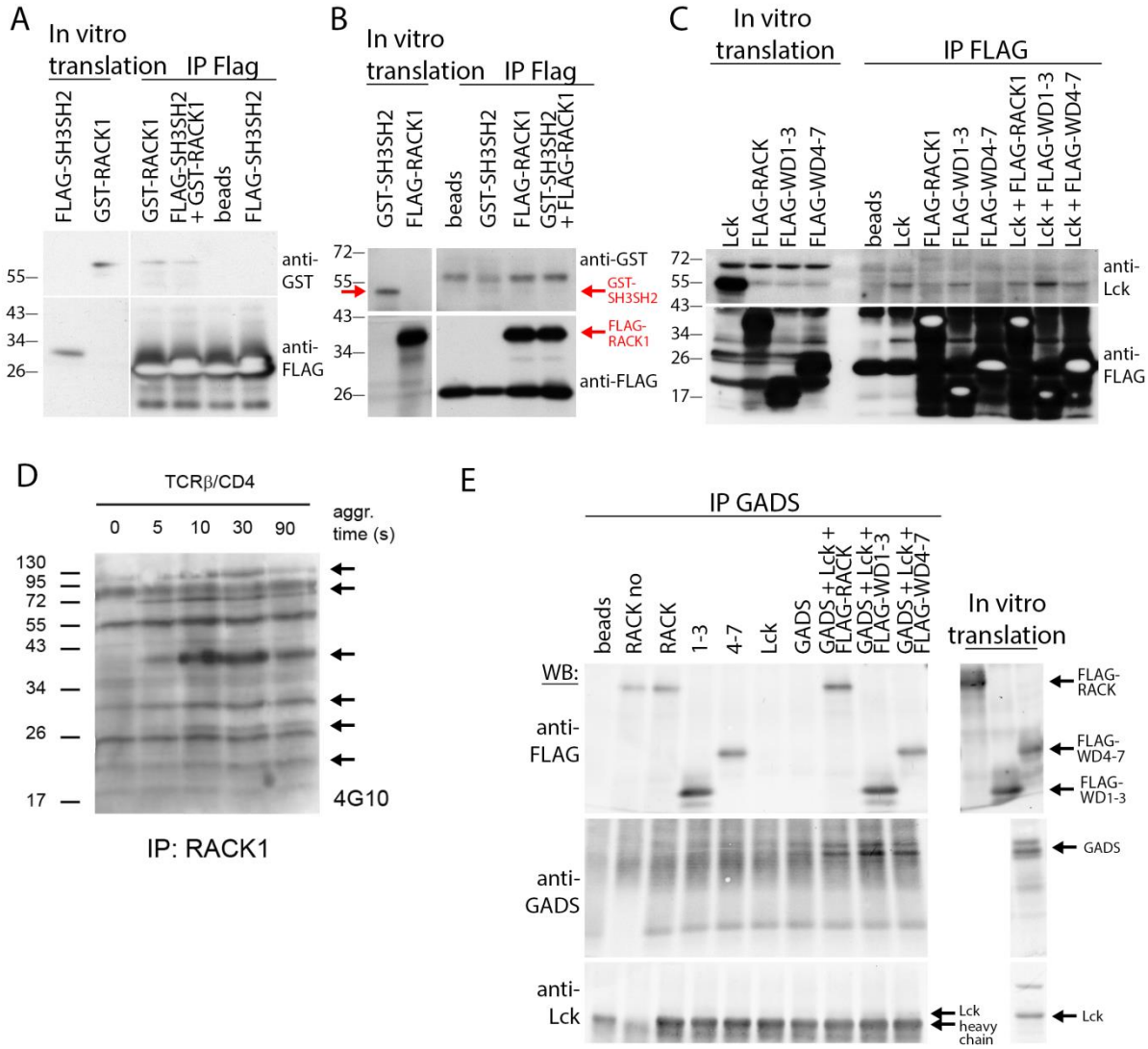



**Figure 3.3 Interaction of Lck and RACK1 in in-vitro translation system.** Plasmids with T7 promotor was used to cloned 3xFLAG-SH3SH2, GST-SH3SH2, GST-SH3, GST-SH2, 3xFLAG-RACK1, GST-RACK1, 3xFLAG-WD1-3 (first three WD40 domains of RACK1) and 3xFLAG-WD4-7 (last four WD40 domains of RACK1). In-vitro translations of 3xFLAG-SH3SH2 and GST-RACK1 were loaded on anti-FLAG beads separately or together, let to interact and consequently stained, showing no specific interaction (a). The same experiment was performed in a reciprocal configuration, using 3xFLAG-RACK1 and GST-SH3SH2, again with the negative result (b). We then decided to test interaction of full-length Y505F Lck with 3xFLAG-RACK1, 3xFLAG-WD1-3 and 3xFLAG-WD4-7. Increased interaction of RACK1 with WD1-3 can be observed, but it might be also non-specific nature (c). Several proteins can be detected to associate with RACK1 and change their phosphorylation status during T-cell activation as shown by RACK1 immunoprecipitation and subsequent staining with phosphotyrosine-specific antibody 4G10 (e). One of the interacting proteins has been shown to be adaptor protein GADS. We therefore tested GADS as possible mediator of interaction between RACK1 and Lck. The product of in vitro translation of Y505F Lck and GADS were mixed with either 3xFLAG-RACK1, or 3xFLAG-WD1-3 or 3xFLAG-WD4-7, and then immunoprecipitated using anti-GADS magnetic beads,. Bound proteins were then stained against FLAG, GADS and Lck (e). High non-specific background interactions of RACK1-derived proteins limit the sensitivity of detection. Despite these difficulties, the signal of RACK1 binding to GADS is increased over the control with only RACK1 on the beads. Faint bands with molecular size of Lck can be seen in samples containing both GADS and Lck, but due to its close proximity to a strong and very intense signal from the heavy chain of antibodies, it cannot be deemed to be a definite proof of interaction.

Translation reactions containing 3xFLAG-SH3SH2 and GST-RACK1 were admixed, incubated with anti-FLAG beads and the amount of bound GST-RACK was assessed by western blotting. The amount of bound GST-RACK1 was comparable regardless of 3xFLAG-SH3SH2 presence (Fig. 3.3a). This suggests that the binding of GST-RACK1 to the beads was of non-specific nature. The experiment was repeated in a reciprocal configuration, using 3xFLAG-RACK1 and GST-SH3SH2 on anti-FLAG beads. No binding of GST-SH3SH2 could be detected in any sample (Fig. 3.3b). That would suggest that the involvement of Lck SH2 domain in RACK1-Lck interaction is rather not direct.

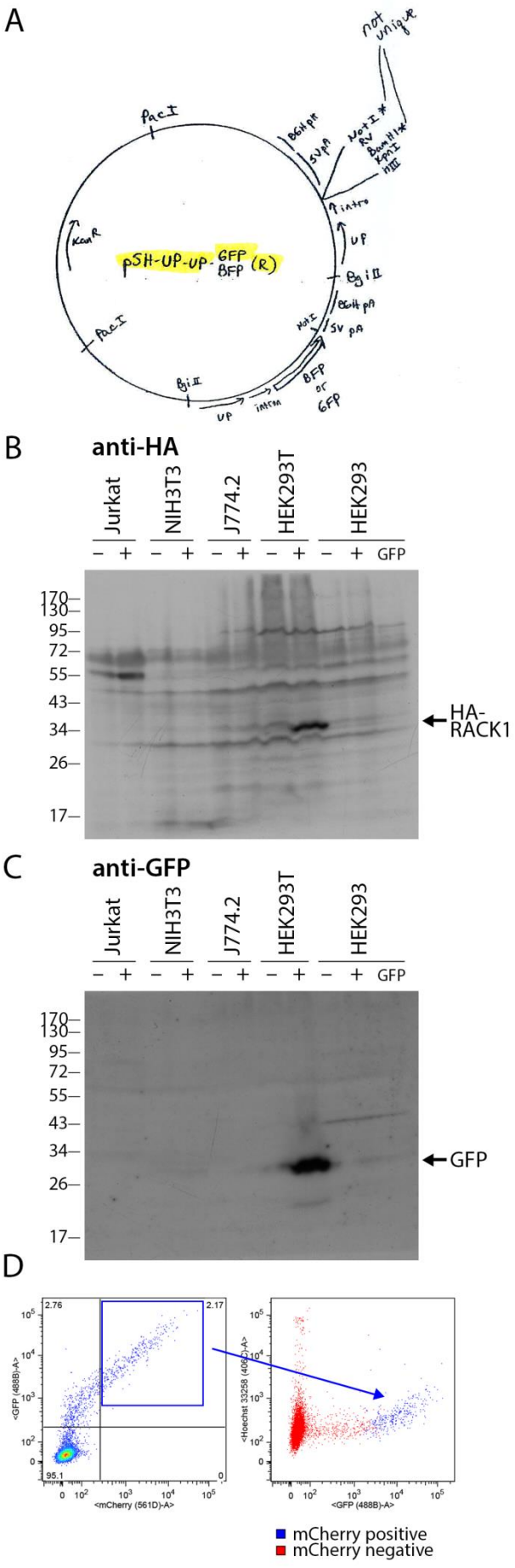
In the next experiment, we decided to use untagged constitutively active Y505F Lck for translation rather than its constructs containing only particular SH2 and/or SH3 domains. We expected that kinase active form of Lck in its full-length version would minimize the risk of faulty interaction caused by the excision of the SH domains from the full sequence. It will also better mimic the situation after the stimulation of T-cells when the kinase gets activated. We also used 3xFLAG-WD1-3 and 3xFLAG-WD4-7 in addition to full 3xFLAG-RACK1, as it has been shown that when separated, the particular moieties of RACK1 can bind their partners more potently then intact full-size RACK1 (Kouba *et al.*, 2012). Unfortunately, these results were again inconclusive because of non-specific interactions that were present (Fig. 3.3c). Specifically, increased signal of Lck was observed when interacting with WD1-3. It is, however, unclear whether it is a sign of interaction or a sum of non-specific bands observed in unmixed Lck and WD1-3.

Since we failed to obtain any convincing evidence in support of a direct interaction between Lck and RACK1, and the fact that at least three structural elements are involved in their complex formation, we proposed that RACK1 and Lck interaction was likely mediated by additional proteins present in the complex. In this context, Ondřej Ballek, a PhD student in our lab, demonstrated the presence of other proteins in RACK1-Lck complex. Notably, when he immunoprecipitated RACK1 complexes at various intervals after TCR triggering by TCR/CD4 aggregation, he could distinguish several proteins that dynamically changed their phosphorylation status (Fig. 3.3d). MS analysis of these immunoprecipitated proteins performed in the laboratory of Prof. Jiří Stulík (Institute of Molecular Pathology, Faculty of Military Health Sciences, Hradec Králové), identified them as α-actinin, GADS, LASP1 and α-tubulin. Because GADS is a well-known adaptor protein which has been previously

described to constitutively interact with RACK1 (Ellis *et al.*, 2000), we decided to assess whether it could function as a linker between RACK1 and Lck. A construct containing GADS under control of T7 promoter was prepared. Then we translated GADS and Y505F Lck, mixed with translated 3xFLAG-RACK, 3xFLAG-WD1-3 or 3xFLAG-WD4-7, and performed IP GADS (Fig. 3.3e). Again, we encountered the problem with a high nonspecific background binding as in previous experiments. Thus, even though these data suggest that GADS interacts with RACK1 because the amount of pulled down RACK1 in the presence of GADS is somewhat higher that in its absence, no definite conclusions can be drawn. The same applies for the complex formation between RACK1-GADS and Lck. Notably, the faint band of Lck is seen in IPs containing GADS, Lck and all three RACK1-related constructs, but due to a strong signal from heavy chain of antibodies with a similar molecular weight, its relevance cannot be unambiguously interpreted.

### *9.2. Adenovirus-mediated expression of RACK1*

Our next goal was to assess the physiological significance of RACK1 in the proximal TCR signalling and during the activation of primary T-cells. Because primary naïve T-cell are very hard to transfect, we decided to use TgCAR mouse strain expressing coxsackie/adenovirus receptor (CAR) under CD2/Lck promotor. The receptor is therefore expressed almost exclusively on T-cells. This ectopic receptor enables specific infection of naïve T-cells by adenovirus (Wan *et al.*, 2000b). Another advantage of this mouse strain is also the fact that it expresses transgenic DO11.10 TCR specific for OVA323-339 peptide. T-cells isolated from these mice can therefore be activated by dendritic cells pulsed with this peptide. Infection by adenovirus enabled us to induce overexpression of RACK1 in naïve T-cells, which were then examined for changes in the activation mechanisms. We also created plasmids containing shRNA that will be used in future experiments for the production of viruses and subsequent downregulation of RACK1 in primary naïve T-cells. This part of my thesis was performed in close collaboration with Ondrej Ballek, where I assisted him in the preparation of several recombinant constructs and adenoviral particles.




## 9.2.1. Virus preparation

For its overexpression in T-cells, we used RACK1 construct tagged by HA for easier detection. We cloned it into shuttle plasmid vector containing two ubiquitin promoters, first controlling EGFP, the other HA-RACK (Fig. 3.4a). As the negative control, we used the shuttle plasmid without any gene cloned under the second promoter. Successful function of the plasmid was assessed by transient transfection into cells. The expression was dependent on the cell type used for transfection, as it was readily detectable only in HEK293T cells (Fig. 3.4b, c). Due to an unusual construction of the plasmid, we created the control vector for the assessment of function of both ubiquitin promoters. Specifically, in the multiple cloning site under second ubiquitin promoter we inserted the fluorescent

**Figure 3.4 Preparation of plasmid vectors for the generation of recombinant adenoviruses.** For the preparation of adenoviruses we used the shuttle vector where EGFP and gene of interest is under the control of two separate ubiquitin promotors. (a). We inserted HA-RACK1 and tested expression of HA-RACK1 (b) and EGFP (c) in four types of cells. Only HEK293T could express the proteins. The properties of the two promotors were tested by cloning mCherry under the control of second promotor. First promotor was stronger, as mCherry expression appeared only in cells with a relatively high expression of GFP (d).

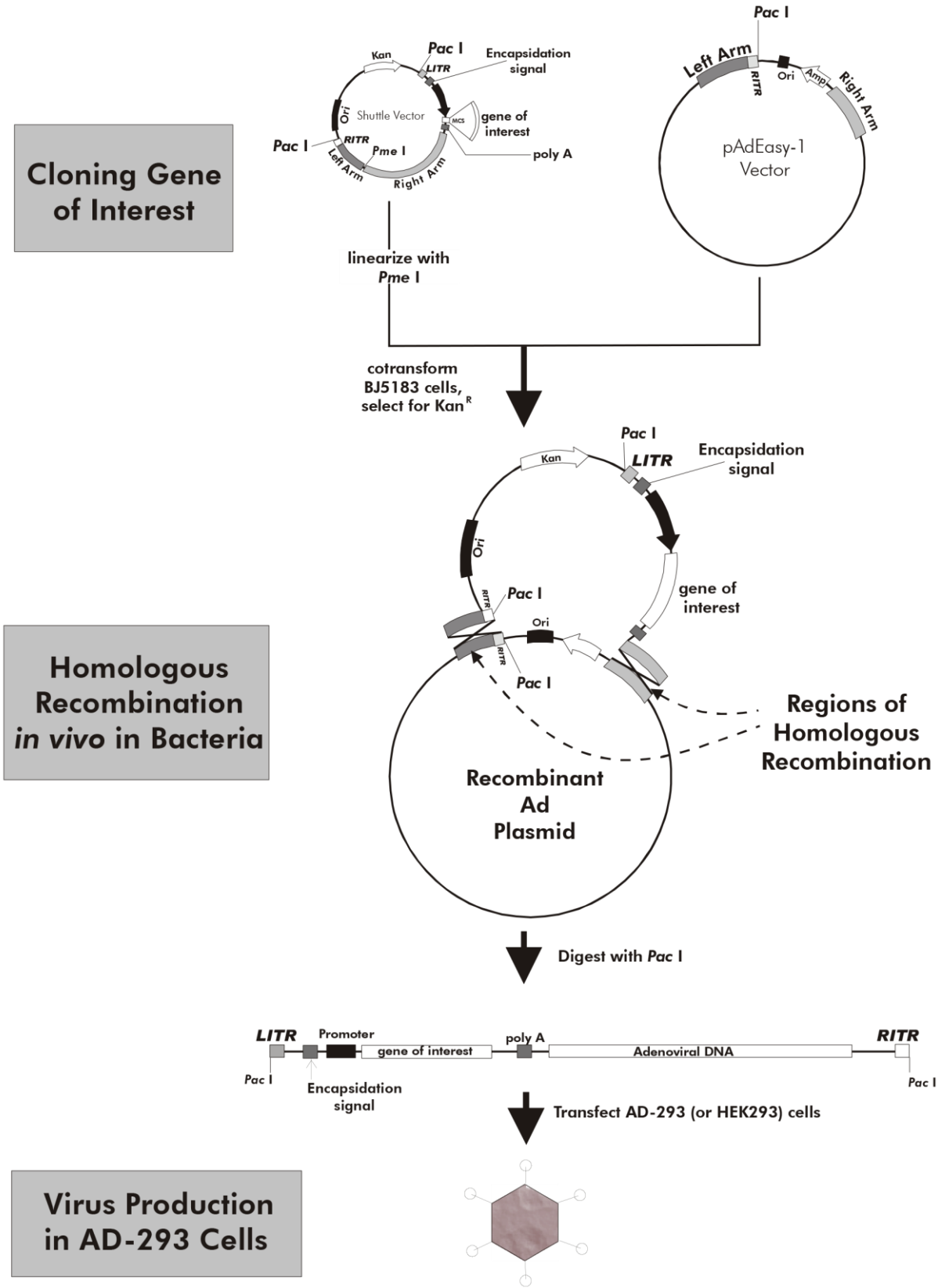



**Figure 3.5 Preparation of adenovirus.** After cloning of HA-RACK1 into the shuttle vector, it was used for the transformation of a special type of bacteria that mediate insertion of EGFP and HA-RACK1 into adenoviral genome by homologous recombination. The genome was then isolated, linearized and transfected into AD-293 cells. This packaging cell line is then used for the production and amplification of the virus, which is then harvested and used for the infection of naïve T-cells. Taken from AdEasy™ Adenoviral Vector System manual.

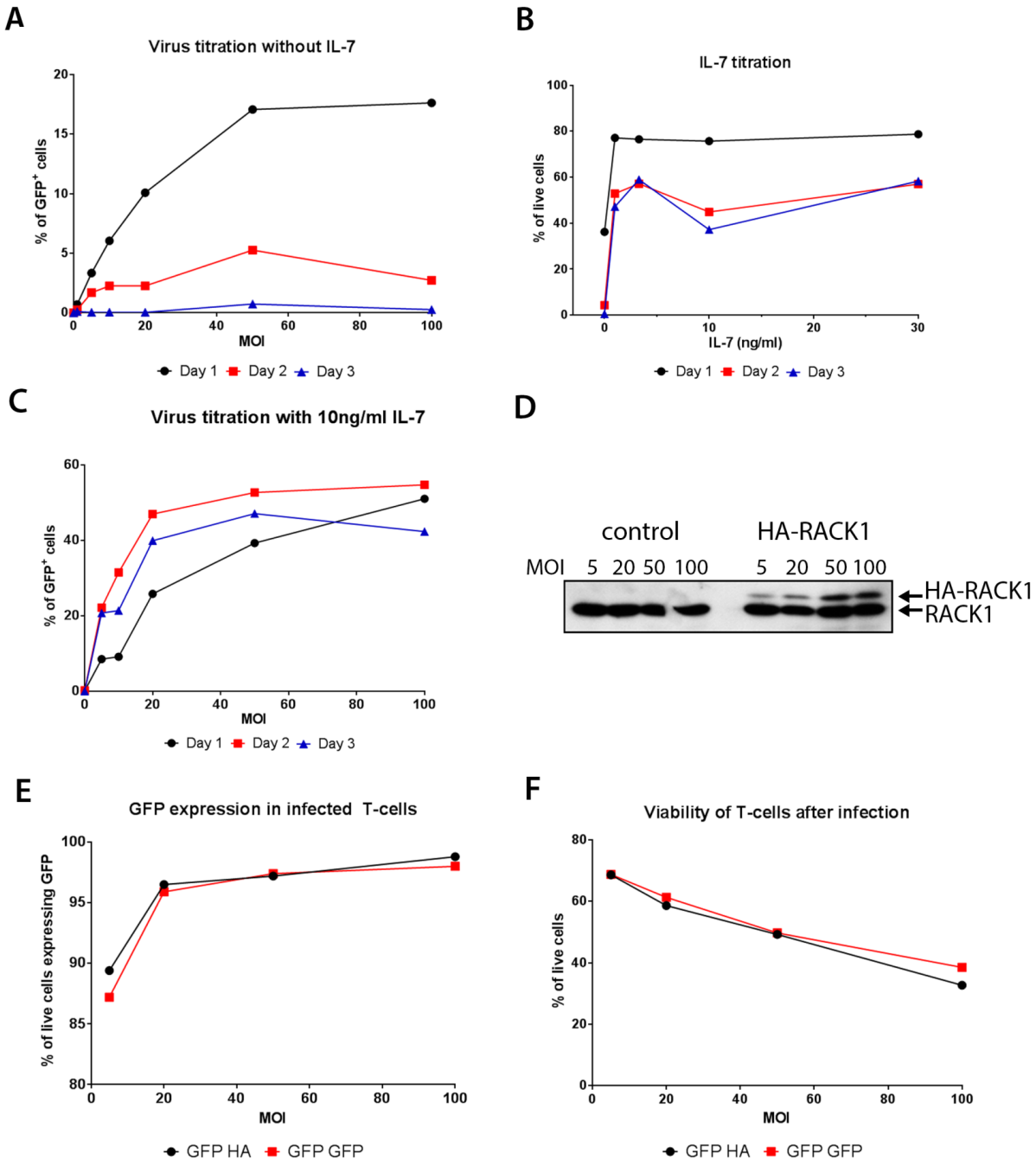



**Figure 3.6 Optimalization of infection conditions.** The adenoviral-mediated expression of GFP by the T-cells was rapidly decreasing over a few day period due to poor viability of cells. Within three days, $GFP^+$ cells were virtually gone (a). To enhance viability, the medium was supplemented with IL-7 cytokine. Its amount was titrated (b) and 2 ng/ml was determined as the sufficient amount. The percentage of surviving $GFP^+$ cells was significantly improved with addition of IL-7 (c). The amount of HA-RACK produced in the infected cells was dependent on multiplicity of infection (MOI) (d), although the production of EGFP was saturated even in low MOI (e). Increasing MOI caused impaired viability of the T-cells (f).

protein mCherry. The expression of both fluorescent probes was detected using FACS analysis. mCherry from the second promoter was expressed only when certain level of EGFP

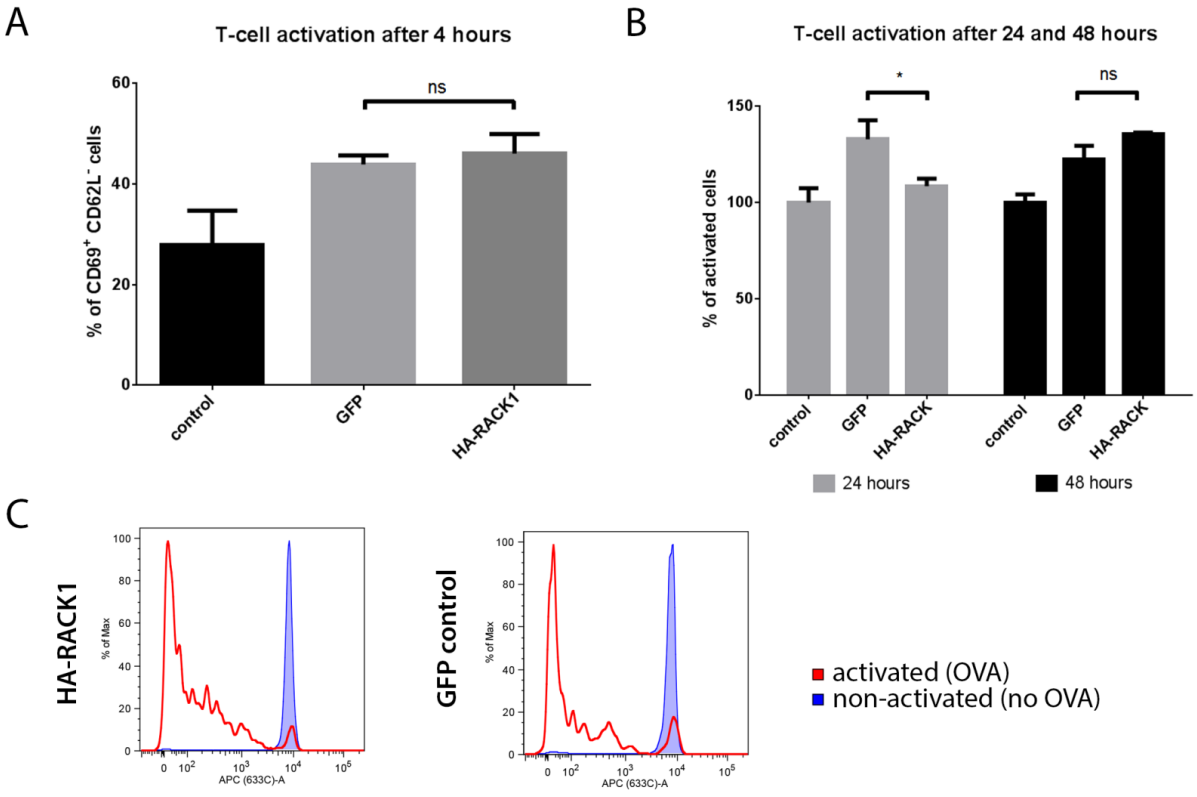



**Figure 3.7 Functional testing of HA-RACK1 overexpression**. The infection by adenovirus coexpressing HA-RACK and EGFP or EGFP only (mock control) at MOI 5 caused enhanced the activation phenotype of T-cells, and the presence of HA-RACK had undistinguishable effect from that of the control sample. The early phase of activation was determined by the expression of CD69 and loss of CD62L expression; level of activation by both viruses was comparable (a). The second phase of activation was observed by production of CD25 after 24 and 48 hours. Activation after 24 hours was higher in cells infected by control virus, but no significant difference was detectable after 48 hours (b). The proliferation of the cells was measured after 72 hours, displaying no difference between HA-RACK and control. * $p<0.05$.

expression from the preceding ubiquitin promoter was achieved, indicating that expression from the second promotor is relatively weak (Fig. 3.4d).

In order to produce the virus causing desired expression level of RACK1, two separate shuttle plasmids with either HA-RACK1 or EGFP had to be inserted into the adenoviral genome (Fig. 3.5). For this purpose, we used the shuttle plasmid to cotransform bacteria with pAdEasy-1 vector, where they could recombine by homologous recombination (Fig. 3.5). Adenoviral plasmids containing our transgenes were then isolated, linearized and

electroporated into AD-293 cells. AD293 cells are more adhesive version of HEK293 cells used specifically for the production and amplification of virus particles to desired viral titers.

### 9.2.2. HA-RACK1 overexpression

With the viruses ready, we had to optimize conditions for our experiments. Crucial is the survival of primary naïve T-cells for extended time. For this purpose we decided to use antiapoptotic cytokine IL-7. Its optimal concentration was determined (Fig. 3.6b). The titration showed that 2 ng of IL-7 per ml of medium is sufficient to improve T-cells survival.

Other important variable is multiplicity of infection (MOI), the mean number of virus particles in medium that could infect one cell. The amount of isolated virus particles was experimentally determined. Various MOI were then tested on the cells. After infection, the expression of GFP in surviving cells was observed for three consecutive days with or without IL-7 (Fig. 3.6c and a, respectively). It is recommended to use MOI as low as possible, as the excess of virus particles can negatively affects cell survival. The experiment identified MOI 20 as relatively low and yet providing sufficient expression of GFP.

Next, we tested the production of HA-RACK1 on protein level in addition to GFP fluorescence and viability of the cells. With increasing MOI, the production of HA-RACK1 rose (Fig. 3.6d), although GFP fluorescence appeared to be saturated (Fig. 3.6e). However, the viability of cells was reversibly correlated with MOI used, ranging from 70 % live cells with MOI 5 to 40 % with MOI 100 (Fig. 3.6f).

After determining optimal conditions for our experiments we proceeded to functional tests of T-cells infected by virus expressing either GFP, or HA-RACK1 or empty vector. $CD4^+$T-cells isolated from TgCAR mice were infected by the virus at MOI 5. Higher MOI caused spontaneous activation of the T-cells to the extent that it would prevent determination of effect of HA-RACK. After infection and incubation of T-cells, we mixed them with the bone marrow-derived dendritic cells pulsed with OVA peptide. We tested different stages of T-cell activation. First stage can be identified by changes in the expression of CD69 and CD62L after 4 hours from activation. While non-activated T-cells express CD62L and not CD69, activated

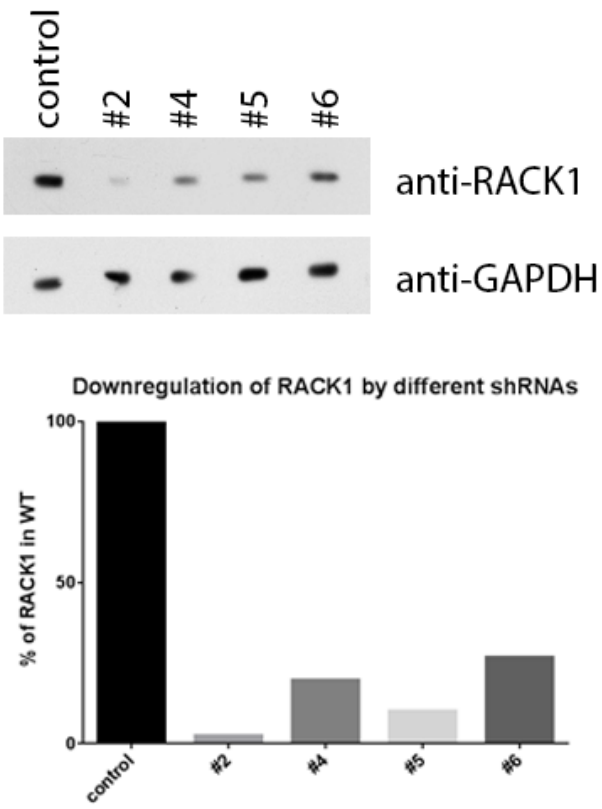



**Figure 3.8 Downregulation of RACK1 in NIH3T3 cells.** Four different shRNAs were tested for their ability to downregulate RACK1 in NIH3T3 cells. They were able to decrease the amount of RACK1 to levels ranging from 3 % (#2) to 27 % (#6) in comparisomn to control, untransfected cells.

T-cells show the opposite pattern. The portion of activated $CD62L^{-}CD69^{+}$ cells increased markedly in cultures with virus, but there was no difference whether the virus contained HA-RACK1 or not (Fig. 3.7a). The second stage of activation was determined by expression of CD25 after 24 and 48 hours (Fig. 3.7). We observed analogous situation, both viruses caused increased activation to extend disregarding virus type. The final step of activation is proliferation, which was detected by staining the cells with proliferation dye (Fig. 3.7c). However, in conclusion no significant difference between GFP and HA-RACK1 viruses could be observed.

### 9.2.3. RACK1 knock-down

The lack of observable effect of HA-RACK1 overexpression could be explained by low level of HA-RACK1 expression compared to endogenous RACK1 (Fig. 3.6d). We therefore decided to try different approach, decreasing the amount of RACK1 rather than increasing it. To this end, four different shRNA have been designed by Ondřej Ballek. Their negative effect on RACK1 transcript and protein levels was confirmed in NIH3T3 fibroblast cell line by transfection (Fig. 3.8). The production of viruses and functional tests are currently under way.

# 10. Discussion

T-cell activation is a complex process, and despite the extensive research conducted in this field some of its aspects remain poorly understood. One of them is the initiation of the most proximal TCR signalling and the mechanisms how the engagement of TCR is translated into a productive activation of Lck and delivery of its function. That includes the membrane redistribution of the main signaling components observed upon TCR triggering. Our previous work identified adaptor protein RACK1 as a candidate interaction partner involved in the activation-induced redistribution of Lck. In this thesis, we performed the structure-function analysis of RACK1-Lck interaction and assessed the functional consequences of RACK1 up- and downregulation of its gene expression. We examined our proposition that C-terminal tail of Lck functions as a novel regulator of protein-protein interactions with ability to affect the its targeting of downstream targets as well as its kinase activity. The premise of this investigation was that the C-terminal end of Lck plays a role in the interaction with adaptor protein RACK1 which mediates the activation-induced translocation of Lck into lipid rafts by the mechanism involving microtubular cytoskeleton. Such repartitioning of kinase active Lck to lipid rafts could result in the phosphorylation and activation of other members of the TCR signalling machinery and the initiation of T-cell activation process. Indeed, based on our previous results, we have suggested the Lck-dependent Fyn activation model where the activation of Lck and subsequent translocation to the lipid rafts leads to activation of raft-resident Fyn and other downstream signalling proteins (Filipp et al., 2003).

Our laboratory previously reported a negative influence of sequential truncations of the first half of C-terminal tail of Lck (ΔYQPQP) on global tyrosine phosphorylation status in cell SYF cells (Filipp et al., 2008). Results associated with this experiment showed a gradual diminishment of Lck to interact with RACK1 and to phosphorylate its intracellular targets, culminating in the complete ablation of phosphorylation with ΔYQPQP Lck. Yet, the kinase activity of Lck itself was comparable or reduced to only 50% compared to the WT Lck. However, natural and recombinant Lck mutants lacking the C-terminal end are fully active as the inhibitory binding of SH2 domain to its own pY505 does not occur. Thus, in presented work, we extended the set of the truncation mutants to gradually cover the entire tail sequence of Lck and expected that the negative effect of these truncations on RACK1 interaction and phosphorylation of target proteins will be reversed.

When the complete panel of these truncations was examined, different results were obtained compared to those published previously (Filipp et al., 2008). Specifically, all Lck truncation mutants, including ΔYQPQP and ΔC11, displayed comparable levels of tyrosine phosphorylation of many intracellular proteins in comparison to that of the constitutively active Y505F Lck, the template of which was used for the generation of these truncations (Filipp et al., 2008). Thus, we failed to confirm the previously published results. New results showed consistent levels of global tyrosine phosphorylation (Fig. 1d). Possible explanation for this discrepancy could lay in mutation found in ΔYQPQP truncate causing this negative effect. Our newly generated ΔYQPQP construct behaved as the constitutively active Y505F Lck. Another explanation can be the unfavourable integration site of the retrovirus which could intercalate into a part of genome that would silence the transgene.

Of note is also the loss of tyrosine phosphorylation in cells with Lck with mutated SH2 domain (R154K). In the original experiment (Filipp et al., 2008), this mutant showed high levels of global phosphorylation, which is consistent with the fact that proper function of both SH2 and SH3 domains is necessary for effective Lck inhibition (Cowan-Jacob et al., 2005). Our current experiment showed completely opposite results as R154K Lck containing cells lacked the phosphotyrosine signals almost entirely. At this moment, the reason for such discrepancy is not known, but the verification of the sequence of the previously and currently used SH2 Lck mutant construct will be performed in a very near future to confirm or disapprove their identity.

The main reason for the creation of C-terminus truncates was to verify the hypothesis that this part of Lck contributes to the translocation of Lck to lipid rafts via association with RACK1. This assumption was supported by the fact that QP-based C-terminal sequence has been shown to affect localization in lipid rafts (Navratil et al., 2006). A decrease in the interaction of RACK1 with ΔC11 (Fig. 1c) (Ballek, 2009) and ΔYQPQP Lck (Ballek, unpublished data) was also observed before. The immunoprecipitation of Lck truncates with RACK1, however, showed only a limited influence of the C-terminal truncation on the amount of Lck associated with RACK1. That specifically concerns the ΔC9 Lck truncate, which displayed approximately a 50% reduction in the complex formation with RACK1 compared to other Lck mutants. While these experiments suggest that Lck-RACK1 interaction can be affected by the C-terminal end of the former, the mechanism and conditions under which it occurs remain to be determined in the future.

Marked decrease in the interaction was also clear in mutants with inactive SH2 and SH3 domains as well as in Y394F, kinase inactive Lck with mutation in the activatory tyrosine positioned in the activatory kinase loop. Interestingly, the interaction of another kinase inactive Lck, K273A, with RACK1 was not impaired, which suggests that this interaction is independent of the kinase activity. While the K273 is a part of the N-lobe of the kinase domain and is located inside the catalytic cleft where it coordinates the phosphate group of ATP and helps to stabilize the proper position of the C helix, the Y394 is located on C-lobe and its phosphorylation leads to the reorganization of the activation loop, creating a substrate-binding surface on the opening of the catalytic cleft (Xu et al., 1997). It is possible that this latter surface, accessible to other proteins, is by some unknown mechanism involved in the interaction with RACK1. In this context, it remains to be determined the level of pY394 in K273 Lck mutant to exclude or consider the possibility that this phosphorylated residue could be involved in interaction with RACK1.

Although we showed that Lck interacts with RACK1, details of this interaction remained largely uncharacterized. We tested whether the interaction was direct, and if so, whether it was mediated by one or both SH domains. RACK1 contains 6 tyrosines that can be phosphorylated and therefore can serve as a binding site for SH2 domains. It was described that Src binds to pTyr246 of RACK1 (Chang et al., 2001), but interaction with Lck has not been mechanistically described. We were unable to detect specific interaction of any SH domain with RACK1, as the detected binding of SH2 turned out to be nonspecific. Even after transferring these experiments to a cell-free system, detected interactions were of non-specific nature or largely inconclusive. The proteins were binding to the beads without the presence of the immunoprecipitated protein, and in the case of magnetic beads, regardless of the presence or absence of precoated antibody (data not shown). Additionally, the detection of precipitated proteins was problematic and required unusually long exposures of the film. The most likely explanation is a very low level of bound protein or insufficient antibody binding.

The fact that mutations in both SH2 and SH3 (and in previous experiments also truncated C-terminus) caused partial, but not complete, loss of interaction with RACK1 suggests that the complex translocated to lipid rafts upon activation could consist of multiple proteins. At least some of these additional proteins are susceptible to tyrosine phosphorylation and their phosphorylation proceeds rapidly during T-cell activation (Fig. 3d). The number of proteins

detected in these complexes by phosphotyrosine-specific antibody also favours the idea of larger multiprotein complex translocation.

This finding is also consistent with the fact that only high molecular weight complexes containing Lck are able to translocate to lipid rafts upon activation (Ballek et al., 2012). The complexes were divided according to its size by gel filtration. The comparison of samples from T-cells activated for 30 seconds and non-activated ones showed that high molecular weight fractions exclusively participated in Lck redistribution. Furthermore, active Lck (phosphorylated on Tyr394) can be found exclusively in high molecular weight complexes. Further analysis of the behaviour of the complexes showed that their translocation to lipid rafts relies on the microtubular network, rather than actin or vimentin filaments. Specifically, the addition of nocodazol to T-cells abolished translocation of the high molecular weight complexes to lipid rafts, while latrunculin B displayed no such effect (Ballek et al., 2012). The utilization of microtubule network in the translocation event is further supported by the fact that the MS analysis of RACK1 immunoprecipitates found α-tubulin present. With additional proteins such as α-actinin, LASP and GADS identified as a part of RACK1-Lck complex, its precise multiprotein organization and kinetics of its formation remains to be elucidated.

At least for now, the question how the interaction of Lck and RACK1 is mediated remains unanswered. As an adaptor protein which interacts with RACK1 directly and constitutively (Ellis et al., 2000), GADS could be the missing element mediating RACK1-Lck interaction. We therefore tried to reconstitute the possible core formation of the complex by coincubating these three proteins or their moieties which were produced by in-vitro translation and to pull down the complex (Fig. 3e). Unfortunately, due to a high background nonspecific binding, we were unable to detect complex formation between RACK1-GADS-Lck. We only detected an increase in the amount of RACK1 bound to anti-GADS-precoated beads in the presence of GADS protein over that determined in the absence of GADS. However, this would just confirm the previously reported binding of RACK1 with GADS. Definitely, more experiments are needed to work out the precise conditions and the protein composition of this complex. Additional proteins are very likely to be required in the mixture to form this multiprotein complex. Other intriguing possibility is that the RACK1-Lck complex formation can occur only in the presence of microtubular network or other cytoskeletal components and thus, the assembly of this complex in in vitro conditions is experimentally unattainable.

We would also like to make several technical comments. First, in the immunoprecipitation experiments we switched from agarose to magnetic beads because the agarose beads have a large internal surface, while magnetic beads bind proteins only on the outside surface of the beads. We hoped that this fact would lead to decrease in a non-specific protein binding. Unfortunately, this was not the case, as constructs derived from RACK1 were still bound to the beads even in the absence of GADS which should mediate this interaction. The same was observed when no anti-GADS antibody was used to precoat the beads. Under such conditions, if the RACK1-GADS interaction indeed occurs but is of weak nature, it is likely masked by a non-specific binding, precluding us to make any reasonable conclusion. Second, for the detection of the proteins, we used fluorescent WB imaging. This method enables observing two distinct proteins on a single blot; the whole sample can therefore be loaded on one gel rather than split on two, and even the weaker signal can be reliably detected. In our case, however, the fluorescent secondary antibodies caused high background by staining heavy chains of the antibodies used for the immunoprecipitation. As the heavy chain signal, which is usually very strong and "fat", is of similar molecular weight as Lck, it extend to the area where Lck is detected, thus interfering with its detection. A faint band corresponding to the size of Lck can be observed in samples containing recombinant GADS, RACK1 and Lck, but it is difficult to verify its identity. Third, as these pulldown experiments with three recombinant constructs contain the triple amount of these products compared to their individual control samples, non-specific binding cannot be ruled out. As discussed above, it would be maybe naïve to assume that mixing RACK1, Lck and GADS together, they start to form a biochemically detectable complex. The process could be much more complicated involving more structural components and of highly transient nature. Thus, even though we consider it likely that GADS, RACK1 and Lck are critical components of this complex, they are likely not sufficient to form its core capable of translocation of Lck to the lipid rafts. Additional components of the complex will have to be carefully examined to elucidate the interplay between the proteins involved in this translocation mechanism.

We also tried to examine the physiological role of RACK1 in T-cell activation. The main idea was to change the level of RACK1 expression in primary naïve T-cells isolated from lymph nodes on the protein level and determine the effect on Lck translocation and early T-cell response. We first used HA-RACK1 construct with a prospect to express of functional mutants of RACK1, should the system prove effective. However, using a relatively low MOI,

we were only able to induce a very mild overexpression of RACK1 over its endogenous counterpart with little tangible effect on T-cell proximal signalling (Fig. 7). Higher MOI led to the higher level of HA-RACK1 expression, but even with MOI 100, the amount of HA-RACK1 did not reach its endogenous levels. Additionally, high MOIs of a mock only GFP-containing construct reduced the viability of the T-cells and caused their spontaneous activation to the extent where its effect became undistinguishable from that promoted by HA-RACK1 recombinant construct, thus preventing us from reaching any conclusion about its physiological function. The likely cause for only a marginal increase in the expression of HA-RACK1 could be that endogenous RACK1 is abundantly expressed protein and a robust expression with recombinant construct using adenoviral expression system is at this moment unachievable. In addition, a massive overexpression of RACK1 is not well tolerated by at least some target cells, such as fibroblast, and leads to their sudden apoptosis (Tomáš Vomastek, personal comunication).

In light of these results, the opposite approach, the downregulation of endogenous RACK1, seems to be a more appropriate and promising way of determining the function of RACK1 during T-cell activation. Our pilot experiments with shRNA-mediated knock-down of RACK1 in cell lines are encouraging, as various shRNAs are able to decrease the production of endogenous RACK1 to 3-27 % of its levels in untreated cells (Fig. 8). However, it is also likely that the inhibition will not be as effective in the primary T-cells. Immortalized NIH3T3 cells grow continuously, divide and therefore have a very active metabolism. On the other hand, naïve T-cells are quiescent type of cells which resist division until they get activated. Due to this slower turnover of RNA synthesis and protein production, it will take them longer to diminish or deplete intracellular RACK1. Another aspect of this RACK1 downregulation experiment is the survival of T-cells. The fact that RACK1 knock-out is embryonically lethal (Volta et al., 2013) and RACK1 itself is required for the ribosome assembly and the initiation of translation, casts doubt whether downregulation of RACK1 is compatible with cell survival. Our experiments with cell lines with downregulated RACK1 however suggest that cells can survival for several days even with severely decreased level of RACK1. While the experiments with RACK1 downregulation have not been completed, we prepared all necessary reagents, target cells, virion particles, tested the methodology and protocols for successful accomplishment of this task in the near future.

Taken together, we performed numerous experiments in order to perform the structure-function analysis of RACK1-Lck interaction which seems to be a prerequisite for TCR-induced translocation of Lck to lipid rafts. On this experimental journey, we obtained several very encouraging results and prepare a whole battery of reagents which are necessary for further advancement of this project. As it usually happens with a new type of project like this, we also faced many technical difficulties which hold us back from determining the precise mechanisms of complex formation between RACK1 and Lck and how RACK1 mediates Lck redistribution. It is likely that the role of RACK1 in T-cell signalling is similar to its function in other signalling pathways, where it mediates the intracellular relocalization of other effector signalling proteins. Even though we detected the interaction between RACK1 and Lck and identified SH2 and SH3 domains as important structural components required for their complex formation, we were unable to show how these interacting structures assemble together. We also cannot rule out that C-terminal tail of Lck has some role in this complex formation; however its impact on global tyrosine phosphorylation and interaction with RACK1 is less profound than originally expected. Moreover, our experiments strongly suggest that Lck does not bind RACK1 directly, but rather through several intermediary products. At least some of them were identified in this work and includes α-actinin, LASP, α-tubulin and GADS. We showed that adaptor protein GADS is not sufficient to mediate interaction between RACK1 and Lck, at least under experimental conditions used in here. Unfortunately, our data concerning the functional role of RACK1 in activation of T-cells are inconclusive, but additional experiments are already underway. It is clear that while our work provided important information concerning the mechanism of Lck redistribution during early T-cell activation, much more research has to be performed on this new and interesting topic in order to fully understand the mechanisms of proximal T-cell signalling.

## 11. Conclusion

- SH2 and SH3 domains of Lck play a critical role in the complex formation with RACK1.
- The C-terminal truncation of Lck exerted only a mild and sequence-specific effect on the RACK1-Lck complex formation. Specifically, last 9 aminoacid truncation of the C-terminus of Lck decreases its interaction with RACK1, other truncates were ineffective.
- The interaction between RACK1 and Lck is most likely to be indirect, mediated by other proteins. Several of these proteins were identified. Their involvement in the formation of RACK1-Lck complex has to be further evaluated. One of these proteins, the adaptor protein GADS, is unable to mediate this interaction.
- Adenovirus mediated overexpression of RACK1 in primary T-cells failed to produce sufficient levels of RACK1 protein to assess its effect on T-cell activation process. Moreover, the viral infection of T-cells caused unspecific activation of these cells which precluded us from performing intended functional tests.
- For an alternative approach, all reagents necessary for the downregulation of RACK1 by RNA interference has been prepared and tested and will be used for the assessment of the role of RACK1 in T-cell activation in the near future.

# 12. References


**Abraham, N. & Veillette, A. (1990)**. ACTIVATION OF P56LCK THROUGH MUTATION OF A REGULATORY CARBOXY-TERMINAL TYROSINE RESIDUE REQUIRES INTACT SITES OF AUTOPHOSPHORYLATION AND MYRISTYLATION. *Molecular and Cellular Biology* **10**, 5197-5206.

**Acuto, O., Di Bartolo, V. & Michel, F. (2008)**. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. *Nature Reviews Immunology* **8**, 699-712.

**Ballek, O. (2009)**.RACK1 as a candidate protein involved in the regulation of translocation of Lck to lipid rafts (diploma thesis).

**Ballek, O., Brouckova, A., Manning, J. & Filipp, D. (2012)**. A specific type of membrane microdomains is involved in the maintenance and translocation of kinase active Lck to lipid rafts. *Immunology Letters* **142**, 64-74.

**Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P. & Alitalo, K. (1992)**. THE HUMAN P50(CSK) TYROSINE KINASE PHOSPHORYLATES P56(LCK) AT TYR-505 AND DOWN REGULATES ITS CATALYTIC ACTIVITY. *Embo Journal* **11**, 2919-2924.

**Billadeau, D. D., Nolz, J. C. & Gomez, T. S. (2007)**. Regulation of T-cell activation by the cytoskeleton. *Nature Reviews Immunology* **7**, 131-143.

**Brandon, N. J., Jovanovic, J. N., Smart, T. G. & Moss, S. J. (2002)**. Receptor for activated C kinase-1 facilitates protein kinase C-dependent phosphorylation and functional modulation of GABA(A) receptors with the activation of G-protein-coupled receptors. *Journal of Neuroscience* **22**, 6353-6361.

**Brdicka, T., Pavilstova, D., Leo, A. & other authors (2000)**. Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase Csk and is involved in regulation of T cell activation. *Journal of Experimental Medicine* **191**, 1591-1604.

**Brdickova, N., Brdicka, T., Andera, L., Spicka, J., Angelisova, P., Milgram, S. L. & Horejsi, V. (2001)**. Interaction between two adapter proteins, PAG and EBP50: a possible link between membrane rafts and actin cytoskeleton. *Febs Letters* **507**, 133-136.

**Brownlie, R. J. & Zamoyska, R. (2013)**. T cell receptor signalling networks: branched, diversified and bounded. *Nature Reviews Immunology* **13**, 257-269.

**Carrillo, D. R., Chandrasekaran, R., Nilsson, M., Cornvik, T., Liew, C. W., Tan, S. M. & Lescar, J. (2012)**. Structure of human Rack1 protein at a resolution of 2.45 A. *Acta Crystallographica Section F-Structural Biology and Crystallization Communications* **68**, 867-872.

**Ceci, M., Gaviraghi, C., Gorrini, C., Sala, L. A., Offenhauser, N., Marchisio, P. C. & Biffo, S. (2003)**. Release of eIF6 (p27(BBP)) from the 60S subunit allows 80S ribosome assembly. *Nature* **426**, 579-584.

**Chang, B. Y., Conroy, K. B., Machleder, E. M. & Cartwright, C. A. (1998)**. RACK1, a receptor for activated C kinase and a homolog of the beta subunit of G proteins, inhibits activity of Src tyrosine kinases and growth of NIH 3T3 cells. *Molecular and Cellular Biology* **18**, 3245-3256.

**Chang, B. Y., Chiang, M. L. & Cartwright, C. A. (2001)**. The interaction of Src and RACK1 is enhanced by activation of protein kinase C and tyrosine phosphorylation of RACK1. *Journal of Biological Chemistry* **276**, 20346-20356.

**Chang, B. Y., Harte, R. A. & Cartwright, C. A. (2002)**. RACK1: a novel substrate for the Src protein-tyrosine kinase. *Oncogene* **21**, 7619-7629.

**Choudhuri, K. & van der Merwe, P. A. (2007)**. Molecular mechanisms involved in T cell receptor triggering. *Seminars in Immunology* **19**, 255-261.

**Cloutier, J. F. & Veillette, A. (1996)**. Association of inhibitory tyrosine protein kinase p50(csk) with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells. *Embo Journal* **15**, 4909-4918.

**Cordoba, S. P., Choudhuri, K., Zhang, H., Bridge, M., Basat, A. B., Dustin, M. L. & van der Merwe, P. A. (2013)**. The large ectodomains of CD45 and CD148 regulate their segregation from and inhibition of ligated T-cell receptor. *Blood* **121**, 4295-4302.

**Cowan-Jacob, S. W., Fendrich, G., Manley, P. W., Jahnke, W., Fabbro, D., Liebetanz, J. & Meyer, T. (2005)**. The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. *Structure* **13**, 861-871.

**Cox, E. A., Bennin, D., Doan, A. T., O'Toole, T. & Huttenlocher, A. (2003)**. RACK1 regulates integrin-mediated adhesion, protrusion, and chemotactic cell migration via its Src-binding site. *Molecular Biology of the Cell* **14**, 658-669.

**Coyle, S. M., Gilbert, W. V. & Doudna, J. A. (2009)**. Direct Link between RACK1 Function and Localization at the Ribosome In Vivo. *Molecular and Cellular Biology* **29**, 1626-1634.

**Davis, S. J. & vanderMerwe, P. A. (1996)**. The structure and ligand interactions of CD2: Implications for T-cell function. *Immunology Today* **17**, 177-187.

**Delon, J., Gregoire, C., Malissen, B., Darche, S., Lemaitre, F., Kourilsky, P., Abastado, J. P. & Trautmann, A. (1998)**. CD8 expression allows T cell signaling by monomeric peptide-MHC complexes. *Immunity* **9**, 467-473.

**Ding, Y. H., Baker, B. M., Garboczi, D. N., Biddison, W. E. & Wiley, D. C. (1999)**. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity* **11**, 45-56.

**Ellis, J. H., Ashman, C., Burden, M. N., Kilpatrick, K. E., Morse, M. A. & Hamblin, P. A. (2000)**. GRID: A novel Grb-2-related adapter protein that interacts with the activated T cell costimulatory receptor CD28. *Journal of Immunology* **164**, 5805-5814.

**Faure, S., Salazar-Fontana, L. I., Semichon, M., Tybulewicz, V. L. J., Bismuth, G., Trautmann, A., Germain, R. N. & Delon, J. (2004)**. ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. *Nature Immunology* **5**, 272-279.

**Feng, S. B., Chen, J. K., Yu, H. T., Simon, J. A. & Schreiber, S. L. (1994)**. 2 BINDING ORIENTATIONS FOR PEPTIDES TO THE SRC SH3 DOMAIN - DEVELOPMENT OF A GENERAL-MODEL FOR SH3-LIGAND INTERACTIONS. *Science* **266**, 1241-1247.

**Filipp, D., Zhang, J., Leung, B. L., Shaw, A., Levin, S. D., Veillette, A. & Julius, M. (2003)**. Regulation of Fyn through translocation of activated Lck into lipid rafts. *Journal of Experimental Medicine* **197**, 1221-1227.

**Filipp, D. & Julius, M. (2004)**. Lipid rafts: resolution of the 'fyn problem"? *Molecular Immunology*, 645-656.

**Filipp, D., Moemeni, B., Ferzoco, A., Kathirkamathamby, K., Zhang, J., Ballek, O., Davidson, D., Veillette, A. & Julius, M. (2008)**. Lck-dependent Fyn activation requires C terminus-dependent targeting of kinase-active Lck to lipid rafts. *Journal of Biological Chemistry* **283**, 26409-26422.

**Gil, D., Schamel, W. W. A., Montoya, M., Sanchez-Madrid, F. & Alarcon, B. (2002)**. Recruitment of Nck by CD3 epsilon reveals a Ligand-Induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* **109**, 901-912.

**Gimmi, C. D., Freeman, G. J., Gribben, J. G., Gray, G. & Nadler, L. M. (1993)**. HUMAN T-CELL CLONAL ANERGY IS INDUCED BY ANTIGEN PRESENTATION IN THE ABSENCE OF B7 COSTIMULATION. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 6586-6590.

**Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. & Dustin, M. L. (1999)**. The immunological synapse: A molecular machine controlling T cell activation. *Science* **285**, 221-227.

**Harada, Y., Tanabe, E., Watanabe, R. & other authors (2001)**. Novel role of phosphatidylinositol 3-kinase in CD28-mediated costimulation. *Journal of Biological Chemistry* **276**, 9003-9008.

**Harrison, S. (2003)**. Variation on an Src-like theme. *Cell*, 737-740.

**Hashimoto-Tane, A., Yokosuka, T., Sakata-Sogawa, K., Sakuma, M., Ishihara, C., Tokunaga, M. & Saito, T. (2011)**. Dynein-Driven Transport of T Cell Receptor Microclusters Regulates Immune Synapse Formation and T Cell Activation. *Immunity* **34**, 919-931.

**He, D. Y., Vagts, A. J., Yaka, R. & Ron, D. (2002)**. Ethanol induces gene expression via nuclear compartmentalization of receptor for activated C kinase 1. *Molecular Pharmacology* **62**, 272-280.

**Horejsi, V. (2005)**. Lipid rafts and their roles in T-cell activation. *Microbes and Infection* **7**, 310-316.

**Hui, E. F. & Vale, R. D. (2014)**. In vitro membrane reconstitution of the T-cell receptor proximal signaling network. *Nature Structural & Molecular Biology* **21**, 133-+.

**Huppa, J. B. & Davis, M. M. (2003)**. T-cell-antigen recognition and the immunological synapse. *Nature Reviews Immunology* **3**, 973-983.

**Inaba, K., Witmerpack, M., Inaba, M. & other authors (1994)**. THE TISSUE DISTRIBUTION OF THE B7-2 COSTIMULATOR IN MICE - ABUNDANT EXPRESSION ON DENDRITIC CELLS IN-SITU AND DURING MATURATION IN-VITRO. *Journal of Experimental Medicine* **180**, 1849-1860.

**Irvine, D. J., Purbhoo, M. A., Krogsgaard, M. & Davis, M. M. (2002)**. Direct observation of ligand recognition by T cells. *Nature* **419**, 845-849.

**Jannot, G., Bajan, S., Giguere, N. J., Bouasker, S., Banville, I. H., Piquet, S., Hutvagner, G. & Simard, M. J. (2011)**. The ribosomal protein RACK1 is required for microRNA function in both C. elegans and humans. *Embo Reports* **12**, 581-586.

**Kaizuka, Y., Douglass, A. D., Varma, R., Dustin, M. L. & Vale, R. D. (2007)**. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 20296-20301.

**Kiely, P. A., Baillie, G. S., Barrett, R., Buckley, D. A., Adams, D. R., Houslay, M. D. & O'Connor, R. (2009)**. Phosphorylation of RACK1 on Tyrosine 52 by c-Abl Is Required for Insulin-like Growth Factor I-mediated Regulation of Focal Adhesion Kinase. *Journal of Biological Chemistry* **284**, 20263-20274.

**Kim, P. W., Sun, Z. Y. J., Blacklow, S. C., Wagner, G. & Eck, M. J. (2003)**. A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. *Science* **301**, 1725-1728.

**Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A. & Soriano, P. (1999)**. Src family kinases are required for integrin but not PDGFR signal transduction. *Embo Journal* **18**, 2459-2471.

**Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A. & Magee, A. I. (1994)**. PALMITOYLATION OF MULTIPLE SRC-FAMILY KINASES AT A HOMOLOGOUS N-TERMINAL MOTIF. *Biochemical Journal* **303**, 749-753.

**Kouba, T., Rutkai, E., Karaskova, M. & Valasek, L. S. (2012)**. The eIF3c/NIP1 PCI domain interacts with RNA and RACK1/ASC1 and promotes assembly of translation preinitiation complexes. *Nucleic Acids Research* **40**, 2683-2699.

**Kuroha, K., Akamatsu, M., Dimitrova, L., Ito, T., Kato, Y., Shirahige, K. & Inada, T. (2010)**. Receptor for activated C kinase 1 stimulates nascent polypeptide-dependent translation arrest. *Embo Reports* **11**, 956-961.

**Lenschow, D. J., Walunas, T. L. & Bluestone, J. A. (1996)**. CD28/B7 system of T cell costimulation. *Annual Review of Immunology* **14**, 233-258.

**Li, D. & Roberts, R. (2001)**. WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases. *Cellular and Molecular Life Sciences* **58**, 2085-2097.

**Li, Y. C., Chen, B. M., Wu, P. C., Cheng, T. L., Kao, L. S., Tao, M. H., Lieber, A. & Roffler, S. R. (2010)**. Cutting Edge: Mechanical Forces Acting on T Cells Immobilized via the TCR Complex Can Trigger TCR Signaling. *Journal of Immunology* **184**, 5959-5963.

**Lin, J. & Weiss, A. (2001)**. Identification of the minimal tyrosine residues required for linker for activation of T cell function. *Journal of Biological Chemistry* **276**, 29588-29595.

**Lopez-Bergami, P., Habelhah, H., Bhoumik, A., Zhang, W. Z., Wang, L. H. & Ronai, Z. (2005)**. Receptor for RACK1 mediates activation of JNK by protein kinase C. *Molecular Cell* **19**, 309-320.

**Ma, Z. Y., Janmey, P. A. & Finkel, T. H. (2008)**. The receptor deformation model of TCR triggering. *Faseb Journal* **22**, 1002-1008.

**Mempel, T. R., Henrickson, S. E. & von Andrian, U. H. (2004)**. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature* **427**, 154-159.

**Murphy, K. M., Heimberger, A. B. & Loh, D. Y. (1990)**. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* **250**, 1720-1723.

**Mustelin, T. & Altman, A. (1990)**. DEPHOSPHORYLATION AND ACTIVATION OF THE T-CELL TYROSINE KINASE PP56LCK BY THE LEUKOCYTE COMMON ANTIGEN (CD45). *Oncogene* **5**, 809-813.

**Neer, E. J., Schmidt, C. J., Nambudripad, R. & Smith, T. F. (1994)**. THE ANCIENT REGULATORY-PROTEIN FAMILY OF WD-REPEAT PROTEINS. *Nature* **371**, 297-300.

**Nika, K., Soldani, C., Salek, M. & other authors (2010)**. Constitutively Active Lck Kinase in T Cells Drives Antigen Receptor Signal Transduction. *Immunity* **32**, 766-777.

**Nolz, J. C., Gomez, T. S., Zhu, P. M., Li, S. X., Medeiros, R. B., Shimizu, Y., Burkhardt, J. K., Freedman, B. D. & Billadeau, D. D. (2006)**. The WAVE2 complex regulates actin cytoskeletal reorganization and CRAC-mediated calcium entry during T cell activation. *Current Biology* **16**, 24-34.

**Norcross, M. A. (1984)**. A SYNAPTIC BASIS FOR LYMPHOCYTE-T ACTIVATION. *Annales D Immunologie* **D135**, 113-134.

**Osmanagic-Myers, S. & Wiche, G. (2004)**. Plectin-RACK1 (receptor for activated C kinase 1) scaffolding - A novel mechanism to regulate protein kinase C activity. *Journal of Biological Chemistry* **279**, 18701-18710.

**Paster, W., Paar, C., Eckerstorfer, P., Jakober, A., Drbal, K., Schutz, G., Sonnleitner, A. & Stockinger, H. (2009)**. Genetically Encoded Forster Resonance Energy Transfer Sensors for the Conformation of the Src Family Kinase Lck. *Journal of Immunology*, 2160-2167.

**Pear, W. S., Miller, J. P., Xu, L. W. & other authors (1998)**. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* **92**, 3780-3792.

**Peggs, K. S. & Allison, J. P. (2005)**. Co-stimulatory pathways in lymphocyte regulation: the immunoglobulin superfamily. *British Journal of Haematology* **130**, 809-824.

**Perlmutter, R. M., Marth, J. D., Lewis, D. B., Peet, R., Ziegler, S. F. & Wilson, C. B. (1988)**. STRUCTURE AND EXPRESSION OF ICK - TRANSCRIPTS IN HUMAN LYMPHOID-CELLS. *Journal of Cellular Biochemistry* **38**, 117-126.

**Robles, M. S., Boyault, C., Knutti, D., Padmanabhan, K. & Weitz, C. J. (2010)**. Identification of RACK1 and Protein Kinase C alpha as Integral Components of the Mammalian Circadian Clock. *Science* **327**, 463-466.

**Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E. & Mochlyrosen, D. (1994)**. CLONING OF AN INTRACELLULAR RECEPTOR FOR PROTEIN-KINASE-C - A HOMOLOG OF THE BETA-SUBUNIT OF G-PROTEINS. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 839-843.

**Roose, J. P., Mollenauer, M., Ho, M., Kurosaki, T. & Weiss, A. (2007)**. Unusual interplay of two types of Ras activators, RasGRP and SOS, establishes sensitive and robust Ras activation in lymphocytes. *Molecular and Cellular Biology* **27**, 2732-2745.

**Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L. & Schlossman, S. F. (1988)**. THE CD4 RECEPTOR IS COMPLEXED IN DETERGENT LYSATES TO A PROTEIN-TYROSINE KINASE (PP58) FROM

HUMAN LYMPHOCYTES-T. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 5190-5194.

**Russell, R. B., Sasieni, P. D. & Sternberg, M. J. E. (1998)**. Supersites within superfolds. Binding site similarity in the absence of homology. *Journal of Molecular Biology* **282**, 903-918.

**Schilham, M. W., Fungleung, W. P., Rahemtulla, A., Kuendig, T., Zhang, L., Potter, J., Miller, R. G., Hengartner, H. & Mak, T. W. (1993)**. ALLOREACTIVE CYTOTOXIC T-CELLS CAN DEVELOP AND FUNCTION IN MICE LACKING BOTH CD4 AND CD8. *European Journal of Immunology* **23**, 1299-1304.

**Schmedt, C., Saijo, K., Niidome, T., Kuhn, R., Aizawa, S. & Tarakhovsky, A. (1998)**. Csk controls antigen receptor-mediated development and selection of T-lineage cells. *Nature* **394**, 901-904.

**Sengupta, J., Nilsson, J., Gursky, R., Spahn, C. M. T., Nissen, P. & Frank, J. (2004)**. Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. *Nature Structural & Molecular Biology* **11**, 957-962.

**Shor, B., Calaycay, J., Rushbrook, J. & McLeod, M. (2003)**. Cpc2/RACK1 is a ribosome-associated protein that promotes efficient translation in Schizosaccharomyces pombe. *Journal of Biological Chemistry* **278**, 49119-49128.

**Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. (2009)**. T Cell Activation. In Annual Review of Immunology, pp. 591-619. Palo Alto: Annual Reviews.

**Stirnimann, C. U., Petsalaki, E., Russell, R. B. & Mueller, C. W. (2010)**. WD40 proteins propel cellular networks. *Trends in Biochemical Sciences* **35**, 565-574.

**thebiogrid.org** Interaction summary for GNB2L1 (Homo sapiens). Available "http://thebiogrid.org/115671/summary/homo-sapiens/gnb2l1.html" (Accessed: 10 Feb 2014).

**Thomas, S. & Brugge, J. (1997)**. Cellular functions regulated by Src family kinases. *Annual Review of Cell and Developmental Biology*, 513-609.

**Thornton, C., Tang, K. C., Phamluong, K., Luong, K., Vagts, A., Nikanjam, D., Yaka, R. & Ron, D. (2004)**. Spatial and temporal regulation of RACK1 function and N-methyl-D-aspartate receptor activity through WD40 motif-mediated dimerization. *Journal of Biological Chemistry* **279**, 31357-31364.

**Tsun, A., Qureshi, I., Stinchcombe, J. C., Jenkins, M. R., de la Roche, M., Kleczkowska, J., Zamoyska, R. & Griffiths, G. M. (2011)**. Centrosome docking at the immunological synapse is controlled by Lck signaling. *Journal of Cell Biology* **192**, 663-674.

**Vallabhapurapu, S. & Karin, M. (2009)**. Regulation and Function of NF-kappa B Transcription Factors in the Immune System. In Annual Review of Immunology, pp. 693-733. Palo Alto: Annual Reviews.

**van der Merwe, P. A., Davis, S. J., Shaw, A. S. & Dustin, M. L. (2000)**. Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition. *Seminars in Immunology* **12**, 5-21.

**van der Merwe, P. A. & Dushek, O. (2011)**. Mechanisms for T cell receptor triggering. *Nature Reviews Immunology* **11**, 47-55.

**Veillette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. (1988)**. THE CD4 AND CD8 T-CELL SURFACE-ANTIGENS ARE ASSOCIATED WITH THE INTERNAL MEMBRANE TYROSINE-PROTEIN KINASE P56LCK. *Cell* **55**, 301-308.

**Volta, V., Beugnet, A., Gallo, S., Magri, L., Brina, D., Pesce, E., Calamita, P., Sanvito, F. & Biffo, S. (2013)**. RACK1 depletion in a mouse model causes lethality, pigmentation deficits and reduction in protein synthesis efficiency. *Cellular and Molecular Life Sciences* **70**, 1439-1450.

**Vyas, J. M., Van der Veen, A. G. & Ploegh, H. L. (2008)**. The known unknowns of antigen processing and presentation. *Nature Reviews Immunology* **8**, 607-618.

**Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D. & Kuriyan, J. (1993)**. BINDING OF A HIGH-AFFINITY PHOSPHOTYROSYL PEPTIDE TO THE SRC SH2 DOMAIN - CRYSTAL-STRUCTURES OF THE COMPLEXED AND PEPTIDE-FREE FORMS. *Cell* **72**, 779-790.

**Wan, Y. Y., Leon, R. P., Marks, R., Cham, C. M., Schaack, J., Gajewski, T. F. & DeGregori, J. (2000a)**. Transgenic expression of the coxsackie/adenovirus receptor enables adenoviral-mediated gene delivery in naive T cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13784-13789.

**Wan, Y. Y., Leon, R. P., Marks, R., Cham, C. M., Schaack, J., Gajewski, T. F. & DeGregori, J. (2000b)**. Transgenic expression of the coxsackie/adenovirus receptor enables adenoviral-mediated gene delivery in naive T cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13784-13789.

**Wange, R. L., Malek, S. N., Desiderio, S. & Samelson, L. E. (1993)**. TANDEM SH2 DOMAINS OF ZAP-70 BIND TO T-CELL ANTIGEN RECEPTOR-ZETA AND CD3-EPSILON FROM ACTIVATED JURKAT T-CELLS. *Journal of Biological Chemistry* **268**, 19797-19801.

**Xu, W. Q., Doshi, A., Lei, M., Eck, M. J. & Harrison, S. C. (1999)**. Crystal structures of c-Src reveal features of its autoinhibitory mechanism. *Molecular Cell* **3**, 629-638.

**Yaka, R., Thornton, C., Vagts, A. J., Phamluong, K., Bonci, A. & Ron, D. (2002)**. NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 5710-5715.

**Yatime, L., Hein, K. L., Nilsson, J. & Nissen, P. (2011)**. Structure of the RACK1 Dimer from Saccharomyces cerevisiae. *Journal of Molecular Biology* **411**, 486-498.

**Zha, Y., Shah, R., Locke, F., Wong, A. & Gajewski, T. F. (2008)**. Use of Cre-adenovirus and CAR transgenic mice for efficient deletion of genes in post-thymic T cells. *Journal of immunological methods* **331**, 94-102.

**Zhang, J., Shehabeldin, A., da Cruz, L. A. G. & other authors (1999)**. Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. *Journal of Experimental Medicine* **190**, 1329-1341.

**Zhang, J., Zhu, F., Li, X. & other authors (2012)**. Rack1 protects N-terminal phosphorylated c-Jun from Fbw7-mediated degradation. *Oncogene* **31**, 1835-1844.

**Zhang, W. G., Trible, R. P. & Samelson, L. E. (1998)**. LAT palmitoylation: Its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* **9**, 239-246.

**Zhou, S. Y., Shoelson, S. E., Chaudhuri, M. & other authors (1993)**. SH2 DOMAINS RECOGNIZE SPECIFIC PHOSPHOPEPTIDE SEQUENCES. *Cell* **72**, 767-778.