Positive and negative regulation of T cell responses by Lck-dependent signaling pathways

Trond Methi

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“To do is to do.” – *Homo Erectus*

“To be is to be.” – *René Descartes*

"To be is to do." – *Immanuel Kant*

"To do is to be." – *Friedrich Nietzsche*

"Do be do be do." – *Frank Sinatra*

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"Videre er det således mulig å forestille seg at dette intet er lik et utall unike kombinasjoner, som alle ved subtraksjon er lik null, beskrevet som X – X = 0. Quod erat demonstrandum." (*Andreas Wensaas*)

Trond Methi
October 2007

1. Abbreviations

AP-1	activated protein 1
APC	antigen presenting cell
Cbl	casitas B-lineage lymphoma
Cbp	Csk binding protein
CD	cluster of differentiation
Csk	C-terminal Src kinase
ERK	extracellular signal regulated kinase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor bound protein 2
IKK	IκB kinase
IL-2	interleukin 2
IS	immunological synapse
ITAM	immunoreceptor tyrosine based activation motif
kd	knockdown
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein-tyrosine kinase
LIME	Lck interacting membrane protein
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor κB
PAG	phosphoprotein associated with glycosphingolipid enriched microdomains
PI3K	phosphatidylinositol 3 kinase
PKC	protein kinase C
PLC	phospholipase C
PKR	protein kinase RNA
Ras	rat sarcoma
SFK	Src family kinase
SH	Src homology
SHP-1	SH2 domain containing protein tyrosine phosphatase 1
SLP-76	SH2 domain containing leukocyte protein of 76 kD
SOS	son of sevenless
Src	sarcoma (from Rous sarcoma)
TCR	T cell receptor
ZAP-70	ζ -chain-associated protein of 70 kD

2. List of publications included

Paper I Methi T, Ngai J, Mahic M, Amarzguioui M, Vang T and Taskén K.
Short-interfering RNA-mediated Lck knockdown results in augmented downstream T cell responses.
Journal of Immunology 2005, 175:7398-7406.

Paper II Methi T, Ngai J, Vang T, Torgersen KM and Taskén K.
Hypophosphorylated TCR/CD3-zeta signals through a Grb2-SOS1-Ras pathway in Lck knockdown cells.
European Journal of Immunology 2007, 37:2539-2548.

Paper III Methi T, Torunn Berge, Torgersen KM and Taskén K.
Reduced Cbl phosphorylation and CD3-zeta degradation in T cells with low Lck levels.
Manuscript submitted.

Paper IV Ngai J, Methi T, Andressen KW, Levy FO, Torgersen KM, Vang T, Wettschureck N and Taskén K.
Gαq regulates TCR-mediated immune response in T cells through an Lck-dependent pathway
Manuscript submitted.

Abstract

We have targeted Lck with siRNA-mediated RNA interference and investigated signaling properties in T cells with Lck knockdown. Proximal signaling was reduced in Lck-kd cells, as determined by overall tyrosine phosphorylation, CD3ζ phosphorylation and Ca^{2+} mobilization. Despite this, NFAT-AP-1 activation and IL-2 secretion was increased (Paper I). We found that Grb2-SOS1 was recruited to hypophosphorylated CD3-zeta in these cells, leading to sustained Ras-Raf-1-ERK1/2 activation (Paper II). Furthermore, endocytosed TCR/CD3-containing vesicles were not targeted to lysosomes, and CD3-zeta levels remained elevated in Lck-kd cells (Paper III). This indicates that prolonged survival of internalized TCR/CD3-complexes may lead to sustained signaling through the Grb2-SOS1-Ras-ERK pathway. If allowed to operate in the absence of negative feedback mediated by SHP-1 (Paper I), Ras-GAP (Paper II), and c-Cbl (Paper III) Lck-kd may thus result in hyperresponsive signaling in T cells with low Lck levels.

We have also investigated the involvement of G proteins in TCR-mediated immune responses, and the possible interplay between Gαq and Lck (Paper IV). TCR engagement activated Gαq and Gαs but not Gαi-2. Targeting of Gαs, Gαi-2 and Gαq using siRNA-mediated knockdown demonstrated a specific role of Gαq in T cell activation with an increase in the transcriptional activation of NFAT-AP1, despite reduced proximal signaling. The same was found in primary T cells from Gαq deficient mice which displayed hyperproduction of IL-2, but a reduced ability to activate proximal signaling events.

3. Introduction

3.1. The immune system

The human body is under constant attack from infectious agents such as viruses, bacteria, fungi, protozoa and parasites, and many mechanisms have evolved to protect us against these threats. Very few infectious agents can penetrate the barrier of intact skin, but the epithelia and mucus membranes of the nasopharynx, lungs, gastrointestinal and urogenital tracts are more vulnerable to entry. Microorganisms come in many different forms, and they continually change structure and strategy to breach or evade our first line defences. When infectious agents gain access to the body, they may cause disease, which can be fatal if left unchecked. A flexible immune response is therefore required to deal with the large variety of microorganisms. Recognition of pathogens is essential for the immune system, and

especially separation from self and harmless material. The two main categories of pathogen recognition are the innate (non-adaptive) and adaptive immune responses. The former employs a fixed set of mechanisms which have co-evolved with emerging microbes, and detect classical patterns in foreign material, such as bacterial carbohydrates (lipopolysaccharide), nucleic acids (bacterial or viral DNA or RNA) and peptidoglycans. In the adaptive immune response, T and B lymphocytes are responsible for specific immune recognition. T cells regulate, direct or execute immune effector functions, and are the subject of the next chapter. B cells produce antibodies. The hallmark of adaptive immunity is a custom tailored response to each microorganism. The response is therefore slower in onset but more potent than innate immunity. The adaptability of the system is achieved by somatic mutations and irreversible recombination of antigen receptor gene segments. These mechanisms allow a small number of genes to generate a vast number of different antigen receptors, which are then expressed on individual lymphocytes. Lymphocytes with self-reacting receptors are eliminated, and the body thereby generates a receptor repertoire for essentially all antigens that are non-self. Triggering of a particular receptor gives rise to clonal selection and proliferation of the lymphocyte, and because the gene rearrangements have lead to an irreversible change in the DNA of each cell, all of the progeny will inherit genes encoding the same receptor specificity. This includes the T and B memory cells which are keys to long-lived specific immunity.

3.2. T cells

The main classes of T cells are the CD4+ helper (Th), CD8+ cytotoxic (Tc) and FOXP3+ regulatory (Treg) cells. All T cells develop from lymphoid bone marrow progenitor cells and are educated in the thymus. Naïve T cells migrate through lymph nodes, spleen and other secondary lymphoid organs, ready to be activated by the interaction with professional antigen presenting cells (APCs), such as macrophages, B cells and dendritic cells. CD4+ T cells regulate and direct cytotoxic (Th1) or humoral (Th2) immune responses depending on the type of infection encountered. The former is typically mounted against virus infections and cancer cells, whereas the latter targets bacterial and protozoan infections. The Th1 and Th2 responses are characterized mainly by the profile of cytokines produced, which in turn activate specific subsets of immune cells. The Th1 response involves IFNγ, TNF and IL-2, and triggers cell-mediated inflammatory reactions executed by NK cells, macrophages and Tc cells. Tc cells eliminate virus infected cells and cancer cells through direct intervention

after recognition of foreign antigen presented on MHC class I molecules. The Th2 response involves IL-4, IL-5, IL-10 and IL-13, and activate B cells. B cells produce and secrete antibody molecules, which bind infectious material and mark it for destruction. Tregs suppress immune responses after an infection has been purged, and thereby maintain immune system homeostasis and tolerance to self. Aberrant or disturbed activation of T cells may lead to autoimmune diseases or immunodeficiency.

3.3. T cell receptor signaling

The T cell receptor (TCR) complex consists of the heterodimeric αβ chains and a set of six polypeptides, the CD3 chains, designated γε ζζ δε (Fig.1). APCs continously process exogenous peptides and present them on MHC class II molecules. The TCR interacts with peptide-loaded MHC class II molecules but will only be engaged with sufficient strength if there are matching antigen epitopes present (Reviewed in Kuhns et al. 2006, and Rudolph et al., 2006). Many factors are involved in proper activation of T cells. The strength and duration of the TCR-MHC class II engagement is important, but also the presence of a co-stimulatory signal mediated through CD28. CD28 interaction with B7 proteins on APCs ensures that the TCR is engaged by professional immune cells, and not accidentally by other cells or by soluble antigen. Absence of a secondary CD28 signal will lead to T cell anergy, a state characterized by a lack of T cell responsiveness to otherwise effective antigens. Anergy has emerged as a fundamental mechanism for preventing autoimmune responses to self-antigens.

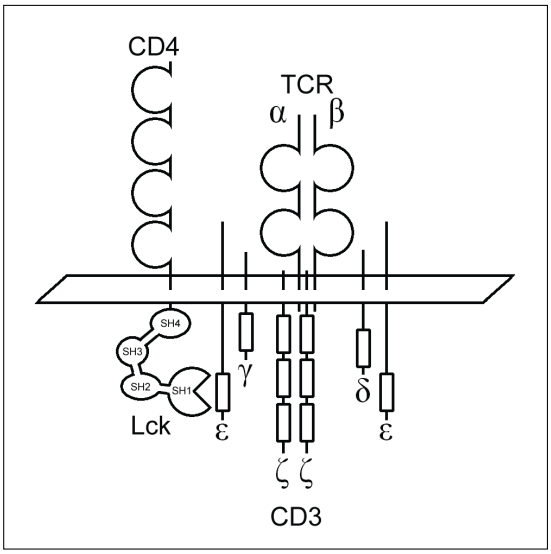

Figure 1. The TCR/CD3-complex

The TCR is located in specialized plasma membrane microdomains, termed lipid rafts. Lipid rafts consist of cholesterol and sphingolipid-enriched platforms which segregate

various signaling molecules, and are postulated to function in signaling and membrane trafficking (Ilangumaran et al., 2000; Marmor and Julius, 2001; Harder, 2001; Horejsi, 2003). The immunological synapse is a term used to denote the complex array of protein-protein interactions which arises between a T cell and an APC (Monks et al. 1998; Grakoui et al., 1999; Reviewed in Dustin et al., 2005). The formation and organization of an immunological synapse is constituted by the coalescence of lipid rafts and recruitment of signaling proteins, the binding of integrins and adhesion molecules, as well as the TCR-MHC class II and CD28-B7 contacts. Altogether, this platform relays coordinated signals from the exterior to the interior of the T cell. Intracellular TCR signaling will be elaborated in the following chapters. Briefly summarized, Lck becomes activated upon TCR engagement, and phosphorylates the intracellular portions of the TCR/CD3-complex. ZAP-70 binds and phosphorylates LAT, to which many adaptor and effector proteins are recruited. This culminates with the mobilization of intracellular Ca^{2+} and activation of PKC and Ras. In turn, transcription factors translocate to the nucleus of the T cell. Activated T cells secrete a plethora of cytokines which lead to proliferation and differentiation of immune cells.

3.4. Lck and Src family kinases

The most membrane proximal event to take place after engagement of the TCR is the activation of Lck (Lymphocyte specific tyrosine kinase). Lck is a 509 amino acids long, 56-kD nonreceptor protein-tyrosine kinase of the Src oncogene family. The Src family of kinases (SFKs) also contain the members Src, Yes, Fyn, Lyn, Hck, Fgr and Blk, but only Lck, Fyn and Yes are expressed in T cells (Kitamura et al., 1982; Marth et al., 1985; Voronova and Sefton, 1986; Semba et al., 1986; Cooke and Perlmutter 1989; Olszowy et al., 1995). The SFKs share many structural and functional features of Lck, and since Lck is is the main topic of this thesis, it will serve as a model example of a Src kinase in the following presentation.

3.4.1 Structure of Lck

Lck is composed of SH3, SH2 and SH1 domains arranged sequentially from the N-terminus to the C-terminus (see Fig. 2). The SH3 domain is 57 residues long and interact with proline rich regions with a central PXXP motif (Cohen et al., 1995). The SH2 domain is 91 residues long and interacts with certain phospho-tyrosines (the Src SH2 domain prefer the sequence pYEEI/L) (Cohen et al., 1995; Pawson, 1995). The SH1 domain is a 250 residue-long tyrosine kinase. Although Fyn and Lck both have SH2 and SH3 domains, the binding specificities of these domains are significantly different, and confer unique functions to these SFKs in T cell signaling (Lin et al., 2000; and reviewed in Zamoyska et al., 2003). Lck functions primarily as a tyrosine kinase, but both the SH2 (Straus et al., 1996; Lewis et al., 1997) and the SH3 domain (Denny et al., 1999) have been shown to be important for signaling in T cells.

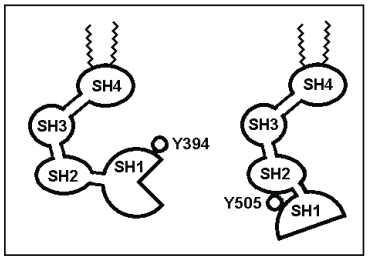

Figure 2. Lck

Lck is anchored to the plasma membrane through lipid modifications (Marchildon et al., 1984; Marth et al., 1985, Voronova and Sefton, 1986). The N-terminal residues G2 and C3/5 are myristoylated and palmitoylated respectively (Marchildon et al., 1984; Johnson et al., 1994, Paige et al., 1993; Koegl et al., 1994; Yurchak et al., 1995). Fyn also contains lipid anchors, but the localizations of Lck and Fyn are non-overlapping in T cells, suggesting spatial organization by other mechanisms (Ley et al., 1994; Lin et al., 2000; Filipp et al., 2003; Filipp et al. 2004). Lck interacts with two cysteines within an CXCP motif in the intracellular domains of CD4 and CD8 through a unique SH4 domain in a zinc-dependent manner (Veillette et al., 1988; Rudd, et al., 1988; Rudd et al., 1989; Luo and Sefton, 1990; Turner et al., 1990; Kim et al., 2003). Approximately 50% of Lck in T cells interact with CD4 in this way (Veillette et al., 1988), and since CD4 is normally excluded from lipid rafts, so is Lck. In fact, up to 95% of Lck to resides in soluble membrane fractions, whereas more than 98% of Fyn concentrates within lipid rafts, some of which associates with the TCR/CD3-complex (Samelson et al., 1990; Timson Gauen et al., 1992; Filipp et al., 2003). There is some controversy in the field regarding Lck and lipid raft translocation. It has been reported that a large fraction of Lck resides in lipid rafts even in resting cells, and that CD28 but not CD4 stimulation results in more Lck recruitment into rafts (Tavano et al., 2004).

3.4.2. Lck and Fyn in T cell development

Lck is primarily expressed in T cells where it serves many functions, both in signaling and T cell ontogeny. TCR signaling affects the selection and survival of T cells at every stage of development (Shortman and Wu, 1996, Sebzda et al., 1999; Kruisbeek et al., 2000). Lck knockout mice display thymic atrophy and diminished double positive (CD4+CD8+) thymocytes (Molina et al., 1992). Mature, single positive thymocytes could not be detected in these animals and peripheral T cell counts were very low Similar findings have been reported with transgenic mice expressing a dominant-negative version of Lck (Levin et al., 1993). This underscores an important role of Lck in T cell development for which Fyn can not compensate. Thymocytes from Fyn-deficient mice undergo normal maturation (Stein et al., 1992; Appleby et al., 1992). However, Fyn is not completely dispensable since knockout of both Lck and Fyn leads to a complete arrest in T cell development at the double negative (CD4-CD8-) thymocyte stage (van Oers et al., 1996a; Groves et al., 1996).

3.4.3. Regulation of Lck by phosphorylation and dephosphorylation

Lck is phosphorylated on two main residues, Y394 (Marth et al., 1988; Veillette et al., 1989a; Veillette et al., 1989b; Lou and Sefton, 1990; Abraham and Veillette, 1990) and Y505 (Courtneidge, 1985; Cooper et al., 1986; Cooper and King, 1986; Amrein and Sefton, 1988; MacAuley and Cooper, 1989). The former is an autophosphorylation site which leads to 2-4 fold increased tyrosine kinase activity. The latter is phosphorylated by C-terminal Src Kinase (Csk) which inhibits Lck (Okada and Nakagawa 1989; Thomas et al., 1991; Nada et al., 1991; Okada et al., 1991; Bergman et al., 1992). Phosphorylated Y505 interacts with the intrinsic SH2 domain of Lck, thus folding the protein onto itself. This inhibits the kinase domain indirectly by inducing conformational changes in the catalytic site (Yamaguchi and Hendrickson, 1996; Sischeri et al., 1997; Xu et al., 1997; Xu et al., 1999). pY394 is dephosphorylated primarily by the PTPases SHP-1, CD45 and LYP (Wu et al., 2006), wheras pY505 is dephosphorylated mainly by CD45. SHP-1 is a central negative regulator of T cell signaling and is presented in chapter 3.7.2. CD45 is expressed in all nucleated hematopoietic cells, but not in other cell types (Mustelin et al., 1989; Mustelin et al., 1990; Mustelin et al., 1992). In CD45-deficient lymphocytes both Lck and Fyn are hyperphosphorylated on Y505 and Y528 respectively, and these cells show diminished responses to antigen stimulation (Pingel and Thomas 1989; Ostergaard et al., 1989; Koretzky et al., 1990; Kishihara et al., 1993; Stone et al., 1997). Membrane targeting or

overexpression of Csk is sufficient to inhibit TCR induced phosphorylation and IL-2 production through phosphorylation of Y505 (Chow et al., 1993). In contrast, deletion or mutation of Y505 in Lck results in a constitutively active enzyme (Marth et al., 1988; Abraham et al., 1991; Chow et al., 1993). Dephosphorylation of pY505 is therefore thought to be a critical positive regulator of Lck and Fyn in T cells. Lck is also phosphorylated on S59 (Winkler et al., 1993), and this regulates the specificity of the SH2 domain (Joung et al., 1995). Weak TCR ligands may trigger a negative feedback loop dependent on the recruitment of SHP-1 to Lck. This is prevented when stronger ligands bind though ERK-dependent phosphorylation of S59 in Lck (Stefanova et al., 2003). Phosphorylation of Y192 within the SH2 domain of Lck disrupts SH2 binding, thereby negatively regulating its participation in T cell antigen receptor signaling (Couture et al., 1996).

3.4.4. Activation and function of Lck

The TCR/CD3-complex lacks enzymatic activity and is therefore dependent on external kinase activity for signal transmission. Lck is considered to be the principal mediator of this function (Veillette et al., 1989a; Veillette et al., 1989b; Veillette et al., 1989c; Barber et al., 1989; Abraham et al., 1991; Luo and Sefton, 1992). However, the exact nature of how Lck becomes activated after engagement of the TCR is still elusive. Several mechanisms have been proposed: Release from tonic inhibition bt Csk, dephosphorylation of the inhibitory pY505 by CD45, clustering within the immunological synapse and juxtaposition of Lck with the TCR/CD3-complex through CD4-Lck interaction with MHC class II molecules. Other forms of SFK activation have also been described. For example, it has been shown that engagement of the SH3 domain of Lck by a proline-rich sequence in CD28 increases the phosphotransferase activity of Lck (Holdorf et al., 1999). Furthermore, engagement of the IL-2 receptor increases the activity of bound Lck (Horak et al., 1991), although IL-2 signaling is not dependent on Lck (Karnitz et al., 1992). In fact, T cells from Lck $^{-/-}$ mice exibit enhanced proliferative responses to IL-2 stimulation (Molina et al., 1992). Src interaction with small G proteins is discussed in chapter 3.8., and examined in more detail in Paper IV. The kinetics and discrete regulation of Y394 and Y505 dephosphorylation by CD45 is not fully elucidated, and it is likely that CD45 has a role as both positive and negative regulator of T cell signaling, which is underscored by recent data (McNeill 2007). It has also been reported that CD4-Lck clusters outside the immunological synapse, away from the TCR-CD3 complex (Ehrlich et al., 2002). Furthermore, stimulation with anti-CD3ε

antibodies (OKT3) is sufficient to trigger robust Lck activation in Jurkat and primary T cells, but the link between CD3ε engagement and Lck activation is still unknown.

Although different mechanisms of activation exist, it is evident that Lck becomes activated upon TCR engagement. The main substrates for Lck are the CD3-chains of the TCR-complex (Barber et al. 1989), and several lines of evidence suggest a pivotal role for Lck in signal transmission through the TCR. Pharmacological inhibition of Lck and Fyn with PP2, or new specific Lck inhibitors, abolish anti-CD3-induced T cell signaling (Hanke et al., 1996; Rapecki et al., 2002). The Jurkat cell line JCaM1, which is deficient in Lck, does not respond to anti-CD3 engagement, and reconstitution of these cells with Lck restores T cell signaling (Goldsmith and Weiss, 1987; Straus and Weiss, 1992). Fyn may also phosphorylate the TCR/CD3-complex, but overexpression of Fyn in JCaM1 cells only leads to a partial recovery (Denny et al., 2000). On the other hand, it has been shown that Syk is activated independently of Lck upon CD3-stimulation (Couture et al., 1994; Chu et al., 1996), and reconstitution of JCaM1 cells with Syk, but not ZAP-70, could also restore signaling to ERK (Williams et al., 1997). Knockout mice have been generated for both Lck and Fyn, and data from these animals indicate that Lck is important, but not indispensible for the TCR/CD3 signaling pathway. The proliferative response to anti-CD3 or TCRαβ crosslinking was substantially reduced in Lck $^{-/-}$ T cells, but was still higher than in unstimulated cells (Molina et al., 1992). Phosphorylation of CD3ζ and ZAP-70 was virtually absent in murine Lck $^{-/-}$ thymocytes, but several phosphoproteins were induced, although to a lower degree and with slower kinetics than in wild-type cells (van Oers et al., 1996b). TCR engagement resulted in CD69 expression in Lck deficient mice, albeit to a substantially lower level than in control animals. Furthermore, peripheral T cells from Lck $^{-/-}$ mice showed normal responsiveness to allostimulation (allo-MHC antigens), suggesting that Lck is not required for this T cell effector function (Yamada et al., 1997). A major problem with Lck knockout animals is that compensatory mechanisms may have developed, and that the few T cells that do mature may represent a population that has adapted to the loss of Lck. Lck$^{-/-}$ mice may therefore not be the best model to investigate all aspects of Lck signaling *in vivo*, and conditional transgenic mice (Lck1^{ind}) that express Lck by a T cell-specific tet-inducible mechanism have been developed. This model has reveled a central role for Lck in the differentiation of CD4 and CD8 thymocytes, but no detectable change in T cell numbers in peripheral lymphoid organs was observed even 9 weeks after loss of Lck (Legname et al., 2000; Tewari et al., 2006). Interestingly, it has been shown that the absence

of Lck actually increased the long-term survival of naïve T cells (Seddon et al., 2000), and that Lck expression was not essential for responses of memory $CD8^+$ T cells to secondary antigen stimulation *in vivo* or *in vitro* (Tewari et al., 2006). One report has used mice expressing a Lck transgene in the thymus to overcome the developmental block associated with the $Lck^{-/-}$ background (Trobridge and Levin 2001). It was shown that primary $Lck^{-/-}$ T cells from these animals had an impaired Ca^{2+} mobilization and defective proliferation. However, the authors also showed that T cells expressing an excess of a catalytically inactive Lck were capable of inducing Ca^{2+}-fluxes, suggesting that Lck kinase activity is not essential in T cell activation. TCR signaling in $Lck1^{ind}$ mice with or without the Fyn $^{-/-}$ background has recently been investigated (Lovatt et al., 2006). In this system, T cells with low levels or complete absence of Lck was compared to wild-type cells. It was shown that Lck controls the threshold of T cell activation by specifically activating the CD3ζ-ZAP-70-LAT-PLCγ1-pathway, leading to Ca^{2+} dependent induction of IL-2 synthesis. Surprisingly, crosslinking CD4 with the TCR enhanced signal transduction even in the absence of Lck, resulting in Ca^{2+} flux and phosphorylation of some LAT residues in Lck-deficient T cells. This occured without ZAP-70 or PLCγ1 phosphorylation. Furthermore, the activation of MEK and ERK was surprisingly high in Lck-low or Lck-deficient mice, although reduced compared to wild type animals. Fyn was shown to be responsible for these effects, and the authors concluded that Lck and Fyn target the ERK1/2-pathway through distinct Ras activators. Interestingly, when intracellular IL-2 was visualized, a significant proportion of Lck-deficient cells were found to produce IL-2 even though secretion was reduced. This indicates that signals received in the absence of Lck are sufficient to open the IL-2 locus but insufficient to induce normal levels of IL-2 secretion. Work included in this thesis investigate further how low levels of Lck may result in T cell signaling (Paper I and II).

3.4.5 Summary of Lck interaction partners and/or substrates

Table 1. Lck interaction partners and/or substrates

Protein		Reference
Cbl	I	Rao et al., 2002, Hawash et al., 2002
CD28	I	Holdorf et al., 1999
CD3ζ/ε/γ/δ	S	Barber et al., 1989, Straus and Weiss 1992, van Oers 1996b
Ezrin	S	Autero et al., 2003
Fyn	S	Filipp et al., 2003
IL-2 receptor	I/S	Hatakeyama et al., 1991, Delespine-Carmagnat 1999
Itk	S	Heyeck et al., 1997
LAT	S	Jiang and Cheng, 2007
LIME	I/S	Brdickova et al., 2003, Hur et al., 2003
PKCδ/θ	S	Konishi et al., 2001, Liu et al., 2000
PLCγ1	S	Liao et al., 1993
Ras-GAP	S/I	Amrein et al., 1992, Amrein et al., 1994
Shc	S/I	Walk et al., 1998, Fukushima et al., 2005
SHP-1	S/I	Lorenz et al., 1994
Unc119	I	Gorska et al., 2004
Vav1	S	Gulbins et al., 1993
ZAP-70	S/I	Duplay et al., 1994, Pelosi et al., 1999

I = Interaction partner; S=Substrate

3.4.6 Fyn

Fyn has been shown to be important for signal transduction in T cells (Cooke et al., 1991; Tsygankov et al., 1992). Peripheral T cells from Fyn $^{-/-}$ animals display relatively normal proliferative responses, but have blunted Ca^{2+} mobilization and IL-2 production (Stein et al., 1992; Appleby et al., 1992). Although Fyn plays a role in T cell signaling, it does not have the same impact as Lck, since overexpression of Fyn can not fully substitute for the loss of Lck (Denny et al., 2000). It has been shown that Fyn requires Lck recruitment to lipid rafts for its activation, and Fyn thus appears to be dependent on and function subsequently to Lck upon TCR engagement (Filipp et al., 2003; Filipp et al., 2004). Some lines of evidence have suggested that one of the roles of Fyn is to dampen T-cell responses, while Lck acts to amplify them (Lovatt et al., 2006; Filby et al., 2007). Fyn provides incomplete signals, such as those delivered by antagonistic ligands (Utting et al., 1998), and Fyn interacts specifically with some negative regulators of T-cell activation, such as c-Cbl (Tsygankov et al., 1996; see chapter 3.7.4.) and Cbp/PAG (Yasuda et al., 2002). Cbp/PAG is a transmembrane protein which binds Csk when phosphorylated (Brdicka et al., 2000; Kawabuchi et al., 2000). TCR stimulation leads to transient dephosphorylation of Cbp/PAG, probably in a CD45-dependent manner, therby releasing Csk from its plasma membrane anchor, and thus facilitating T cell activation (Torgersen et al., 2001; Davidson et al., 2003). Fyn-mediated

rephosphorylation of Cbp/PAG leads to the recruitment of Csk, and thereby constitutes a negative feedback mechanism. The function of Cbp/PAG is, however, still under investigation. In contrast to Csk null mutation, which leads to embryonic lethality (Imaoto and Soriano, 1993; Nada et al., 1993), Cbp/PAG-deficient mice appear normal (Dobenecker et al., 2005; Xu et al., 2005). Dobenecker et al. reported that Csk was still recruited to lipid rafts in these animals, indicating that other Csk adaptors compensate for the loss of Cbp/PAG. On the other hand, Xu. et al. reported that raft localization of Csk was greatly reduced, but that this did not lead to any detectable functional defects in T cell signaling. A recent report showed that siRNA-mediated knockdown of Cbp/PAG enhanced SFK and Ras activation (Smida et al., 2007). It has also been shown that Fyn-dependent phosphorylation of Cbp/PAG is involved in the induction of anergy (Davidson et al., 2007). Other interaction partners of Fyn in T cell signaling are SAP/SLAM (Chan et al., 2003; Latour et al., 2003; Davidson et al., 2004), Fyb (Da Silva et al., 1997) and the focal adhesion kinase family (Kanazawa et al., 1996). Phosphorylation of Pyk2 links Fyn to cytoskeletal organization, cell spreading and migration (Qian et al., 1997)

3.4.7. Lck and human pathophysiology

Aberrant expression or regulation of Lck has been associated with several diseases. Some patients with systemic lupus erythematosus (SLE) have T cells with decreased levels of Lck due to increased ubiquitin-mediated degradation. The specific Lck activity, however, was increased, and the T cells displayed augmented apoptosis and lipid raft abnormalities (Matache et al., 1999; Matache et al., 2001; Jury et al., 2003).

Patients with common variable immunodeficiency (CVID) or severe combined immuno deficiency (SCID) have been reported to have reduced Lck levels (Goldman et al., 1998; Sawabe et al., 2001). In both patients it was shown that the *lck* transcript lacked the entire exon 7 resulting in reduced or almost complete loss of Lck expression. Interestingly, JCaM1 cells exibit the exactly same defect, but the responsible mutation is yet to be found. Surprisingly, anti-CD3-induced tyrosine phosphorylation, ERK1/2 phosphorylation and calcium mobilization was intact in the SCID-patient, yet CD69 and most proliferative responses were reduced. However, the proliferative responses to allo-antigen were intact (Goldman et al., 1998) as reported previously (Yamada et al., 1997). Altogether, these studies indicate that loss of Lck can result in congenital immunodeficiency and selective

CD4 lymphopenia. This separates human T cell development from that of mice, since mice deficient in p56lck show a substantial reduction in $CD4^+/CD8^+$ thymocytes with the development of only a small number of peripheral single positive T cells (Molina et al., 1992).

Lck is the target of many viral proteins, especially the tyrosine kinase interacting protein Tip of Herpesvirus saimiri (HVS), and Nef encoded by the Human immunodeficiency virus (HIV). The oncoprotein Tip physically interacts with Lck in HVS transformed cells, and augments Lck activity independently of Y394 and/or Y505 phosphorylation (Wiese et al., 1996; Hartley et al., 1999). Tip induces peripheral T-cell lymphoma in transgenic mice (Wehner et al. 2001). Nef contributes to HIV disease pathogenesis by augmenting virus replication and disturbing T cell function. Nef binds and inhibits Lck, and MAPK kinase activity (Greenway et al., 1996), but simultaneous overexpression of Nef has been shown to increase T cell ERK1/2 phosphorylation in a calcium-independent manner (Schrager et al., 2002). Furthermore, Nef synergizes with PMA in inducing NFAT activation, an effect that is independent of Lck (Manninen et al., 2002). According to these data it appears that the Ras pathway rather than the calcium pathway is the rate-limiting step in TCR-mediated NFAT induction. The suceptibility of T cells to HIV infection has been inversely correlated with Lck activity (Yousefi et al., 2003). HIV binds to CD4 through gp120 (Juszczak et al., 1991), and internalization of gp120 is associated with down-modulation of membrane CD4 and Lck together with impairment of T cell activation (Cefai et al., 1992). Reduced levels of Lck but increased levels of Fyn may play a role in the anergic response observed early during HIV infection (Cayota et al., 1994).

Reduced Lck levels have also been shown in some type 1 diabetic patients. Interestingly, both ZAP-70 and PLCγ1 recruitment and phosphorylation was relatively normal, despite reduced phosphorylation of CD3ζ (Nervi et al., 2000). Furthermore, T cells from the synovial fluid of rheumatoid arthritis patients displayed lower levels of Lck protein, and components of the TCR/CD3-complex (Romagnoli et al., 2001). This downregulation correlated with hyporesponsiveness of the T cells. Lastly, Lck has also been shown to be downregulated in Alzheimer disease, and was identified as a risk gene for this disease (Zhong et al., 2005).

3.5. CD3 chain ITAMs

Immunoreceptor tyrosine-based activation motifs (ITAMs) are found within the intracellular portions of the CD3 chains, and are the principal substrates for Lck in T cell signaling (Barber et al. 1989; Irving et al., 1993, Iwoshima et al., 1994, van Oers et al., 1996b). ITAMs have the concensus sequence YXXL/IX(6-8)YXXL/I (Reth, 1989), and CD3ζ contains three repititions of of this motif, whereas the γ, δ and ε-chains contain only one (Reviewed in Pitcher et al., 2003, and Pitcher and van Oers, 2003). Each doubly phosphorylated ITAM may recruit the tandem SH2 (Src homology 2) domains of ZAP-70 (Chan et al., 1991; Wange et al., 1992; Wange et al., 1993; Iwashima et al., 1994) (Fig. 3). ZAP-70 is a Syk family tyrosine kinase which is activated through phosphorylation by Lck on Y443 (Chan et al., 1995; Kong et al., 1996) and autophosphorylation on many sites, most importantly Y319 (Pelosi et al., 1999; Di Bartolo et al., 1999; Williams et al., 1999). The main substrate of ZAP-70 is the Linker for Activation of T cells (LAT) (Zhang et al., 1998) (see chapter 3.6). Loss of ZAP-70 disrupts TCR signaling and T cell development (Negishi et al., 1995; Williams et al., 1998), although ZAP-70-independent signaling has also been shown (Shan et al. 2001).

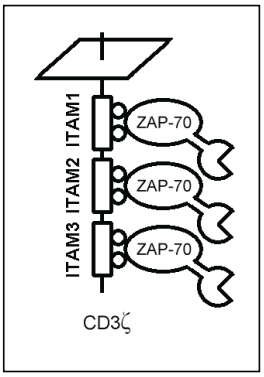

Figure 3. CD3ζ

In thymocytes and peripheral T cells, ITAMs are constitutively tyrosine phosphorylated on some residues (van Oers et al., 1993; van Oers et al.; 1994; Pitcher et al., 2003), and it appears that Lck or Fyn may fulfill the function of partly phosphorylating ITAMS in resting T cells equally well (Seddon and Zamoyska, 2002). ZAP-70 binds to these phosphorylated ITAMs but is not activated. Whether this pool of ZAP-70 transduces low level signals necessary for T cell survival, primes T cells for activation, or merely protects the ITAMs from other proteins, is currently unknown (reviewed in Zamoyska et al., 2003).

Mutational analysis has revealed redundancy between various chains of the CD3 complex. CD3 γε/δε provides normal T cell functions in the absence of CD3ζ (Wegener et al., 1992; van Oers et al., 1998, Ardouin et al., 1999; van Oers et al., 2000), and TCR signaling is not qualitativly affected by crippling of the CD3ε ITAM (Sommers et al., 2000). The migration of unphosphorylated CD3ζ is 16 kD, and depending on increasing tyrosine phosphorylation, 21 and 23 kD forms also appear. The six tyrosine residues in ITAM1-3 are numbered Y1-6

counting from the N-terminal membrane proximal part. The kinetics of CD3ζ phosphorylation has been studied to great extent, with some conflicting findings. It is thought that a hierarchic pattern of phosphorylation exists, where phosphorylated ITAM2 and 3 correspond to p21, whereas full phosphorylation correspons to p23 (Kersh et al., 1998a; van Oers et al., 2000). Differential signaling occurs from truncated or tyrosine-to-phenylalanine mutations in CD3ζ ITAMs, and partially phosphorylated CD3ζ can inhibit (Kersh et al., 1999), variably affect (Chae et al., 2004) or not affect (Ardouin et al., 1999) T cell activation. Some reports indicate that proteins such as Grb2, Shc and SOS may be recruited to hypophosphorylated ITAMs (Ravichandran et al., 1993; Osman et al., 1995; Nel et al., 1995; Labadia et al., 1996; Zenner et al., 1996; Chau and Madrenas 1999). This kind of signaling from CD3ζ may be relevant under certain physiological settings, for example, it has been shown that various TCR binding affinities may mediate differential phosphorylation of the CD3ζ chains, leading to anergy in some cases (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Kersh et al., 1998b). Hypophosphorylation of CD3ζ and alternative signaling mechanisms are addressed in paper II.

3.6. LAT and downstream signaling

Activated CD3-bound ZAP-70 maintains the tyrosine phosphorylation cascade by phosphorylating LAT (Zhang et al., 1998; Weber et al., 1998) and SLP-76 (Wardenburg et al., 1996). LAT is a transmembrane protein which concentrates in lipid rafts (Lin et al., 1999). It contains ten pY residues, where Y127, Y132, Y171 Y191 and Y226 (human sequence) are the most important (Zhang et al., 2000; Sommers et al., 2001; Paz et al., 2001; Lin and Weiss, 2001). LAT serves as a docking site to which a number of adapter and signaling molecules bind through SH2 domains (Grb2, Gads, PLCγ1 and PI3K), or indirectly via adapters (SLP-76, Vav-1, SOS and Itk) (Zhang et al., 1998; Finco et al., 1998; Zhang et al., 1999a; Liu et al., 1999; Shan and Wange, 1999; Zhang et al., 2000; Bunnell et

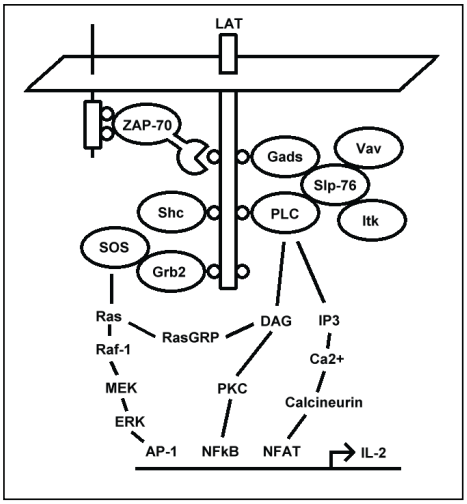

Figure 4. LAT signaling

al., 2000; Ching et al., 2000; Paz et al., 2001; Lin and Weiss, 2001) (See Fig. 4). LAT is essential for TCR signaling as LAT-deficient mice have a block in thymocyte development at the immature stage and completely lack mature peripheral T cells (Finco et al., 1998; Zhang et al., 1999). SLP-76 is of particular importance for the LAT signaling scaffold. It binds LAT through Gads and is phosphorylated by ZAP-70 (Wardenburg et al., 1996). Phosphorylated SLP-76 provides binding for the Tec family kinase Itk which is imperative for full PLC γ1 activation (Yablonski et al., 1998; Irvin et al., 2000; Bogin et al., 2007). As such, from SLP-76 and Itk knockout mice have similar phenotypes, and display disturbed PLCγ1 activity, Ca^{2+} mobilization and IL-2 production (Liao and Littman, 1995; Clements et al., 1998; Pivniouk et al., 1998). Fully activated PLCγ1 (pY783) catalyses the formation of IP3 and DAG (Downward et al., 1990), where the former leads to intracellular Ca^{2+} influx, and the latter recruits conventional and novel PKCs and RasGRP. RasGRP and SOS are guanine nucleotide exchange factors (GEFs), and catalyse the binding of GTP on the small G protein Ras. RasGRP has been shown to be the most important GEF in T cells (Ebinu et al., 2000; Priatel et al., 2002; Roose et al., 2005), although Grb2-SOS has also been implicated (Ravichandran et al., 1993), and especially upon partial agonist stimulation (Chau and Madrenas, 1999). Recent data indicate interplay between SOS1 and RasGRP in T cell Ras activation (Roose et al., 2007). Ras-GTP activates Raf-1, which phoshorylates MEK, leading to ERK phosphorylation. Phosphorylated ERK in turn activates c-Fos and c-Jun which constitute the AP-1 transcription factor dimer (Su and Karin, 1996). The novel type calcium-independent PKCθ is critical in the activation of NFκB in T cells (Sun et al., 2000). PKCθ is unique in its ability to localize to the supramolecular activation clusters of the TCR/CD3 complex (Monks et al., 1997; Monks et al., 1998). where it is phosphorylated and activated by Lck (Liu et al., 2000). Once active, PKCθ activates IkB kinase (IKK) (Coudronniere et al., 2000, Lin et al., 2000), through the phosphorylation of CARMA1 (Sommer et al., 2005; Matsumoto et al., 2005) and recruitment of the Bcl10–MALT1 complex. Activated IKK phosphorylates IκB (Brown et al., 1995; Traenckner et al., 1995), marking it for ubiquitination and proteosomal degradation (Chen et al., 1995; Scherer et al., 1995). When released from inhibition by IκB, the transcription factor NFκB is free to translocate into the nucleus (May and Ghosh, 1998). On the other hand, the transcription factor NFAT translocates to the nucleus upon dephosphorylation by Calcineurin, a phosphatase activated by Ca^{2+} bound to calmodulin (Jain et al., 1993; Aramburu et al. 1998; Peng et al. 2001). NFAT, AP-1 and NFκB promote the transcription of a number of genes, most importantly IL-2, a cytokine which promotes long term proliferation of activated T

cells (Zhang and Nabel, 1994). Signal transduction from the TCR and CD28 also lead to cytoskeletal rearrangements, involving proteins such as Vav1, Cdc42, Rac-1 and WASP (Stowers et al., 1995; Holsinger et al., 1998; Sedwick et al., 1999; Snapper and Rosen, 1999). These rearrangements are important for integrin signaling and formation of the immunological synapse.

3.7. Lck and inhibition of T cell signaling

Interestingly, Lck has also been linked to negative regulation of TCR signaling (fig. 5). Some early reports suggested that the kinase function of Lck was not required for CD4-dependent T cell activation (Collins and Burakoff, 1993) or that deletion of the kinase domain even lead to hyperactivity (Xu and Littman, 1993). Engagement of the TCR with antagonists increased the activity of CD4-associated Lck without leading to T cell activation (Racioppi et al., 1996; Haughn et al., 1992). Such T cells become anergic, and this indicates a negative role for Lck. Interestingly, it has been shown that triggering of the T cell antigen receptor by superantigens, such as staphylococcal enterotoxins, occurs independently of Lck (Yamasaki et al., 1997). In fact, JCaM1 cells lacking Lck, or human T cells pretreated with the PP2, are strongly hyperresponsive to SEE stimulation (Criado and Madrenas, 2004). This reveals a negative contribution of Lck to T cell activation, an effect that is dependent on its kinase activity. It was recently shown that superantigen triggering of the TCR activated PLCβ in a Gα11-dependent manner, and this resulted in robust ERK1/2 activation in Lck-deficient cells (Bueno et al., 2006). Other examples of Lck-independent signaling and negative regulation can also be found in the literature. For example, inhibition of Lck by antisense RNA in Th2 cells leads to elevated levels of lymphokine mRNAs, including IL-4, IL-5, and IL-10, and these cells were capable of secreting IL-4 upon activation through the TCR (Al-Ramadi et al., 1996). This occured despite abolished phosphorylation of CD3ζ and ZAP-70 and defective Ca^{2+} mobilization to antigenic stimuli. These data indicate that Lck-independent pathways of gene induction exist, a concept which we explored further in papers I and II. The negative role implicated for Lck is emphasized by its involvement in ligand-induced

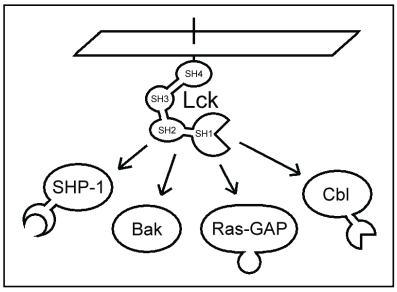

Figure 5. Negative regulation of TCR signaling through Lck

TCR internalization (Luton et al., 1994; D'Oro et al., 1997; Lee et al., 1997; Salio et al., 1997) and activation-induced cell death (Yu et al., 2004). These data suggest that Lck may be important for regulating the availability of TCR and the magnitude of T cell signaling. Apoptotic pathways are also dependent on Lck (see chapter 3.7.5.). In paper III we investigated TCR turnover in T cells with low levels of Lck.

3.7.1. Negative regulators of TCR signaling

Inhibitory signaling is an important aspect of TCR signaling. In resting cells, inhibitory mechanisms keep the cell at rest, preventing aberrant immune activation. When the TCR of primary T cells is engaged without the secondary CD28 signal, negative feedback mechanisms trigger pathways rendering the cell anergic. T cells undergoing thymic education become apoptotic if triggered by autoantigens (clonal deletion). T cells react differently depending on the strength and duration of TCR engangement, or to paracrine cytokines and cell-cell contacts with other immune cells. A balance of activating and inhibiting pathways are important for this fine tuning of T cell responses. The tyrosine phosphorylation cascade outlined in chapters 3.3-3.6 reaches its peak after about 2-3 minutes of TCR/CD3 stimulation. Likewise, the MAP kinases ERK1/2 reach their maximum phoshorylation within 3 minutes. After this peak most phosphorylation levels decline rapidly through the action of phosphatases and protein degradation, and are down to basal after about 30 minutes of TCR stimulation. Failures in these inhibitory mechanisms may lead to aberrant T cell responses, facilitating autoimmune diseases or cancer developement.

3.7.2. SHP-1

The function of the tyrosine phosphatase SHP-1 is an instructive example of negative T cell signaling (reviewed in Zhang J. et al., 2000). It is expressed at high levels in hematopoietic cells of all lineages, and altered expression and/or structure of SHP-1 plays a role in the progress of many forms of leukemia. Thymocytes from moth-eaten mice with reduced levels of SHP-1 show increased activation of Lck and Fyn, and develop a severe autoimmune and immunodeficiency syndrome with hyperactive T cells (Lorenz et al., 1996). SHP-1 is activated by engagement of its tandem SH2 domains by phosphotyrosines (Pei et al., 1994), and Lck-mediated phosphorylation of Y536 and Y564 (Lorentz et al., 1994, Frank et al., 2004). SHP-1 in turn serves to deactivate Lck by dephosphorylating

Y394 (Chiang et al., 2001), and also dephosphorylates other effectors of T cell signaling such as ZAP-70 (Plas et al., 1996, Brockdorff et al., 1999), Vav1 (Pani et al., 1996; Kon-Kozlowski et al., 1996), SLP-76 (Binstadt et al., 1998) and CD3ζ (Sozio et al. 2004). Furthermore, SHP-1 participates in fine tuning of TCR signals, as demonstrated by low affinity ligands which trigger binding of the SHP-1 to Lck. This negative feedback is circumvented by stronger ligands through phosphorylation of S59 in Lck in an ERK-dependent manner, which prevents SHP-1 binding and inhibition (Stefanova et al., 2003). The impact of Lck-kd on SHP-1 activity was addressed in Paper I.

3.7.3. Ras-GAP

The Ras GTPase p120 Ras-GAP is a negative regulator of Ras signaling acting by accelerating the hydrolysis of Ras-GTP to Ras-GDP. Ras-GAP is thought to be activated by membrane recruitment and tyrosine phosphorylation on several residues, most importantly Y460 (Liu and Pawson, 1991; Amrein et al., 1992, Park et al., 1993). There has been some controversy regarding the effects of Ras-GAP phosphorylation by Lck (Amrein et al., 1994, Giglione et al., 2001). Recently it was shown that Ras-GAP interacts with Cbp/PAG in stimulated T cells, suppressing Ras activation (Smida et al., 2007). This effect was shown to be independent of Csk binding. The phosphorylation and recruitment of Ras-GAP in the context of Lck knockdown was investigated in Paper II.

3.7.4. Cbl

The E3 ubiquitin ligase Cbl functions as a negative regulator of many signaling pathways. Cbl exists in two main isoforms, c-Cbl and Cbl-b, with a high level of sequence conservation. c-Cbl is activated by tyrosine phosphorylation on several residues, most importantly Y700, Y731 and Y774 (Hunter et al., 1999; Donovan et al., 1994; Feshchenko et al., 1998; Kassenbrock and Anderson, 2004). These residues are not, however, substrates of Lck or ZAP-70, but of Fyn and Syk (Hunter et al., 1999; Feshchenko et al., 1998; Tsygankov et al., 1996; Deckert et al., 1998), which are activated directly or indirectly by Lck (Filipp et al., 2003). Engagement of the TCR leads to activation of T cells, but also internalization and lysosomal degradation of the receptor complex (Valitutti et al., 1997). This serves to terminate signaling, and this process is dependent on the tyrosine kinase activity of Lck (Luton et al., 1994; D'Oro et al., 1997) and ubiquitination by c-Cbl (Hou et al., 1994; Cenciarelli et al., 1996; Wang et al., 2001, Naramura et al., 2002). Ubiquitination

marks active enzymes and receptors for degradation (reviewed in Thien and Langdon, 2005 and Swaminathan and Tsygankov, 2006). Cbl-dependent ubiquitination requires interaction with the target protein. For example, c-Cbl and Lck interact through binding of proline-rich seqences in Cbl with the SH3 domain in Lck. The SH2 domain of Lck also binds to phosphorylated sites in Cbl, and the Cbl TKB domain binds to the activation loop phosphorylation site in Lck after stimulation of T cells (Rao et al., 2002). Other notable targets for c-Cbl-mediated ubiquitination are Vav (Miura-Shimura et al., 2003), Fyn (Yokouchi et al., 2001; Andoniou et al., 2000; Hunter et al., 1999), ZAP-70 (Lupher et al., 1996), as well as the already mentioned TCR/CD3-complex. Consistent with the negative regulation assigned to the Cbl family of proteins, T cells from c-Cbl$^{-/-}$ and Cbl-b$^{-/-}$ mice were hyperactive upon TCR engagement, although some biochemical distinctions between the phenotypes existed (Murphy et al., 1998; Naramura et al., 1998; Thien et al., 1998; Bachmaier et al., 2000; Chiang et al., 2000). T cells from double-knockout (dKO) mice lacking both c-Cbl and Cbl-b, failed to modulate surface TCR after ligand engagement, resulting in sustained TCR signaling and ERK1/2 phosphorylation. However, signaling through the major TCR pathways were not increased (Naramura et al., 2002). The impact of reduced Lck levels on c-Cbl activity and TCR/CD3 turnover was investigated further in Paper III.

3.7.5. Bak

Bak is a pro-apoptotic member of the Bcl-2 family of proteins, and participates in the mitochondrial pathway of apoptosis. JCaM1 cells were shown to completely lack Bak, an effect that was reversed upon reconstitution with Lck (Samraj et al., 2006). This and other studies have shown that T cells lacking Lck are resistant to apoptosis induced by many kinds of stimuli (Di Somma et al., 1995; Oyaizu et al., 1995; Belka et al., 2003; Hur et al., 2004; Gruber et al., 2004). Interestingly, the effect of Lck on Bak transcription was independent of the kinase domain of Lck and classical mediators of T-cell signaling such as ZAP-70 and LAT (Samraj et al., 2006). Most research regarding Lck has focused on its capacity as a tyrosine kinase, but these data indicate that Lck also participates in other kinds of signaling, where the presence of Lck is required for the transcription of other proteins.

3.8. Heterotrimeric G proteins and T cell signaling

Heterotrimeric G proteins consist of an α-subunit that binds and hydrolyzes GTP, and β- and γ-subunits that form an undissociable βγ-complex. Coupling between an activated G-protein-coupled receptor (GPCR) and its G protein promotes the exchange of GDP for GTP on the α-subunit. This allows the α-subunit and the βγ-complex to dissociate and modulate the activity of a variety of effectors such as ion channels and enzymes (reviewed in Offermanns, 2000). T cells express most Gα-subunits of the Gαs, Gαi, Gαq/o and Gα12 families (Offermanns et al., 2001), and heterotrimeric G proteins have been implicated in T cell signaling in several reports (Cenciarelli et al., 1992; Sancho et al., 1993; Stanners et al., 1995; Zhou et al., 1998; Tsoukas et al., 2000; Lippert et al., 2000). For example, it has been shown that activation of primary T cells lead to recruitment of Gαs and Gαq to lipid rafts (Abrahamsen et al., 2004), and crosstalk between heterotrimeric G-proteins and different tyrosine kinases has been reported in several cell types (Luttrell et al., 1996; Luttrell et al., 1997; Luttrel et al., 1999; Luttrell et al., 2004). Src can be activated directly by Gαs or Gαi through binding binding of the G protein to the catalytic domain of Src (Ma et al., 2000), and Lck can also be regulated positively by the same Gαs-dependent mechanism (Gu et al., 2000). Cross-talk between the Gαq and Lck has been reported (Inngjerdingen et al. et al., 2002). Transfection with a function deficient mutant of Gαq/11 impared phosphorylation of CD3ζ and ZAP-70 (Stanners et al. 1995). A recent report shows that JCaM1 cells lacking Lck may be activated through a Gα11-dependent mechanism, mediated through PLCβ1 activation (Bueno et al., 2006). The involvement of G proteins in T cell signaling and the interplay between G proteins and Lck was explored in paper IV.

4. Aims of the present study

In this thesis we wanted to investigate the molecular mechanisms responsible for regulation of TCR-induced signaling mediated through the Src kinases Lck and Fyn, and the heterotrimeric G proteins Gαs, Gαi-2 and Gαq.

The main objectives of the present study were to:

1. Generate siRNAs for the SFKs Lck and Fyn, as well as the heterotrimeric G proteins Gαs, Gαi-2 and Gαq, and validate these siRNAs with respect to potency and specificity in both Jurkat and primary human T cells.

2. Explore the impact of Lck knockdown in T cells, and hereunder:

- **a)** Characterize the observed paradoxical hyperresponsive Lck-kd phenotype using standard biochemical assays.
- **b)** Investigate possible alternative mechanisms of T cell activation.
- **c)** Study the involvement of Lck in negative T cell signaling.

3. Investigate the involvement of heterotrimeric G proteins in T cell signaling, with particular emphasis on their possible crosstalk with Lck.

5. Synopsis of papers

Paper I:

Short-interfering RNA-mediated Lck knockdown results in augmented downstream T cell responses. Here, we designed, synthesized and validated four siRNAs and one control siRNA for Lck and Fyn respectively. We obtained highly efficient knockdown in both Jurkat and primary human T cells using low doses of siRNA (100 nM). By using other siRNAs we controlled for off-target effects. As expected, proximal signaling was reduced with Lck-kd as determined by overall tyrosine phosphorylation, CD3ζ phosphorylation and Ca^{2+} mobilization. Surprisingly, we observed augmented activation of NFAT-AP-1 and sustained phosphorylation of ERK1/2 in Jurkat cells, and increased secretion of IL-2 in primary T cells. We found that the phosphatase activity of SHP-1 was reduced in Lck-kd cells, and we speculated whether alternative mechanisms of activation could be in operation, possible mediated through ERK as we observed sustained phosphorylation of this MAPK. Fyn did not seem to be responsible for this mechanism since co-knockdown of Fyn did not reverse hyperresponsiveness in Lck-kd cells.

Paper II:

Hypophosphorylated TCR/CD3-zeta signals through a Grb2-SOS1-Ras pathway in Lck knockdown cells. In this study we presented an alternative mechanism of TCR signaling in T cells with Lck-kd. We reported the recruitment of Grb2-SOS1 to CD3ζ after prolonged stimulation of Lck-kd cells. In line with this, Ras, Raf-1 and ERK1/2 displayed sustained activation. In a solid phase assay, Grb2 bound to incompletely phosphorylated ITAM1 with the pY-Y configuration, but was competed by ZAP-70 with respect to binding to the doubly phosphorylated pY-pY conformation of the ITAMs. We also found that the phosphorylation of Ras-GAP was strongly suppressed in Lck knockdown cells, indicating that a Ras negative feedback mechanism is dependent on Lck.

Paper III:

Normal TCR/CD3 endocytosis but reduced CD3-zeta degradation despite diminished Cbl phosphorylation in T cells with low Lck levels. In this report we investigated further how low levels of Lck may result in aberrant T cell signaling. c-Cbl phosphorylation was strongly reduced in T cells with Lck knockdown, as was ubiquitination of CD3ζ. Surprisingly, endocytosis of the T cell receptor complex occured normally, but confocal microscopy showed that CD3ζ containing vesicles were not targeted for lysosomal

degradation to the same degree as in control cells. This led to reduced degradation of CD3ζ, and we hypothesized that prolonged cytosolic subsistence of the T cell receptor complex could result in extended signaling in T cells with Lck knockdown.

Paper IV:

Gαq regulates TCR-mediated immune response in T cells through an Lck-dependent pathway. In this paper we showed that Gαs and and Gαq, but not Gαi-2 were activated upon anti-CD3 stimulation, and we targeted Gαs, Gαi-2 and Gαq with siRNA-mediated knockdown to examine their roles in TCR sigaling. Gαs-kd and Gαi-2-kd did not result in any significant changes, but Gαq-kd reduced Lck activity significantly. Despite this, NFAT-AP-1 and ERK1/2 phosphorylation were augmented. Similarly, primary T cells from $G\alpha q^{-/-}$ mice displayed reduced proximal signaling, but augmented secretion of IL-2, IL-5, IL-12 and TNF-α. The hyperresponsivenss in Gαq-kd cells was reversible when cells were transfected with constitutively active Lck Y505F or pretreated with PP2. We speculated that Gαq has a key regulatory role in T cell signaling acting at the level of Lck.

6. Discussion

6.1. The Lck knockdown phenotype

6.1.1. Low levels of Lck and alternative signaling mechanisms

Recruitment of other proteins than ZAP-70 to hypophosphorylated CD3ζ ITAMs has been suggested previously, but not in the context of Lck knockdown. (Ravichandran et al., 1993, Osman et al., 1995, Nel et al., 1995, Labadia et al., 1996, Zenner et al., 1996, Chau et al., 1999). Chau and Madrenas (1999) proposed that partial agonists may trigger Grb2-SOS binding to hypophosphorylated CD3ζ in the absence of LAT phosphorylation. Similarly, we propose that low levels of Lck may give rise to hyperresponsive signaling through CD3ζ-Grb2-SOS1 when negative feedback through SHP-1, Ras-GAP and c-Cbl is displaced (Paper I-III). An important question is why T cells treated with pharmacological inhibitors to Lck such as PP2, or cells overexpressing Csk, do not display a similar hyperactivity in response to anti-CD3 ligation. Such treatments also reduce the kinase activity of Lck, but they do not result in an hyperactive response as described for Lck-kd.

It is becoming clear that Lck does not function exclusively as a tyrosine kinase. Lck is also an important adapter protein, and participates in signaling pathways not directly related to the TCR. It was recently reported that the transcriptional activation of the pro-apoptotic protein Bak was dependent on Lck (Samraj et al., 2006). The authors concluded that this effect was independent of the kinase domain of Lck, PP2 treatment, and classical mediators of T-cell signaling such as ZAP-70 and LAT. How Lck confers its influence on Bak expression remains undetermined. In another report, JCaM1 cells expressing a mutated form of Lck lacking the SH3 domain, failed to activate ERK1/2 despite normal TCRζ chain phosphorylation, ZAP-70 recruitment, and ZAP-70 activation (Denny et al., 1999). In contrast, we show that ERK1/2 becomes activated despite reduced TCRζ phosphorylation and ZAP-70 activation (Papers I and II). These results suggest that Lck participates in more than one signaling pathway, and that removal of Lck, or parts of Lck, may result in different outcomes than mere inhibition of the protein. This notion is of interest in T cells since most of the research conducted on Lck has focused on its capacity as a tyrosine kinase. The siRNA-mediated Lck-kd phenotype we have investigated is paradoxical in this respect, and alludes to alternative or complementary signaling mechanisms. Whether or not such responses are aberrant or physiologically relevant is not known. Acute knockdown of a protein is unlikely to occur in a physiological setting. T cells with reduced levels of Lck

have been reported from humans in the literature, but these patients have endured long lasting lack of Lck activity. The pathologies implicated ranged from immunodeficiency (CVID, SCID) to autoimmunity (lupus, diabetes, reumatoid arthritis). Common for most, but not all of these cases, was reduced proximal T cell signaling. In some cases, various forms of activity was observed, although not hyperresponsiveness as we report. It is therefore possible that siRNA-mediated knockdown of Lck results in an experimentally induced artificial phenotype. What is interesting, however, is that we have detected an alternative signaling mechanism, which may be important for signaling in T cells in other contexts. In the study published by Lovatt et al., low levels of Lck produced the same degree of MEK and ERK1/2 phosphorylation as Lck-deficient cells. This effect was shown to be dependent on Fyn, and they speculated that SOS could be involved in producing Ras activation (Lovatt et al., 2006). Clearly, such a notion is interesting in respect to the data presented in Paper II. In the classical paradigm of T cell activation, Lck is the exclusive conveyer of signal transmission mediated through the TCR. In this framework, Lck is activated by TCR engagement and its primary function is to phosphorylate the TCR/CD3-complex. Genetic, mutational and pharmacological data assembled from fifteen years of research indicate that Lck plays an important role, but it is not indispensable for T cell signaling, and Lck also participates in negative regulation. Lck-independent signaling mechanisms have been shown especially for partial agonist effects (Yamasaki et al., 1997; Chau and Madrenas, 1999; Criado and Madrenas, 2004; Bueno et al., 2006). It has also been shown that defective Ca^{2+} signaling blocks NFAT1 translocation, while selectively activating NFAT2 in anergic T cells (Srinivasan and Frauwirth, 2007). The field now increasingly acknowledges the non-linear dynamics of cellular systems, with increasing focus on differential signaling mechanisms, and the more complex role of signal transducers such as Lck.

6.1.2. Lck and apoptosis

Since Lck has been implicated in apoptosis and activation induced cell death (AICD), we hypothesized that reduced apoptosis and/or AICD, could contribute to sustained signaling i T cells with low levels of Lck. However, during the course of the experiments conducted in Jurkat TAg cells (6 h for the NFAT-AP-1-luciferase assay), no induction of apoptosis or reduction of cell viability was observed after OKT3 stimulation, even with low or high dosages (TM, unpublished observations). This was surprising as we found Bak expression

to be severely affected by Lck knockdown (Fig. 6), in agreement with the report by Samraj et al. (2006). Activation of Caspase-3 was even reduced upon OKT3-stimulation in both control and Lck-kd cells, and there was no difference in Caspase-3 activation in the positive control (FasL treatment) (TM, unpublished observations).

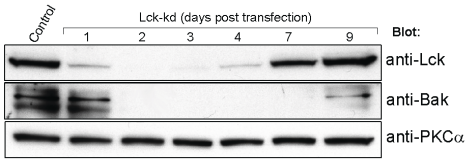

Figure 6. Bak expression in Lck-kd cells

6.1.3. Endocytosis of TCR/CD3

As reported in Paper III, we observed no difference in endocytosis of engaged TCR/CD3-complex in control and Lck-kd cells. This was surprising as previous reports had indicated this process to depend on Lck and/or Cbl-mediated ubiquitination for this process to occur (Luton et al., 1994, D'Oro et al., 1997, Hou et al., 1994, Cenciarelli et al., 1996, Wang et al., 2001, Naramura et al., 2002). Seemingly, endocytosis can take place with very low Lck and/or Cbl activity, or other mechanisms are involved. The latter scenario seems plausible as internalization of the TCR/CD3-complex was completely unaffected by the loss of Lck. However, we did observe a significant reduction in lysosomal sorting of CD3-containing vesicles in the Lck-kd cells. This suggests that Cbl-mediated ubiquitinylation is required for this process, but is dispensable for endocytosis of the TCR. Similar data have been reported for the EGF receptor (Duan et al., 2003, Padrón et al., 2007).

6.2. The involvement of heterotrimeric G proteins in T cell signaling

As shown in paper IV, triggering of the TCR leads to GTP-binding and thus activation of Gαq. The mechanism whereby this occurs is unknown, but probably involves the recruitment of a GEF for heterotrimeric G proteins to the membrane, or cross-talk between the TCR and a GPCR, perhaps CXCR4 (Peacock and Jirik 1999; Kumar et al., 2006; Patrussi et al., 2007). Knockdown of Gαq inhibited Lck autophosphorylation, whereas overexpression of a constitutively active mutant of Gαq augmented Lck activity. These data alludes to some kind of interplay beween Lck and Gαq, which has been shown previously for Gαs and Lck (Gu et al. 2000). Surprisingly, Gαq-kd and Gαq KO augmented downstream T cell responses, despite reduced proximal signaling. This effect was reversed

to basal levels by PP2, and to the level of stimulated control cells by co-transfection with Lck Y505F. This indicates that both reduction and overexpression of Gαq may produce Lck-dependent hyperactivity. Furthermore, since Lck Y505 reduced the response in Gαq-kd cells, but augmented signaling in unstimulated cells, it is likely that Lck contributes both negatively and positively to T cell activation. We postulate that TCR stimulation activates Gαq which augments Lck activity, whereas Gαq-kd inhibits Lck activity and results in signaling through other pathways.

6.3. Methodological considerations

6.3.1. siRNA-mediated RNAi

A publication in 1990 triggered the discussion of co-suppression of endogenous genes by transgenes: Overexpression of chalcone synthase, which is responsible for violet coloration in petunias, rendered the petunia flowers white (Napoli et al., 1990). dsRNA-mediated gene silencing of target mRNA was documented in C.elegans eight years later (Fire et al., 1998), a work for which the principal investigators Andrew Fire and Craig Mello received the Nobel Prize in 2006. This method has since been employed with success in mammalian cells (Elbashir et al., 2001), and T lymphocytes (McManus et al., 2002a, McManus et al., 2002b), and is now routinely used in biomedical research. Per october 2007, a PubMed search returns more than 12.800 hits for “siRNA”.

The siRNAs used in this thesis are 21-nt duplex oligomers with a 2-nt overhang on each end. They were designed and synthesized in-house based on an in-house developed algorithm by Amarzguioui et al., (2004). The utility of the RNAi approach depends on target specificity and side effects of the treatment. For example, indifferent design or secondary RNA structures may cause off-target down regulation of other genes (Bridge et al., 2003). siRNAs utilize a molecular machinery already present in the cell, that probably evolved to combat RNA viruses. The complete effects of this system, appart from mRNA clevage, are still not known in full detail. When introduced into mammalian cells, long dsRNAs are fragmented by the protein Dicer. The shorter pieces are used as templates by RNA induced silencing complex (RISC) to cleave any complementary RNA sequence in the cell, thus disrupting protein translation. However, virus-infected cells may also trigger a PKR-interferon response when exposed to dsRNA. This shuts down all protein production and induces the production of INFγ, a cytokine which activates cytotoxic immune cells.

This classical anti-viral defence is instigated to avoid transcription of viral proteins and to ensure elimination of infected cells. siRNAs are thought to be short enough to evade the PKR-interferone effects in vertebrates, but 21-nt siRNAs have been shown to activate these mechanisms in a concentration-dependent manner *in vitro* (Sledz et al., 2003). Such problems must be considered whenever using siRNAs, and they must be controlled for as thoroughly as possible in biomedical research. The siRNAs used in this thesis were examined and validated by the following means:

1.) All siRNA seqences were blasted towards the human genome to avoid overlap with other proteins.

2.) Control siRNAs were designed for all siRNAs employed. The control siRNAs contain a triple G/C switch, and were tested with standard biochemical assays (monitoring of NFAT-AP-1 reporter assay, and ERK1/2 phosphorylation), against mock transfected cells and another control siRNA (Csk2033M3), to ensure that they did not significantly influenced signaling.

3.) Knockdown cells were compared to control transfected cells in all experiments, thus ensuring that introduction of siRNAs *per se* does not generate unwanted side effects.

4.) Dosage and incubation time were optimized for each siRNA to ensure minimum RNA load in each cell. For Lck232 and Fyn1059, 100 nM of siRNA and 48 hours of incubation post transfection was found to produce optimal knockdown. For knockdown of G-proteins, 400 nM siRNA was used. Compared to other studies, both 100 nM and 400 nM are low (Peter et al., 2007) when taking into account transfection efficiency in electroporation and nucleofection used on T cells. This indicates high potency of the designed siRNAs.

5.) We monitored several other proteins to verify that knockdown was specific. For Lck-kd, the expression of Fyn, Csk, PKCα, PLCγ1, LAT, FAK and Pyk2 was not disturbed in siRNA concentrations up to 1500 nM, which was the maximum tested.

6.) For Lck-kd and Gαq-kd, another siRNA was used to reproduce the main findings. No difference in results between the different siRNAs was found, thus minimizing the risk of observations being influenced by off-target effects due to secondary structures.

7.) For Lck-kd we also performed a selection experiment. Cells were co-transfected with a surface marker, and positive cells were selected from control and Lck knockdown populations for NFAT-AP-1 luciferase reporter assays. Selected cells

were compared to control and negatively selected cells, but no difference in relative signaling between Lck knockdown and control cells was observed. As expected, the absolute luciferase numbers were higher in the selected population as they contained more NFAT-AP-1 cDNA. However, since transfection with siRNAs are about 3-fold more efficient than cDNA transfections, and knockdown efficiency is so high, the selected cells behaved essentially as the whole cell population, and indeed, the knockdown of Lck was similar between the two groups (Paper I).

8.) We also transfected cells and monitored them for several days. The hyperresponsive effect seen in Lck-kd cells was induced upon protein knockdown and was completely reversible when protein levels returned to normal, approximately 7-9 days post-transfection (Paper III).

Based on these assays, we propose that the siRNAs used in our studies are properly validated, and that the results obtained are primarily due to knockdown of specific proteins, not unwanted side effects. Given these premises, siRNA-induced gene silencing have many advantages over gene knockout by homologous recombination. Firstly, it is a faster and cheaper method, and one can relatively easily knock down more than one protein simulatneously. Secondly, the method gives acute knockdown in mature human cells. The last point is important as it opposes to knockout animals who may suffer embryonic lethality or long term defects from their knockouts. For example, compensatory mechanisms may develop over time. The siRNAs used in this thesis have provided potent knockdown consistently, and the cells could be used in experiments just 48 h after transfection.

This does not preclude that other problems might arise with siRNA induced gene silencing. An important factor is that ranges of knockdown are obtained. Since we operated with knockdown efficiencies close to 100% this factor is minimized, but it is still possible to envision the potential problem: In principle, if western blotting of whole cell lysates show 90% knockdown of a protein, one can not be sure whether 100% of the cells have 90% protein knockdown, or 90% of the cells have 100% knockdown. The most plausible scenario is a range of knockdowns ranging from 0% in untransfected cells and close to 100% in cells with high transfection efficiency. Most biochemical assays are conducted on whole cell populations, and effects from certain levels of knockdown may dominate the final result. For example, it is possible that certain ranges of Lck knockdown produce hyperactive responses, while untransfected cells behave as normal cells, and some cells with

very high knockdown are nonresponsive. The end result may indicate augmented signaling, even though this is not true for all the cells in the experiment.

6.3.2. The Jurkat cell model

In immunology, the Jurkat T cell line (Weiss et al., 1984) has been used along side other T cell lines and primary T cells from mice and humans, as model systems for investigation of T cell signaling. A large body of research using both Jurkat cell lines and primary T cells, has demonstrated the crucial role of Lck in T cell development and TCR signaling. It is therefore surprising to note that Jurkat TAg T cells with siRNA mediated Lck knockdown display hyperresponsiveness. Jurkat TAg T cells are Jurkat E6.1 cells stably transfected with Simian virus 40 (SV40) large T antigen (TAg). The complete nature of SV40 oncogenic cellular transformation is not completely understood, but it involves the manipulation of tumor suppressors and cell cycle regulatory proteins such as the retinoblastoma family of proteins, p53 and the transcriptional co-activators p300 and CBP (reviewed in Ali et al., 2001). Jurkat TAg and E6.1 cells are thought of as identical in regard to TCR signaling characteristics, but TAg cells are more viable and easier to transfect. As a control, knockdown of Lck was conducted in Jurkat TAg and E6.1 cells in parallell, and NFAT-AP-1 activation was monitored. Surprisingly, Jurkat E6.1 Lck-kd cells were completely non-responsive to anti-CD3 ligation, wheras Jurkat TAg Lck-kd cells were hyperresponsive (Fig. 7). These conflicting data are not unique to Jurkat cells. A similar scenario was reported for HEK293 cells by Lefkowitz et al. in 2002, in which the Ptx-sensitivity to β-adrenergic ERK1/2 activation was shown to vary from 0 to 100% in cell lines obtained from various sources (Lefkowitz et al. 2002). How is it possible that Jurkat TAg cells and Jurkat E6.1 cells with Lck-kd give rise to completely different phenotypes? Based on data from primary human T cells where observations are similar to those in obtained in Jurkat TAg we have continued to investigate Lck knockdown in Jurkat TAg cells, but only reproduction of these results by other groups can validate our conclusions. Cell lines may accumulate

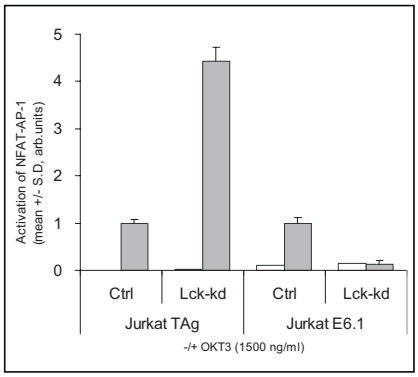

Figure 7. Jurkat TAg vs Jurkat E6.1

differences over time, and batches of cells may vary from laboratory to laboratory. We have reproduced the results from Jurkat TAg cells with other sources to rule out specific disorders with our batch. Relative to other cell types, the Jurkat TAg and E6.1 cells are very similar, and the two cell lines have been used side by side in experiments for many years. This discrepancy warrants a discussion regarding the validity of cell lines, not only in T cell signaling, but also in experimental research generally. This also illustrates the necessity of validating observations from cell lines in primary human cells or mouse models. We have used primary human T cells in Papers I and II, and mouse T cells from knockout mice in Paper IV.

6.4. Future perspectives

Many interesting questions were raised during the work on this thesis, and further investigation of complementary or alternative signaling mechanisms in T cells is of interest. It is becoming clear that the strictly linear signaling concept of the Lck-CD3-ZAP70-LAT pathway is an oversimplification. Various ligand affinities and durations of TCR, CD3, CD4 and CD28 engagements, may produce different kinds of signaling. Within the Lck knockdown model, it would be interesting to monitor more closely differences in activation patterns for various of Lck protein levels. For example, it is possible to transfect T cells with siRNAs, withdraw cells after various periods of incubation, and analyse the status of many phosphoproteins by flow cytometry. By doing this, changes in signaling depending on reducing or increasing amounts of Lck can be observed with higher fidelity.

Furthermore, since the expression of Bak is dependent on the presence of Lck, it would be interesting to perform microarray analysis of Lck-kd cells to monitor whether other proteins are affected. If other proteins are also regulated, it may bring new insight to the paradoxical hyperresponsive signaling we have observed in Lck-kd cells. The mechanism by which Lck controls the promoter activity and expression of other proteins is also of importance, since this opens up new a aspect of Lck-mediated signaling.

In Paper III we reported that endocytosis of the TCR/CD3-complex occured normally despite reduced Lck and Cbl activity. Seemingly, internalization of the receptor was not dependent on Lck/Cbl, or very low levels of activity from these proteins was sufficient for this process to occur. Furthermore, even though reduced, colocalization of CD3-containing

vesicles with lysosomes was not completely abolished in Lck-kd cells, indicating that vesicles can merge with lysosomes independently of ubiquitination. Further investigation of the mechanism by which TCR/CD3 is endocytosed, and how these vesicles localize with lysosomes, is warranted. The Lck-kd model, and siRNA-mediated knockdown of other protens, can be used as a valuable tool for such experiments. For example, what phenotypes will knockdown of c-Cbl, or the concurrent knockdown of c-Cbl and Lck produce in this respect?

The question of how the TCR activates Gαq, and how the activated subunit exerts its effect on Lck, and vice versa, will be interesting to pursue in future studies. Our data indicate that interplay between Lck and Gαq exist, and that they interact physically in T cells. Furthermore, signaling through, and crosstalk between TCR and CXCR4 would be interesting to investigate using siRNA-mediated knockdown of Lck and G-proteins.

Finally, the striking difference in signaling observed in Jurkat TAg and Jurkat E6.1. cells with Lck-kd should be investigated further. That two similar cell lines can generate completely different phenotypes is disturbing, and this finding should be addressed more thoroughly in the wider scientific community.

7. Conclusions

1. siRNA-mediated knockdown of the Src family kinase Lck, but not Fyn, renders T cells hyperresponsive.

2. An alternative signaling pathway is activated in T cells with low levels of Lck. Prolonged stimulation leads to recruitment of Grb2-SOS1 to hypophosphorylated CD3ζ, resulting in signaling through Ras-Raf-1-MEK-ERK.

3. Colocalization of CD3-containing vesicles with lysosomes is reduced in stimulated T cells with Lck knockdown, resulting in prolonged subsistence of CD3ζ.

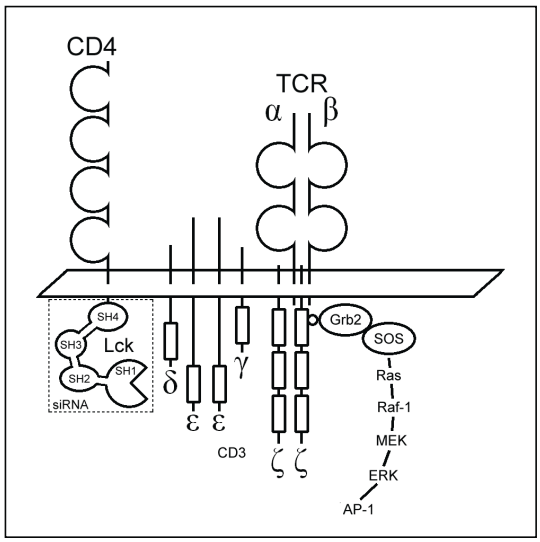

Figure 8. Alternative signaling pathway in T cells with siRNA-mediated Lck knockdown.

4. Negative regulators of T cell signaling, such as SHP-1, Ras-GAP and Cbl display reduced activity in Lck-kd cells. We propose that sustained signaling from CD3ζ-Grb2-SOS1-Ras-ERK1/2 in the absence of the negative feedback constraints normally imposed by Lck, may result in hyperresponsiveness in T cells with siRNA-mediated Lck knockdown (Fig. 8).

5. We report interplay between Lck and Gαq. Gαq appears to enhance Lck activation upon TCR triggering. Knockdown of Gαq renders T cells hyperresponsive possibly due to sustained ERK signaling. Altogether this indicates a regulatory role of this G protein in T cell signaling.

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