# Analysis of the TCR-mediated signaling dynamics

## Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium
(Dr. rer. nat.)

genehmigt durch die Fakultät für Naturwissenschaften
der Otto-von-Guericke-Universität Magdeburg


von M. Sc. Mateusz Pawel Poltorak

geb. am 23.03.1985 in Lublin, Polen


Gutachter: PD Dr. Luca Simeoni


eingereicht am: 16.12.2013

verteidigt am: 16.07.2014

# Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Luca Simeoni who was supporting me constantly throughout my whole PhD studies. From him I learned not only how to perform experiments, but also how to analyze results, judge critically obtained data, and present it in an understandable way. Without his guidance I wouldn't be able to become a true scientist.

I am also very grateful to Prof. Burkhart Schraven not only for giving me the opportunity to work in his institute but also for continuous and intensive support of my scientific development. Despite many responsibilities he always had time to discuss, comment or solve every difficult issue.

Of course, I cannot forget to mention the great help of our technical assistants Ines Meinert and Camilla Merten, who aided me in countless experiments. I am also extremely grateful to colleagues from my group and from the institute, especially Katrin Deiser and Amelie Witte for making my work and writing this thesis as painless as possible.

Lastly, I would like to thank my friends and family for taking care of me and my life after I exit the institute.

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# Abstract

T cells are key components of the defense system protecting our body from invading pathogens. Upon antigen recognition, T cells become activated and subsequently initiate specific cellular programs leading to proliferation, differentiation and acquisition of effector functions (e.g. cytokine production, cytotoxicity). Alterations in T-cell activation may lead to diseases such as, chronic inflammation, immunodeficiency, allergy, and cancer. Therefore in my study, I investigated how T-cell activation is regulated. In particular, I focused on the analysis of the dynamics of T-cell activation. As a model system, I used primary human T cells. Cells were stimulated to induce either transient or sustained T-cell activation. These two different activation dynamics correlate with apoptosis/unresponsiveness (anergic-like state) or proliferation, respectively. I studied how the execution of these two cellular programs is regulated at the molecular level. I found that transient signaling corresponds with strong activation of tyrosine kinases and phospho-tyrosine-dependent signaling, which induce negative feedback loops thereby terminating T-cell activation. Conversely, sustained signaling is associated with a positive feedback circuit between Lck (a crucial tyrosine kinase involved in initiation of T-cell activation) and ERK, which is required to modulate Lck activity, hence prolonging signaling.

In the second part of my work, I focused my investigation on how the dynamics of Ras-ERK activation are regulated. This cascade is critical for the specification of cellular responses in many cell types. In T cells, the Ras-ERK cascade is activated by the combined action of two guanine nucleotide exchange factors, Sos1 and RasGRP1. I found that RasGRP1 is necessary for the activation of ERK under conditions inducing both transient and sustained signaling, whereas Sos1 appears to be dispensable for transient, but required for sustained ERK signaling. In conclusion, I showed for the first time how TCR-mediated signaling dynamics are regulated in primary human T cells.

# 1. Introduction

## 1.1. The immune system

The natural environment is filled with a plethora of microorganisms, which may induce infections or diseases. In order to protect our body from these pathogens, we are equipped with a sophisticated network of defense mechanisms known as the immune system. The immune system can be divided into two major parts: non-specific or innate immunity and specific or adaptive immunity.

Innate immunity is the first line of defense. It responds very fast to invading pathogens, as the defenses of the innate immune system are constitutively expressed and hence they can instantly react. However, the innate immunity is not specific. This means that cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs), which are common to many pathogens. Phagocytic cells, natural killer (NK) cells, the complement system, and other secreted soluble factors constitute the innate immune system. Additionally, physical barriers like the skin and mucosa are also components of innate immunity. This type of defense is evolutionary older than adaptive immunity and it does not possess immunological memory (see below).

On the other hand, the adaptive immunity is induced upon infection and thus represents the second line of defense. It is composed of highly specialized cells, such as B and T lymphocytes, and soluble factors such as antibodies. Lymphocytes synergize together in order to protect the body from foreign pathogens. In fact, T lymphocytes are required for proper activation of B cells and antibody production. Moreover, the adaptive immunity is characterized by the presence of immunological memory. This means that after the first encounter with a pathogen the adaptive immunity develops experienced T- and B-cell subsets, which are able to rapidly respond upon a re-encounter with the same pathogen (Chaplin, 2006).

## 1.2. T lymphocytes

T cells are essential for adaptive immunity, as they directly recognize and respond against pathogens. Moreover, T cells are also required for the activation of other immune cells. All T cells arise from hematopoietic stem cell progenitors that are generated in the bone marrow. T-cell progenitors migrate from the bone marrow to the thymus, where they undergo a tightly regulated maturation process (Fig. 1.1). T-cell development in the thymus can be monitored by measuring the expression of surface markers defined as clusters of differentiation (CD), such as CD4 and CD8. Upon entering the thymic cortex via the post-capillary venules, lymphoid progenitors differentiate into immature T cells (thymocytes), which are characterized by the lack of CD4 and CD8 expression and are, therefore, called double

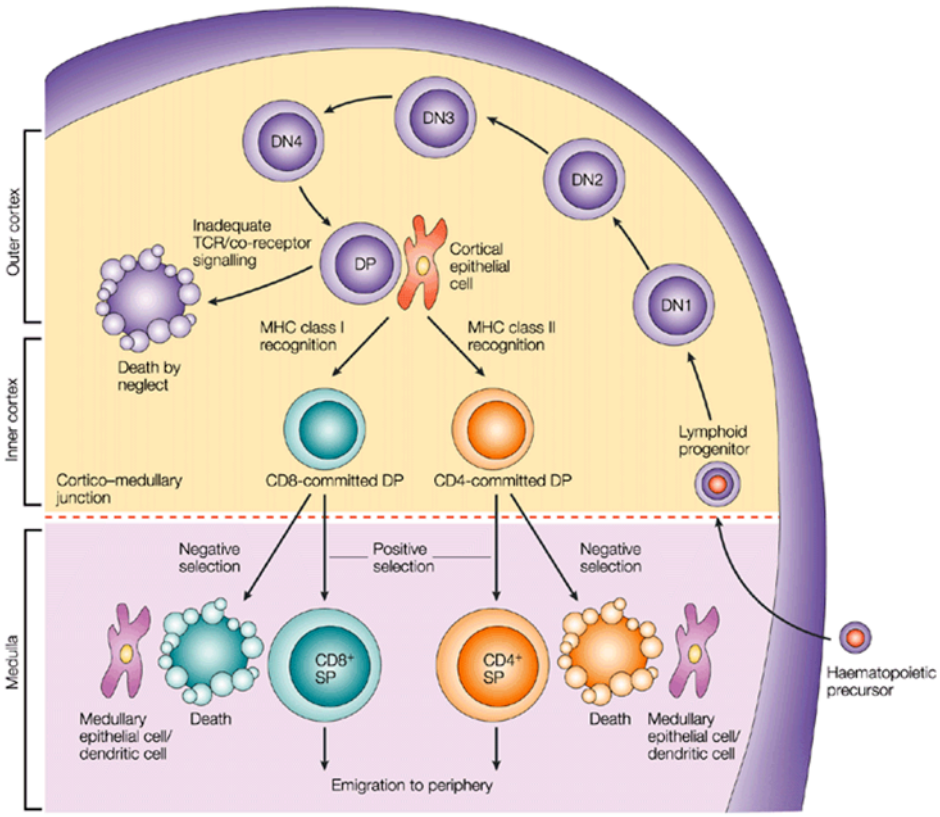


**Figure 1.1. T-cell development.** T-cell progenitors enter the thymus and undergo several stages of maturation. In the cortex they develop into DN (double negative) thymocytes and subsequently mature into DP (double positive) cells. DP cells are surveyed for their ligand binding affinity and they undergo either positive or negative selection. Cells expressing functional TCRs are then selected and commit to either the CD4 or CD8 lineage. Finally, fully mature SP (single positive) $CD4^+$ and $CD8^+$ T cells leave the thymus and migrate into the periphery (the figure was adopted from Germain, 2002).

negative (DN) thymocytes. DN thymocytes can be further subdivided by the expression of CD25 and CD44 into DN1 ($CD25^-$, $CD44^+$), DN2 ($CD25^+$, $CD44^+$), DN3 ($CD25^+$, $CD44^-$) and DN4 ($CD25^-$, $CD44^-$) (Fig. 1.1) (Godfrey et al, 1993). Chemokines induce the migration of the most immature thymocytes (DN1/2) from the cortex to the subcortex where they further mature into DN3 cells (Fig. 1.1).

At the DN3 stage, thymocytes express the β chain of the T-cell receptor (TCR), which pairs with an invariant pre-Tα chain and with CD3ε, CD3γ, CD3δ, and TCRζ molecules to form the pre-TCR. Signaling via the pre-TCR is ligand independent, but indispensable for further maturation of DN3 thymocytes. Cells that fail to express a functional pre-TCR are arrested at the DN3 stage and die by apoptosis. This process is one of two major checkpoints during T-cell development and is called β-selection (Germain, 2002).

The pre-TCR transduces signals inducing proliferation and further maturation to the DN4 stage. Moreover, it also initiates allelic exclusion that will ensure that only one TCR β chain will be expressed in each cell. Subsequently, DN4 cells upregulate both CD4 and CD8 molecules to become double positive (DP) thymocytes (Fig. 1.1). At this point, the β chain of the T-cell receptor forms a heterodimer with a randomly rearranged mature TCRα chain. At the end of this process, each cell bears a unique TCR. To verify the functionality of the mature TCR, a second developmental checkpoint, the so-called αβ-selection, takes place in the thymic cortex. Here, DP thymocytes are exposed to MHC (major histocompatibility complex) class I and II molecules complexed with self-antigens (Fig. 1.1). If a mature TCR interacts with weak/moderate affinity with self-peptide-MHC molecules, then DP cell will undergo positive selection. Positively selected cells will mature further and will be committed to either the CD4 or CD8 single positive (SP) T-cell lineages (Fig. 1.1). Conversely, if the TCR is not able to recognize self-peptide-MHC molecules, the DP thymocyte will undergo apoptosis (“death by neglect”) (Fig. 1.1). If DP thymocytes strongly react with self-peptide-MHC molecules, which indicates that they have the potential to become autoreactive, negative selection will take place. These autoagressive thymocytes will be eliminated by apoptosis, thus preventing the development of autoreactive T cells (Fig. 1.1) (Germain, 2002). These selection events are also known as central tolerance.

Nevertheless, not all autoreactive T cells are deleted during negative selection. Therefore, other safety systems, called peripheral tolerance, will keep these autoaggressive T cells under control. Only a minor fraction of DP cells complete maturation and migrate to the periphery, where they circulate as naïve T cells until exposure to pathogens. Upon antigen encounter, naïve T cells will proliferate and become activated effector cells. Some of these cells will eventually differentiate into long-lasting memory T lymphocytes.

As I have mentioned before (see 1.1), memory T cells are antigen-experienced and, therefore, respond much faster to reoccurring infections, thus enhancing the adaptive

immune response. T-cell activation is tightly regulated in order to ensure the clearance of the pathogens without causing chronic inflammation and autoimmunity.

Mature naïve and memory T cells circulating in the periphery are divided into two major subsets, T helper ($T_h$) and T cytotoxic ($T_c$) cells. These subpopulations can be distinguished by the expression of the surface markers CD4 and CD8. $T_h$ cells express CD4, whereas $T_c$ cells express CD8, respectively.

$T_h$ cells function as mediators and regulators of immune responses. They orchestrate the activation of other immune cells (e.g. B lymphocytes, macrophages etc.) by producing cytokines (i.e. interleukins). There are several subsets of $T_h$ cells, which are divided according to their function and cytokine profile: (i) $T_{h1}$ cells produce IFNγ (interferon gamma) and activate macrophages in order to increase killing of intracellular pathogens and further support the activation of $T_c$ cells; (ii) $T_{h2}$ secrete a variety of interleukins to augment antibody production by B cells; (iii) $T_{h17}$ mainly produce IL-17 (interleukin 17) and provide anti-microbial defense; (iv) $T_{regs}$ (regulatory T cells) produce IL-10 and TGFβ (transforming growth factor beta) to suppress immune responses thereby limiting chronic inflammation and autoimmunity (Zhu et al, 2008).

$T_c$ cells, the second T-lymphocyte subset, are responsible for the generation of cell-mediated immunity against intracellular pathogens, such as viruses. $T_c$ cells recognize foreign antigens presented on MHC class I molecules, which are expressed, for example, on virus-infected cells. They kill the target cell by inducing programmed cell death (apoptosis). Apoptosis of the target cell can be induced either by the release of soluble factors such as perforin, granzymes, and granulysin, or by the engagement of the Fas receptor expressed on the target cell.

In summary, mature naïve T cells and antigen-experienced memory T cells create a versatile defense mechanism crucial for adaptive immunity.

### 1.3. Molecular events occurring during T-cell activation

#### 1.3.1. TCR engagement

Given the importance of T cells within the immune system, I would like to describe the molecular events occurring during T-cell activation. First, I would like to focus my attention on the TCR, a surface receptor crucial for T-lymphocyte biology. The TCR regulates T-cell development, homeostasis, and activation. It is a heterodimer consisting of two highly variable chains, TCRα and TCRβ (or TCRγ and TCRδ in a minor T-cell population) connected by disulfide bond (Fig. 1.2). It is able to recognize peptides presented together

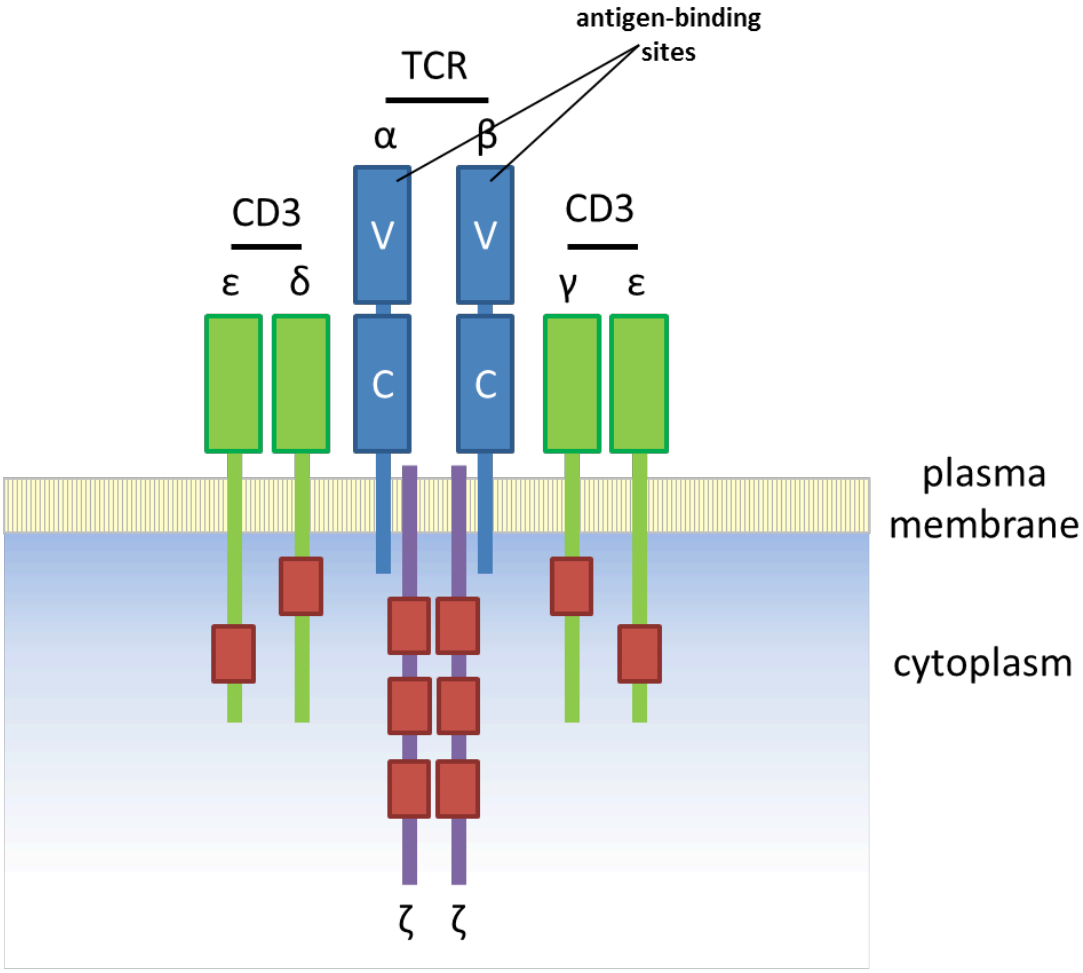


**Figure 1.2. The structure of T-cell receptor (TCR/CD3) signaling complex.** The TCR consists of two chains TCRα and TCRβ (shown in blue) containing constant (C) and variable (V) regions. The TCR/CD3 complex is formed from CD3δε and CD3γε heterodimers (shown in green), and two TCRζ chains (shown in violet). The ITAMs (immunoreceptor tyrosine-based activation motifs) required for signal initiation are indicated in red.

with MHC class I and II molecules. The TCRα and TCRβ chains of the TCR are composed of constant (C) and variable (V) regions (Fig. 1.2). The constant part is required for anchoring of the TCR to the plasma membrane, whereas the variable part contains the antigen-binding site (Fig. 1.2). V regions are generated by random joining of gene segments, thus creating the unique antigen specificity of the TCR. The TCRαβ heterodimer is associated with the CD3 and TCRζ molecules required for signal transduction. The full TCR/CD3 complex consists of a TCRαβ heterodimer, CD3εδ and CD3εγ heterodimers, and a TCRζζ homodimer (Fig. 1.2). Signaling downstream of the TCR/CD3 complex depends on the phosphorylation of distinct tyrosine motifs called immunoreceptor tyrosine-based activation motifs (ITAMs) located within the CD3 and TCRζ chains (Fig. 1.2). ITAMs include two characteristic amino acid sequences (YxxL or YxxI) separated from each other by 6 to 8 amino acids. The TCR/CD3 complex contains 10 ITAMs.

T-cell activation is initiated upon the binding of the TCR to peptide antigens presented by MHC molecules expressed on antigen presenting cells (APCs). However, despite intense investigations, it is not yet fully understood how TCR-mediated signaling is initiated and several models have been hypothesized. The widely accepted hypothesis, called the segregation model, postulates that signals are triggered upon spatial reorganization of TCR/CD3 complexes and effector molecules (Fig. 1.3) (Davis et al, 2006). According to this model, TCR engagement leads to TCR oligomerization and to the formation of signaling microclusters also including crucial effector molecules such as Lck (lymphocyte-specific protein tyrosine kinase), but not negative regulators such as the phosphatase CD45 and the tyrosine kinase Csk (c-Src tyrosine kinase) (Fig. 1.3) (Torgersen et al, 2001; Choudhuri et al, 2010; Borger et al, 2013; Rossy et al, 2013). Thus, according to this model, signaling is initiated upon the segregation of positive and negative regulatory molecules.

### 1.3.2. Initiation of the TCR-mediated signaling by Src and Syk family kinases

The TCR has no intrinsic catalytic activity and therefore it is closely associated with tyrosine kinases belonging to the Src and Syk family. Src (sarcoma tyrosine kinase) family kinases (SFKs) such as Lck and Fyn (feline yes-related protein) phosphorylate the ITAMs within the CD3 and TCRζ chains. It has been proposed that both Lck and Fyn are constitutively active (40% - 50% of the total pool) in T cells and maintain the basal level of TCR phosphorylation (Nika et al, 2010; Brownlie et al, 2013). This is necessary to provide tonic signaling required for T-cell survival (Seddon et al, 2002). In T cells, Lck can be associated with the CD4 or CD8 co-receptors expressed on the surface of $T_h$ or $T_c$ cells, respectively. Upon TCR engagement, Lck is brought into close proximity of the ITAM chains in both a co-receptor-dependent and -independent manner (Artyomov et al, 2010). According to the segregation

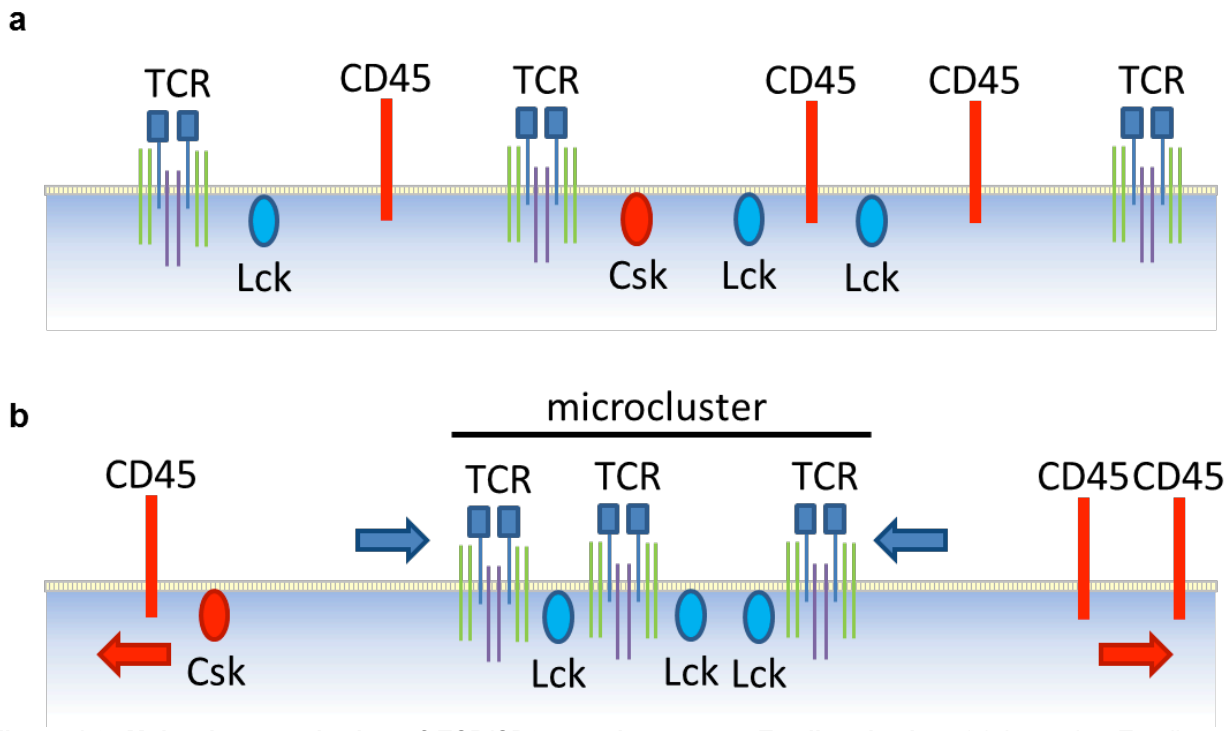


**Figure 1.3. Molecular organization of TCR/CD3 complexes upon T-cell activation.** (a) In resting T cells, TCR/CD3 complexes, effector molecules (e.g. Lck), and negative regulators (e.g. Csk, CD45) are randomly distributed throughout the plasma membrane. (b) Upon T-cell activation, TCR complexes oligomerize with effector molecules to form microclusters, whereas negative regulators are excluded from this clustering zone.

model discussed above (see 1.3.1), Lck and TCR/CD3 complexes are then sequestered from inhibitory molecules into microclusters (Fig. 1.3), where Lck will phosphorylate the ITAMs, thus initiating TCR-mediated signaling (Fig. 1.4).

Lck is the apical tyrosine kinase in the signaling cascade and the mechanisms regulating Lck activation have been under intense investigation for more than 20 years. However, how Lck is activated upon TCR triggering is still not well-understood. According to the model proposed by Nika et al., and others, signaling is initiated upon translocation of the active Lck pool to the engaged TCR without the need of *de novo* Lck activation (Paster et al, 2009; Nika et al, 2010). However, a very recent study from our institute has challenged this model and proposed that a fraction of Lck is indeed activated at the triggered TCR (Stirnweiss et al, 2013).

Lck contains an N-terminal unique region (with two palmitoylation and one myristoylation sites required for lipid raft anchoring), an SH3 (Src homology 3) domain (necessary for interaction with other proteins via their proline-rich regions) followed by an SH2 domain (which binds phosphorylated tyrosine residues) and the kinase domain followed by a C-terminal tail (Fig. 1.5a). In addition, Lck possesses two critical tyrosine residues, $Y^{505}$ located in the C-terminus and $Y^{394}$ located within the activatory loop of the kinase domain, which control Lck activation (Fig. 1.5a). Phosphorylation of Lck on $Y^{505}$ results in the binding of the C-terminal tail to the Lck-SH2 domain thereby, generating a “closed” conformation and an inactive enzyme (Fig. 1.5b) (Xu et al, 1999). The phosphorylation of $Y^{505}$ is mediated by Csk, which is a master negative regulator of TCR-mediated signaling (Schoenborn et al, 2011). Conversely, the phosphatase CD45 is known to dephosphorylate Lck at the C-terminal tyrosine, leading to the so-called “primed” non-phosphorylated Lck (Fig. 1.5b) (Hermiston et al, 2003; Salmond et al, 2009). At this point, Lck can cluster and *trans*-phosphorylate on $Y^{394}$ leading to the “opened” conformation, which corresponds to an active enzyme (Fig. 1.5b). It has been proposed that the “opened” conformation can be reverted by the phosphatases PTPN22 (protein tyrosine phosphatase non-receptor type 22) bound to Csk, SHP1 (SH2 domain-containing phosphatase 1), or CD45 (Cloutier et al, 1999; Salmond et al, 2009). Finally, “opened” Lck can be further phosphorylated on $Y^{505}$ resulting in double phosphorylated, active form (Nika et al, 2010). Only “opened” and active Lck is capable to phosphorylate ITAMs and therefore initiate signaling.

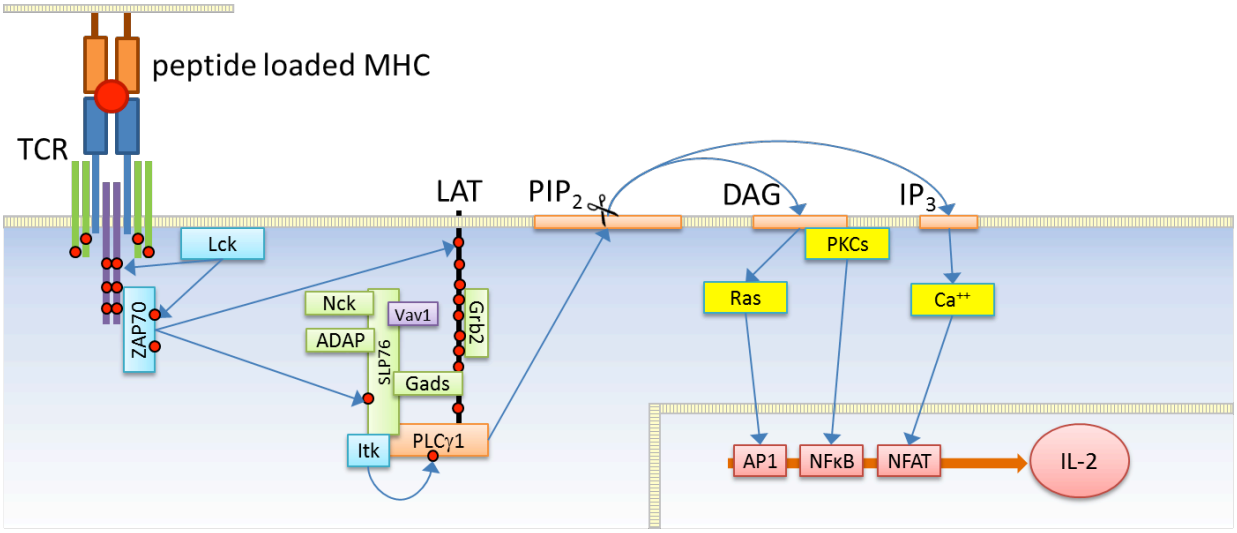


**Figure 1.4. Organization of TCR-mediated proximal signaling.** After T-cell activation Lck phosphorylates the ITAMs of the TCR/CD3 complex, allowing recruitment and Lck-mediated activation of ZAP70. Subsequently, activated ZAP70 phosphorylates LAT and SLP76 adaptor proteins, thus facilitating formation of the LAT signalosome. The assembled LAT signalosome consists of LAT, Grb2, Gads, SLP76, ADAP, PLCγ1, Itk, Nck, and Vav1. Here, Itk is able to phosphorylate PLCγ1 which subsequently hydrolyzes $PIP_2$ to DAG and $IP_3$, second messengers required for the activation of PKC-, Ras-, and $Ca^{++}$-mediated downstream pathways necessary for activation of the transcription factors AP1, NFκB, and NFAT, respectively. Activated AP1, NFκB, and NFAT drive the synthesis of the cytokine IL-2 to further support T-cell activation and proliferation. Phosphorylation of crucial molecules is indicated by red dots.

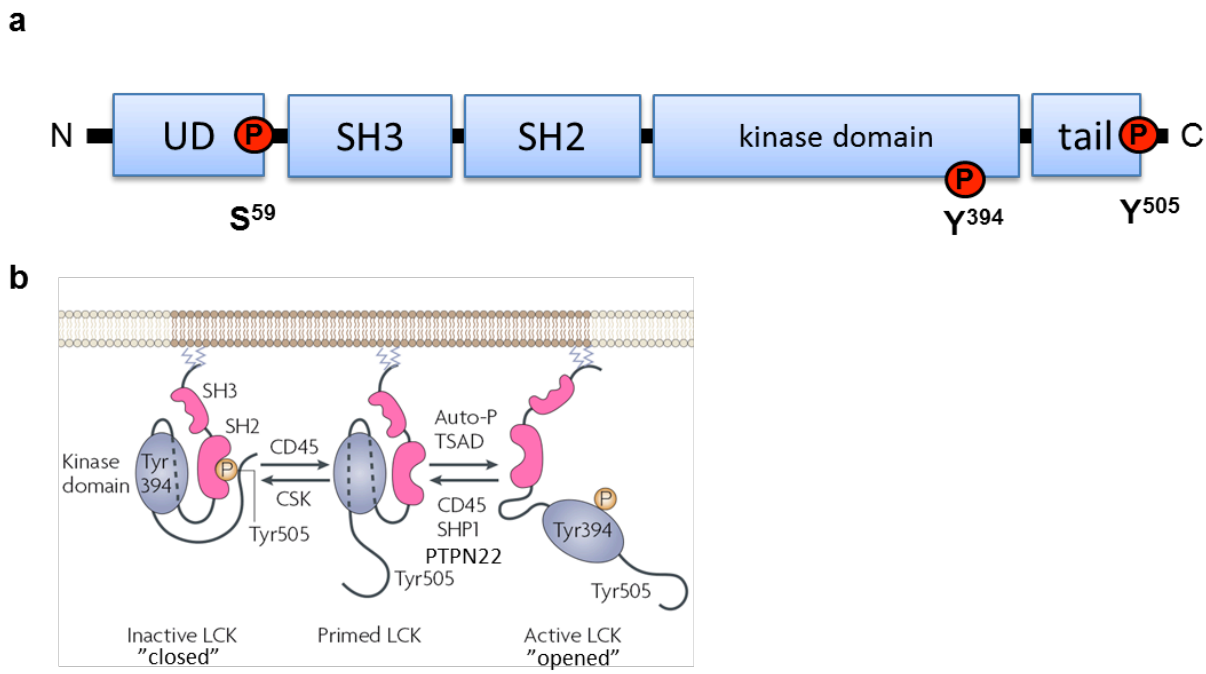


**Figure 1.5. Regulation of Lck.** (a) Schematic representation of the Lck protein. Blue squares represent domains and red circles indicate possible phosphorylation sites with the corresponding amino acid indicated below. The structure of Lck is as follows: unique domain (UD), Src homology domain 3 (SH3), Src homology domain 2 (SH2), kinase domain (also known as SH1), and tail region. (b) Conformational changes of Lck representing "closed", "primed" or "opened" form (figure 1.5b was modified from Acuto et al, 2008).

TCR-mediated signaling is further propagated upon recruitment of ZAP70 (zeta-chain-associated protein kinase 70 kDa), a member of the Syk family, to the phosphorylated ITAMs (Fig. 1.4) (Isakov et al, 1995). Binding of its tandem SH2 domains to the ITAMs unlocks ZAP70, which is subsequently phosphorylated on $Y^{315}$ and $Y^{319}$ by Lck. Further *auto*-phosphorylation on $Y^{493}$ completes ZAP70 activation (Pelosi et al, 1999; Deindl et al, 2007; Acuto et al, 2008; Yan et al, 2013). Catalytically active ZAP70 is necessary for the phosphorylation of two adaptor proteins, LAT (linker of activated T cells) and SLP76 (SH2 domain-containing leukocyte protein of 76 kDa), which constitute the center of an anchoring platform (LAT signalosome), which recruits additional cytosolic signaling molecules that are required to propagate signaling to the nucleus (Fig. 1.4) (Bubeck-Wardenburg et al, 1996; Paz et al, 2001).

### 1.3.3. Assembly of the LAT signalosome and activation of downstream signaling

LAT belongs to the transmembrane adaptor protein (TRAP) family and has nine tyrosine residues, which are phosphorylated upon TCR triggering (Fuller et al, 2011; Balagopalan et al, 2010). Phosphorylated LAT binds effector proteins such as PLCγ1 (phospholipase C gamma 1), the p85 subunit of PI3K (phosphoinositide 3-kinase), and the cytosolic adaptors - Grb2 (growth factor receptor-bound protein 2) and Gads (Grb2-related adapter protein downstream of Shc) (Fig. 1.4). Gads is constitutively associated with SLP76 (Liu et al, 1999). Upon recruitment of Gads to LAT, SLP76 interacts with PLCγ1, the proto-oncogene Vav1, the Tec family kinase Itk (IL-2-inducible T-cell kinase), and the adaptor proteins - Nck (non-catalytic region of tyrosine kinase adaptor protein) and ADAP (adhesion and degranulation promoting adapter protein) (Fig. 1.4). Thus, by recruiting several cytosolic effector molecules, LAT and SLP76 coordinate the activation of different cellular signaling pathways (e.g. $Ca^{++}$, PKC, Ras), which will ultimately culminate in gene expression, cytoskeletal reorganization, and T-cell activation (Smith-Garvin et al, 2009).

The activation of the phospholipase PLCγ1 is of particular importance during T-cell activation. Upon binding to LAT and SLP76, PLCγ1 is phosphorylated on $Y^{783}$ and hence activated by Itk. Subsequently, PLCγ1 hydrolyzes the membrane lipid $PIP_2$ (phosphatidylinositol-4,5-bisphosphate), producing the second messengers $IP_3$ (inositol 1,4,5-trisphosphate) and diacylglycerol (DAG) (Fig. 1.4). $IP_3$ activates $Ca^{++}$-dependent signaling, whereas DAG will initiate two major intracellular signaling pathways involving protein kinase C (PKC) and Ras (rat sarcoma) (Fig. 1.4) (Smith-Garvin et al, 2009). These pathways lead to the activation of three transcription factors – NFAT (nuclear factor of activated T-cells), NFκB (nuclear factor kappa light chain enhancer of activated B cells), and AP1 (activator protein 1), which are required for IL-2 synthesis (Fig. 1.4). IL-2 is an important cytokine for activated T cells, which enhances T-cell activation and proliferation. Therefore,

given the importance of NFAT, NFκB, and AP1, I will briefly describe the molecular events leading to their activation.

$Ca^{++}$-NFAT

PLCγ1-generated $IP_3$ activates $IP_3$ receptors on the endoplasmic reticulum (ER) and leads to the release of $Ca^{++}$ from cytoplasmic stores. Depletion of intracellular calcium opens calcium release activated channels (CRAC) on the plasma membrane, thus allowing the entry of extracellular $Ca^{++}$. Rise of intracellular $Ca^{++}$ is sensed by calmodulin (CaM), which binds and activates other proteins such as, calmodulin kinase (CaMK) and the calmodulin-dependent phosphatase calcineurin. Calcineurin dephosphorylates members of the NFAT transcription factor family, thus allowing their translocation into the nucleus to activate gene transcription (Smith-Garvin et al, 2009, Robert et al, 2011).

PKCθ-NFκB

The NFκB pathway is initiated by the generation of DAG at the plasma membrane. DAG production results in the membrane localization and activation of PKCθ. PKCθ is then able to phosphorylate CARMA1 (CARD-containing MAGUK protein 1), a member of a trimolecular CBM complex (CARMA1, Bcl10, and MALT1). Upon phosphorylation CARMA1 oligomerizes and associates with Bcl10 (B-cell lymphoma 10), and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1). The CBM complex contributes to the degradation of the regulatory subunit of IKK (IκB kinase), thus unlocking the IKK catalytic site. Activated IKK phosphorylates IκB (inhibitor of kappa B) which keeps NFκB inactive. Phosphorylation of IκB leads to its degradation and release of NFκB. Ultimately, free NFκB molecules enter the nucleus and initiate gene transcription (Smith-Garvin et al, 2009).

Ras-ERK-AP1

Activation of the AP1 transcription complex, consisting of two proto-oncogenes Jun and Fos, depends on the Ras-ERK pathway. Ras is a small guanine nucleotide-binding protein that hydrolyzes GTP into GDP (GTPase). Ras is active in the GTP-bound state and inactive when loaded with GDP. Transition from the inactive to the active state is mediated by GEFs (guanine nucleotide exchange factors), which facilitate the release of GDP and promote the binding of GTP. During T-cell activation, GEFs are induced upon the assembly of the LAT signalosome (see 1.4.1).

In activated T cells, RasGTP (active Ras) interacts with Raf (rat fibrosarcoma, MAP3K), a MAPK (mitogen-activated protein kinase) through its Ras binding domain (RBD). This association is necessary to unlock autoinhibited Raf from its “closed” conformation. “Opened” Raf can dimerize and *trans*-phosphorylate to achieve full activation. In turn, activated Raf phosphorylates and activates the dual specificity kinases MEK1/2 (mitogen-activated protein kinase kinase 1/2). MEKs are required for the phosphorylation of the crucial serine/threonine-specific protein kinases ERK1/2 (extracellular signal-regulated kinase 1/2). Activated ERK is

essential for the generation of the AP1 transcription complex. ERK1/2 can modulate gene transcription directly (by phosphorylating and stabilizing Jun and Fos) or indirectly (by phosphorylating RSK [ribosomal s6 kinase] and ELK [ETS domain-containing protein] transcription activators). The phosphorylated AP1 complex controls the transcription of several genes and drives the expression of CD69, which is commonly used as a marker of T-cell activation (Smith-Garvin et al, 2009).

### 1.3.4. Additional signals supporting T-cell activation

*CD28-mediated co-stimulation*

In order to induce full T-cell activation, co-stimulatory signals are also required. In fact, ligation of the TCR alone (defined as signal 1) does not induce T-cell activation, but results in a non-responsive state called anergy. In addition to the TCR, co-stimulatory receptors deliver signals (called signal 2) during T-cell activation. Ligands for these receptors are expressed on APCs, such as dendritic cells (DCs), macrophages, and B cells. Among the co-stimulatory receptors in T cells, CD28 is one of the most studied. CD28 interacts with CD80 (B7-1) and CD86 (B7-2) molecules expressed on APCs. CD28-mediated co-stimulation synergies with T-cell receptor signals and promotes survival, clonal expansion, and differentiation (Rudd et al, 2009).

Similarly to the TCR, CD28 lacks intrinsic catalytic activity and signals by recruiting effector proteins to its cytoplasmic tail. Engagement of CD28 by its ligands leads to Lck and Fyn-mediated tyrosine phosphorylation of tyrosine-based signaling motifs (TBSMs) located within the CD28 cytosolic tail. The phosphorylation of the YMNM motif promotes the association of CD28 with the p85 regulatory subunit of PI3K and Grb2 (Okkenhaung et al, 1998). Recruited p85 binds to p110, the catalytic subunit of PI3K, which converts the phospholipid $PIP_2$ into $PIP_3$. In turn, $PIP_3$ serves as a docking site for pleckstrin homology (PH) domain-containing proteins including PDK1 (phosphoinositide-dependent protein kinase 1) and its substrate PKB/Akt (protein kinase B/Ak thymoma). Recruitment of these molecules to the plasma membrane activates signaling pathways involved in cell metabolism and survival (Rudd et al, 2009; Smith-Garvin et al, 2009).

*IL-2-mediated signaling*

In addition to TCR- and CD28-mediated signaling (signal 1 and 2), cytokines such as IL-2 (signal 3) also play a role in T-cell activation and proliferation. TCR- and CD28-mediated transcriptional activation leads to IL-2 production and to the upregulation of the IL-2R (IL-2 receptor). IL-2-IL-2R interaction provides a mitogenic signal leading to clonal expansion of activated T cells (Malek, 2008). The high affinity form of the IL-2 receptor is composed of IL-2Rα (CD25), IL-2Rβ (CD122), and the common γ chain (CD132). The latter is also shared with other cytokine receptors. The IL-2Rα and β chains bind IL-2. This leads to the activation

of Janus kinase 3 (JAK3). Subsequently, the IL-2Rβ chain is phosphorylated by active JAK3, thus enabling the recruitment of JAK1 and Shc (SH2 domain-containing transforming protein) to the receptor. At the activated IL-2R, triple phosphorylated Shc serves as a platform for the binding of Grb2/Sos1 complex required for the activation of the Ras-ERK cascade (see 1.4.1) (Gu et al, 2000). On the other hand, activated JAK1/3 phosphorylate STAT (signal transducer and activator of transcription) proteins, which dimerize and translocate to the nucleus where they can regulate cell growth, survival and differentiation (Vainchenker et al, 2013). The IL-2R can also recruit the p85 subunit of PI3K, which will trigger PKB/Akt activation, thus further promoting T-cell survival. Interestingly, the IL-2R and TCR signaling networks are interconnected. The cross-talk between these two signaling pathways has been recently described (Beyer et al, 2011). This study suggests that for adequate T-cell activation some effector proteins, such as ERK, have to receive all three activatory signals.

## 1.4. Regulation of T-cell activation

### 1.4.1. Feedback regulation of T-cell activation

T-cell activation is regulated by the interplay between upstream activators, such as apical tyrosine kinases, and effector proteins downstream. The interactions between these signaling molecules are defined as positive and negative feedback loops. Feedback regulation determines whether agonist-induced activation will be translated into transient or sustained T-cell signaling, or whether it will be terminated. Below I would like to describe several important examples.

*Negative feedback regulation*

Negative feedbacks circuits are crucial during T-cell activation as they are responsible for fine-tuning and termination of the signal. Negative regulatory mechanisms oppose rapid induction and amplification of biochemical events. They allow signals to be controlled or stopped and guarantee the appropriate response to perturbations. Phosphorylation and dephosphorylation are the main means of dampening signal propagation, although other modifications, such as ubiquitinylation, may also contribute (Acuto et al, 2008).

Very strict and elaborate feedback regulation adjusts proximal signaling. For example, the Lck-SHP1 negative feedback loop controls Lck activity. As I have mentioned previously (see 1.3.2), SHP1 is one of the phosphatases involved in the inhibition of Lck. Upon T-cell activation, Lck is able to phosphorylate SHP1 on several tyrosine residues including $Y^{564}$ which lies within the consensus sequence for binding of the Lck SH2 domain (Stefanova et al, 2003). Phosphorylated SHP1 can deactivate Lck directly by dephosphorylating the activatory $Y^{394}$ residue (Fig. 1.5b). SHP1 can also counteract Lck action by

dephosphorylating Lck substrates or other downstream signaling molecules, such as TCRζ, ZAP70, Vav1, Grb2, and SLP76. (Fig. 1.6a) (Lorenz et al, 1994; Plas et al, 1996; Acuto et al, 2008; Methi et al, 2005).

Another negative feedback loop regulating Lck, and also Fyn, involves the adaptor protein PAG (phosphoprotein associated with GEMs) and Csk (Brdicka et al, 2000; Smida et al, 2007). In resting T cells, membrane-bound PAG is constitutively phosphorylated by Fyn. Phosphorylated PAG binds Csk and localizes it at the plasma membrane, in the proximity of SFKs. There, Csk is able to phosphorylate the inhibitory tyrosines of Lck and Fyn, $Y^{505}$ and $Y^{529}$, respectively (Fig. 1.6a). Upon T-cell activation, PAG is dephosphorylated, likely by CD45, and Csk is released into the cytoplasm, where it no longer inhibits tyrosine kinases (Brdicka et al, 2000).

Other components of proximal signaling are regulated by a negative feedback loop mediated by the SHIP1-Dok2 module (Fig. 1.6a) (Acuto et al, 2008). Upon TCR stimulation, SHIP1 (SH2 domain-containing inositol-5-phosphatase) and Dok2 (docking protein 2) are phosphorylated by Tec family tyrosine kinases and form a complex with Grb2. SHIP1 dephosphorylates $PIP_3$ into $PIP_2$, thus interfering with the recruitment of PH domain-containing proteins, such as PKB/Akt or PDK1, to the plasma membrane (Smith-Garvin et al, 2009; Acuto et al, 2008). In addition, Dok2 negatively regulates signaling by recruiting Csk or RasGAP (Ras GTPase activating protein) – an inhibitor of the Ras-ERK pathway (Schoenborn et al, 2011; Acuto et al, 2008). Furthermore, it has been proposed that Dok2, together with Dok1, may also compete with ZAP70 for binding to the phosphorylated ITAMs or interfere with the assembly of the LAT signalosome, thus negatively regulating T-cell activation in multiple ways (Fig. 1.6a) (Dong et al, 2006; Yasuda et al, 2007).

Ubiquitinylation of components of the TCR/CD3 complex and also other effector molecules plays an additional role in the inhibition of TCR-mediated signaling. It has been shown that members of the CBL (casitas B lineage lymphoma) family (e.g. cCbl, an E3 ubiquitin ligase) are involved in the ubiquitinylation and subsequent degradation of ZAP70, TCRζ chains, and potentially other components of the TCR/CD3 complex. In activated T cells, cCbl is brought into close proximity of the ζ chains upon its binding to phosphorylated ZAP70. Here, cCbl may promote ubiquitinylation of its targets (Fig. 1.6a) (Wang et al, 2001; Naramura et al, 2002; Wang et al, 2008). This process is also thought to be part of the mechanism regulating TCR expression.

*Positive feedback regulation*

In contrast to negative regulation, positive feedbacks promote signaling. Positive circuits are crucial for the prolongation and/or the amplification of the initial signal without continuous presence of the original stimulus. Extending and propagating signals is necessary for

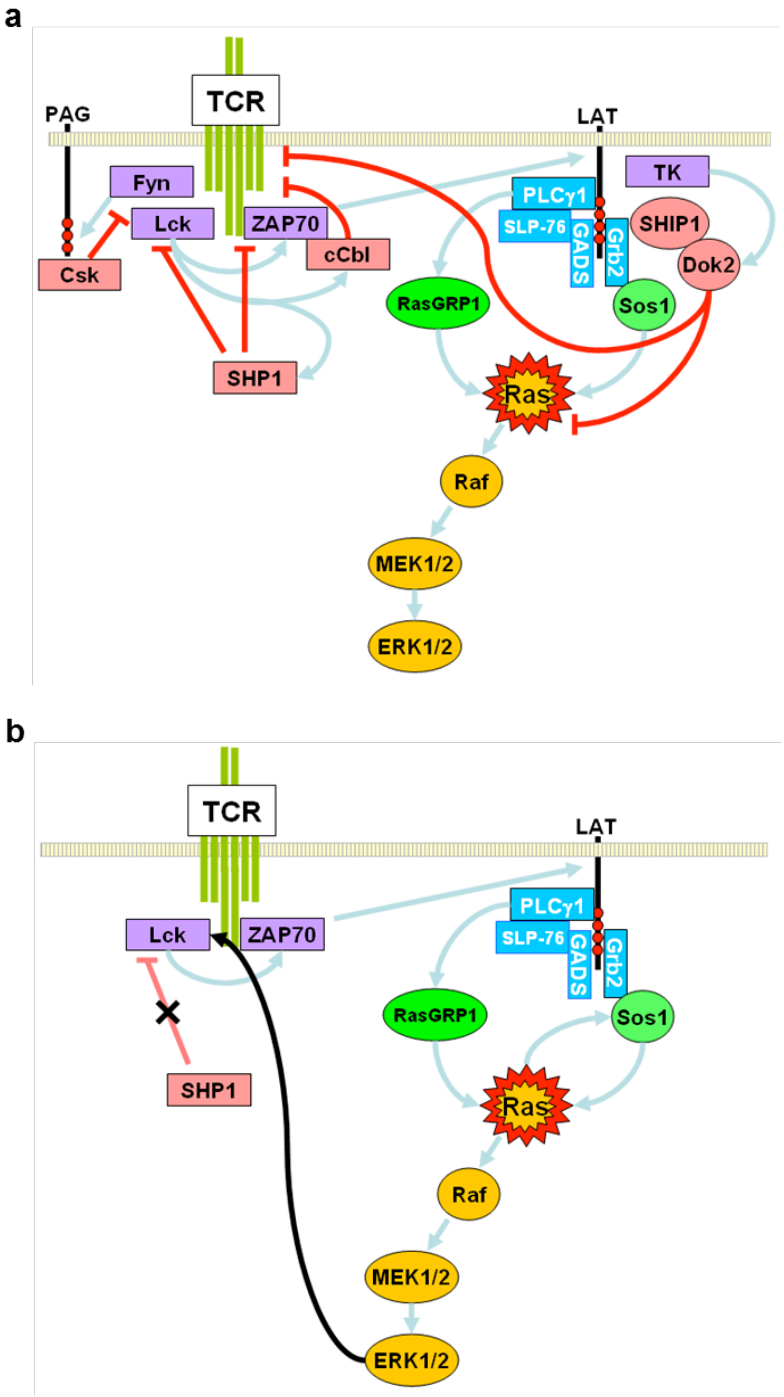


**Figure 1.6. Negative and positive feedback regulation in TCR-mediated signaling.** (a) The following negative regulators are involved in the orchestration of T-cell signaling, PAG-bound Csk (a master tyrosine kinase regulator), SHP1 (a phosphatase able to dephosphorylate Lck, ZAP70, and other signaling molecules), cCbl (an E3 ubiquitin ligase capable of ubiquitinylating TCRζ, ZAP70, and potentially other components of TCR complex), and SHIP1-Dok2 complex (responsible for deactivation of Ras and interferening with ITAMs phosphorylation), which is phosphorylated by Tec kinases (TK). (b) Actions of some negative regulators can be counteracted by positive feedbacks, such as the ERK-Lck feedback loop, which prevents Lck from SHP1-mediated dephosphorylation or Ras-Sos1 feedback necessary to amplify activation of Ras-ERK cascade (the figure was modified from Poltorak et al, 2013).

facilitation of particular molecular processes, for example stabilization of transcription factors. The interplay between positive and negative regulation shapes T-cell responses and consequent functional choices.

As a model molecule, Lck is regulated by both positive and negative feedbacks. A positive feedback between ERK1/2 and Lck has been proposed to block the interaction with SHP1 (see above) (Stefanova et al, 2003; Dong et al, 2010). It has been shown that Lck can be phosphorylated by activated ERK1/2 on serine 59 ($S^{59}$) (Watts et al, 1993; Winkler et al, 1993; August et al, 1996). Phosphorylation of this site leads to a conformational change in Lck, which affects the binding capacity of the Lck SH2 domain and prevents the interaction of Lck with SHP1, thus impeding SHP1-mediated dephosphorylation and hence inactivation of Lck (Fig. 1.6b) (Joung et al, 1995; Stefanova et al, 2003).

In addition, activation of the Ras-ERK cascade is regulated by an unusual interplay of Ras activators involving a positive feedback circuit. T cells express GEFs belonging to two different families, RasGRP1/4 (Ras guanyl-releasing protein 1/4) and Sos1/2 (son of sevenless 1/2) (Kortum et al, 2013; Stone, 2011). Although the function of RasGRP4 is not yet fully understood, it is very well-established that RasGRP1 is the major Ras activator in T cells (Genot et al, 2000; Smith-Garvin et al, 2009). Studies from RasGRP1$^{-/-}$ mice and T-cell lines demonstrated the importance of RasGRP1 for the activation of ERK in both mature and immature T cells (Roose et al, 2005; Roose et al, 2007; Priatel et al, 2010; Kortum et al, 2012). Moreover, defects in the expression of RasGRP1 in humans are contributing factors to autoimmune diseases, such as systemic lupus erythematosus (SLE) (Yasuda et al, 2007; Stone, 2011). RasGRP1 possesses a catalytic domain required for its GEF function composed of a REM (Ras exchange motif) box and a CDC25 (cell division cycle 25) box (Fig. 1.7a). Additionally, RasGRP1 possesses two calcium-binding elements called EF hands, a C1 domain for DAG-binding, and a unique tail (Fig. 1.7a) (Ebinu et al, 1998; Stone, 2011). Upon T-cell activation RasGRP1 is recruited to the plasma membrane via its C1 domain and the unique tail. At the plasma membrane, RasGRP1 is activated by PKCθ-mediated phosphorylation of $T^{184}$ (Carrasco et al 2004; Roose et al, 2005; Fuller et al, 2012). Thus, the activation of RasGRP1 depends on DAG and hence on PLCγ1 activity.

The second Ras activator, Sos, was discovered in *Drosophila melanogaster*, where it is essential for normal eye development (Bonfini et al, 1992). In human T cells, two homologues are expressed, Sos1 and Sos2 (Chardin et al, 1994). Mutations in the Sos1 gene have been recently reported in Noonan syndrome, which is a RASopathy – a developmental disorder caused by alterations in genes related to Ras-MAPK pathways (Pierre et al, 2011). In contrast to RasGRP1, Sos1 is constitutively bound to Grb2, an adaptor which is recruited to phosphorylated LAT (see 1.3.3). Thus, Grb2 is required to bring

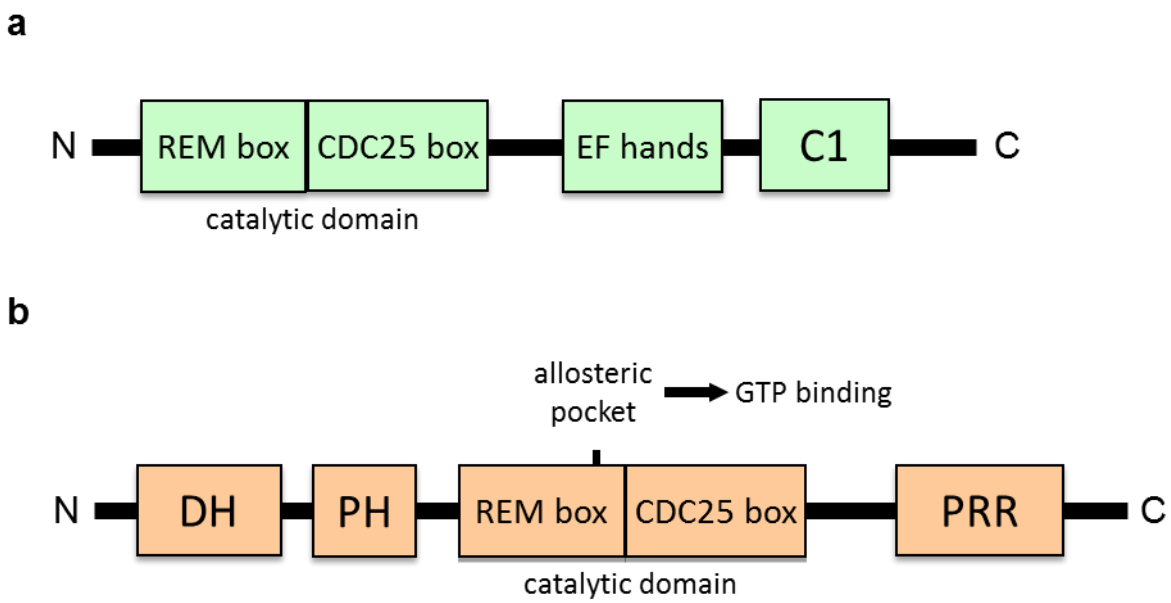


**Figure 1.7. Structure of RasGRP1 and Sos1.** (a) Schematic representation of the RasGRP1 protein. Green squares represent domains. The structure of RasGRP1 is as follows: catalytic domain-containing a REM (Ras exchange motif) box and a CDC25 (cell division cycle 25) box, EF hands, and a C1 domain. (b) Schematic representation of the Sos1 protein. Orange squares represent domains. A unique allosteric pocket required for RasGTP binding is indicated. The structure of Sos1 is as follows: Dbl homology (DH) domain, pleckstrin homology (PH) domain, catalytic domain containing REM box, CDC25 box and unique allosteric pocket, and proline-rich region (PRR).

Sos to the plasma membrane. Sos1 has a Dbl homology (DH) domain, a PH domain followed by a RasGRP1-like catalytic domain with a unique allosteric pocket, and a proline-rich region (PRR) necessary for interaction with the SH3 domain of Grb2 (Fig. 1.7b) (Margarit et al, 2003; Pierre et al, 2011). Upon TCR stimulation, the Grb2/Sos1 complex is recruited to phosphorylated LAT where it can facilitate the activation of Ras (Genot et al, 2000; Smith-Garvin et al, 2009). It is believed that the intrinsic GEF activity of Sos1 can be greatly enhanced upon the loading of active RasGTP in its unique allosteric pocket (Fig. 1.7b) (Margarit et al, 2003; Roose et al, 2007). Moreover, it has been proposed that membrane-recruited Sos1 is in an autoinhibited conformation and priming of Sos1 initially depends on RasGTP generated exclusively by RasGRP1 (Margarit et al, 2003; Sondermann et al, 2004). According to a recently proposed model, it is believed that Ras activation in T cells is orchestrated by the coordinated action of both RasGRP1 and Sos1 (Roose et al, 2007; Das et al, 2009). Initial TCR triggering leads to Ras activation exclusively via RasGRP1. Subsequently, RasGRP1-generated RasGTP primes Sos1, thus enhancing its enzymatic activity up to 80-fold. This interaction creates a positive RasGTP-Sos1 loop and strongly increases the levels of active Ras (Fig. 1.6b) (Roose et al, 2007; Das et al, 2009). It has been proposed that RasGTP generated by RasGRP1 alone regulates T-cell differentiation (positive selection), whereas the high amount of RasGTP generated by both RasGRP1 and Sos is believed to take part in the regulation of apoptosis in developing thymocytes (negative selection) (Priatel et al, 2002; Prasad et al, 2009). However, recent studies suggest that at specific stages of T-cell development, RasGRP1 and Sos1 can activate Ras independently (Kortum et al, 2011; Kortum et al, 2012). For example, RasGRP1$^{-/-}$ mice display only a mild defect in β-selection, but a severe block in positive selection (Table 1.1) (Kortum et al, 2012). On the other hand, Sos1 conditional knock-out mice show a strong defect in β-selection, but normal positive and negative selection (Table 1.1) (Kortum et al, 2011). Therefore these studies indicate that Sos1 can influence T-cell development during particular stages of thymopoiesis independently of RasGRP1 (e g. during β-selection).

| Mouse model | Developmental effect |
|---|---|
| RasGRP1$^{-/-}$ | 20% reduction in thymocyte cellularity<br>Mild defect in β-selection<br>Block in positive selection<br>Normal negative selection |
| Sos1$^{-/-}$ | 50% reduction in thymocyte cellularity<br>Proliferative defect at the β-selection checkpoint<br>Normal positive and negative selection |
| RasGRP1/Sos1$^{-/-}$ | Up to 90% reduction in thymocyte cellularity<br>Block in β-selection<br>Block in positive and negative selection |
| Grb2$^{-/-}$ | Defective positive and negative selection |

**Table 1.1. The role of RasGRP1, Sos1, and Grb2 in T-cell development** (the table modified from Kortum et al, 2013)

### 1.4.2. Regulation of T-cell responses: the mode of Ras-ERK activation

The magnitude/duration of Ras-ERK activation controls cellular responses. In the well-established model system for neuronal differentiation based on the PC12 cell line, it has been shown that moderate and sustained ERK phosphorylation upon NGF (nerve growth factor) treatment causes cell differentiation, whereas strong and transient ERK activation upon EGF (epidermal growth factor) stimulation induces proliferation in the same cells (Santos et al, 2007). In other cell types such as fibroblasts, it has been demonstrated that sustained ERK activation induced by mitogens, such as α-thrombin, leads to cell cycle re-entry, whereas transient signaling triggered by synthetic agonists, such as TMP (thrombin mimicking peptide), results in quiescence (Vouret-Craviari et al, 1993; Murphy et al, 2002). Thus, data from different cell types indicate that receptor stimulation at the plasma membrane is translated into quantitatively and/or qualitatively different activation kinetics of the Ras-ERK module to activate different cellular programs.

In T cells, it has been postulated that the magnitude of ERK activation is important for the regulation of thymic development. For example, strong ERK activation correlates with apoptosis (negative selection), whereas weak ERK activity is associated with differentiation (positive selection) (Daniels et al, 2006). Additionally, it has been shown that also in mature mouse T cells the magnitude of ERK activation is important for the regulation of T-cell responses. For example, strong ERK signal in $CD8^+$ T cells leads to apoptosis, in contrast to low levels of phosphorylated ERK, which result in survival/proliferation (Wang et al, 2008).

The duration of the ERK signal is another important factor for the generation of cellular responses. A dynamic behavior of ERK activity seems to exist in murine thymocytes. Transient ERK activation induces negative selection, whereas sustained ERK activity leads to positive selection of immature T cells (McNeil et al, 2005; Daniels et al, 2006). In mature T cells, induction of transient signals corresponds with unresponsiveness or apoptosis, in contrast to sustained ERK signaling, which results in activation and proliferation (Berg et al, 1998; Wang et al, 2008).

It has been proposed that differences in the magnitude and the duration of the Ras-ERK signaling are regulated by different mechanisms including feedback loops (see 1.4.1). In PC12 cells a strong and transient signal results in activation of a negative feedback circuit between ERK and, most likely, Sos, whereas moderate and sustained ERK phosphorylation is regulated by PKC-mediated ERK-Raf positive feedback loop (Santos et al, 2007). In cytotoxic mouse T lymphocytes transient signaling is mediated by classical PKC isoforms, whereas novel PKCs are involved in prolonged signals (Puente et al, 2006). Additionally, it has been shown in mouse thymocytes that the compartmentalization of Ras activators (Grb2/Sos1 complex and RasGRP1), as well as Ras and ERK themselves may contribute to the activation kinetics. Localization analysis of these molecules indicated that during transient activation, signaling occurs exclusively at the plasma membrane. However, when sustained signaling is induced, the activated proteins localize in cytoplasmic vesicles (Daniels et al, 2006; Wang et al, 2008).

Despite the fact that the regulation of Ras-ERK activation has been well-studied in some cell systems, how the magnitude/duration of the Ras-ERK signal is regulated in primary human T cells is still poorly understood.

In summary, in order to activate the appropriate cellular program and to induce an efficient immune response, T cells are equipped with a multitude of regulatory mechanisms, which serve to integrate, fine-tune, and terminate signals triggered at the plasma membrane.

### 1.5. Aims of the study

Altered T-cell activation is the basis for many human diseases, such as chronic inflammation, immunodeficiency, allergy, and cancer. In my study, I focused on how TCR-mediated signaling is regulated on the molecular level. For my experiments, I used primary human T cells because of their relevance in human disease. I addressed the following scientific questions:

*How are the activation dynamics regulated in T cells?*

In the first part of my work, I analyzed how transient *vs.* sustained TCR signaling is regulated. Transient signaling correlates with apoptosis/unresponsiveness (anergic-like

state), whereas sustained signaling is associated with T-cell activation and proliferation. I found that transient signaling is regulated by negative regulatory loops involving inhibitory molecules such as Dok2 and cCbl, whereas sustained signaling is triggered by a positive regulatory feedback involving the ERK-mediated phosphorylation of Lck.

*How is Ras-ERK activation regulated in T cells?*

In the second part of my work, I focused my attention on the contribution of RasGRP1 and Sos1 to Ras-ERK activation. Using RNAi (RNA interference), I demonstrated that RasGRP1 is a crucial activator of Ras-ERK in primary human T cells. Conversely to RasGRP1, Sos1 contributes only to Ras-ERK activation during sustained signaling. Moreover, I found that Sos2, a homologue of Sos1, and Grb2, an adaptor molecule associated with Sos1, appear to be dispensable for the ERK activation upon TCR-mediated stimulation in human primary T cells.

The results of my studies are presented in the section below.

## 2. Results

In the results section, I characterized stimulation methods to induce transient or sustained TCR-mediated signaling, investigated the activation dynamics of key signaling molecules upon TCR triggering and analyzed the regulation of Ras-ERK activation.

### 2.1. Analysis of transient *vs.* sustained TCR signaling

In order to study how T cells are activated upon physiological stimulation in living organisms, the *in vitro* experimental setup has to mimic as closely as possible the *in vivo* conditions. During the past years, different methods have been developed to stimulate T cells *in vitro*, which have allowed an extensive analysis of the biochemical events occurring during T-cell activation. A variety of studies have employed mouse T cells expressing a transgenic TCR. The advantage of this system is that T cells can be stimulated with physiological ligands (e.g. peptide-MHC complexes specific for the transgenic TCR). However, the mouse immune system is not fully comparable with the human immune system. In fact, a very recent study has analyzed the profiles of genes upregulated in response to inflammatory stress in humans and mice and has come to the conclusion that mouse models are poorly suited to study human inflammatory diseases (Seok et al, 2013). Therefore, in order to better understand the molecular mechanisms underlying human diseases, I decided to employ human T cells for my studies. Unfortunately, with the exception of memory T cells, which can be re-stimulated *in vitro* with specific antigen (e. g. tetanus) (Cellerai et al, 2007), there are no physiological ligands available to stimulate a sufficient number of naïve human T cells *in vitro* for biochemical studies.

Therefore, I took advantage of two available stimulation systems to activate human peripheral T cells *in vitro*. I used antibodies applied in solution (sAbs) and antibodies immobilized on microbeads (iAbs). Both systems are based on antibodies directed against the TCR/CD3 complex and co-stimulatory molecules. Anti-CD3 antibodies recognize epitopes in the extracellular part of the CD3ε chains and induce the aggregation of at least two TCR/CD3 complexes (a process called crosslinking). Antibodies against the CD28 co-stimulatory molecule and the CD4 co-receptor utilize the same principle. In my studies, I used monoclonal IgG antibodies (mAbs) against human CD3ε (clones OKT3, UCHT1), CD28 (clone CD28.2), and CD4 (clone OKT4), which were biotinylated. Biotinylation allows linkage of multiple antibodies upon the addition of streptavidin, thus enhancing TCR/CD3 crosslinking and T-cell stimulation,

One of the most important results of my studies is that sAbs and iAbs induce completely different signaling dynamics and T-cell functional outcomes (Arndt et al, 2013; Poltorak et al, 2013). Therefore, these stimuli have allowed me to study the molecular mechanisms

regulating signaling kinetics and T-cell responses. The major characteristics of transient and sustained signaling triggered by sAbs or iAbs, respectively, are presented below.

*Transient signaling induced by sAbs*

Human peripheral T cells were left unstimulated or stimulated with sAbs for the indicated time periods (Fig. 2.1). The Western blot analysis presented in figure 2.1 clearly show that the maximal signal intensity induced by sAbs peaks at 2 - 5 min and then rapidly declines to the basal levels 15 to 30 min post-stimulation (Fig. 2.1a). These transient phosphorylation kinetics are observed for molecules involved in both proximal (ZAP, LAT, PLCγ1) and more distal (Akt, Raf, MEK, ERK, RSK) TCR signaling (Fig 2.1a and Poltorak et al, 2013).

It has been previously shown that transient TCR-mediated signaling does not induce activation and differentiation of mouse $CD8^+$ T cells (Berg et al, 1998; Wang et al, 2008). To test if sAbs also do not induce T-cell activation in human primary T cells, I measured the expression of CD69 and CD25, two well-known activation markers. CD69 is expressed in response to the ERK-dependent activation of the transcription factor AP1 and CD25 upregulation occurs concomitantly with IL-2 production. When T cells were stimulated with sAbs neither CD69 nor CD25 were upregulated (Fig. 2.1b). In agreement with the impaired activation, crosslinking of the antibodies in solution also did not induce T-cell proliferation (Fig. 2.1c). Interestingly, we found that, conversely to mouse T cells where sAbs induce apoptosis (Wang et al, 2008), stimulation of human T cells with sAbs induces an anergic-like state, characterized by unresponsiveness to re-stimulation (data not shown).

*Sustained signaling induced by iAbs*

Antibodies against the TCR/CD3 complex can be bound to plastic (e.g. 96-well plates or plastic Petri dishes) or to microspheres of different size (Koike et al, 2003; Carpentier et al, 2009; Li et al, 2010). Under these conditions of stimulation, T-cell proliferation will be induced (Berg et al, 1998; Puente et al, 2000; Puente et al, 2006). In my work, I decided to take advantage of antibodies immobilized on SuperAvidin-coated microbeads with the diameter of approximately 10 μm. This size was selected to mimic the dimensions of APCs and to prevent endocytosis of the beads by T cells.

Next, I performed analysis of the signaling kinetics triggered by antibodies immobilized on microbeads (Fig. 2.2). In stark contrast to sAbs, iAbs stimulation triggered sustained phosphorylation of proximal and distal signaling molecules for up to 12 h (Fig. 2.2a and 2.2b). It has been previously shown that sustained signaling corresponds with T-cell activation and differentiation in mouse $CD8^+$ T cells (Berg et al, 1998; Wang et al, 2008). Therefore, I tested whether iAbs also induce activation/proliferation of human T cells. As presented in figure 2.2c, microbeads were able to induce strong expression of both the

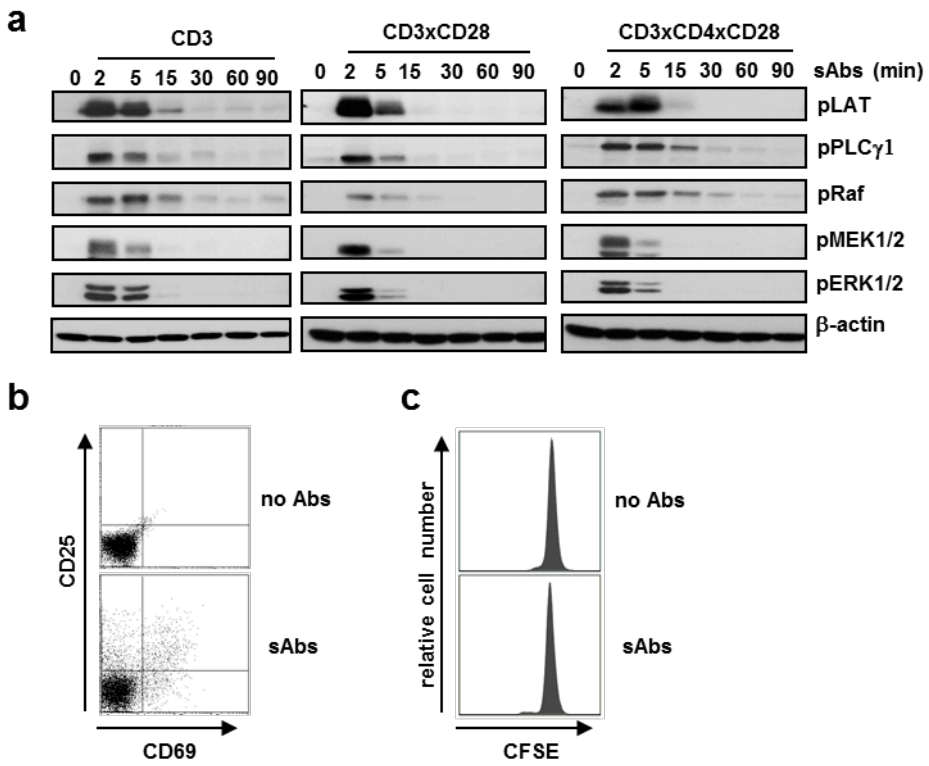


**Figure 2.1. sAbs induce transient signaling and T-cell unresponsiveness.** Purified primary human T cells were treated with CD3, CD3xCD28 or CD3xCD4xCD28 mAbs cross-linked in solution (sAbs) as indicated. (a) Samples were analyzed by Western blotting using the indicated Abs. For each condition one representative immunoblot of at least four independent experiments is shown. (b) 24 h after stimulation with CD3xCD28 sAbs, the expression of CD25 and CD69 was analyzed by flow cytometry. (c) T cells were labeled with CFSE and stimulated as indicated in (b). Proliferation was assessed after 72 h by analyzing CFSE content by flow cytometry (the figure was modified from Arndt et al, 2013).

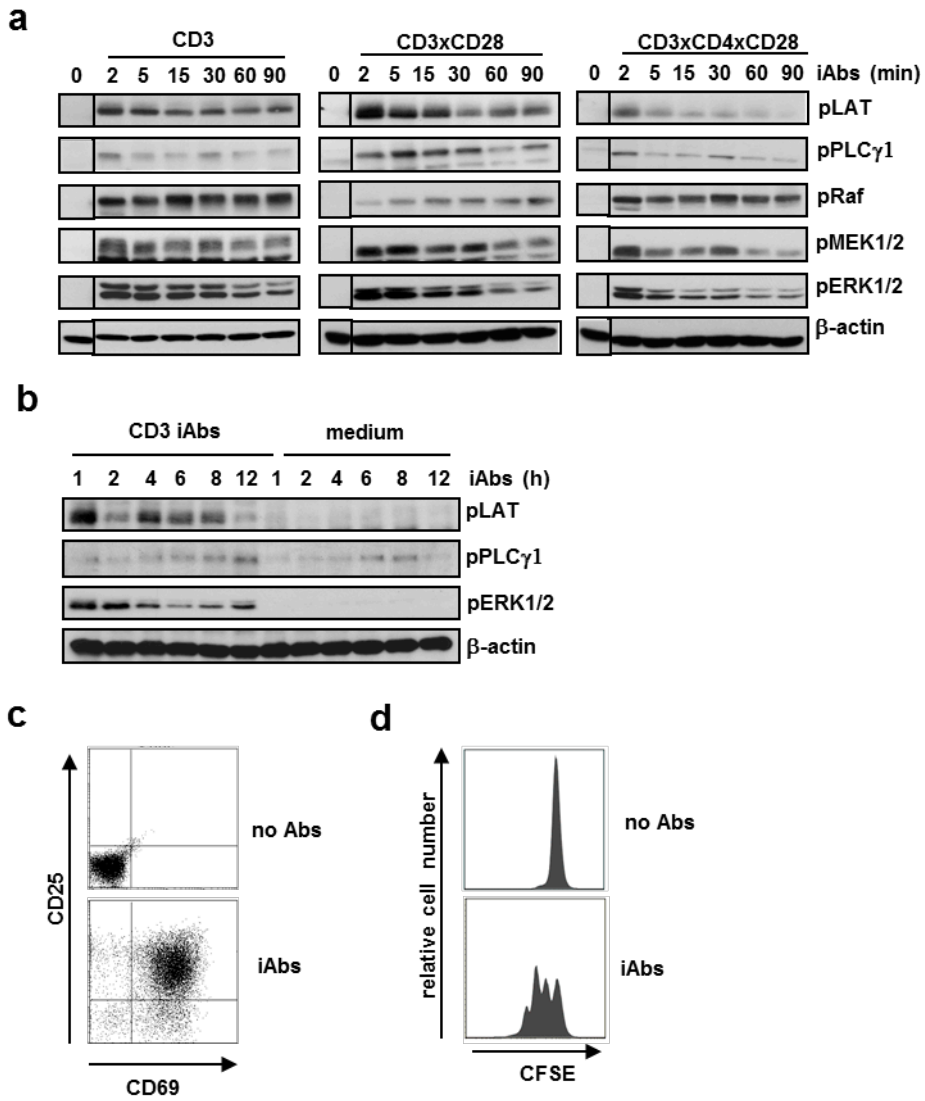


**Figure 2.2. iAbs trigger sustained signaling kinetics, T-cell activation and proliferation.** Purified primary human T cells were treated with CD3, CD3xCD28 or CD3xCD4xCD28 mAbs immobilized on microbeads (iAbs) as indicated. (a and b) Samples were analyzed by Western blotting using the indicated Abs. For each condition one representative immunoblot of at least four independent experiments is shown. (c) 24 h after stimulation with CD3xCD28 sAbs, the expression of CD25 and CD69 was analyzed by flow cytometry. (d) T cells were labeled with CFSE and stimulated as indicated in (c). Proliferation was assessed after 72 h by analyzing CFSE content by flow cytometry (the figure was modified from Arndt et al, 2013).

activation markers CD69 and CD25. Moreover, flow cytometric analysis of CFSE-labeled T cells clearly showed that iAbs stimulation triggers proliferation (Fig. 2.2d).

Collectively, the data show that sAbs stimulation correlates with transient signaling that leads to unresponsiveness, whereas iAbs stimulation corresponds with sustained signaling and productive T-cell responses, such as CD69 and CD25 upregulation and proliferation.

### 2.2. Analysis of feedback regulation in transient *vs.* sustained T-cell activation

The stimuli I employed activate the same receptor and trigger the same cellular pathways. Nevertheless, they induce different activation dynamics, thus resulting in different cellular responses. Therefore, I performed additional studies to shed light onto how the activation of the same receptor (i. e. the TCR) results in different signaling kinetics.

#### 2.2.1. Transient signaling correlates with a strong activation of Src family kinases

I initially focused my attention on proximal TCR signaling events (see 1.3.2). To this end, I measured the phosphorylation of TCRζ upon sAbs *vs.* iAbs treatment (Fig. 2.3a). TCRζ immunoprecipitates were prepared from either unstimulated or stimulated T cells and probed with an anti-pan-phospho-tyrosine antibody (clone 4G10). As shown in figure 2.3a, transient signaling (sAbs) correlates with increased tyrosine phosphorylation of TCRζ, as suggested by the appearance of two phosphorylated bands running at 21 and 23 kDa. In stark contrast, I did not observe any significant change in TCRζ chain phosphorylation during sustained signaling (iAbs) (Fig. 2.3a). These data imply that the activity of SFKs, which are responsible for TCRζ phosphorylation (see 1.3.2), is likely differentially regulated upon transient *vs.* sustained activation. Therefore, to further test this hypothesis, I analyzed the activity of Lck and Fyn. I focused on the fractions of Lck and Fyn which are associated with the TCR/CD3 complex and hence are directly involved in signaling. To this aim, I performed immunoprecipitations of TCRζ under mild detergent conditions to pull-down intact TCRζ chains associated with effector molecules. Subsequently, taking advantage of the phospho-Src-$Y^{416}$-specific antibody, which recognizes phosphorylation of the activatory tyrosines $Y^{394}$ and $Y^{416}$ in Lck and Fyn, respectively, I tested TCRζ immunoprecipitates for the presence of active SFKs. The results presented in figure 2.3b show that, in contrast to sustained signaling, the amount of active Lck and Fyn associated with the TCR significantly increases during transient activation. These data demonstrate that SFKs activity is strongly enhanced during transient signaling. In agreement with these findings, I observed that the global tyrosine phosphorylation pattern is strongly induced during transient activation (Fig. 2.3c). Conversely, tyrosine phosphorylation does not appear to be as strongly induced during sustained as during transient signaling (Fig. 2.3c).

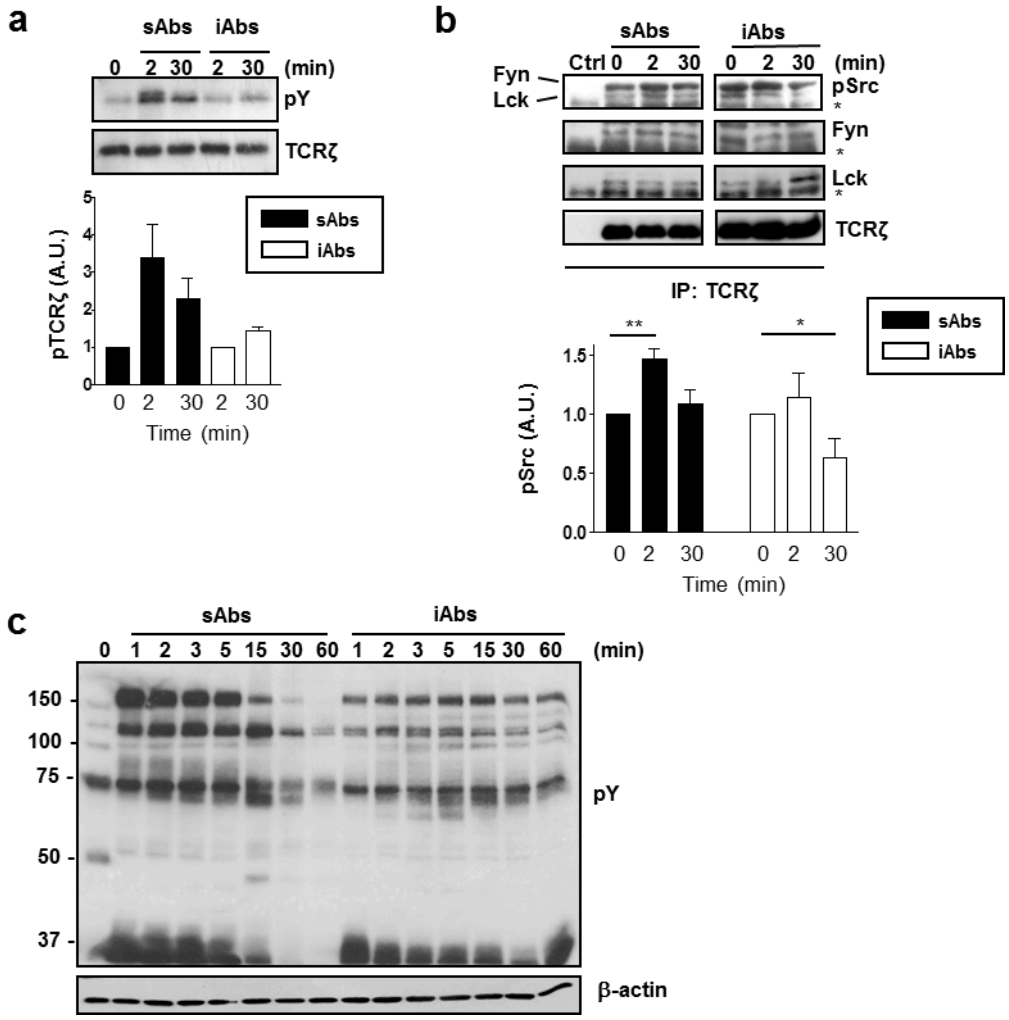


**Figure 2.3. Transient signaling correlates with a strong activation of Src family kinases.** Purified human T cells were treated with either soluble (sAbs) or immobilized (iAbs) CD3xCD28 mAbs for the indicated time periods. TCRζ immunoprecipitates (a and b) or total cell lysates (c) were prepared and analyzed by Western blotting using the indicated Abs. In (b), anti-TCRζ-coated agarose beads were incubated in lysate buffer without cells as a control (Ctrl). One representative immunoblot of at least three independent experiments is shown. The phosphorylation of TCRζ (a) or Fyn and Lck (b) was quantified using the 1D ImageQuant software and the values were normalized to the corresponding total TCRζ or Lck and Fyn signal, respectivelyl. Data represent the mean of phosphorylation levels shown as arbitrary units ± SEM of at least three independent experiments. Asterisk in (b) indicates the antibody heavy chain (figures 2.3a and b were modified from Poltorak et al, 2013).

In summary, these data suggest that a quantitative difference in the activation of Src family kinases distinguishes transient from sustained activation.

### 2.2.2. Activation of negative regulators during transient TCR-mediated signaling

The enhanced activation of Lck and Fyn implies that also the activation of downstream signaling molecules is augmented under conditions of stimulation inducing transient signaling. This hypothesis is further supported by the enhanced tyrosine phosphorylation pattern (Fig. 2.3c). Indeed, I found that the phosphorylation of ZAP70, LAT, and PLCγ1 (Fig. 2.1) is enhanced upon sAbs stimulation. I hypothesized that, in addition to positive regulators, also the activation of negative regulators of TCR signaling may be strongly augmented during transient signaling. If this holds true, an imbalance in the equilibrium between positive and negative regulators may explain why signaling is rapidly terminated upon transient stimulation.

It is known that some negative regulators depend on tyrosine phosphorylation for their activation. One of these is cCbl, an E3 ubiquitin ligase involved in the ubiquitinylation and degradation of TCRζ and ZAP70 (see 1.4.1). I analyzed cCbl phosphorylation under conditions of stimulation inducing either transient or sustained signaling. The results presented in figure 2.4 clearly show that stimulation with sAbs greatly increases cCbl phosphorylation above the basal level, whereas treatment with iAbs does not. In agreement with these findings, we found that ZAP70 expression was reduced upon sAbs treatment, likely indicating degradation (Poltorak et al, 2013). This hypothesis is further supported by the observation that anti-ZAP70 immunoblots revealed a particular pattern of ZAP70 migration on SDS-PAGE gel corresponding with ubiquitinylation (Wang et al, 2008). Thus, activation of cCbl and the subsequent degradation of signaling molecules such as ZAP70 upon sAbs stimulation may lead to a dampening of the TCR-mediated signaling and hence may contribute to the observed transient activation under this condition of stimulation (Poltorak et al, 2013).

Next, I tested whether other negative regulators are also activated during sAbs stimulation. Another inhibitory molecule activated by tyrosine phosphorylation is Dok2 (see 1.4.1). As described in 1.4.1, Dok2 can interfere with TCR signaling at several levels. Therefore, it was important to test whether Dok2 is also phosphorylated/activated under sAbs treatment. As expected, Dok2 phosphorylation was strongly enhanced upon transient stimulation (Fig. 2.4). In contrast, in iAbs-treated T cells, Dok2 phosphorylation was only slightly increased above the basal level (Fig. 2.4).

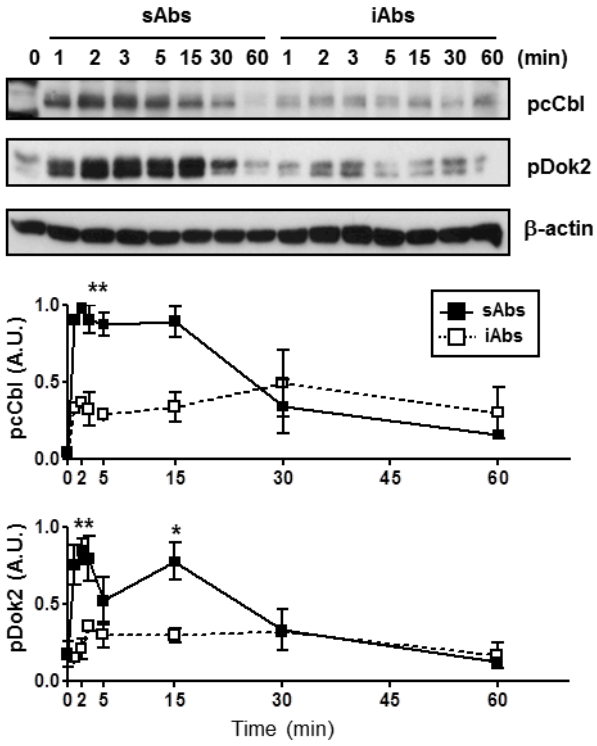


**Figure 2.4. Activation of negative regulators during transient TCR-mediated signaling.** Purified naïve $CD4^+$ human T cells were left untreated or treated with either soluble (sAbs) or immobilized (iAbs) mAbs for the specified time periods. Samples were analyzed by Western blotting using the indicated Abs. The phosphorylation of cCbl and Dok2 was quantified using the 1D ImageQuant software and the values were normalized to the corresponding β-actin signal. Data represent the mean of the phosphorylation levels shown as arbitrary units ± SEM of three independent experiments. One representative experiment of four is shown.

These findings support the hypothesis that strong activation of SFKs may unbalance the equilibrium between positive and negative regulators of TCR-mediated signaling thereby leading to termination of T-cell activation.

### 2.2.3. Positive feedback regulation under sustained TCR signaling

It has been shown that sustained signaling in other cell types, such as PC12 cells, is regulated by positive feedback loops (see 1.4.2). Therefore, I investigated whether sustained signaling in T cells upon iAbs stimulation is also regulated by positive circuits. It has been suggested that active ERK1/2 phosphorylates Lck to initiate signaling in thymocytes (Stefanova et al, 2003). Hence, I assessed whether an ERK-Lck positive feedback mediates sustained signaling in mature peripheral T cells.

Sustained, but not transient, TCR signaling correlates with the appearance of a Lck form migrating approximately at 59 kDa on SDS-PAGE (p59Lck) (Fig. 2.5a). It is believed that this retarded mobility of Lck results from the phosphorylation of serine residues that affect Lck conformation (Watts et al, 1993; Gold et al, 1994). Previous reports proposed that Lck could be phosphorylated on serine residues by both ERK- and/or PKCs (Winkler et al, 1993; Schroeder et al, 2000). Thus, I decided to shed more light onto how Lck is regulated during sustained signaling in primary human T cells. To this end, I used well-characterized MEK inhibitors to block ERK activation and evaluated the effect on Lck. As expected, pre-incubation with MEK Inhibitor I or U0126 completely abolished ERK1/2 phosphorylation (Fig. 2.5b). Subsequently, I tested how Lck migrates in SDS-PAGE. As shown in figure 2.5b, both MEK inhibitors abolished the generation of p59Lck. These results confirmed that activated ERK1/2 phosphorylates Lck in primary human T cells. To test if PKCs also phosphorylate Lck, I induced PKC activation upon treatment of T cells with the DAG analog, PMA. DAG may activate both ERK and PKCs. Therefore, T cells were stimulated in the presence of a MEK inhibitor to block ERK, but not PKC activation. I found that upon strong PKC activation and in the presence of inactive ERK, Lck appears as a unique band migrating at 56 kDa (Fig. 2.5c). Collectively, these data suggest that ERK, but not PKC, phosphorylates Lck in primary human T cells.

Next, I decided to investigate which serine residues in Lck are phosphorylated by ERK. Several studies indicated that $S^{42}$ and $S^{59}$ in the N-terminal part of Lck might be the targets for serine/threonine kinases (Watts et al, 1993; Winkler et al, 1993; Joung et al, 1995). To assess this issue, I took advantage of Lck constructs carrying S-to-D and S-to-A mutations, either on $S^{42}$ or $S^{59}$. Substitutions from serine to aspartic acid mimic constitutive phoshorylation, and hence Lck should migrate as a unique 59 kDa band on SDS-PAGE. Conversely, serine to alanine substitution prevents phosphorylation and thus Lck should

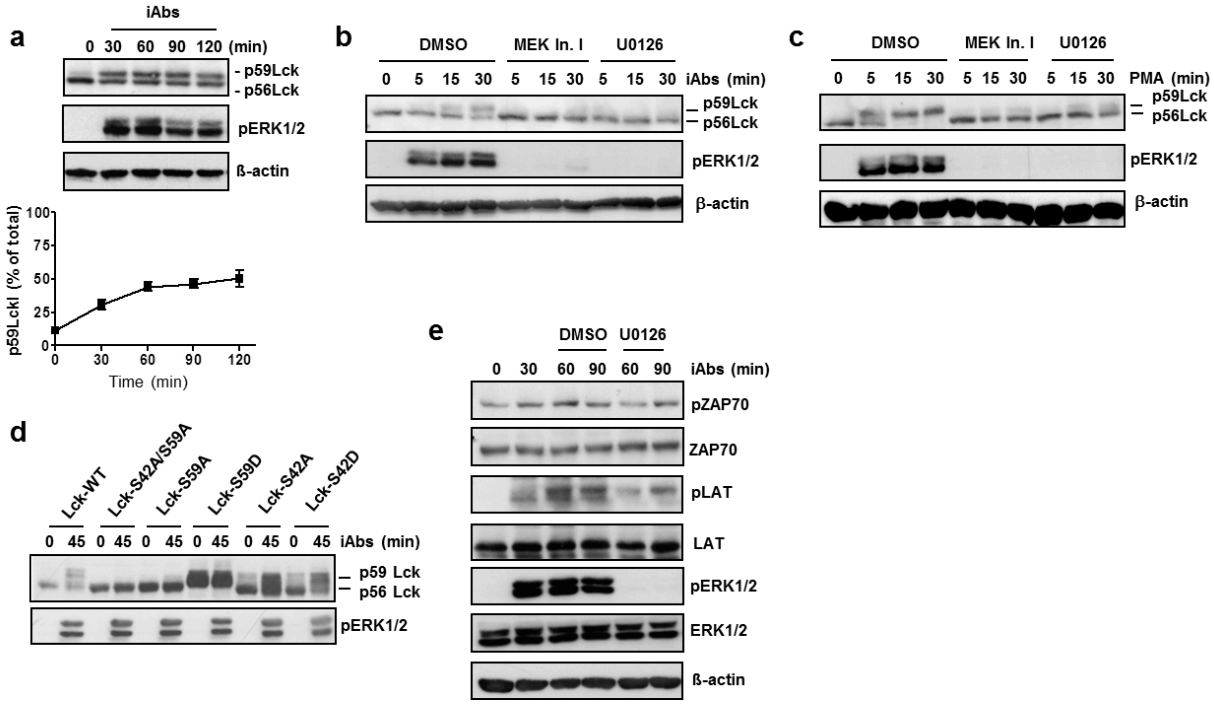


**Figure 2.5. Positive feedback regulation under sustained TCR signaling.** Purified primary human T cells were left untreated or treated with either immobilized CD3xCD28 mAbs (a and b) or with 27 ng/ml PMA (c) for the indicated time periods in the presence or absence of MEK Inhibitor I or U0126. Samples were analyzed by immunoblotting using the specified Abs. One representative immunoblot of at least three independent experiments is shown. Bands in (a) were quantified using the 1D ImageQuant software. The graph represents the mean of p59Lck signal intensity normalized to the total amount of Lck shown as percentage ± SEM of ten independent experiments. (d) J.CaM1.6 cells were transfected with constructs carrying different Lck mutations (S42A, S42D, S59A, S59D, S42A/S59A). 24 h after transfection, cells were either left unstimulated or stimulated with CD3xCD28 iAbs. Samples were analyzed by Western blotting using the indicated Abs. One representative immunoblot of five independent experiments is shown. (e) Purified human T cells were treated with iAbs alone for 30 min, then either DMSO, MEK inhibitor I, or U0126 was added and samples were incubated for additional 30 to 60 min. Cell lysates were analyzed by Western blotting using the indicated Abs. One representative immunoblot of four independent experiments is shown (figures 2.5b and d were modified from Poltorak et al, 2013).

migrate exclusively as a 56 kDa protein. I used the following mutants, S42A, S42D, S59A, S59D, and S42A/S59A. The mutants were transfected into the Lck-deficient Jurkat T-cell variant (J.CaM1.6). 24 h after transfection, cells were harvested and stimulated with iAbs (Fig. 2.5d). Constructs carrying the S42A/S59A and S59A Lck mutations migrated at 56 kDa upon iAbs stimulation, whereas the S59D mutant resulted in the presence of the p59Lck form only (Fig. 2.5d). On the other hand, both S42A and S42D mutants showed mobility on SDS-PAGE comparable to that of wild type Lck and hence appeared as both 56 and 59 kDa bands (Fig. 2.5d). Thus, these findings indicate that $S^{59}$, but not on $S^{42}$, is the amino acid residue phosphorylated by ERK.

Finally, I investigated whether ERK-mediated phosphorylation of Lck has functional consequences. To test this, I measured the phosphorylation of the Lck substrate ZAP70 and other downstream signaling molecules such as LAT, in the presence of U0126 (Fig. 2.5e). Human primary T cells were first stimulated with iAbs for 30 min, to activate the ERK-Lck feedback loop, and then, ERK1/2 activation was blocked by the addition of the inhibitor U0126. As shown in figure 2.5e, both ZAP70 and LAT phosphorylation were decreased in the presence of U0126. This decrease is not due to a reduction in the expression levels of LAT or ZAP70 (Fig. 2.5e). To further show that the ERK-Lck feedback loop is only required for sustained TCR signaling, the same experiments were repeated using sAbs. As mentioned above sAbs do not induce the appearance of p59Lck, thus indicating that they do not induce the ERK-Lck feedback loop. Treatment with U0126 did not affect ZAP70 and LAT phosphorylation upon sAbs stimulation (Poltorak et al, 2013). These results indicate that (i) U0126 has no off-target effects on ZAP70 and LAT and (ii) that the ERK-Lck feedback loop regulates the activation of Lck downstream targets and hence sustained TCR signaling (Poltorak et al, 2013).

### 2.2.4. ERK-Lck feedback regulates Lck activity

Next, I decided to investigate how ERK regulates Lck function. First, I evaluated whether ERK influences the activity of Lck. I measured Lck activation taking advantage of the phospho-Src$Y^{416}$-specific antibody which detects phosphorylation of the activatory tyrosine residue $Y^{394}$ (Fig. 2.6a).

I found that the phosphorylation of total Lck on $Y^{394}$ was significantly reduced in primary human T cells at the later stages of stimulation (Fig. 2.6a). To exclude the possibility that the observed reduction in p59Lck phosphorylation was due to protein degradation, I analyzed Lck expression in T cells in which the ERK-Lck feedback loop was either activated or inhibited. Results presented in figure 2.6b show that expression of total Lck is comparable between untreated and U0126 treated primary human T cells, suggesting that degradation is not triggered by the ERK-Lck feedback loop.

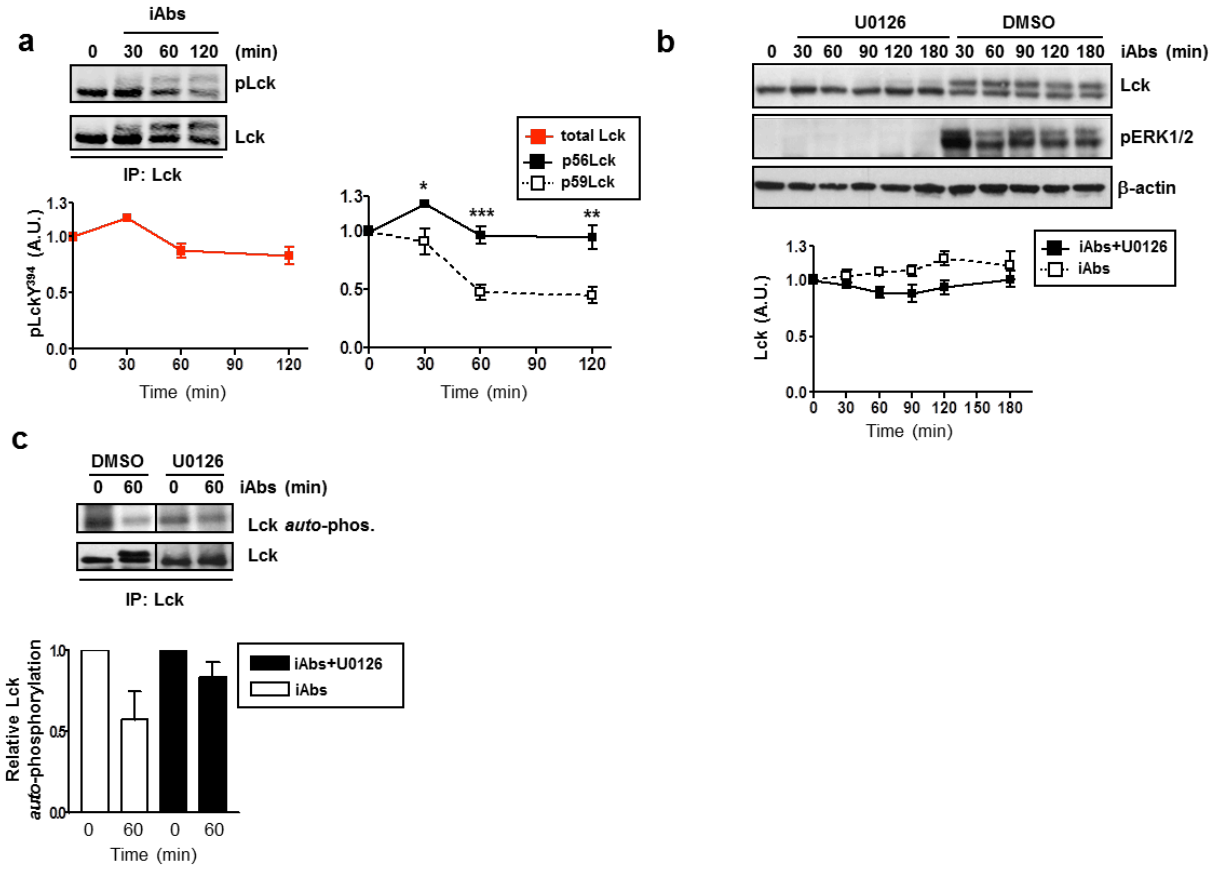


**Figure 2.6. ERK-Lck feedback regulates Lck activity.** Purified human T cells were left unstimulated or stimulated with CD3xCD28 iAbs for indicated time periods in the presence or absence of U0126. Lck immunoprecipitates (a) or total cell lysates (b) were prepared and analyzed by Western blotting using the specified Abs. One representative immunoblot of at least four independent experiments is shown. Bands intensity was quantified using the 1D ImageQuant software. Graphs in (a) represent the mean of the phospho-specific Lck, p56Lck, or p59Lck signal normalized to the total amount of Lck shown as arbitrary units ± SEM of eight independent experiments. Data in (b) represent the mean of Lck expression levels normalized to β-actin shown as arbitrary units ± SEM of four independent experiments. (c) Lck immunoprecipitates were subjected to a classical *in vitro* kinase assay using radiolabelled $^{32}$γ-ATP. *In vitro* labeled IPs were resolved on the SDS-PAGE gel, transferred to a nitrocellulose membrane, and analyzed by an autoradiography. One representative autoradiograph is depicted. Autoradiographs were quantified using the 1D ImageQuant software. Data on graph represent the mean of the relative Lck *auto*-phosphorylation shown as arbitrary units ± SEM of three independent experiments.

Next, I looked at the phosphorylation of the two Lck forms. I found that the phosphorylation of p56Lck was not significantly changed upon iAbs stimulation. Conversely, the phosphorylation of p59Lck on $Y^{394}$ was strongly decreased (up to 50%) (Fig. 2.6a). Collectively, these results indicate that reduced $Y^{394}$ phosphorylation of p59Lck accounts for the decreased phosphorylation of total Lck during sustained TCR signaling.

To investigate whether the reduced phosphorylation on $Y^{394}$ correlates with a decreased enzymatic activity of Lck, I performed a classical *in vitro* kinase assay. Briefly, Lck immunoprecipitates from unstimulated or iAbs-stimulated primary human T cells in the presence or absence of U0126 were incubated with radiolabelled $^{32}$γ-ATP. Subsequently, IPs were washed, resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to autoradiography. The data shown in figure 2.6c indicate that generation of p59Lck in iAbs-stimulated T cells correlated with an almost 50% reduction in *auto*-phosphorylation of Lck. These findings corroborated previous results showing that recombinant mouse Lck has approximately two-fold lower kinase activity when incubated in the presence of purified ERK (Watts et al, 1993). Thus, Lck phosphorylated on $S^{59}$ by ERK appears to be less active than non-phosphorylated Lck.

### 2.3. Regulation of the Ras-ERK cascade in transient *vs.* sustained T-cell signaling

The striking differences in feedback regulation during transient *vs.* sustained signaling prompted me to further investigate signaling events occurring during T-cell activation. In particular, I focused my attention on the regulation of the Ras-ERK module. The current model for Ras-ERK activation proposes that Ras can be triggered by a positive feedback between RasGRP1 and Sos, the GEFs expressed in T cells (see 1.4.1). As this model is based on data from lymphoid cell lines, it is not yet clear how RasGRP1 and Sos contribute to Ras activation in primary human T cells. Moreover, the proposed model does not address how the Ras-ERK module is regulated during transient *vs.* sustained signaling.

#### 2.3.1. RasGRP1 is required for transient and sustained ERK activation

Experimental evidence suggests that RasGRP1 is the crucial activator of Ras in T cells (see 1.4.1). This has been demonstrated in different systems including Jurkat T cells and RasGRP1$^{-/-}$ thymocytes (Roose et al, 2007; Stone, 2011; Kortum et al, 2013). Indeed, RasGRP1-deficient T cells have a severe block in Ras-ERK activation (Priatel et al, 2002; Roose et al, 2007; Kortum et al, 2012). However, the importance of RasGRP1 for Ras activation in mature human T cells has not yet been demonstrated. Therefore, I used primary human T cells as model system. To investigate the role of RasGRP1 in these cells, I performed RNAi (RNA interference) to suppress RasGRP1 expression. Peripheral human T cells were transfected with RasGRP1-specific short interfering RNA (siRNA) or a non-

relevant siRNA duplex (control) and stimulated with sAbs or iAbs (Fig. 2.7). Western blot analysis revealed that ERK1/2 phosphorylation upon suppression of RasGRP1 expression was diminished in comparison to controls under both transient and sustained signaling (Fig. 2.7a and 2.7b). These data demonstrated the importance of RasGRP1 for Ras-ERK activation also in primary human T cells. Moreover, my experiments demonstrate for the first time that RasGRP1 is required for both transient and sustained ERK activation.

The observed reduction in ERK1/2 phosphorylation indicates that RasGRP1 downregulation might also have an impact on T-cell activation. Thus, I investigated if T-cell activation and proliferation were affected upon suppression of RasGRP1 expression. I found that both CD69 upregulation (a readout for T-cell activation) and proliferation were significantly reduced in T cells expressing low RasGRP1 levels (Fig. 2.7c and 2.7d). These findings further corroborate the assumption that RasGRP1 is crucial for T-cell activation.

Nevertheless, despite the fact that I was able to achieve efficient suppression of RasGRP1 expression, the block in ERK1/2 phosphorylation and T-cell activation was not complete. This would suggest that other factors, such as Sos, are are involved in the regulation of the Ras-ERK cascade.

### 2.3.2. Sos1 is dispensable for transient but required for sustained ERK activation

The results presented above (see 2.3.1) suggest the involvement of other factors in the activation of the Ras-ERK pathway. The obvious candidate, which in addition to RasGRP1 may be involved in Ras activation, is Sos1. Therefore, I assessed the role of Sos1 in the regulation of Ras-ERK activation, using a similar experimental approach as for RasGRP1 (Fig. 2.8). Surprisingly, I discovered that suppression of Sos1 expression does not affect ERK1/2 phosphorylation upon sAbs stimulation (Fig. 2.8a). In marked contrast, upon iAbs treatment ERK activation was significantly attenuated in T cells were Sos1 expression was suppressed (Fig. 2.8b). Importantly, the decreased ERK1/2 phosphorylation in Sos1 knock-down cells was more apparent at later stages of T-cell activation (Fig. 2.8b, lower panel). This observation suggests that Sos1 is required for the sustained activation of the Ras-ERK cascade, whereas it is dispensable for transient ERK signaling.

Next, I assessed whether Sos1-mediated reduction in sustained ERK activation translates into decreased T-cell activation. To this end, I measured expression of CD69 and proliferation in Sos1-low cells. As shown in figure 2.8c and 2.8d, both parameters of T-cell activation were significantly affected.

Collectively, these data suggest that, similar to RasGRP1, Sos1 is required for ERK phosphorylation and T-cell activation upon iAbs stimulation. This is an important finding of my studies as I was able to show for the first time that Sos1 is necessary for the regulation of ERK activity in human primary T cells.

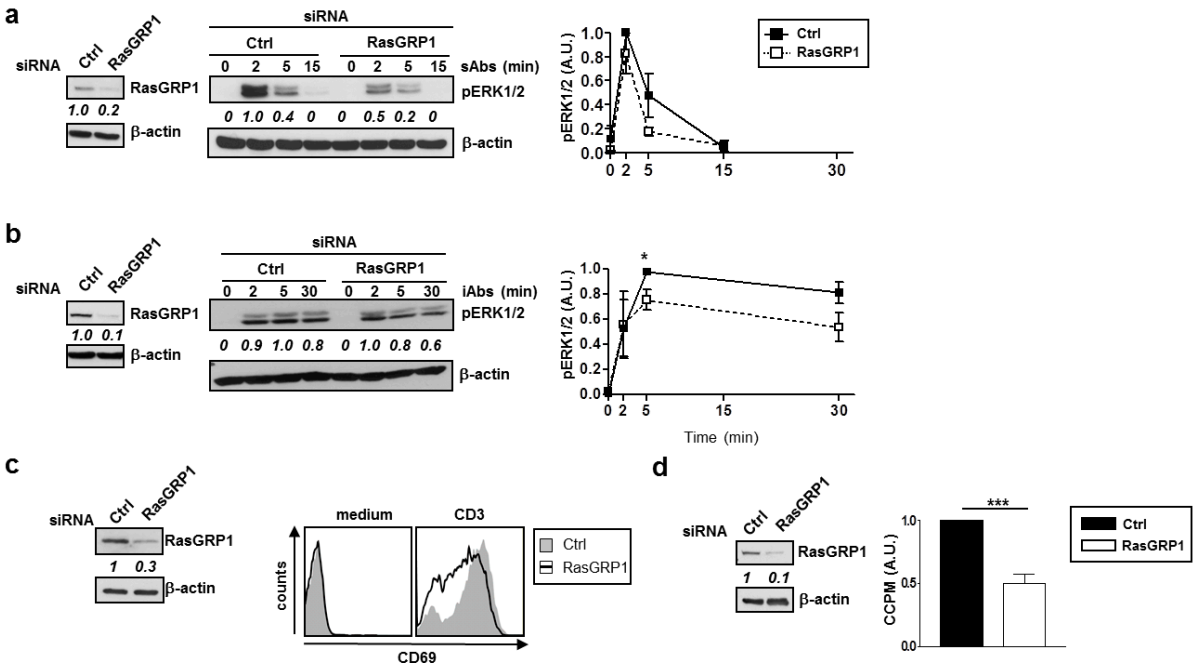


**Figure 2.7. RasGRP1 is required for both transient and sustained ERK activation.** Peripheral human T cells were transfected with control (Ctrl) or RasGRP1-specific siRNA and cultured for 72 h. Subsequently, cells were stimulated with either sAbs (a) or iAbs (b) for the indicated time periods. Cell lysates were analyzed by immunoblotting using the indicated Abs. Bands in (a) and (b) were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in (a) and (b) show the phosphorylation levels of ERK1/2 as arbitrary units ± SEM of at least three experiments. (c) Transfected human T cells were treated as indicated. 3 h after stimulation, CD69 expression was analyzed by flow cytometry. One representative experiment of four is shown. (d) T cells were transfected with siRNA duplexes and stimulated with plate-bound CD3. After 2 days, cells were pulsed with [$^3$H]-thymidine and processed for standard scintillation counting. Graph shows proliferation expressed as arbitrary units ± S.E.M. of RasGRP1-low T cells compared to control from three independent experiments. For (a), (b), (c), and (d) immunoblots verifying RasGRP1 downregulation are shown. Numbers below the bands indicate expression levels or fold induction of phosphorylation compared to controls (figures 2.7a, b, and c were adopted from Poltorak et al, 2014; figure 2.7d was modified from Warnecke et al, 2012).

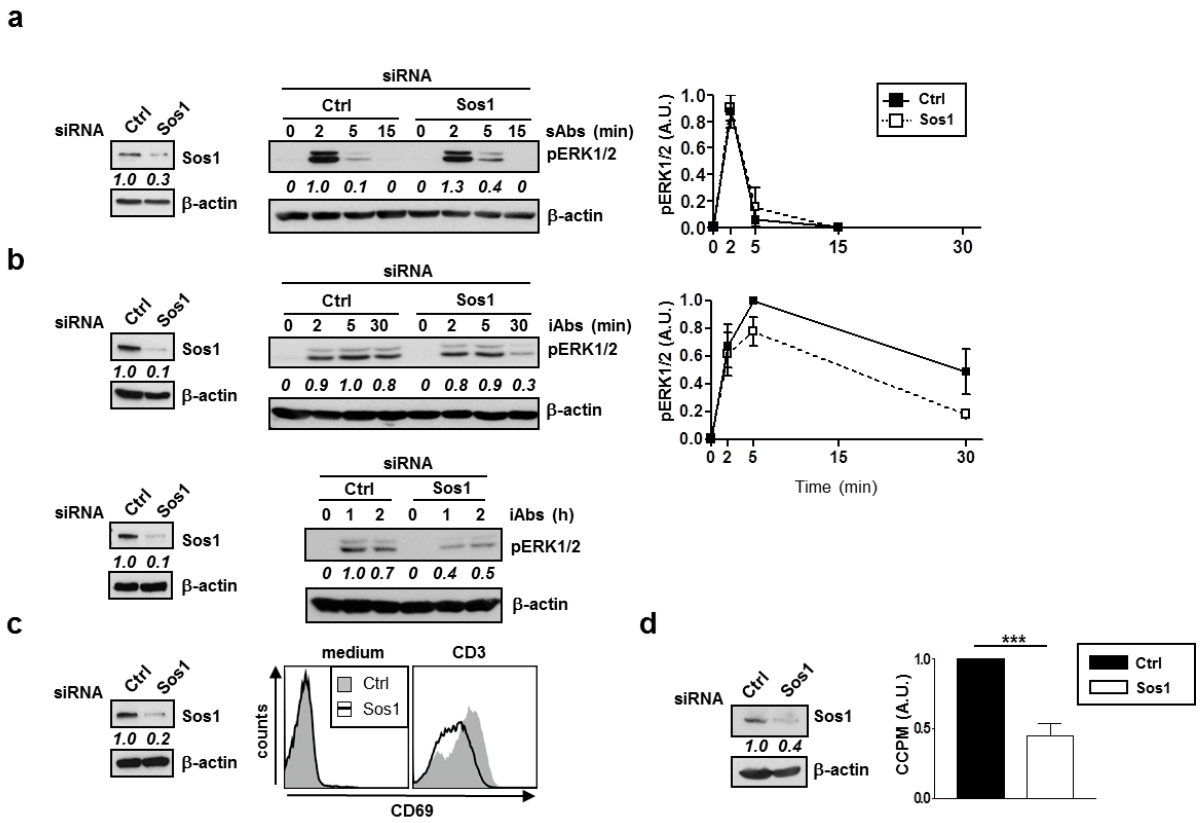


**Figure 2.8. Sos1 is dispensable for transient, but required for sustained ERK activation.** Peripheral human T cells were transfected with control (Ctrl) or Sos1-specific siRNA and cultured for 72 h. Subsequently, cells were stimulated with either sAbs (a) or iAbs (b) for the indicated time periods. Cell lysates were analyzed by immunoblotting using the indicated Abs. Bands in (a) and (b) were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in (a) and (b) show the phosphorylation levels of ERK1/2 as arbitrary units ± SEM of at least three experiments. (c) Transfected human T cells were treated as indicated. 3 h after stimulation, CD69 expression was analyzed by flow cytometry. One representative experiment of four is shown. (d) T cells were transfected with siRNA duplexes and stimulated with plate-bound CD3. After 2 days, cells were pulsed with [$^{3}$H]-thymidine and processed for standard scintillation counting. Graph shows proliferation expressed as arbitrary units ± S.E.M. of Sos1-low T cells compared to control from four independent experiments. For (a), (b), (c), and (d) immunoblots verifying Sos1 downregulation are shown. Numbers below the bands indicate expression levels or fold induction of phosphorylation compared to controls (figures 2.8a, b, and c were adopted from Poltorak et al, 2014; figure 2.8d was modified from Warnecke et al, 2012).

### 2.3.3. Sos2 is dispensable for both transient and sustained ERK activation

T cells express also Sos2, a homologous of Sos1. Therefore, it is possible that loss of Sos1 can be, at least partially, compensated by the presence of Sos2. In order to analyze whether Sos1 and Sos2 possess redundant functions, I suppressed Sos2 expression using RNAi and measured ERK1/2 phosphorylation in T cells stimulated with either sAbs or iAbs, as described above. An efficient suppression of Sos2 has no effect on ERK activation upon both transient and sustained signaling (Fig. 2.9a and 2.9b).

To exclude that the influence of Sos2 on the activation of ERK1/2 might be masked by the dominant role of Sos1 in cells expressing low Sos2 levels, I performed simultaneous suppression of both Sos1 and Sos2 in human peripheral T cells (Fig. 2.9c). T cells in which the expression of both Sos proteins was suppressed displayed similar levels of ERK1/2 phosphorylation as single Sos1 knockdowns (Fig. 2.7b, 2.9c, and Warnecke et al, 2012). These results suggest that Sos2 is dispensable for Ras-ERK activation in TCR-mediated signaling.

### 2.3.4. Grb2 is dispensable for both transient and sustained ERK activation

Sos1 is constitutively associated with Grb2, which is required for the recruitment of Sos to the LAT signalosome (see 1.4.1). To test the role of Grb2 in TCR-mediated ERK activation, I suppressed Grb2 expression using RNAi (Fig. 2.10). Surprisingly, suppression of Grb2 expression did not influence ERK activation regardless of the type of stimulation (Fig.2.10a, 2.10b, and Warnecke et al, 2012). Although unexpected, these data were in line with previous observations made in Grb2-deficient mice. In fact, in these mice the loss of Grb2 had no effect on the activation of either Ras or ERK1/2 in thymocytes (Jang et al, 2010). Thus, the data suggests that these two molecules may have independent and non-redundant functions (Kortum et al, 2013).

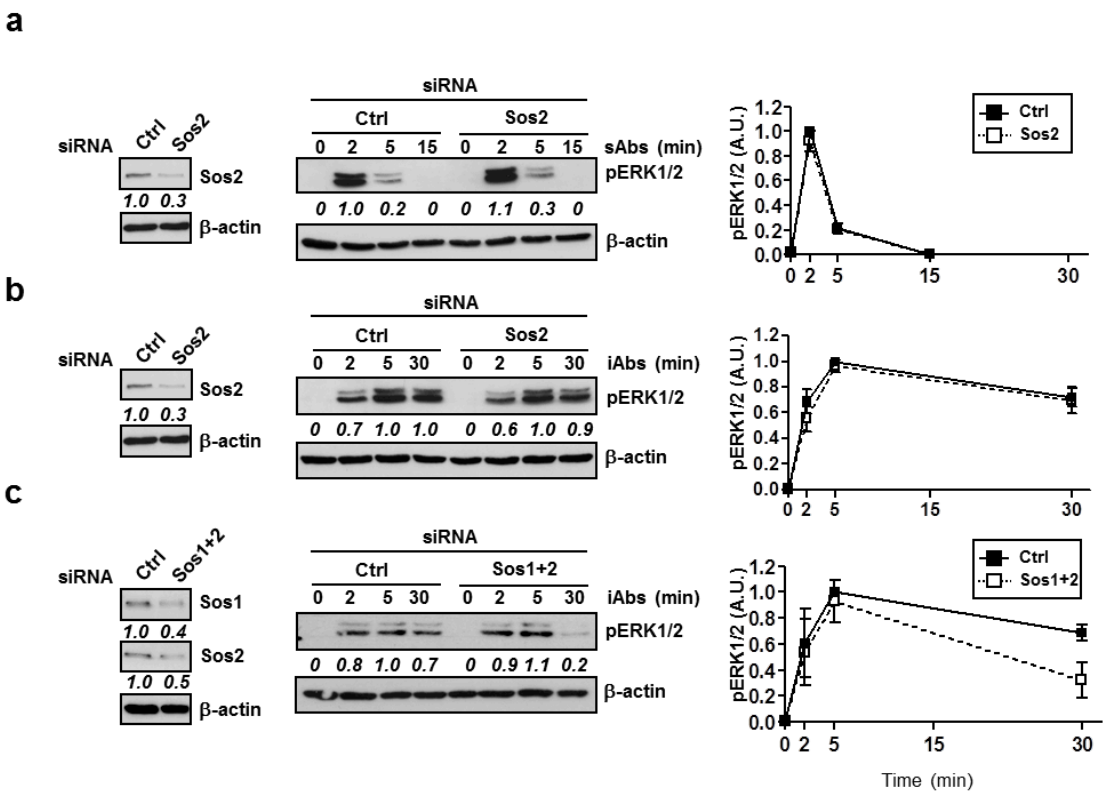


**Figure 2.9. Sos2 is dispensable for both transient and sustained ERK activation.** Peripheral human T cells were transfected with control (Ctrl), Sos2-specific or Sos1- and Sos2-specific siRNA and cultured for 72 h. Subsequently, cells were stimulated with either sAbs (a) or iAbs (b and c) for the indicated time periods. Cell lysates were analyzed by immunoblotting using the indicated Abs. Bands in (a), (b), and (c) were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in (a), (b), and (c) show the phosphorylation levels of ERK1/2 as arbitrary units ± SEM of at least three independent experiments. For (a), (b), and (c) immunoblots verifying Sos1 and Sos2 downregulation are shown. Numbers below the bands indicate expression levels or fold induction of phosphorylation compared to controls (the figure 2.9 was adopted from Poltorak et al, 2014).

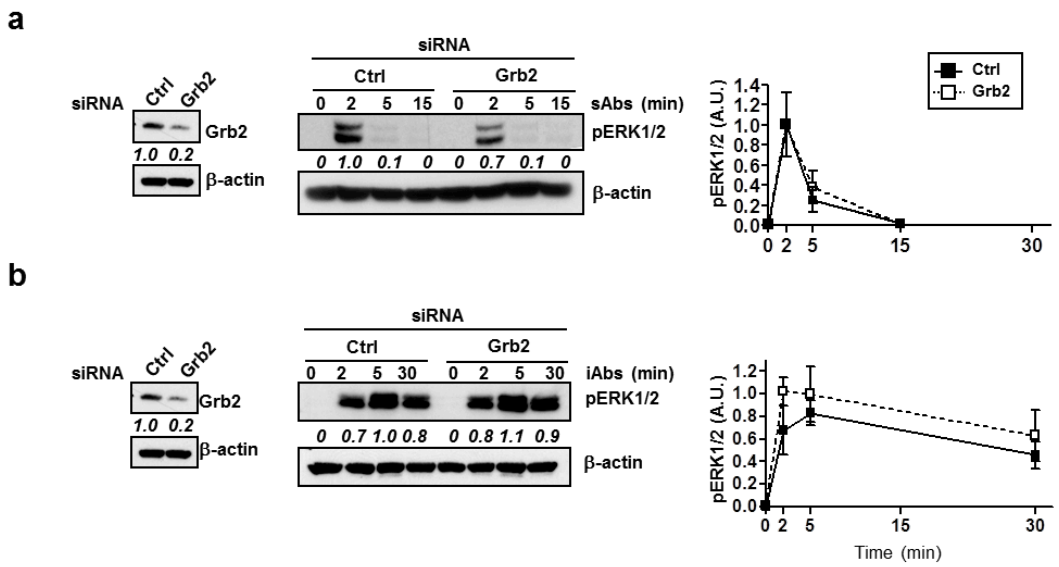


**Figure 2.10. Grb2 is dispensable for both transient and sustained ERK activation.** Peripheral human T cells were transfected with control (Ctrl) or Grb2-specific siRNA and cultured for 72 h. Subsequently, cells were stimulated with either sAbs (a) or iAbs (b) for the indicated time periods. Cell lysates were analyzed by immunoblotting using the indicated Abs. Bands in (a) and (b) were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Graphs in (a) and (b) show the phosphorylation levels of ERK1/2 as arbitrary units ± SEM of at least four experiments. For (a) and (b) immunoblots verifying Grb2 downregulation are shown. Numbers below the bands indicate expression levels or fold induction of phosphorylation compared to controls.

## 3. Discussion

### 3.1. Differential regulation of proximal signaling in transient *vs.* sustained T-cell activation

Ligation of the TCR generates a signal which will be ultimately translated into a specific cellular response. In my study, I performed a detailed comparison of the signaling dynamics correlating with two different cellular outcomes induced upon TCR stimulation in primary human T cells. I observed marked differences in the molecular regulation of an anergic-like state induced by monoclonal antibodies in solution (sAbs) *vs.* proliferation triggered by antibodies immobilized on microbeads (iAbs). My analyses indicate that the anergic-like state corresponds with transient signaling, whereas proliferation correlates with sustained activation. These two different cellular responses and signaling dynamics likely originate from the activation of distinct regulatory feedback loops.

These two distinct types of stimulation induce qualitative differences already in the apical part of the TCR signaling cascade. Lck appears to be a key factor in the regulation of signaling dynamics and cellular outcomes. I propose that Lck can function as a "signal splitter", a molecule which senses and discriminates the signal emanating from the TCR (Neilson et al, 2004). The existence of a "signal splitter" in the apical part of the cascade could be beneficial for the cell because it could decipher the nature of the signal induced by a particular ligand almost instantly, thus requiring only a limited number of molecules regulating downstream signaling. In another study LAT has been proposed as a protein directing T-cell responses via modulating Lck activation (Dong et al, 2010). Dong et al., suggested that the active pool of Lck in the proximity of the TCR is controlled by LAT and that LAT promotes T-cell activation induced by TCR/MHC-peptide interactions of low affinity (Dong et al, 2010). Although we and others agree that Lck is a decisive factor positioned in the epicenter of T-cell activation, whether it is a "signal splitter" is still a matter of debate.

In my experiments, transient signaling corresponded with substantial phosphorylation of Lck on $Y^{394}$, the well-know activatory site (see 1.3.2). Furthermore, in the study by Stirnweiss et al., we have detected a 20% increase in Lck kinase activity upon stimulation with soluble antibodies (Stirnweiss et al, 2013). Parallel observations were made in another very recent study where the "opened"/active fraction of Lck correlated with condensation of TCR microclusters (Rossy et al, 2013). I was able to correlate the activation of Lck with augmented general tyrosine phosphorylation of cellular proteins and enhanced phosphorylation of the Lck substrates, TCRζ and ZAP70. Collectively, these data suggest that a fraction of Lck, or more generally SFKs, are *de novo* activated upon stimulation. Noteworthy, in my study only transient signaling results in the increased Lck activation, whereas iAbs stimulation does not elevate Lck activity.

I hypothesize that changes in the magnitude of Lck activation have a tremendous impact on downstream molecular events. In fact, I observed a strong phosphorylation of inhibitory molecules such as cCbl and Dok2 upon transient, but not sustained activation. Furthermore, others have reported that during transient signaling another negative regulator, the phosphatase SHP1, is activated (Methi et al, 2005). Since transient activation corresponds with a very strong activation of Lck and Fyn, it is likely that the high activity of SFKs correlates with the phosphorylation of phospho-tyrosine-dependent negative regulators. Hyperphosphoryation of inhibitory proteins may disturb the equilibrium between positive and negative regulators and therefore may lead to signal termination (Fig. 3.1, left side). Indeed, active cCbl, Dok2, and SHP1 are capable of deactivating Lck and other TCR proximal signaling molecules thereby terminating signaling (see 1.4.1).

To confirm that Lck plays a role in the activation of inhibitory feedback loops, we have suppressed Lck expression by RNA interference (Poltorak et al, 2013). Indeed, Jurkat T cells expressing low levels of Lck exhibited prolonged ERK1/2 phosphorylation upon soluble antibody stimulation in comparison to controls (Poltorak et al, 2013). Others have shown that reduced Lck expression correlated with impaired activation of cCbl as well as SHP1 and with extended activation of the transcription factors NFAT and AP1 (Methi et al, 2005; Methi et al, 2008). Therefore, fine-tuning of Lck activity appears to be crucial for the signaling dynamics and for the generation of a productive T-cell response. On the basis of these considerations, we hypothesized that by augmenting Lck activity a sustained signal can be converted into a transient one (Poltorak et al, 2013). In order to test this hypothesis, cells were stimulated with iAbs, which do not induce a substantial increase in Lck activation, and with soluble CD4. It is known that crosslinking of CD4 strongly increases Lck phosphorylation. As anticipated, enhancement of Lck phosphorylation upon CD4 cross-linking led to a reduced ERK1/2 activation, decreased CD69 expression, and reduced proliferation upon iAbs stimulation (Poltorak et al, 2013). Collectively, our data show that suppression of Lck activity (e.g. by RNAi) extends signaling, thus converting transient into sustained signaling. Conversely, increase in Lck activity terminates sustained signaling (Fig. 3.1, left side), thus resulting in the conversion from sustained to transient signal.

In stark contrast to transient signaling, sustained signaling does not dramatically increase Lck activity. Furthermore, I found that after the initial phase of T-cell activation, Lck activity is rather gradually downmodulated. *In vitro* kinase assays revealed that the ability of Lck

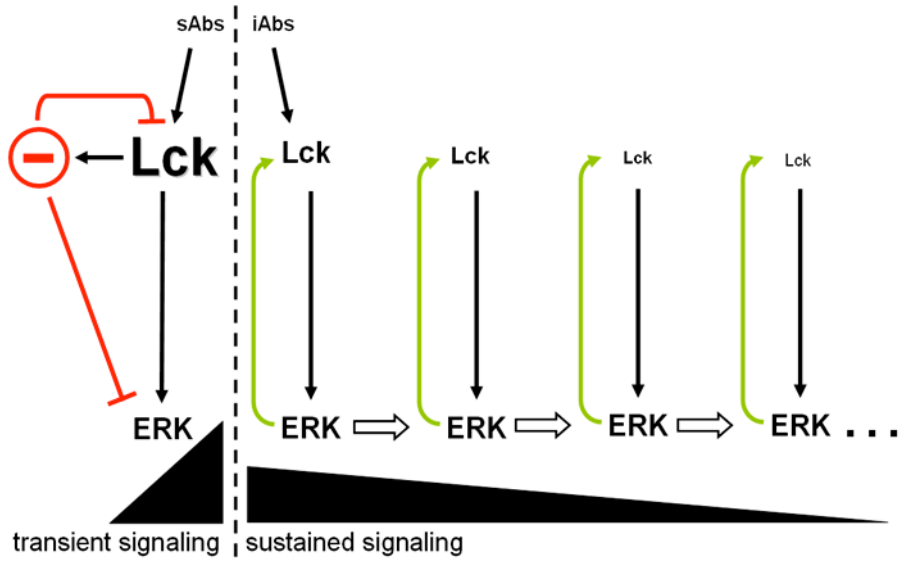


**Figure 3.1. Regulation of Lck activity and signal duration.** sAbs stimulation (left side) results in hyperactivation of Lck and activation of negative regulators concluding in rapid termination of signaling. iAbs stimulation (right side) leads to establishment of ERK-Lck feedback, which is necessary to modulate Lck activity and to sustain signaling.

to *auto*-phosphorylate was greatly reduced 60 min after stimulation. These results further corroborate the hypothesis that hyperactivation of Lck may be detrimental for T-cell activation. It is reasonable to assume that SFKs have to be kept "in check" during the course of T-cell activation. If not controlled, a slow but continuous increase in Lck activity would lead to enhanced tyrosine phosphorylation and to the activation of negative regulators (as in the case of stimulation with sAbs). Moreover, reduced Src kinase activity could increase the activation threshold and desensitize T cells, thus preventing spontaneous re-activation.

Noteworthy, in support of our data, similar observations and conclusions were made by an independent group in different experimental settings (Lee et al, 1997). Lee et al., have described that mouse $CD4^+$ $T_{h1}$ clones expressing a transgenic TCR specific for pigeon cytochrome c (3C6) stimulated for longer time periods with APCs (P13.9 cells) show decreased global and TCR-specific tyrosine phosphorylation. Biochemically, these observations correlated with increased serine phosphorylation of Lck, decreased Lck kinase activity, and loss of cytosolic Lck. Lee et al., proposed that the downregulation of Lck activity is an important regulatory mechanism necessary to prevent unwanted TCR signaling and activation that can lead to cell death (Lee et al, 1997).

These observations raise the question of how Lck activity is downmodulated during sustained signaling. I propose that the ERK-Lck positive feedback loop regulates Lck activity. I was able to show that during the course of iAbs treatment, Lck is phosphorylated on $S^{59}$ by activated ERK1/2. It has been observed by other groups that ERK can bind directly to Lck via the SH3 domain (August et al, 1996). Subsequent phosphorylation of $S^{59}$ by active ERK has been implicated in a change in the binding specificity Lck's SH2 domain, which favors the binding of a different set of proteins to Lck (Joung et al, 1995). For example, Stefanova et al., proposed that ERK-mediated phosphorylation of Lck protects Lck from binding to its negative regulator, SHP1 (Stefanova et al, 2003). Therefore, the ERK-Lck circuit may be involved in promoting positive signals, on the one hand, by preventing Lck from inactivation (e.g. by SHP1) and on the other, by allowing slow downmodulation of Lck by other regulators (Fig. 3.1, right side).

Phosphatases responsible for gradual dephosphorylation/deactivation of Lck are not yet known. However, one possible candidate is PTPN22 (also known as LYP), which forms a complex with Csk (Cloutier et al, 1996). The PTPN22-Csk complex has a dual function: PTPN22 is able to dephosphorylate $Y^{394}$ while Csk phosphorylates $Y^{505}$ of Lck (Brownlie et al, 2013). Their combined actions force the conversion of the kinase into a "closed" and inactive conformation (Vang et al, 2012). It is tempting to speculate that $S^{59}$ phosphorylation and subsequent alteration in the binding properties of the SH2 domain may favor the interaction of PTPN22 with Lck over SHP1 leading to gradual dephosphorylation instead of a rapid inactivation. Low active, but not hyperactive, Lck is then able to sufficiently phosphorylate its

substrates for continuous signaling. In line with this hypothesis, PTPN22$^{-/-}$ mice have an augmented SFK signaling in effector cells and increased tendency to develop autoimmune inflammatory diseases (Hasegawa et al; 2004; Brownlie et al, 2012). Furthermore, R620W polymorphism of PTPN22 in humans has been associated with high risk for autoimmune diseases (Bottini et al, 2006). Nevertheless, the role of the PTPN22-Csk complex or other phosphatases in Lck inactivation during iAbs treatment requires further investigation.

In summary, I was able to show that transient activation results in abnormal increase in activity of Src kinases and consequent strong phosphorylation of negative regulators of TCR signaling leading to premature termination of the T-cell activation signal (Fig. 3.1, left side). Phosphorylation of cCbl, Dok2, and probably other negative regulators, appears to be a fail-safe mechanism to prevent improper activation of T cells. On the other hand, sustained signaling results in the activation of an ERK-Lck positive feedback loop which changes the binding specificity of Lck thereby protecting Lck from inactivation (e.g. by SHP1) and promotes a slow and subtle downmodulation of its activity (likely by PTPN22) (Fig. 3.1, right side). Thus, activation of the ERK-Lck loop appears to be necessary to support continuous signaling and to induce productive T-cell response. Nevertheless, in both types of activation, Lck plays a crucial role in regulating signals at the very apical part of the TCR cascade.

### 3.2. Regulation of the Ras-ERK cascade differs in transient *vs.* sustained T-cell activation

Differences in the activation of proximal signaling molecules are translated into differential activation of downstream pathways. I focused my attention on the Ras-ERK cascade which plays an important role in T-cell development and activation (Kortum et al, 2013). Regulation of the Ras-ERK pathway has an enormous therapeutic potential because perturbations in the activation of this cascade have been described in many pathological conditions (Kortum et al, 2013). Ras-ERK is one of the central cascades because many signals propagated from different receptors converge in the activation of Ras and ERK. How cells are able to distinguish these signals and respond to them accordingly is still a matter of debate. It seems that the mode of ERK activation (e.g. magnitude and duration) can be interpreted and translated into different functional outcomes (Murphy et al, 2004: Ebisuya et al, 2005; von Kriegsheim et al, 2009). Therefore, changes in the strength or kinetics of Ras-ERK signaling may result in different T-cell responses, like positive and negative selection in the thymus (see 1.4.2) (Daniels et al, 2006). Taking these facts into account, the understanding of the regulation of Ras and ERK is of great importance.

Hence, I decided to investigate how the Ras-ERK cascade is activated under transient or sustained TCR signaling in human peripheral T cells. I focused my studies on two well-know activators of Ras, the lymphoid-specific RasGRP1 and ubiquitously expressed Sos1. My aim

was to validate a widely accepted model proposing an interplay between RasGRP1 and Sos1, which is required to prime Sos1 by RasGRP1-produced Ras (RasGTP-Sos1 positive feedback loop) (see 1.4.1) (Roose et al, 2007; Das et al, 2009).

First, I investigated the role of RasGRP1 in the activation of the Ras-ERK cascade in primary human T cells. I demonstrated that RasGRP1 is required for both transient and sustained ERK1/2 phosphorylation (Fig. 3.2) as well as for CD69 expression and T-cell proliferation. These results are in line with the data from RasGRP1$^{-/-}$ mice, where reduction in ERK signaling was reported in DP thymocytes (Kortum et al, 2012) and CD8$^{+}$ T cells (Priatel et al, 2010). Also a similar effect on TCR-mediated ERK activation was observed in RasGRP1-deficient Jurkat T cells (Roose et al, 2005; Roose et al, 2007).

Next, I analyzed the impact of Sos1, and of its homologue Sos2, on the phosphorylation of ERK1/2 upon transient or sustained activation. Suppression of Sos1 and/or Sos2 by RNA interference did not affect ERK activation in cells stimulated with sAbs. Also in Sos1$^{-/-}$ mice, the loss of Sos1 led to a very modest (10 - 15%) reduction in ERK1/2 phosphorylation in mature T cells after soluble anti-CD3 stimulation (Kortum et al, 2012). However, iAbs stimulation of human T cells, in which Sos1 expression was suppressed, significantly affected ERK activation. Noteworthy, the reduction in ERK1/2 phosphorylation was more pronounced at later time-points of T-cell activation (30 min and longer). The reduced ERK activation corresponded with a reduction in the expression of early activation genes (i.e. CD69) and decreased proliferation. Importantly, Sos1 appeared to play a role only in sustained ERK activation in mature T cells. This observation would support the model, where RasGRP1 is required for the initiation of the Ras-ERK pathway and for priming of Sos1. Subsequently, triggered Sos1 can contribute to prolongation of the ERK signaling (Fig. 3.2, right side). Nevertheless, the cooperation between RasGRP1 and Sos1 in primary cells has still to be demonstrated. In a recently proposed model, the relative expression levels of RasGRP1 and Sos1 determine their participation in thymic selection (Kortum et al, 2013). At the DN3 stage, Sos1 is expressed at much higher level than RasGRP1, hence Ras is preferentially activated via Sos1. Here, RasGRP1 acts as a fail-safe mechanism to ensure transition of sufficient numbers of DN thymocytes to the DP stage in case of Sos1 malfunction (Kortum et al, 2013). At the TCR checkpoint (DP stage), RasGRP1 expression increases up to five-fold, whereas Sos1 expression decreases about five-fold (Dower et al, 2000; Prasad et al, 2009; Kortum et al, 2011). Therefore, positive selection mainly depends on RasGRP1-mediated Ras activation and only in negative selection, which requires full Ras-ERK activation, both RasGRP1 and Sos1 are needed (Kortum et al, 2013). Since the ratio between RasGRP1 and Sos1 does not change dramatically from DP thymocytes to SP T cells, ERK activation in mature cells also depends preferentially on RasGRP1, whereas Sos1 is required to sustain the Ras signal.

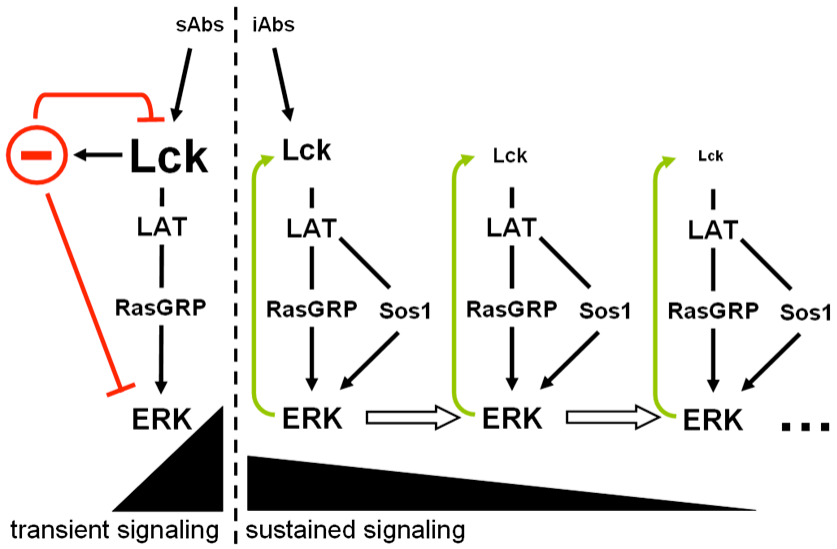


**Figure 3.2. Regulation of the Ras-ERK cascade during transient and sustained signaling.** sAbs stimulation (left side) results in activation of ERK solely via RasGRP1, whereas iAbs stimulation (right side) leads to combined actions of RasGRP1 and Sos1 to sustain ERK phosphorylation. Sustained ERK activation triggers ERK-Lck positive feedback (right side) which is necessary for further support of the continuous signaling.

In summary, data generated by us and others suggest a more elaborate and dynamic interplay between these two GEFs than initially predicted.

In contrast to Sos1, Sos2 downregulation does not influence ERK upon either transient or sustained activation. Additionally, combined suppression of Sos1 and Sos2 expression does not further affect ERK1/2 phosphorylation compared to suppression of Sos1 alone. Comparable observations were made in $Sos2^{-/-}$ and $Sos1/2^{-/-}$ mice, where deletion of Sos2 did not further affect ERK activation and T-cell development (Kortum et al, 2012). Therefore, it appears that Sos2 is dispensable for ERK activation and T-cell development.

I also explored the function of Grb2 in the activation of Ras-ERK. Grb2 is required to bring Sos to the plasma membrane. However, I was not able to show any effect on ERK1/2 phosphorylation upon suppression of Grb2 expression. My results are in agreement with the data obtained from T-cell specific conditional Grb2 knock-out mice. In fact, the activation of both Ras and ERK is unaffected in these mice. It has to be taken into consideration that Grb2 interacts with many molecules such as SHIP1, RasGAP, Gab2, Themis (thymocyte-expressed molecule involved in selection), and cCbl, which may have an inhibitory effect on the Ras-ERK cascade (see 1.4.1) (Yamasaki et al, 2003; Dong et al, 2006; Fu et al, 2009; Smith-Garvin et al, 2009; Gascoigne et al, 2011; Paster et al, 2013). In fact, $Sos1^{-/-}$ and $Grb2^{-/-}$ mice have different phenotypes. Grb2 knock-outs have defective positive and negative selection whereas positive and negative selections are normal in Sos1-deficient mice (see Table 1.1). Data from Grb2-deficient thymocytes further suggest that Grb2 may play an unexpected role in the activation of Src kinases. However, I didn't observe any effect on the tyrosine phosphorylation pattern in primary human T cells upon the suppression of Grb2. Thus, Grb2 may play a different role in mature *vs.* immature T cells.

In addition to the TCR, it has been shown that the Grb2/Sos1 complex is involved in the regulation of the Ras-ERK cascade downstream of other receptors, such as the IL-2R (see 1.3.4). In IL-2R-mediated signaling, both STAT and ERK pathway are activated. Activation of STATs depends on JAK kinases, whereas activation of ERK requires the Grb2 and Sos1 (Beyer et al, 2011). We have shown that downregulation of both Grb2 and Sos in T-cell blasts resulted in attenuated ERK1/2 phosphorylation (Warnecke et al, 2012). In contrast, suppression of RasGRP1 had no impact on ERK1/2 activation downstream of the IL-2-R (Priatel et al, 2010, Warnecke et al, 2012).

Thus, Grb2 appears to be required for the activation of the Ras-ERK cascade downstream of the IL-2R, but not of the TCR. On the other hand, Sos1 appears to be crucial for both TCR- and IL-2R-mediated ERK activation. Conversely, RasGRP1 activates Ras-ERK downstream of the TCR, but not downstream the IL-2-R (Table 3.1). Regardless of the receptor, all three proteins are necessary for proper ERK-dependent T-cell activation as the loss of one of them impairs proliferation (Poltorak et al, 2013).

| Molecule | TCR-mediated transient ERK activation | TCR-mediated sustained ERK activation | IL-2R-mediated ERK activation |
|---|---|---|---|
| RasGRP1 | required | required | dispensable |
| Sos1 | dispensable | required | required |
| Sos2 | dispensable | dispensable | dispensable |
| Grb2 | dispensable | dispensable | required |

**Table 3.1. Importance of RasGRP1, Sos1/2, and Grb2 in ERK activation.**

It is important to mention that, despite the fact that RasGRP1 and Sos1 are crucial for Ras activation, other mechanisms to activate Ras may exist in T cells. Recently, it has been reported that the B-cell adaptor molecule of 32 kDa (Bam32) activates ERK in T cells in a Ras-independent manner (Sommers et al, 2008; Rouquette-Jazdanian et al, 2012). Study shows that a Bam32-PLCγ1-Pak1 complex activates ERK via Pak1-mediated phosphorylation of Raf1 and MEK (Rouquette-Jazdanian et al, 2012). Furthermore, in LAT-Y136F knock-in mice, a PLCγ1-independent Lck-PKCθ-RasGRP1-Ras pathway has been also described (Kortum et al, 2013). When the LAT binding site for PLCγ1 is mutated, RasGRP1 is presumably phosphorylated by PKCθ.

Collectively, I was able to show for the first time that activation and regulation of the Ras-ERK cascade in primary human T cells depends on cooperative actions of RasGRP1 and Sos1 downstream of the TCR (Fig. 3.2). However, it appears that either during different stages of thymic development or upon different stimulatory conditions, the importance of RasGRP1 and Sos1 may vary. Moreover, recent developments in the field suggest that other pathways can also contribute into the Ras-ERK activation. Thus, interactions between RasGRP1 and Sos1 have proven to be very dynamic, complex, and until now are not fully resolved.

## 4. Materials and Methods

### 4.1. Ethics

Approval for these studies involving the analysis of TCR-mediated signaling in primary human T cells was obtained from the Ethics Committee of the Medical Faculty at the Otto-von-Guericke University, Magdeburg, Germany with the permission number [107/09]. Informed consent was obtained in writing in accordance with the Declaration of Helsinki.

### 4.2. Materials

#### 4.2.1. Reagents and recipes

Human culture medium

RPMI 1640 liquid medium with $NaHCO_3$ and stable glutamine (Biochrom)

10% FCS (PAN Biotech)

2 µg/ml Ciprobay (Bayer Schering Pharma)

Cell-lines culture medium

RPMI 1640 liquid medium with $NaHCO_3$ and stable glutamine (Biochrom)

10% FCS (PAN Biotech)

100 U/ml of Penicilin/Streptavidin (Biochrom)

Standard lysis buffer

1% lauryl maltoside (N-dodecyl β-maltoside) (Calbiochem)

1% IGEPAL CA-630 (Sigma Aldrich)

1 mM $Na_3VO_4$ (Sigma Aldrich)

1 mM PMSF (Sigma Aldrich)

10 mM NaF (Sigma Aldrich)

10 mM EDTA (Roth)

50 mM Tris pH 7.5 (Roth)

150 mM NaCl (Roth)

Mild lysis buffer

1% Brij58 (Thermo Scientific)

1% IGEPAL CA-630 (Sigma Aldrich)

1 mM $Na_3VO_4$ (Sigma Aldrich)

1 mM PMSF (Sigma Aldrich)

10 mM NaF (Sigma Aldrich)

10 mM EDTA (Roth)

50 mM Tris pH 7.5 (Roth)

150 mM NaCl (Roth)

5 x Reducing sample buffer

50% glycerol (Merck)

330 mM Tris pH 6.8 (Roth)

10% SDS (Serva)

0.01% bromphenolblue (Roth)

10% 2-mercaptoethanol (Merck)

SDS-PAGE resolving 7.5% gel

7,2 ml $ddH_2O$

3,8 ml 30% Acrylamid (Bio-Rad)

3,8 ml 1,5 M Tris pH 8.8 (Roth)

150 µl 10% SDS (Serva)

150 µl 10% APS (Roth)

15 µl TEMED (Roth)

SDS-PAGE resolving 10% gel

4,5 ml $ddH_2O$

3,8 ml 30% Acrylamid (Bio-Rad)

2,9 ml 1,5 M Tris pH 8.8 (Roth)

113 µl 10% SDS (Serva)

113 µl 10% APS (Roth)

11 µl TEMED (Roth)

SDS-PAGE resolving 12% gel

3,5 ml $ddH_2O$

3,8 ml 30% Acrylamid (Bio-Rad)

2,5 ml 1,5 M Tris pH 8.8 (Roth)

100 µl 10% SDS (Serva)

50 µl 10% APS (Roth)

5 µl TEMED (Roth)

SDS-PAGE stacking gel

2,1 ml $ddH_2O$

0,6 ml 30% Acrylamid (Bio-Rad)

0,9 ml 1,5 M Tris pH 8.8 (Roth)

38 µl 10% SDS (Serva)
38 µl 10% APS (Roth)
4 µl TEMED (Roth)

Running Buffer
25 mM Tris pH 8.3 (Roth)
192 mM glycine (Roth)
0.1% SDS (Serva)

Blotting Buffer
39 mM glycine (Roth)
48 mM Tris (Roth)
0.037% SDS (Serva)
20% methanol (Merck)

TBS Buffer
10 mM Tris pH 7.5 (Roth)
150 mM NaCl (Roth)

Blocking Buffer
10 mM Tris pH 7.5 (Roth)
150 mM NaCl (Roth)
5% Milk (Roth)

Washing Buffer
20 mM Tris pH 7.5 (Roth)
150 mM NaCl (Roth)
0.02% Tween 20 (Roth)

IP Washing Buffer
0.1% lauryl maltoside (N-dodecyl β-maltoside) (Calbiochem)
0.1% IGEPAL CA-630 (Sigma Aldrich)
1 mM PMSF (Sigma Aldrich)
50 mM Tris pH 7.4 (Roth)
10 nM NaF (Sigma Aldrich)
0.16 M NaCl (Roth)

Kinase Buffer

50 mM Tris pH 7.4 (Roth)

10 mM $MnCl_2$ (Sigma Aldrich)

0.1% IGEPAL CA-630 (Sigma Aldrich)

10 µCi [$\gamma$-$^{32}$P] ATP (Perkin Elmer)

### 4.2.2. siRNA sequences

Grb2

5' – CAU GUU UCC CCG CAA UUA UTT – 3'

3' – AUA AUU GCG GGG AAA CAU GTT – 5'

RasGRP1

5' – GGG UGA GGA GUU ACA UUG CTT – 3'

3' – GCA AUG UAA CUC CUC ACC CTT – 5'

5' – CAG CCC AGG AUA CUC UAU AUG UGC U – 3'

3' – AGC ACA UAU AGA GUA UCC UGG GCU G – 5'

Renilla Luciferase (siRNA negative control)

5' – CCA AGU AAU GUA GGA UCA ATT – 3'

3' – UUG AUC CUA CAU UAC UUG GTT – 5'

Sos1

5' – UUG CCC AUU UAU CAA UUG GTT – 3'

3' – CCA AUU GAU AAA UGG GCA ATT – 5'

Sos2

5' – UCA GCU AAU GAA GAG UCU CUC UAU U – 3'

3' – AAU AGA GAG ACU CUU CAU UAG CUG A – 5'

All siRNA duplexes were purchased from Invitrogen (Life Technologies) and used according to manufacturer's recommendations.

### 4.2.3. Antibodies

Antibodies used for stimulation

Biotin conjugated mouse anti-human CD28 (clone CD28.2, eBioscience)

Biotin conjugated mouse anti-human CD3ε (clone UCHT1, eBioscience)

Biotin conjugated mouse anti-human CD4 (clone OKT4, eBioscience)

Biotin conjugated mouse $IgG_1$ K isotype control (clone P3.6.2.8.1, eBioscience)

Biotin conjugated mouse $IgG_{2a}$ K isotype control (clone eBM2a, eBioscience)

Mouse anti-human CD3ε MEM92 hybridoma supernatants (kindly provided by Vaclav Horejsi, Academy of Sciences of the Czech Republic, Prague, Czech Republic)

Antibodies used for immunoblotting

anti-β-actin (clone AC-15, Sigma-Aldrich)

anti-ERK1/2 (#V1141, Promega)

anti-Fyn (clone Fyn01, kindly provided by Vaclav Horejsi, Academy of Sciences of the Czech Republic, Prague, Czech Republic)

anti-Grb2 (clone C-23, Santa Cruz Biotechnology)

anti-LAT (#06-807, Milipore)

anti-Lck (clone 28/Lck, BD Biosciences)

anti-Lck (clone Y123, Epitomics)

anti-phosphotyrosine HRP conjugate (clone 4G10, Millipore)

anti-phospho(p)$S^{217/221}$ MEK1/2 (clone 41G9, Cell Signaling Technology)

anti-$pS^{338}$ cRaf (clone 56A6, Cell Signaling Technology)

anti-$pT^{202}/Y^{204}$ ERK1/2 (#9101, Cell Signaling Technology)

anti-$pY^{171}$ LAT (#3584, Cell Signaling Technology)

anti-$pY^{351}$ Dok2 (#3911, Cell Signaling Technology)

anti-$pY^{416}$ Src (#2101, Cell Signaling Technology)

anti-$pY^{493}$ ZAP70 (#2704. Cell Signaling Technology)

anti-$pY^{731}$ cCbl (#3554, Cell Signaling Technology)

anti-$pY^{783}$ PLCγ1 (#2821, Cell Signaling Technology)

anti-RasGRP1 (kindly provided by James C. Stone, University of Alberta, Edmonton, Canada)

anti-Sos1 (clone C-23, Santa Cruz Biotechnology)

anti-Sos2 (clone C-19, Santa Cruz Biotechnology)

anti-ZAP70 (clone 29/ZAP70, BD Biosciences)

Each antibody was diluted in TBS buffer (see 4.2.1) supplemented with 5% BSA (Sigma Aldrich) or 5% Milk (Roth) according to manufacturer's recommendation.

Antibodies used for immunoprecipitation

agarose-conjugated anti-CD3ζ (clone 6B10.2, Santa Cruz Biotechnology)

anti-Lck (clone 3A5, Santa Cruz Biotechnology)

anti-Lck (clone 3A5, Milipore)

normal goat control IgG (Santa Cruz Biotechnology)

normal rabbit control IgG (Santa Cruz Biotechnology)

Each antibody was added into cell lysate in the presence of Protein A or G agarose beads (Santa Cruz Biotechnology) and 2% of BSA (Sigma Aldrich) in the concentration recommended by manufacturer.

Antibodies used for flow cytometry

APC conjugated anti-human CD69 (clone FN50, BD Biosciences)

FITC conjugated anti-human CD25 (clone M-A25, BD Biosciences)

For cell staining antibodies were diluted in PBS (Biochrom) in the concentration recommended by the manufacturer.

### 4.3. Methods

#### 4.3.1. Human T-cell purification and culture

Peripheral blood mononuclear cells were isolated by Ficoll gradient (Biochrom) centrifugation of heparinized blood collected from healthy volunteers. Total population of human T cells or $CD4^+$ subpopulation were further purified by non-T cell depletion using T-cell isolation kits and AutoMacs magnetic separation system (all from Miltenyi Biotec). The purity of T cells, determined by flow cytometry, was usually more than 96%. After isolation, T cells were cultured at 37°C and 5% $CO_2$ in human culture medium (see 4.2.1).

#### 4.3.2. T-cell line culture

The Lck-deficient variant of the Jurkats (J.CaM1.6) were maintained at 37°C and 5% $CO_2$ in cell-lines culture medium (see 4.2.1).

#### 4.3.3. T-cell transfection

For protein downregulation in human primary T cells or T-cell blasts siRNA duplexes purchased from Invitrogen (Life Technologies) were used (see 4.2.2). 8 x $10^6$ cells were washed with PBS containing $Mg^{++}$ and $Ca^{++}$ ions (BIochrom), resuspended in 200 µl of Opti-MEM transfection medium (Life Technologies), and transferred into transfection cuvette (Bio-Rad). Subsequently, 50 µM of siRNA duplex was added 3 min before electroporation. To achieve efficient downregulation, primary human T cells or T-cell blasts were electroporated using the Gene Pulser Xcell (Bio-Rad) (one 500V pulse for 3 ms). Cells were collected 72 h (in case of primary T cells) or 24 h (in case of T-cell blasts) after electroporation and stimulated accordingly.

For Lck-deficient variant of the Jurkats (J.CaM1.6) transfection, pEF-BOS expression plasmid encoding various Lck constructs (S42A, S42D, S59A, S59D, S42A/S59A) were used. 2 x $10^7$ cells were washed, resuspended in 350 µl of PBS containing $Mg^{++}$ and $Ca^{++}$

ions (Biochrom), and transferred into transfection cuvette (Bio-Rad). Subsequently, 30 µg of cDNA was added. To achieve efficient transfection, cells were electroporated using the Gene Pulser II (Bio-Rad) (230V; 950 µF). 24 h after electroporation cells were collected, stimulated with iAbs or sAbs as indicated.

#### 4.3.4. T-cell stimulation

T cells were stimulated with either soluble or immobilized mAbs as follows. For soluble Ab stimulation, 2 x $10^6$ cells were loaded with 5 µg/ml biotinylated anti-human CD3 alone or in combination with 5 µg/ml biotinylated anti-human CD28, and 5 µg/ml biotinylated anti-human CD4 mAbs (see 4.2.3) in 100 µl RPMI 1640 medium (Biochrom) for 15 min on ice. After washing, receptors were cross-linked by addition of 25 µg/ml NeutrAvidin (Pierce). For microbead stimulation, SuperAvidin-coated polystyrene microspheres (Ø~10 µm, Bangs Laboratories) were coated with biotinylated CD3 alone or in combination with CD28 and CD4 mAbs as indicated (5 µg/ml each) for 30 min at 37 °C in PBS (Biochrom). Antibody-coated microbeads were washed twice with PBS, resuspended in RPMI 1640 medium and incubated with T cells in a 1:1 ratio. Stimulation of T cells was facilitated and synchronized by centrifuging samples for about 10 s at 100xg. Biotinylated $IgG_{2a}$ and $IgG_1$ mouse immunoglobulins were used as a control (see 4.2.3). Stimulation with plate-bound antibodies was performed as follows, 10 µg/ml of CD3 and 10 µg/ml CD28 Abs were coated on 6-well plates for 2 h at 37°C. After washing, 2 x $10^6$ cells were added to the plates, rapidly centrifuged at 100xg and incubated at 37°C for the indicated time periods.

Stimulations in the presence of either the MEK inhibitor I, U0126 (Calbiochem) or DMSO (Sigma Aldrich) were performed by pre-incubating T cells for 30 min with 10 µM of the compounds before stimulation with mAbs. For indicated microbead stimulation, 10 µM of either U0126 or DMSO were added 30 min after stimulation.

#### 4.3.5. Immunoblotting

After specific period of time stimulation of cells was stopped by addition of 1 ml ice-cold TBS buffer (see 4.2.1) and brief centrifugation at 10,000xg. Subsequently, cells were lysed in 30 µl of standard lysis buffer (see 4.2.1) for 25 min on ice. Afterwards, nuclear content was separated from protein suspension by 10 min centrifugation at 16,000xg. To unwind proteins 7,5 µl of 5x reducing sample buffer (see 4.2.1) was added and samples were heated for 5 min at 95°C. Post-nuclear lysates were separated by SDS-PAGE electrophoresis system (Bio-Rad) and transferred using semi-dry Western blotting onto nitrocellulose membranes (Amersham) (for used reagents see 4.2.1). Membranes were probed with the indicated primary antibodies (see 4.2.3) and the appropriate HRP-conjugated secondary antibodies (Dianova) and developed using the ECL detection system on the Hyperfilm MP (Amersham).

For quantifications of the immunoblots, the intensity of the detected bands was acquired using the Perfection V700 Photo Scanner (Epson) and analysis was performed using 1D ImageQuant software (Kodak). Unless indicated otherwise, β-actin was used as a loading control (typical loading error in the experiment: ±13%).

For dual-color analysis, nitrocellulose membranes were first incubated with rabbit anti-$pY^{416}$ Src antibody (see 4.2.3) and goat anti-rabbit IRDye 680LT (LI-COR Biosciences), stripped for 20 min in Restore PLUS Western Blot Stripping Buffer (Thermo Scientific), and then incubated with rabbit antibody against Lck (see 4.2.3) and goat anti-rabbit IRDye 800CW (LI-COR Biosciences). Signal intensities were determined by scanning the membranes with an Odyssey infrared imager (LI-COR Biosciences) and analyzing the data with the Odyssey application software.

#### 4.3.6. Immunoprecipitation

Primary human T cells (3 x $10^7$) were either left untreated or stimulated with sAbs or iAbs for the indicated periods of time (see 4.3.4). Cells were processed as for immunoblotting (see 4.3.5), lysed in standard or mild lysis buffer (see 4.2.1), and cleared by centrifugation. Proteins of interest were immunoprecipitated from lysates with specific antibodies (see 4.2.3) conjugated with recombinant 40 µl of protein A or G agarose beads (Santa Cruz Biotechnology) in the presence of 2% BSA (Sigma Aldrich) at 4°C overnight. After washing thoroughly with IP washing buffer (see 4.2.1), immunoprecipitates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane (Amersham), and analyzed by immunoblotting with the indicated antibodies (see 4.2.3).

#### 4.3.7. Flow cytometric measurements

To determine the efficiency of T-cell activation, T cells were stimulated as described previously (see 4.3.4). After 24 h, T cells were stained with specific antibodies against activation markers (see 4.2.3) for 15 min at 4°C and analyzed by flow cytometric analysis using a BD LSRFortessa, FACSDiva Software 6.1.3 (BD Biosciences), and FlowJo 7.6.5 (Tree Star).

Proliferation experiments were carried out in 96-well plates (Costar). Purified human T cells were labeled with 2.5 µM CFSE (Molecular Probes) for 10 min at 37°C. After washing, 2 x $10^5$ cells were seeded in a total volume of 200 µl to each well and cultured in human culture medium (see 4.2.1). T cells were either left unstimulated or stimulated with sAbs or iAbs as indicated. T cells were cultured for 72 h at 37°C and 5% $CO_2$. Proliferation was assessed by CFSE dilution using a BD LSRFortessa, FACSDiva Software 6.1.3 (BD Biosciences), and FlowJo 7.6.5 (Tree Star).

### 4.3.8. Proliferation assay

To assess the proliferative capacity T cell, cells were transfected with siRNA duplex as described above (see 4.3.3), rested overnight and stimulated in 96-well round-bottomed tissue culture plates (Corning Life Sciences) coated with CD3 monoclonal antibody (MEM92) (see 4.2.3). Cells were plated at 5 x $10^4$ cells per well in quadruplicates and cultured for 48 h. [$^3$H]-Thymidine (0.3 mCi per well; specific activity 50 Ci/mmol, ICN) was added for the last 8 h, and the plates were collected using a PHD cell harvester (Inotech). Thymidine incorporation was measured by liquid scintillation counting using liquid scintillator 1450 Microbeta Wallac (Perkin Elmer).

### 4.3.9. *In vitro* kinase assay

After stimulation with iAbs (see 4.3.4), T cells were lysed in standard lysis buffer (see 4.2.1). Subsequently, lysates were subjected to immunoprecipitation with anti-Lck antibody (see 4.2.3 and 4.3.6). Immunoprecipitates were washed 5 times with IP washing buffer (see 4.2.1) and 10% was taken for Western blot analysis (see 4.3.5). Remaining 90% was resuspended in 40 µl kinase reaction buffer containing [γ-$^{32}$P]-ATP (Perkin Elmer) (see 4.2.1). The reaction was allowed to proceed for 5 min at room temperature and stopped by adding 10 µl 5x reducing sample buffer (see 4.2.1) and heating at 95°C for 5 min. Samples were analyzed on 7.5% SDS-PAGE (see 4.2.1). Gels were dried and exposed to Hyperfilm MP (Amersham) for 12 h at -70°C with intensifying screen.

### 4.3.10. Statistical analysis

Graphical representation of the data and statistical analysis were performed using GraphPad Prism 3.02 (GraphPad Software Inc). Significance was calculated by paired two-tailed student's t-test (* P≤0.05, ** P≤0.01, *** P≤0.001).

**5. Used abbreviations**

| | |
|---|---|
| A | alanine |
| Abs | antibodies |
| ADAP | adhesion and degranulation promoting adapter protein |
| Akt | Ak thymoma |
| AP1 | activator protein 1 |
| APC | antigen presenting cell |
| Bam32 | B cell adaptor molecule of 32 kDa |
| Bcl10 | B-cell lymphoma 10 |
| C | constant region |
| CaM | calmodulin |
| CaMK | calmodulin kinase |
| CARMA1 | CARD-containing MAGUK protein 1 |
| Cbl | casitas B lineage lymphoma |
| CD | cluster of differentiation |
| CDC25 | cell division cycle 25 |
| cPKC | classical protein kinase C |
| CRAC | calcium release activated channels |
| Csk | c-src tyrosine kinase |
| cSMAC | central supramolecular activating cluster |
| D | aspartic acid |
| DAG | diacylglycerol |
| DC | dendritic cell |
| DH | Dbl homology |
| DN | double negative |
| Dok2 | docking protein 2 |
| DP | double positive |
| dSMAC | distal supramolecular activation cluster |
| EGF | epidermal growth factor |
| ELK | ETS domain-containing protein |
| ER | endoplasmic reticulum |
| ERK1/2 | extracellular signal-regulated kinase |
| FasL | Fas ligand |
| Fyn | feline yes-related protein |
| Gads | Grb2-related adapter protein downstream of Shc |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| Grb2 | growth factor receptor-bound protein 2 |
| GSF | GDI-like solubilizing factor |
| GTP | guanosine triphosphate |
| iAbs | immobilized anitbodies |
| IFNγ | interferon gamma |
| IκB | inhibitor of kappa B |
| IKK | IκB kinase |
| IL | interleukin |
| IL-2R | IL-2 receptor |
| $IP_3$ | inositol 1,4,5-trisphosphate |
| IS | immunological synapse |
| ITAM | immunoreceptor tyrosine-based activation motif |
| Itk | IL-2-inducible T-cell kinase |
| JAK | janus kinase |
| JNK | c-Jun N-terminal kinase |
| LAT | linker of activated T cells |
| Lck | lymphocyte-specific protein tyrosine kinase |
| mAbs | monoclonal antibodies |

| | |
|---|---|
| MALT | mucosa-associated lymphoid tissue lymphoma translocation protein 1 |
| MAP3K | mitogen-activated protein kinase kinase kinase |
| MAPK | mitogen-activated protein kinases |
| MEK | mitogen-activated protein kinase kinase |
| MEKK1 | mitogen-activated protein kinase kinase kinase 1 |
| MHC | major histocompatibility complex |
| MKK | mitogen-activated protein kinase kinase |
| MW | molecular weight |
| Nck | non-catalytic region of tyrosine kinase adaptor protein |
| NFAT | nuclear factor of activated T-cells |
| NFκB | nuclear factor kappa light chain enhancer of activated B cells |
| NGF | nerve growth factor |
| nPKC | novel protein kinase C |
| OVA | ovalbumin |
| OT | ovalbumin-specific TCR |
| PAMP | pathogen-associated molecular patterns |
| PAG | phosphoprotein associated with GEMs |
| PC12 | pheochromocytoma of the rat adrenal medulla cell line |
| PH | pleckstrin homology |
| $PIP_2$ | phosphatidylinositol-4,5-bisphosphate |
| $PIP_3$ | phosphatidylinositol-3,4,5-triphosphate |
| PDK1 | phosphoinositide-dependent protein kinase 1 |
| PI3K | phosphoinositide 3-kinase |
| PKB | protein kinase B |
| PKC | protein kinase C |
| PLCγ1 | phospholipase C gamma 1 |
| PRR | proline-rich region |
| pSMAC | peripheral supramolecular activation cluster |
| PTP | protein tyrosine kinase |
| PTPN22 | protein tyrosine phosphatase, non-receptor type 22 |
| R | arginine |
| Rac | Ras-related C3 botulinum toxin substrate |
| Raf | rat fibrosarcoma |
| Ras | rat sarcoma |
| RasGAP | Ras GTPase activating protein |
| RasGRP1 | Ras guanyl-releasing protein 1 |
| RBD | Ras binding domain |
| REM | Ras exchange motif |
| RNAi | RNA interference |
| RSK | ribosomal s6 kinase |
| S | serine |
| sAbs | soluble antibodies |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SFK | Src family tyrosine kinase |
| Shc | SH2 domain-containing transforming protein |
| SH2 | Src homology 2 |
| SH3 | Src homology 3 |
| SHP1 | SH2 domain-containing phosphatase 1 |
| SHIP1 | SH2 domain-containing inositol-5-phosphatase |
| siRNA | short interfering RNA |
| SLE | systemic lupus erythematosus |
| SLP76 | SH2 domain-containing leukocyte protein of 76 kDa |
| Sos | son of sevenless |
| SP | single positive |
| Src | sarcoma tyrosine kinase |

| | |
|---|---|
| STAT | signal transducer and activator of transcription |
| Syk | spleen tyrosine kinase |
| TBSM | tyrosine-based signaling motif |
| $T_c$ | cytotoxic T cell |
| TCR | T-cell receptor |
| TGFβ | transforming growth factor beta |
| $T_h$ | T helper cell |
| Themis | thymocyte-expressed molecule involved in selection |
| TMP | thrombin mimicking peptide |
| TRAP | transmembrane adaptor protein |
| $T_{reg}$ | regulatory T cell |
| TSAd | T-cell specific adapter protein |
| Ub | ubiquitin |
| Unc119 | uncoordinated 119 |
| W | tryptophan |
| WT | wild type |
| V | variable region |
| Vav1 | proto-oncogene vav 1 |
| Y | tyrosine |
| ZAP70 | zeta-chain-associated protein kinase 70 kDa |

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# Curriculum Vitae


| | |
|---|---|
| Name & Surname: | Mateusz Paweł Półtorak |
| Birth Date: | 03/23/1985 |
| Place of Birth: | Lublin, Poland |
| e-mail: | mateusz.poltorak@med.ovgu.de |


**Education:**

| | |
|---|---|
| 02/2010 – present | Ph.D. studies (specialization immunology) in Institute of Molecular and Clinical Immunology at Otto-von-Guericke University in Magdeburg, Germany |
| 10/2007 – 05/2009 | Master's degree undergraduate biological studies (specialization microbiology) at Maria Curie-Sklodowska's University in Lublin, Poland (Certificate of Appreciation) |
| 10/2004 – 06/2007 | Bachelor's degree undergraduate biological studies (specialization microbiology) at Maria Curie-Sklodowska's University in Lublin, Poland |

**Certificates:**

| | |
|---|---|
| 09/2012 | Merck Milipore: Seminar über Western Blot |
| 12/2008 | English Certificate TELC B2 (advanced level) |

**Scientific Experience:**

| | |
|---|---|
| 02/2010 – present | Research Program: "Molecular Organization of Cellular Communication in the Immune System" (CRC854, TP-19), Institute of Molecular and Clinical Immunology, Otto-von-Guericke University, Magdeburg, Germany (PhD student) |
| 06/2010 – present | Research Training Group: "Cell-Cell-Communication in Neural and Immune Systems: Topological Organization of Signal Transduction" (GRK1167), Leibniz Institute for Neurobiology, Magdeburg, Germany (Associated member) |
| 12/2009 – 02/2010 | Institute of Agrophysics in Lublin, Polish Academy of Sciences, Lublin, Poland (Research assistant) |
| 06/2009 – 10/2009 | Department of Microbiology Molecular Biology and Biochemistry, University of Idaho, Moscow ID, USA (Research assistant) |
| 04/2008 | "SSPK nr 1" Public Hospital in Lublin, Poland (Laboratory assistant) |
| 01/2008 – 05/2009 | Department of Comparative Anatomy and Anthropology, Maria Curie-Sklodowska's University in Lublin, Poland (master's degree student) |

**Publication Record:**

"Sos1 regulates sustained TCR-mediated Erk activation" **Poltorak M***, Meinert I, Stone JC, Schraven B, Simeoni L. Eur J Immunol. 2014 May;44(5):1535-40. doi: 10.1002/eji.201344046.

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* - first authorship

**Presentations:**

05/2014 EMBO Conference Series: Lymphocyte signaling, Bertinoro, Italy "Sos1 is dispensable for transient but required for sustained TCR-mediated Erk activation"

11/2013 17th Joint Meeting of the Signal Transduction Society, Weimar, Germany "Analysis of post-translational modifications of the Src-family tyrosine kinase Lck" (Oral and poster presentation)

09/2013 43rd Annual Meeting German Society for Immunology, Mainz, Germany "Sos1 regulates Erk phosphorylation during productive T-cell activation" (Poster presentation)

08/2013 15th International Congress of Immunology, Milan, Italy "Regulation of the Ras-Erk cascade during T-cell activation" (Oral presentation)

11/2012 16th Joint Meeting of the Signal Transduction Society, Weimar, Germany "Sos1 is dispensable for transient but required for sustained TCR-mediated Erk activation" (Oral and poster presentation)

11/2011 15th Joint Meeting of the Signal Transduction Society, Weimar, Germany "Grb2/Sos1 are not required for TCR-mediated Erk activation in peripheral human T cells" (Oral and poster presentation)

09/2011 2011 Joint Annual Meeting SIICA DGfI, Riccione, Italy “Analysis of TCR Activation Kinetics and Feedback Regulation of the Ras-Erk Cascade in T Cells” (Poster presentation)

12/2010 2nd International Symposium DFG Research Training Group 1167, Magdeburg, Germany “Regulation of the ERK signaling module in T cells” (Poster presentation)

10/2010 2nd Autumn School, Bad Schandau, Germany
“Positive feedback loop in Ras-ERK cascade in human primary T-cells”
(Oral presentation)

# Declaration

I, Mateusz Pawel Poltorak, hereby declare that the work contained herein has been created independently and has not been submitted elsewhere for any other degree or qualification. The research work was carried out from February 2010 to December 2013 at the Institute of Molecular and Clinical Immunology, Otto-von-Guericke University, Magdeburg.
All sources of information are clearly marked, including my own publications.

In particular I have not consciously:

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