Dynamics of proximal signaling events after TCR/CD8-mediated induction of proliferation or apoptosis in mature T-cells

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von Dipl.-Biol. Xiaoqian Wang

geb. am 24.12.1973 in Shaanxi, China

Gutachter: Prof. Dr. Burkhart Schraven

     Prof. Dr. Ottmar Janssen

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1. Introduction

The function of the immune system is to prevent and eradicate infections by means of layered defenses of increasing specificity. Physical barriers prevent pathogens from entering the organism at the first stage. However, if a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response, called innate immune response. Only when the innate immune responses are bypassed an adaptive immune response is required. The adaptive immune response is long-lived and specialized protective imunity and is triggered when microbes and microbial antigens enter into secondary lymphoid organs following a simple diffusion or capture by APCs (Antigen Presenting Cells). Secondary lymphoid organs, including lymph nodes, spleen and mucosal-associated lymphoid tissues, form a complex system that supports the interaction between antigen and antigen-specific lymphocytes. There are two types of adaptive immunity, humoral immunity and cell mediated immunity. Humoral immunity is mediated by antibodies produced by B lymphocytes. Antibodies are secreted into the circulation and mucosal fluids, where they participate in host defense to eliminate microbes in different ways including neutralization, opsonization, and complement activation. Cell mediated immunity refers to the host defense against intracellular microbes. It involves the activation of macrophages, NK (Natural Killer) cells, T cells which subsequently differentiated into antigen-specific CTLs (Cytotoxic T Lymphocytes), and the release of various cytokines in response to an antigen [1].

1.1. T cell development

T cells arise from hematopoietic stem cells and the progenitors migrate from the bone marrow to the thymus, where they develop into naive T cells. Upon entering into the thymus, the progenitors lack the expression of the co-receptors CD4 and CD8, and therefore, are called DN (Double Negative) thymocytes. The development of thymocytes follows sequential steps that are defined by the expression of CD4 and CD8 and the developing thymocytes are divided into four major subsets, namely, $CD4^-CD8^-$ (DN), $CD4^+CD8^+$ (DP-Double Positive), $CD4^-CD8^+$ (SP-Single Positive) and $CD4^+CD8^-$ (SP) thymocytes. Based on the cell-surface expression of CD25 and CD44, the DN population can be further divided into the DN1, DN2, DN3 and DN4 stages (Figure 1) [2]. It has also been demonstrated that the different stages of thymocyte development occur within defined thymic regions. The hematopoietic progenitors enter the thymus through post-capillary venules and mature to $CD44^+$ (DN1), cells. As they

migrate outward towards the thymic capsule in response to chemokine signaling, they upregulate CD25 (DN2) and then downregulate CD44 (DN3) [3]. At this stage, the TCR (T Cell Receptor) β chain is expressed and assembles with the invariant pre-Tα (pre-TCR α) chain and the CD3 signal transducing molecules. This complex is called the pre-TCR. The failure of the formation of a pre-TCR complex leads to cell death by apoptosis. Pre-TCR signaling leads to the inhibition of further TCR β chain rearrangement (allelic exclusion), cell proliferation and the expression of CD4 and CD8. This process is called β-selection (Figure 1). The rearrangement of the TCR α chain occurs at the DP stage and results in the expression of a mature and functional TCR on the cell surface. DP cells undergo positive or negative selection. Positive selection occurs in the cortex and is triggered by weak interactions between the mature TCR and MHC–self peptide complexes expressed on cTECs (cortical Thymic Epithelial Cells). In response to CCR7 signaling, positively selected thymocytes migrate to the medulla, which is the main site for negative selection [4]. Negative selection involves strong interactions between the TCR and MHC-self peptides displayed on medulla epithelial cells and DCs (Dendritic Cells) within the thymus, which results in apoptosis. Negative selection protects the body from possible autoimmunity by removing T cells that recognize self peptides with high affinity (Figure 1).
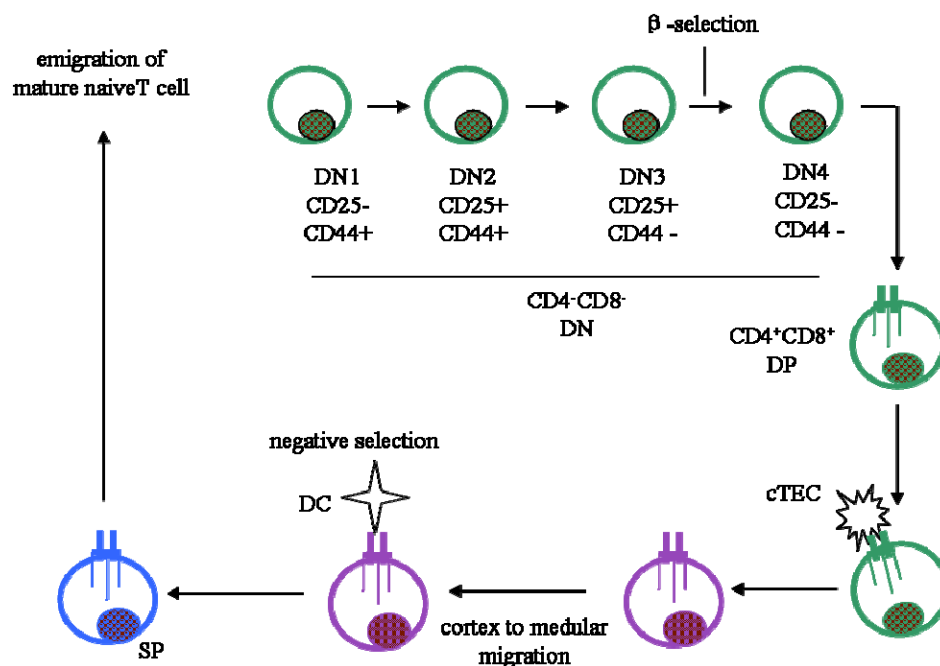
Figure 1. Thymic T cell development

Hematopoeitic progenitors enter the thymus and differentiate into DN cells. Thymocyte maturation and differentiation is characterized by the expression of different cell-surface markers, including CD4, CD8, CD44 and CD25 and the TCR. Thymocytes undergo positive selection in the cortex through the interaction between the TCR and MHC–self peptide complexes expressed on cTECs (cortical Thymic Epithelial Cells). The positively selected thymocytes are then screened for reactivity to tissue restricted self peptides by means of negative selection.

1.2. T cell activation

Naive T cells constantly re-circulate throughout the peripheral lymphoid organs to scan for foreign antigens expressed on APCs, such as B cells, dendritic cells, and macrophages. Specific recognition of antigen by the TCR initiates an adaptive immune response [5, 6].

1.2.1. The TCR

The majority of T cells bear TCRs composed of an α chain and a β chain, which are transmembrane proteins. The extracellular portion of each chain consists of two domains, resembling immunoglobulin V and C domains, respectively. The juxtaposition of the immunoglobulin V domains in both α chain and β chain forms the antigen binding site. In addition to the α and β chains, the αβTCR contains the signal-transducing CD3 complex and the homodimer ζ chains [7]. The CD3 complex consists of one CD3γ, one CD3δ, and two CD3ε subunits (Figure 2). The association between the αβ heterodimers, the CD3 complex and the ζ chains is important for T cell activation because the intracellular domains of the α- and the β- chains are short and do not contain signaling motifs. Hence, after the interaction with antigen/MHC, the CD3 complex and ζ chains transmit the signal into the cytoplasm of the T cell to trigger T cell activation.
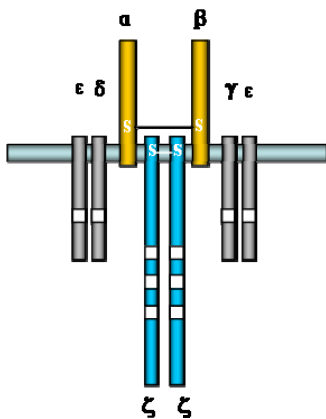
Figure 2. The TCR complex

The TCR complex is composed of the highly variable, antigen-binding TCR heterodimer (α and β chain) and the invariant signaling proteins CD3γ, CD3δ, CD3ε and ζ chain, which are responsible for signal transduction. ITAMs (Immuno receptor Tyrosine-based Activation Motifs) are indicated by white boxes.

Signaling from the TCR complex depends on the presence of ITAMs (Immuno receptor Tyrosine-based Activation Motifs), which are present in the cytoplasmic tails of both the CD3 complex and ζ chains. ITAMs are composed of two tyrosine residues which are separated by 6-8 amino acids. Although each of the CD3 subunits contains at least one ITAM, the ζ chains are believed to be the dominant component during T cell signaling because they contain 6 of the 10 ITAMs (three in each ζ chain) [8].

Following TCR engagement, one of the first detectable biochemical events is the phosphorylation of the ITAMs. The multiple ITAMs in the ζ chains confer flexibility in signaling [9]. By using sequence-specific phosphotyrosine antibodies, Kersh et al. found that the ζ chains undergo a series of ordered phosphorylation events upon TCR engagement [10, 11]. Complete phosphorylation depends on the nature of the TCR ligand. For example, recognition of a less potent ligand only leads to phosphorylation of a subset of tyrosines on the ζ chains, and complete phosphorylation of the ITAMs is only observed with a strong agonist ligand that fully activates the T cell.

1.2.2. TCR signaling

It is known that tyrosine phosphorylation in ITAMs is the first event after TCR ligation and that it is mediated by Src-family tyrosine kinases (Lck, Fyn). Phosphorylation of the ITAMs allows their interaction with the SH2 domains of ZAP-70 (Zeta-chain-Associated Protein kinase 70) which is important for transmitting the signal onwards (see below). Once bound to ITAMs, ZAP-70 is activated and further contributes to the phosphorylation of multiple downstream molecules, such as LAT (Linker for Activation of T cells), SLP-76 (SH2 domain containing Leukocyte Protein of 76 kDa) and PLCγ1 (Phospholipase C γ1). This signaling cascade initiates calcium flux and MAP kinase activation and ultimately culminates in proliferation and differentiation of T cells.

1.2.2.1. Src kinases

In contrast to the classical receptor tyrosine kinases, the TCR complex does not possess any intrinsic enzymatic activity. However, ITAMs in the TCR complex can be phosphorylated by Src kinases. The phosphorylated ITAMs further transduce the activation signals from the membrane to the nucleus. Therefore, ITAM phosphorylation *via* Src kinases is the first crucial biological event following TCR engagement. T cells primarily express the Src kinases Lck and Fyn, both of which contain N-terminal lipid modifications (targeting them to the membrane), a unique region, an SH3 domain, an SH2 domain, a proline rich region, and a tyrosine kinase domain. Lck constitutively interacts with either the CD4 or the CD8 coreceptor *via* a dicysteine motif present in its unique domain [12]. The enzymatic activity of the Src kinase is regulated by the phosphorylation of two tyrosines. Tyrosine 394 (Y^{394}) is located within the kinase domain and is phosphorylated when Lck is active

(autophosphorylation). Tyrosine 505 (Y^{505}) lies in the C-terminal region and has a negative regulatory function. In resting T cells, Y^{505} is phosphorylated and interacts with the SH2 domain of Lck, thereby leading to a closed and inactive conformation of this kinase [13, 14]. Phosphorylation of Y^{505} is regulated in two steps. The constitutive phosphorylation of Y^{505} in resting T cells is mediated by the protein tyrosine kinase Csk (COOH-terminal Src Kinase). TCR engagement triggers the dissociation of Csk from the plasma membrane and the dephosphorylation of Y^{505} by the protein tyrosine phosphatase CD45, leading to the formation of an open conformation of Lck and its activation (Figure 3). Therefore, Csk is considered to be a critical negative regulator of Lck while CD45 is regarded as a positive one following TCR engagement [15].
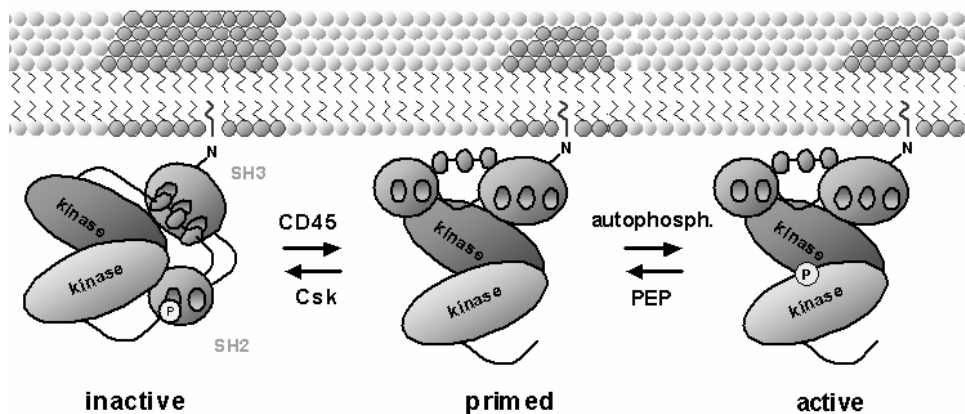
Figure 3. Regulation of Lck activity by CD45 and Csk

Lck activation is regulated by both positive and negative mechanisms. In resting T cells, Lck is deactivated through Csk mediated phosphorylation of its negative regulatory tyrosine Y^{505}. TCR stimulation induces the dissociation of Csk from its substrate and Lck is in a primed state. CD45 can dephosphorylate both autocatalytic and inhibitory tyrosines of Lck while the primed Lck is able to undergo autophosphorylation of Y^{394} generating an active kinase that subsequently phosphorylates the ITAMs of the CD3 and ζ chain. This figure is provided by Smida M.

1.2.2.2. ZAP-70

Once the ITAMs within the TCR complex are phosphorylated by Src-family kinases, they will recruit the tyrosine kinase ZAP-70 to the plasma membrane. ZAP-70 is a 70 kDa protein expressed throughout thymocyte development and at high level in peripheral T cells. ZAP-70 deficiency leads to a block in T cell development at the transition from the DP to the SP stage [16, 17]. The SH2 domains in ZAP-70 binds to the phosporylated tyrosines in the ITAMs of the TCR complex and thus targets ZAP-

70 to the plasma membrane [18]. Once ZAP-70 is targeted to the plasma membrane, it undergoes tyrosine phosphorylation either by autophosphorylation or phosphorylation by Lck leading to its activation. The tyrosine phosphorylation sites in ZAP-70 include Y^{315}, binding to the SH2 domain of Vav1 (Guanine nucleotide exchange factor for the GTPases Rac and CDC42) [19, 20], Y^{319}, associating with SH2 domain of Lck [21, 22] and Y^{413} [23]. The activated kinase is then able to phosphorylate its substrates, such as adaptor protein LAT (Linker for Activation of T cells) [24, 25].

1.2.2.3. Adaptor proteins

Adaptors are proteins that do not have enzymatic or transcription-regulating activities but rather possess tyrosine residues as well as multiple protein protein interaction domains such as SH2 domains, SH3 domains, PH domain, PTB domains or proline rich regions. These structures enable adaptors to associate with other proteins and thereby allow the formation of multi-component signaling complexes. In T cells, specialized adaptor proteins, such as LAT and SLP-76 relay the signals from the TCR to intracellular signaling components.

LAT is a transmembrane protein with the molecular weight of 36-38 kDa. It is expressed in T lymphocytes, NK and mast cells, platelets, and pre-B cells. There are nine conserved tyrosine residues within the cytoplasmic tail of LAT. LAT is a substrate of ZAP-70 in response to T cell activation [25]. The sites of ZAP-70 phosphorylation in LAT have been mapped to the tyrosines 127, 132, 171, 191, and 226 [26]. Upon TCR activation, these tyrosines become phosphorylated and provide docking sites for multiple downstream signaling molecules including Grb2 (Growth factor Receptor-Bound protein 2), PLCγ1, and Gads (Grb2 related Adaptor Downstream of Shc) [26]. The phosphorylated LAT dependent interactions are essential to target these downstream molecules to the plasma membrane and in turn, to couple the TCR to downstream signaling cascades. A LAT deficient T cell line exhibits tyrosine phosphorylation defects including significantly diminished phosphorylation of PLCγ1, Vav, and SLP-76. These cells fail to flux intracellular calcium and cannot activate the Ras/Raf/ERK1/2 signaling cascade after TCR engagement [27, 28]. Re-expression of LAT reconstitutes all these events, demonstrating that LAT not only mediates TCR-induced tyrosine phosphorylation of many substrates but is also required for calcium flux, activation of Ras, and subsequent

downstream events leading to IL-2 gene expression. LAT also plays a crucial role in T cell development since LAT-deficient thymocytes are developmentally blocked at the DN stage [29].
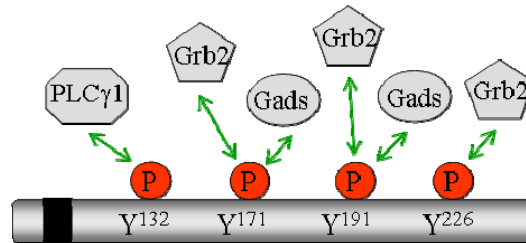
Figure 4. The binding partners of phosphorylated LAT after TCR stimulation.
LAT is a substrate of the protein tyrosine kinase ZAP-70 in T cells. Following phosphorylation at the sites of Y^{132}, Y^{171}, Y^{191}, Y^{226}, LAT recruits Grb2, Gads, and PLCγ1 *via* their SH2 domains.

Unlike LAT, SLP-76 is a cytoplasmic adaptor protein that contains an N-terminal domain with several tyrosine phosphorylation sites, a central proline-rich region, and a C-terminal SH2 domain [30, 31]. Tyrosine phosphorylation of SLP-76 at positions 113, 128, and 145 leads to its association with the SH2 domains of Vav, Nck (adaptor protein), and Itk (IL-2 inducible T cell Kinase) [32-35]. Mutation of these three tyrosines results in a severely diminished activation of downstream signaling cascades [36]. The proline-rich region of SLP-76 constitutively binds the SH3 domain of Gads, through which SLP-76 is targeted to phosphorylated LAT after TCR stimulation. The SH2 domain at the C-terminus of SLP-76 mediates its interaction with the cytosolic adaptor protein ADAP (Adhesion and Degranulation-promoting Adaptor Protein) [37] and the serine/threonine kinase HPK1 (Hematopoietic Progenitor Kinase 1) [38]. The critical function of SLP-76 in T cell activation has been demonstrated in cell lines and *in vivo*. In the SLP-76-deficient Jurkat T cell line J14, TCR activation fails to induce PLCγ1 phosphorylation, Ca^{2+} mobilization, ERK1/2 phosphorylation, and the activation of TCR-dependent transcription factors such as NF-AT and AP-1 [39]. *In vivo*, SLP-76 deficiency results in a complete arrest of T cell development at the DN3 stage [40, 41].
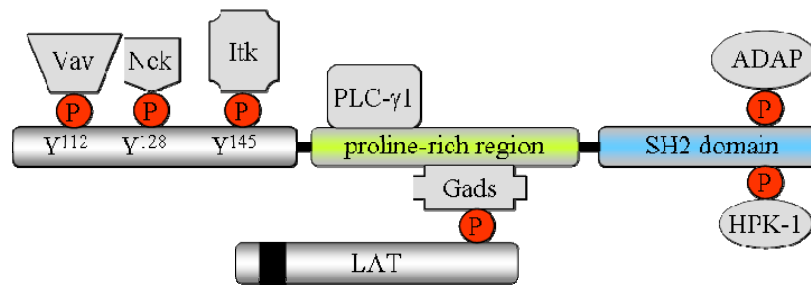
Figure 5. The structure of SLP-76 and its binding partners important for T cell activation

SLP-76 is a cytoplasmic adaptor protein. It contains tyrosine phosphorylation sites (Y^{112}, Y^{128}, Y^{145}) at the N-terminal domain mediating its interaction with Vav, Nck, and Itk. The central proline-rich domain is required for its constitutive interaction with Gads and PLCγ1, while the SH2 domain at the C-terminal is essential for the association with ADAP and HPK-1.

1.2.2.4. PLCγ1 activation and calcium flux

The LAT/SLP-76 complex is important for plasma membrane recruitment and activation of PLCγ1 after TCR stimulation. The current model for the activation PLCγ1 postulates that in response to TCR stimulation, LAT is phosphorylated at Y^{132} which mediates an inducible interaction with the SH2 domain of PLCγ1. This interaction serves to recruit PLCγ1 to the plasma membrane. Phosphorylated LAT also associates with the Gads/SLP-76 complex, which in turn binds to Itk after TCR stimulation. The close proximity of Itk and PLCγ1 results in the phosphorylation and activation of PLCγ1 [42, 43]. Activated PLCγ1 hydrolyzes PIP_2 (phosphatidylinositol 4,5-bisphosphate) producing two second messengers, DAG (diacylglycerol) and IP_3 (inositol 1,4,5-trisphosphate) [44-46]. IP_3 induces a transient increase in free intracellular Ca^{2+}, which binds to calmodulin that, in turn, activates calcineurin, a Ca^{2+}/calmodulin dependent protein phosphatase [47]. Activated calcineurin dephosphorylates NF-AT, thus allowing its translocation to the nucleus where the latter participates in IL-2 (Interleukin-2) transcription. Generation of DAG in response to TCR stimulation is also essential for the initiation of T cell activation. Proteins with a C1 domain, such as PKC (Protein Kinase C) [44, 48, 49] and RasGRP (Ras Guanyl nucleotide-Releasing Protein) [50], interact with DAG [51]. DAG is also required for the activation of PKD1 (Protein Kinase D1). Wood et al. have shown that the requirement of DAG for PKD1 activation integrates two DAG signals [52]. The first is

that DAG is required for PKC activation, which then colocalizes with PKD1 and phosphorylates serine 744 and 748 within PKD1. The second reflects a requirement for DAG binding to the CRD (Cysteine-Rich Domain) of PKD1, thereby relieving the inhibition of the catalytic domain of PKD1. PKD1 activity can be determined by analyzing S^{916} phosphorylation, which was previously reported to modify the conformation of the kinase and to influence the duration of its kinase activity [53].
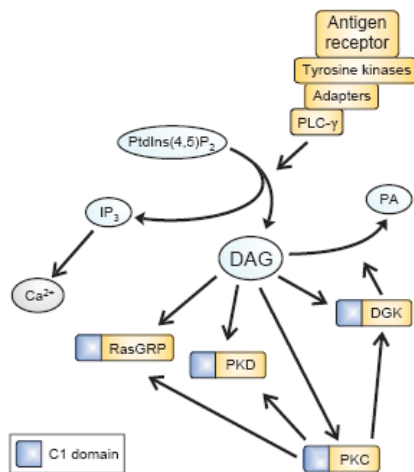
Figure 6. The network of DAG-binding proteins.

Antigen receptor–coupled tyrosine kinases phosphorylate adaptors that trigger DAG (diacylglycerol) production *via* PLC-mediated hydrolysis of PtdIns(4,5)P_2. DAG binds to conserved C1 domains in many different molecules, including various isoforms of PKC (Protein Kinase C), PKD1 (Protein Kinase D1), Ras-GRP (activator of the GTPase Ras) and DGK (Diacylglycerol Kinase). Binding of DAG to C1 domains is essential in the regulation of protein localization but does not operate by a simple 'on or off' switch to stimulate the catalytic function of C1 domain–containing molecules. Instead, evidence is emerging that many DAG-binding proteins are substrates for and are regulated by PKC.

1.2.2.5. Ras-Raf-ERK1/2 activation

PLCγ1 phosphorylation is also required for the activation of the Ras-Raf-ERK1/2 cascade. The GTPase Ras is a guanine nucleotide binding protein that cycles between an inactive GDP-bound and an active GTP-bound state. Ras activity can be regulated in a positive way by GEFs (Guanine nucleotide Exchange Factors) that promote the transition from the GDP-bound state to the active GTP-bound conformation and in a negative way by GAPs (GTPase-Activating Proteins), which stimulate Ras GTPase activity resulting in the hydrolysis of GTP to GDP and the accumulation of inactive Ras–GDP complexes. There are two GEFs proposed to activate Ras in T cells in response to TCR ligation. One is SOS (Son of Sevenless) which constitutively interacts with the adaptor protein Grb2 *via* binding of the SH3 domain in Grb2 to a proline rich region in SOS [54]. TCR cross-linking leads to LAT phosphorylation and its binding to Grb2, which correspondingly recruits SOS to the plasma membrane to trigger Ras activation. RasGRP is the other GEF in T cells. Recruitment of RasGRP to the membrane by DAG also leads to Ras activation [55]. GTP-bound Ras next recruits Raf-1 to the plasma membrane. There, Raf-1 is activated in a series of incompletely

understood steps[56]. Activated Raf-1 then phosphorylates the cytosolic kinase MEK, which is responsible for the subsequent activation of the MAP kinase ERK1/2. Activated ERK1/2 translocates to the nucleus to target the ternary complex factor Elk-1, which is important for IL-2 production and T cell activation. Figure 7 represents some elements involved in TCR induced T cell activation.
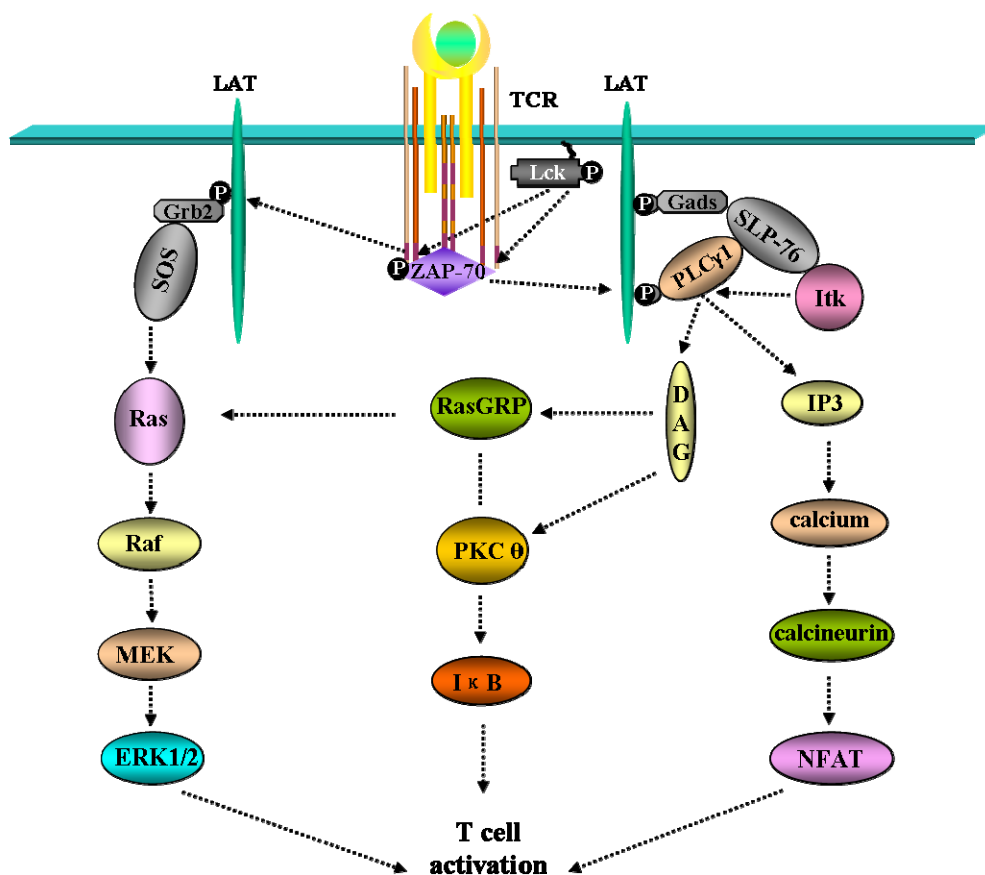
Figure 7. The TCR signaling pathway

Engagement of the TCR leads to the activation of Lck and ZAP-70 and in turn results in the phosphorylation of SLP-76 and LAT. Phosphorylated SLP-76 and LAT serve as docking elements for numerous cytoplasmic signaling molecules such as Grb2/SOS and PLCγ1. These molecules are involved in the activation of the Ras/MAP-kinase cascade, in the combination of calcium flux, in the activation of PKC and ultimately in the induction of trancriptional activity.

1.2.3. The role of TCR signaling in thymocyte development

TCR signalling in mature T cells, as descirbed above, results in the induction of T cell effector functions, such as cytokine secretion and cell mediated cytoxicity. In the thymus, on the other hand, TCR-induced signals lead to positive or negative selection of DP thymocyte. Although it is widely accepted that the affinity of ligands dictates the outcome of thymocyte development, the molecular mechanisms involved are still

unknown. Initial studies have demonstrated that ERK1/2 plays an important role in positive selection [57, 58]. In ERK1^{-/-} mice, thymocyte maturation beyond the CD4^{+}CD8^{+} stage is reduced by half, with a similar diminution in the thymocyte subpopulation expressing high levels of TCR (CD3 high). However, more recent studies identified ERK1/2 as a potential point where the signals of positive and negative selection quantitatively diverge [57, 59]. The current model indicates that both the duration and the level of ERK1/2 activation influence negative selection as well as positive selection. Werlen et al. reported that weak but sustained activation of ERK1/2 occurs in response to low affinity ligands and results in positive selection. In contrast, negative selectors induce strong, but transient ERK1/2 activation [60]. In addition, Daniels et al. found that a small increase in ligand affinity for the TCR leads to a marked change in the activation and subcellular localization of Ras and ERK1/2 and the induction of negative selection [61].
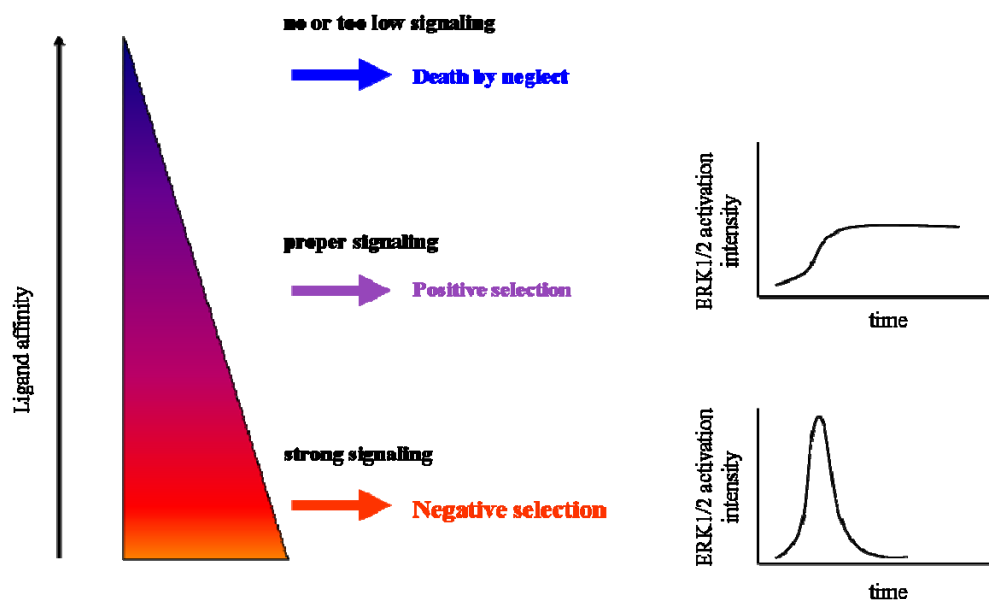
Figure 8. TCR affinity model and the role of ERK1/2 activation during T cell development

The affinity model predicts that high affinity ligands trigger strong but transient ERK1/2 activation corresponding to negative selection whereas low affinity ligands induce a weak and sustained ERK1/2 activation correlating with positive selection.

1.2.4. Negative regulation of antigen receptor signaling in T cells

1.2.4.1. PTPases (Protein Tyrosine Phosphatases)

Since protein tyrosine phosphorylation plays an essential role in TCR signaling, removing the phosphorylation provides an easy way to inhibit T cell activation. It is generally accepted that PTPases are crucial for keeping T cells in a resting state in the absence of antigen. Several PTPases are reported to be capable of inhibiting T cell activation, such as Shp1 (SH2 domain containing PTP1) and CD148.

Shp1 is a cytoplasmic tyrosine phosphatase that is primarily expressed in hematopoietic cells [62]. Shp1 works as an important negative regulator of immune functions. The *motheaten mice,* which show point mutations within the Shp1 gene, develop a progressive inflammatory disease characterized by infiltrates of neutrophils and macrophages in the lung, skin, and other tissues [63]. Thymocytes and peripheral T cells from *motheaten mice* exhibit a markedly increased proliferative response to TCR stimulation compared to normal cells. Shp1 deficient thymocytes also show increased constitutive tyrosine phosphorylation of the TCR complex and enhanced and prolonged TCR-induced tyrosine phosphorylation of the ζ chain and CD3ε, as well as a number of cytosolic proteins [64]. Shp1 is reported to attenuate the earliest events in TCR signaling, since Lck and ZAP-70 have been identified as possible substrates for Shp1. Upon T cell activation, Shp1 binds to ZAP-70, resulting in an increase in Shp1 phosphatase activity and a decrease in ZAP-70 kinase activity [65, 66]. In addition, Stefanova et al. suggested that Shp1 can also help T cells to distinguish between high and low affinity ligands. Following TCR stimulation, activated Lck triggers Shp1 phosphorylation at Y^{564}. This promotes Shp1 recruitment to the TCR *via* its binding to the Lck-SH2 domain, where Shp1 then dephosphorylates and inactivates Lck, thus terminating signals. However, high affinity ligands not only induce Shp1 phosphorylation, but also evoke ERK1/2-mediated Lck phosphorylation at S^{59}, preventing Shp1 recruitment and thus Lck inactivation [66] (Figure 9).
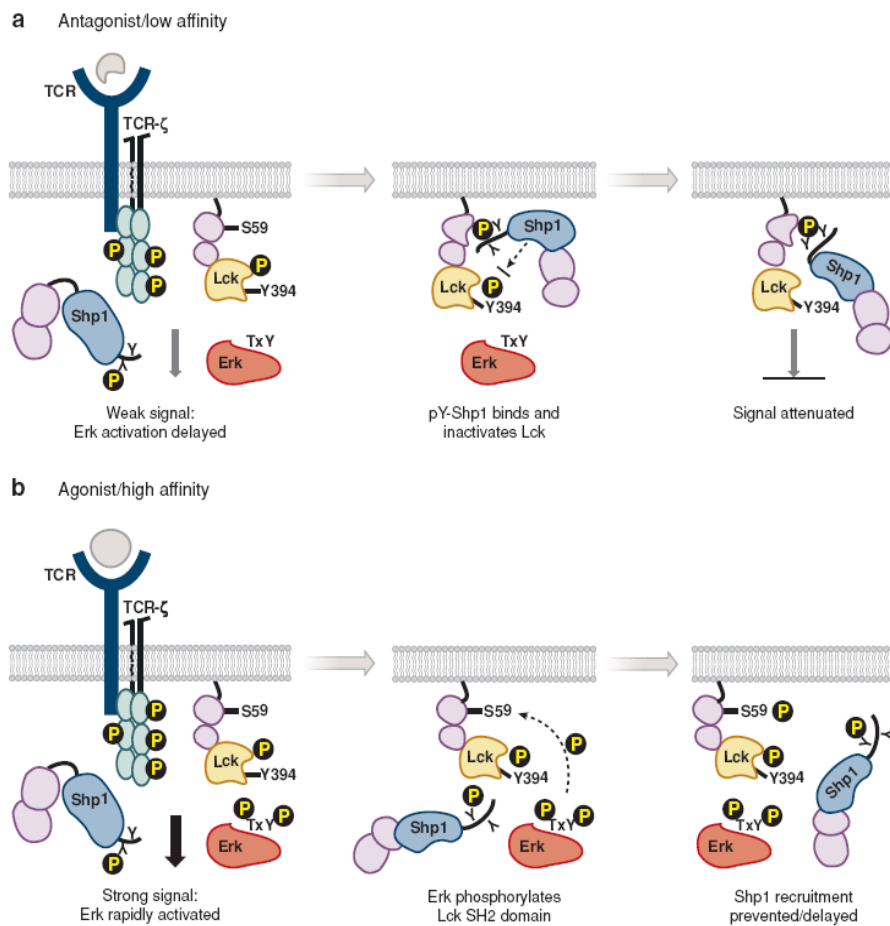
Pao, 2007

Figure 9. Shp1/Lck/ERK1/2-mediated feedback loops in TCR signaling.

(A) Upon TCR engagement with an agonist, Lck is activated, resulting in Shp1 phosphorylation at Y^{564} (pShp1) by Lck. This promotes pShp1 recruitment to the TCR *via* its binding to the Lck-SH2 domain (pink), where pShp1 then dephosphorylates and inactivates Lck to terminate signaling. (B) If TCR signal strength exceeds a critical threshold (high affinity ligand), TCR-evoked ERK1/2 activation results in ERK1/2-mediated Lck phosphorylation at S^{59}, preventing Shp1 recruitment and thus Lck inactivation.

CD148 is another protein tyrosine phosphatase that negatively regulates TCR signaling. CD148 was found to be weakly expressed on B and T cells, platelets, natural killer cells, certain dendritic cells as well as on mature thymocytes. CD148 is upregulated following *in vitro* activation of human peripheral T cells [67]. Overexpression of CD148 in Jurkat T cells inhibits TCR-induced NF-AT activation, ERK1/2 phosphorylation, calcium flux, PLCγ1 activation, and LAT phosphorylation [68]. Following stimulation with APCs loaded with staphylococcal enterotoxin superantigen (SAg), CD148 is excluded from the immunological synapse, sequestering it from its potential substrates. Once the T cell has disengaged from the APCs, CD148 can access

its substrates, such as LAT, PLCγ1, leading to their dephosphorylation, thereby downregulating T cell activation. Targeting the CD148 phosphatase domain to the immunological synapse potently inhibits NF-AT activation by TCR triggering [69].

1.2.4.2. Negative regulation of T cell activation by ubiquitination and degradation

TCR signaling can also be inhibited by removing critical mediators of activation through selective protein ubiquitination and degradation. Ubiquitination is a posttranscriptional modification of cellular proteins in which ubiquitin (Ub) monomers are either attached to lysine residues of target proteins or to Ub itself (to form poly-Ub chains). Attachment of a single ubiquitin motif to the substrate results in monoubiquitination while addition of ubiquitin chains to the substrate leads to the formation of polyubiquitination [70]. Two types of ubiquitin chains are abundant: K48 (lysine48 linked chain) and K63 (lysine63 linked chain). It has been suggested that K48 linked Ub chains serve as recognition signals for proteosomal degradation whereas K63 linked polyubiquitin chains regulate several cellular processes, such as DNA repair, signaling, endocytosis, vesicular trafficking, and cell-cycle progression [71]. The process of ubiquitination involves three enzymes: an Ub-activating enzyme (E1), an Ub-conjugating enzyme (E2), and an Ub ligase (E3) [72].

Cbl proteins are a family of E3 ligases of three related gene products c-Cbl, Cbl-b, and Cbl-c. All three Cbls have an N-terminal TKB (Tyrosine Kinase Binding) domain which mediates the binding to specific phosphotyrosine motifs on target molecules and a Ring finger domain that interacts with the E2 conjugase and position it so that the ubiquitin moiety can be transferred from E2 conjugase to the substrate *via* the E3 ligase (Figure 10). c-Cbl and Cbl-b have additional C-terminal tails composed of proline rich regions that contain functional SH3 binding sites and tyrosine residues that enable Cbl to interact with the SH2 domains of other proteins [73]. Cbl appears to be essential for the negative regulation of TCR signaling, since T cells from c-Cbl/Cbl-b double knockout mice are hyperresponsive towards TCR stimulation. This is partly due to the fact that TCR internalization into the lysosomes was blocked in the double knockout T cells [74].
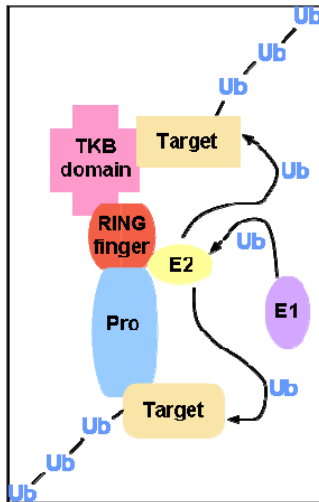
Figure 10. Model of Cbl Ubiquitin Ligase Function
An ubiquitin-activating enzyme (E1, purple) activates Ub and transfers it to an ubiquitin-conjugating enzyme (E2, yellow) which interacts with a ubiquitin ligase (E3) and transfers Ub to the target protein (peach). The RING finger of the E3 ligase mediates its binding to the E2 conjugase. Multiple motifs in Cbl proteins, such as TKB (pink) domain, the proline-rich region (blue), or the phosphorylated tyrosine residues (not shown) serve to recruit the substrates for ubiquitinylation. The induced ubiquitinylation may be in the form of monoubiquitin units or polyubiquitin chains, leading to lysosomal or proteasomal targeting, respectively.

Duan, 2004

1.3. T cell apoptosis

In response to antigens, which are processed and presented to T cells by APCs, antigen specific T cells begin to proliferate and differentiate. This process is essential for the conversion of naïve T cells into effector T cells, whose function is to eliminate invading microbes. The induction of apoptosis is another central issue during T cell activation. Apoptosis is responsible for maintaining homeostasis within the immune system, since unlimited proliferation of lymphocytes results in autoimmunity. Apoptosis involves a series of biochemical reactions leading to a variety of morphological changes including DNA cleavage, nuclear condensation, and fragmentation. It is also accompanied by surface exposure of phosphatidylserine, shrinking and blebbing of the plasma membrane, and formation of apoptotic bodies containing the condensed cell organelles and chromatin fragments. Caspases, a family of cysteinyl proteases, play an essential role in the regulation and execution of apoptosis [75, 76]. Caspases are synthesized as inactive enzyme precursors with a prodomain of variable length followed by a large subunit of 20 kDa (p20) and a small subunit of about 10 kDa (p10). The inactive caspases must undergo processing and activation before they can mediate apoptosis. The mechanism for procaspase activation involves procaspase cleavage at a specific asp-X bond resulting in the formation of the active caspase as a tetramer of two p20-p10 heterodimers and the release of the prodomain [77]. Among these caspases, caspase-3 serves as a downstream "apoptosis executor". Its activation is involved in both the intrinsic and extrinsic apoptosis pathways. Caspase-3-defective peripheral T cells are less susceptible to CD3ε- and Fas

receptor induced apoptosis and activation-induced cell death than similar cells from wild-type mice [78].
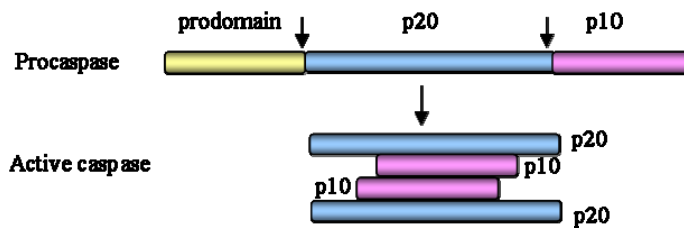
Figure 11. Mechanism of caspase activation

Procaspase contains a small amino-terminal prodomain, a p20 and a p10 subunit. The interfaces of these domains contain critical aspartate residues that are caspase cleavage sites. These are cleaved by active caspases and form p10−p20 heterodimers in a tetrameric structure containing two active caspase sites.

1.3.1. Two signaling pathways leading to apoptosis

T cells have two different apoptotic pathways which are mediated by distinct initiator caspases but converge into the same executor, caspase-3 (Figure 12). The first pathway also called the "extrinsic" apoptosis signaling pathway is mediated by death receptors [79, 80]. The death receptors are transmembrane proteins. Death domains, which are responsible for apoptotic signal transduction, are located in the cytoplasmic tail of the receptor. Six members of this subfamily are known so far [81]. In T cells only TNF-receptor and FasR (also known as CD95) are essential for T cell apoptosis [82, 83]. The ligation of death receptors by their ligand causes the formation of DISC (Death-Inducing Signaling Complex), which involves the oligomerized death receptors and their death domain containing adaptor FADD, which is recruited to the cytoplasmic tail of the death receptor. Through the homotypic interaction of DEDs (Death Effector Domains), FADD can further recruit procaspase-8 to the DISC. Upon recruitment, caspase-8 undergoes processing by cleavage and forming dimers [84]. Once activated, caspase-8 will induce the activation of caspase-3 and T cell apoptosis.
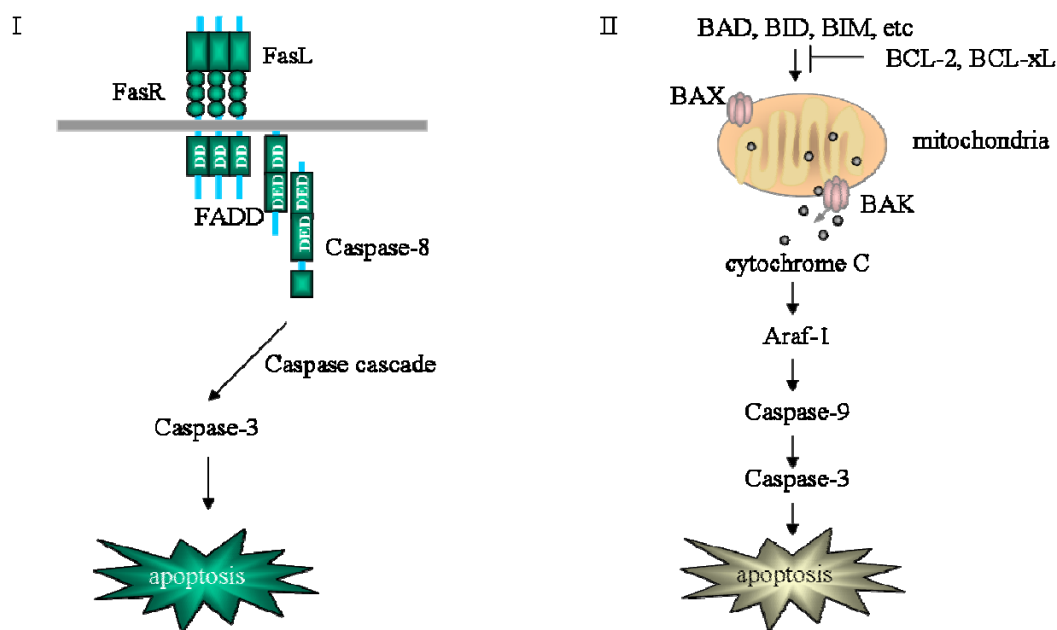
Figure 12. Illustration of apoptosis driven by death receptors or Bcl-2 family members in activated T cells.

In type I apoptosis oligomerization of death receptors by their ligands induces recruitment of adaptor proteins such as FADD. These adaptor proteins bind to the cytoplasmic tail of receptors through homologous DD interactions. Procaspase-8 is then recruited into the complex and activated. Once activated, caspase-8 cleaves and activates caspase-3, which in turn cleaves other caspases, thus leading to apoptosis. In type II apoptosis, high levels of BAD, BID, and BIM by unknown mechanism signal BAX or BAK to form pores in mitochondria and cause the release of cytochrome C from mitochondria. Once released, cytochrome C interacts with procaspase-9. This complex favors the activation of caspase-9 which, when activated, cleaves and activates caspase-3, thus leading to apoptosis. The effects of pro-apoptotic molecules can be blocked by anti-apoptotic molecules, such as Bcl-2 or Bcl-xL.

In the other pathway, also called the "intrinsic" apoptosis pathway, apoptosis is initiated through regulating mitochondrial membrane integrity. The presence of apoptotic stimuli leads to the disruption of the mitochondrial membrane and the leakage of the pro-apoptotic molecule cytochrome C into the cytosol. In the presence of ATP, cytochrome C can bind Apaf-1 (Apoptosome scaffold Protein Apoptotic protease-activating Factor 1), which further results in the activation of caspase-9 [85, 86]. The events leading to the disruption of the mitochondrial membrane and release of pro-apoptotic molecules are not clear. However, the Bcl-2 family proteins represent checkpoint that regulate the integrity of the mitochondrial membrane. The Bcl-2 family consists of two groups of molecules that are either pro-apoptotic or anti-apoptotic. Anti-apoptotic molecules, such as Bcl-2 and Bcl-xL [87] are characterized by four

conserved BH domains in addition to a C-terminal hydrophobic transmembrane domain through which the anti-apoptotic molecules are localized in the membranes of the mitochondria, the endoplasmic reticulum (ER), and the nucleus. Anti-apoptotic molecules are supposed to protect the cell from apoptosis. The pro-apoptotic group includes molecules like Bid, Bim, Bak, and Bax [88-91]. In resting cells, the pro-apoptotic molecules are located in the cytosol or loosely attached to the endoplasmic reticulum membrane. In response to apoptotic stimuli, they translocate to the mitochondria, where they oligomerize, integrate into the membrane and form holes in the mitochondrial membrane, thus inducing the release of apoptogenic proteins. The anti-apoptotic molecules prevent the oligomerization and insertion of some pro-apoptotic molecules into the mitochondrial membrane. Of note the BH3-protein Bid is one cirtical determinant of a crosstalk between the intrinsic and the extrinsic apoptotic pathway [92].

1.4 Aims of the present research

Engagement of the TCR can induce different functional outcomes such as differentiation, survival, or apoptosis. However, despite the progress in our understanding of the signaling pathways regulating T cell function, how the TCR can transmit signals leading to distinct cellular responses is so far not completely understood. Thus, to shed light on this issue, I firstly identify ligands that induce two different cellular outcomes, namely proliferation or apoptosis. Successively, I investigated signaling pathways triggered by ligands inducing proliferation or apoptosis, by using antibodies raised against phosphorylated sites. An increasing body of evidences indicates that both the amplitude and the duration of signaling events can regulate cellular responses. For example, EGF transiently activates ERK1/2, and thus stimulates cell proliferation, whereas NGF stimulation leads to sustained ERK1/2, resulting in neuronal differentiation. Thus, I subsequently address the activation kinetics of signaling molecules under proliferation *versus* apoptosis condition. Finally, it is known that the subcellullar localization of signaling molecules could also regulate cellular outcomes. Therefore, I also examined the subcellular localization of molecules mediating the TCR induced ERK1/2 activation.

2. Materials and methods

2.1. Materials

2.1.1. Instruments

AutoMACS Separator (Miltenyi Biotec)

ELISA reader: Expert Plus Microplate Reader (ASYS)

Pipette (Eppendorf)

Power PAC 200 (Bio-Rad)

Nova Blot (Amersham)

Centrifuge 5415R (Eppendorf)

Multifuge 1 S-R Centrifuge (Heraeus)

Incubator (Heraeus)

Kodak Image Station 2000R (Kodak)

Leica TCS-NT laser-scanning confocal microscope (Leica Microsystems)

LSR flow cytometer (BD Bioscience)

FACS Calibur flow cytometer (BD Bioscience)

PCR machine (Bio-Rad)

Pipette boy (Eppendorf)

Scintillation counter (1450 MicroBeta Trilux; PerkinElmer)

Thermomixer compact (Eppendorf)

Documentation station (Herolab GmbH)

Whell shaker Duomax 1030 (Heidolph)

NeoLab Rotator 2-1175 (NeoLab)

Bio-Rad Mini DNA system (Bio-Rad)

Bio-Rad Mini protein system (Bio-Rad)

Neubauer counting chamber (Marienfeld)

Coverslip 24x50 mm (for immunofluorescence) (Roth)

Coverslip (for Neubauer counting chamber) (Roth)

12 spot slide (precoated with poly-L-Lysine) (Marienfeld)

2.1.2. Plastic ware

Eppendorf tubes (Eppendorf)

PCR soft tubes (Biozym)

FACS tubes (Falcon)

Plastic pipettes (5 ml, 10 ml, 25 ml, 50 ml) (Costar)

Eppendorf tips (10 µl, 200 µl, 1 ml) (Eppendorf)

Syringes (2 ml, 20 ml) (BD Bioscience)

15 ml and 50 ml tubes (Greiner)

96 well cell culture plates (TPP)

96 well cell culture plates (U bottom) (Costar)

48 well cell culture plates (Nunclon)

40 µm diameter pore-size strainers (BD Bioscience)

2.1.3. Kits

Nucleospin Tissue kit (Macherey-Nagel, # 740 952.250)

Rh Annexin V/FITC kit (Bender MedSystems, # bms306FICE)

Caspase-3 detection kit (Calbiochem, # QIA91)

Pan T cell isolation kit, mouse (Miltenyi Biotec, # 130-096-861)

CD8 T cell isolation kit, mouse (Miltenyi Biotec, # 130-090-859)

Taq-polymerase kit (PeqLab, # 01-1030)

ECL Western Blotting detection reagent (Amersham, # 2106)

2.1.4. Chemical Reagents

30% Acrylamide/BIS, 37.5:1 (Bio-Rad, # 161-0158)

Acetic acid (Roth, # 3738.1)

Agarose (Peqgold universal agarose) (PeqLab, # 35-1020)

APS (Ammoniumpersulfat) (Roth, # 9592.3)

2-mercaptoethanol (Sigma, # M7154)

Bromphenolblue (Roth, # A512.1)

BSA (Bovine Serum Albumin) (Sigma, # A9647)

dNTP 25 mM (Fermentas, # 0161)

DMSO (Dimethyl Sulfoxide) (Roth, # A994.2)

EDTA (Ethylenediaminetetraacetic acid) (Sigma, # E5134)

Ethanol, 99% (Roth, # 9065.1)

Ethidium bromide, 10 mg/ml (Roth, # 2218.1)

Fetal Bovine Serum (FBS) (Pan Biotech GmbH, # P30-3302)

GeneRuler 50 bp DNA Ladder (Fermentas, # SM0371)

Glycerol (Sigma, # G6279)

Glycine (Roth, # 3908.2)

Horse serum (Biochrom AG, # 9133)

Indo-1 (Invitrogen, # I1223)

Ionomycin (Sigma, # I3909)

Igepal CA-630 (Nonidet P-40 (NP-40)) (Sigma, # I3021)

LM (lauryl maltoside / n-Dodycyl-β-D-maltoside) (Calbiochem, # 324355)

Methanol, 99.9% (Roth, # 4627.1)

Milk powder (Roth, # T145.2)

Mowiol 4-88 (Calbiochem, # 475904)

NaF (Sodium Fluoride) (Sigma, S-7920)

NaN$_3$ (Sodium Azide) (Roth, # K305.02)

NaCl (Sodium Chloride) (Roth, # 3957.2)

NH$_4$Cl (Ammonium Chloride) (Roth, # A4514)

Pageruler prestained protein ladder (Fermentas, # SM 0671)

PFA (Paraformaldehyde) (Merke, # 1.04005.1000)

PBS (Phosphate Buffered Saline) (Biochrome, # L1825)

Penicillin/Streptomycin 10000U/10000 µg/ml (Biochrom AG, # A2213)

Phalloidin-TRITC (Tetramethylrhodamine B isothiocyanate) (Sigma, # P1951)

PMA (Phorbol Myristate Acetate) (Sigma, # P8139)

PMSF (Phenylmethylsulfonyl Fluoride) (Roth, # 6367.1)

Ponceau S (Sigma, # P-3504)

Protein-A agarose beads (Santa Cruz, # sc-2001)

Roti Nanoquant reagent (Roth, # K880.1)

RPMI 1640 medium (Biochrome, # FG1215)

RPMI 1640 medium without phenol red (Invitrogen, # 11835)

SDS (Sodium Dodecyl Sulfate) (Roth, # 2326.2)

Streptavidin (Dianova, # 016-000-113)

TEMED (Tetramethylethylenediamine) (Roth, # 2367.3)

[^{3}H]-Thymidine (MP Biomedicals, # 24043)

Tris (Tris (hydroxymethyl)-aminomethan) (Roth, # 4855.2)

Triton X-100 (Sigma, # T9284)

Trypan blue solution 0.4% (Sigma, # T8154)

Tween 20 (Roth, # 9127.2)

Xylenxylanol (Roth, # A513.1)

2.1.5. Antibodies

2.1.5.1. Antibodies for stimulation

Biotin-conjugated anti-mouse CD3ε monoclonal antibody (mAb) (hamster) (BD Bioscience, # 553060)

Biotin-conjugated anti-mouse CD8α mAb (rat) (BD Bioscience, # 553029)

Biotin-conjugated anti-mouse CD8β mAb (rat) (BD Bioscience, # 553039)

Biotin-conjugated anti-mouse TCR β chain mAb (hamster) (BD Bioscience, # 553168)

2.1.5.2. Antibodies for flow cytometric analysis

FITC (Fluorescein-5-isothiocyanate)-conjugated anti-mouse Vα2 T-cell receptor mAb (mouse) (BD Bioscience, # 553288)

FITC-conjugated anti-mouse CD8α mAb (rat) (BD Bioscience, # 553031)

FITC-conjugated anti-Fas ligand mAb (mouse) (Kamiya Biomedical Co, # MC-136)

FITC-conjugated anti-Fas receptor mAb (hamster) (BD Bioscience, # 15404D)

FITC-conjugated anti-mouse LAMP1 mAb (rat) (BD Bioscience, # 553793)

FITC-conjugated anti-mouse CD69 mAb (mouse) (BD Bioscience, # 553236)

PE (Phycoerythrin)-conjugated anti-mouse CD3ε mAb (hamster) (BD Bioscience, # 553063)

PE-conjugated anti-mouse CD25 mAb (rat) (BD Bioscience, # 553866)

PE-conjugated anti-mouse CD4 mAb (rat) (BD Bioscience, # 553049)

Cy5 (Cyanine 5)-conjugated anti-mouse CD4 mAb (rat) (BD Bioscience, # 553050)

2.1.5.3. Antibodies for Western blotting and Immunoprecipitation (IP)

Anti-phospho-ZAP-70 (Tyr 319) /Syk (Tyr 352) antibody (rabbit) (Cell Signaling, # 2701)

Anti-phospho-PLCγ1 (Tyr 783) antibody (rabbit) (Santa Cruz, # 12943)

Anti-phospho-LAT (Tyr 171) antibody (rabbit) (Cell Signaling, # 3581)

Anti-phospho-PKD (Ser 916) antibody (rabbit) (Cell Signaling, # 2054)

Anti-phospho-p44/p42 MAP kinase (Thr 202/Tyr 204) antibody (rabbit) (Cell Signaling, # 9101)

Anti-phospho-PKB (Ser 473) mAb (rabbit) (Cell Signaling, # 9271)

Anti-ubiquitin mAb (mouse) (Cell Signaling, # 3936)

Anti-ZAP-70 mAb (mouse) (BD Bioscience, # 610239)

Anti-ERK1/2 antibody (rabbit) (Cell Signaling, # 9102)

Anti-LAT mAb (mouse) (BD Bioscience, # 611108)

Anti-PKB antibody (rabbit) (Cell Signaling, # 9272)

Anti-PLCγ1 antibody (rabbit) (Santa Cruz, # sc-81)

Anti-Bcl-xL antibody (rabbit) (BD Bioscience, # 556361)

Anti-CD107a (LAMP-1) mAb (rat) (BD Bioscience, # 553792)

Anti-Bim antibody (rabbit) (BD Bioscience, # 559685)

Anti-CD3ζ mAb (mouse) (Santa Cruz, # sc-1239)

Anti-β-actin mAb (mouse) (Sigma, # A1978)

Anti-Rab5 mAb (mouse) (BD Bioscience, # 610281)

Anti-Ras mAb (mouse) (Oncogene, # AB-3)

2.1.5.4. Other antibodies

Anti-IL-2 mAb (rat) (BD Bioscience, # 554375)

2.1.5.5. Secondary antibodies

Peroxidase-conjugated affinipure goat anti-mouse IgG+IgM (H+L) (Jackson Immunoresearch, #115-035-068)

Peroxidase-conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson Immunoresearch, # 115-035-045)

Peroxidase-conjugated affinipure goat anti-rat IgG (H+L) (Jackson Immunoresearch, # 112-035-167)

FITC-conjugated affinipure donkey anti-rabbit IgG (H+L) (Jackson Immunoresearch, # 711-095-152)

FITC-conjugated affinipure goat anti-rat IgG (H+L) (Jackson Immunoresearch, # 112-095-167)

FITC-conjugated affinipure donkey anti-mouse IgG (H+L) (Jackson Immunoresearch, # 715-095-150)

Cy3 (Cyanine3)-conjugated affinipure goat anti-rat IgG (H+L) (Jackson Immunoresearch, # 112-165-167)

Cy3-conjugated affinipure goat anti-rabbit IgG (H+L) (Jackson Immunoresearch, # 111-165-144)

Cy3-conjugated affinipure donkey anti-mouse IgG (H+L) (Jackson Immunoresearch, # 715-165-151)

Cy5-conjugated affinipure donkey anti-mouse IgG (H+L) (Jackson Immunoresearch, # 715-175-151)

2.1.6. Streptamers

MHC-I-Strep H-2K^b Ovalbumin (IBA, # 6-7015-001)

Strep-Tactin (IBA)

Strep-Tactin PE (IBA, # 6-5000-001)

Strep-Tactin APC (Allophyocyanin) (IBA, # 6-5000-002)

2.1.7. Mouse strains

OT1-TCR transgenic (tg) mice were kindly provided by Dr. Percy Knolle (Institute of Molecular Medicine and Experimental Immunology, University of Bonn, Germany). Perforin knock-out (perforin$^{-/-}$) mice were kindly provided by Dr. Andreas Ambach (Department of Dermatology and Venerology, Otto-von-Guericke-University, Germany).

2.2. Methods

2.2.1. Animal experimentation

2.2.1.1. Mice condition and handling

Mice were kept in the central animal facility at the Otto-von-Guericke-University of Magdeburg and maintained in pathogen-free conditions. All experiments involving mice were performed according to the guidelines of the State of Sachsen-Anhalt, Germany.

2.2.1.2. Genomic DNA isolation from mouse tails

Genomic DNA was purified according to the manufacturer's protocol. Briefly, a 1 cm piece of mouse tail was provided from the animal facility and placed into a 1.5 ml eppendorf tube. The mouse tail was pre-digested by incubation with 180 μl buffer T1 (cell lysis solution) and 25 μl proteinase K solution at 56°C for 3-5 h. The mouse tail was further digested by incubating with 200 μl of lysis buffer for 10 min at 70°C, under constant shaking. The condition for DNA binding to the silica membrane was adjusted by adding 210 μl 99% ethanol and vortexing vigorously. The lysis solution was added next onto the column and centrifuged for 1 min at 11,000 g leading to DNA

binding to the silica membrane. The flow-through was discarded and the column was placed back into the collecting tube. The silica membrane was washed with 500 µl buffer BW and 600 µl buffer B5, respectively. The silica membrane was dried by centrifugation for 1 min at 11,000 g. DNA was eluted from the silica membrane with 100 µl prewarmed elution buffer BE (70°C) and centrifuged at 11,000 g for 1 min.

2.2.1.3. Genotyping of OT1-transgenic mice strain by PCR (Polymerase-Chain Reaction)

Genotyping of the OT1-transgenic mice was performed according to the PCR protocol established by Jackson Laboratories.

Primers used for genotyping (Metabion):

Primer 1 (internal control F): 5'- CAA ATG TTG CTT GTC TGG TG -3'

Primer 2 (internal control R): 5'- GTC AGT CGA GTG CAC AGT TT-3'

Primer 3 (OT1-TCR F): 5'- AAG GTG GAG AGA GAC AAA GGA TTC-3'

Primer 4 (OT1-TCR R): 5'- TTG AGA GCT GTC TCC-3'

The following reaction mix was pipetted into the PCR tube according to the manufacturer's protocol.

Primer 1	0.25 µl (1 µM)
Primer 2	0.25 µl (1 µM)
Primer 3	0.125 µl (0.5 µM)
Primer 4	0.125 µl (0.5 µM)
Taq polymerase	0.5 µl (2.5 U)
5 x enhancing buffer	5 µl
10 x reaction buffer	2.5 µl
dNTP (mix)	0.25 µl (200 µM)
dH$_2$O	up to 23.5 µl

To each reaction 1.5 µl DNA was added.

The following program was run in a PCR machine:

Hot start	94°C, 3 min	
Denaturation	94°C, 30 sec	
Hybridization	52°C, 30 sec	38 cycles
Elongation	72°C, 30 sec	

Final elongation   72°C, 2 min

2.2.1.4. Gel electrophoresis of nucleic acids

1 x TAE buffer: 10 mM Tris

                                                    0.1142% acetic acid

                                                    1 mM EDTA pH 8.0

Agarose gel 2%:   2 g agarose

                                                    100 ml 1 x TAE buffer

                                                    10 µl ethidium bromide (10 mg/ml)

6 x loading buffer: 30% glycerol

                                                    20 mM Tris (pH 7.6)

                                                    2 mM EDTA

                                                    0.02% bromphenolblue

                                                    0.02% xylenxylanol

Marker: GeneRuler 50 bp DNA Ladder

The reaction was analyzed by mixing the PCR products with 6 x loading buffer and loaded on a 2% agarose gel supplemented with ethidium bromide. Electrophoresis was carried out with a Bio-Rad Mini DNA system in TAE-buffer at 100 V for 30 min. DNA fragments were visulized by UV light and picture was taken by the documentation station.

2.2.2. T cell purification

2.2.2.1. Preparation of single cell suspension from mouse spleen

Mice were sacrificed and the spleens were removed. To disrupt the organs and to release the cells, the spleen was mashed through a 40 μm diameter pore-size strainer with a syringe plunger at RT (Room Temperature). Cells were collected in a 15 ml tube and washed by adding ice-cold PBS to a final volumn of 15 ml and centrifuged for 10 min, 370 g, at 4°C. The pellet was resuspended in 10 ml ice-cold PBS and the cell number was determined.

2.2.2.2. Cell counting

Trypan blue solution: 0.4% in PBS

Trypan blue solution was first diluted to 0.1% with PBS. The cell number was determined using a Neubauer counting chamber. An aliquot of the cell suspension was mixed with an equal volume of 0.1% trypan blue solution in PBS. Cell solution was pipetted into the Neubauer counting chamber. Cells in 1×10^{-4} ml were counted and the concentration of cells was determined using the following formula:

Negative trypan blue cells in one quadrant x 2 x dilution factor x 10^4 = cells /ml.

Mean values of four quadrants were always calculated.

2.2.2.3. Purification of CD8$^+$ T cell

Because OT1-TCR tg mice contain only CD8 T cells, a Pan T cell isolation kit was used to purify these cells. The single cell suspension described in 2.2.2.1 was centrifuged at 370 g for 10 min. The cell pellet was resuspended in ice-cold PBS at a concentration of 10×10^6 cells/45 µl. Staining was performed with 5 µl Biotin-Antibody Cocktail (cocktail of biotin-conjugated monoclonal antibodies against CD11b (Mac-1), CD45R (B220), DX5 and Ter-119) and incubated for 10 min on ice. Subsequently, the cell suspension was mixed together with 40 µl of ice-cold PBS per 10×10^6 cells and 10 µl of anti-Biotin MicroBeads. The mixture was incubated for 15 min on ice. The cells were next washed once with 10 ml of ice-cold PBS and centrifuged at 370 g for 10 min. The supernatant was removed and the cell pellet was resuspended in PBS at the concentration of 1×10^8 cells/ml. The magnetic separation was performed by using the Auto-MACS separator. The purity of isolated cells was determined by flow cytometry.

Since perforin$^{-/-}$ mice were not crossed onto the OT1-transgenic background, a CD8$^+$ T cell isolation kit was used to isolate CD8$^+$ T cells from perforin$^{-/-}$ mice. The purification procedure was the same as described above.

2.2.3. T cell stimulation *in vitro*

2.2.3.1. T cell stimulation in vitro with OT1-streptamers

Mouse medium: RPMI-1640

                                                       10% FBS

                                                       1% antibiotics (penicillin and streptomycin)

                                                       1.75 µl 2-mercaptoethanol (in 500 ml RPMI)

Prior to stimulation of T cells with streptamers, 0.75 µg Strep-Tactin was incubated with recombinant monomeric biotinylated-MHC-I (1 µg) in a final volume of 50 µl with PBS or mouse medium at 4°C for 45 min according to the manufacturer's instructions. Recombinant monomeric biotinylated H-2K^b molecules presenting the ovalbumin SIINFEKL peptide for the OT1–TCR were used in this study. 2 x 10^6 purified T cells were resuspended in 50 µl PBS or mouse medium. Subsequently, the cell solution was mixed with the OT1-streptamers (pMHC-strep-tactin-PE) at 37°C for the indicated time points. Stimulation was stopped by adding 1 ml ice-cold PBS. The procedure was continued according to the specific purpose.

2.2.3.2. CD3/CD8 mAbs stimulation

2 x 10^6 purified T cells were resuspended in 100 µl of PBS or mouse medium. Before stimulation, the cells were pre-incubated with biotinylated CD3ε mAb and biotinylated CD8 mAb (both ranging from 10 µg/ml to 1 µg/ml) at 37°C for 1 min. Stimulation started when cells were crosslinked with 50 µg/ml streptavidin. 1 ml of ice-cold PBS was added to stop the reaction. The procedure was continued according to the specific purpose.

2.2.3.3. Plate-bound CD3/CD8 mAbs stimulation

Fourty-eight well plates were coated with 10 µg/ml of CD3/CD8 mAbs, overnight at 4°C. Subsequently, the plates were washed three times with PBS. Purified T cells were cultured in mouse medium at the concentration of 2 x 10^6/300 µl. Cell suspension was added to the antibody-coated plate and maintained in culture for the indicated time at 37°C and 5% CO_2. The stimulation was stopped by adding 1 ml ice-cold PBS. The procedure was continued according to the specific purpose.

2.2.4. Surface staining and FACS (Fluorescence Activated Cell Sorting) analysis

2.2.4.1. Extracellular staining

1 x 10^6 cells were centrifuged for 5 min, 400 g, at 4°C. The pellet was resuspended in 100 µl of the indicated antibody solution. After 30 min of incubation in the dark at 4°C, the cells were washed with PBS. The cell pellet was resuspended in 300 µl PBS and measured by the FACS Calibur machine. Data were analyzed using the CellQuest software.

2.2.4.2. TCR internalization assay

Purified T cells were left untreated or stimulated with CD3/CD8 mAbs or OT1-streptamers for 30 min, 1 h and 2 h at 37°C. Cells were then incubated with FITC-conjugated TCR-Vα2 antibody for 15 min at 4°C in the dark. The expression of TCR-Vα2 on the cell surface was analyzed by flow cytometry.

2.2.5. Proliferation assay

Purified T cells were cultured in mouse medium using U-bottomed 96-well plates at a concentration of 2.5×10^4 cells/well. Cells were left either untreated or stimulated with OT1-streptamers (1 μg), CD3/CD8α (10 μg/ml), CD3/CD8β (10 μg/ml), CD3/CD8α/CD8β (10 μg/ml), TCR/CD8α (10 μg/ml), and PMA (40 ng/ml)/ionomycin (100 ng/ml) for 72 h at 37°C. Cells were then labeled with [^{3}H]-thymidine (0.3 μCi/well from ICN) for 8 h, harvested onto glass fiber filter and counted with a scintillation counter.

2.2.6. Apoptosis assay

Cell apoptosis was analyzed by the ability of annexin V to bind to exposed phosphatidylserine residues at the outer leaflet of the plasma membrane in combination with the application of PI (Propidium Iodide). Purified T cells were resuspended in mouse medium at the concentration of 1×10^6 cells/ml in a 48-well cell culture plate. Cells were left untreated or treated with OT1-streptamers (1 μg) or CD3/CD8α (10 μg/ml) at 37°C for 8 h and 24 h, respectively. Cells were next harvested and washed with PBS. The cell pellet was resuspended in 195 μl of 1x binding buffer. The staining was performed by mixing the cell solution with 5 μl of annexin V-FITC and incubating for 10 min at RT in the dark. The cells were washed once with PBS and resuspended in 190 μl 1x binding buffer together with 10 μl PI. Samples were analyzed by flow cytometry within one hour. The apoptosis analysis was performed according to the protocol established by Bender MedSystems.

2.2.7. Caspase-3 activity assay

To detect activated caspase-3 in living cells, the caspase-3 detection kit was used. Caspase-3 activity was measured using FITC-DEVD-FMK, which is a cell-permeable, non-toxic inhibitor that binds irreversibly to the activated caspase-3 in apoptotic cells. T cells were resuspended in mouse medium at the density of 1×10^6 cells/ml and left untreated or treated with 10 μg/ml CD3/CD8 mAbs or 1 μg OT1-streptamers, for 8 h

and 24 h at 37°C. Cells were then transferred into a FACS tube, washed with PBS and resuspended in 1 ml PBS. Caspase-3 activity was assessed by incubation wih 1 µl of FITC-DEVD-FMK for 45 min at 37°C in the dark. Cell were washed once with PBS and resuspended in 300 µl PBS. Caspase-3 activity was assessed by flow cytometry.

2.2.8. Calcium flux determination

Calcium flux measurements were performed by measuring the ratio of the fluorescent signals given by staining with the Ca^{2+}-binding fluorochrome Indo-1AM to either saturated or free Ca^{2+} molecules. $CD8^+$ T cells (2 x 10^7 cells/ml) were resuspended in RPMI-1640 medium (phenol-red free; supplemented with 10% FCS) and loaded with 3.75 µg/ml Indo-1-AM at 37°C for 45 min. After washing, the cells were incubated in the same medium at 37°C for additionally 45 min followed by centrifuging for 10min, 400 g. Cell pellets were resuspended in the same medium at the concentration of $2x10^6$ /100 µl. Cell suspension was incubated either with CD3/CD8 mAbs (each 10 µg/ml) at 37°C for 2 min to establish the baseline and cross-linked with streptavidin (50 µg/ml) or with OT1-streptamers (1 µg/reaction). As a cell-autonomous Ca^{2+} "charge" control, full-scale deflection of the calcium flux was measured by addition of ionomycin (10 µg/ml). Data for calcium mobilization were acquired on an LSR flow cytometer and ratiometric analysis was performed using the Flow Jo software.

2.2.9. Immunoblotting

2.2.9.1. Cell lysis

Lysis buffer: 1% LM

                                                 1% NP-40

                                                 1 mM Na-monovanadate

                                                 1 mM PMSF

                                                 50 mM Tris-HCl (pH 7.4)

                                                 10 mM NaF

                                                 10 mM EDTA

                                                 0.16 M NaCl

After stimulation, $2x10^6$ cells were washed once with ice-cold PBS. Cells were resuspended in 40 µl lysis buffer and incubated for 20 min on ice. Samples were then centrifuged for 10 min at 16,000 g, 4°C and the post-nuclear supernatant was transferred into a new eppendorf tube.

2.2.9.2. Protein concentration measurement

BSA standard (0-100 μg/ml)

Roti-Nanoquant

Protein concentrations were determined by using the Bradford protein assay. According to the manufacturer's protocol, a working solution was prepared by diluting the 5x Roti-Nanoquant to 1x with dH_2O. Samples were pre-diluted with dH_2O. 50 μl of the prediluted samples were then transferred to a 96-well plate and incubated with 200 μl of 1x Roti-Nanoquant. Absorption was measured using an ELISA reader at 570 nm. BSA (0-100 μg/ml) was used as standard and the protein concentration was calculated on the basis of the derived standard curve.

2.2.9.3. SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

SDS is an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures and applies a negative charge to proteins in proportion to their mass. In SDS-PAGE, the migration of proteins is related to their molecular weight.

10% and 12% SDS-PAGE separating gel: 4.2 ml (10%) or 3.44 ml (12%) dH_2O

2.46 ml 1.5 M Tris-HCl, pH 8.8

3.2 ml (10%) or 3.94 ml (12%) 30% acrylamide/BIS

0.1 ml 10% SDS

0.05 ml 10% APS

5 μl TEMED

SDS-PAGE stacking gel: 2.4 ml dH_2O

0.5 ml 30% acrylamide/BIS

1 ml 0.5 M Tris-HCl pH 6.8

0.04 ml 10% SDS

0.04 ml 10% APS

0.004 ml TEMED

SDS-PAGE running buffer (1x):   25 mM Tris,

250 mM glycine,

0.1% SDS

5x reducing loading buffer :	50% glycerol
	330 mM Tris, pH 6.8
	10% SDS
	0.01% bromphenolblue
	10% 2-mercaptoethanol

| Marker: | pageruler prestained protein ladder |

Cell lysates and 5x sample buffer were mixed and boiled at 99°C for 5 min. The samples were then loaded and resolved on a 10% or 12% SDS-PAGE gel. For each lane, 20 µg of total protein were loaded. Electrophoresis was conducted with Bio-Rad Protein system. Gels were run at 120 V for 90 min.

2.2.9.4. Western blotting analysis and immunoblotting

Protein transfer buffer (1x):	39 mM glycine
	48 mM Tris
	0.037% SDS
	20% methanol

| TBS | 0.01M Tris |
| | 0.15M NaCl |

| Blocking buffer: | 5% milk powder in TBS |

| Washing buffer: | 0.02% Tween 20 in TBS |

| Ponceau S solution: | 0.1% Ponceau S in 5% acetic acid |

| HRP inactivation buffer: | 1% NaN_3 in PBS |

Stripping buffer:	0.7% 2-mercaptoethanol
	2% SDS
	62.5 mM Tris-HCl (pH 6.8)

The proteins seperated by gel electrophoresis were then transferred to a nitrocellulose membrane, where they were detected using antibodies specific to the target proteins. In some experiments, equal loading was controlled by incubation of the membrane with Ponceau S solution for 2 min. The staining was removed by washing with TBS. Blocking of the membrane was performed using blocking buffer for 30 min to prevent unspecific binding to the membrane. Subsequently, membranes were probed with a primary antibody for 1 h at RT. After washing 3 times with washing buffer, the membrane was incubated with the appropriate peroxidase-conjugated secondary antibody for 1 h at RT. The membrane was further washed 3 times with washing buffer and the bound antibodies were then visualized using an ECL (Enhanced Chemiluminescence) detection system according to the manufacturer's instructions. The intensity of the detected bands was acquired using the Kodak Image station 2000R and analysis was performed using ID Image software (Kodak). The membranes can be stripped and reprobed a number of times by incubating with stripping buffer for 20 min at 50°C. The stripping buffer was used to remove the bound primary and secondary antibodies from the membrane. Prior to the addition of the next primary antibody, the membrane was blocked for 30 min with blocking buffer. In cases where the primary antibodies were derived from different species, the peroxidase of the secondary antibodies was inactivated with 1% NaN_3 in PBS for 45 min at RT.

2.2.9.5. Immunoprecipitation

Washing buffer: 0.1% LM

                                                                 0.1% NP-40

                                                                 1 mM PMSF

                                                                 50 mM Tris-HCl pH 7.4

                                                                 10 nM NaF

                                                                 0.1% NP-40

                                                                 0.16 M NaCl

Immunoprecipitation (IP) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. The antibody is coupled to a solid substrate such as protein A or protein G agarose beads. In our experiments, 40 x 10^6 cells were left untreated or stimulated with OT1-streptamers or CD3/CD8 mAbs for 3 min. Cells were then lysed in 500 µl ice-cold lysis buffer for 20 min on ice. After centrifugation (16,000 g, 10 min, 4° C), the post-

nuclear supernatant was taken and 2 µl of anti-ZAP-70 antibody as well as 30 µl protein-A agarose beads were added. To reduce non-specific binding, 1/10 volume of 10mg/ml BSA was added. The samples were incubated on a rotating wheel for 2 hours at 4° C. After washing 5x with 1 ml ice cold NP-40 washing buffer, the beads were incubated with 2x reducing loading buffer at 99°C for 5 min.

2.2.10. Immunofluorescence

12 spot slide (precoated with poly-L-Lysine)

Fixation solution:	3.5% PFA in PBS
Permeabilisation solution:	0.3% Triton X-100 in PBS
Blocking solution:	5% horse serum in PBS
Mounting medium:	2.4 g mowiol-488
	6 g glycerol dissolved in 12 ml 0.2 M
	Tris (pH 8.5)

Cells were suspended in PBS/BSA (0.5%) at a concentration of $2 \times 10^6/100$ µl. 20 µl of the cell suspension was dropped per spot of a 12-spot slide and incubated at 4°C for 15 min to allow the cells to attach. Unattached cells were removed by washing in PBS. Cells were next fixed for 15 min followed by permeabilization for 10 min at RT. After washing for 3 times, the slide was blocked with 5% horse serum/PBS for 15 min at RT. The cells were then stained with the primary antibody for 60 min at RT. After 3 times washing, the cells were further incubated with the secondary antibody for 60 min in the dark at RT. Following three additional washing steps the samples were embedded in mounting media and the coverslips were fixed to the slide with nail polish. F-actin was detected by incubating with TRITC-phalloidin for 1 h at RT in the dark. The cells were imaged on a LEICA TCS SP2 laser-scanning confocal system and analyzed with the LEICAsoftware

3. Results

The affinity of the TCR for its ligand is one of the critical parameters regulating both thymocyte development and peripheral T cell activation (see Figure 8). In the thymus, intermediate affinity ligands induce survival and positive selection whereas high affinity stimuli direct thymocytes to apoptosis, a process known as negative selection. Thus, one receptor can induce two distinct cellular programs (differentiation or cell death). However, despite the progress in our understanding of the signaling pathways regulating T cell function, how the TCR can transmit signals leading to distinct cellular responses is so far not completely understood. Therefore, in this study, I utilized OT1-TCR tg mice as these mice possess a TCR of defined specificity. The OT1-TCR contains TCR-Vα2 and TCR-Vβ5 chains which recognize ovalbumin residues 257-264 (SIINFEKL) in the context of H2K^b. T cells were stimulated with two classical stimuli: (1) biotinylated CD3ϵ and CD8α mAbs that were cross-linked with streptavidin in solution and (2) the more physiological stimuli OT1-streptamers (Figure 13). OT1-streptamers are composed of biotinylated H-2K^b molecules loaded with the ovalbumin peptide and are cross-linked with Strep-Tactin. Both stimuli only bind the TCR and the costimulatory molecule CD8, however with different affinity and avidity. These cross-linked antibodies in solution have a much higher affinity (K_D=2.4 x 10^{-9} M) than streptamers (K_D=7 x 10^{-6} M) [93, 94] and, thus result in a much stronger stimulation of the cells.

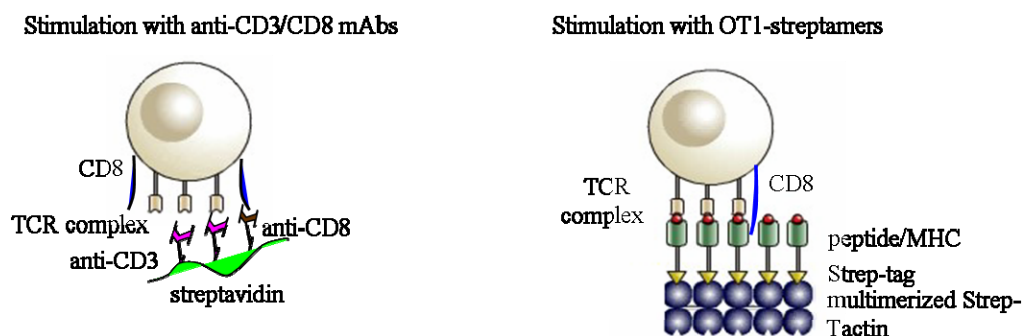*Figure 13. Model of TCR triggering with either anti-CD3/CD8 mAbs or OT1-streptamers*

CD8 positive T cells were either treated with soluble biotinylated CD3ϵ mAbs together with biotinylated CD8α mAbs, which were subsequently cross-linked with streptavidin or they were incubated with biotinylated H-2Kb molecules loaded with the high-affinity peptide SIINFEKL and cross-linked with Strep-Tactin (OT1-streptamers).

3.1. TCR triggering with soluble CD3/8 mAbs causes apoptosis while OT1-streptamers induce proliferation

3.1.1. Quality control of OT1-streptamers

To evaluate the specific binding of OT1-streptamers to the OT1-TCR, purified splenic T cells from OT1-TCR tg mice were incubated with OT1-streptamers labeled with PE. After 30 min incubation at 4°C, the cells were further stained with CD8-FITC and analyzed for binding to the OT1-streptamers by flow cytometry. As shown in Figure 14, 95.1% of the gated CD8$^+$ T cells bind to OT1-streptamers. I speculated that the remaining 5% CD8$^+$ T cells that do not bind the OT1-streptamers are not transgenic cells, but rather possess endogenous TCR since the OT1-transgenic mice used here are not on the Rag$^{-/-}$ background. Rag is a family of proteins that are critical for the recombination and the generation of the TCR during thymocyte development. On the Rag$^{-/-}$ background, the endogenous TCR rearrangement is blocked at DN3 stage, thus there are no peripheral T cells [95, 96].
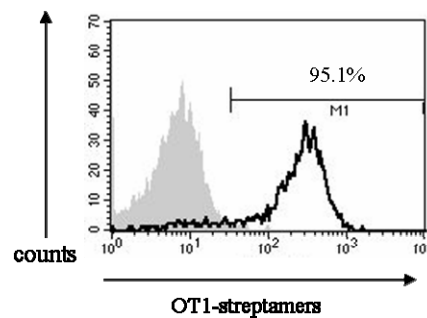

Figure 14. Specific binding of OT1-TCR to OT1-streptamers

Splenic T cells were isolated from OT1-tg mice and incubated with PE-conjugated OT1 streptamers for 30 min on ice. After washing, the cells were further stained with CD8-FITC. The gated CD8+ T cells were analyzed for binding of OT1-streptamers by means of flow cytometry. Data are representative of three individual experiments.

It is reported that the SIINFEKL peptide induces negative selection in thymocytes [61]. To characterize the OT1-streptamers functionally, freshly isolated thymocytes from OT1-tg mice were left untreated or treated with OT1-streptamers for 24 h. Soluble CD3/CD8 mAbs, also known to induce negative selection [97], were used as positive control. The viability of thymocytes was determined by trypan blue staining and the number of viable thymocyte in the untreated sample was taken as 100%. Figure 15A demonstrates that following streptamer stimulation, the rate of cell survival was reduced to 70% compared to that without stimulation. Only 40% of cells survived after antibody stimulaton. To further confirm that OT1-streptamers induce negative

selection, thymocytes were stained with CD4 and CD8 antibodies and the gated living cells were analyzed by flow cytometry 24 h after stimulation. An alteration in the composition of thymocyte subpopulations was observed, with a clear reduction of $CD4^+CD8^+$ DP immature thymocytes and a strong enhancement of $CD4^-CD8^-$ DN thymocytes following OT1-streptamer stimulation (Figure 15B). Moreover, streptamers reduced the propotion of $CD8^+$ SP thymocytes. These data indicate that OT1-streptamers not only trigger the negative selection of DP thymocyte, but also induce the second thymic selection of SP thymocytes in thymic medulla. The second thymic selection is important for deletion of self-reactive cells as well as of dysfunctional cells [98]. As expected, soluble anti-CD3/CD8 mAbs lead to deletion of both DP and $CD8^+$ SP thymocytes.
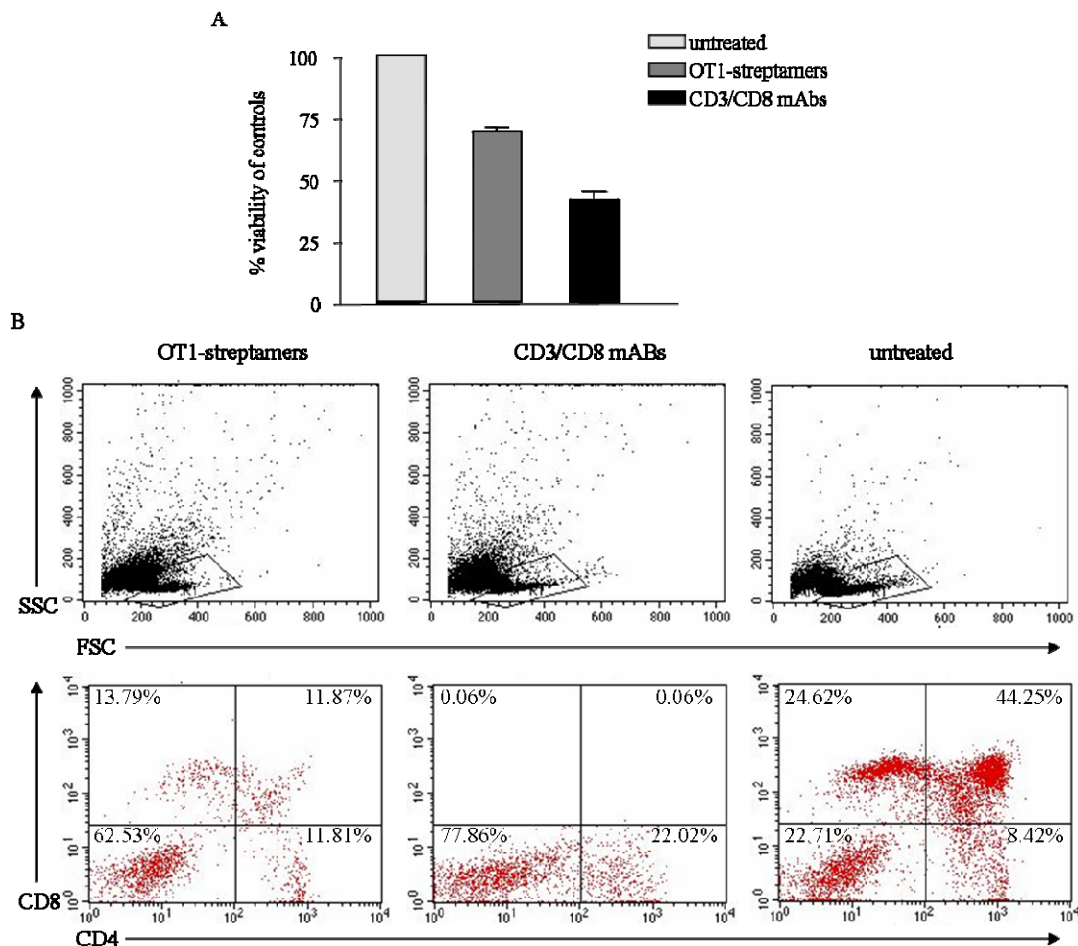
Figure 15. *OT1-streptamers trigger negative selection*

(A). Freshly isolated thymocytes were left untreated (left) or treated for 24 h with OT1-streptamers (1 µg) (middle) or soluble CD3/CD8 mAbs (10 µg/ml) (right). Viability was determined by trypan blue staining and the number of viable thymocyte in untreated sample was set as 100%. (B). Following 24 h incubation, the thymocytes were stained with CD4-PE and CD8-FITC. The gated living cells were analyzed for the expression of CD4 and CD8 by flow cytometry. Data are representative of three individual experiments.

3.1.2. TCR triggering of peripheral T cells with OT1-streptamers leads to T cell proliferation

To elucidate how one receptor can induce two distinct cellular responses in peripheral T cells, I attempted to identify the ligands that distinguish proliferation and apoptosis. Peripheral T cells were stimulated with either soluble antibodies or OT1-streptamers. After 72 h stimulation, CD3/CD8 mAbs treatment failed to induce proliferation of the splenic T cells, while OT1-streptamers led to robust proliferation (Figure 16A).

To address whether lower concentrations of antibodies might induce T cell proliferation, I tested a range of antibody concentrations from 10 μg/ml to 0.3 μg/ml and assessed proliferation by [^{3}H]-thymidine incorporation after 72 h treatment. PMA/ionomycin was used as positive control for proliferation. Figure 16B shows that reducing the concentration of antibodies still did not result in proliferation. Therefore, OT1-streptamer stimulation induces vigorous T cell proliferation while a similar effect cannot be observed with soluble antibodies independent of their concentration. As expected, PMA/ionomycin induced vigorous proliferation. Note that higher concentration of streptamers still triggers strong proliferation (data now shown).
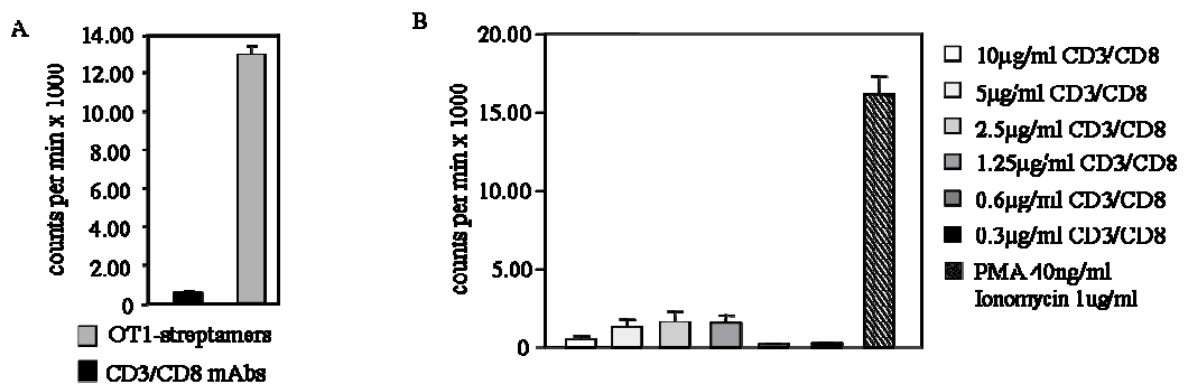
Figure 16. OT1-streptamer stimulation leads to proliferation

(A). Equal numbers of CD8+ T cells were cultured with soluble anti-CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for 72 h. Proliferation was subsequently assessed by [^{3}H]-thymidine incorporation. (B). Purified T cells were stimulated with different concentrations of antibodies (as indicated). PMA/ionomycin (40 ng/ml/1 μg/ml) were used as positive control to induce proliferation. Data represent the averages ± SEM from triplicate cultures of three individual experiments.

The TCR complex is composed of an α chain, a β chain, the CD3 complex, and the ζ chains while the coreceptor CD8 comprises CD8α and CD8β, which recognize non-

polymorphic regions within MHC-I molecules [99]. To further investigate the function of different components of the CD8 and the TCR complex, I treated purified OT1-TCR tg T cells with different antibody combinations, such as soluble CD3ε/CD8α mAbs, CD3ε/CD8β mAbs, CD3ε/CD8α/CD8β mAbs, and TCRβ/CD8α mAbs for 72 h. However, none of the antibody combinations tested induced T cell proliferation (Figure 17). In contrast, PMA/ionomycin gave rise to strong proliferation. Therefore, the engagement of different components of the TCR complex and the CD8 subunits with soluble antibodies cannot induce proliferation.
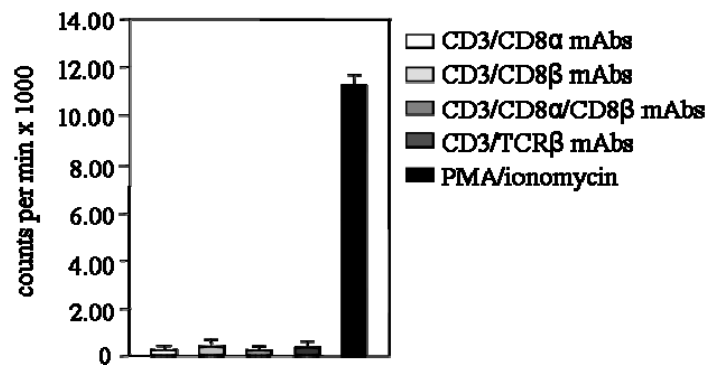
Figure 17. Engagement of different components of the TCR complex and CD8 subunit by antibodies does not induce proliferation

Equal numbers of CD8+ T cells were cultured with soluble CD3ε/CD8α mAbs, CD3ε/CD8β mAbs, CD3ε/CD8α/CD8β mAbs, and TCRβ/CD8α (10 μg/ml) for 72 h. Proliferation was assessed by [3H]-thymidine incorporation. Data represent the averages ± SEM from triplicate cultures of three individual experiments. PMA/ionomycin (40 ng/ml/1 μg/ml) was used as positive control for proliferation.

To exclude the possibility that the presence of excessive antibody is toxic to the cells due to continuous restimulation of the cells, I pre-incubated OT1-TCR tg T cells with cross-linked antibodies for different periods of time. The T cells were subsequently washed to remove unbound antibodies, excluding the continuous restimulation, and incubated in medium for another 72 h. As shown in Figure 18, the removal of unbound antibodies did not induce proliferation. Therefore, soluble anti-CD3/CD8 mAbs cannot induce proliferation.

Figure 18. Removal of unbound antibodies does not induce proliferation.

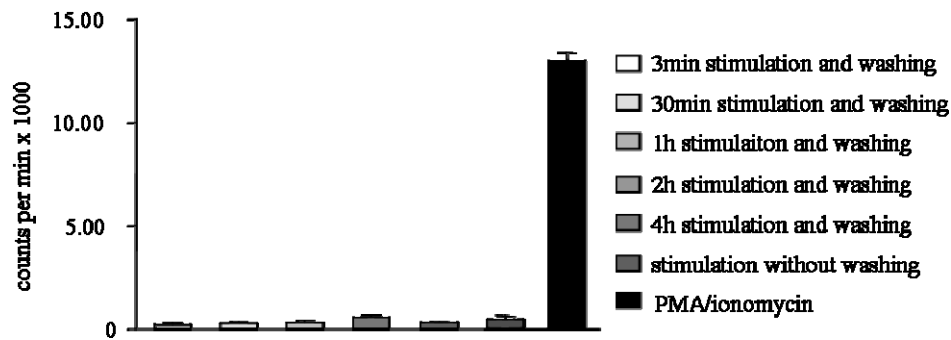Purified CD8^{+} T cells were stimulated with CD3/CD8 mAbs (10 µg/ml) for the indicated periods of time. The cells were then washed with PBS to remove the unbound antibodies. Cell pellets were resuspended in medium and incubated for another 72 h. Proliferation was then assessed by [³H]-thymidine incorporation. Data represent the averages ± SEM from triplicate cultures of one experiment. PMA/ionomycin (40 ng/ml/1 µg/ml) was used as positive control for proliferation.

3.1.3. The susceptibility of CD8^{+} T cells to apoptosis in response to antibody stimulation

3.1.3.1. Antibody stimulation induces apoptosis

The lack of proliferation in response to CD3/CD8 mAbs could be due to either the induction of T cell unresponsiveness or apoptosis. To distinguish between these two possibilities, purified T cells from OT1-tg mice were stimulated with either CD3/CD8 mAbs or OT1-streptamers for 8 h and 24 h. Apoptosis was subsequently determined by staining with annexin V and PI (Propidium Iodide). Annexin V is a protein that has a high affinity for phosphatidylserine, which translocates from the inner face of the plasma membrane to the cell surface whenever apoptosis has been initiated. PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centred around 600 nm. Viable cells with an intact plasma membrane exclude PI, whereas membranes of dead and damaged cells are permeable to this dye. Figure 19 shows an increase in the annexin V[+] and PI[-] early apoptotic cell population (40.84%) after 8 h stimulation with soluble antibodies. In contrast, after stimulation with OT1-streptamers only 9.55% of the cell population showed signs of early apoptosis. After 24 h stimulation with antibodies the total amount of the apoptotic fraction (PI[+]) reached up to 80.01%,

whereas the majority of the cells remained viable after incubation with OT1-streptamers (71.90%) (annexin V$^-$ / PI$^-$). Hence, antibody stimulation induces apoptosis. It is known that mouse T cells rapidly undergo apoptosis when they are cultured *in vitro* in the absence of any stimuli [100]. Therefore, it was not clear whether the apoptosis observed after antibody stimulation was not simply due to the *in vitro* culture conditions. To answer this question, I compared T cell apoptosis after 8 h and 24 h stimulation in the absence or presence of antibody. The effects of soluble antibodies to induce apoptosis was not apparent compared to the untreated control after 24 h stimulation, since there was only 10% difference in apoptosis between the untreated cells (68%) (PI$^+$) and antibody stimulated cells (80%) (PI$^+$) (Figure 19B). However, the effects of antibodies to induce apoptosis were more evident when cells were cultured for 8 h. Indeed, only 23% of cells (annexin V$^-$ / PI$^-$) survived after antibody stimulation, compared to 65% (annexin V$^-$ / PI$^-$) in the untreated sample (Figure 19A). The data clearly demonstrated that antibody stimulation actively induced apoptosis.
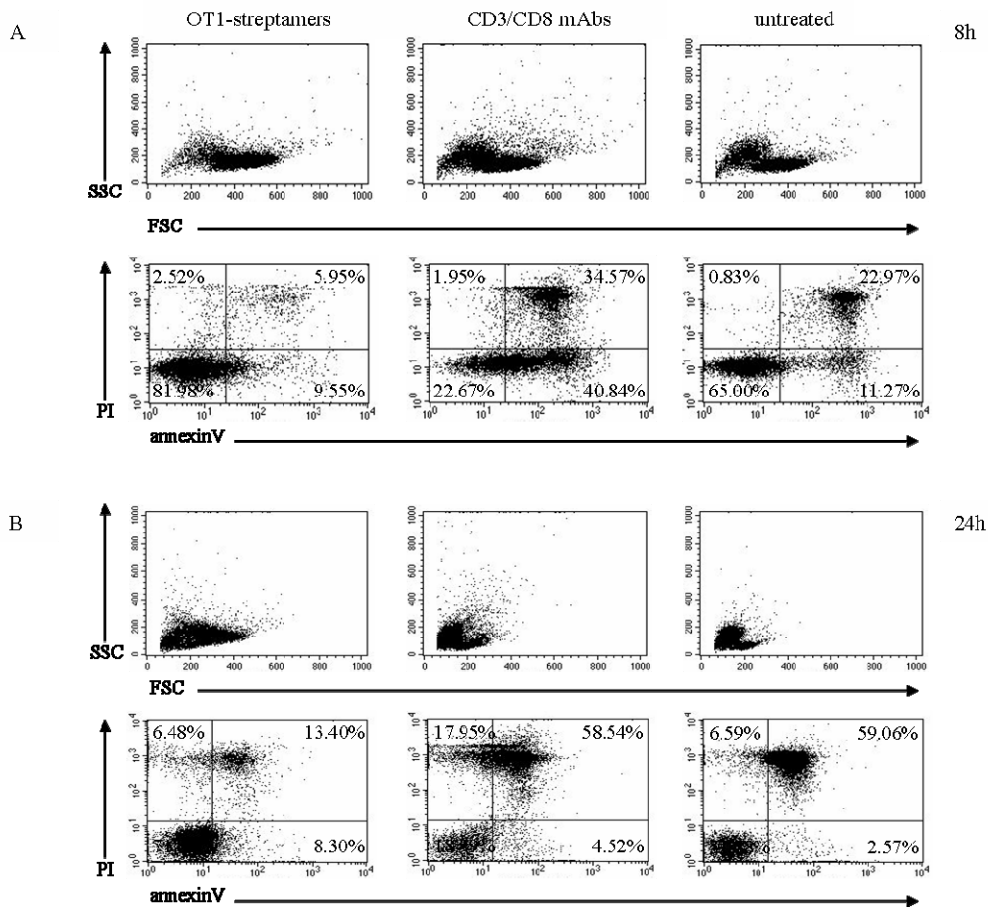

Figure 19. TCR triggering with soluble CD3/CD8 mAbs induces apoptosis

CD8$^+$ T cells were left untreated or incubated with OT1-streptamers (1 μg) and CD3/CD8 mAbs (10 μg/ml) for 8 h (A) and 24 h (B). Apoptosis was assessed by annexin V and PI staining. One representative experiment out of three individual experiments is shown.

Caspase-3 is an executor caspase that induces apoptosis [76]. To further confirm the active induction of apoptosis in response to antibody stimulation, I directly examined the activity of caspase-3. Cells were either left untreated or stimulated with CD3/CD8 mAbs or OT1-streptamers for 8 h and 24 h and then labelled with FITC-DEVD-FMK to monitor the activity of caspase-3. Active caspase-3 cleaves after the aspartate resudue within the 4 amino acid sequence DEVD in a substrate molecule [101]. The fluorescent marker, FITC-DEVD-FMK, is a cell-permeable, non-toxic inhibitor that binds irreversibly to activated caspase-3 whenever it gets into contact with active caspase-3 in apoptotic cells allowing for the detection of active caspase-3. As shown in Figure 20 (upper panel), after 8 h stimulation, cells treated with soluble CD3/CD8 mAbs exhibited an approximate 30% increase in caspase-3 activity compared to OT1-streptamer stimulated cells. Furthermore, after 24 h stimulation, the difference in caspase-3 activity between soluble antibody and OT1-streptamer stimulation was even more dramatic (55% enhancement) (Figure 20 lower panels). In addition, I also compared caspase-3 activity in cells untreated or stimulated with soluble antibodies and detected a 2-fold increase in caspase-3 activity in antibody treated cells (Figure 20). These results further confirm that soluble antibodies actively induce caspase-3 activity.
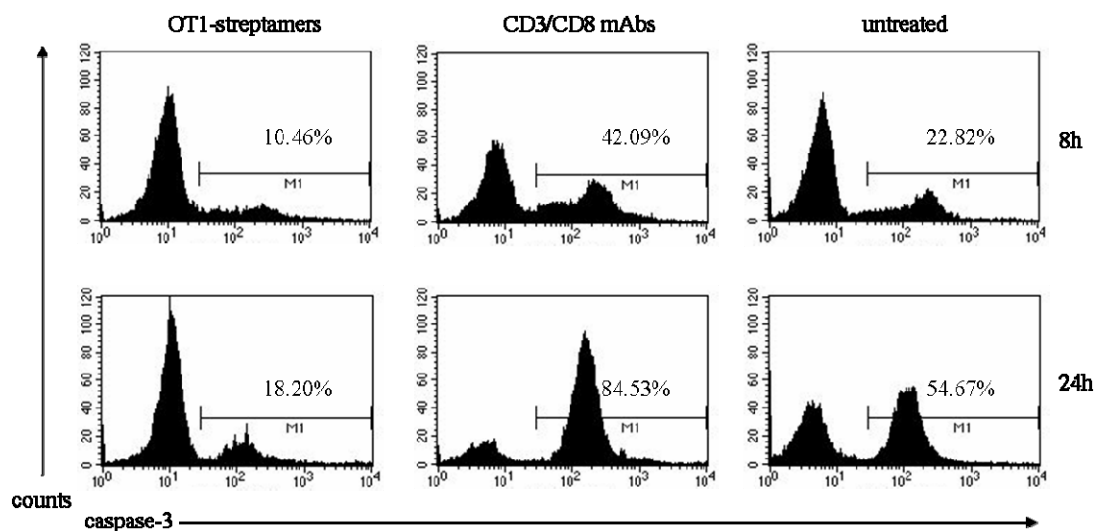

Figure 20. T cells stimulated with soluble CD3/CD8 mAbs exhibit enhanced caspase-3 activity

T cells were cultured in the absence or presence of OT1-streptamers (1 μg) or CD3/CD8 mAbs (10 μg/ml) for 8 h and 24 h. Activated caspase-3 was detected using FITC-conjugated DEVD-FMK. One representative experiment of three individual experiments is shown.

In response to peptide/MHC-I complexes on APCs, CD8^{+} T cells proliferate and differentiate into effector T cells (CTLs-Cytotoxic T Lymphocytes) [102]. CTLs migrate to sites of infection and interact with target cells, which are killed mainly through the release of cytotoxic granules. Two granular proteins are essential for killing, granzyme and perforin. Perforin has been shown to be involved in activation induced cell death in mouse T cells [103]. The proteolytic maturation of perforin under acidic conditions is required for its activity [104]. Blockade of perforin expression by incubation with perforin antisense oligonucleotides results in increased viability of activated T cells [105]. To test whether antibody induced apoptosis is perforin dependent, I analyzed T cell apoptosis from perforin$^{-/-}$ mice. Purified CD8^{+} T cells from WT and perforin$^{-/-}$ mice were stimulated with soluble CD3/CD8 mAbs for 24 h and apoptosis was assessed by PI and annexin V staining. Nearly 70% of antibody-stimulated T cells from either WT or perforin$^{-/-}$ mice stained positive for PI (Figure 21A). Consistent with these data, stimulation with soluble antibodies did not induce proliferation in T cells from WT or perforin$^{-/-}$ mice (Figure 21B). These data indicate that soluble CD3/CD8 mAb induced apoptosis is independent of perforin. It is important to note that in this experiment the effect of the antibodies to induce apoptosis was more dramatic compared to untreated control since only 25-30% of untreated T cells from both WT and perforin$^{-/-}$ mice underwent apoptosis. The lower extent of apoptosis in the untreated T cells from either WT or perforin$^{-/-}$ could be due to the fact that these mice are not on OT1-TCR tg background.
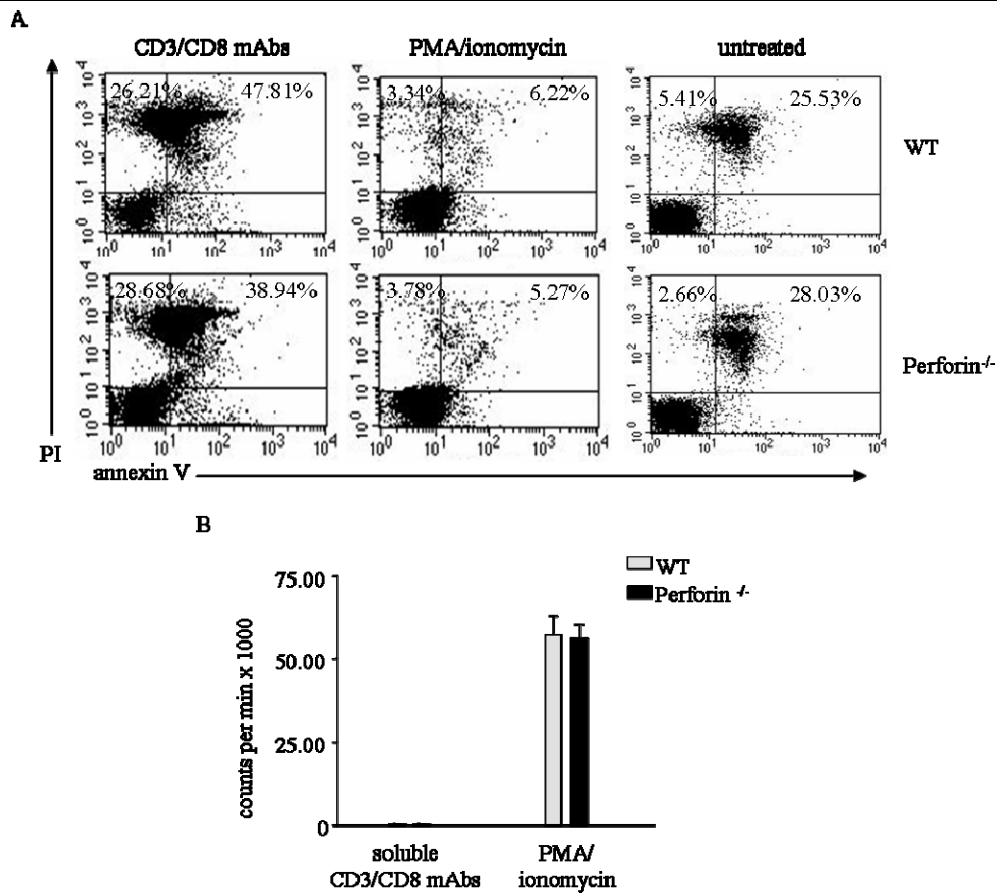
Figure 21. Apoptosis induced by soluble anti-CD3/CD8 mAbs is independent of perforin.

Purified CD8^{+} T cells from WT and perforin^{-/-} mice were treated with soluble CD3/CD8 mAbs (10 μg/ml). (A). Apoptosis was assessed after 24 h stimulation by using annexin V and PI staining. Data are representative of three independent experiments. (B). Proliferation was determined after 72 h by [³H]-thymidine incorporation. Data represent the averages ± SEM from triplicate cultures of three individual experiments. PMA/ionomycin (40 ng/ml/1 μg/ml) was used as positive control.

3.1.3.2. The intrinsic versus extrinsic signaling pathways of apoptosis and their involvement in TCR induced apoptosis

Apoptotic T cell death and the activation of caspase-3 can be induced *via* the extrinsic death-receptor-mediated pathway or through the intrinsic mitochondrial pathway. To explore the possible mechanisms for antibody induced apoptosis, I first investigated the extrinsic pathway by determing the up-regulation of FasR (Fas Receptor) and FasL (Fas Ligand). Purified T cells were stimulated with either CD3/CD8 mAbs or OT1-streptamers for 24 h and the surface expression of FasL and FasR was analyzed by flow cytometry. Surprisingly however, I found that the expression of FasR is much higher in cells stimulated with OT1-streptamers compared to cells treated with soluble antibodies. Indeed, the expression of FasR was hardly detectable following antibody stimulation. In addition, an upregulation of FasL was only observed after streptamer

triggering (Figure 22), although the expression level was low. Thus, I conclude that the extrinsic death-receptor-mediated pathway (FasR/FasL) may not contribute to the apoptosis induced by antibody stimulation. However, to further confirm this conclusion, it would be necessary to assess apoptosis when FasR is blocked by CD95-Fc after antibody stimulation.
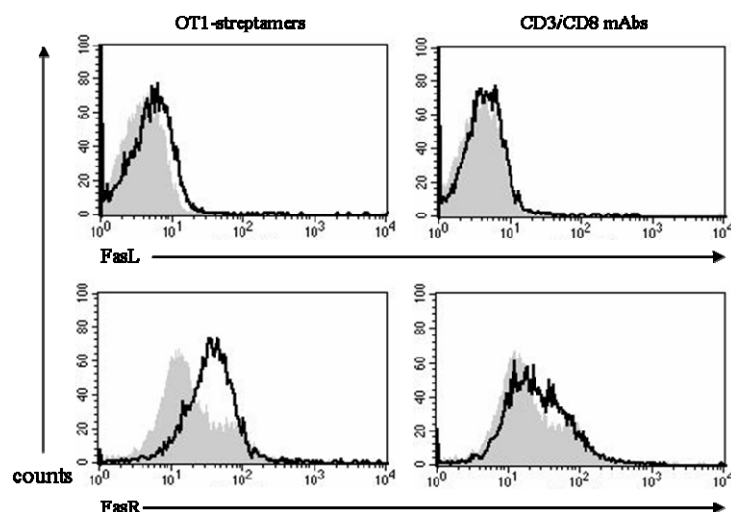

Figure 22. Antibody induced apoptosis is independent of Fas receptor ligation

Purified T cells were exposed to CD3/CD8 mAbs (10 μg/ml) and OT1-streptamers (1 μg) for 24 h. Cells were collected and FasR and FasL expression were analyzed by flow cytometry. The histograms represent profiles of untreated (shaded) and stimulated (thick lines) cells. A representative figure from two independent experiments is shown.

Besides FasR/FasL, molecules of the intrinsic mitochondrial pathway could connect the TCR to caspase-3 activation. The intrinsic apoptosis pathway is initiated by activation of BH3 only pro-apoptotic molecules, resulting in the disruption of the mitochondrial membrane and the release of pro-apoptotic proteins such as cytochrome C [87]. Pro-apoptotic molecule Bim is an important BH3-protein that appears to be critical for T cell development and T cell activation [91]. The activity of Bim is modulated both at the transcriptional and the posttranslational level. Recently, it has been reported that phosphorylation of Bim leads to its ubiquitination and degradation, thus regulating its expression [106].

To analyze whether Bim might play a role in antibody-induced apoptosis, purified T cells were stimulated with CD3/CD8 mAbs and OT1-streptamers for different periods of time. Bim expression was analyzed by Western blotting using an anti-Bim antibody. Figure 23 shows that treatment with CD3/CD8 mAbs induced a rapid and transient shift in the electrophoretic mobility of Bim on SDS-PAGE. The shift started at 3 min

and declined to basal levels after 30 min stimulation. In contrast, T cell stimulation with OT1-streptamers induced a prominent and sustained mobility shift of Bim, starting at 30 min and lasting for 24 h. According to the literature, the mobility shift of Bim represents its phosphorylation [107]. Parallel to its phosphorylation, the amount of Bim protein was reduced after OT1-streptamer stimulation. This reduction started at 30 min and the protein level was reduced by more than 60% after 4-24 h incubation, compared to untreated cells. In contrast, only a transient reduction of Bim expression was detected after antibody stimulation. Collectively, streptamers trigger the phosphorylation and degradation of the pro-apoptotic molecule Bim, which correlates with the resistance of T cells to apoptosis after streptamer stimulation. It is important to note that Bim can not be detected after 24 h stimulation with antibodies. This is most likely due to the apoptotic loss of cells and hence total proteins, since 70-80% of cells underwent apoptosis under this stimulation condition (see Figure 19). This is also reflected by a substantially lower β–actin signal upon antibody stimulation after 24 h (Figure 23 lower panel).
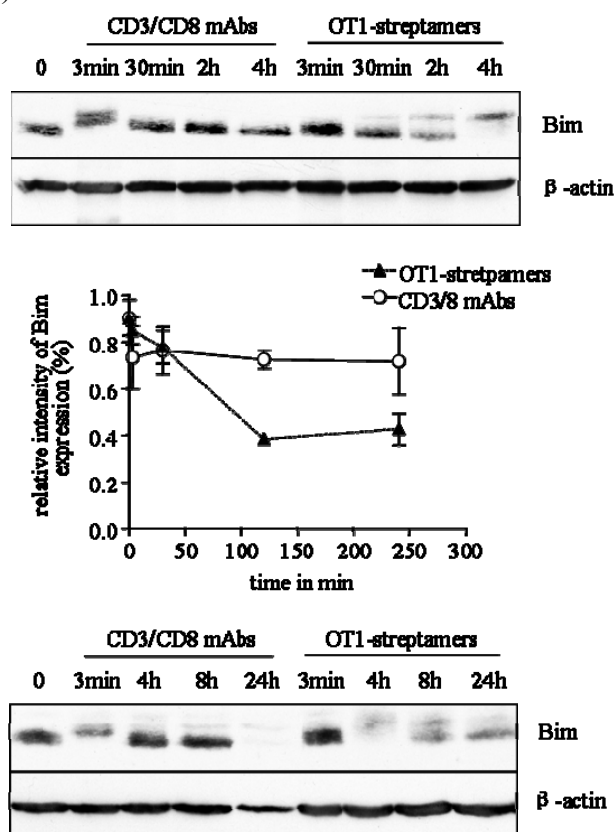
Figure 23. TCR triggering with OT1-streptamers leads to Bim phosphorylation and its subsequent degradation

CD8+ T cells were incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated periods of time. Western blot analysis of cell lysates was performed using anti-Bim and anti-β-actin antibodies. The Bim bands were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. The figure represents one of three independent experiments

Apoptosis induced after disruption of the mitochondrial membrane is determined by the ratio between pro-apoptotic and anti-apoptotic molecules because anti-apoptotic molecules, such as Bcl-2 and Bcl-xL, help to sequester pro-apoptotic molecules from the mitochondria [108]. To further explore the mechanism responsible for antibody induced apoptosis, I examined the expression of the anti-apoptotic molecule Bcl-xL. As shown in Figure 24, in resting T cells the expression of Bcl-xL was not detectable. Treatment of T cells with OT1-streptamers led to the induction of Bcl-xL expression, which started at 4 h, reached its maxium after 24 h, and was still detectable after 48 h of stimulation. In contrast, TCR triggering with CD3/CD8 mAbs did not induce the expression of Bcl-xL. Taken together, these data indicate that antibody induced apoptosis correlates with the stable expression of the pro-apoptotic molecule Bim (Figure 23) and a failure to induce the expression of the anti-apoptotic molecule Bcl-xL.
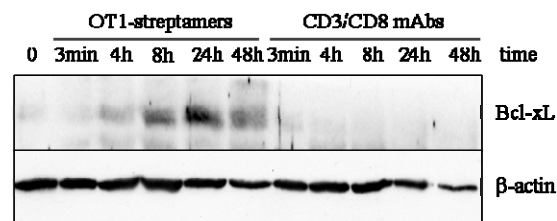
Figure 24. OT1-streptamers induces the expression of Bcl-xL

Purified CD8^{+} T cells were treated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μgl) for the indicated time periods. Post nuclear lysates were prepared and analyzed by Western blotting using antibodies against Bcl-xL and β-actin. The figure shows one representative experiment out of three.

The different expression pattern of Bcl-xL between antibody and streptamer treated cells prompted me to investigate next the activation kinetics of PKB (Protein Kinase B, also known as Akt), which is reported to regulate the expression of Bcl-xL [109]. PKB, a serine/threonine kinase, is a downstream target of PI3K, which becomes activated after TCR stimulation and is a key molecule involved in regulating cell survival [109]. PKB activation was examined by using a phosphospecific antibody against phosphorylated S^{473} (which is a known phosphorylation site in active PKB [110]). Surprisingly, both stimuli triggered PKB activation (Figure 25). However, CD3/CD8 mAbs induced a strong, but transient phosphorylation of PKB, which peaked at 3 min and rapidly declined thereafter. In contrast, OT1-streptamers induced a much weaker, but sustained phosphorylation of PKB, which was still detectable after 24 h of

stimulation. Thus, it appears as if a strong, but transient activation of PKB alone is not sufficient to protect T cells from apoptosis as to induce the expression of Bcl-xL.
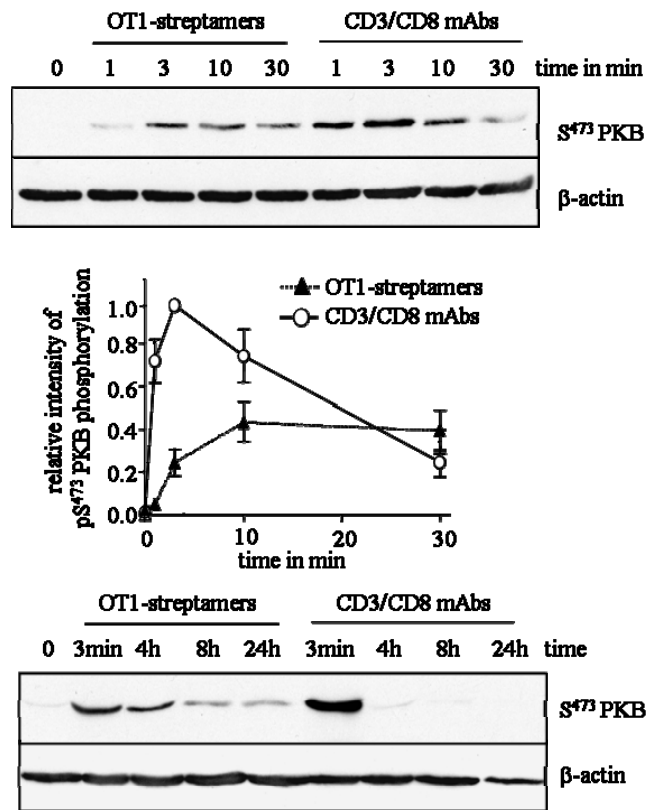
Figure 25. OT1-streptamers induce sustained PKB phosphorylation

Purified CD8$^+$ T cells were treated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated time periods. Cell lysates were prepared and analyzed by Western blotting using antibodies against phosphorylated PKB (S^{473}) and β-actin. The phosphorylated PKB bands were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM of three independent experiments.

3.1.4. OT1-streptamer stimulation induces CD8$^+$ T cell activation and differentiation into effector T cells

To further confirm that OT1-streptamer stimulation induces T cell activation, the expression of activation markers, namely CD25 and CD69, was determined by flow cytometry. In line with the proliferation data shown in Figure 16, I observed a strong up-regulation of both CD25 and CD69 expression in response to OT1-streptamers. In contrast, TCR triggering with CD3/CD8 mAbs only induced low levels of either CD25 or CD69 expression (Figure 26).
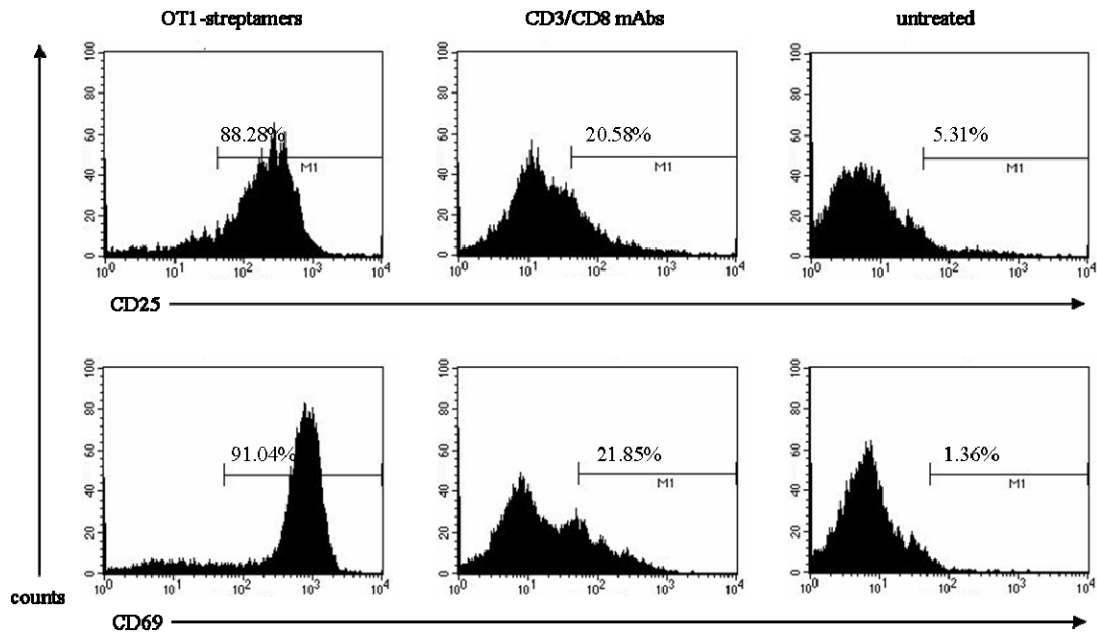
Figure 26. T cell treatment with OT1-streptamers enhances the expression of CD69 and CD25

The expression of CD69 or CD25 in T cells stimulated with OT1-streptamers (1 µg) or CD3/CD8 mAbs (10 µg/ml) (for 24 h) was analyzed by flow cytometry. Data are representative of three individual experiments.

To further confirm that CD8$^+$ T cells are activated in response to OT1-streptamers, I compared the ability of CD3/CD8 mAbs and OT1-streptamers to induce T cell degranulation. TCR-induced translocation of LAMP1 to cell surface has been used as marker for T cell degranulation [111]. T cells were stimulated with either soluble antibodies or OT1-streptamers for 5 h and 24 h and the increase in the cell surface expression of LAMP was determined by flow cytometry using an antibody against LAMP1. As shown in Figure 27, after 5 h both stimuli triggered the translocation of LAMP1 to the plasma membrane, although the effects were more dramatic upon streptamer stimulation. In addition, the recruitment of LAMP1 to the plasma membrane was more evident and sustained for 24 h in response to OT1-streptamer stimulation. In contrast, the majority of T cells displayed low level of surface expression of LAMP1 after antibody stimulation for 24 h.
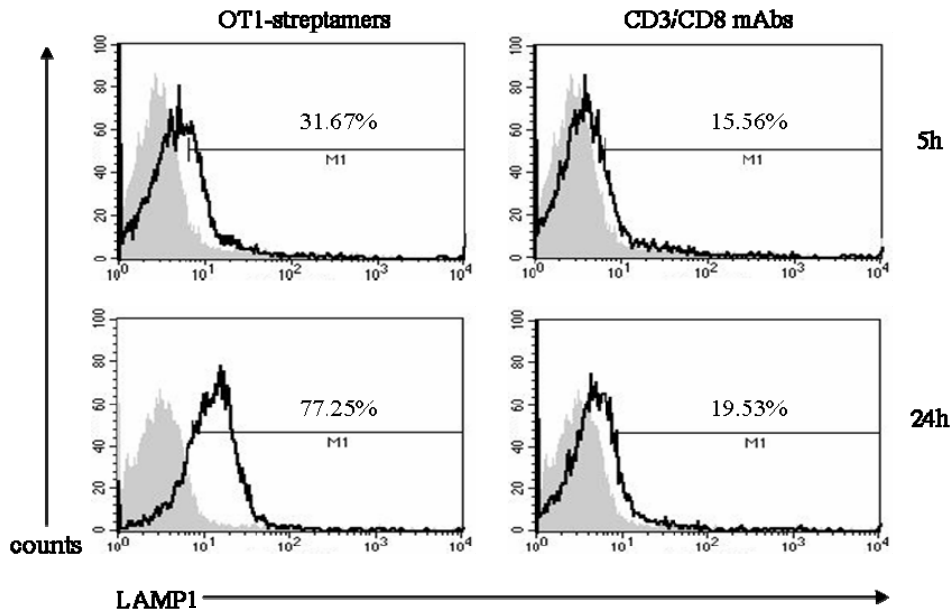
Figure 27. Activation of CD8$^+$ T cells results in degranulation.

Purified T cells were cultured in the presence of OT1-streptamers (1 μg) or antibodies (10 μg/ml). After the indicated periods of time, the translocation of LAMP1 to the plasma membrane was analyzed by flow cytometry. The histograms represent profiles of untreated (shaded) and stimulated (thick lines) cells. Data are representative of three individual experiments.

I also determined the expression level of LAMP1 after TCR stimulation by Western blotting. As shown in Figure 28, OT1-streptamer stimulation induced strong expression of LAMP1, which reached a maximum after 24 h stimulation. In contrast, there was only low level of LAMP1 upregulation after antibody stimulation. Together with the plasma membrane translocation of LAMP1, these data indicate that OT1-streptamer stimulation leads to the activation and differentiation of naive CD8$^+$ T cells into effector CD8$^+$ T cells.
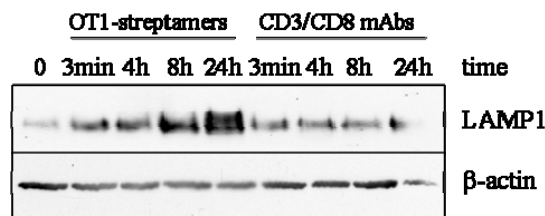
Figure 28. OT1-streptamer stimulation induces enhanced expression of LAMP1

Purified CD8$^+$ T cells were incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1 streptamers (1 μg) for the indicated periods of time. Cell lysates were prepared and analyzed by Western blotting using antibodies against LAMP1 or β-actin. The figure represents one of three representative experiments.

Taken together, in the first section, I characterised the biological outcomes of two stimuli, streptamers and antibodies that trigger the same receptor, in T cell activation. In response to streptamer stimulation, OT1 T cells undergo sustained PKB phosphorylation, upregulation of the activation markers CD25 and CD69, proliferation and differentiation into lytic effectors. In contrast, anti-CD3/CD8 mAbs actively induce apoptosis and the induction of caspase-3 activity, which correlates with the stable expression of the pro-apoptotic molecule Bim and the failure to upregulate the anti-apoptotic molecule Bcl-xL.

3.2. Analysis of signaling pathways regulating proliferation after TCR stimulation

TCR engagement leads to the activation of Lck and ZAP-70, which further result in the phosphorylation of the adaptor proteins LAT and SLP-76. LAT and SLP-76 next serve as docking sites for numerous cytoplasmic signaling molecules including Grb2, Vav, and PLCγ1. These proteins are required for the generation of downstream signaling events, such as the activation of the Ras/MAP-kinase cascade. The amplitude and duration of the intracellular signaling has been implicated in dictating appropriate biological outcome [61]. Thus, to account for the different biological responses described in the first part of the results section, I will address the activation kinetics of molecules mediating TCR induced ERK1/2 activation, such as ZAP-70, LAT, PLCγ1, PKD1, and ERK1/2, under both proliferation (streptamers) and apoptosis (anti-CD3/CD8 mAbs) inducing conditions, by using phospho-specific antibodies

3.2.1. Streptamers induce sustained activation of ERK1/2

ERK1/2 are serine/threonine kinases and the duration of ERK1/2 activation is essential for both positive and negative selection [61, 112]. To assess whether the same holds true in peripheral T cells, the kinetics of ERK1/2 activation under survival *versus* apoptosis-inducing conditions was examined. CD3/CD8 mAbs gave rise to a strong, but transient ERK1/2 activation. Under this condition, the activation of ERK1/2 occurred as early as 1 min and reached a maximum between 1 and 3 min. The signal declined at 10 min and was no longer detectable after 30 min of stimulation. In contrast, OT1-streptamer stimulation induced a weak, but sustained ERK1/2 activation, that even after 30 min was still as strong as it was at 3 min. An extended kinetic analysis revealed that ERK1/2 phosphorylation was still detectable after 24 h stimulation. Hence, similar to the selection in the thymus, soluble CD3/CD8 mAbs induce a strong,

but transient ERK1/2 activation whereas OT1-streptamers generate a weak, but sustained ERK1/2 activation.
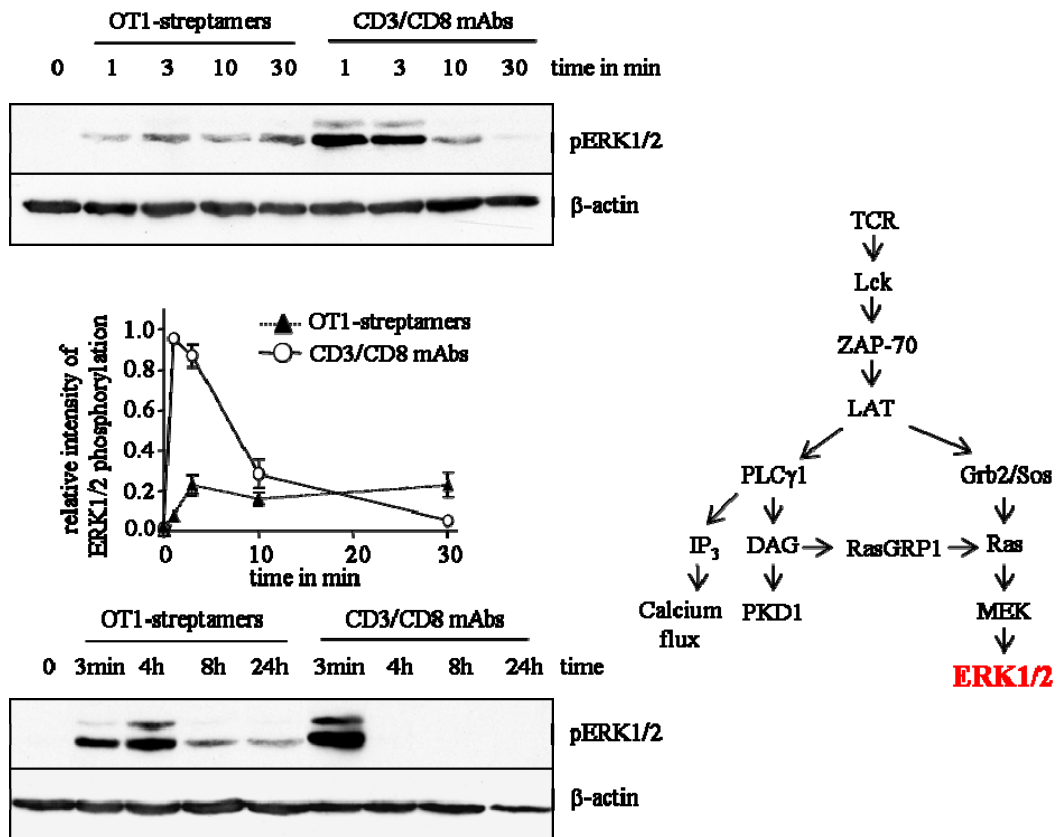
Figure 29. OT1-streptamers induce sustained ERK1/2 activation

Purified CD8⁺ T cells were treated with soluble CD3/CD8 mAbs (10 μg/ml) or with OT1-streptamers (1 μg) for the indicated periods of time. Lysates were prepared and analyzed by Western blotting using the indicated antibodies. The pERK1/2 bands were quantified using ImageQuant software and values were normalized to the corresponding β-actin signals. Data represent the mean ± SEM from three independent experiments.

It is important to note that in multiple experiments a substantial increase in ERK1/2 phosphorylation was observed after 4 h of streptamer stimulation (Figure 29 lower panel). This "second wave" of ERK1/2 phosphorylation could result from the production and release of cytokines, such as IL-2. To elucidate this hypothesis, I blocked IL-2 by applying a neutralizing anti-IL-2 antibody to the cells. As shown in Figure 30, the strength of ERK1/2 phosphorylation was reduced to 50% in the presence of the neutralizing antibodies compared to the control. These data indicate that IL-2 at least partially contributes to the second wave of ERK1/2 phosphorylation.
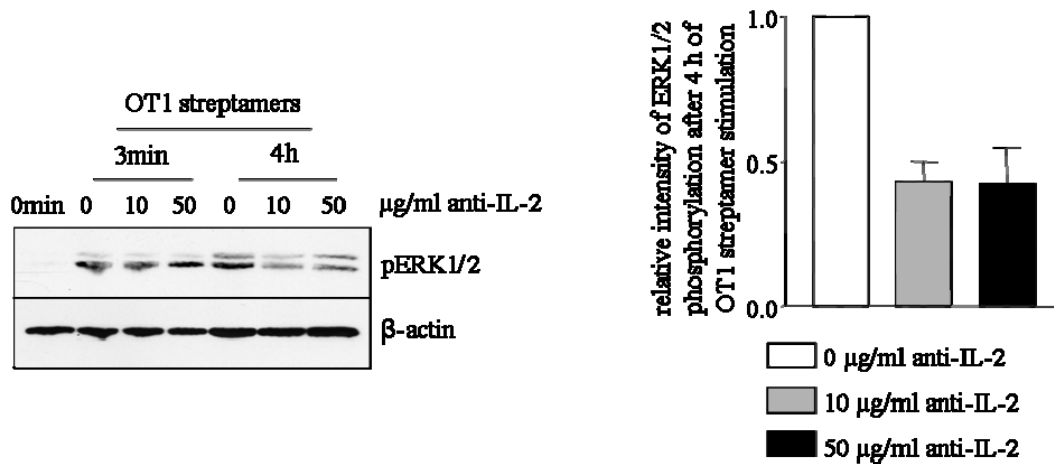
Figure 30. IL-2 partially contributes to the second wave of ERK1/2 stimulation after OT1-streptamer stimulation

T cells were stimulated with OT1 streptamers (1 µg) for the indicated time points in the absence or presence of neutralizing anti-IL-2 antibodies. Lysates were prepared and analyzed by Western blotting using antibody against pERK1/2 and β-actin. The pERK1/2 bands were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments.

3.2.2. Activation-induced degradation of ζ chain, Lck, and ZAP-70 after antibody stimulation contributes to transient ERK1/2 activation

Despite the mounting evidence suggesting that both the amplitude and the duration of ERK1/2 activation govern the cellular response [61, 113], the mechanism of how transient *versus* sustained ERK1/2 activation is induced by TCR triggering still remains intriguing. In response to ligand binding, the TCR complex is internalized, followed by either delivery to lysosomes (for degradation) or recycling back to the plasma membrane [114]. Hence, I first asked whether the transient ERK1/2 activation after antibody stimulation might result from a loss of TCRs on the cell surface due to CD3/CD8 mAb induced internalization. I assessed the expression of the TCR levels upon stimulation with either antibodies or streptamers by flow cytometry. As shown in Figure 31, the cells exhibited similar level of TCR on the surface under both stimulation conditions (Figure 31). Thus, difference in cell surface expression of the TCR apparently do not account for the distinct kinetics of ERK1/2 activation after application of the two stimuli.
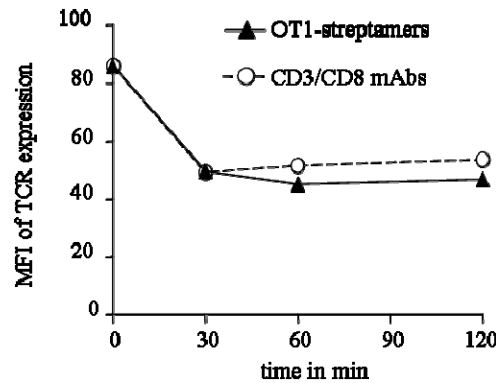
Figure 31. Comparable level of TCR expression on cell surface

CD8$^+$ T cells were treated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated times points. The expression of surface OT1-TCR was assessed by flow cytometry using FITC-conjugated anti-TCR-Vα2 mAbs. Data represent the MFI (Mean Fluorescence Intensity) of the TCR expression of three independent experiments.

The TCR complex is composed of the TCRα and β chains that are noncovalently associated with the CD3γεδε and a homodimer of ζ chains. The similar level of TCR-Vα2 expression on the cell surface in response to both stimuli prompted me to investigate the fate of other components of the TCR complex. Hence, the total cellular content of the ζ chain was analyzed by Western blot. A biphasic pattern of ζ chain expression was observed in response to streptamer stimulation: between 30 min and 4 h following stimulation 60% of the ζ chain was degraded. The protein level of the ζ chain remained low for at least 8 h of continous stimulation. Only after 24 h did the ζ chain protein level start to return to the basal level. The recovery of the expression of ζ chain could result from the upregulation of ζ mRNA, since it is reported that T cell activation induces the upregulation of ζ mRNA [115]. In contrast, antibody stimulation induced the rapid and pronounced degradation of the ζ chain. Between 3 min and 24 h following stimulation, 80% of the ζ chain was degraded. There was no recovery during the observation period.

Since ζ chain is essential for TCR expression at the plasma membrane, an interesting question arised from my data is how TCR expression is maintained (Figure 31) when ζ chain is degraded (Figure 32). A possible hypothesis to this observation is that ζ chain is required for the targeting of the TCR to the plasma membrane, but once at the plasma membrane, ζ chain separates from the rest of the TCR complex. Indeed, Gruta La et al. suggested that TCR ligation results in the exposure of the ζ chain NH$_2$

terminus, which is ordinarily buried in the complex [116]. Such architectural changes might ultimately lead to dissociation of the ζ chain from the rest of TCR complex.
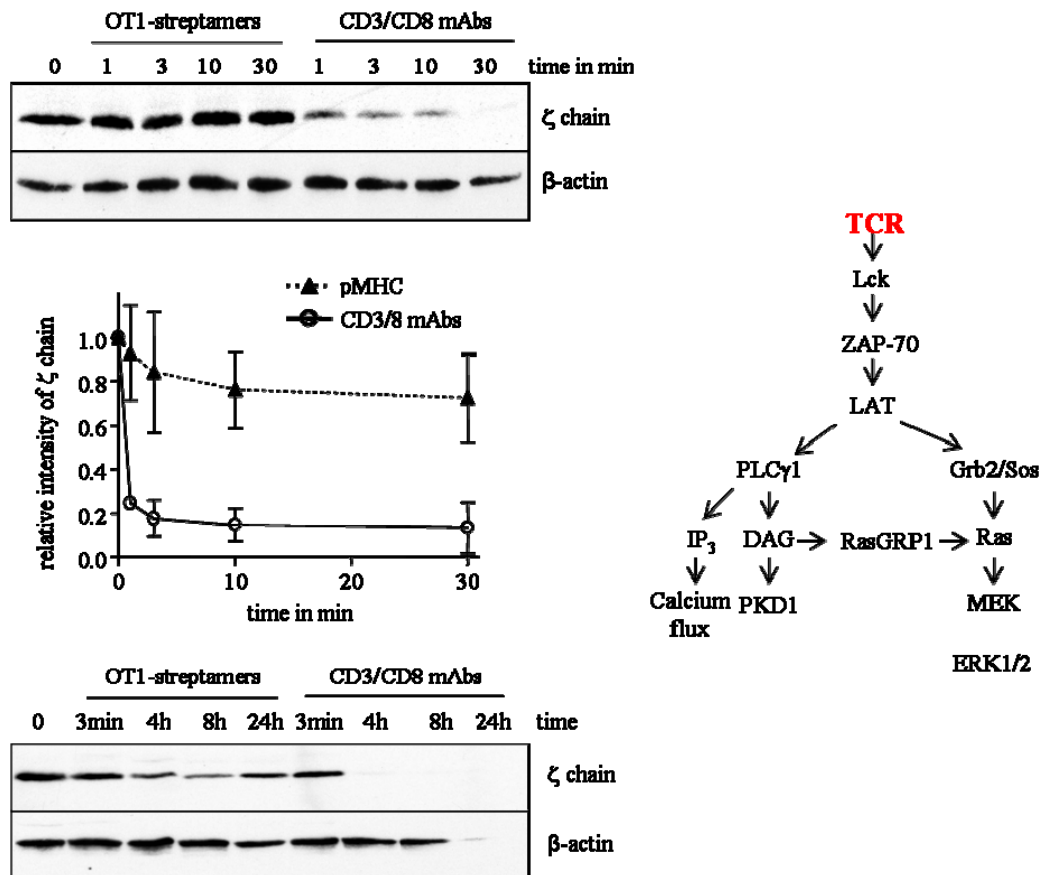
Figure 32. Soluble CD3/CD8 mAb stimulation induces more degradation of the ζ chain

CD8+ T cells were incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated time periods. Western blot analysis of cell lysates was performed using anti-ζ chain and anti-β-actin antibodies. The ζ chain bands were quantified using the ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments.

Upon TCR triggering several important signaling molecules in the signaling cascade are internalized and/or degraded, such as the ζ chain, ZAP-70, and Lck. This process is called activation-induced degradation which is an effective way for T cells to control the activation process [117-119]. Since I observed a rapid degradation of the ζ chain upon antibody stimulation, I asked whether activation induced degradation of Lck and ZAP-70 could also contribute to the transient activation kinetics of ERK1/2 in response to antibody. To test this, I analyzed the expression levels of Lck, which is one of the first molecules to become activated upon TCR stimulation and is essential for T cell activation [13], in response to OT1-streptamer *versus* antibody stimulation by Western

blot. I observed a rapid downregulation of Lck expression which dropped to 50% of the initial levels already after 3 min of antibody stimulation. The decline continued until about 4 h, after which Lck expression stabilized at around 20-30% of the initial level (Figure 33). In contrast, an activation-induced loss of Lck was not observed upon stretamer stimulation. However, I observed a decrease in the electrophoretic mobility of Lck on SDS-PAGE, which is reported to correlate with serine phosphorylation of Lck [120, 121]. The decrease in the electrophoretic mobility occurred at 4 h and lasted for 24 h. Several studies have reported that serine phosphorylation of Lck inhibits its enzymatic activity [122-124]. However, Stefanova et al. suggested that phosphorylation of serine 59 *via* ERK1/2 positively regulates Lck activity by preventing Shp1 recruitment and thus Lck inactivation [66]. Therefore, it will be necessary to identify which sites of serine in Lck were phosphorylated following OT1-streptamer stimulation. The decrease in the electrophoretic mobility was not observed after antibody stimulation.
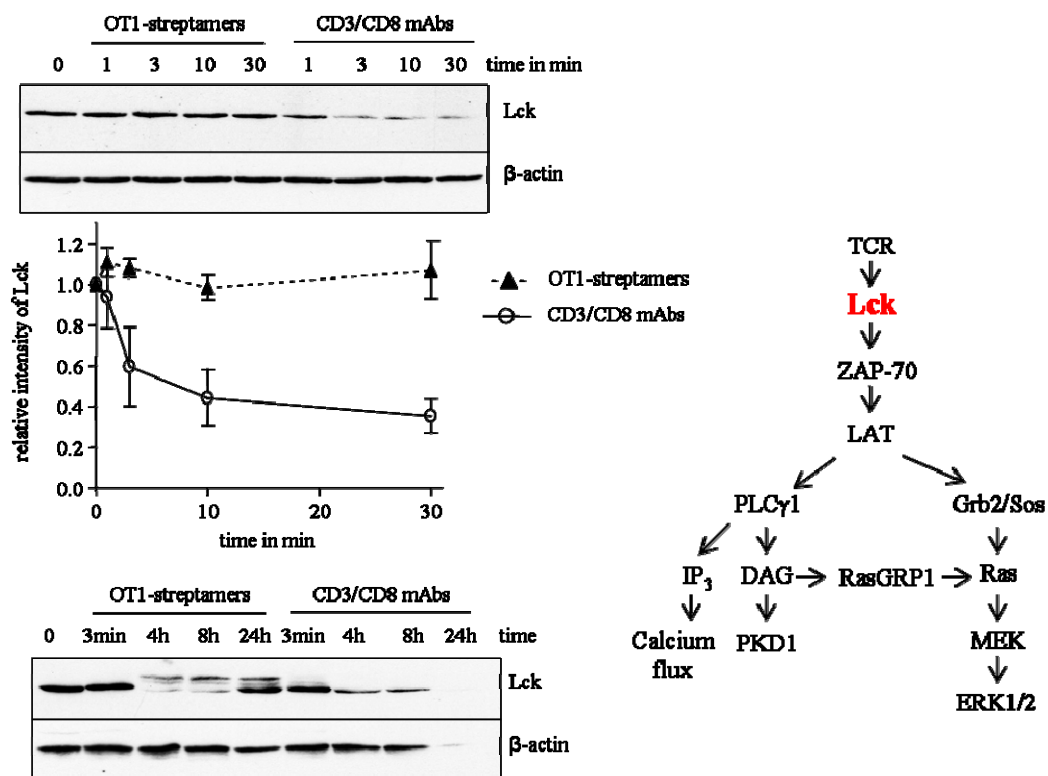
Figure 33. OT1-streptamer stimulation causes serine phosphorylation while CD3/CD8 mAb stimulation induces Lck degradation.

Purified T cells were incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated periods of time. The expression of Lck was determined by incubation with anti-Lck antibodies. The Lck bands were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments.

As I mentioned before, TCR engagement induces a Lck mediated phosphorylation of the ITAMs, which leads to the recruitment of ZAP-70 to the plasma membrane, where ZAP-70 is subsequently activated by Lck. One question emerging from the finding shown in Figure 32 and 33 was whether the rapid degradation of the ζ chain and Lck would interfere with ZAP-70 activation? To address this question, I analyzed the phosphorylation status of ZAP-70 at Y^{319}, which is the autophosphorylation site and correlates with its activation [21, 22]. I found that CD3/CD8 mAbs caused a rapid and robust phosphorylation of ZAP-70, which peaked at 1 min and rapidly declined afterwards. Supprisingly, in marked contrast to the sustained activation kinetics of ERK1/2, streptamers only triggered a transient phosphorylation of ZAP-70. Indeed, after 4 h of stimulation, no signal was observed. The intensity of ZAP-70 phosphorylation at Y^{319} induced by streptamers was only 20-30% of that after antibody stimulation. These data indicate that despite the activation-induced degradation of the ζ chains and Lck, anti-CD3/CD8 mAbs still caused a strong, but transient ZAP-70 activation. However, the mechanism underlying this phenomenon is still unclear.
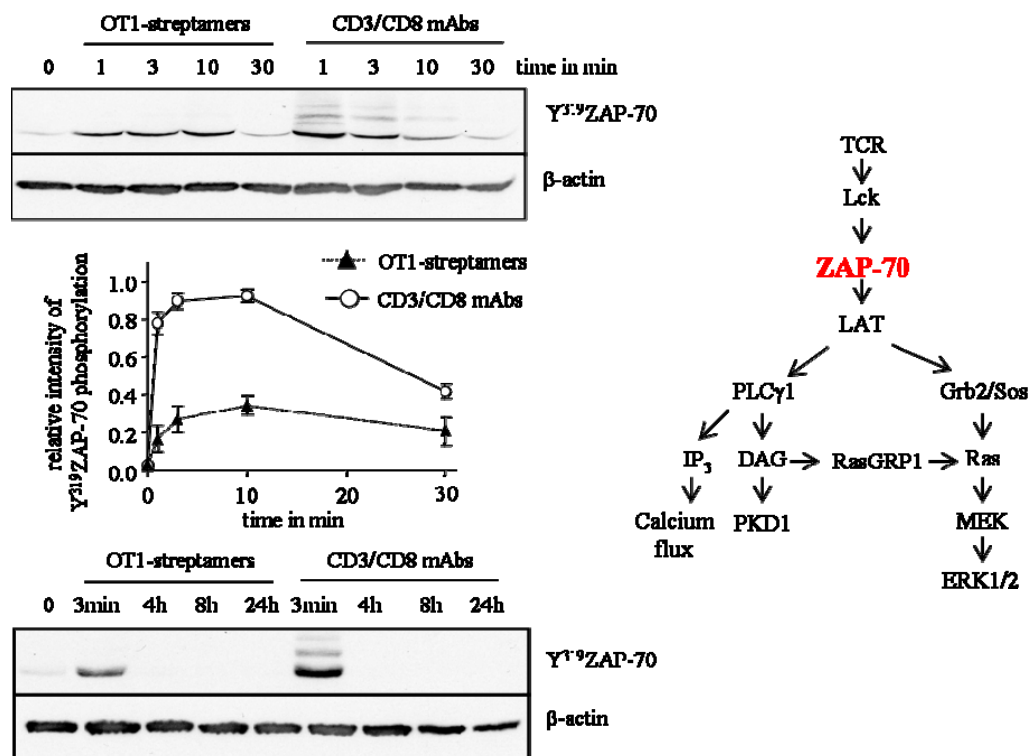
Figure 34. The kinetics of ZAP-70 activation

CD8+ T cells were incubated with either soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated periods of time. Western blot analysis of cell lysates was performed using antibodies against phosphorylated ZAP-70 at Y^{319}, total ZAP-70 and β-actin. The pZAP-70, ZAP-70, and β-actin bands were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments.

In Figure 34, besides the major band corresponding to Y^{319}, I observed additional ZAP-70 bands displaying a decrease in electrophoretic mobility on SDS-polyacrylamide gels exclusively after antibody stimulation. This observation suggests that antibody stimulation not only caused a very strong activation of ZAP-70, but also induced its ubiquitination. To address this point, I stimulated T cells for 3 min with either CD3/CD8 mAbs or with OT1-streptamers. Subsequently, ZAP-70 was immunoprecipitated and blotted with anti-ubiquitin or anti-ZAP-70, respectively. As expected, antibody stimulation indeed induced a strong ubiquitination of ZAP-70 (Figure 35A). In contrast, no ubiquitination of ZAP-70 was observed after streptamer stimulation. Hence, antibody stimulation not only induces a strong activation of ZAP-70, but also its ubiquitination.

In line with the observed ubiquitination of ZAP-70, anti-ZAP-70 blots of whole cell lysates showed a decrease in ZAP-70 protein level after CD3/CD8 mAb stimulation. Changes in ZAP-70 expression are not observed upon OT1-streptamer stimulation (Figure 35B). Taken together, the activation-induced degradation of ζ chain, Lck, and ZAP-70 in response to soluble CD3/CD8 mAbs could contribute to terminating the signal transduction process and thereby control the duration of ERK1/2 activation.
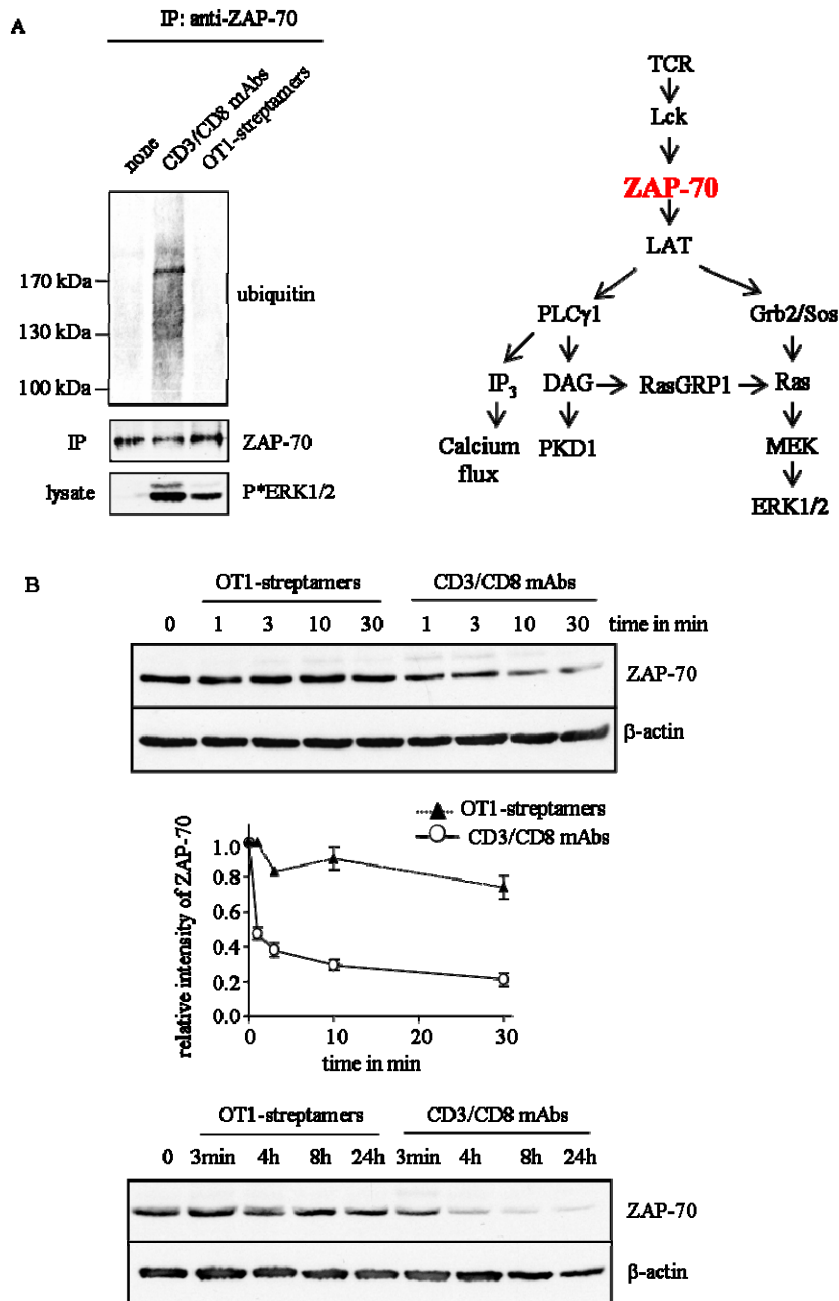
Figure 35. Soluble CD3/CD8 mAb stimulation induces ubiquitination and degradation of ZAP-70

(A). Purified T cells were left untreated or incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for 3 min. ZAP-70 was immunoprecipitated and Western blotting analysis was performed using the indicated antibodies. To control for successful stimulation, lysates were analyzed by Western blot for the presence of phosphorylated ERK1/2. Data are representative of two independent experiments. (B). Purified T cells were incubated with soluble CD3/CD8 mAbs or OT1-streptamers for the indicated periods of time. The ZAP-70 bands were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments.

3.2.3. Kinetics of LAT phosphorylation

I was next interested how the differential activation/expression of ZAP-70 affects the phosphorylation of its substrate LAT. To investigate this point, I first examined the total tyrosine phosphorylation kinetics of LAT by Western blot using the anti-phosphorylated tyrosine antibody (4G10). Figure 36A shows that antibody stimulation induced an approximately 5-fold stronger phosphorylation of LAT compared to streptamer stimulation. However, the kinetics of LAT phosphorylation differed only slightly between the two stimuli and neither stimulus induced a sustained phosphorylation of LAT. Note that similar results were obtained when a specific tyrosine residue phosphorylation within LAT-Y^{171}, which serves as docking site for Grb2 and Gads, was examined (Figure 36B).
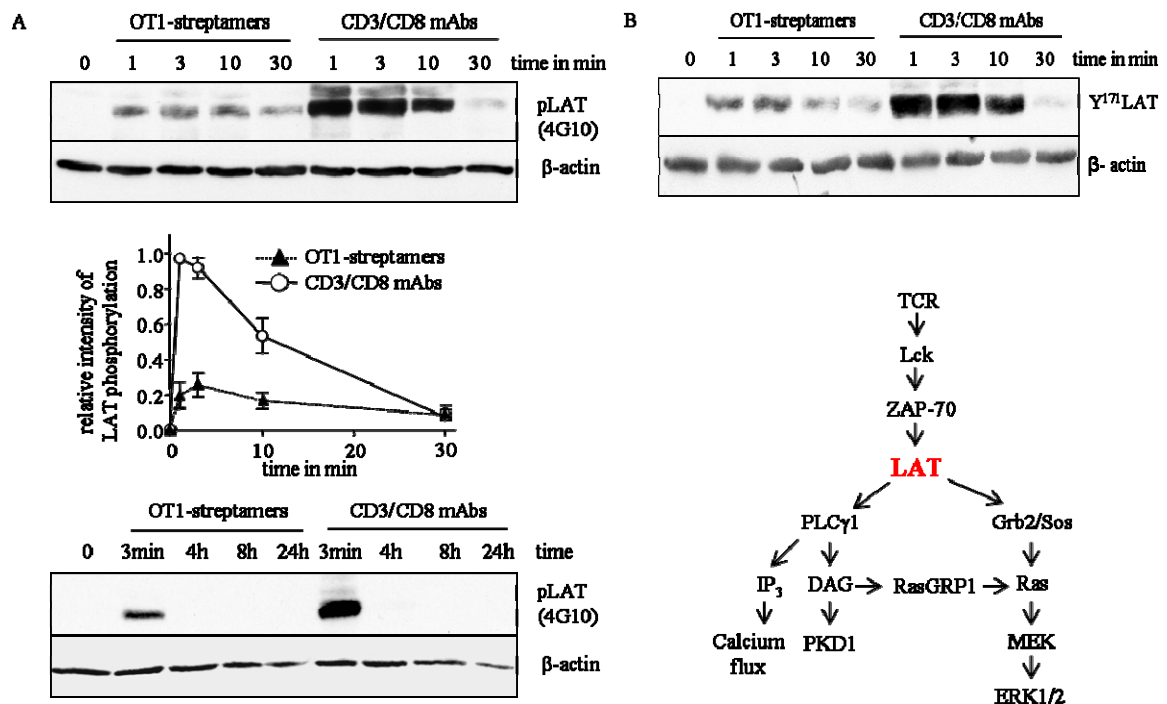
Figure 36. Transient LAT phosphorylation by CD3/CD8 mAb or OT1-streptamer treatment

CD8^{+} T cells were incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated time points. Western blotting analysis of cell lysates was performed using antibody against total tyrosine phosphorylation (4G10) (A), phosphorylated LAT (Y^{171}) (B), and β-actin. The band corresponding to phosphorylated LAT and β-actin were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM of three independent experiments.

3.2.4. OT1-streptamers induce a weak calcium mobilization but sustained phophorylation of PLCγ1

Phosphorylated LAT then serves as a docking site for several molecules including PLCγ1, Grb2, and Gads. The recruitment of PLCγ1 to LAT results in its access for the Tec kinase-Itk, thus initiating the phosphorylation and activation of PLCγ1 [42]. The striking difference in the magnitude of LAT phosphorylation after antibody *versus* streptamer stimulation prompted me to next compare PLCγ1 phosphorylation. Since PLCγ1 phosphorylation at Y^{783} correlates with its activation [42], I analyzed the phosphorylation status of PLCγ1 at Y^{783} by Western blotting of whole cell lysates. As shown in Figure 37A, CD3/CD8 mAbs induced a strong phosphorylation of PLCγ1, whereas the signal was much weaker after streptamer stimulation. Surprisingly, the activation of PLCγ1 appeared to be much more sustained in response to streptamers and considerable phosphorylation of Y^{783} was still detectable even after 24 h stimulation.

It is important to underline here that signaling kinetics upstream of LAT are transient in both stimulations. However, downstream of LAT the signals diverge. In cells stimulated with streptamer, indeed, I observed the weak and sustained activation of PLCγ1 and ERK1/2, whereas in T cells stimulated with soluble antibodies the activation kinetics of these molecules are strong and transient. Thus, streptamers and antibodies clearly have different signaling signature downstream of LAT.

Activated PLCγ1 converts PIP_2 to DAG and IP_3. IP_3 is critical for modulating the calcium response within the cell through binding to its receptors (IP_3R) on the endoplasmic reticulum [45]. Having observed different PLCγ1 phosphorylation kinetics after CD3/CD8 mAb and OT1-streptamer stimulation, I next examined IP_3 mediated calcium flux. Corresponding to the strong phosphorylation of Y^{783} of PLCγ1, soluble CD3/CD8 mAbs generated a strong calcium flux (Figure37 B). In contrast, the calcium flux was much weaker after OT1-streptamer stimulation.
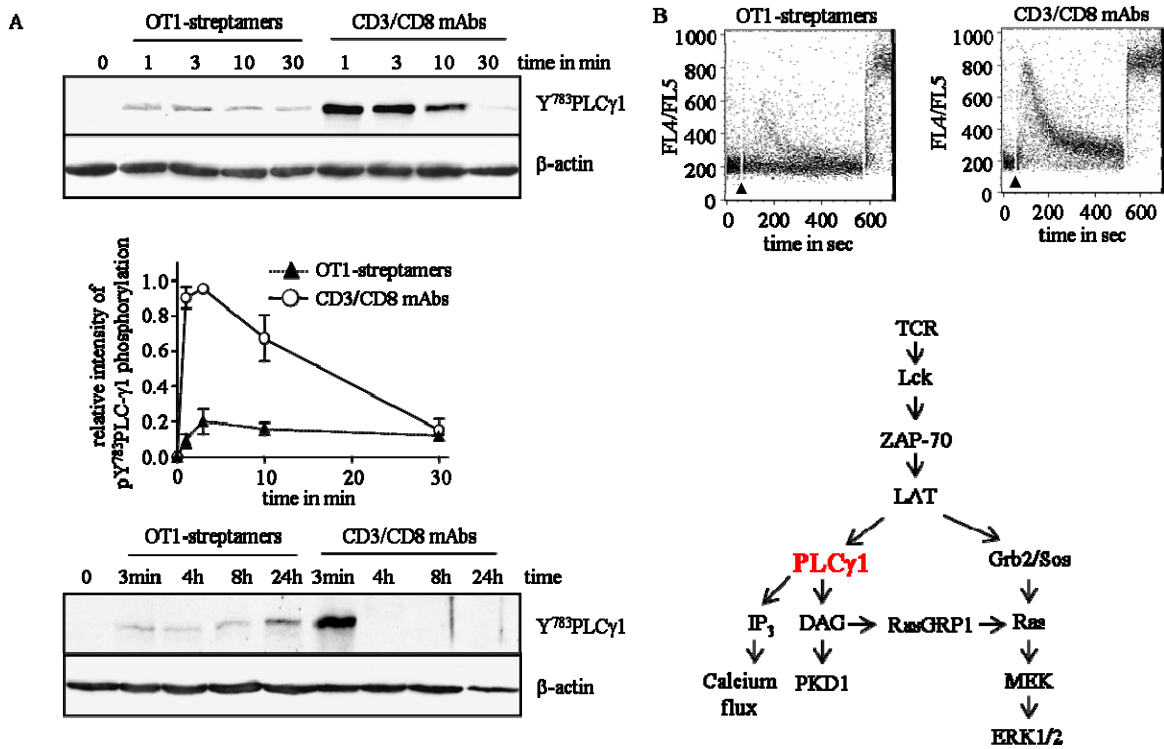
Figure 37. OT1-streptamers cause low but sustained PLCγ1 phosphorylation

(A). Purified splenic T cells were treated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the different periods of time indicated. Cells were lysed and equivalent amounts of protein extract were analyzed for phosphorylated PLCγ1 (Y^{783}). The bands corresponding to phosphorylated PLCγ1 (Y^{783}) were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM of three independently performed experiments. (B). T cells were loaded with Indo-1-AM and calcium release was triggered by soluble CD3/CD8 mAbs or OT1-streptamers, respectively. Calcium influx was determined by flow cytometry based on the change in the FL4/FL5 ratio. Ionomycin was added to the end of each experiment to verify proper Indo-1-AM loading. Data are representative of three independent experiments.

DAG is also generated by activated PLCγ1 and represents a key second messenger in the initiation of T cell activation in response to TCR triggering. The DAG binding protein RasGRP1 plays an essential role in the activation of the Ras-ERK1/2 pathway. To address the question, if the weak, but sustained activation of PLCγ1 induced after streptamer stimulation was sufficient to produce DAG (and, hence to activate Ras), I assessed the phosphorylation status of PKD1 on S^{916}, which was previously shown to be dependent upon the production of DAG [125]. As shown in Figure 38, stimulation with CD3/CD8 mAbs induced a strong, but transient phosphorylation of PKD1 at S^{916}, whereas PKD1 phosphorylation was sustained (albeit weaker) after streptamer stimulation. Therefore, although OT1-streptamers were only weak activators of PLCγ1,

they were sufficient to induce a sustained production of DAG, which likely contributed to the sustained activation of the Ras-ERK1/2 pathway. Given the fact that neither antibody nor streptamer-stimuli induced an extended activation/phosphorylation of ZAP-70 and LAT, these data strongly suggeste that under the experimental conditions used here, the activation kinetics of key molecules involved in the regulation of ERK1/2 activity bifurcate at the level of the LAT / PLCγ1.
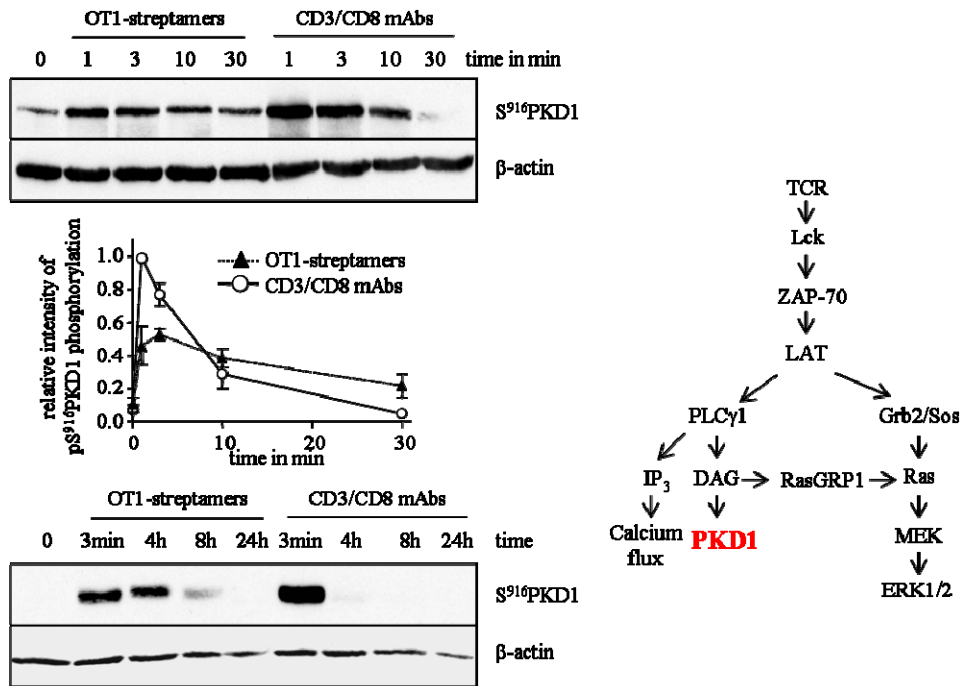
Figure 38. OT1-streptamer stimulation leads to sustained activation of PKD1.

CD8+ T cells were either incubated with soluble CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg). The phosphorylation of S^{916} of PKD1, along with the expression level of β-actin, was determined by Western blotting. The bands corresponding to pPKD1 (S^{916}) were quantified using ImageQuant software and values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM of three independently performed experiments.

Collectively, the second part of the results reveals major differences in the phosphorylation dynamics of PLCγ1, PKD1, and ERK1/2. The input signals leading to apoptosis induce a strong, but transient activation of these molecules, whereas stimuli promoting survival/proliferation generate a rather weaker, but sustained activation kinetics. The transient activation of PLCγ1, PKD1, and ERK1/2 under pro-apoptotic conditions of stimulation maybe partially due to the rapid degradation of the ζ chain, Lck, and ZAP-70. Taken together, our data indicate that the duration of activation of intracellular signaling molecules correlates with dinstint outcomes.

3.3. Spatial compartmentalization and distinct subcellular localization of ERK1/2

The previous data clearly demonstrated that the duration of signal activation correlates with the biological outcomes. Furthermore, the activation-induced degradation of ζ chain, Lck, and ZAP-70 in response to soluble CD3/CD8 mAbs could contribute to the transient ERK1/2 activation. However, it is still remain elusive how the sustained activation kinetics is maintained in the peripheral T cells after streptamer stimulation. Daniels et al. have proposed that compartmentlazation of signaling molecules endow thymocytes with the ability to convert a small change in ligand-affinity into positive *versus* negative selection (correlating with distinct activation kinetics of signaling molecules) [61]. Thus, to examine the role of cellular localization of signalling molecules in peripheral T cell activation, T cells were stimulated as in Figure 39 and analysed by confocal microscopy.

Figure 39 demonstrates that after 3 min, CD3/CD8 mAbs induced a strong clustering of phosphorylated ERK1/2 and F-actin at the plasma membrane. In addition, phosphorylated ERK1/2 was no longer detectable after 30 minutes of antibody-mediated stimulation, thus confirming its transient activation under apoptosis inducing conditions (as shown in Figure 29). Since streptamers gave rise to sustained ERK1/2 activation (Figure 29), I was wondering whether the sustained phosphorylated ERK1/2 also localized on the plasma membrane. Indeed, phosphorylated ERK1/2 was recruited to the plasma membrane and colocalized with F-actin after 3 min stimulation. Surprisingly however, in cells treated with streptamers for 30 min, the major phosphorylated ERK1/2 was no longer found on the plasma membrane, but rather in small vesicular structures, which localized close to the plasma membrane as well as in the cytosol (Figure 39). This particular subcellular localization of phosphorylated ERK1/2 was sustained at least for 4 hours. These results show that under conditions inducing T cell death, phosphorylated ERK1/2 transiently accumulates at the plasma membrane, whereas under conditions promoting T cell survival and proliferation, activated ERK1/2 shuttles from the plasma membrane to vesicular subcellular compartments within the cell.
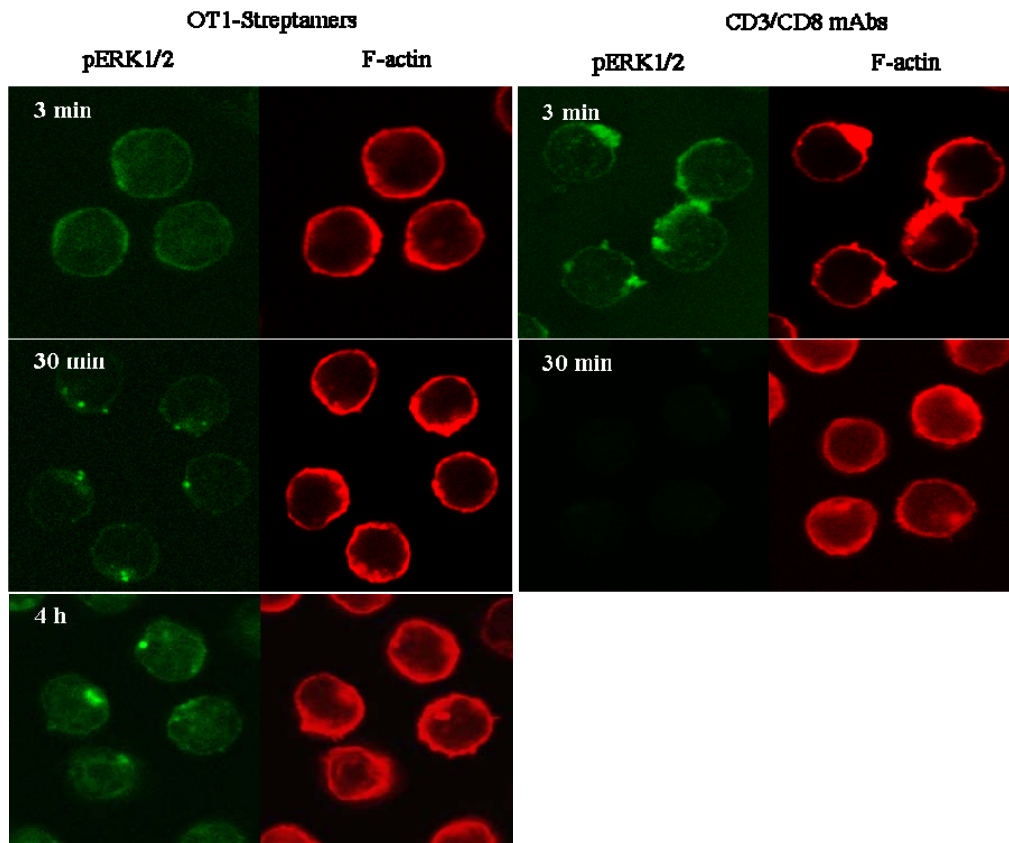
Figure 39. Different subcellular localization of phosphorylated ERK1/2 in response to OT1-streptamres or anti-CD3/Cd8 mAbs

Confocal laser scanning microscopic analysis was performed on CD8^{+} T cells stimulated with either CD3/CD8 mAbs (10 μg/ml) or OT1-streptamers (1 μg) for the indicated times. Cells were permeabilized and subjected to intracellular staining with the indicated antibodies. Cells shown in each panel are representative of at least 40 stimulated cells from two independent experiments.

Since ERK1/2 activation is the result of TCR ligation, I was next interested to investigate whether pERK1/2 colocalized with the TCR complex in such small vesicles. By using confocal microscopy, I found that the major phosphorylated ERK1/2 and TCR-Vα2 colocalized in the small vesicles after 30 min and 4 h stimulation (Figure 40).
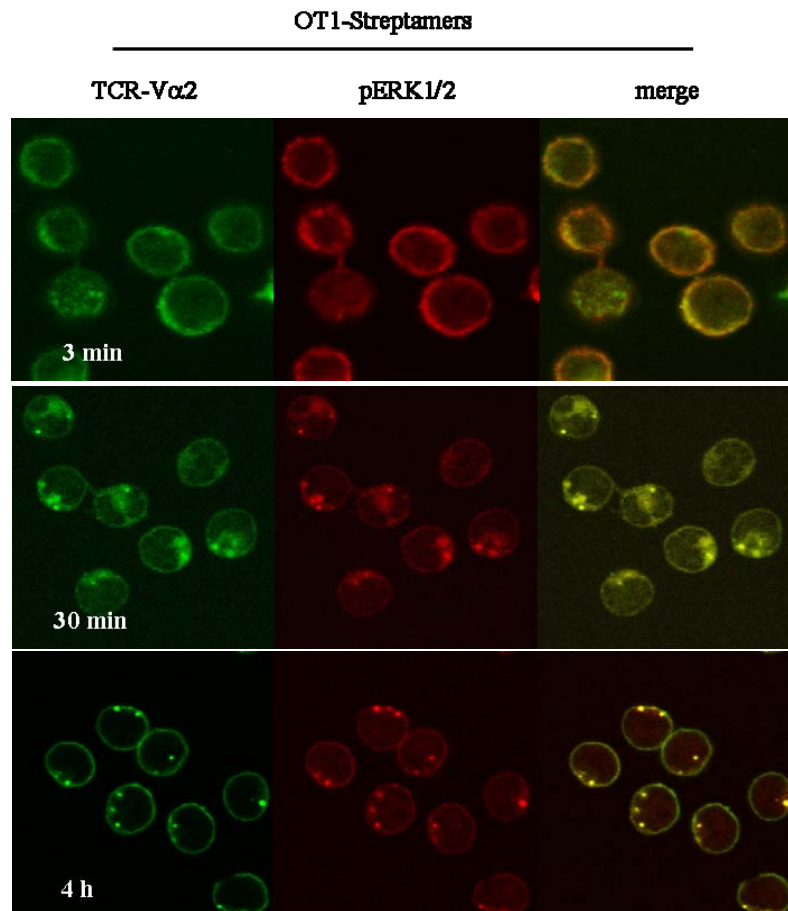
Figure 40. Colocalization of phosphorylated ERK1/2 and TCR-Vα2 in small vesicular structures after streptamer stimulation

Confocal laser scanning microscopic analysis was performed on CD8+ T cells stimulated with OT1-streptamers (1 μg) for the indicated times. Cells were permeabilized and subjected to intracellular staining with the indicated antibodies. Cells shown in each panel are representative of at least 40 stimulated cells from two independent experiments.

It is known that receptor internalization and degradation serves to downregulate the signal elicited upon ligand binding. However, in neurons, the internalization of TrkA (Tyrosine Receptor Kinase) triggers the communication of signal from axon terminals in the target region back to the neuron cell body by forming the "signaling endosomes". The "signaling endosomes" contain NGF (Nerve Growth Factor) bound to its activated receptor-TrkA and the signaling proteins that participate in the Ras-ERK1/2 pathway [126, 127] (Figure 41). It is important to note that the "signaling endosomes" are required for the sustained ERK1/2 activation after NGF stimulation.
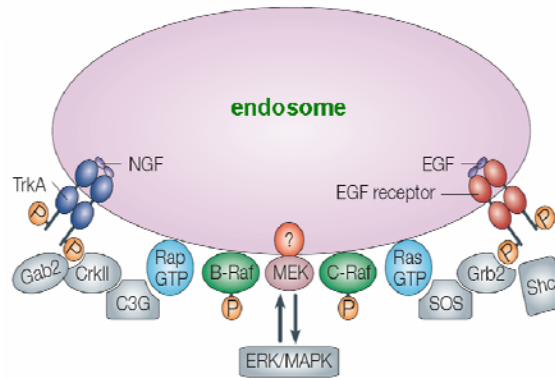
Sorkin, 2002

Figure 41. Hypothetical model of signaling complexes in endosomes

Adaptor proteins, Grb2 and Crk, guanine nucleotide exchange factors (SOS and C3G), and small GTPases (Ras and Rap1) can translocate to endosomes with activated TrkA. Binding of Raf to GTP-loaded Ras contributes to the recruitment of Raf to endosomes. Docking of ERK1/2 and MEK to the endosomal membrane might involve an unidentified anchoring protein.

To address whether the activated ERK1/2 that I detected in small vesicular structures after 30 min and 4 h of OT1-streptamer stimulation was localized within the endosomal compartment, I used Rab5 as an endosomal marker. Rab5 is a small GTPase that associates with the plasma membrane and with endosomes [128]. It regulates clathrin-coated-vesicle-mediated transport from the plasma membrane to the early endosomes [129]. Indeed, the majority of Rab5 colocalized with phosphorylated ERK1/2 in the small vesicular structures following 30 min and 4 h treatment with streptamers, thus identifying them as endosomes (Figure 42A). To further confirm that pERK1/2 localized in the endosomes and not in other vesicles, I stained the cells with LAMP1, a lysosomal marker. Since the patterns for pERK1/2 localization were similar after 30 min stimulations and 4 h treatment, I examined the colocalization of LAMP1 and pERK1/2 following 30 min treatment. As indicated by the arrow, pERK1/2 did not colocalize with LAMP1 after streptamer stimulation, indicating that the ERK1/2 positive vesicles are not lysosomal compartments (Figure 42B).
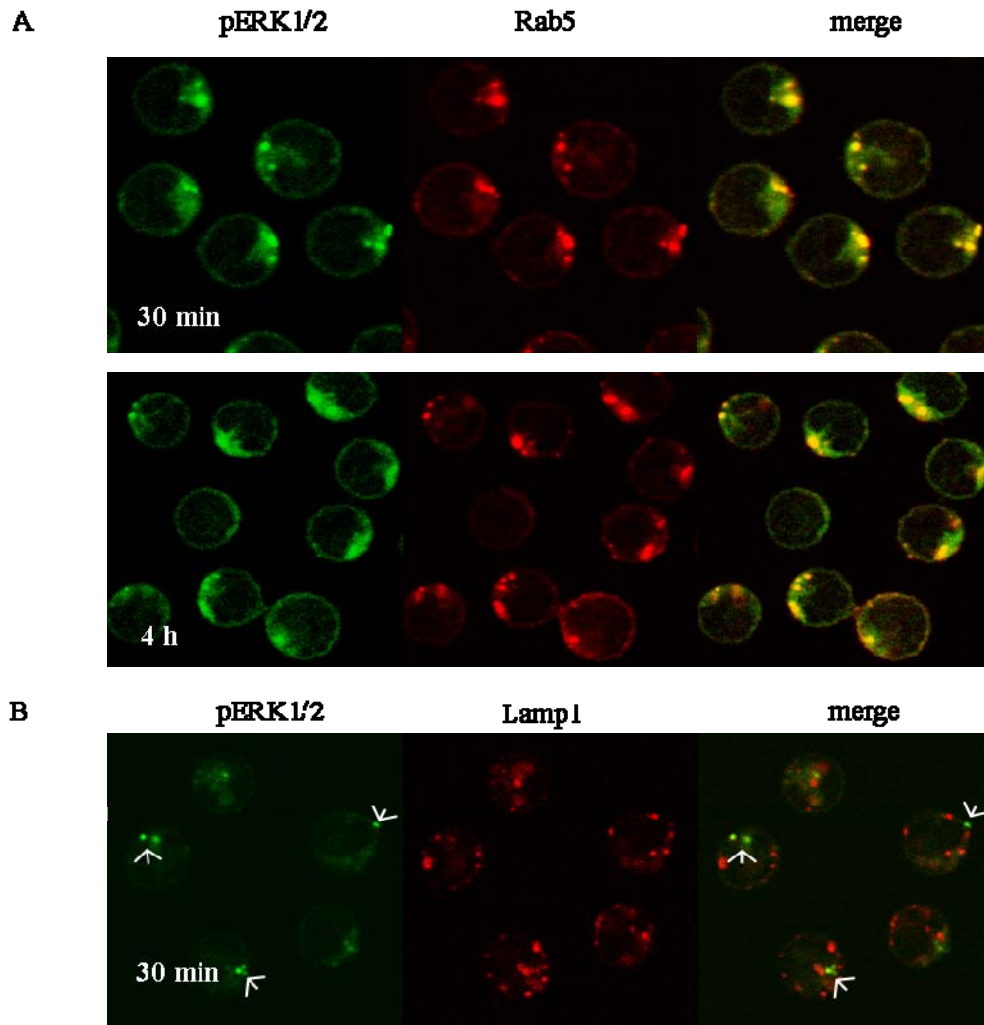
Figure 42. Phosphorylated ERK1/2 localizes in Rab5 positive vesicles (endosomes) after OT1-streptamer stimulation

Confocal laser scanning microscopic analysis was performed on CD8^{+} T cells stimulated with OT1-streptamers (1 μg) for 30 min and 4 h, respectively. Cells were permeabilized and subjected to intracellular staining to detect phosphorylated ERK1/2, Rab5 or LAMP1. Cells shown in each panels are representative of at least 40 stimulated cells from two independent experiments.

The recruitment of activated ERK1/2 into endosomes raised the possibility that OT1-streptamer stimulation induced the formation of "signaling endosomes". To prove this hypothesis, I firstly examined the localization of TCR complex. After 30 min stimulation TCR-Vα2 indeed colocalized with Rab5 (Figure 43A).

The ζ chain is another component of the TCR complex and it is important for signal transduction. Thus, I was next interested to determine ζ chain localization after streptamer stimulation. However, since the antibodies against the ζ chain and Rab5 are raised in the same species, the cells were stained with antibodies against the ζ chain and pERK1/2 instead (recall that pERK1/2 completely colocalized with Rab5 and thus can serve as a marker for endosomes). Corresponding to the localization of TCR-Vα2, the major ζ chain colocalized with pERK1/2 after 30 min stimulation with OT1-streptamers (Figure 43B), confirming that the TCR complex was localized in endosomes. Since the TCR complex was observed in Rab5 positive vesicles, we further wanted to address whether the ligand for the TCR, the streptamers, were also targeted to endosomes. To answer this question, I treated the T cells with OT1-streptamers conjugated with APC (Allophyocyanin). After 30 min stimulation, the major internalized ligands (OT1-streptamers) localized in Rab5 positive vesicles as did the ζ chain and TCR-Vα2 (Figure 43C). To exclude the possibility that endosomal localization observed before was unspecific, cells were incubated with antibodies against Rab5 and the transmembrane protein CD45. Although a small part of CD45 localized in Rab5 containing vesicles near the plasma membrane after 30 min stimulation with streptamers, the majority of CD45 remained on the plasma membrane (Figure 43D). In addition, the vesicles inside the cells did not contain CD45 (as indicated with arrow). Taken together, these data indicate that OT1-streptamer stimulation induces the formation of "signaling endosomes", containing the TCR / ζ chain complex, its ligand-the streptames and the activated ERK1/2.
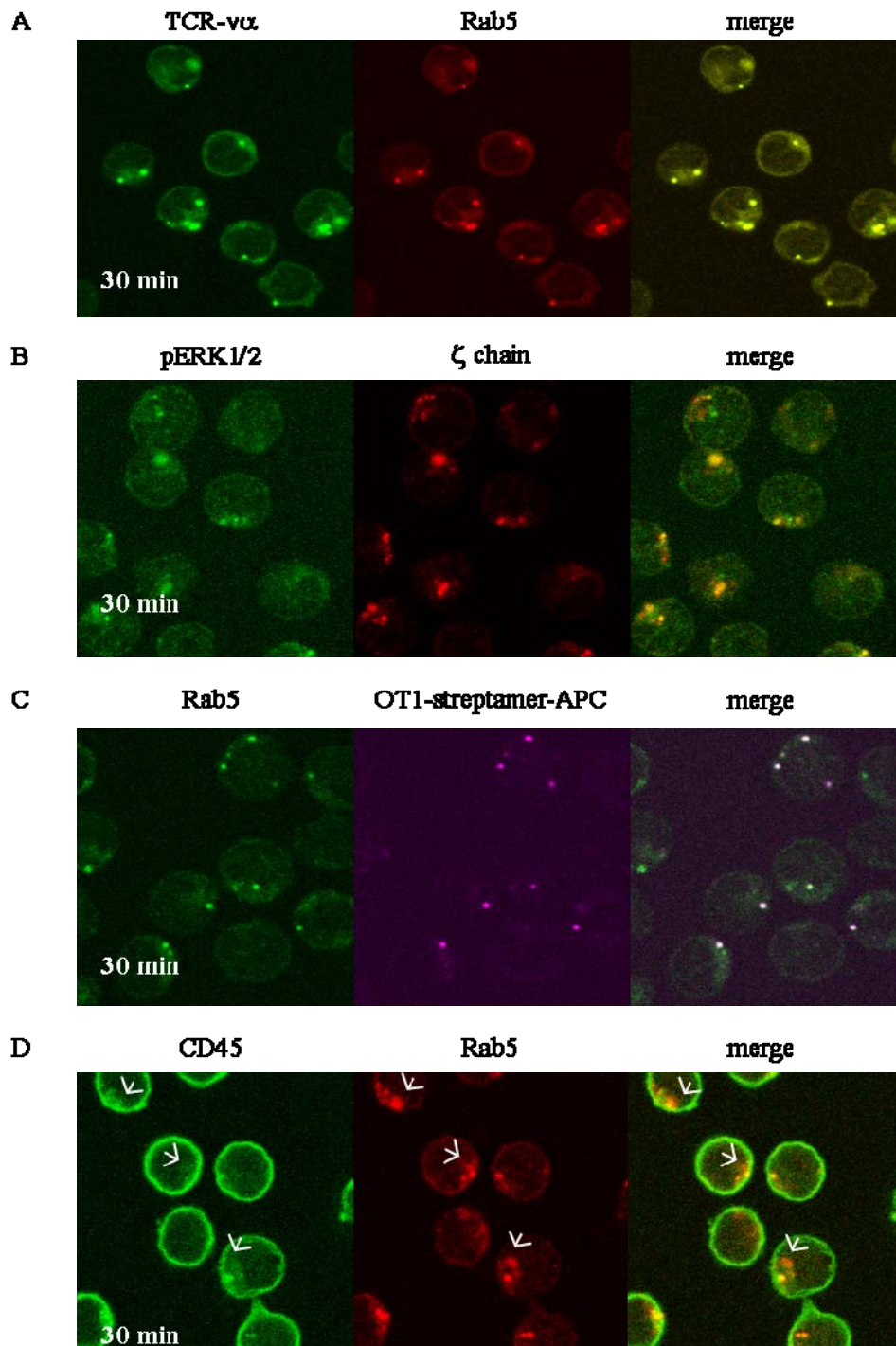
Figure 43. The TCR complex and its ligands translocate to the endosome after OT1-streptamer stimulation

T cells were stimulated for 30 min with OT1-streptamers (A, B, D) or OT1-streptamers-APC (C). Cells were subjected to immunofluorescence with the indicated antibodies. Cells shown in each panel are representative of at least 40 stimulated cells from two independent experiments.

To further explore the components of "signaling endosomes" in T cells, I next examined Ras, which has been described to localize in the endosomal compartment in response to NGF treatment in PC12 cells [130]. Unfortunately, since the available antibodies against Ras and Rab5 were from same species, I could not directly examine the localization of Ras in endosomal vesicles. Therefore, I stained the cells with anti-Ras and anti-pERK1/2 antibodies. As expected, the majority of Ras colocalized with pERK1/2 in endosomes after 30 min and 4 h stimulation with OT1-streptamers (Figure 44A). It would be interesting to assess next whether the phosphorylated PLCγ1 also localized in endosome. Unfortunately, our attempts to visualize phosphorylated PLCγ1 in Rab5 containing vesicles by confocal laser canning microscopy were so far not successful. This may be due to the low phosphorylation status of these molecules or the inaccessibility of different phospho-specific antibodies tested for immunofluorescence. To overcome this problem, I examined the localization of total PLCγ1. As shown in Figure 44B and 44C, PLCγ1 colocalized with Ras in Rab5 positive vesicles indicating that PLCγ1 is another component of the "signaling endosome" after streptamer stimulation.

Taken together, these data indicate that OT1-streptamer stimulation induced the formation of "signaling endosomes". The molecules with sustained activation kinetics, such as PLCγ1 and ERK1/2, shuttle from the plasma membrane to the "signaling endosomes", which could serve as a second platform for molecule activation.
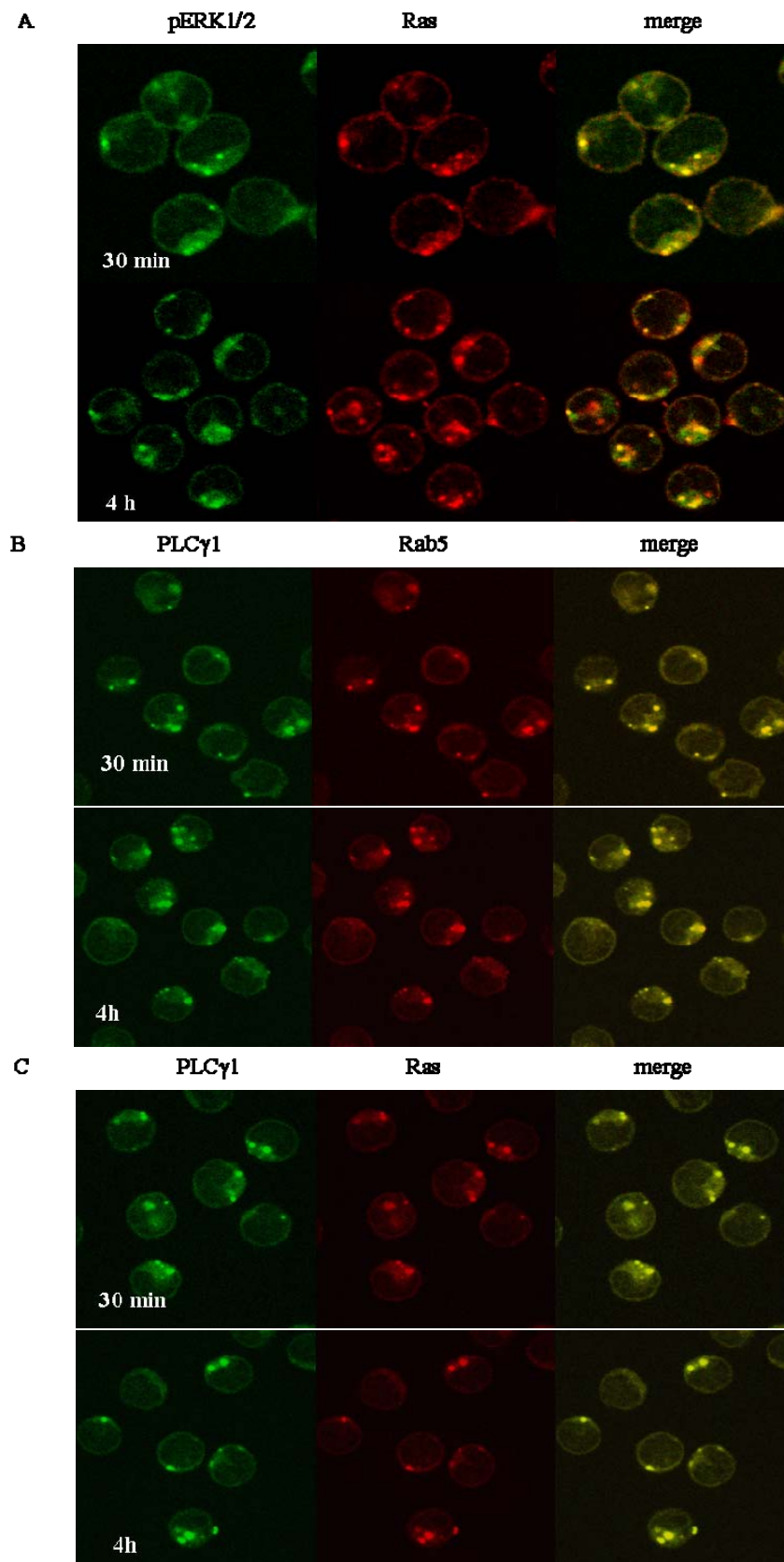
Figure 44. Identification of signal-transduction related proteins (Ras and PLCγ1) in endosomes after streptamer stimulation

Signaling molecules involved in ERK1/2 activation were identified in endosomes by confocal laserscanning microscopic analysis. CD8⁺ T cells stimulated with OT1-streptamers (1 μg) for 30 min and 4 h and cells were permeabilized and subjected to intracellular staining to detect Ras, PLCγ1 or Rab5. Cells shown in each panels are representative of at least 40 stimulated cells from two independent experiments.

Upon binding to their ligands, cell surface receptors and the associated signaling machinary are cleared from the plasma membrane by endocytosis. During this process, some of these endosomes recycle to the cell surface, while others are targeted for degradation. Based on the formation of "signalling endosome" upon streptamer stimulation and degradation of ζ chain following antibody stimulation, it is tempting to speculate that both stimuli induce the endocytosis of TCR signaling machinery. However, upon streptamer stimulation, these endosomes do not convert into lysosomes, thus leading to sustained signaling, whereas antibody induced endosomes are targeted to degradation. This idea is supported by the ubiquitination and degradation of proximal signals following antibody stimulation. To further prove this hypothesis, I investigated the localization of ζ chain after antibody stimulation. As shown in Figure 45, after 5 min of stimulation with soluble antibodies, ζ chain colocalized with LAMP1, a marker for lysosomes. This result indicates that the localization of proximal molecules in lysosomes might contribute to the transient signalling.
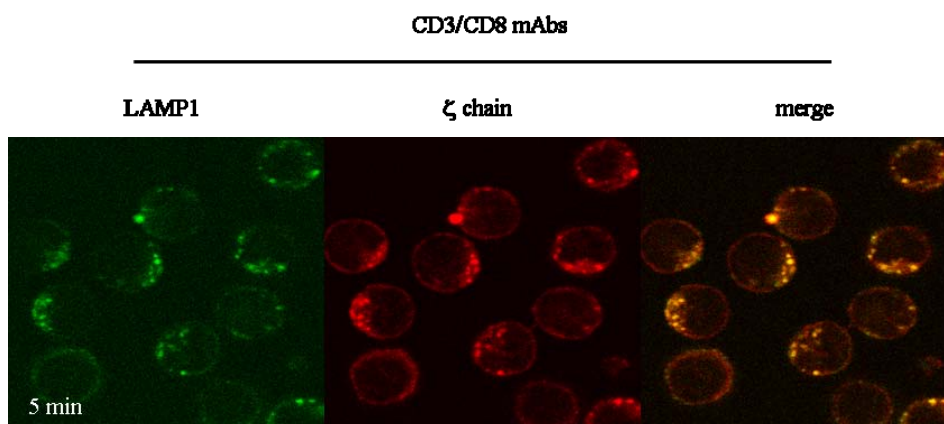
Figure 45. ζ chain localizes in lysosomes following antibody stimulation

T cells were stimulated for 5 min with CD3/CD8 mAbs. Cells were subjected to immunofluorescence with the indicated antibodies. Cells shown in each panel are representative of at least 40 stimulated cells from two independent experiments.

3.4. The conversion from apoptosis to cell survival by addition of lysosome inhibitor in response to antibody stimulation

In part 3.2 of my thesis, I showed that antibody stimulation induced the degradation of proximal signaling molecules (ζ chain, Lck, and ZAP-70), a transient activation of important signal components (pERK, pPLCγ1, pPKD1, and pPKB) and apoptosis. In this part of my thesis, I tried to convert apoptosis to survival by inhibition of protein degradation, which has been reported to occurr either in the proteasome or in the

lysosome [117, 119]. Since proteasomal inhibitors are toxic for cells (data not shown), I focused on inhibition of lysosomal function by using NH_4Cl [117]. To my surprise, the addition of NH_4Cl not only reduced the extent of antibody-induced apoptosis (20% reduction), but also diminished caspase-3 activity from 80% to 50% after 24 h stimulation (Figure 46). As a control, NH_4Cl had no effects on T cell apoptosis or caspase-3 activity induced by *in vitro* culture condition.
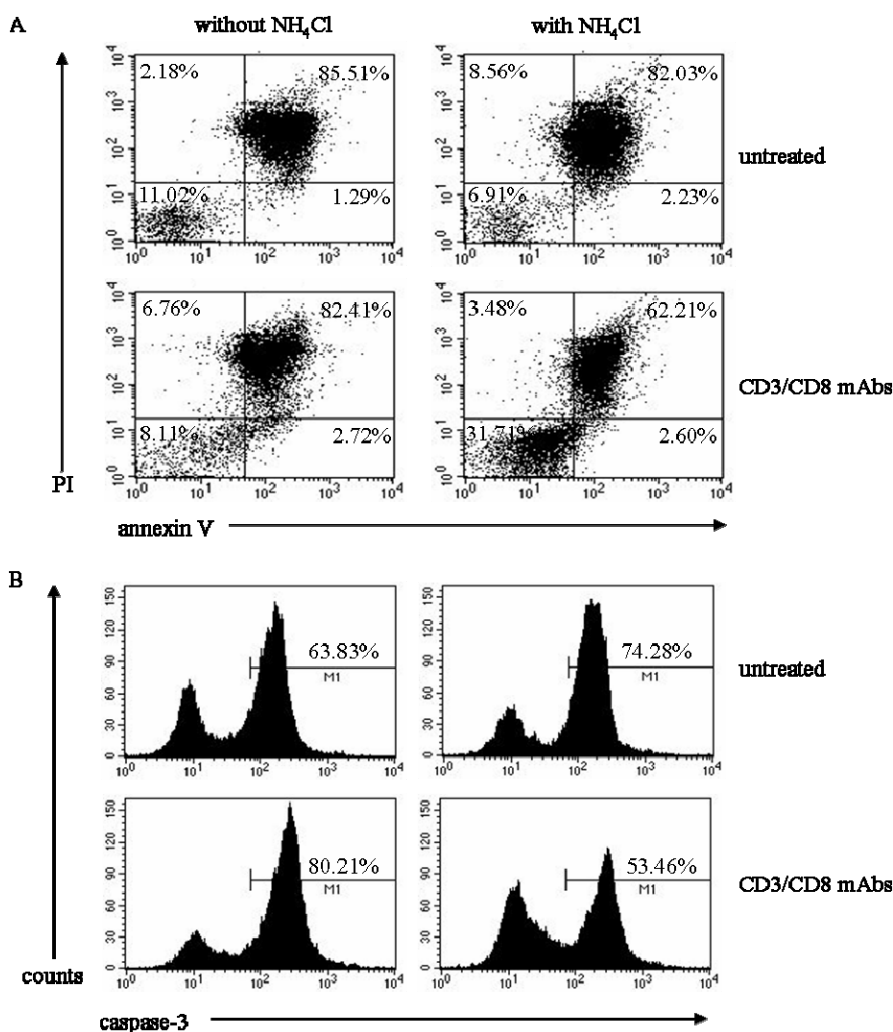
Figure 46. The effects of NH_4Cl on T cell apoptosis induced by soluble antibody stimulation

Purified CD8^{+} T cells were left untreated or pre-incubated with 10 mM NH_4Cl for 30 min. Cells were then cultured in 48-well plates in the absence or presence of soluble CD3/CD8 mAbs (10 μg/ml) for 24 h. Apoptosis was assessed by annexin V and PI staining (A) and caspase-3 activity was determined using FITC-conjugated DEVD-FMK (B). Data are representative of two independent experiments.

It is not clear whether the reduced apoptosis and caspase-3 activity following NH_4Cl treatment correlate with T cell activation. Thus, I next analyzed the expression of the activation markers CD69 and CD25. As shown in Figure 47, following antibody

stimulation, the addition of NH$_4$Cl induced the upregulation of both CD69 and CD25, which cannot be observed without antibody stimulation. Thus, inhibition of lysosomal function by NH$_4$Cl can partially convert T cell apoptosis into T cell survival and activation upon antibody stimulation.
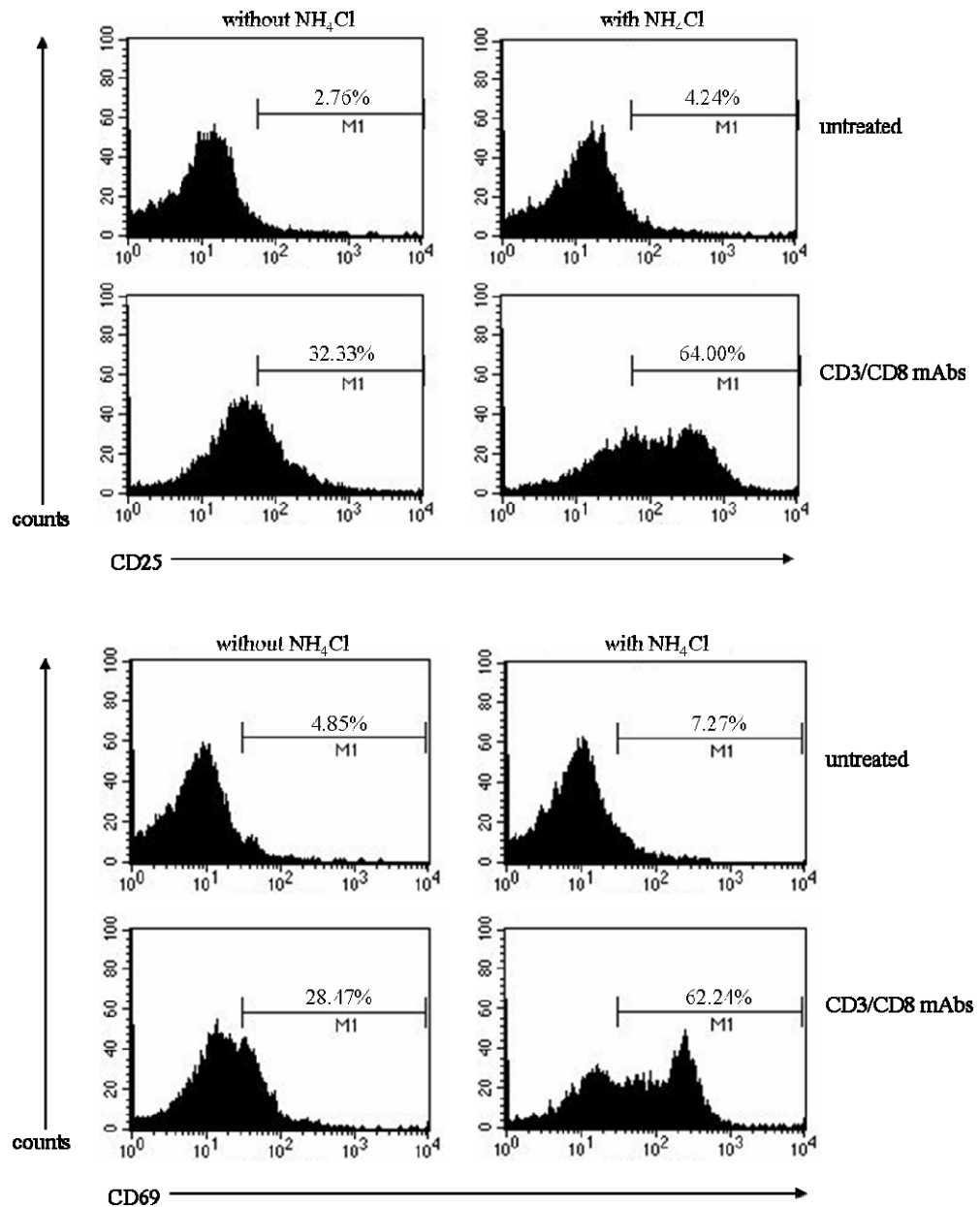
Figure 47 Lysosome inhibition induces the upregulation of activation markers after antibody stimulation.

Purified T cells were left untreated or pre-treated with 10 mM NH$_4$Cl followed by incubation with CD3/CD8 mAbs (10 µg/ml) or medium for 24 h. The expression of CD25 and CD69 were analyzed by flow cytometry. Data are representative of three individual experiments.

The reduction in apoptosis and the upregulation of CD25 and CD69 in NH$_4$Cl treated cells prompted me to investigate the effects of NH$_4$Cl on ERK1/2 activation after

antibody stimulation. As shown in Figure 48, pre-treatment with NH_4Cl did not alter the short term activation kinetics of ERK1/2 although the phosphorylation intensity was slightly reduced. ERK1/2 was still phosphorylated after 3 min stimulation in NH_4Cl treated cells and the signal declined after 30 min in response to antibody stimulation. However, after 4 h stimulation, a second wave of ERK1/2 phosphorylation was observed in NH_4Cl treated cells. I next examined the expression of the ζ chain, whose degradation also occurs in lysosomes. As expected, the addition of NH_4Cl partially prevented ζ chain degradation after 4 h of antibody stimulation (Figure 48). However, NH_4Cl had no effects on the degradation of ZAP-70. Moreover, it did not convert the transient activation kinetics of ZAP-70, PLCγ1, PKB, and PKD1. Hence, it is tempting to speculate that the second wave of ERK1/2 phosphorylation in the presence of NH_4Cl might result from the cytokine receptor triggering instead of TCR activation.
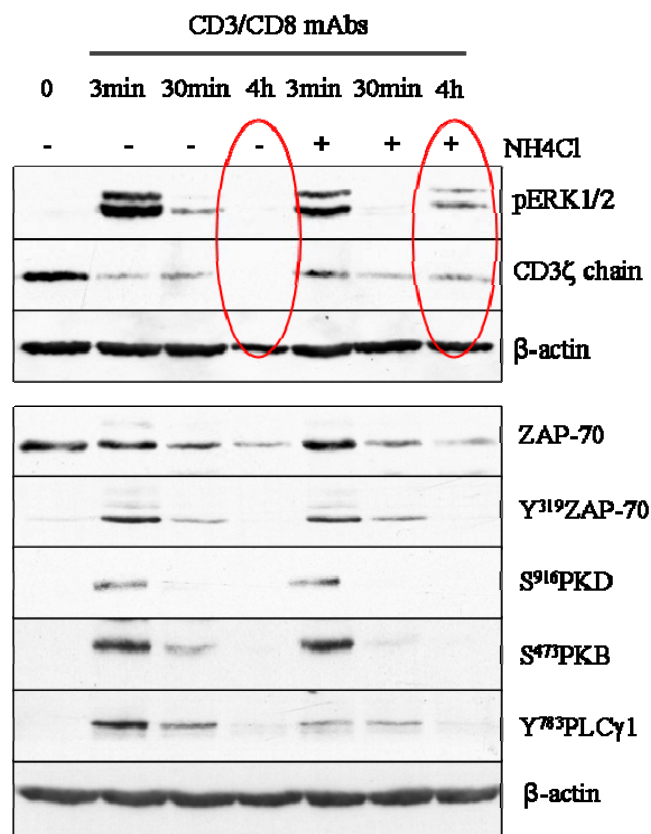
Figure 48. Inhibition of the lysosome leads to sustained ERK1/2 phosphorylation

T cells were preincubated with NH_4Cl for 30 min followed by stimulation with soluble CD3/CD8 mAbs for the different periods of time. Samples were analyzed by Western blotting with the indicated antibodies. The results are representative for three independent experiments.

Taken together, the data in this section suggest that it is possible to convert apoptosis into T cell activation and survival after antibody stimulation.

4. Discussion

Depending on the quality of the ligand, engagement of the TCR can result in different cellular responses, such as differentiation, proliferation or apoptosis. In the thymus, intermediate affinity ligands induce positive selection, whereas high affinity stimuli direct thymocytes to negative selection [60, 131, 132]. However, how the triggering of one receptor initiates two different cellular responses and how these responses are organized on the molecular level is so far not understood. In an attempt to shed light on these important questions, I utilized peripheral T lymphocytes obtained from OT1-TCR tg mice and established an experimental system that can induce either cell proliferation or apoptosis by using stimuli of different affinity/avidity: soluble CD3/CD8 mAbs *versus* streptamers loaded with the OT1-TCR specific agonistic OVA peptide SIINFEKL. I demonstrated that input signals leading to apoptosis induce a strong, but transient activation of ERK1/2 mainly at the plasma membrane, whereas stimuli promoting survival/proliferation generate a low and sustained activation of ERK1/2 localized in the endosomes, which contain the TCR complex, Ras, and PLCγ1 (Figure 39-44). The transient activation of ERK1/2 under pro-apoptotic conditions correlates with the rapid degradation of the ζ chain, Lck, and ZAP-70 (Figure 32-35). The sustained activation of ERK1/2 under survival promoting conditions is paralleled by the induction/phosphorylation of anti-apoptotic molecules, such as PKB (Figure 25) and Bcl-xL (Figure 24). Collectively, these data provide insight into the signaling signatures that are associated with proliferation or apoptosis in T cells.

4.1. Soluble CD3/CD8 mAbs induce apoptosis *via* Bim-caspase-3 mediated pathway

It was somehow unexpected that soluble antibodies induce rapid apoptosis (Figure 19) and activation of caspase-3 (Figure 20), whereas peptide loaded streptamers induce the expression of the anti-apoptotic molecule Bcl-xL. While the induction of Bcl-xL expression by peptide loaded streptamers can be explained by sustained activation of PKB, the induction of apoptosis by soluble antibodies is more difficult to understand. T cell apoptosis and activation of caspase-3 can be induced *via* the extrinsic death-receptor-mediated pathway or through the intrinsic mitochondrial pathway. Currently, I do not know which of the two pathways becomes activated by soluble antibodies, but I

clearly favour a mechanism that involves the mitochondrial pathway for the following reasons. First, it was reported that naïve or short term activated T cells (for one day) are resistant to FasR-mediated apoptosis [133]. Second, an expression of FasR/FasL after antibody stimulation was hardly detectable. Third, the time frame between the application of the death-inducing stimulus and the first signs of cell death (8 hours) appeared to be too short to be mediated *via* the FasR/FasL-system in primary T cells. Therefore, I think that molecules involved in the intrinsic mitochondrial pathway such as Bim, Bid, or Bad might be more likely the candidates that connect the TCR to the activation of caspase-3.

Bim is a member of the BH3-only Bcl-2 family and promotes apoptosis in both thymocytes and peripheral T cells [134]. Bim activity is regulated in several ways. The first mechanism operates at the protein level. In different cell types, the expression of Bim is induced after stimulation [135-137]. The second mechanism is through the translocation of Bim to the mitochondria after release from the dynein motor complex [138]. Bim can also be phosphorylated *via* activated ERK1/2 [139, 140]. Bim phosphorylation leads to its degradation *via* the proteasomal pathway. In fact, it has been shown that Bim degradation is blocked by proteasome inhibitors and is defective in a temperature sensitive mutant of the E1 component in the ubiquitin conjugating system [139]. Furthermore, in PC12 cells, Bim phosphorylation and degradation correlates with sustained ERK1/2 activation, which lasts for several days after NGF treatment [141]. Consistant with the third mechanism, I observed sustained ERK1/2 activation, Bim phosphorylation and degradation in peripheral T cells in response to streptamers (Figure 23). Thus, I speculate that the degradation of Bim mediated by sustained activated ERK1/2, together with the upregulation of Bcl-xL, can contribute to cell survival in response to OT1-streptamer stimulation. Additional experiments will be needed to directly test the function of ERK1/2 (i.e. by applying MEK inhibitors and examining Bim phosphorylation and degradation in streptamer treated cells).

It is interesting to note that Bim phosphorylation parallels with the transient ERK1/2 activation upon antibody stimulation. Indeed, Bim was phosphorylated after 3 min of stimulation and the phosphorylation declined to basal level after 30 min of stimulation (Figure 23), a time point when ERK1/2 activation could no longer be observed. According to the transient Bim phosphorylation, I also observed a transient Bim

degradation in response to antibody stimulation, which occurred within the first 30 min. This might indicate that the transient ERK1/2 activation after antibody stimulation is not sufficient to induce the sustained phosphorylation and degradation of Bim. Thus, my data are suggestive for the fact that there is sufficient Bim to trigger apoptosis in T cells stimulated with soluble antibodies.

By using the Bax/Bak double knock out mice, Thompson et al. and Korsmeyer et al. showed that Bim requires the apoptotic effecter molecules Bax and Bak to mediate pro-apoptotic functions, although the mechanism remains unknown [142, 143]. Under apoptosis-inducing conditions, Bax and Bak undergo conformational changes, leading to their homodimerization and translocation to the mitochondrial membrane [144, 145]. There they trigger mitochondrial dysfunctions and induce the release of apoptogenic factors, such as cytochrome C, Endonuclease G, Smac/DIABLO, and AIF (Apoptosis Inducing Factor) [146-149].

Under survival promoting conditions, the apoptotic function of Bim is blocked by anti-apoptotic molecules, such as Bcl-2 and Bcl-xL, which are found in a constitutive association with Bim on the mitochondrial membrane [150, 151]. Therefore, it appears as if the ratio of Bcl-2 and Bcl-xL *versus* Bim could regulate T cell life or death. In line with this assumption, I demonstrated that under the survival-favoring TCR stimulation conditions (streptamers) Bcl-xL expression is upregulated. Thus I would like to propose here that upregulated Bcl-xL blocks the function of Bim after streptamer stimulation.

In addition to its association with Bim, Bcl-xL exerts its anti-apoptotic function in different ways: the heterodimerization between Bcl-xL and pro-apoptotic molecule Bax is considered to be essential for the anti-death activity of Bcl-xL [152]. Another mechanism by which Bcl-xL can prevent apoptosis is by sequestering pro-caspase-9 from Apaf. Bcl-xL binds to both pro-caspase-9 and Apaf-1 and this association prevents the Apaf-1 mediated caspase-9 activation. Release of Apaf-1 from Bcl-xL might be essential for determining the life or death of a cell [153, 154].

4.2. Molecular mechanism leading to sustained ERK1/2 activation

It has been shown that TCR engagement and induction of tyrosine phosphorylation **alone** are not sufficient for T cell activation but that the duration of TCR stimulation is also critical to obtain a functional T cell response. Indeed, Berg et al. demonstrated that TCR stimulation by plate-bound antibodies induces sustained ERK1/2 activation and degranulation of CTLs [113], whereas soluble cross-linked antibodies to the TCR/CD3 complex fail to induce T cell degranulation although they can trigger transient ERK1/2 activation. My data confirme and further extend these observations. In fact, I showed that sustained ERK1/2 activation in response to OT1-streptamer stimulation correlates with the upregulation of CD25 and CD69 (Figure 26). Moreover, the lysosomal marker LAMP1 [155], a molecule that is transiently exposed on the cell surface upon fusion of the lytic granules with the plasma membrane [156, 157], localized to the plasma membrane only after OT1-streptamer stimulation (Figure 27). This indicates that upon streptamer stimulation naïve CD8$^+$ T cells differentiate into cytolytic T lymphocytes. In contrast, I could not observe an upregulation of CD25 and CD69 and translocation of LAMP1 to the plasma membrane after antibody stimulation.

The principal effector function of cytotoxic T cells is the release of lytic granules, containing perforin, granzymes, and granulysin [158]. Although the release of cytotoxic effector proteins induces the apoptosis of target cells, the cytotoxic mediators, such as granzyme B, have also been described to initiate apoptosis in activated CD8$^+$ T cells [103, 159, 160]. An intriguing question arised here is that streptamers generate "full" CTLs, but the cells do not undergo granzyme B mediated apoptosis. One possibility to explain this discrepancy lies in the integrity of mitochondria. It is known that the critical substrate of granzyme B is Bid [160, 161] and the cleavage of Bid by granzyme B results in its activation, mitochondrial permealibilization, and cytochrome C release [88, 160]. However, mitochondria integrity is regulated by both pro-apoptotic and anti-apoptotic molecules. Indeed, Bid function can be inhibited by anti-apoptotic molecules such as Bcl-2 and Bcl-xL [87]. Based on the upregulation of Bcl-xL following streptamer stimulation, I would like to propose that the expression of Bcl-xL neutralizes Bid, thereby providing protection from the rapid apoptosis induced by granzyme B.

It is yet not clear how ERK1/2 activation can be maintained for several hours after OT1-streptamer stimulation. In multiple experiments I observed a biphasic ERK1/2 activation after OT1-streptamer stimulation (see Figure 29). The first phase started at 3 min and lasted for 30 min while the second wave began after 2-4 h. Biphasic activation of ERK1/2 has been observed previously. For example, AND TCR tg CD4^{+} peripheral T cells demonstrate a biphasic ERK1/2 activation after long-term stimulation with a high-affinity peptide [112, 162]. However, the nature of the second wave of ERK1/2 activation remains unclear. I assume that cytokine release is responsible for the second wave of ERK1/2 phosphorylation under survival promoting conditions. I tested this assumption by adding neutralizing antibodies against IL-2 (Figure 30). I clearly demonstrated that the presence of neutralizing antibodies reduced the second wave of ERK1/2 activation to 40-50% after 4 h stimulation. Hence, I showed that IL-2 signaling at least partially contributes to the second wave of ERK1/2 activation observed in response to OT1-streptamers. Moreover, IL-2 expression can be detected 4 h after streptamer stimulation (data not shown).

Three hypotheses were proposed to explain why anti-IL-2 neutralizing antibodies failed to completely abolish the second wave of ERK1/2 activation upon streptamer stimulation. The first hypothesis is based on the high affinity of the IL-2R for IL-2. IL-2R has three chains: α, β, and γ [163]. Resting T cells express a form of this receptor composed of β and γ chains that binds IL-2 with moderate affinity [163]. Streptamer stimulation tiggers the upregulation of the α chain (CD25, Figure 26), which associates with the β and γ heterodimer, creating a receptor with a much higher affinity for IL-2 (10 pM) [163]. T cells expressing the high affinity IL-2R can respond to very low concentration of IL-2. However, the affinity of anti-2 neutralizing antibodies is 1000-fold less than that of IL-2R. Thus, it is likely that neutralizing antibodies can not compete with IL-2R for IL-2. In addition, T cells produce IL-2, which acts in an autocrine manner [164]. The rapid uptake of IL-2 by IL-2R can prevent the neutralization of IL-2 by anti-IL-2 neutralizing antibodies. Therefore, both the high affinity of IL-2R and the autocrine action of IL-2 may prevent the complete neutralization of IL-2. Finally, it is possible that in the presence of anti-IL-2 antibody, the IL-2 signaling cannot be prevented.

The second possibility to explain how the second wave of ERK1/2 activation is maintained in the presence of neutralizing anti-IL-2 antibodies is that part of the ERK1/2 activation is mediated *via* FasR or TNFR (Tumor Necrosis Factor Receptor). The TNFR and FasR are physiological mediators of apoptosis. However, several groups demonstrated that activation of the FasR also induces ERK1/2 activation [165], which subsequently suppresses Fas-induced apoptosis by deflecting DISC signaling from activating caspase-8 and Bid in activated T cells [166]. Inhibition of ERK1/2 activation markedly sensitized the cells to FasR mediated apoptosis [166]. Consistant with these data, I observed an enhanced expression of FasL/FasR upon streptamer stimulation. Thus, I would like to propose that sustained ERK1/2 activation partially results from FasR triggering. This could represent a negative feedback to regulate FasR mediated apoptosis.

The third possibility that could explain the second wave of ERK1/2 activation might result from the prolonged TCR signals, which occurs at the endosomes. In support of this statement, I detected the formation of "signaling endosomes" which are positive for TCR and its ligand, PLCγ1, Ras, and pERK1/2. The signaling endosomes may serve as the second platform leading to the sustained ERK1/2 activation and will be discussed later.

4.3. The divergence of signals for T cell activation at the level of LAT / PLCγ1

One of the most surprising findings of this thesis is that signals involved in TCR-induced ERK1/2 activation following antibody *versus* streptamer stimulation diverge downstream of LAT. Both stimuli induce transient phosphorylation of ZAP-70 and LAT. Conversely, downstream of LAT the activation of PLCγ1, PKD1, and ERK1/2 is sustained upon streptamer stimulation, but remain transient after antibody stimulation. The mechanism for such divergence remains unclear. Here, several possibilities will be proposed. The first hypothesis is that upon antibody stimulation Gab2 mediates the suppression of PLCγ1 activation. Gab2 is an adaptor protein that is tyrosine phosphorylated by ZAP-70 following TCR ligation [167]. Gab2 can function as an inhibitory adaptor protein by competing with SLP76 for Gads binding *via* its PXXXR motif [168]. Indeed, increased amounts of Gab2 prevent the association between SLP76 and Gads in heterologous expression systems [168]. The formation of LAT-Gads-Gab2 ternary complex was also observed following TCR ligation. However, the

mechanisms regulating the binding of Gads-Gab2 or Gads-SLP76 to LAT are still unknown. SLP76 plays an important role for the recruitment of PLCγ1 to the plasma membrane and positioning in close proximity to Itk. Subsequently, Itk phosphorylates and activates PLCγ1 [42, 43]. On the basis of these data, it is intriguing to speculate that the molecular ratio of LAT-Gads-Gab2 and LAT-Gads-SLP76 complexes might be different upon antibody stimulation *versus* streptamer stimulation. Antibody stimulation could induce higher amounts of LAT-Gads-Gab2 complexes, which might subsequently prevent PLCγ1 activation and give rise to its transient phosphorylation kinetics. In contrast, upon streptamer stimulation, more LAT-Gads-SLP76 complexes are formed, allowing sustained PLCγ1 activation.

It was surprising to find that PLCγ1 remains active despite the absence of detectable LAT phosphorylation. A positive feedback loop might support PLCγ1 phosphorylation independent of LAT. This positive feedback loop includes PLCγ1, DAG, PKCθ, and Tec (a kinase belong to the Tec kinase family), based on the following observations. First, activated PLCγ1 hydrolyzes PIP_2, resulting in the production of IP_3 and DAG [42, 43]. DAG activates PKCθ, which according to Altman et al., function as a positive regulator of PLCγ1 [169]. In fact, it has been reported that activation of PLCγ1 is significantly impaired in PKCθ-deficient T cells [169]. Additionally, PKCθ may also associate and support Tec, which is required for PLCγ1 activation. Indeed, Tec constitutively binds to PKCθ in both Jurkat T cells and in primary mouse T cells. PKCθ-deficient T cells display a reduced tyrosine phosphorylation of Tec kinase [169]. Thus, I would like to propose that streptamer stimulation may induce the formation of PLCγ1-DAG-PKCθ-Tec-PLCγ1 positive feedback loop, thus allowing sustained activation of PLCγ1. This hypothesis is further supported by the observation that PKD1 activation, indicative of DAG production, is also sustained following streptamer stimulation. In the case of antibody stimulation, the strong phosphorylation of PLCγ1 leads to the activation of PKCθ/Tec, which might positively regulate PLCγ1 activation. However, since local PIP_2 is used up quickly, by a strong activated PLCγ1, DAG production is no longer possible, thus subsequently preventing the recruitment of PKCθ/Tec to the plasma membrane, ultimately leading to transient PLCγ1 activation.

One interesting question that emerges is how PLCγ1 is targeted to the plasma membrane in the absence of LAT phosphorylation. The PH domain of PLCγ1 has been shown to bind to PIP_3, thus allowing its recruitment to the plasma membrane [170]. Therefore, I propose a two step mechanism to regulate the localization of PLCγ1 at the plasma membrane. Immediately after stimulation, phosphorylated LAT target PLCγ1 to the plasma membrane. Successively, once that enough PIP_3 is produced by PI3K, PLCγ1 could remain at the plasma membrane by binding to PIP_3 without further requirement for association to LAT. In line with this hypothesis, I observed that upon streptamer stimulation, PKB phosphorylation, indicative of PI3K activation, is sustained, allowing for the membrane localization of PLCγ1 in the absence of LAT phosphorylation. Moreover, it is important to note that the generation of PIP_3 might still occur in endosomal membrane by PIP_2 phosphorylation. Indeed, although the major pool of PIP_2 is in the plasma membrane, PIP_2 is also present in endosomal membrane [171]. Thus, this makes it possible for the recruitment of PLCγ1 to the endosomal membrane, which will be discussed later.

4.4. Negative regulation of T cell activation

An important question that still remains to be answered is how the transient activation of ERK1/2 under apoptosis-promoting conditions is regulated at the molecular level. Here several possibilities can be envisioned. First, I observed that antibody stimulation induces rapid ubiquitination and degradation of ZAP-70 (Figure 35). Candidates mediating ZAP-70 degradation include members of the Cbl family, which exert E3-dependent ubiquitin ligase activity and have been shown to interact with ZAP-70 [73]. However, the role of Cbl molecules in terms of ZAP-70 ubiquitination and degradation is controversial. For example, it has been shown that degradation of ZAP-70 can be induced after overexpression of c-Cbl [172, 173]. Conversely, the expression levels of ZAP-70 are not substantially enhanced in c-Cbl deficient T cells although the enzymatic activity of ZAP-70 is augmented [174, 175]. Hence, the regulation of ZAP-70 expression by c-Cbl remains elusive. Another candidate that can regulate the expression of ZAP-70 is calpain. Sustained T cell-APC interaction results in Ag dose- and time-dependent degradation of ZAP-70 *via* a calpain-dependent mechanism and ZAP-70 degradation in antigen stimulated T cells is inhibited by calpain inhibitors [118]. Calpains are a family of ubiquitous, Ca^{2+}-dependent, cytosolic cysteine

proteases and their expression and proteolytic activity are increased following TCR stimulation [176]. Therefore, it is tempting to speculate that the strong calcium flux following antibody stimulation enhances proteolytic activity of calpains and thus supports the degradation of ZAP-70. Indeed, treatment of CD8 T cells with ionomycin alone triggered degradation of ZAP-70 [119]. In future experiments, I will employ calpain inhibitors and examine their influence of ZAP-70 expression after antibody stimulation. Since calpains are Ca^{2+}-dependent, I will also analyze the effects of calcium on ZAP-70 degradation.

In addition to ubiquitination and degradation of ZAP-70, I also found a rapid degradation of Lck and of the ζ chain after antibody stimulation (Figure 32, 33). Since antibody mediated degradation of the ζ chain and Lck was very rapid, the issue arised how ZAP-70 activation, which is downstream of ζ chain phosphorylation and Lck activation, could nevertheless occur? It has been reported that the phosphorylation of ZAP-70 and the ζ chain occurs quite rapid following TCR ligation, being clearly inducible after only 15 s of activation [177]. Therefore, it is possible that ζ chain phosphorylation within 1 min is sufficient to target ZAP-70 to the plasma membrane leading to its phosphorylation and activation after anti-CD3/CD8 mAbs stimulation.

Another hypothesis to explain why ZAP-70 is activated in the absence of ζ chain expression may lie in ITAM multiplicity in the TCR complex [8]. As I mentioned before, each of the CD3 components contains a single ITAM within its cytoplasmic tail while each of the ζ chain contains three tandem ITAMs [8]. All the ITAMs in TCR complex can bind to the SH2 domain of ZAP-70, although the ITAMs in the the ζ chain have much more affinity [178]. Thus, it might be that the ITAMs in the ζ chain initiate T cell activation by interacting with ZAP-70. Once ζ chain underwent degradation, the ITAMs in other CD3 complex can also drive ZAP-70 activation, but with lower capacity. Finally, a relative stability of ZAP-70 to counteract the effects of phosphatase could also help to maintain the activation of ZAP-70 when ζ chain and Lck undergo degradation.

In addition to the degradation of Lck, the ζ chain, and ZAP-70, other mechanisms may also contribute to the transient activation of ERK1/2 under pro-apoptotic conditions of

stimulation. One possibility could be that the strong activation of membrane proximal tyrosine kinases (e.g. ZAP-70, Lck, Fyn, Itk, Rlk) not only induces the assembly of positive-regulatory but also of negative-regulatory signaling complexes at the plasma membrane. In this regard, it is important to mention a recent study which revealed a negative regulatory role for Lck in T cells by showing that TCR-mediated stimuli are stronger/sustained after suppression of Lck expression by siRNA in Jurkat T cells and in primary human T lymphocytes [179]. Additionally, a negative regulatory role for LAT in T cell signaling has also been suggested [180, 181]. Knock-in approaches have demonstrated that the deletion of particular tyrosine residues within LAT leads to the development of autoimmune diseases [182-184]. Thus, it is tempting to speculate that the strong phosphorylation of LAT after antibody-stimulation induces the assembly of negative regulatory complexes, which prevent a sustained activation of ERK1/2. Conversely, weaker input signals (= less strong activation of membrane proximal protein tyrosine kineses) would not induce the activation of these negative regulatory loops, thus allowing a sustained activation of ERK1/2.

Other important molecules that negatively regulate the TCR signal pathway are the DGKs (Diacylglycerol Kinases). DGKs convert DAG to PA, which in turn acts as a lipid second messenger [185]. DGKα is highly expressed in T cells and translocates to the plasma membrane in response to TCR stimulation, a process that requires calcium flux [51]. Overexpression of a catalytically inactive form of DGKα induces sustained signaling through RasGRP1 [55]. DGKα contains Ca^{2+}-binding EF hand motifs, which presumably mediate Ca^{2+} mediated activation of this enzyme by inducing conformational changes in DGKα [186]. The strong calcium flux following antibody stimulation might induce a conformational change of DGKα and facilitate its activation. Activated DGKα could then terminate TCR initiated activation of RasGRP and cause the transient ERK1/2 activation. To address this hypothesis, I pretreated T cells with R59949, a specific inhibitor of DGKα, followed by antibody stimulation. In preliminary experiments, I could indeed demonstrate that inhibition of DGKα by R59949 leads to an increase in ERK1/2 activation compared to untreated cells. Therefore, the activation of DGKα may also contribute to the transient ERK1/2 activation. It is also worth to investigate whether the addition of R59949 can convert apoptosis into survival following antibody stimulation.

Taken together, my data indicate that "optimal" membrane proximal signals, such as a strong Ca^{2+} flux or maximal LAT phosphorylation, may not be automatically interpreted as a sign of a productive T cell response. Rather, it appears that only a detailed analysis of the dynamics of membrane proximal signaling events together with the assessment of late events of T cell activation, such as proliferation, upregulation of activation marker, or apoptosis, allows a correct interpretation of the biochemical data.

4.5. Distinct subcellular localization of molecules

During thymocyte development positive selecting ligands trigger a sustained activation of ERK1/2 that leads to its accumulation throughout the cell. In contrast, negative selecting ligands induce a transient ERK1/2 activation and target ERK1/2 to the plasma membrane [61]. This study indicated that the cell fate correlates with the distinct subcellular localization of signals. In line with the data, the localization of phosphorylated (and hence activated) ERK1/2 was markedly different under apoptosis *versus* proliferation inducing conditions in my study. I found that under both stimulation conditions, pERK1/2 localized at the plasma membrane 3 min after stimulation (Figure 39). However, after 30 min, proliferation inducing stimuli led to an accumulation of activated ERK1/2 within the endosomes. Howe et al. demonstrated that after NGF stimulation endosomes not only contain pERK1/2, but also Ras, Raf, and MEK. They further claimed "signaling endosomes" whose existence has been debated for more than a decade [130]. Experiments with rat liver cells showed that in response to EGF, the EGF receptor is rapidly internalized, but still associates with Shc, Grb2, and SOS in endosomes. These events could be linked to Ras activation [187-189]. In endocytosis defective cells, EGF-dependent tyrosine phosphorylation of EGFR and ERK1/2 is suppressed [188, 190]. Morever, in HEK293 cells expressing dominant suppressor mutants of β-arrestin or dynamin (both are needed to initiate receptor internalization), β2-adrenergic receptor-mediated activation of ERK1/2 is inhibited. The inhibition of receptor internalization specifically blocks Raf-mediated activation of MEK [190]. Collectively, these data strongly support the existence of signaling endosomes.

The existence of "signaling endosomes" in T cells was also indicated in our study. After streptamer stimulation, I found that many of the components that link the TCR complex to ERK1/2 activation localized in the endosome. These molecules included

the TCR complex, PLCγ1, and Ras (Figure 43, 44). To further prove the existence of "signaling endosomes" in T cells, I shall isolate enriched endosomal fractions from CD8$^+$ T cells following streptamer stimulation and analyze the samples by Western blot. The method has been already established in our laboratory. It will allow us to confirm the endosomal localization of molecules. In addition, some molecules can not be detected by using confocal laser canning microscopy, such as pLAT, pPLCγ1, and pPKB. This may be due to the low phosphorylation status of these molecules or the inaccessibility of different phospho-specific antibodies tested for immunofluorescence. Thus, the purification of endosomes will mike it possible to investigate the localization of these molecules.

What is the function of "signaling endosomes" for T cell activation? "Signaling endosomes" could provide another platform for signal transduction and help to sustain activation of ERK1/2 and other signaling molecules in response to physiological ligand. Furthermore, appropriate trafficking of the activated TCR through endosomes could control the spatial and temporal regulation of ERK1/2 signaling, thus defining a unique biological response. Indeed, mislocalizing late endosomes to the cell periphery was shown to cause a prolonged EGFR activation on late endosomes and sustained ERK1/2 and p38 signaling and to result in hyperactivation of nuclear targets in Hela cells [191]. Finally, endosomal association might protect phosphorylated ERK1/2 as well as the signaling components contributing to ERK1/2 activation from cytosolic or plasma membrane targeted phosphatases and other negative regulators.

How can phosphorylated ERK1/2 translocate to the nucleus when it is localized in endosomes after OT1-streptamer stimulation? It has been reported that receptor-mediated endocytosis coordinates the nuclear transport of signaling proteins through the endosomal compartments. Indeed, Rho B is important for late endosome trafficking to the perinuclear region and for efficient nuclear trafficking of activated PKB and ERK1/2 in response to PDGF [192]. Using NIH-3T3 cells, Bild et al. showed that receptor endocytosis is essential for Stat3 nuclear translocation and Stat3-dependent gene regulation [193]. Following growth factor stimulation, Stat3 localizes to endocytotic vesicles that move sequentially in a time-dependent manner from the plasma membrane through the cytosol to the perinuclear region. Inhibition of receptor mediated endocytosis abrogates the transport of Stat3 to the perinuclear region,

excludes Stat3 DNA-binding activity, and suppresses Stat3-mediated transcriptional events. On the basis of these reports and the results demonstrated above, I propose that pERK1/2 could also be transported from the plasma membrane through the cytosol to the perinuclear region by receptor mediated endocytosis in response to streptamers. To further confirm this hypothesis, additional experiments should be performed to assess whether after long term stimulation (24 h), activated ERK1/2 translocate to the nucleus in response to OT1-streptamer stimulation.

Upon binding to their ligands, cell surface receptors are cleared from the plasma membrane by endocytosis through sequential endosomal compartments. Some of these endosomes recycle to the cell surface, while others are targeted for degradation. The ubiquitination of receptors serves as a signal targeting them to lysosomes for degradation. Therefore, the same receptor can mediate different kinetics of activation of signaling pathway by using different endocytic vesicles strategy. In response to OT1-streptamer stimulation, activated ERK1/2 localizes in endosomes after 30 min and 4 h of stimulation (Figure 39). On the contrary antibody stimulation induced the ubiquitination of ZAP-70 as well as the degradation of ζ chain and Lck, which may be mediated by Cbl. Based on the same rate of TCR internalization (Figure 31), I speculate that both stimuli induce the TCR endocytosis, while the ubiquitination of the proximal molecules in response to anti-CD3/CD8 mAbs can serve to sort the endosomes to lysosomes for degradation, thus leading to the transient activation kinetics. However, in response to streptamers, the TCR and the associated signaling molecules could be rapidly recycled from early endosomes into late endosomes and accumulate in the perinuclear region, where some molecules, such as pERK1/2, could be released from endosomes and subsequently imported into the nucleus, thus leading to gene transcription and activation. My hypothesis is summarized in Figure 49. Indeed, I already showed that ζ chain localizes in lysosomes after antibody stimulation. In addition, the inhibition of lysosomal function (by NH_4Cl) led to a sustained ERK1/2 activation. It will be interesting to investigate the localization of other molecules mediating TCR induced ERK1/2 activation after antibody stimulation in the absence or presence of NH_4Cl.
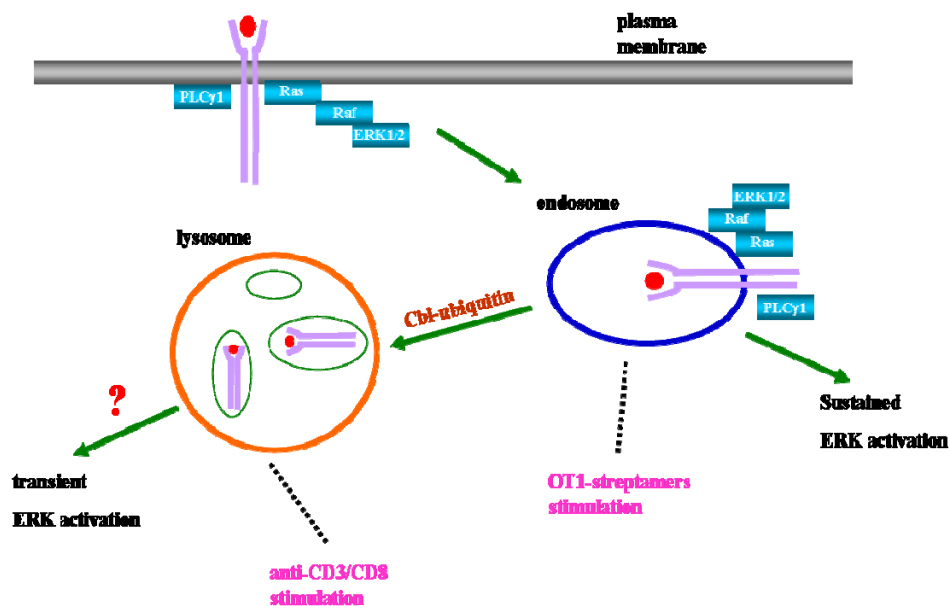
Figure 49. Summary and assumption of trafficking of signalling molecules in response to OT1-streptamers or anti-CD3/CD8 mAbs

OT1-streptamer stimulation induces TCR endocytosis together with its ligands. PLCγ1, Ras, and ERK1/2 also translocate to the same endosomes and further transmit the signals even after TCR internalization. In contrast, the ubiquitination of the TCR complex, which might occur after antibody stimulation, could cause the transition of the signaling complex from the endosome to the lysosome, induce the degradation of the TCR complex, and subsequently leading to transient ERK1/2 activation.

In summary, I have shown that triggering of the TCR by different stimuli can induce either survival/proliferation or alternatively apoptosis of mature CD8 T lymphocytes. I have further demonstrated that these cellular outcomes correlate with different activation/phosphorylation kinetics of key molecules involved in T cell activation, which appear to bifurcate at the level of LAT / PLCγ1. My data corroborate and extend previous experiments performed in thymocytes [61] and suggest that the molecular mechanisms underlying these two systems are to a large extent comparable. How mature T cells upregulate thresholds for T cell activation to convert a negative selecting signal within the thymus into a proliferation-inducing signal in the periphery requires further analysis. In addition, careful kinetic and quantitative activation/phosphorylation studies are required to fully elucidate how transient *versus* sustained signaling events close to the membrane are converted into distinct cellular responses. These experiments will also have to take into account of the differential compartmentalization of signaling molecules. Moreover, a methmatical model will be established based on the data produced in this thesis. Finally, our data as well as the

data reported by Daniels et al. suggest that analysis of the "strength" of membrane proximal events by biochemical methods should be interpreted with care as this does not automatically allow one to conclude whether a productive T cell activation (in the sense of proliferation) is initiated or not.

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Abbreviations

ADAP	Adhesion and Degranulation promoting Adaptor Protein
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumine
Cbl	Casitas B-lineage Lymphoma
Csk	C-terminal Src Kinase
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
EDTA	Ethylene-Diamine Tetra-acetic Acid
ER	Endoplasmatic Reticulum
Erk	Extracellular signal-Regulated protein Kinase
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GADS	Grb2 related Adaptor protein
Grb2	Growth factor Receptor Bound protein 2
H	Hour
IP	ImmunoPrecipitation
IP$_3$	Inositol-1,4,5-triphosphate
IS	Immunological Synapse
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
Itk	Interleukin-2 inducible T cell Kinase
LAT	Linker for Activation of T cells
LAX	Linker for Activation of X-cells
Lck	Lymphocyte-specific Cytoplasmic protein tyrosine Kinase
LIME	Lck-Interacting Membrane protein
MAPK	Mitogen Activated Protein Kinase
Min	Minute
Nck	Non-Catalytic region of tyrosine Kinase
NFκB	Nuclear Factor κB
NF-AT	Nuclear Factor of Activated T cells
NTAL	Non-T cell Activation Linker

PAG	Phosphoprotein Associated with Glycosphingolipid enriched microdomains
PBS	Phosphate-Buffered Saline
PH	Pleckstrin Homology
PI3K	Phosphatidylinositol-3 Kinase
PIP	Phosphatidylinositol Phosphate
PIP$_2$	Phosphatidylinositol-4,5-biphosphate
PIP$_3$	Phosphatidylinositol-1,4,5-triphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLCγ1	Phospholipase Cγ1
PRR	Proline-Rich Region
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
RasGRP	Ras Guanyl-Releasing Protein
SDF	Stromal cell-Derived Factor
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SFKs	Src Family Kinases
SH2	Src Homology 2 domain
SH3	Src Homology 3 domain
SHP-1	SH2 domain Containing PTP-1
siRNA	small interfering RNA
SIT	SHP-2 Interacting Transmembrane adaptor protein
SLP-76	SH2 domain containing Leukocyte Protein of 76 kDa
SOS	Son of Sevenless
TCR	T cell receptor
TRAP	Transmembrane Adaptor Protein
TRIM	T cell Receptor Interacting Molecule
WASP	Wiskott-Aldrich Syndrome Protein
ZAP-70	Zeta-Associated Protein of 70 kDa
μ	Micro

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Summary

Engagement of the TCR can result in different functional outcomes such as activation, proliferation, survival, or apoptosis. However, it is not yet completely understood how signal specificity is achieved when different signals are relayed by the common intracellular signal transduction pathways. The aim of this project is to elucidate how a single-specificity TCR in peripheral T cells distinguishes between ligands which trigger distinct biological responses. Thus, an experimental system was established in which either proliferation (by treatment with OT1-streptamers) or apoptosis (by treatment with anti-CD3/CD8 mAbs) can be induced in mature OT1 TCR transgenic T cells. The cellular outcomes correlate with distinct activation/phosphorylation kinetics of key molecules involved in T cell activation, which appeared to bifurcate at the level of LAT / PLCγ1. The input signals leading to apoptosis induced a strong, but transient activation of PLCγ1, PKD1 and ERK1/2. Among these molecules, ERK1/2 phosphorylation occurs mainly at the plasma membrane and its transient nature of activation is partially due to the "activation-induced degradation" of ζ chain, Lck, and ZAP-70. In support of this observation, the inhibition of ζ chain degradation (by NH_4Cl) led to sustained ERK1/2 phosphorylation and T cell activation instead of apoptosis upon antibody stimulation. In contrast, stimuli promoting proliferation generate a low and sustained activation of PLCγ1, PKD1 and ERK1/2. Phosphorylated ERK1/2 shuttles from the plasma membrane to the endosomes, which are also positive for the TCR, PLCγ1, and Ras. These endosomes serve as the second platform for sustained signal activation. Moreover, OT1-streptamers induce the expression of the anti-apoptotic molecule Bcl-xL and the degradation of the pro-apoptotic molecule Bim, which are required to prevent T cell apoptosis. Collectively, these data provide signaling signatures that are associated with proliferation or apoptosis of T cells.

Curriculum viate

Name:	Xiaoqian Wang
Birthday:	24.12.1973
E-mail:	xqwangmail@yahoo.com
	xiaoqian.wang@med.ovgu.de
Tel.:	Office: 0049 391 6714448
	Home: 0049 391 40491850
	Cell: 0049 176 20526889
Address:	Fermersleber Weg 45A / 0402,
	39112, Magdeburg, Germany
Gender:	Female
Marital status:	Married
	Husband: Liquan Yang
Nationality:	Chinese

Education and work Experience:

Dec. 2008—Now	Ph.D. student (LOM). Institute of Molecular and Clinical Immunology, Medical Faculty, Otto-von-Guericke-University, Magdeburg, Germany.
Dec. 2005—Nov. 2008	Ph.D. student (GRK1167). Project 1: "Dynamics of proximal signaling events after TCR/CD8-mediated induction of proliferation or apoptosis in mature CD8 T cells". Project 2: "The role of ADAP/SKAP55/RIAM module for CXCR4 mediated migration". Institute of Molecular and Clinical Immunology, Medical Faculty, Otto-von-Guericke-University, Magdeburg, Germany.
Jun. 2004.—Nov. 2005	Ph.D. student. Project: "Dynamics of proximal signaling events after TCR/CD8-mediated induction of proliferation or apoptosis in mature CD8 T cells". Max-Planck Institute of Dynamics of Complex Technical Systems, Magdeburg, Germany and Institute

	of Molecular and Clinical Immunology, Medical Faculty, Otto-von-Guericke-University, Magdeburg, Germany.
Feb. 2004—Jun. 2004	Practice course in the Institute of Molecular and Clinical Immunology, Medical Faculty, Otto-von-Guericke-University, Magdeburg, Germany.
Sep. 2002—Oct. 2003	Research assistant Shanghai Institute of Biological Products, Shanghai, China
Sep. 1999—Aug. 2002	Master of Science in Molecular Biology. Project: "The characterization and purification of Fibronectin from blood plasma". Shanghai Institute of Biological Products, Shanghai, China
Sep. 1994—Jul.1998	Bachelor of Science in Biological Pharmacology. China Pharmaceutical University, Nanjing, China

Publications and presentations

1. Publications

Horn J[#], **Wang XQ**[#]**,** Reichardt P, Stradal TEB, Warneck N, Simeoni L, Gunzer M, Yablonski D, Schraven B, Kliche S. SLP76 is mandatory for TCR mediated inside-out signalling but dispensable for CXCR4 induced LFA-1 activation, adhesion and migration. J Immunol. [In revision].   (#: equal contribution to this work)

Wang XQ, Simeoni L, Lindquist J, Saez-Rodrigues J, Ambach A, Gilles ED, Kliche S, and Schraven B. Dynamics of proximal signaling events after TCR/CD8-mediated induction of proliferation or apoptosis in mature CD8^{+} T-cells. J Immunol, 2008. 180:6703-12

Kliche S, Breitling D, Togni M, Pusch R, Heuer K, **Wang XQ**, Freund C, Kasirer-Friede A, Menasche G, Koretzky GA, Schraven B. The ADAP/SKAP55 signaling module regulates T-cell receptor-mediated integrin activation through plasma membrane targeting of Rap1. Mol Cell Biol. 2006. 26:7130-44

2. Talk

Wang XQ, Kliche S, Saez-Rodriguez J, Gilles ED, Schraven B. Quantitative analysis of the dynamics of membrane proximal signaling events regulating the activation and proliferation of peripheral T cells. 36[#] Joint annual meeting of the Germany and Scandinavian societies for Immunology. Kiel, Germany. 2005.

3. Posters

Wang XQ, Busse M, Saez-Rodriguez J, Gilles ED, Schraven B, Lindquist JA, Kliche S. The conversion of apoptosis to cell survival by the inhibition of lysosomal function. Poster on German symposium on systems biology 2009. Heidelberg, Germany. 2009.

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