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Dynamics of Proximal Signaling Events after TCR/CD8-Mediated Induction of Proliferation or Apoptosis in Mature CD8+ T Cells

Xiaoqian Wang,* Luca Simeoni,* Jonathan A. Lindquist,* Julio Saez-Rodriguez,‡ Andreas Ambach,† Ernst D. Gilles,‡ Stefanie Kliche,2* and Burkhart Schraven2*

Engagement of the TCR can induce different functional outcomes such as activation, proliferation, survival, or apoptosis. How the TCR-mediated signaling cascades generating these distinct cellular responses are organized on the molecular level is so far not completely understood. To obtain insight into this question, we analyzed TCR/CD8-mediated signaling events in mature OT-I TCR transgenic T cells under conditions of stimulation that lead to either proliferation or apoptosis. These experiments revealed major differences in the phosphorylation dynamics of LAT, ZAP70, protein kinase B, phospholipase C-γ1, protein kinase D1, and ERK1/2. Moreover, input signals leading to apoptosis induced a strong, but transient activation of ERK1/2 mainly at sites of TCR-engagement. In contrast, stimuli promoting survival/proliferation generated a low and sustained activation of ERK1/2, which colocalizes with Ras in recycling endosomal vesicles. The transient activation of ERK1/2 under pro-apoptotic conditions of stimulation is at least partially due to the rapid polyubiquitination and subsequent degradation of ZAP70, whereas the sustained activation of ERK1/2 under survival promoting conditions is paralleled by the induction/phosphorylation of anti-apoptotic molecules such as protein kinase B and Bcl-xL. Collectively, our data provide signaling signatures that are associated with proliferation or apoptosis of T cells. *The Journal of Immunology, 2008, 180: 6703–6712.

Stimulation of the TCR/CD3/ζ-complex can result in a variety of different cellular responses such as differentiation, cytokine secretion, survival, proliferation, or apoptosis. According to the currently accepted model, signaling downstream of the engaged TCR is initiated after ITAMs within the TCR-associated CD3/ζ-complex have been phosphorylated by the Src-family protein kinases Lck and Fyn (1). Phosphorylation of the ITAMs induces the recruitment of ZAP70, which is in turn phosphorylated and activated by Lck and Fyn (2). Activated ZAP70 then phosphorylates several downstream molecules, including the key adapter proteins LAT and SLP-76 (3, 4). The formation of a signalosome containing LAT and the adapter proteins Gads and SLP-76 provides a membrane-associated signaling platform that facilitates the recruitment and activation of PLC-γ1 which subsequently converts phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG)3 and inositol 1,4,5-trisphosphate. 1,4,5-trisphosphate mediates the release of Ca2+ from intracellular stores whereas DAG activates PKC as well as RasGRP, a nucleotide exchange factor for Ras (5). GTP-loaded Ras (active form) binds to and activates the serine/threonine kinase Raf-1 which subsequently activates the MEK1/2-ERK1/2 pathway (6, 7).

The kinetics of ERK1/2 activation after receptor engagement have been correlated with specific functional outcomes, including differentiation, proliferation, survival, or apoptosis (7). During thymocyte development, the fate of DP thymocytes is determined by a single receptor, which can differentially modulate ERK1/2 activity, depending on the affinity of the ligand. TCR-engagement by either positive selecting (low-affinity) peptide ligands induces a weak but sustained activation of ERK1/2 correlating with differentiation and survival of thymocytes, whereas negative selecting (high-affinity) peptides generate a strong but transient activation of ERK1/2 presumably responsible for the induction of thymocyte apoptosis (8, 9). Recently, it was shown that the different kinetics of ERK1/2 activation also correlate with distinct subcellular localization of this kinase. Thus, negative selecting ligands appear to target activated ERK1/2 to the plasma membrane whereas positive selecting ligands induce the recruitment of activated ERK1/2 to the Golgi apparatus (10). However, it is currently not clear whether different localization and/or activation kinetics of ERK1/2 are indeed directly responsible for regulating positive vs negative selection.

Abs recognizing site-specific phosphorylation motifs of key molecules have been successfully used to study the kinetics and sequence of early events leading to T cell activation. During the past years several research groups have addressed the question how the TCR regulates the activation of intracellular signaling cascades. Experimental systems included in vitro stimulation of primary T cells or Jurkat T cell lines with either soluble or immobilized Abs that recognize the TCR-associated CD3ζ-chains, peptide-loaded APCs, or MHC-I/MHC-II tetramers carrying high- or low-affinity peptides. However, the number of detailed studies that

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3 Abbreviations used in this paper: DAG, diacylglycerol; WT, wild type; tg, transgenic; p, phospho; PI, propidium iodide; PKB, protein kinase B; PKD1, protein kinase D1.

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correlate phosphorylation kinetics of key molecules involved in T cell activation with different cellular responses is very low (10-12).

In the present study, we have addressed the question how different T cell responses (proliferation vs apoptosis) correlate with intracellular signaling events in mature OT-1 TCR-transgenic CD8⁺ T cells. Stimulation conditions were chosen in a way that engagement of the TCR and the CD8 coreceptor either induces proliferation/survival or cell death/apoptosis. We show that these two conditions of stimulation lead to dynamically distinct patterns of phosphorylation/activation of key molecules involved in T cell activation. Furthermore, we also demonstrate that these different cellular responses correlate with distinct subcellular localizations of ERK1/2 in peripheral T cells.

Materials and Methods

**Mice, T cell purification, and activation**

OT-1 TCR transgenic (tg) mice were provided by Percy Knolle (University of Bonn, Bonn, Germany). Perforin knock out (perforin⁻/⁻) mice were obtained from Jackson Laboratories. The mice were maintained in pathogen free conditions. All experiments involving mice were performed according to the guidelines of the State of Sachsen-Anhalt, Germany. Splenic CD8⁺ T cells from OT-1 TCR tg mice were purified using a pan T cell isolation kit and AutoMacs magnetic separation system (Miltenyi Biotec) according to the manufacturer’s instructions. Purification of splenic CD8⁺ T cells from wild-type (WT) or perforin⁻/⁻ mice was performed using CD8⁺ T cell isolation kit (Miltenyi Biotec). Cells were stimulated with biotinylated anti-CD3e (10 μg/ml) and biotinylated anti-mouse CD8α (10 μg/ml) mAb (both from BD Biosciences) and cross-linked with streptavidin (50 μg/ml; Dianova). Before stimulation of cells with streptamers, either PE-conjugated or nonconjugated Strept-Tactin was incubated with recombinant monomeric biotinylated-MIC-1 (1 μg/reaction) (IBA GmbH) at 4°C according to the manufacturer’s instructions. Recombinant monomeric biotinylated H-2Kb molecules presenting the OVA SIINFEKL peptide specific for the OT-1-tg TCR were used in this study.

**Flow cytometry and TCR internalization**

CD8⁺ T cells were stained with FITC-labeled mAbs against CD25 and CD69 (BD Biosciences). Cell-associated fluorescence was analyzed on a FACS Calibur using the Cell Quest software (BD Biosciences). To determine TCR internalization, 2 × 10⁶ cells were either left untreated or stimulated with CD3/CD8 mAbs or OT-I-streptamers as mentioned above at 37°C for 30–120 min. Cells were stained with FITC-conjugated TCR-Vα-2 mAb (BD Biosciences) for 30 min at 4°C and analyzed by flow cytometry.

**Proliferation assay**

CD8⁺ T cells were cultured in RPMI 1640 medium containing 10% FCS (PAN Biotech), 100 U/ml penicillin, 100 μg/ml streptomycin (all from Biochrom AG), and 50 μM 2-ME in 96-well plates at a concentration of 2.5 × 10⁵ cells/well. Cells were left either unstimulated or stimulated as described above for 72 h at 37°C. Cells were then labeled with [³H]thymidine (0.3 μCi/well from ICN) for 8 h, harvested onto glass fiber filter and recounted with a scintillation counter (1450 MicroBeta Trilux; PerkinElmer).

**Cell survival assay and caspase-3 activity**

To quantify cell survival under different stimulation conditions, T cells were resuspended in RPMI 1640 containing supplements (as described above) at a density of 1 × 10⁶ cells/ml in a 48-well tissue culture plates. Cells were stimulated with either CD3/CD8 mAbs or OT-I-streptamers (as described before) at 37°C. Cells were harvested after 8 and 24 h, respectively. The percentage of cells undergoing apoptosis was measured by flow cytometry using FITC-annexin V and propidium iodide (PI) (r annexin V/FITC kit; Bender MedSystems) according to the manufacturer’s instructions. To detect activated caspase-3 in living cells, we used the caspase-3 detection kit according to the manufacturer’s instructions (Calbiochem). In brief, T cells were treated as described above with OT-I-streptamers and CD3/CD8 mAbs for 8 and 24 h, respectively. Cells were washed and 1 × 10⁶ cells were incubated with 1 μg FITC-conjugated DEVD-FMK in 30 μl PBS for 1 h at 37°C. After washing, the activated caspase-3 inside the cells was analyzed by flow cytometry.

**Western blotting and immunoprecipitation**

CD8⁺ T cells were either left untreated or stimulated with CD3/CD8 mAbs or OT-I-streptamers, lysed as described in (13) and protein concentration was determined using the Röti-Nanoquant reagent (Roth) according to manufacturer’s instructions. The samples were analyzed by Western blotting. Anti-phospho(sy)-ERK1/2, anti-phospho(ser)96 protein kinase D1 (PKD1) (all from Cell Signaling Technology), anti-PLC-γ1, anti-phospho(ser)783 PLC-γ1, anti-PTK70 (all from Santa Cruz Biotechnology), anti-ZAP70 (clone IE7.2, anti-Bcl-xL (all from BD Biosciences), anti-pTyr (clone 4G10) (Upstate Biotechnology), and anti-β-actin (clone AC-15) (Sigma-Aldrich) were used for Western blotting. The density of the detected bands was measured using Kodak Image software (Kodak). To assess the ubiquitination of ZAP70, 4 × 10⁶ T cells were either left unstimulated or treated with either CD3/CD8 mAbs or OT-I-streptamers (as mentioned before) for 3 min and resuspended in 500 μl lysis buffer. ZAP70 was immunoprecipitated using 2 μg of rabbit anti-ZAP70 Ab (Santa Cruz Biotechnology) in combination with 30 μl protein-A agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. Samples were analyzed by Western blotting using an anti-ubiquitin (Cells Signaling Technology) and anti-ZAP70 Ab (BD Biosciences).

**Calcium flux**

CD8⁺ T cells (2 × 10⁵ cells/ml) resuspended in RPMI 1640 medium (phenol-red free; Invitrogen) supplemented with 10% FCS were loaded with 3.75 μg/ml Indo-1-AM (Molecular Probes) at 37°C for 45 min. After loading, the cells were incubated in the same medium at 37°C for an additionally 45 min. The measurement was performed on a LSR flow cytometer (BD Biosciences). Cells were incubated with CD3/CD8 mAbs at 37°C for 0.5 min to establish the baseline and cross-linked with streptavidin or treated with OT-I-streptamers (as mentioned before) followed by ionomycin (10 μg/ml). Data for calcium mobilization were acquired on a LSR flow cytometer and a ratiometric analysis was performed using the Flow Jo software (BD Biosciences).

**Confocal microscopy**

CD8⁺ T cells were stimulated as described above with either OT-I-streptamers or CD3/CD8 mAbs. Cells (2 × 10⁵) were placed onto poly-l-lysine coated slides, fixed, permeabilized, and blocked as described in (10). Cells were stained with anti-p-ERK1/2, anti-RAβ5 mAb (BD Biosciences) or anti-pan Ras mAb (Oncogene) in combination with FITC/cy3-conjugated goat anti-rabbit IgG, Cy3-conjugated goat anti-mouse, FITC-conjugated anti-TCR-Vα-2 mAb, or TRITC-phalloidin (Sigma-Aldrich). A minimum of 40 cells were imaged with LEICA TCS SP2 laser-scanning confocal system and analyzed with the LEICA software.

**Results**

The fate of mature CD8⁺ T cells differs depending on the stimuli

The aim of this study was to assess how the TCR translates different signals into diverse cellular responses such as proliferation or apoptosis. For this purpose, we used purified CD8⁺ T cells isolated from spleens of OT-I TCR-transgenic (tg) mice and applied two different stimuli. T cells were either activated with soluble biotinylated CD3ε mAbs in combination with biotinylated CD8α mAbs and subsequently cross-linked with streptavidin or they were incubated with biotinylated H-2Kb molecules loaded with the peptide SIINFEKL and cross-linked with Strep-Tactin (OT-I-streptamer) (14). For this study, we used 10 μg/ml each mAb or 1 μg OT-I-streptamers to activate OT-I T⁺ CD8⁺ T cells, the binding of 1 μg OT-I-streptamers was close to saturation under these stimulation conditions (data not shown).

To determine whether the two stimuli induce T cell proliferation we assessed [³H]thymidine incorporation after 72 h of in vitro culture. Fig. 1A shows that CD8⁺ T cells stimulated with CD3/CD8 mAbs did not proliferate. Importantly, stimulation of the same cells with lower doses of Abs (ranging from 10 μg/ml to 1.25 μg/ml) also did not induce proliferation (data not shown). Furthermore, preincubation of the cells with different concentration of cross-linked Abs (30 min to 2 h) followed by washing (to remove unbound antibodies) also did not result in a proliferative response.
The lack of proliferation after stimulation with CD3/CD8 Abs, but not peptide-loaded streptamers, induced a strong activation of caspase-3 (Fig. 1D). Notably, treatment of these cells with lower doses of Abs (ranging from 10 μg/ml to 1.25 μg/ml) still induced apoptosis whereas increasing concentrations of streptamers did not induce cell death (data not shown). In summary, the results shown in Fig. 1 indicate that OT-I-streptamers induced proliferation and activation of peripheral T cells, whereas CD3/CD8 mAbs induced apoptosis.

Activation of CD8⁺ T cell leads to its differentiation into CTLs. CTLs kill targeted cells mainly through the perforin/granzymes system. To assess whether Ab induced apoptosis is mediated via perforin, we stimulated purified CD8⁺ T cells from WT and perforin⁻/⁻ mice with soluble anti-CD3/CD8 mAbs and PMA/ionomycin for 24 h. Cell survival was assessed by propidium iodide and annexin V staining. Fig. 1E showed that nearly 70% of Ab-stimulated T cells from WT or perforin⁻/⁻ mice stained positive for propidium iodide. Consistent with these data, stimulation with soluble Abs did not induce proliferation (Fig. 1F). These data indicated that induction of apoptosis by soluble anti-CD3/CD8 mAbs occurred independent of perforins.

**OT-I-streptamers induce sustained activation of PKB and expression of Bcl-x<sub>L</sub>**

The above system allowed us to compare signaling events leading to either T cell survival or apoptosis in more detail. We first focused on PKB (also known as Akt), a serine threonine kinase, which is critically involved in T cell survival (15). As shown in Fig. 2A, triggering of the TCR with CD3/CD8 Abs induced a strong, but transient phosphorylation of PKB which peaked at 3
and rapidly declined thereafter (Fig. 2A). In contrast, OT-I-streptamers induced a much weaker, but sustained phosphorylation of PKB that was still detectable after 24 h of stimulation (Fig. 2, A and B).

Previously, it was reported that expression of the anti-apoptotic member of the Bcl2-family, Bcl-xL, prevents TCR-mediated apoptosis and promotes T cell survival (16). Therefore we analyzed whether OT-I-streptamers enhance the expression of Bcl-xL. Fig. 2C showed that this is indeed the case. Although Bcl-xL expression was not detectable after CD3/CD8 mAb stimulation, T cells activated with OT-I-streptamers started to express Bcl-xL after 4 h of stimulation. Bcl-xL expression was maximal at 24 h of stimulation and was still detectable after 48 h (Fig. 2C). Thus, Ab mediated T cell stimulation activates pro-apoptotic molecules, such as caspase-3 and induces apoptosis whereas peptide-loaded streptamers lead to the expression of anti-apoptotic molecules and promote cell survival. Moreover, the data shown in Figs. 1 and 2 suggest that a strong and transient activation of PKB alone is not sufficient to protect T cells from apoptosis.

**FIGURE 2.** OT-I-streptamers induce sustained phosphorylation of PKB and expression of Bcl-xL. A, Purified CD8+ T cells were treated with soluble CD3/CD8 mAbs or OT-I-streptamers for the indicated time periods. Samples were analyzed by Western blotting using the indicated Abs. 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. B, For long term stimulation, CD8+ T cells were triggered with soluble Abs or OT-I-streptamers and analyzed for PKB-pS473 phosphorylation as described above. The data are representative of three independent experiments. Note the expression levels of PKB were not affected either in Ab- or streptamer-stimulated lymphocytes over the various time points investigated (data not shown). C, Purified CD8+ T cells were incubated with soluble CD3/CD8 mAbs or OT-I-streptamers for the indicated periods of time. The expression of either Bcl-xL or β-actin was assessed by Western blotting. Data are representative of three independent experiments.

**FIGURE 3.** OT-I-streptamers induce sustained ERK1/2 activation. A, CD8+ T cells were treated with soluble CD3/CD8 mAbs or with OT-I-streptamers for the indicated periods of time. Lysates were prepared and analyzed by Western blotting using the indicated Abs. The p*ERK1/2 bands were quantified using ImageQuant software and values normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments. B, For long term stimulation, CD8+ T cells were triggered with soluble CD3/CD8 Abs or OT-I-streptamers and subsequently analyzed for ERK1/2 phosphorylation as described above. The data are representative of three independent experiments. Note the expression levels of ERK1/2 were not affected either in Ab- or streptamer-stimulated lymphocytes over the various time points investigated. Importantly, phosphorylated ERK1/2 was not detected in the absence of any stimuli after 24 h of incubation (data not shown). C, CD8+ T cells were treated with soluble CD3/CD8 mAbs or OT-I-streptamers for the indicated times. The expression of the OT-I TCR was assessed by FITC-conjugated anti-TCR-Vα-2 mAb staining analysis by flow cytometry. Data represent the mean fluorescence intensity (MFI) of the TCR expression of three independent experiments.

**OT-I-streptamers induce sustained activation of ERK1/2**

The ubiquitously expressed serine/threonine kinases ERK1/2 belong to the MAPK family and are important for numerous cellular responses including proliferation and survival (7). We next investigated the kinetics of activated MAPK under survival/proliferation vs apoptosis-inducing conditions of stimulation. As shown in Fig. 3A, CD3/CD8 mAbs induced a strong but transient phosphorylation of ERK1/2 which peaked at 1 min, rapidly declined thereafter and was no longer detectable after 30 min of stimulation. Similar kinetics of ERK1/2 phosphorylation, but with lower maximal intensity were observed with lower doses of the stimulating Ab (data not shown), thus excluding the possibility of a concentration effect. In contrast to Ab-mediated stimulation, engagement
The phosphorylation of pY319 of ZAP70 was calculated for the indicated time points. Data represent the mean ± SEM from three independent experiments. B. Besides the main pY319 ZAP70 band as shown in A, all bands above in Ab-stimulated cells were quantified and the ratio between phosphorylated and nonphosphorylated ZAP70 was calculated for the indicated time points. Data represent the mean ± SEM from three independent experiments. C. Purified T cells were left untreated or incubated with soluble CD3/CD8 mAbs or OT-I-streptamers for 3 min, respectively. ZAP70 was immunoprecipitated and Western blot analysis was performed using the indicated Abs. To control successful stimulation, lysates were analyzed by Western blotting for the presence of phosphorylated ERK1/2. Data are representative of two independent experiments. D. The total ZAP70 (middle panel shown in A) were quantified using ImageQuant software and values normalized to the corresponding β-actin signal. Data represent the mean ± SEM from three independent experiments. E. CD8 T cells were incubated with soluble CD3/CD8 mAbs or OT-I-streptamers for the indicated times. Western blot analysis of cell lysates was performed using anti-phosphotyrosine mAb (4G10) and the band corresponding to phosphorylated LAT was quantified as described for Fig. 2. Data represent the mean ± SEM of three independent experiments.

of the TCR/CD8 with OT-I-streptamers induced a weak, but sustained activation of ERK1/2 (Fig. 3A). Indeed, the level of phosphorylation after 30 min was as strong as after 3 min of stimulation. An extended kinetic analysis revealed that ERK1/2 phosphorylation was still detectable after 24 h of streptamer stimulation (Fig. 3B). It is important to note that in multiple experiments we observed a substantial increase in ERK1/2 phosphorylation after 4 h of streptamer stimulation. The molecular basis for the latter observation is unclear.

A rapid internalization and thus a decrease in the number of available TCR molecules on the cell surface could explain the transient activation of ERK1/2 after Ab stimulation. To assess this point, we compared the expression levels of the TCR after stimulation with either soluble CD3/CD8 mAbs or OT-I-streptamers by flow cytometry. As shown in Fig. 3C, treatment of T cells with either soluble Abs or OT-I-streptamers induced almost identical rates of TCR-internalization (Fig. 3C). Thus, differences in TCR internalization do not account for the different kinetics of ERK1/2 activation. In summary, the data shown in Fig. 3 indicate that survival and proliferation of mature T cells after streptamer stimulation correlates with a low and sustained activation of ERK1/2.

Abs induce strong activation, ubiquitination, and rapid degradation of ZAP70

Having excluded an altered rate of TCR internalization as the cause of the transient phosphorylation of ERK1/2 after Ab stimulation, we next investigated upstream signaling events involved in the activation of ERK1/2. ZAP70 activation is one of the first signaling events occurring after TCR triggering (2) and is required for the activation of ERK1/2 (17). We assessed the activation kinetics of ZAP70 after Ab vs streptamer stimulation by analyzing the phosphorylation status of pY319, which appears to be required for TCR-mediated downstream signaling (17).

As shown in the upper panel of Fig. 4A, in terms of onset and duration, the kinetics of ZAP70 phosphorylation were comparable after CD3/CD8 mAb and streptamer-stimulation. In marked contrast to the differential activation kinetics of ERK1/2 neither of the two stimuli induced a prolonged activation of ZAP70 (data not shown). However, when normalized to the expression levels of ZAP70, Ab-mediated stimulation appeared to induce a 4- to 5-fold stronger activation of ZAP70 than streptamer-stimulation (Fig. 4B). In line with these data, we found a strikingly higher kinase activity of immunoprecipitated ZAP70 in Ab-stimulated T lymphocytes compared with streptamer-stimulated cells (data not shown).

The Western blot images depicted in Fig. 4A further showed that besides the major band corresponding to Y319, additional ZAP70 bands displaying retarded migration in SDS-PAGE became visible exclusively after Ab stimulation. This observation led us to suspect that Ab stimulation not only resulted in a very strong activation of ZAP70, but also induced its ubiquitination and subsequent degradation. To address this point, T cells were stimulated for 3 min with either CD3/CD8 Abs or with streptamers. Subsequently, ZAP70 immunoprecipitates were obtained and analyzed by antibody or anti-ZAP70 Western blotting. Fig. 4C demonstrates...
that Ab-stimulation indeed induced a strong ubiquitination of ZAP70 which correlated well with the rapid loss of detectable ZAP70 protein (see Fig. 4, middle panels and Fig. 4D for quantification). In contrast, no ubiquitination of ZAP70 was observed after streptamer-stimulation. Thus, Ab stimulation not only induces a strong activation of ZAP70, but also its ubiquitination and rapid degradation. The latter mechanism might contribute to the transient activation of ERK1/2.

We were next interested how the differential activation/express-

FIGURE 5. OT-I-streptamers mediate low calcium mobilization but sustained PLC-γ1 and PKD1 phosphorylation. A, T cells were loaded with Indo-1-AM and calcium release was triggered by soluble CD3/CD8 mAbs or OT-I-streptamers, respectively. Calcium influx was determined by cytometry based on the FL4/FL5 ratio. Ionomycin was added to the end of each experiment to verify proper Indo-loading. Data are representative of three independent experiments. B, Purified splenic T cells were treated with soluble anti-CD3/CD8 mAbs or OT-I-streptamers, respectively. Cells were lysed and equivalent amounts of protein from whole cell extracts were analyzed for phosphorylated PLC-γ1 (pY783) and were quantified using ImageQuant software. Values were normalized to the corresponding β-actin signal. Data represent the mean ± SEM of three independently performed experiments. C, For long term stimulation, CD8+ T cells were triggered with soluble CD3/CD8 Abs or OT-I-streptamers and subsequently analyzed for PLC-γ1 phosphorylation as described above. The data are representative of three independent experiments. D, CD8+ T cells were either incubated with soluble CD3/CD8 mAbs or OT-I-streptamers. The phosphorylation of serine 916 (S916) of PKD1, along with the expression level of β-actin, was determined by Western blotting and quantified as described for Fig. 2. Data represent the mean ± SEM of three independent experiments. E, For long term stimulation, CD8+ T cells were triggered with soluble Abs or OT-I-streptamers and subsequently analyzed for phosphorylation of S916 as described above. Data are representative of three independent experiments. Note, the expression levels of PLC-γ1 or PKD1 were not affected either in Ab- or streptamer-stimulated lymphocytes over the various time points investigated. Importantly, phosphorylated PLC-γ1 or PKD1 was not detected in the absence of any stimuli after 24 h of incubation (data not shown).

OT-I-streptamers mediate low calcium mobilization but sustained phosphorylation of PLC-γ1

The strikingly different magnitude of LAT or ZAP70 phosphorylation after Ab vs streptamer stimulation prompted us to compare intracellular calcium flux after application of the two stimuli. As shown in Fig. 5A, Ab stimulation generated a strong calcium signal, whereas this signal was barely detectable after streptamer stimulation. Thus, the strong phosphorylation of LAT or ZAP70 induced by the Abs appears to correlate with a strong Ca2+-response.

Upon binding to LAT PLC-γ1 becomes phosphorylated on several tyrosine residues, including Y783, which is known to be vital for PLC-γ1 activity (18). To address the question whether the strong phosphorylation of LAT and the strong induction of calcium-flux that occurs after Ab-mediated stimulation correlated with the activation of PLC-γ1, we analyzed the phosphorylation status of PLC-γ1 at Y783 by Western blotting of whole cell lysates. As expected, CD3/CD8 mAbs induced a strong phosphorylation of PLC-γ1 whereas the signal was much weaker after streptamer stimulation (Fig. 5B). Surprisingly however, the activation of
PLC-γ1 appeared to be weak but sustained after streptamer-stimulation. Indeed, even after 24 h of streptamer stimulation minor phosphorylation of Y783 was detectable (Fig. 5C). Given the fact that neither Ab- nor streptamer-stimulation induced an extended activation/phosphorylation of ZAP70 and LAT, these data strongly suggest 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 PLC-γ1.

The second messenger DAG is also generated by PLC-γ1 and is important to activate PKC and RasGRP, which in turn leads to activation of the Ras-ERK1/2 pathway (5). To address the question, whether the sustained activation of PLC-γ1 induced after streptamer stimulation is sufficient to produce DAG, we assessed the phosphorylation status of PKD on pS916, which was previously shown to be dependent upon the production of DAG (19). Stimulation with CD3/CD8 mAbs induced a strong but transient phosphorylation of S916, whereas S916 phosphorylation was sustained, albeit weaker, after streptamer stimulation (Fig. 5, D and E). Therefore, although OT-I-streptamers are only weak activators of PLC-γ1, they induce an extended production of DAG, which likely contributes to the sustained activation of the Ras-ERK1/2 pathway.

**Distinct subcellular localizations of ERK1/2**

A recent study has shown that activated ERK1/2 resides in different subcellular compartments when thymocytes undergo positive vs negative selection (10). To examine whether a similar situation also occurs in peripheral T cells undergoing apoptosis or proliferation we assessed the localization of phosphorylated ERK1/2 after Ab or streptamer-mediated T cell activation using confocal laser scanning microscopy. Fig. 6A demonstrates that ~80% of Ab-stimulated T cells showed an intensive localization of phosphorylated ERK1/2 close to the plasma membrane after 3 min of stimulation. Additional experiments revealed that under these conditions of stimulation activated ERK1/2 colocalized to a large extent with clustered TCR-complexes (Fig. 6B). In line with the quantification of the Western blot analysis shown in Fig. 3A, phosphorylated ERK1/2 was no longer detectable after 30 min of Ab-mediated stimulation thus confirming its transient activation under apoptosis inducing conditions of stimulation (Fig. 6A).

Although OT-I-streptamers induced a weaker clustering of the TCR compared with Ab-stimulation in the majority of cells, they also led to membrane targeting of phosphorylated ERK1/2 in ~80% of the cells (Fig. 6A). As expected from the quantification of the Western blot analysis (Fig. 3A) the signal was of a much lower intensity but even under these conditions phosphorylated ERK1/2 partially colocalized with the transgenic TCR (Fig. 6B). However, after 30 min and 4 h of stimulation in ~60–70% of streptamer-treated cells phosphorylated ERK1/2 was no longer
found at the plasma membrane but rather in small vesicular structures which were located close to the plasma membrane as well as in the cytosol.

It has been described in different cellular systems that after insulin receptor or TrkA receptor triggering, phosphorylated ERK1/2 accumulates in the recycling endosomes (20, 21). To address whether the activated ERK1/2 that we detected in vesicular structures after 30 min and 4 h OT-I-streptamer stimulation localizes to the endosomal compartment, we used Rab5 as a marker for confocal laser scanning microscopy. The upper two panels of Fig. 7 depicted that the vesicles indeed stained positive for Rab5, thus identifying them as endosomes.

Other components of the ERK1/2 signaling pathway such as the GTPase Ras have also been found in the endosomal compartment (20, 21). In line with these data we found that Ras also colocalized with phosphorylated ERK1/2 in Rab5-positive vesicles after streptamer stimulation (Fig. 7, lower panels). Collectively, these data indicate that under conditions promoting T cell survival and proliferation activated ERK1/2 shuttles from the plasma membrane to the recycling endosomes, whereas under conditions inducing T cell death phosphorylated ERK1/2 only transiently accumulates at sites of TCR-engagement.

**Discussion**

Engagement of the TCR can result in different cellular responses such as differentiation, survival, proliferation, or apoptosis. However, how the triggering of one receptor initiates different responses and how these responses are organized on the molecular level is so far not understood. To address this question we used peripheral T lymphocytes obtained from OT-I TCR-tg mice and applied two different stimuli soluble CD3/CD8 mAbs or OT-I-streptamers loaded with the agonistic OVA peptide SIINFEKL, which selectively induce either apoptosis (mAbs) or proliferation (streptomers) (Fig. 1).

Biochemical analysis of the pathways initiated by these two types of stimuli revealed that signals inducing apoptosis produce strong, rapid and transient phosphorylation/activation of key molecules involved in T cell activation including ZAP70, LAT, PKB, PLC-γ1, PKD1, and ERK1/2. In contrast to the strong and transient signals elicited by soluble mAbs, most signals that were induced by peptide loaded streptamers were of much lower intensity, but were also more sustained. This applied to the activation of ERK1/2 and of PLC-γ1, PKB, and PKD1.

Our study corroborates and extends recent experiments that had assessed selection events in OT-I TCR-tg thymocytes (10). Hence, it appears that independent of the system a strong and transient activation of ERK1/2 correlates with the induction of apoptosis, whereas a weak and sustained activation of ERK1/2 correlates with survival and proliferation of T lymphocytes.

The molecular events that translate the different activation kinetics of ERK1/2 into distinct cellular responses are still unclear. One hypothesis is that the differential localization of activated ERK1/2 within the cells contributes to these effects. Indeed, similar to the study performed by Daniels et al. (10) we also found the localization of phosphorylated (and hence activated) ERK1/2 to be markedly different under apoptosis vs proliferation inducing conditions. Thus, pro-apoptotic stimuli induced rapid targeting of activated ERK1/2 to the plasma membrane, whereas proliferation inducing stimuli led to an accumulation of activated ERK1/2 within the recycling endosomes (Fig. 6 and Fig. 7). Localization of activated ERK1/2 at the endosomes after insulin receptor or TrkA receptor stimulation is a well-documented phenomenon (20, 21).

Furthermore, in insulin- or NGF-treated cells other signaling molecules required for the activation of ERK1/2 have also been described to localize in the endosomal compartment. These include Ras, Raf, MEK1, PLC-γ1, and PI3K (important for activation of PKB) (20, 21). In line with these data, we also found Ras and phosphorylated ERK1/2 in Rab5-containing vesicles after OT-I-streptamer stimulation (Fig. 7). Therefore, it is reasonable to speculate that the sustained phosphorylation kinetics of PLC-γ1 and PKB after OT-I-streptamer stimulation might also be due to targeting of these proteins to the endosomal compartment. Unfortunately, our attempts to visualize phosphorylated PLC-γ1 or PKB in Rab5-containing activated ERK1/2 vesicles by confocal laser scanning microscopy were so far not successful. This may be due to the low phosphorylation status of these proteins in streptamer-treated cells or the inaccessibility of the different phospho-specific Abs tested for immunofluorescence. Nevertheless, our data indicated that compartmentalization of ERK1/2 into the endosomes after OT-I-streptamer treatment correlates with sustained activation of this kinase. One mechanism might be that endosome-associated ERK1/2 is protected from cytosolic or membrane associated phosphatases. However, how OT-I-streptamer triggering of the TCR/CD8 regulates the differential shuttling of activated ERK1/2 at the molecular level is still unclear. Experiments are currently set up in our laboratory to address this question.

Another important question that remains to be resolved is how the transient activation of ERK1/2 under apoptosis-promoting conditions is regulated on the molecular level. Here several possibilities can be envisaged. First, we observed that stimulation of T cells with CD3/CD8 mAbs results in a rapid poly-ubiquitination and degradation of ZAP70 (Fig. 4). Moreover, the Valitutti group has previously demonstrated that T cell activation induces ubiquitination events at the immunological synapse (22). Therefore, pro-apoptotic signals initiate a negative feedback loop that rapidly down-regulates the input signal by eliminating ZAP70. Several reports have shown that the ZAP70 related protein tyrosine kinase Syk serves as a substrate for ubiquitin-ligases (23). However, to our knowledge a TCR-mediated ubiquitination and subsequent degradation of ZAP70 has so far not been reported. Also, the enzyme(s) that is/are responsible for ZAP70 ubiquitination have yet to be determined. Obvious candidates would be members of the Cbl family which exert E3-dependent ubiquitin ligase activity and which have been shown to interact with ZAP70 (24). However, the role of Cbl in ZAP70 ubiquitination and degradation is controversial and further work is needed to address if and how Cbl contributes to ZAP70 ubiquitination.

In addition to the degradation of ZAP70, other mechanisms may also contribute to the transient activation of ERK1/2 under pro-apoptotic conditions of stimulation. One possibility would be that the strong activation of membrane proximal tyrosine kinases (e.g., ZAP70, Lck, Fyn, Itk, and 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 (25). Additionally, a negative regulatory role for LAT in T cell signaling has been suggested (26, 27). Thus, it is tempting to speculate that the strong phosphorylation of LAT after Ab-stimulation induces the assembly of negative regulatory complexes, which prevent a sustained activation of ERK1/2. Conversely, weaker input signals (a less strong activation of membrane proximal PTKs) would not induce the activation of these negative regulatory loops, thus allowing for a sustained activation of ERK1/2. In line with the idea that cell fate decisions are determined at the level of the LAT complex, would be our finding that the signaling molecules which...
are located immediately downstream of LAT (i.e., PLC-γ1 and PKD1) already show markedly different phosphorylation kinetics in our experimental system.

Finally, ERK1/2 itself could play a major role in inhibiting its prolonged activation by inducing the phosphorylation of negative regulatory proteins close to the membrane. Indeed, recent studies (28, 29) have revealed the importance of serine phosphorylation of key molecules for T cell homeostasis (SLP-76 by HPK1). Clearly, further studies are required to elucidate the events that lead to the transient activation of ERK1/2 under apoptosis-inducing conditions of stimulation. Nevertheless, our data indicate that “optimal” membrane proximal signals, such as a strong Ca²⁺-flux or maximal LAT phosphorylation, may not automatically be interpreted to mean that these events induce a productive T cell activation. 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 or apoptosis, allows a correct interpretation of biochemical data.

It was somewhat unexpected that soluble Abs induced a rapid apoptosis and activation of caspase-3 whereas peptide loaded streptamers induced the expression of the anti-apoptic molecule Bcl-x<sub>L</sub>. Although the induction of Bcl-x<sub>L</sub> expression by peptide loaded streptamers can be explained by a sustained activation of PKB (30), the induction of apoptosis by soluble Abs is more difficult to understand. Apoptotic T cell death and activation of caspase-3 can be induced via the extrinsic death-receptor-mediated pathway or through the intrinsic mitochondrial pathway (31). Currently we do not know which of the two pathways becomes activated by soluble Abs, but we clearly favor a mechanism that involves the mitochondrial pathway for the following reasons. The expression levels of CD95 were almost identical after Ab- vs streptamer-stimulation and we also could not detect enhanced expression of CD95L after either mode of stimulation (data not shown). Moreover, the time frame between the application of the death-inducing stimulus and the first signs of cell death (8 h) appears to be too short to be mediated via the CD95/CD95L-system in primary T cells. Candidate molecules that could connect the TCR to the activation of caspase-3 via the mitochondrial pathway are members of the BH3-only proteins that have been shown to promote apoptosis in T cells for example Bim (32), Bid (33), or Bad (34). Therefore, further work is needed to address which of the BH3-only proteins contribute to Ab-induced apoptosis and how these molecules organize pro-apoptotic pathways after Ab-mediated apoptosis.

In multiple experiments, we observed a substantial increase of ERK1/2 phosphorylation after 4 h of stimulation with OT-I-streptamers (Fig. 3F). Such a “second wave” of ERK1/2 phosphorylation has previously been observed after long-term stimulation of AND TCRtg CD4<sup>+</sup> T cells using a high-affinity peptide (35). It might be that the production and release of cytokines, such as IL-2, is responsible for the second wave of ERK1/2 phosphorylation under survival promoting conditions of stimulation. Preliminary data obtained in our laboratory indicated that IL-2 could indeed partially contribute to the second wave of ERK phosphorylation after OT-I-streptamer stimulation. Thus, in the presence of neutralizing against IL-2 Ab, the phosphorylation of ERK1/2 was reduced up to 50% after 4 h of stimulation with OT-I-streptamers (data not shown). This data indicated that not only IL-2, but also other cytokines, e.g., IL-7, may also facilitate activation, survival and proliferation of peripheral CD8<sup>+</sup> T cells (36). Therefore, we would speculate that the initial TCR-mediated signals leading to a first wave of ERK1/2 activation under survival promoting conditions are replaced at later time points by cytokine receptor-mediated signaling events. These signals may then contribute to the sustained activation of ERK1/2 and may also be involved in the sustained activation of PKB thereby supporting the expression of survival promoting molecules such as Bcl-x<sub>L</sub>.

In summary, we have shown that triggering of the same set of receptors by different ligands can induce either survival/proliferation or apoptosis/cell death of mature CD8<sup>+</sup> T lymphocytes. We have further demonstrated that these cellular outcomes correlate with different activation/phosphorylation kinetics of key molecules involved in T cell activation which seem to bifurcate at the level of PLC-γ1. Our data corroborate and extend previous experiments (10) performed in thymocytes and suggest that the molecular mechanisms in the two systems are to a large extent comparable. How mature T cells up-regulate TCR-mediated thresholds to convert the 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 vs sustained signaling events close to the membrane are converted into distinct cellular responses. Finally, these experiments will also have to take the differential compartmentalization of signaling molecules into account.

**Disclosures**

The authors have no financial conflict of interest.

**References**


