Chemical Inhibitors when Timing Is Critical: A Pharmacological Concept for the Maturation of T Cell Contacts

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Cellular signal transduction proceeds through a complex network of molecular interactions and enzymatic activities. The timing of these molecular events is critical for the propagation of a signal and the generation of a specific cellular response. To define the timing of signalling events, we introduce the combination of high-resolution confocal microscopy with the application of small-molecule inhibitors at various stages of signal transduction in T cells. Inhibitors of Src-family tyrosine kinases and actin dynamics were employed to dissect the role of the lymphocyte-specific tyrosine kinase Lck in the formation and maintenance of T cell receptor/CD3-dependent contacts. Anti-CD3ε-coated coverslips served as a highly defined stimulus. The kinetics of the recruitment of the yellow fluorescent protein-tagged signalling protein ZAP-70 were detected by high-resolution confocal microscopy. The analysis revealed that at 5 min after receptor engagement, Lck activity was required for maintenance of contacts. In contrast, after 20 min of receptor engagement, the contacts were Lck-independent. The relevance of the timing of inhibitor application provides a pharmacological concept for the maturation of T cell–substrate contacts.

Introduction

The interaction of a T lymphocyte with an antigen-presenting cell (APC) is a primary example of regulation of an intercellular interaction. Research in the past few years has provided a wealth of information on molecules in the plasma membrane and inside T cells and target cells that participate in and modulate these interactions. Now, the timing and contribution of individual molecular events to contact formation and generation of a T cell response need to be addressed in detail.

Binding of T cell receptors (TCR) to agonistic major histocompatibility (MHC) complexes initiates a cascade of signalling events central to the execution of the adaptive immune response.[1] Upon TCR engagement, the Src-family kinase (SFK) Lck phosphorylates tyrosines of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCR/CD3 complex.[2] The cytoplasmic Syk family tyrosine kinase ZAP-70 is recruited to the TCR/CD3 complex by binding to phosphorylated ITAMs. Activation of ZAP-70 leads to the progagation of the signal through multiple pathways that culminate in gene expression and the formation of a highly ordered supramolecular structure at the interface of the T lymphocyte and the APC, the "immunological synapse".[3] Formation of the immunological synapse occurs through the rearrangement of transmembrane molecules in the T cell–APC contact.[4] Concomitant with the redistribution of transmembrane molecules, proteins involved in cell adhesion and intracellular signalling, as well as microfilaments and associated proteins are concentrated at the contact site.

Given the prominence of the redistribution of membrane proteins to this point, research on the immunological synapse has been based on T cell–APC contacts or on model systems in which the APC was replaced by lipid bilayers incorporating membrane proteins; these are experimental approaches that allow for the lateral diffusion of membrane proteins.[3] As a consequence synapse maturation has been associated with the morphological reorganization of the T cell–APC contact. Limited information exists on the individual contributions of signalling molecules to the transformation of the contact from a premature early stage into a mature late stage.

In order to focus on the role of signalling activities in contact maturation, we selected anti-CD3ε antibody-coated cover-

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slips as a well-defined stimulus for the activation of T cells.[5] In contrast to stimuli provided by APCs or lipid bilayers, surface-coated anti-CD3 antibodies are immobile; this precludes the rearrangement of transmembrane receptors by lateral diffusion as a mechanism for contact maturation. As a consequence, stimulation of T cells with immobilized antibodies eliminates one level of complexity in the analysis of CD3-dependent signalling events. Coverslips coated with anti-CD3ε antibodies reproduce major events of T cell receptor-dependent signalling and enable the analysis of the recruitment of signalling molecules to the CD3 complex by high-resolution confocal microscopy.[6]

We applied chemical inhibitors to investigate the role of Lck in the maintenance of T cell contacts at various stages after engagement of receptors.[7,8] It has previously been shown that active Lck is associated with the immature immunological synapse.[9] However, cells lacking this kinase fail to establish contacts with anti-CD3-coated coverslips (unpublished results). Therefore, application of small-molecule inhibitors after contact formation is the only way to analyze the functional role of Lck activity at later stages of signalling.

T cell hybridoma cells stably expressing a ZAP-70–yellow fluorescent protein (YFP) fusion protein alone or in combination with a CD3ζ–cyan fluorescent protein (CFP) were exposed to anti-CD3ε-coated coverslips, and inhibitors were added at different time points before and after formation of contacts. ZAP-70 is recruited to the TCR signalling complexes within the first minutes of T cell activation and remains associated with the synapse throughout the early stages of immunological synapse formation.[9,10] We therefore expected ZAP-70–YFP localization to be a sensitive readout for any changes in activities that have an impact on the engagement and activation of CD3 complexes.

Results

Using anti-CD3ε-coated coverslips as a stimulus, we intended to i) elucidate the functional role of active Lck in the early stages of CD3 engagement and ii) determine whether a spatial rearrangement of transmembrane molecules was required for the transformation of a T cell contact from an immature to a mature stage. Hybridoma cells coexpressing the ZAP-70–YFP fusion protein alone or in combination with a CD3ζ–CFP fusion protein were used as readouts for signalling events affecting the CD3-complex.

Functional integrity of fusion proteins

ZAP-70–YFP and CD3ζ–CFP fusion proteins were overexpressed in the 3A9 hybridoma cell line by using a retroviral infection system with an efficient and highly stable expression. Western blotting of total cell lysates with an anti-GFP antibody revealed that both proteins were expressed at the expected molecular weights of 95 kDa and 41 kDa. No degradation products could be detected. Incubation of the coexpressing cells with 0.5 mM sodium pervanadate followed by anti-phosphotyrosine immunoprecipitation and Western blotting against GFP revealed that both ZAP-70 and CD3ζ fusion proteins were phosphorylated in an activation-dependent manner (Figure 1). Furthermore, by using anti-CD3ε-coated coverslips as a stimulus, the recruitment of the ZAP-70 fusion protein to the CD3 complex was demonstrated by immunoprecipitation (see below).

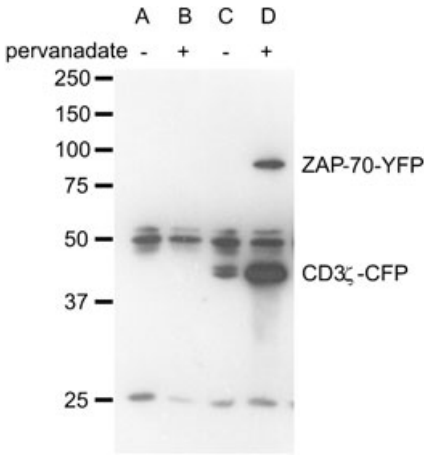

Figure 1. *Activation-dependent tyrosine phosphorylation of CD3ζ–CFP and ZAP-70–YFP. A) and B) lysates from 3A9 cells alone or, C) and D) from cells co-expressing CD3ζ–CFP and ZAP-70–YFP were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody (mAb) before (–) or after (+) a 5 minute treatment with sodium pervanadate (0.5 mM). Immunoprecipitates were subsequently blotted with anti-GFP mAb. The bands at 25 and 50 kDa probably result from cross-reactivity of the secondary anti-mouse antibody with the antibody used for immunoprecipitation.*

Transient recruitment of ZAP-70–YFP to CD3 clusters

First, the dynamics of cell spreading and ZAP-70 recruitment were investigated by exposing 3A9 ZAP-70–YFP transfectants to coverslips coated with anti-CD3ε antibodies. In our case, the adsorption of the antibodies was enhanced by rendering the surface of the coverslip hydrophobic by silanization with octadecyl-trimethoxysilane, in contrast to work reported previously.[11] In order to prevent surface functionalization with extracellular matrix proteins within the cell suspension, which could mediate cell attachment through integrins, coverslips were blocked with 1% BSA in PBS after incubation with antibody. In addition, cells were washed and added to the coverslips in serum-free medium. Upon contacting the anti-CD3ε-functionalized coverslip, the cells spread rapidly and small, discrete clusters of ZAP-70–YFP (approx. 0.5 μm) began to form (Figure 2). Analysis of the surface distribution of the anti-CD3ε antibodies by immunofluorescence excluded inhomogeneities as the origin of ZAP-70–YFP clustering (data not shown). No cell spreading or ZAP-70–YFP clusters were observed with control coverslips coated with polylysine, anti-CD4 antibodies or BSA.

Maximum cell spreading was detected within 5 min. Concomitant with cell spreading, clusters appeared at the leading edge of the cells, culminating in a near complete peripheral ring of ZAP-70–YFP after about 10 min. Time-lapse imaging demonstrated that the formation of clusters at the periphery resulted from engagement of CD3 complexes at newly formed

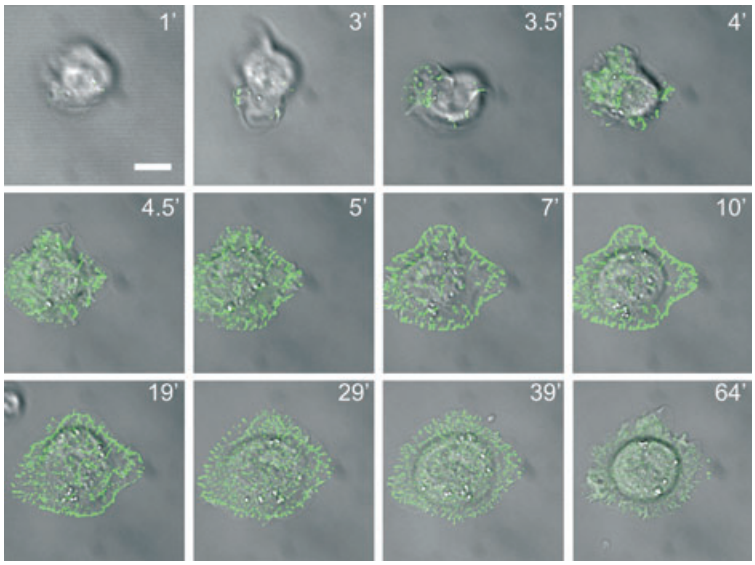

Figure 2. *Cell spreading and clustering of ZAP-70–YFP fusion proteins in a ZAP-70–YFP transfected 3A9 cell that is in contact with an anti-CD3ε-coated coverslip. The cell was imaged by time-lapse confocal microscopy. In order to ensure recording of coverslip-proximal fluorescence, a confocal stack consisting of five slices was recorded for each time point and slices containing surface-proximal fluorescence were selected for the figure. Time in minutes; the bar denotes 10 μm.*

contact sites, rather than lateral diffusion or transport of aggregated proteins along the proceeding cell edge. The ring of clusters started to disappear after about 15 min and was completely undetectable about 25 min after formation, that is, about 30 to 40 min after the initial cell–substrate contact. At the same time, the cells slowly started to retract their extensions from the coverslip. Furthermore, a dissipation of central ZAP-70–YFP clusters was observed, until they were barely visible after 60 min.

The time frame of ZAP-70–YFP recruitment observed in our system resembles the dynamics of ZAP-70 association in a T cell–APC immunological synapse.[9] The dissipation of ZAP-70 clusters indicates the initiation of down-regulatory events that limit the duration of the ZAP-70-dependent signal. Functional TCR engagement by surface-coated anti-CD3ε antibodies was further confirmed for this system by the detection of nuclear translocation of the transcription factor NFATc (data not shown). For experiments requiring fixed cells, the 40 min time point was chosen. At this stage, the cells still fully adhered but ZAP-70 clusters had partially dissipated. When using signalling inhibitors, this time point should therefore reliably reflect changes in signalling activities that influence ZAP-70 recruitment and cell attachment.

ZAP-70–YFP colocalizes with CD3 receptor complexes

In order to identify the molecular basis for the formation and dissipation of ZAP-70 clusters, we first determined whether the dynamics of ZAP-70–YFP clustering correlated with the formation of CD3ζ–CFP clusters. For this purpose, 3A9 hybridoma cells coexpressing the ZAP-70–YFP and a CD3ζ–CFP fusion protein were examined by confocal microscopy by using the same stimulus (Figure 3). CD3ζ–CFP formed discrete clusters that fully colocalized with ZAP-70–YFP in the early stages of stimulation. This colocalization suggests that ZAP-70–YFP clustering resulted from recruitment to phosphorylated CD3 ITAMs, notably the CD3ζ chain. In contrast to ZAP-70, the CD3ζ clusters persisted over the whole 60 min time course of the experiment. The persistence of CD3ζ clusters indicates that the dissipation of ZAP-70–YFP clusters was not due to CD3ζ internalization but to other down-regulatory mechanisms that modulate ZAP-70 recruitment to the plasma membrane. These experiments, for which fixed cells were used, also confirmed that the loss of YFP fluorescence in the live-cell experiments was not due to photobleaching. The confocal optics restricted the detection of fluorescence to an optical section next to the coverslip. It was therefore difficult to determine, whether the loss of YFP fluorescence was due to redistribution of the fusion protein throughout the cell or to degradation. In order to answer this question, cells were seeded onto anti-CD3ε-coated coverslips, and the levels of the fusion protein and endogeneous protein were probed by Western blotting. The ZAP-70 levels remained unchanged throughout the experiment (Figure 3B).

Continuous Src-family kinase activity is required for maintenance of cell contacts only in the early phase of cell attachment

Up to this point we have established that anti-CD3ε-coated coverslips induce a focal engagement of CD3 complexes that leads to a transient recruitment of ZAP-70 with a time course similar to that of the T cell–APC contact.[9] In order to reveal the role of Lck in the formation and maintenance of the clusters, an inhibitor-based strategy was chosen. Small-molecule inhibitors represent powerful tools for rapidly interfering with cellular-signalling processes. The specificity of the effects observed for inhibition of Lck activity was established through a combination of results obtained with the Src-family kinase inhibitors PP2 and SU6656. Of the kinases involved in early signalling in T lymphocytes, PP2 inhibits the Src-family kinases Lck and Fyn with an IC_{50} value of 4–5 nM.[12] However, additional kinases might also be inhibited.[13] In contrast, for SU6656, the IC_{50} value for the inhibition of Lck is 6.9 μM, while Src, Fyn, Yes and Lyn are inhibited with IC_{50} values in the mid to upper nanomolar range.[14] In addition, SU6656 and PP2 vary greatly in their activity profiles for the inhibition of non-Src-family kinases.[13]

Pretreatment of the cells with 20 nM PP2 inhibited their capacity to adhere and spread on the anti-CD3ε-coated coverslips. Signalling-active Lck is associated with the immature immunological synapse for the first 10 min after contact formation.[9] When PP2 was added 5 min after contact formation, ZAP-70 clusters, especially those in the periphery of the cells, dissipated. The cells retracted their contacts, and the contour of the cell changed from convex to concave within 5 min (Fig-

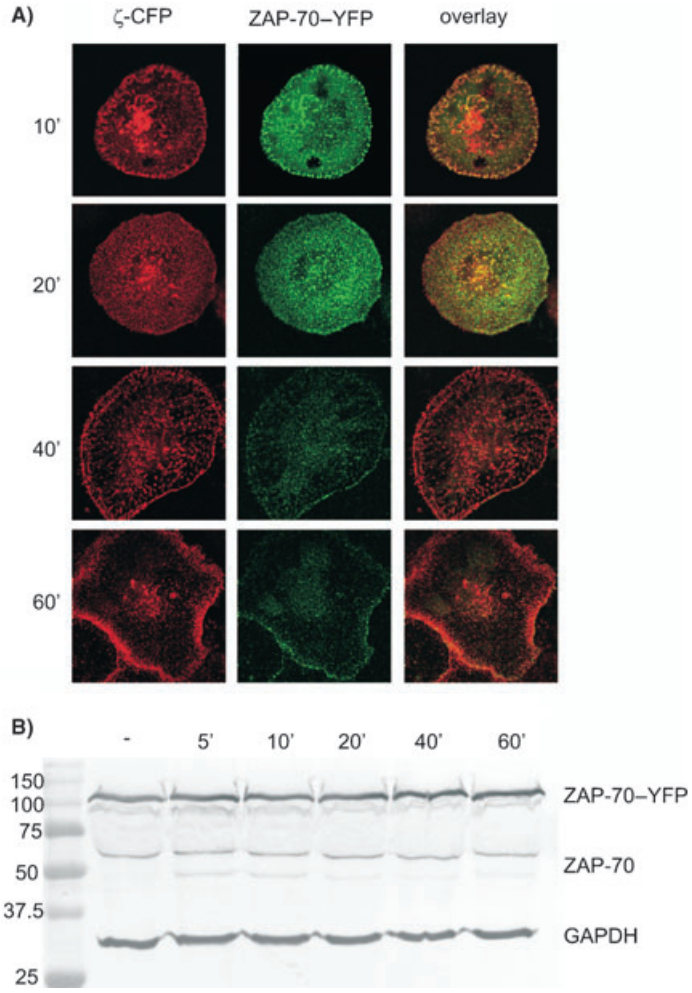

Figure 3. *Corecruitment of TCR CD3ζ-chains and ZAP-70. A) 3A9 cells cotransfected with CD3ζ–CFP and ZAP-70–YFP were exposed to anti-CD3ε-coated coverslips, fixed after 10, 20, 40, and 60 min of stimulation and imaged by confocal laser scanning microscopy. Left panels: CFP fluorescence (red); centre panels: YFP fluorescence (green); right panels: superposition of both channels. A confocal section corresponding to the surface of the coverslip is shown in each case. The bar denotes 10 μm. B) An anti-ZAP-70 Western blot was performed in order to determine whether the decrease in fluorescence was due to a degradation of the fusion protein. 3A9 cells expressing ZAP-70–YFP and CD3ζ–CFP were left unstimulated (–), or stimulated on CD3ε-functionalized coverslips for 5, 10, 20, 40 and 60 min. Lysates were subsequently blotted with an anti-ZAP-70 antibody. An anti-GAPDH antibody served as a control for the amount of cellular protein. The band at 50 kDa likely resulted from the cross-reactivity of the secondary antibody with residual stimulatory antibody that was washed off the coverslip during cell lysis.*

ure 4A). This rapid and strong retraction was a phenomenon observed exclusively for more than 60% of the PP2-treated cells at this stage. The detachment of cells prompted us to also add the inhibitor at a time point when the ZAP-70 clusters started to dissipate. We hypothesized that contacts might be functionally different when early signalling events had terminated. In fact, in contrast to addition after 5 min, addition of PP2 20 min after contact formation had no significant effect on cell attachment. The size and distribution of the ZAP-70–YFP clusters corresponded to those of untreated cells (Figure 4B). Furthermore, the kinetics of cluster dissipation were comparable to those of the control. Apparently, in the early phase of CD3 engagement, continuous Src-family kinase activity is required to maintain cell attachment and ZAP-70 clustering. Later, this requirement vanishes. In order to biochemically validate the inhibitor effects at different time points, the interaction of the CD3-complex with ZAP-70 was probed with anti-CD3ε immunoprecipitation followed by Western blotting with an anti-ZAP-70 antibody. Given that the addition of PP2 (20 nM) caused a dissipation of clusters over a time course of several minutes, the concentration dependence of the effect of PP2 was first assessed (Figure 4C). This inhibitor has been used at very different concentrations in different cellular model systems, from the lower nanomolar to the micromolar range.[6,12,15,16] Cells were stimulated on the same anti-CD3ε-coated coverslips used for cell biological experiments and lysed after a total of 10 min stimulation time. As expected, the recruitment of ZAP-70 to the CD3 complex was stimulus-dependent. To our surprise, at 20 nM PP2, no effect on ZAP-70 recruitment was detected, either when the inhibitor was added before initiation of stimulation or after. When the inhibitor was added at 200 nM or higher concentrations before the initiation of stimulation, the ZAP-70 signal was strongly reduced. Interestingly, the inhibitory effect was even more pronounced when 200 nM PP2 was applied 5 min after initiation of stimulation than at 20 min after or before initiation of stimulation (Figure 4D).

Exposure of cells to SU6656 at a concentration of 1 μM had no effect either on the attachment and spreading of cells or on ZAP-70 clusters; this indicates that the PP2-dependent effects were specifically related to the inhibition of Lck. In order to confirm that SU6656 had the potential to exert a specific inhibitory activity, a human Jurkat T cell lymphoma cell line was exposed to coverslips coated with an antibody directed against the integrin LFA-1. This integrin acts as a coreceptor in T cell signalling.[17] The propagation of integrin-dependent signals is Src-kinase dependent. As expected, preincubation with both PP2 (20 nM) and SU6656 (1 μM) strongly reduced the attachment of Jurkat cells. On anti-CD3-coated coverslips, PP2 exerted a significant effect, while SU6656 showed only a weak effect (not shown).

Cell detachment is actin independent

In order to address the molecular basis for PP2-dependent cell detachment, we decided to investigate the role of actin reorganization on cell attachment, ZAP-70 clustering and cell spreading. Reorganization of F-actin at the immunological synapse has been related to the stabilization of the T cell–APC contact.[18] Both Lck and Fyn have been implicated in the T cell stimulation-dependent actin reorganization.[19,20] T cell spreading on a functionalized surface is accompanied by the rearrangement of F-actin into circumferential rings.[5,11]

When cell detachment was induced by addition of PP2 after 5 min of stimulation, a fragmentation of F-actin was observed (Figure 5A and B). In contrast, little effect on F-actin was observed when PP2 was added to cells after 20 min stimulation (Figure 5C). Again, the characteristic detachment was visible

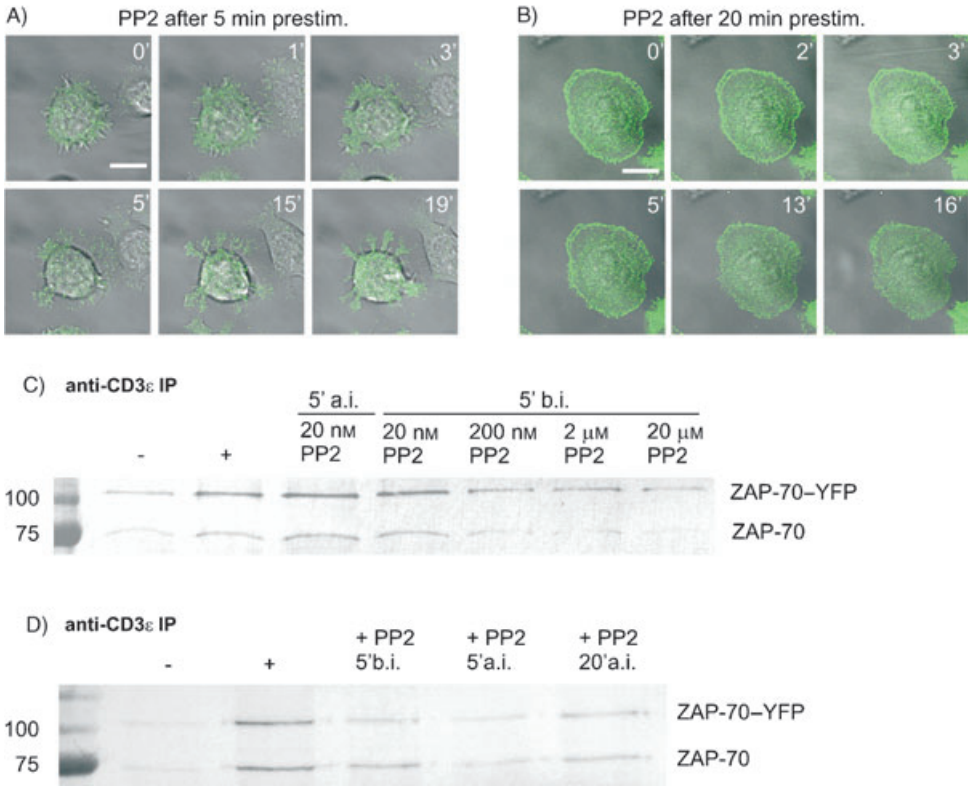

Figure 4. *Effect of Src-family kinase inhibition on cell attachment and ZAP-70 clustering. Time-lapse confocal microscopy of ZAP-70–YFP expressing cells before and after addition of the Src-family kinase inhibitor PP2 (20 nM). Cells were allowed to establish contacts with the coverslips for A) 5 min and B) 20 min before addition of the inhibitor. A confocal section corresponding to the surface of the coverslip is shown in each case. The time points after the addition of the inhibitor are shown. The bar denotes 10 μm. C) Biochemical validation of the effect of PP2 on ZAP-70 recruitment. 3A9 cells expressing ZAP-70–YFP were left unstimulated (−), stimulated on CD3ε-functionalized coverslips without inhibitor (+), treated with PP2 (20 nM) added 5 min after initiation of stimulation (a.i.) or treated with PP2 added at the concentrations of 20 nM, 200 nM, 2 μM, and 20 μM 5 min before initiation of stimulation (b.i.). Cells were stimulated for a total time of 10 min. Lysates were immunoprecipitated with antibodies against CD3ε and subsequently blotted with an anti-ZAP-70 antibody. D) Dependence of the inhibition of ZAP-70 recruitment on the timing of inhibitor application. Anti-CD3ε immunoprecipitates of lysates of cells left unstimulated (−), stimulated on anti-CD3ε-coated coverslips for a total of 30 min, without (+) or with PP2 (200 nM) added at the indicated time points were probed for ZAP-70 by Western blotting.*

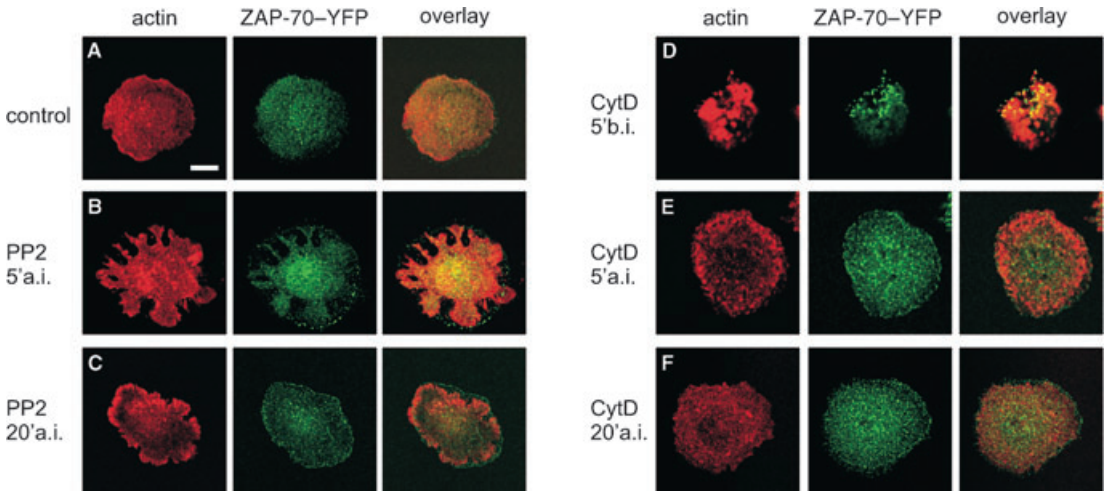

Figure 5. *Effect of inhibitors on F-actin, cell attachment and ZAP-70 clusters. A) Untreated controls, B) cells exposed to PP2 (20 nM) after 5 min (5′ a.i.) and C) 20 min (20′ a.i.) after initiation of stimulation, D) cells pretreated with CytD (10 μM) 5 min before exposure to the coverslips (5′ b.i.), E) and incubated with CytD after 5 min and, F) 20 min after initiation of stimulation. In the figure, the time point when the inhibitor was added is given in each panel. In each case, including the control, the cells were incubated for a total of 40 min before fixation, that is, a further 35 min when inhibitor was added after 5 min, or a further 20 min when inhibitor was added after 20 min. Actin was visualized by using biotin-conjugated phalloidin (50 μg mL^{-1}) in combination with Cy5-conjugated streptavidin. The left panels (red) represent actin, the centre panels (green) ZAP-70–YFP. In the right panels a superposition of both channels is shown.*

for the majority of the PP2 treated cells, but only rarely seen in untreated cells. In order to ascertain whether the fragmentation of F-actin was the reason for cell detachment, cells were incubated with CytD, an inhibitor of actin polymerization. In contrast to PP2, preincubation of cells with CytD (10 μM) did not inhibit the formation of ZAP-70–YFP clusters at the cell-substrate contact or cell adhesion (Figure 5D). However, consistent with results reported previously for Jurkat cells, the actin-mediated spreading of cells on the surface was inhibited.[11] Unlike PP2, CytD treatment of cells contacting the coverslips for 5 min, did not cause detachment or retraction of the cells from the surface. Instead, the cells were spread out on a smaller area; this demonstrated that only cell extension was inhibited (Figure 5E). No peripheral actin ring formed, and actin did not colocalize with ZAP-70–YFP clusters. For cells treated with CytD after 20 min, the only visible effect was a mild disruption of F-actin compared to the controls (Figure 5F). In addition, CytD inhibited not only the spreading of the cells, but also prevented the dissipation of ZAP-70–YFP clusters, since the ZAP-70–YFP clusters appeared brighter in CytD-treated cells than in control cells stimulated for the same length of time. In contrast to PP2-treated cells, ZAP-70 clusters in CytD-treated cells persisted over the entire 60 min time course of the experiment. In order to address whether this persistence of clusters was due to an inhibition of actin-dependent internalization, confocal stacks of CytD-treated cells and controls were acquired. No evidence for actin-dependent internalization of ZAP-70-rich vesicles was obtained. The observations made for the inhibitors and the loss of colocalization in controls at the 40 min time point support the notion that ZAP-70 clustering is only indirectly coupled to F-actin polymerization. However, cell spreading driven by stimulation-dependent actin reorganization is a prerequisite for the formation of peripheral contacts and clusters.

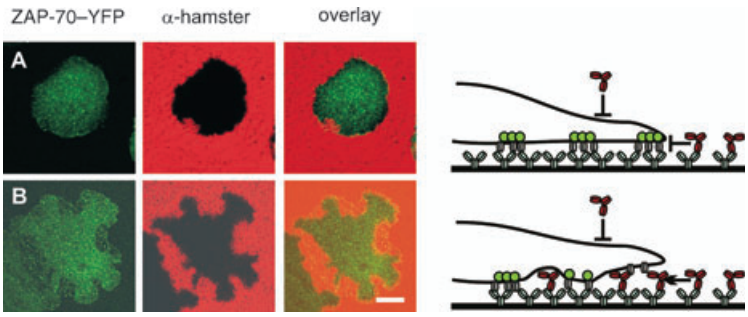

Figure 6. *Analysis of the mechanism of PP2-induced cell detachment. Tightness of cell-substrate contacts as determined by the accessibility of surface-bound Armenian hamster anti-CD3ε antibody to Cy5-labelled anti-Armenian hamster antibody (α-hamster). Cells were stimulated for 5 min, followed by incubation for 20 min A) in the absence B) or presence of PP2 (20 nM). ZAP-70–YFP fluorescence is shown in green (left panels), Cy5 fluorescence of anti-Armenian hamster antibody in red (centre panels). Right panels: superpositions of both channels. To the right, schemes of surface staining with Cy5-labelled anti-Armenian hamster antibodies are shown. The cells were fixed but not permeabilized. Surface bound Armenian hamster anti-CD3 antibodies in the cell-substrate contact were therefore only accessible to the anti-Armenian hamster antibody by lateral diffusion of the molecules into the contact area. The bar denotes 10 μm.*

Cell retraction upon Src-family kinase inhibition occurs by detachment of contacts

The results shown so far indicate that the PP2-induced detachment of cells is not due to an inhibition of actin polymerization. Instead, Lck might exert a direct effect on the CD3 complex or CD3-associated proteins that act upstream and/or are independent of actin polymerization and that destabilize the cell-substrate contact. In order to probe the intimacy of cell–substrate contact, cells were fixed, but not permeabilized, and coverslips were incubated with a Cy5-conjugated anti-Armenian hamster antibody. In the absence of permeabilization, the surface of the coverslip underneath the cell is accessible to the Cy5-labelled antibody only from the side (Figure 6). In control cells, the area of cell contact with the coverslip was completely inaccessible to the antibody; this is indicative of a tight cell–substrate contact (Figure 6). In contrast, in the periphery of PP2-treated cells, the anti-CD3ε fluorescence colocalized with ZAP-70–YFP fluorescence. This distribution of fluorescence strongly suggests that cell retraction induced by PP2 occurs by disengagement of cell contacts from the antibody-coated surface. The peripheral CD3 contacts were destabilized to the point that, even for the contacts themselves, the surface became accessible to the antibody. Finally, detachment occurred in a highly disordered fashion. Instead of a uniform retraction of the cell edge, some contacts were maintained and large bay-like shapes formed. Formation of these shapes can be explained by a generalized destabilization of contacts across the surface and their random detachment.

Discussion

In this paper, chemical inhibitors were used in combination with high-resolution confocal microscopy for the analysis of the timing of signalling activities in cellular signal-transduction pathways. The application of inhibitors of Src-family kinases as well as microfilament dynamics at various stages before and after contact formation provided new insights into the initial steps of CD3-dependent signal transduction in T cells.

Maturation of signalling clusters on anti-CD3ε-coated surfaces

In our experiments, glass coverslips homogeneously coated with anti-CD3ε antibodies were used as a minimal stimulus engaging CD3 complexes in a manner that precludes the ordered rearrangement of transmembrane molecules by lateral diffusion. Three different stages of susceptibility to inhibition of Src-family kinases by PP2 could be distinguished. First, cells pretreated with PP2 failed to establish contacts; this is consistent with the finding that PP2 pretreatment inhibits induction of tyrosine phosphorylation upon subsequent TCR/CD3 cross-linking.[21] Second, cells that had established contacts for 5–10 min continued to be sensitive to PP2. Third, at 20 min after

initial engagement, the ZAP-70 cluster dynamics were no longer sensitive to the inhibitor and the dissipation, and detachment followed its normal time course. By combining inhibitors with different specificities, especially by taking advantage of the recently described inhibitor SU6656, we were able to show that the effect of PP2 could most likely be attributed to inhibition of Lck (Figure 7). While PP2 acts as an inhibitor of

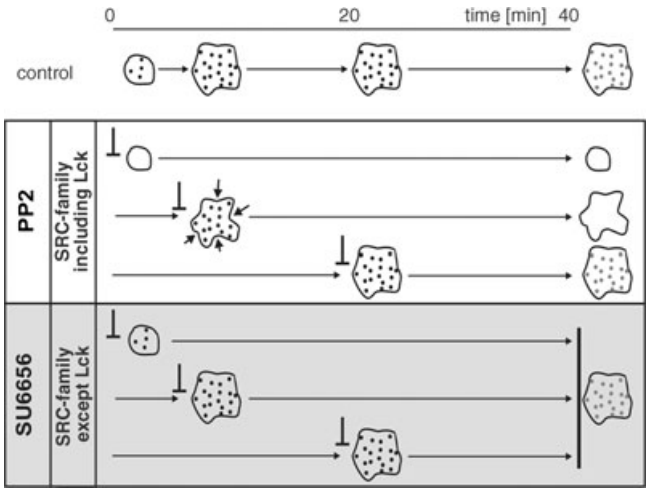

Figure 7. *Schematic summary of the effects of inhibitors on cell morphology and ZAP-70 aggregates observed for addition of compounds before, 5 min and 20 min after contact of cells with the anti-CD3-coated coverslips.*

the Src-family kinases Lck, Fyn, Hck and Src, SU6656 is thought to act on the Src-family kinases Src, Fyn, Yes and Lyn, but not on Lck at the concentration used in our experiments.[14] In addition, at a concentration of 20 nM, Lck and Fyn are the primary activities relevant for T cell attachment inhibited by PP2. Moreover, the Lck-deficient Jurkat cell line JCaM1.6 failed to spread on anti-CD3ε-functionalized coverslips, while Lck-retransfected cells were able to spread (data not shown).[22] The failure of Fyn to compensate for Lck mutations in cell adhesion further stresses the significance of Lck in the maintenance of ZAP-70 clusters.[19] Since Lck is the primary kinase involved in CD3-dependent signalling, validation of the inhibitory activity of SU6656 had to rely on an independent assay. Using a very similar experimental design, we could demonstrate that this compound inhibited the attachment of Jurkat T cell lymphoma cell line on anti-LFA-1-coated coverslips. As expected, given the activity profiles of the compounds, both PP2 and SU6656 had the same effect.

Molecular mechanism of contact maturation and detachment

Actin remodelling has been described as a hallmark of both immunological synapse formation and contact formation of T cells with anti-CD3-coated surfaces. Both Lck and Fyn have been implicated in these processes.[11,20,23] Consistent with these prior results, addition of PP2 affected the distribution of F-actin, both at the early and late time points. However, inhibition of microfilament dynamics by CytD only affected cell spreading. Neither cell attachment nor ZAP-70 clustering were affected by this compound. Apparently, the Lck-dependent regulation of CD3 engagement is controlled by mechanisms acting upstream of actin polymerization at the contact site. At present, we can only speculate how such disengagement occurs on the molecular level. The failure of cells preincubated with PP2 to adhere to anti-CD3-coated coverslips indicates that the presence of receptor and ligand is insufficient for establishing strong receptor–ligand contacts and suggests that some sort of feedback in early intracellular events is necessary for adhesion, even to such a simple and highly stimulatory surface. The observations made for the CD3-dependent attachment resemble the modulation of integrin-mediated cell adhesion.[24–26] The results suggest that Src-family kinases act on the CD3 complex in a way that affects the avidity of these receptors for a polyvalent ligand.

Analysis of T cell contacts by chemical inhibitors

Given the importance that has been attributed to the lateral mobility of receptor molecules, the formation and maturation of the immunological synapse has most often been investigated in the T cell–APC contact or for T cells exposed to ligands incorporated into supported lipid bilayers.[1] Therefore, up to this point, contact maturation between a T cell and an APC has been based largely on morphological criteria. Our results show that stimulation by a well-defined and highly simplified nondiffusive stimulus can also induce transformation of contacts from a stage sensitive to Src-family/kinase inhibition to one that is inhibition insensitive.

The demonstration that T cell contacts acquire an insensitivity to the inhibition of Lck introduces a pharmacological concept of contact maturation. Moreover, the different phenotypes obtained for the inhibition of Lck before and at various stages after formation of contacts, stress a major advantage of chemical inhibitors over alternative methods for interfering with the function of a protein. Knock-down strategies as well as the down-regulation of protein expression by small interfering RNAs (siRNA) preclude the analysis of protein signalling activities that are vital for the initiation of a signal.

In the analysis of T cell signal transduction, there is evidence to suggest that the kinetics of signalling critically determine the functional response of a T cell.[27] Chemical inhibitors represent a powerful means to investigate in detail the functional implications of such signalling kinetics. As an alternative to the application of an inhibitor after a given period of time, for low affinity compounds one could also imagine removing a chemical signalling block by "washing-out". In addition to investigating the function of a gene product with compounds of known activity, screens of small-molecule inhibitors with unknown targets for the induction of phenotypes in chemical genetics are also being performed, for example, in the embryonic development of organisms,[8] particularly as kinetics also play a role in these cases. Such screens, therefore, greatly benefit from the addition of compounds at different time points.[8]

Interestingly, the cellular phenotype provided a more sensitive sensor to the presence of the inhibitor than the biochemical analysis of the recruitment of ZAP-70. Incubation of cells with 200 nM PP2 5 min after initiation of stimulation had the strongest effect on ZAP-70 recruitment, in accordance with the results obtained by microscopy. However, when cells were preincubated with 20 nM of PP2, ZAP-70 was still recruited to the CD3 complex. In contrast, at this inhibitor concentration, no spreading of 3A9 cells on anti-CD3-coated coverslips was detected. At present, the molecular details of this phenomenon remain elusive. We can only speculate that subtle changes in the balance of kinase and phosphatase activities are translated into different degrees of cell spreading. Given that T cells react sensitively to differences in MHC–peptide complexes and that the kinetics and balance of the recruitment of signalling proteins have been discussed as the basis for this discriminatory ability, these results are not completely surprising.[28] In our analyses, we aimed at using the same anti-CD3-coated coverslips as used for cellular experiments for the stimulation of cells in biochemical experiments. A larger number of cells and more detailed analysis of the pattern of phosphorylation might resolve the molecular details of this observation.

One possible limitation is the lack of specificity of some inhibitors. However, for the Lck-dependent signalling addressed in this study, only two members of the group of SFK target proteins have been implicated in the investigated signalling pathway. Of these two proteins (Lck and Fyn) Lck was singled out as the relevant target by incubation of cells with an inhibitor with a different activity profile. Given the availability of diverse compound collections with different activity profiles, a strategy in which the specificity of an observed effect is confirmed by application of different inhibitors at various time points might be realized for a large number of molecular targets. In order to perform such analyses efficiently, a robust stimulation protocol that provides maximum information on the respective signalling pathway is required. For the analysis of T cell signalling, this prerequisite was fulfilled by exposure of cells to anti-CD3-coated coverslips, a stimulus that reproduces major characteristics of TCR-dependent signalling events initiated by more-complex stimuli. We are well aware that cellular experiments that use such simplified stimuli cannot replace the analysis of T cell signalling in physiological T cell–APC contacts. However, our approach could complement the latter in several ways. First, by providing highly defined stimuli that reveal new and interesting characteristics of components of the signalling machinery. Second, by acting as a filter to screen for experimental conditions, for a further detailed analysis. The considerable extra effort required for the analysis of T cell–APC contacts precludes the systematic testing of a large number of experimental conditions.

The stimulation and image acquisition protocols presented in this study can readily be implemented into automated experimental procedures. Initial experiments have shown that cell attachment, size and kinetics of signalling clusters respond sensitively to inhibition of a large number of downstream events. Moreover, the approach could be extended to stimuli other than CD3-directed antibodies. The anti-LFA-1-coated coverslips used to confirm the activity of SU6656 were a first step in this direction. By taking advantage of a large repertoire of available inhibitors targeting distinct steps in T cell signalling, it would be interesting to systematically address the involvement and timing of downstream signalling components controlling T cell adhesion, cytoskeletal reorganization and the dynamics of signalling clusters.

Experimental Section

Cells and reagents: 3A9 hybridoma T cells and derivatives were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal calf serum (FCS) (10%, PAN, Aidenbach, Germany), sodium pyruvate (100 μM), nonessential amino acids, β-mercaptoethanol (45 nM), L-glutamine (200 μM), penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}).[29] Plat-E packaging cells were cultured with the addition of puromycin (1 μg mL^{-1}) and of blasticidin (10 μg mL^{-1}). Supplements were obtained from Gibco BRC (Karlsruhe, Germany) and Sigma (Deisenhofen, Germany). Jurkat, Jurkat JCaM1.6 and derived cell lines were cultured in RPMI 1640 (PAN) with FCS (10%).[30]

Generation of fusion constructs: To insert EYFP into the retroviral expression vector pMFG, the *Sac*I–*Not*I fragment from the EYFP-N1 vector (Clontech, Heidelberg, Germany) containing the EYFP-coding region was ligated into the *Sac*I-*Not*I site of pBluescript KS$^+$. The EYFP-coding region was then recovered as a *Nco*I-*Bam*HI fragment and cloned into the corresponding unique sites of the retroviral vector pMFG.[31] The murine ZAP-70 gene was amplified by PCR from murine thymic cDNA by using the forward primer: 5′ata catgtccgatcccgcggcgca containing an *Afl*III restriction site (underlined) at its start codon. The reverse primer: 5′ccacatgttggcc gatccccaccgccagacccgccacatgcagcctcggccac includes the last six codons of ZAP-70 fused to a linker sequence (GGSGGGGSAN) and an *Afl*III restriction site (underlined). The PCR product was purified, ligated into pGEM-TZ and sequenced (Genome Express, Grenoble, France). The ZAP-70 *Afl*III fragment was recovered from pGEM-TZ and cloned into the *Nco*I site of pMFG-YFP to give ZAP-70 with YFP fused to its C terminus (ZAP-70–YFP). The CD3ζ–CFP fusion was derived from a pMFG-CD3ζ–YFP (pAPVR1, M. Malissen, CIML) construct by exchanging YFP for CFP by the unique *Nco*I-*Not*I sites.

Retroviral infection and cell characterization: 3A9 hybridoma T cells were infected with retroviruses generated from the pMFG vector by using the Plat-E packaging cells as described by Morita.[32] YFP fusion protein expression was monitored by flow cytometry 48 h post infection. Cells were expanded, sorted by flow cytometry and subcloned by limiting dilution. For double labelling with CD3ζ–CFP, the wild type 3A9 cells were first infected with CD3ζ–CFP DNA, as described above. Immediately after infection, cells were cloned by limiting dilution. Clones expressing labelled CD3ζ–CFP at the plasma membrane were selected by visual inspection under an epifluorescence microscope. These cloned populations were then infected with pMFG–ZAP-70–YFP, sorted for YFP expression and subcloned. To verify the integrity of the YFP and CFP chimeras expressed in the 3A9 cells, post-nuclear cell lysates were prepared, run on SDS-PAGE (10%), transferred to PVDF membrane and probed for GFP expression with an anti-GFP monoclonal antibody (mAb; Clontech), bound with a donkey anti-mouse horseradish peroxidase conjugated secondary antibody (Immunotech Beckman Coulter, Fullerton, USA) and visualised with ECL/autoradiography. Anti-phosphotyrosine mAb, 4G10, was a kind gift from Dr. H.-T. He, CIML, Marseille, France.

Functionalization of coverslips: Silanization of 12 mm coverslips with trimethoxyoctadecylsilane (Fluka, Seelze, Germany) was carried out as described previously.[33] Silanized coverslips were coated with anti-CD3ε mAb (200 µl; 5 µg mL^{-1} mAb145–2C11, BD Pharmingen, San Diego, USA) for 16 h at 4 °C. After being coated, the surface was washed twice for 5 min with PBS followed by blocking with PBS containing bovine serum albumine (BSA; 1%) for 30 min at RT. Finally, coverslips were washed three times for 5 min with PBS and stored at 4 °C until use. For control experiments, uncoated silanized slides, silanized slides coated with BSA (Sigma), anti-CD4 (Pharmingen) or with poly-L-lysine (Sigma) were used. Anti-LFA-1 antibodies were purified from TS1/22 hybridoma supernatant (Developmental Studies Hybridoma Bank, Iowa City, USA).[34]

Inhibitors: The Src-family kinase inhibitor PP2 (Calbiochem) was added from an aqueous solution to a final concentration of 20 nM. The Src-family kinase inhibitor SU6656 (Sigma) was applied at 1 µM, a concentration that inhibits the kinases Src (IC_{50} = 0.28 µM) and Fyn (IC_{50} = 0.17 µM), but is insufficient to inhibit Lck (IC_{50} = 6.88 µM).[14] Cytochalasin D (Sigma) was added to a final concentration of 10 µM.

Immunofluorescence: Cells were exposed to the coverslips at 37 °C and fixed after the indicated time points or observed by confocal time-lapse microscopy in a custom-made chamber, by using CO_2-independent serum-free DMEM (Gibco). Cells on coverslips were fixed with 3.5% paraformaldehyde (PFA) in *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)-buffered saline (HBS) first for 10 min at 4 °C, then for 15 min at RT, followed by washing and permeabilization with saponin buffer (HBS containing saponin (0.1%, Sigma) and BSA (0.1%)). Incubations with antibodies were carried out for 1 h at RT in saponin buffer. Actin was detected by using biotin-labelled phalloidin (50 µg mL^{-1}, Alexis, Grünberg, Germany) in saponin buffer in combination with Cy5–streptavidin (1 µg mL^{-1}, Dianova). After washing with saponin buffer, samples were fixed once more with PFA for 15–20 min. Prior to mounting in Mowiol (Fluka), any remaining fixative was quenched by 3 washes with Tris-acetate buffer (100 mM, pH 8) for 5 min. Immobilized stimulatory antibody was detected by using Cy5-labelled anti-Armenian hamster antibody (3 µg mL^{-1}, Dianova).

Fluorescence microscopy: Confocal microscopy was performed on an inverted LSM 510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) by using a C-Apochromat 63×1.4 Oil lens (Carl Zeiss). YFP was imaged by excitation at 514 nm and detection with a BP 530–600 nm band pass filter. For the parallel detection of CFP and YFP, the spectrally resolving META detector of the instrument was used. CFP was excited at 458 nm and detected at 465–518 nm when using the META detector or with a BP 475–525 nm band pass filter. YFP was excited at 514 nm and detected at 518–604 nm when using the META detector or with a 530–600 nm band pass filter. For parallel detection of YFP and Cy5 the 514 nm line of the Argon ion laser and the light of the 633 nm HeNe laser were directed over an HFT 514/633 beam splitter in combination with an NFT 635vis beam splitter and a BP 530–600 nm band pass filter for YFP detection and an LP 650 long pass filter for detection of Cy5.

Western blots: In order to analyze the cellular ZAP-70–YFP content at different time points of cell stimulation, 2.5×10^5 cells per coverslip were stimulated as described above for the stated times and lysed (5×10^6 cells mL^{-1}) in lysis buffer (Tris pH 7.5 (20 mM), Triton X-100 (1%), EDTA (1 mM), NaCl (150 mM), Na_3VO_4, (1 mM), protease inhibitor cocktail (Roche, Mannheim, Germany)) on ice for 30 min. The crude lysates were clarified by centrifugation at 20000 *g* for 15 min, separated by SDS-PAGE (10%) and transferred to a PVDF membrane.[35] The blots were probed for ZAP-70–YFP expression by using a ZAP-70 antibody (BD Transduction Labs, Franklin Lake, USA) in combination with a goat anti-mouse–alkaline phosphatase conjugated secondary antibody for detection (Sigma). In order to analyze the activation-dependent recruitment of ZAP-70 to the CD3/TCR complex, cells were stimulated as described above. Anti-CD3ε antibodies (final concentration of 10 µg mL^{-1}) and µMACS-beads functionalized with protein G (Miltenyi Biotec, Bergisch Gladbach, Germany, dilution 1:20) were added to the lysates. Samples were incubated on ice for 20 h and precipitated according to the manufacturer's directions. The pellets were washed twice with lysis buffer and once with PBS, and analyzed by Western blotting as described above.

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