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# In the Immune Synapse, ZAP-70 Controls T Cell Polarization and Recruitment of Signaling Proteins but Not Formation of the Synaptic Pattern

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## Summary

**Recognition by T cells of their ligands at the surface of antigen-presenting cells (APCs) leads to T cell activation, polarization of the T cell toward the APC, and formation of an immune synapse. Using ZAP-70-deficient T cells expressing ζ-GFP, we show that ZAP-70 signaling drives the TCR-dependent reorientation of the microtubule-organizing center thus leading to relocation of a ζ-GFP$^+$ intracellular compartment close to the APC. ZAP-70 is also necessary to supply the synapse with the signaling molecules PKC-θ and LAT. In contrast, ZAP-70 is not required for clustering of ζ-GFP and CD2 or exclusion of CD45 and CD43 from the synapse. These data show that ZAP-70-dependent signaling is required for formation of a functional immune synapse.**

## Introduction

The T cell receptor (TCR) interaction with its ligand, i.e., complexes of peptides and MHC molecules expressed at the surface of an antigen-presenting cell (APC), triggers a cascade of intracellular signals that culminate in cytokine gene expression, proliferation, and execution of the T cell effector functions. This cascade of events requires the sequential activation of several protein tyrosine kinases such as ZAP-70, and this key role has been highlighted by the identification of a familial form of severe combined immunodeficiency caused by mutations in the gene-encoding ZAP-70 (Arpaia et al., 1994; Elder et al., 1994; Hivroz and Fischer, 1994). Patients suffering from this immunodeficiency lack mature CD8$^+$ T cells whereas their CD4$^+$ T cells are nonresponsive to stimulation by TCR ligands.

In the past few years, interaction of the TCR with its ligand has been shown to take place within an organized contact zone known as the immune synapse. It consists of highly organized interaction sites at the interface between the T cell and the APC in which TCRs, coreceptors, adhesion, and signaling molecules are assembled (Grakoui et al., 1999; Monks et al., 1998) in concentric zones, also known as supramolecular activation clusters (SMACs), with an inner circle of TCR (Monks et al., 1998) and CD2 (Anton van der Merwe et al., 2000; Dustin et al., 1998; Leupin et al., 2000), an outer ring of LFA-1, and a distribution of CD45 and CD43, which is largely excluded from these concentric zones (Allenspach et al., 2001; Delon et al., 2001; Johnson et al., 2000; Leupin et al., 2000; Roumier et al., 2001). Actin cytoskeleton plays a critical role in the formation of SMACs by orchestrating molecular movements of the T cell surface molecules (Grakoui et al., 1999; Monks et al., 1998) and by recruiting specialized membrane domains enriched in glycolipids and signaling molecules (Viola et al., 1999; Xavier et al., 1998).

Formation of the immune synapse is also accompanied by a profound remodeling of the microtubule cytoskeleton, characterized by the polarization of the T cell microtubule-organizing center (MTOC) toward the APC. This phenomenon is critical since it allows the relocation of the T cell's secretory machinery (Kupfer and Dennert, 1984) and thus a polarized delivery of cellular mediators, such as lymphokines, at the central synapse (Kupfer et al., 1991; Poo et al., 1988; Reichert et al., 2001).

Relatively little is known about MTOC polarization. Lowin-Kropf et al. have shown that anti-TCR-coated beads induce MTOC reorientation (Lowin-Kropf et al., 1998). They further showed that phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by p56$^{Lck}$ was required for MTOC polarization and that a dominant-negative mutant of ZAP-70 inhibited this polarization (Lowin-Kropf et al., 1998). However, mechanisms governing MTOC reorientation in a T cell interacting with an APC are not known, and a potential role of MTOC polarization in immune synapse formation and recruitment of signaling molecules at the synapse has not been investigated.

Moreover, a potential role of signaling in SMACs formation has been challenged by mathematical modelings of these processes which demonstrated that the synaptic pattern observed in living cells could result from spontaneous self-assembly processes with no need of active cellular interventions (Qi et al., 2001).

In the present study, we directly addressed the role of TCR signaling in the immune synapse formation by studying the role of ZAP-70 in the genesis of the synapse. We show that ZAP-70 kinase activity controls the T cell's MTOC polarization. The defective MTOC reorientation observed in ZAP-70-deficient T cells is accompanied by a defective polarization of an intracellular compartment containing ζ-GFP. Moreover, we demonstrate that ZAP-70 activity is not required for TCR-driven clustering of the plasma membrane pool of ζ and patterning of T cell surface molecules at the immune synapse. In contrast, we show that ZAP-70 is required for the recruitment of signaling molecules such as PKC-θ and LAT at the T/APC interface.

These results demonstrate that ZAP-70 drives the formation of a functional immune synapse characterized by the T cell secretory apparatus relocation and the targeting of signaling proteins toward the APC.

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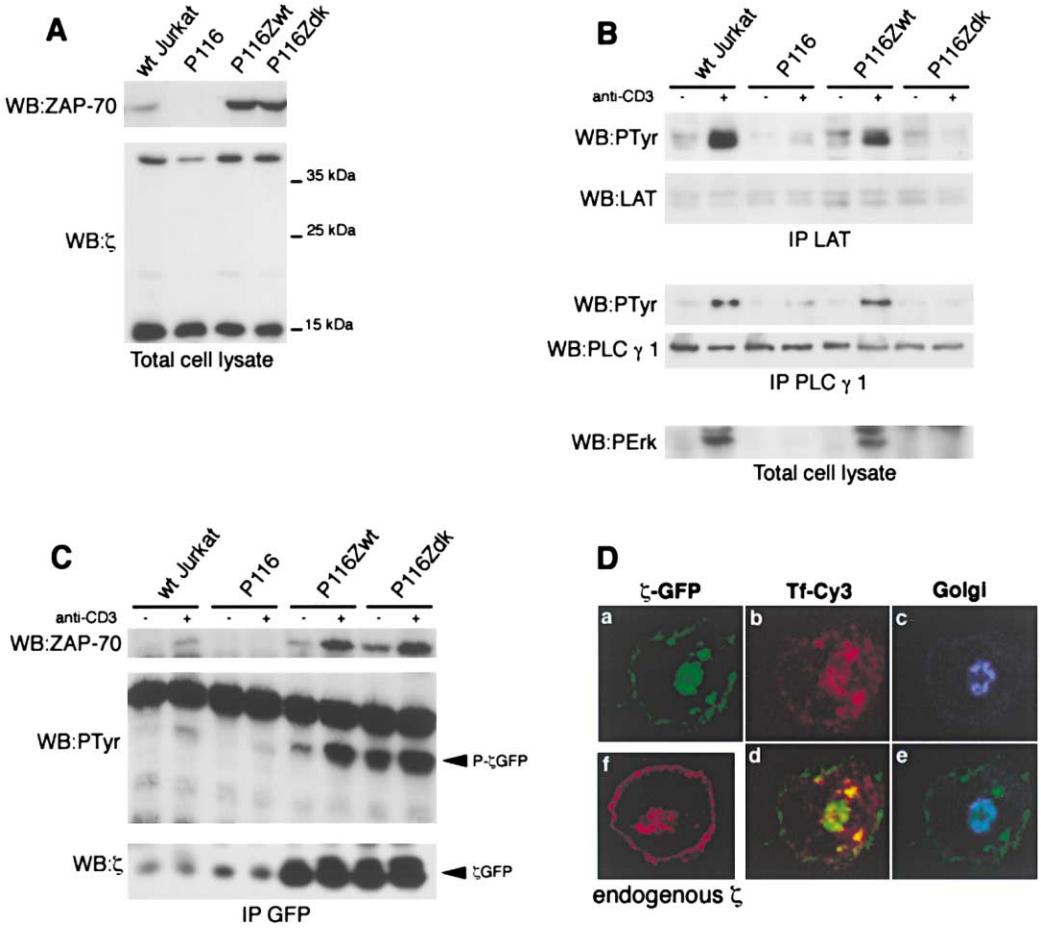


Figure 1. TCR-Mediated Signaling Is Not Affected by Expression of the ζ-GFP Transgene

(A) Total cell lysates for each Jurkat-derived clone were resolved by SDS-PAGE and Western blotted against ZAP-70 (upper panel) and ζ (lower panel).

(B) $10^7$ cells were activated by an anti-CD3ε mAb (UCHT1) for 5 min, lysed, and immunoprecipitated sequentially with rabbit polyclonal Abs against LAT and with murine mAbs anti-PLCγ1. Samples were Western blotted first with anti-phosphotyrosine mAbs, and membranes were reprobed using the anti-LAT and anti-PLCγ1 immunoprecipitating Abs. The lower panel shows the phosphorylation of MAPKs in total cell lysates.

(C) Cells stimulated for 5 min with UCHT1 were lysed and immunoprecipitated with anti-GFP Abs. Western blot with anti-phosphotyrosine mAbs revealed the tyrosine phosphorylated form of ζ-GFP (middle panel), and hybridization with an anti-ZAP-70 Ab showed coprecipitation of ZAP-70 with ζ-GFP (upper panel). The lower panel shows the total amount of ζ-GFP precipitated.

(D) Intracellular localization of ζ-GFP (a) in transfected wild-type Jurkat cells after a pulse-chase with transferrin-Cyanine 3 (b and d) and a labeling with the CTR433 mAb, directed against a marker of the *cis*-Golgi compartment, followed by a Cyanine 5-coupled secondary Ab (c and e). (f) Labeling of endogenous ζ in a nontransfected wild-type Jurkat cell with an anti-ζ mAb followed by a Cyanine 3-coupled secondary Ab.

## Results

### TCR Signaling and Intracellular ζ Localization Are Not Impaired in ζ-GFP-Expressing T Cell Clones

After contact with an APC, several cell surface molecules, such as the CD3/TCR/ζ complex, LFA-1, CD45, and CD43 segregate into distinct areas at the interaction site with the APC. However, the role of signaling in the formation and the spatial organization of this contact zone remains largely unknown. We addressed the question of the involvement of ZAP-70 in the immune synapse genesis by using P116, a Jurkat-derived T cell clone deficient for ZAP-70 (Williams et al., 1998), which we reconstituted with a wild-type or a dead kinase mutant of ZAP-70 (hereafter referred to as P116Zwt and P116Zdk, respectively) (Mege et al., 1996). In order to study the dynamics of the TCR/CD3/ζ complex upon APC recognition, all these cells were stably transfected with a plasmid encoding a C-terminal fusion protein of the ζ chain with the GFP (ζ-GFP).

First, relative expression levels of ZAP-70 in the different ζ-GFP expressing clones are shown in Figure 1A. P116 did not express any ZAP-70 whereas P116Zwt and P116Zdk displayed comparable amounts of the protein but a higher amount than wild-type Jurkat cells. Expression of the ζ-GFP chimeric protein in the total lysates was assessed (Figure 1A). Endogenous ζ had an apparent molecular weight of 16 kDa while the ζ-GFP form migrated at an apparent molecular weight of 45 kDa. ζ-GFP was more strongly expressed by wild-type Jurkat cells, P116Zwt, and P116Zdk than by P116-derived clones. We then studied the phosphorylation of LAT

and PLCγ1, two proteins phosphorylated in a ZAP-70-dependent manner, after CD3 activation (Arpaia et al., 1994; Williams et al., 1998; Zhang et al., 1998a). After 5 min of anti-CD3ε stimulation, LAT was phosphorylated on tyrosine residues only in the wild-type Jurkat cells and in the P116Zwt but neither in the P116 nor in the P116Zdk (Figure 1B). Similarly, PLCγ1 was phosphorylated only in Jurkat-derived cell lines expressing a wild-type form of ZAP-70. We then investigated more distal signaling events, such as MAP kinase activation (Figure 1B). As expected, no Erk phosphorylation was induced by CD3 activation of the P116 cells expressing ζ-GFP; however, Erk phosphorylation was restored by expression of an active form of ZAP-70 but not by expression of a dead kinase form (Figure 1B). CD3 activation of T cells induces ζ phosphorylation and ZAP-70 association with this phosphorylated form (Chan et al., 1992). We checked whether our ζ-GFP chimera behaved similarly. As presented in Figure 1C, CD3 stimulation induced ZAP-70 association with ζ-GFP; furthermore, a ζ-GFP phosphorylated form was detected in all the clones, including P116 where the band was very faint and observed at a lower apparent molecular weight (Figure 1C). These results are consistent with previous results suggesting that ζ did not remain phosphorylated after activation in the absence of ZAP-70 (Williams et al., 1998).

We then verified that the intracellular localization of endogenous ζ and ζ-GFP were the same. Confocal microscopy analysis, shown in Figure 1D, revealed the presence of ζ-GFP at the plasma membrane and in subcellular compartments mainly localized around the centrosome and labeled either with transferrin or with a marker of the *cis*-Golgi. ζ-GFP was thus present in recycling endosomes and the Golgi apparatus. This localization was similar to the one observed for the endogenous ζ (Figure 1Df and data not shown).

### ZAP-70 Does Not Control the Clustering of ζ-GFP but Partially Controls Polarization of a ζ-GFP$^+$ Intracellular Compartment at the T/APC Interaction Site

One of the first phenomena described after TCR activation is the clustering of the TCR/CD3/ζ complex. Several studies have shown that this clustering is dependent on signaling events; however, the role of ZAP-70 has never been directly addressed. We studied the behavior of ζ after interaction with an APC by time lapse videomicroscopy in our ζ-GFP-transfected Jurkat cells. The first movie (Figure 2A and Supplemental Movie S1 at http://www.immunity.org/cgi/content/full/17/4/389/DC1) shows a ζ-GFP-expressing wild-type cell interacting with one SEE-pulsed B cell. At time zero, the chimera localized both at the plasma membrane and in intracellular compartments, defined as recycling endosomes and Golgi apparatus (see Figure 1D). As soon as the first contact occurred, the T cell underwent morphological changes, such as membrane ruffles and reorientation of the ζ-GFP$^+$ compartment toward the APC. Concomitantly, ζ-GFP accumulated at the interaction site while the intracellular compartment (indicated by the white arrowhead) positioned in very close vicinity to the plasma membrane. Similar results were observed with P116Zwt (see Supplemental Movie S3 at http://www.immunity.org/cgi/content/full/17/4/389/DC1). Concentration of ζ at the immune synapse lasted for the whole length of the movie (33 min in real time). In contrast, in the ζ-GFP-expressing P116 cell presented in Figure 2B and in Supplemental Movie S2 (at http://www.immunity.org/cgi/content/full/17/4/389/DC1), intracellular ζ did not polarize toward the interacting B cell, yet ζ-GFP still accumulated at the contact with the APC, forming a cluster, which lasted at least for the time of the observation (33 min). Like in P116, in the majority of P116Zdk, mobilization of the ζ-GFP$^+$ intracellular compartment was not observed, whereas ζ-GFP accumulated at the T/APC interface (see Supplemental Movie S4 at http://www.immunity.org/cgi/content/full/17/4/389/DC1).

These data demonstrate that ZAP-70 is involved in targeting intracellular ζ toward the APC but is not required for clustering of the plasma membrane pool of ζ.

### ZAP-70 Activity Controls the TCR-Dependent Polarization of the T Cell MTOC toward the APC

We reasoned that the absence of reorientation of the ζ-GFP$^+$ intracellular compartment witnessed an absence of MTOC polarization. Indeed, T cells rapidly undergo a reorientation of the MTOC toward the bound APC (Geiger et al., 1982; Ryser et al., 1982), allowing the T cell Golgi apparatus to position into close proximity with the APC (Kupfer and Dennert, 1984; Kupfer et al., 1991). We scored the MTOC polarization in the four different Jurkat-derived cell lines. To do so, B cells were labeled with a red cell tracker and then either left untreated or pulsed with SEE, and Jurkat:B cell conjugates were allowed to form for 15 min at 37°C at a 1:1 ratio. Cells were then fixed and processed for immunofluorescence with anti-centrin Abs. This staining is restricted to the centriole area and marks both the exact position of the T cell centrosome/MTOC with respect to the APC and the location of the ζ-GFP intracellular compartment (see Supplemental Figure S1 at http://www.immunity.org/cgi/content/full/17/4/389/DC1). Importantly, the percentage of conjugated T cells (calculated as the number of T cells in contact with at least one B cell divided by the total number of T cells) did not depend on the presence of ZAP-70 (data not shown). As depicted in the example (Figure 3A), a T cell within a conjugate was scored positive only if its centrosome/MTOC (indicated by the white arrowhead) located within an area directly underlying the B cell membrane. Two to four independent experiments, summarized in Figure 3B, showed that MTOC reorientation occurred in ~30% to 40% of T cells in the absence of SEE; the percentage was not significantly different in the various T cell lines. This MTOC reorientation in the absence of SEE was also visualized by following the ζ-GFP intracellular compartment by time lapse videomicroscopy (see Supplemental Movies S5–S7 at http://www.immunity.org/cgi/content/full/17/4/389/DC1). In the presence of SEE, MTOC reorientation was increased to ~75% in P116Zwt as opposed to ~48% in the P116 ZAP-70-deficient cells. Expression of a dead kinase mutant further impaired the polarization capacity (~43%).

These results indicate that ZAP-70 kinase activity does not control the basal level of MTOC reorientation but does control the TCR-induced polarization of T cells.

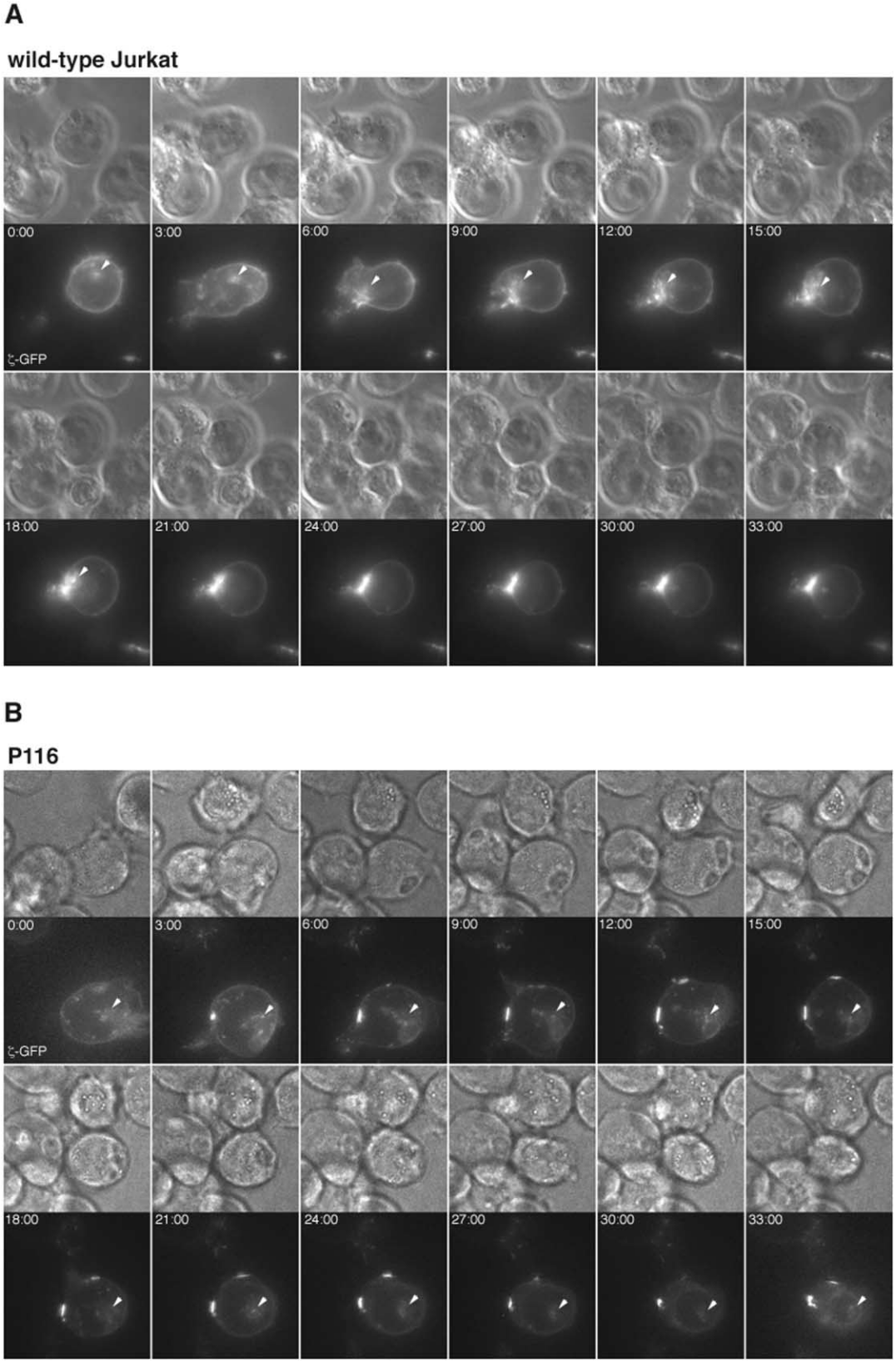


Figure 2. ZAP-70 Is Not Required for the ζ-GFP Clustering at the T/APC Interface but Controls Polarization of Intracellular ζ-GFP toward the Interface

Wild-type Jurkat cells (A) and P116 cells (B) transfected with ζ-GFP were put on a polylysine-coated coverslip and placed at 37°C onto the chamber of a videomicroscope. At time 0, Hom2 pulsed with 0.1 μg/ml SEE were added. Every 30 s, one DIC image and a stack of 8 xy-planes (step 1 μm) were acquired. Presented here are sequences of images obtained every 3 min. Each single picture consists of a DIC image at the top and a projection of the GFP images at the bottom. The white arrowhead points to the MTOC (total length: 33 min).

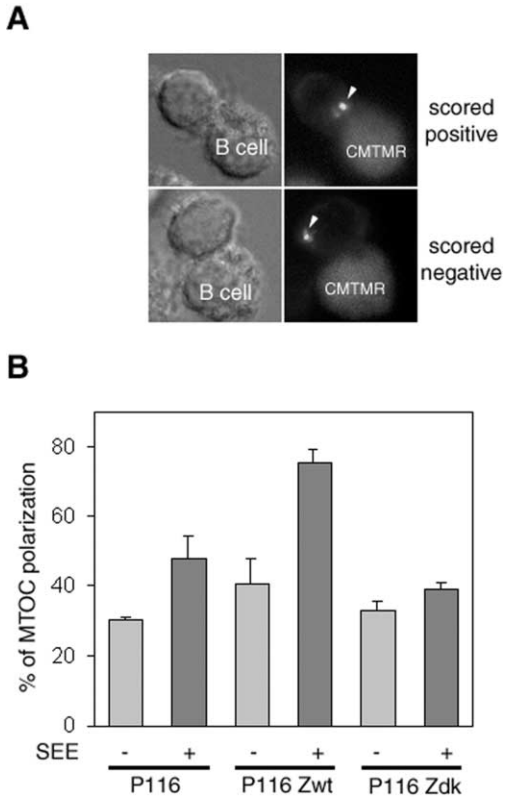


Figure 3. ZAP-70 Controls the T Cell MTOC Polarization toward the SEE-Pulsed APC

(A) Hom2 B cells labeled with 0.3 μM of the orange cell tracker (CMTMR) and pulsed or not with 0.1 μg/ml SEE were let to adhere on a polylysine-coated coverslip. Jurkat-derived cells were added on the B cells and left for 15 min at 37°C. Conjugates were fixed and stained with rabbit anti-centrin Abs followed by Alexa488-coupled secondary Abs to visualize the position of the centrosome/MTOC with respect to the APC. B cells were identified on the basis of the CMTMR labeling and T cells in contact with B cells were counted. They were scored positive (upper panel) only if the centrin labeling (pointed here by the white arrowhead) was located in very close proximity to the B cell membrane.

(B) Results are the mean ± SD of four (with SEE) and two (without SEE) independent experiments; altogether at least 500 (with SEE) and 200 (without SEE) conjugates were blindly scored for each cell type.

### ZAP-70 Does Not Control the Distribution of ζ, CD2, CD45, and CD43 at the T/APC Interaction Site

When encountering an APC, the T cell's surface molecules undergo a massive reorganization; these changes concern not only the TCR/CD3/ζ complexes but also other receptors. We thus studied in more detail the patterning of ζ, CD3, CD2, CD45, and CD43 in conjugates formed between the Jurkat-derived clones described above and the B cell lines (Raji or Hom2, as indicated in the legend), either nontreated or pulsed with SEE. APCs were put on polylysine-coated lamellas, and T cells were added and left for 45 min at 37°C, a time considered to be long enough for the establishment of mature synapses (Grakoui et al., 1999; Lee et al., 2002). After fixation and labeling with Abs specific for CD2, CD45, and CD43, samples were analyzed by confocal microscopy. Serial optical sections along the Z axis were recorded for each of the fluorescent labels, allowing a three-dimensional (3D) reconstruction of the conjugates and the projection of the entire contact region on a YZ plane. The immunofluorescent images (Figures 4A–4C) show an accumulation of ζ-GFP at the T/APC interface both in P116Zwt and P116Zdk whereas no ζ-GFP clustering was observed in the absence of SEE (Figures 4D–4F), although T:B cell conjugates still established. As shown in Supplemental Figure S2 at http://www.immunity.org/cgi/content/full/17/4/389/DC1, CD3ε and TCR β were partially codistributed with the intracellular pool of ζ-GFP, which was polarized toward the APC. However, as reported by several others (for review see Alcover and Alarcon, 2000), TCRβ and CD3ε were detected predominantly in the endoplasmic reticulum wherein the endogenous ζ and ζ-GFP were hardly observed. CD3ε and TCR β were also observed at the plasma membrane, clustered together with ζ-GFP in the central zone of contact with the APC (see Supplemental Figure S2 at http://www.immunity.org/cgi/content/full/17/4/389/DC1). CD2 clustering was observed independently of ZAP-70 activity and of the presence of SEE; however, CD2 accumulation was less intense in the absence of SEE (Figure 4D). In conjugates formed with T cells and SEE-pulsed B cells, a coclustering of ζ-GFP with CD2 in the central zone of contact was observed (Figure 4A). The en face view of the contact area for both cell types showed that this coclustering occurred in overlapping zones. Labeling of CD45 revealed exclusion of this protein from the T/APC contact zone in conjugates formed both in the presence (Figure 4C) and absence of SEE (Figure 4F), independently of the ZAP-70 activity. CD43 was also excluded from the central zone in conjugates formed between P116Zwt or P116Zdk and SEE-pulsed B cells (Figure 4B). In the absence of SEE, the majority of cells displayed no or partial CD43 exclusion as depicted in Figure 4E. Clustering of ζ-GFP and CD2 in and exclusion of CD45 and CD43 from the central zone of contact between SEE-pulsed B cells and the ZAP-70-deficient P116 cells were also observed (data not shown), confirming the results observed in P116Zdk.

Our data thus strongly suggest that ZAP-70 does not play a role in segregation of surface molecules at the T/APC interface even for the events that are dependent on TCR activation by SEE.

As shown earlier, P116Zdk properly polarized toward the SEE-pulsed APC in 43% of the cases. We thus asked whether the correct segregation of surface proteins was dependent on MTOC reorientation. As displayed in Figures 4A–4C, segregation of ζ-GFP, CD2, CD43, and CD45 still occurred in P116Zdk, which did not polarize their MTOC correctly (see the arrow pointing to the centrosome). Therefore, our data show that spatially organized distribution of surface molecules at the immune synapse requires neither ZAP-70 activity nor MTOC reorientation.

### ZAP-70 Is Required for Translocation of PKC-θ and LAT at the Contact Zone between T Cell and APC

PKC-θ, a serine/threonine kinase known to play a role in T cell activation (Sun et al., 2000), translocates to the contact site between Ag-specific T cells and APCs,

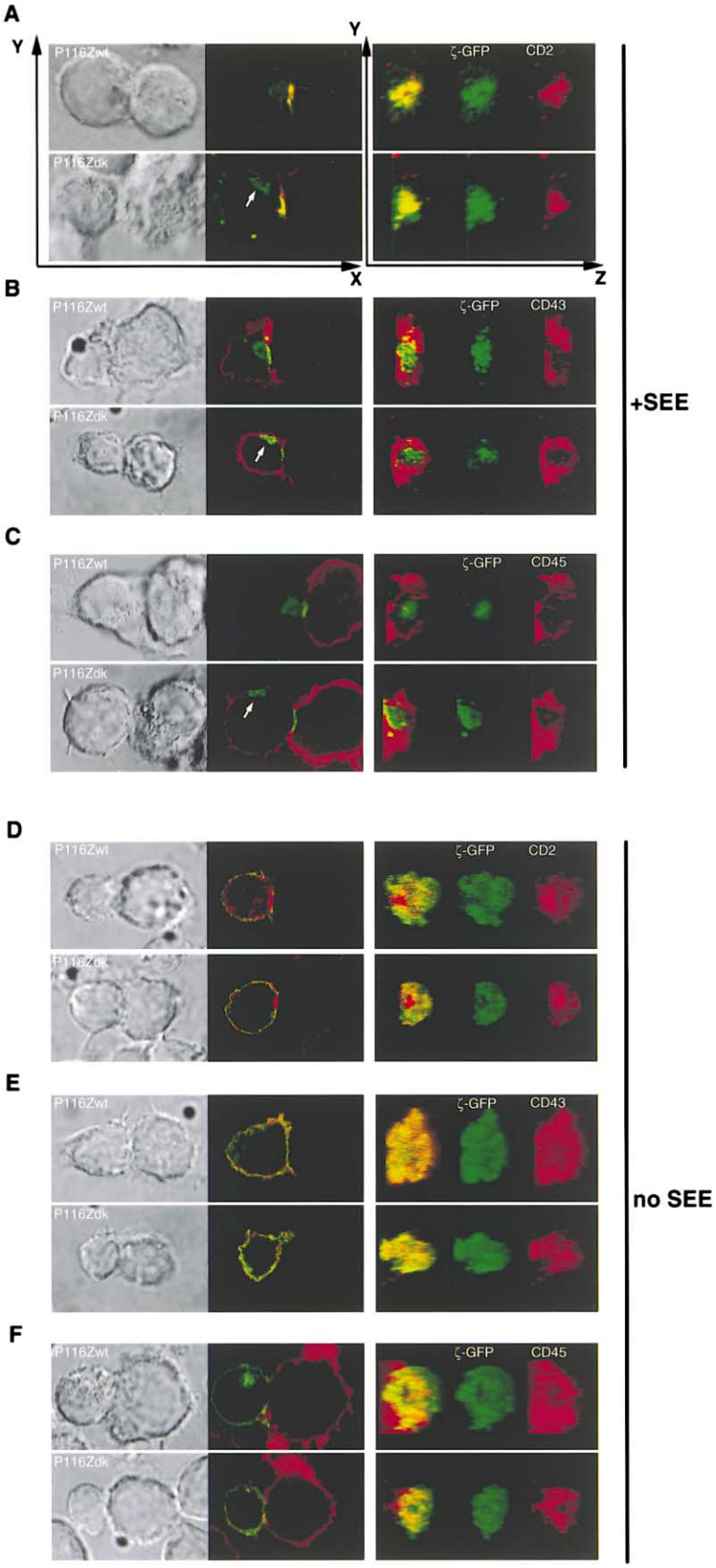


Figure 4. ZAP-70 Is Necessary Neither for the Coclustering of ζ-GFP and CD2 at the T/SEE-Pulsed APC Interaction Site nor for the Exclusion of CD43 and CD45

Conjugates between ζ-GFP-expressing P116Zwt or P116Zdk and B cells were allowed to establish for 45 min, fixed, and processed for immunofluorescence labeling with murine anti-CD2 mAbs ([A], with SEE; [D], without SEE), anti-CD43 mAbs ([B], with SEE; [E], without SEE) (in this case, B cells are Raji cells), or anti-CD45 mAbs ([C], with SEE; [F], without SEE) followed by Cyanine 3-coupled anti-mouse Abs. In each panel, the upper row shows the results obtained for P116Zwt cells whereas the lower one shows the images for P116Zdk. Each row consists of one DIC image (with the T cell always on the left), one representative fluorescent image taken in a medial XY plane, and the projections of the entire cell contact (along the *x* axis), allowing the en face view of the interface. In P116Zdk/B/SEE couples, white arrows point to the intracellular compartment containing ζ-GFP.

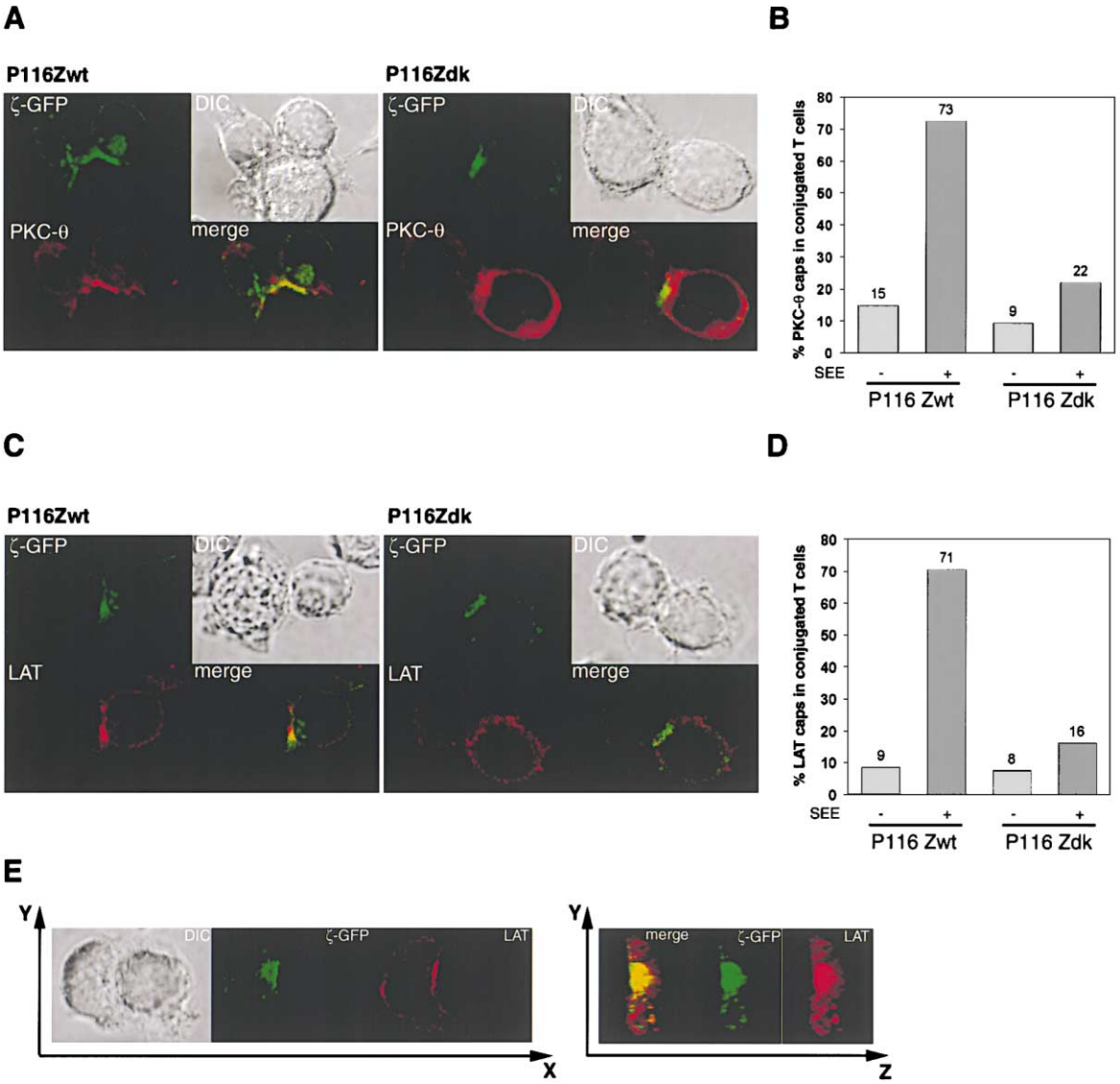


Figure 5. ZAP-70 Is Strictly Required for the TCR-Mediated Translocation of PKC-θ and LAT at the T/APC Interface

P116Zwt or P116Zdk cells expressing ζ-GFP were incubated in the presence of B cells (Hom2) pulsed with SEE or untreated for 45 min at 37°C on a polylysine coverslip. Conjugates were then fixed and labeled with a murine anti-PKC-θ mAb (A) or rabbit anti-LAT Abs (C) followed by Cyanine 3-coupled secondary Abs. In (A) and (C), results for P116Zwt cells are presented on the left whereas results for P116Zdk cells are shown on the right of each panel. The percentage of T cells conjugated to a B cell and exhibiting PKC-θ (B) or LAT (D) recruitment was then quantified in the presence or absence of SEE. Conjugates were selected using the DIC image and scored for the recruitment of PKC-θ ($n > 60$ without SEE, $n > 200$ with SEE) or LAT ($n > 40$ without SEE, $n > 100$ with SEE). (E) LAT labeling of a ζ-GFP-expressing wild-type Jurkat cell in conjugate is shown. Projections of the entire cell contact along the *x* axis are presented in the right panel.

where it localizes together with the TCR (Monks et al., 1997, 1998). More recently in a model similar to ours, Montoya et al. have shown that LAT, an adaptor molecule also playing a crucial role in T cell activation (Zhang et al., 1998a), concentrates at the T/APC contact area (Montoya et al., 2002). We investigated whether ZAP-70 controls PKC-θ and LAT translocation to the immune synapse. Conjugates between P116-derived clones expressing ζ-GFP and Hom2 cells loaded with or without SEE were formed for 45 min, fixed, and labeled with Abs specific for PKC-θ and LAT. As expected, stimulation of P116Zwt cells (Figure 5A), as well as wild-type Jurkat cells (data not shown), by B cells/SEE led to the redistribution of PKC-θ from the cytosol to the central core enriched in ζ-GFP. In sharp contrast to P116Zwt, we rarely detected such a translocation in P116Zdk cells. We further investigated this defect by scoring the percentage of T cells that exhibited a translocation of PKC-θ (Figure 5B). This quantification showed that PKC-θ translocation was induced by triggering of the TCR since translocation in the absence of SEE was observed only in 15% of the P116Zwt/BSEE conjugates whereas 73% of the conjugates showed a translocation when B cells were pulsed with SEE. In P116Zdk PKC-θ translocation was observed in 9% of the conjugates in the absence of SEE and only 22% in the presence of SEE. Similarly,

as shown in Figures 5C and 5D, LAT was recruited at the synapse in a TCR-dependent manner since only 9% of the conjugates formed in the absence of SEE displayed a redistribution of LAT. In the presence of SEE, the percentage of conjugates showing a redistribution of LAT at the synapse was 71% for P116Zwt but only 16% for P116Zdk. The same defect in PKC-θ and LAT recruitment was seen in P116-derived clones (data not shown).

Of note, in P116Zwt, 3D reconstructions and YZ-projected views of the contact region showed a clustering of ζ and LAT within overlapping areas (Figure 5E). Altogether, these results demonstrate that ZAP-70 is required for the TCR-induced translocation of signaling molecules, such as PKC-θ and LAT, at the immune synapse but does not seem to drive the patterning of transmembrane surface proteins.

## Discussion

The essential role of ZAP-70 with respect to the biochemical and transcriptional changes that accompany TCR-mediated T cell activation has been well documented (Hivroz and Fischer, 1994). Dramatic functional changes in terms of cytoskeletal remodeling and organization of the surface and signaling molecules have also been shown when a T cell recognizes its specific ligand at the surface of an APC. The aim of this study was to determine the contribution of ZAP-70 to these structural changes. We developed a model which allowed us to dynamically follow the formation of the immune synapse. We established stable clones expressing a GFP-tagged ζ chimera by transfecting the ZAP-70/Syk-deficient Jurkat cell:P116 (Williams et al., 1998). We studied the immune synapse formation between these clones and, as APC, a MHC class II positive lymphoma B cell untreated or pulsed with the SEE superantigen which binds to the Vβ8 expressed by Jurkat cells.

Our data show that ZAP-70 controls the TCR-induced polarization of T cell's MTOC toward Ag-pulsed APCs and thus also the TCR-induced polarization of an intracellular pool of ζ. Moreover, ZAP-70 is required for recruitment at the synapse of two signaling proteins, PKC-θ and LAT. In contrast, ZAP-70 is required neither for CD2 and TCR/CD3/ζ clustering in the central zone of the synapse nor for exclusion of CD43 from this zone. Although we cannot formally generalize to normal T lymphocytes the data we obtained in Jurkat cells wherein some signaling defects have been reported (Shan et al., 2000), it is worth noting that the immune synapse we describe herein is not different from the ones described in T cell clones (Monks et al., 1998). Moreover, the Jurkat synapse was comparable to the one we observed between T cell blasts derived from control donors and the B lymphoma used in this study (data not shown).

The actin cytoskeleton plays a critical role in immune synapse formation. Actin polymerization is required for the formation of T cell/APC contacts (Delon et al., 1998) (Krummel et al., 2000) and movement of surface proteins in and out of the interaction zone (Wulfing and Davis, 1998; Allenspach et al., 2001; Delon et al., 2001; Roumier et al., 2001). Several potential substrates of ZAP-70 have been implicated in remodeling the actin network. The Rho family GDP/GTP exchange factor Vav1 (Fischer et al., 1998; Holsinger et al., 1998), the Rho family GTPase CDC42 (Stowers et al., 1995), and the CDC42-associated Wiscott Aldrich Syndrome protein (WASP) (Snapper et al., 1998) link the TCR to cytoskeletal reorganization, receptor clustering, and cap formation. ZAP-70 has been strongly suggested to play a role both in Vav activation (Michel et al., 1998) and CDC42 recruitment at the immune synapse (Cannon et al., 2001). Recently, LAT, another substrate of ZAP-70 (Zhang et al., 1998a) has been shown to drive actin cytoskeleton remodeling and cell spreading in T cells (Bunnell et al., 2001). Because these different studies suggested that ZAP-70 plays a critical role in remodeling the actin cytoskeleton, it was thus surprising to observe ζ-GFP clustering and normal exclusion of CD43 from the contact zone in ZAP-70-deficient cells interacting with Ag-pulsed APCs. Strikingly, although the number of T/B/SEE conjugates observed with ZAP-70-deficient Jurkat cells was normal (data not shown and Morgan et al., 2001), the morphology of the conjugated T cells was different. In the case of T cells expressing functional ZAP-70, we often observed projections of the T cell membrane partially engulfing the APC. These images were very few in conjugates containing T cells expressing no ZAP-70 or a dead kinase mutant of ZAP-70 (see Figure 4 and data not shown) suggesting that actin remodeling was not perfectly normal in the absence of functional ZAP-70. This interpretation was corroborated by data showing that in P116ZAPdk, polymerization of actin-GFP induced by interaction with SEE-pulsed B cells was not strictly comparable to the one observed in wild-type Jurkat (data not shown and Morgan et al., 2001).

Our results strongly suggest that ZAP-70, although playing a role in remodeling of the cortical actin, is not strictly required for patterning of T cell surface molecules in the synapse. The initial driving force leading to clustering of the plasma membrane pool of ζ and to exclusion of CD43 from the contact zone may be given by ZAP-70-independent signals. The protein tyrosine kinase $p56^{Lck}$ could be a key player in the initial steps since it regulates TCR clustering, T cell morphological changes, and conjugate formation (Donnadieu et al., 2001; Lowin-Kropf et al., 1998; Morgan et al., 2001). Once initiated, the segregation of proteins may occur spontaneously as proposed by mathematical modeling which takes into account membrane fluidity, protein size, and receptor/ligand affinity (Qi et al., 2001). Of note, our study demonstrates that MTOC reorientation is not required for patterning of the surface molecules at the immune synapse since, as shown in Figure 4, a clustering of ζ and CD2 in the central zone of contact and an exclusion of CD43 were observed in mispolarized P116ZAPdk cells.

It has been shown that antagonists are not able to induce TCR clustering and immune synapse formation and that partial agonists are less efficient than agonists in inducing TCR clustering (Grakoui et al., 1999). Antagonists and partial agonists also induce abortive signaling characterized by an absence of ZAP-70 recruitment and activation (Madrenas et al., 1995). It was tempting to link the absence of synapse formation to the absence of ZAP-70 activation. Our results strongly suggest that this absence of synapse formation is not solely due to the defective ZAP-70 activation.

When encountering an APC, T cells reorientate their MTOC toward the bound APCs (Geiger et al., 1982; Ryser et al., 1982). This MTOC reorientation is essential to position the T cell secretory apparatus into close proximity with the APC, allowing the polarized delivery and concentration of cytokines and cytotoxic mediators at the synapse (Kupfer and Dennert, 1984; Kupfer et al., 1991). Lowin-Kropf and coworkers have shown that anti-CD3 Abs immobilized on beads induce a MTOC polarization which is dependent on $p56^{Lck}$ and ZAP-70 and requires at least one intact ITAM (Lowin-Kropf et al., 1998). We confirmed, in a model using an APC, that ZAP-70 regulates T cell MTOC reorientation. It is worth noting that correct polarization of MTOC is still seen in 50% of the conjugates formed with ZAP-70-deficient P116 cells and is observed in 30%–40% of conjugates formed in the absence of SEE. Despite the random-based proper centrosome positioning with respect to the APC, which we evaluate at 20%–25%, these results still suggest that MTOC polarization is not entirely driven by ZAP-70 activity. Which could be the potential substrates of ZAP-70 that can play a role in MTOC reorientation? As discussed earlier, ZAP-70 may regulate CDC42 activation, which has been shown to control MTOC polarization (Stowers et al., 1995). ZAP-70 also regulates CD2 signaling (Meinl et al., 2000), and it has been reported that CD2 and CD2AP, an adaptor protein associated with CD2, are required for MTOC reorientation (Dustin et al., 1998). Finally, ZAP-70 is necessary for the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) induced by TCR activation (Arpaia et al., 1994; Meinl et al., 2000; Williams et al., 1998), and $[Ca^{2+}]_i$ has been shown to control the reorientation of the cytotoxic T lymphocyte MTOC toward its target (Kupfer and Dennert, 1984).

Finally, we demonstrate that ZAP-70 is required for the translocation at the inner face of the T/APC interface of two crucial signaling proteins, i.e., PKC-θ and LAT which are known to be recruited there (Monks et al., 1997; Montoya et al., 2002). Results concerning PKC-θ are somehow contradictory with the results from Bi et al., who showed that PKC-θ is recruited in the rafts in a ZAP-70-independent manner (Bi et al., 2001); however, a reconcilable hypothesis would be that in ZAP-70-deficient T cells, rafts are not recruited to the immune synapse. This would also explain why LAT, which is found in rafts (Zhang et al., 1998b), is not recruited to the immune synapse in ZAP-70-deficient cells.

The absence of PKC-θ and LAT recruitment at the immune synapse observed in the absence of ZAP-70 activity may somehow be linked to the MTOC mispolarization. Microtubules have been shown to be critical for the spatial organization of signal transduction; indeed many signaling proteins bind to microtubules. Thus, they may represent a scaffold facilitating the interaction of signaling proteins; moreover, association of signaling proteins to microtubule-anchored motors may facilitate their transport (for review see Gundersen and Cook, 1999). Future experiments will assess whether playing with microtubule stability and/or polymerization can modify recruitment of TCR/CD3/ζ and signaling proteins to the immune synapse.

The studies reported here reveal that signaling through ZAP-70 at the immune synapse regulates microtubule polarization and recruitment of transduction proteins but does not control the patterning of surface molecules at the interface between the T cell and the APC. The model we set up for this study should prove useful to characterize the different ZAP-70 substrates implicated in the formation of a functional synapse.

## Experimental Procedures

### Cell Culture, Transfection, and Cloning

All cell lines were grown in Glutamax-containing RPMI (Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS, European Grade, Biological Industries). Hom-2 and Raji (two B lymphoma cell lines) were given by P. Benaroch and F. Mazerolles (Institut Curie and Necker Hospital, Paris, respectively). Wild-type Jurkat cells (Cl20) were kindly provided by A. Alcover (Institut Pasteur, Paris). The Jurkat-derived ZAP-70-deficient (P116) cell line was obtained from R. Abraham (Duke University, Durham, NC). P116 transfected back by wt ZAP cDNA (P116 Zwt) or a cDNA encoding for a ZAP mutated in its kinase domain (P116 Zdk) were established as described (Mege et al., 1996). The selection agent for P116 Zwt and P116 Zdk was puromycin (4 μg/ml). ζ cDNA followed by a short GGG linker was subcloned into the pEGFP-N1 (Clontech) between the BglII and AgeI restriction sites within the multiple cloning site, and cells were transformed by electroporation. Stable clones (for wt Jurkat and P116) or stable bulks (for P116 Zwt and P116 Zdk) expressing the ζ-GFP fusion protein were obtained by continuous growing in the presence of 1 mg/ml G418 (Life Technology).

### Antibodies (Abs) and Reagents

The following Abs were used in this study: UCHT1 (murine IgG1), an anti-CD3ε monoclonal Ab (mAb) (obtained from an hybridoma provided by P. Beverley, University College, London); an anti-ζ mAb (murine IgG1, Santa Cruz); an anti-TCRβ mAb (IgG1, Bioadvance); an anti-CD2 mAb (murine IgG2b, Santa Cruz); an anti-CD45RA mAb (murine IgG1, Immunotech); an anti-PKC-θ mAb (murine IgG2a, Transduction Laboratory); a mixture of two anti-GFP mAbs (murine IgG1, Boehringer Mannheim); 4G10 an anti-phosphotyrosine mAb (murine IgG2b, UBI); a mixture of anti-PLCγ1 mAbs (murine IgG, Upstate Biotechnologies); an anti-CD43 murine mAb (kindly given by F. Sánchez-Madrid, Universidad Autonoma de Madrid, Madrid, Spain); CTR433, an anti-median Golgi murine mAb, and a polyclonal rabbit anti-centrin3 Ab (both kindly provided by M. Bornens, Institut Curie, France); a polyclonal rabbit anti-ZAP-70 Ab (Santa Cruz); a polyclonal rabbit anti-LAT Ab (Upstate Biotechnology.); Cy3-conjugated $F(ab')_2$ goat anti-mouse IgG (H+L) or donkey anti-rabbit (Jackson Immunoresearch); Alexa488-conjugated $F(ab')_2$ anti-rabbit Abs (Molecular Probes); HRP-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Pierce).

### Immunoprecipitation and Western Blot Analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 140 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 0.5% NaDOC, 0.1% SDS, 100 μM $Na_3VO_4$, 1% aprotinin, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 μg/ml of antipain, pepstatin, and leupeptin) for 20 min on ice. All these reagents were purchased from Sigma. The postnuclear supernatant was then obtained by centrifugation of the nuclei and cell debris. For immunoprecipitation, $10^7$ cells were lysed in 500 μl of lysis buffer and activated or not for 5 min with an UCHT1 ascitic fluid (dilution 1/500). Four micrograms/tube of anti-LAT Abs, 2 μg/tube of anti-GFP Abs, or 2 μg/tube of anti-PLCγ1 Abs were then used for the precipitation. Samples were analyzed under reducing conditions by SDS-PAGE and electroblotted on Immobilon P membrane (Millipore). The antibody/antigen complexes were visualized by an enhanced chemiluminescence detection kit according to the manufacturer's instructions (ECL, Amersham-Pharmacia).

### Transferrin Loading, Conjugate Formation, and Immunolabeling

To visualize recycling endosomes, T cells were first starved in RPMI + 0.5% BSA for 1 hr at 37°C, then pulsed for 30 min with Cy3-coupled holo-transferrin (Sigma, labeled using the Amersham Cy3

labeling kit), washed, and chased for 15 min in RPMI + 10% FCS. When indicated and in order to distinguish between T and APCs, Hom2 were labeled with a cytosolic fluorescent red dye according to the manufacturer's instructions (0.3 μM Cell Tracker Orange, CMTMR, Molecular Probes). B cells were then left untreated or pulsed for 1 hr at 37°C with 0.1 μg/ml Staphylococcus Enterotoxin E (SEE, Toxin Technologies), washed, resuspended in RPMI alone, and allowed to adhere onto a poly-L-lysine-coated coverslip for 15 min at room temperature. After medium removal, T cells washed in RPMI were added on the B cells at a 1:1 ratio, and conjugates were allowed to establish at 37°C for 15 or 45 min. Cells were fixed with 3% paraformaldehyde (Carlo Erba) for 20 min and incubated for 10 min in 10 mM PBS glycine to quench free aldehyde groups, except in Supplemental Figure S7 (at http://www.immunity.org/cgi/content/full/17/4/389/DC1) wherein cells were fixed in 100% MetOH for 5 min at −20°C. Cells were then permeabilized and labeled by incubation with the first Ab diluted in PBS, BSA 0.2% (Sigma), saponin 0.05% (ICN Biomedicals Inc.) for 1 hr and with the second fluorescent Ab diluted in the same buffer for 20 min. Coverslips were finally mounted onto glass slides using a Mowiol (Calbiochem) solution.

### Polarization Scoring and 3D Reconstruction

Quantification of the MTOC polarization in the different Jurkat cell types was performed as follows: conjugates formed between red-labeled B cells and T cells were immunostained with an anti-centrin Ab followed by an Alexa488-coupled secondary Ab to visualize the exact position of the centrosome. Images were collected on a Leica DM IRBE epifluorescence microscope controlled by Metamorph software (Universal Imaging) using a 63 × 1.32 NA objective, a cooled CCD camera (MicroMax, Princeton Instruments Inc.), and a halogen lamp. For each field, one DIC image, one image with the red filter set, and a stack of four xy-planes (step 1.5 μm) with the green filter set (to cover most of the cell volume) were acquired. Among all T cells, only cells in contact with one B cell were taken into account. Polarization was then scored positive only if the T MTOC was located in close proximity to the B cell membrane.

Other fluorescence images were acquired using a Leica TCS SP2 confocal scanning microscope equipped with a 100 × 1.4 NA HCX PL APO oil immersion objective, and Ar and HeNe lasers emitting at wavelengths of 488, 543, and 633 nm. Before acquiring a double-staining z series, the intensity of excitation wavelengths and the power of photodetectors were adjusted to avoid crosstalk. Voxel size (x, y, z) of each recorded stack was 0.07 × 0.07 × 0.37 μm (zoom 4). To obtain the en face view of the contact zone, stacks were rotated (using Metamorph software), bringing the T cell on the left of the field, and cropped narrowly around the synapse. The maximum-intensity method was applied to this region encompassing the interface to obtain the projections on the *yz* plane.

### Videomicroscopy

For videomicroscopy experiments, we used the Leica DM IRBE microscope described above with the 100 × 1.4 NA oil immersion objective. Coverslips covered with T cells were placed into a chamber on the microscope at 37°C in a 5% $CO_2$ atmosphere. At time 0, SEE-loaded or untreated B cells were added, and every 30 s one DIC image and a stack of eight sections along the *z* axis (step 1 μm) were collected with the green filter set. Movies consisting of the DIC image and the max-intensity projection of ζ-GFP planes were accelerated 90×.

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