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Itk Controls the Spatiotemporal Organization of T Cell Activation

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Abstract

During T cell activation by antigen-presenting cells (APCs), the diverse spatiotemporal organization of components of T cell signaling pathways modulates the efficiency of activation. Here, we found that loss of the tyrosine kinase interleukin-2 (IL-2)–inducible T cell kinase (Itk) in mice altered the spatiotemporal distributions of 14 of 16 sensors of T cell signaling molecules in the region of the interface between the T cell and the APC, which reduced the segregation of signaling intermediates into distinct spatiotemporal patterns. Activation of the Rho family guanosine triphosphatase Cdc42 at the center of the cell-cell interface was impaired, although the total cellular amount of active Cdc42 remained intact. The defect in Cdc42 localization resulted in impaired actin accumulation at the T cell–APC interface in Itk-deficient T cells. Reconstitution of cells with active Cdc42 that was specifically directed to the center of the interface restored actin accumulation in Itk-deficient T cells. Itk also controlled the central localization of the guanine nucleotide exchange factor SLAT [Switch-associated protein 70 (SWAP-70)–like adaptor of T cells], which may contribute to the activation of Cdc42 at the center of the interface. Together, these data illustrate how control of the spatiotemporal organization of T cell signaling controls critical aspects of T cell function.

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SUPPLEMENTARY MATERIALS

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INTRODUCTION

T cells can be activated through interactions with antigen-presenting cells (APCs). T cell activation is accompanied by the diverse spatiotemporal distributions of T cell signaling intermediates across the entire signaling system (1–5). Distinct spatiotemporal distributions, focused at the site of receptor engagement and extending throughout the entire cell, are a widespread feature of signaling systems, as is also seen, for example, during neuronal and neutrophil activation (6, 7); however, the functional importance of systems-scale spatiotemporal distributions in signaling is essentially unresolved. By relating specific patterns of individual signaling intermediates to T cell function, various roles of spatiotemporal patterning for T cell function have been proposed, including the enhancement of T cell signaling, the termination of T cell signaling, and the control of regulated secretion of cytokines, as well as no substantial roles at all (2, 8–10). For a more comprehensive understanding of the function of spatiotemporal patterning in signaling, consideration of the systems context and causal experimentation is desirable: Spatiotemporal distributions of proteins affect interaction probabilities during signaling and, thus, the efficiency of signaling steps. Two proteins enriched at the same time in the same location are more likely to interact with one another. The insight gained from spatiotemporal distributions for the likelihood of signal progression increases with the number of signaling interactions addressed, that is, by investigation of the spatiotemporal patterning of as many signaling intermediates as feasible in parallel (that is, at the systems level). As causal evidence for the roles of spatiotemporal patterning in the regulation of signaling, the placement of signaling intermediates in distinct subcellular locations should yield functional consequences. Here, we have identified interleukin-2 (IL-2)–inducible tyrosine kinase (Itk) (11) as a systems-scale regulator of the spatiotemporal distributions of signaling molecules during T cell activation. Manipulating the localization of signaling intermediates downstream of Itk may be linked to the established role of Itk in controlling actin dynamics in T cells.

Itk is a tyrosine kinase with an N-terminal recruitment module consisting of a pleckstrin homology (PH) domain, a Tec homology (TH) domain, and Src homology 2 (SH2) and SH3 domains (11). In Itk-deficient mice, development of conventional CD8⁺ T cells is almost completely suppressed (12, 13). Upon activation with antigen *in vitro* and *in vivo*, Itk-deficient CD4⁺ T cells display a defect in their ability to increase the intracellular calcium concentration ($[Ca^{2+}]_i$) and to secrete cytokines (14, 15). The T helper 2 (T_H2) subset of CD4⁺ T cells is the subset most severely affected by a deficiency in Itk, which leads to defective responses to pathogens (16). Itk is linked to proximal T cell receptor (TCR) signaling by its binding to the adaptor protein SH2 domain–containing leukocyte phosphoprotein of 76 kD (SLP-76) (17); thus, Itk substantially contributes to the activation of phospholipase C- γ (PLC- γ) (14). The SH2 domain of Itk is required for Itk-dependent actin accumulation at the interface between the T cell and the APC (which is known as the immunological synapse), possibly by mediating recruitment of parts of the actin-nucleation machinery to the interface (18–20).

Linking the roles of Itk in T cell organization and signaling, Itk-dependent recruitment of the hematopoietic cortactin homolog HS1 regulates the formation of microclusters of PLC- γ 1 (21). A potentially important signaling intermediate in the regulation of T cell organization by Itk is the small Rho family guanosine triphosphatase (GTPase) Cdc42, which is a critical regulator of actin polymerization in T cells (22). In the activation of Itk-deficient transgenic T cells that express the AND TCR, the recruitment of active Cdc42 to the T cell–APC interface is diminished compared to that in wild-type cells (20). Despite all of the genetic and biochemical insights into Itk, many aspects of the mechanism by which it acts remain unresolved. Prominently, how Itk regulates actin dynamics in T cells is still unclear.

Here, we have established Itk as a general regulator of the spatiotemporal organization of signaling molecules in activated T cells. By manipulating the localization of active Cdc42 downstream of Itk, we suggest that the ability of Itk to activate Cdc42 in a defined location, the center of the T cell–APC interface, is required for the Itk-dependent accumulation of actin. Through spatiotemporal analysis of signaling molecules and knockdown experiments, we identified Switch-associated protein 70 (SWAP-70)–like adaptor of T cells (SLAT) as the likely guanine nucleotide exchange factor (GEF) to contribute to the activation of Cdc42 at the center of the T cell–APC interface. The SH2 domain of Itk was critical for its regulation of the spatiotemporal distributions of T cell signaling molecules. Thus, we suggest that critical aspects of T cell function can be controlled by the spatiotemporal organization of T cell signaling.

RESULTS

Itk accumulates at the center of the T cell–APC interface

To study Itk during T cell activation, we used primary in vitro–primed T cells from DO11.10 TCR transgenic mice that were either wild-type for Itk or Itk-deficient (23). The DO11.10 TCR recognizes the ovalbumin peptide 324–340 (Ova), which is presented by the major histocompatibility complex (MHC) molecule I-A^d (24). We activated DO11.10 T cells with A20 B cell lymphoma cells (which act as APCs) in the presence of Ova peptide (10 μ M), which is considered a strong stimulus. To determine the spatiotemporal distribution of Itk, we retrovirally transduced DO11.10 T cells to express a fusion protein of the Itk recruitment domains (that is, the PH, TH, SH3, and SH2 domains) with green fluorescent protein (GFP). Upon T cell activation, this Itk sensor was rapidly recruited to the center of the T cell–APC interface, such that, at the time of tight cell coupling, $80 \pm 6\%$ of cell couples showed accumulation of the sensor at the central interface (Fig. 1, A and B). The accumulation of Itk at the interface was well sustained over time. Itk is a member of the central supramolecular activation complex (cSMAC), a cluster of signaling intermediates found at the center of the T cell–APC interface (2, 3, 25), as was corroborated by our imaging of the Itk sensor during the activation of T cells that have a different TCR, 5C.C7 TCR; data on the spatiotemporal patterning of a larger number of signaling intermediates are available for comparison (fig. S1) (3).

Itk regulates multiple elements of the spatiotemporal organization of T cell signaling

To address potential roles for Itk in regulating the spatiotemporal patterning of signaling molecules in T cells, we imaged 16 sensors during the activation of wild-type and Itk-deficient DO11.10 T cells. These sensors included the TCR; TCR-proximal signaling intermediates, including the kinases Lck and Tec, the adaptor protein linker of activated T cells (LAT), SLP-76, PLC- γ , and protein kinase C- θ (PKC θ); regulators of cytoskeletal dynamics, including Cdc42, Rac, SLAT, Vav1, α -Pix, and PIP₂ (phosphatidylinositol 4,5 bisphosphate); and elements of the cytoskeletal machinery, including cofilin, myosin 1C, and actin. We found substantial differences in sensor patterning between wild-type and Itk-deficient T cells for 14 of the 16 sensors (Fig. 2, A and B). Only the patterns of actin and Vav1 did not show statistically significant differences in their distribution between wild-type and Itk-deficient DO11.10 T cells.

We identified three spatiotemporal patterns that occurred that were consistently reduced in extent in Itk-deficient T cells compared to those in wild-type T cells; the central pattern, the invagination pattern, and the lamellar pattern, as previously defined (3). For example, 1 min after tight cell couples formed between wild-type T cells and APCs, $47 \pm 7\%$ of the cell couples displayed the accumulation of active Rac in the central pattern. In Itk-deficient T cells, however, this percentage was significantly reduced to $8 \pm 5\%$ (fig. S2, G and H; $P <$

0.001). Similarly, 1 min after tight cell couples formed between wild-type T cells and APCs, $22 \pm 5\%$ of the cell couples displayed the accumulation of LAT in what we term an invagination pattern; however, no cell couples between Itk-deficient T cells and APCs displayed such a pattern (fig. S2, A and B; $P = 0.01$). Finally, 1 min after the formation of tight cell couples between wild-type cells and APCs, $53 \pm 8\%$ of the cell couples exhibited the accumulation of PIP₂ in a lamellar pattern, which was significantly more than was displayed by cell couples containing Itk-deficient T cells ($17 \pm 7\%$; $P < 0.005$) (fig. S2, K and L).

At the systems-scale depiction of the patterning data as a heat map (Fig. 2, A and B), we observed consistent reductions in the extent of the accumulation of signaling molecules in central, invagination, and lamellar patterns between wild-type T cells and Itk-deficient T cells. The segregation of signaling intermediates into the central or diffuse, peripheral, and lamellar groups, as determined by cluster analysis, became less distinct. The average dendrogram distance between these groups decreased from 0.62 ± 0.08 in wild-type cells to 0.39 in Itk-deficient cells, with complete loss of the lamellar group in the latter case. In addition, whereas Cdc42 and Lck adopted a central or diffuse pattern in wild-type cells, they accumulated in the peripheral group in Itk-deficient cells, and SLP-76 and cofilin lost their association with groups in Itk-deficient cells. Thus, Itk was a general regulator of the spatiotemporal organization of T cell signaling molecules and was required for the segregation of signaling intermediates into distinct spatiotemporally defined groups.

Itk is a critical regulator of the localization of active Cdc42

To begin to assess the functional consequences of the regulation of spatiotemporal patterning by Itk, we investigated the accumulation of actin at the T cell–APC interface. The accumulation of actin is reduced in Itk-deficient T cells compared to that in wild-type T cells; however, the molecular mechanisms used by Itk are uncertain (18–20). Cdc42 is a critical regulator of the accumulation of actin at the cell interface (18–20, 22). By imaging with a sensor for active Cdc42 (22), we found that active Cdc42 was effectively recruited to the T cell–APC interface in wild-type and Itk-deficient T cells (Fig. 2, C to F); however, the location of active Cdc42 differed between wild-type and Itk-deficient T cells. At each time point ≥ 40 s after the formation of tight cell couples with wild-type T cells, $>30\%$ of cell couples displayed the central accumulation of active Cdc42. In contrast, $<12\%$ of cell couples involving Itk-deficient T cells exhibited such a pattern ($P < 0.01$ at each time point), consistent with the general impairment in the central accumulation of molecules that we characterized earlier. The amount of the sensor of active Cdc42 that was recruited to the T cell–APC interface, which acted as a measure of the total Cdc42 activity there, did not differ between wild-type and Itk-deficient T cells (fig. S2U). The amounts of active Cdc42 in wild-type and Itk-deficient T cells were also comparable in Cdc42 pull-down experiments (fig. S2V). Thus, we suggest that Itk was critical for the central localization of active Cdc42, but not for the extent of Cdc42 activation in the cell as a whole. The central nature of Cdc42 activity and the extent of actin accumulation at the T cell–APC interface are closely related (22). We therefore investigated whether the ability of Itk to mediate Cdc42 activation at the center of the T cell–APC interface was critical for Itk-dependent actin accumulation at the cell interface.

The central localization of active Cdc42 is critical for Itk-dependent actin accumulation at the cell interface

To determine the importance of the central location of Cdc42 activation for Itk-dependent actin accumulation at the interface, we reconstituted Itk-deficient DO11.10 T cells with different amounts of spatially targeted active Cdc42. We used the V12 constitutively active mutant of Cdc42 (Cdc42ca), because we were unsure whether proteins that mediate Cdc42

activation would properly localize in *Itk*-deficient T cells. We used protein transduction with the HIV Tat-derived transduction peptide (tat) (26, 27) to enable dose-dependent delivery of Cdc42ca to cells (Fig. 3). To target Cdc42 to the center of the interface, we fused Cdc42ca to the Tec-PHTHSH3 module. The occurrence of the intended central targeting was experimentally confirmed with retrovirally expressed tat-Tec-PHTHSH3-GFP-Cdc42ca (Fig. 3, A and D). To distribute Cdc42 across the entire interface (to produce a diffuse pattern of expression), we fused Cdc42ca to the tandem SH2 domain of the kinase ζ chain-associated protein kinase of 70 kD (ZAP-70) (Fig. 3, B and E) (3). To address the role of Cdc42 activity throughout the cell, we used a Cdc42ca that lacked a targeting domain ("tat-Cdc42ca"). We found that the accumulation of retrovirally expressed tat-GFP-Cdc42 at the interface was modest, occurring in <50% of cell couples with accumulation in any pattern (Fig. 3, C and F).

In wild-type DO11.10 T cells, actin was rapidly recruited to the T cell-APC interface upon its formation, such that 20 s after tight cell couples were formed, the intensity of actin-GFP at the cell interface was 3.7 ± 0.1 times greater than the background fluorescence of the cell (Fig. 3G and fig. S3D). In *Itk*-deficient DO11.10 T cells, such enrichment was significantly ($P < 0.001$) less intense, resulting in 2.6 ± 0.1 times the background fluorescence of the cell (Fig. 3G and fig. S3E), confirming previous data (18–20). A range of concentrations of tat-Cdc42ca (100 nM to 1 μ M) did not significantly enhance actin accumulation at the interface in either wild-type or *Itk*-deficient T cells (Fig. 3I). Cellular amounts of active Cdc42 thus were not limiting, consistent with the undisturbed cell-wide generation of active Cdc42 in *Itk*-deficient T cells (fig. S2, U and V); however, upon treatment of *Itk*-deficient DO11.10 T cells with tat-Tec-PHTHSH3-Cdc42ca (100 nM), the extent of accumulation of actin at the interface was restored to that seen in wild-type DO11.10 T cells (Fig. 3G), reaching an actin-GFP intensity of 3.5 ± 0.2 times the background fluorescence of the cell 20 s after the formation of tight cell couples, which was indistinguishable from the amount of actin-GFP in wild-type DO11.10 T cells, but was significantly ($P < 0.001$) greater than the amount of actin-GFP in *Itk*-deficient T cells. Similar treatment of wild-type DO11.10 T cells did not enhance the accumulation of actin at the interface (Fig. 3G).

Treatment of *Itk*-deficient DO11.10 T cells with tat-Tec-PHTHSH3 (100 nM) (that is, the targeting domain on its own) (fig. S3A), or with tat-ZAP-70-2SH2-Cdc42ca (100 or 350 nM) (that is, a diffusely targeted Cdc42ca) (Fig. 3H), did not restore actin accumulation at the interface. Thus, active Cdc42 restored the accumulation of actin at the interface in *Itk*-deficient T cells only when it was selectively localized at the center of the T cell-APC interface. Therefore, the localization of active Cdc42, but not its cellular concentration, was critical.

Whereas actin amounts at the cellular interface were restored with centrally targeted Cdc42ca, actin patterning was altered. Treatment of cells with tat-Tec-PHTHSH3-Cdc42ca (100 nM) was accompanied by slightly more peripheral actin accumulation during the activation of wild-type and *Itk*-deficient DO11.10 T cells. In wild-type and *Itk*-deficient DO11.10 T cells treated with buffer alone, the initial actin burst (that is, the actin accumulation that occurred in the first 2 min after formation of tight cell couples) occurred with an even mix of diffuse and peripheral patterns (fig. S3, B to E); however, upon treatment with tat-Tec-PHTHSH3-Cdc42ca (100 nM), peripheral actin accumulation became dominant during this time (fig. S3, F and G). The percentage of cell couples that showed accumulation of actin with a peripheral pattern was significantly enhanced (20% increase) with a concomitant decrease in the extent of diffuse patterning of actin in wild-type and *Itk*-deficient DO11.10 T cells ($P \leq 0.05$, at time points 0 to 120 and 20 to 120, respectively). Treatment of T cells with the recruitment domain alone, that is, with tat-Tec-PHTHSH3 (100 nM), had no substantial effect (fig. S3, H and I). A possible mechanism for

increased peripheral actin accumulation is a reduction in the rate of actin turnover. That is, increased actin nucleation by additional active Cdc42 may not be balanced by increased disassembly of actin. Not surprisingly, these data are indicative of the high degree of complexity of the regulation of actin turnover. Consistent with altered actin dynamics, defective clustering of the TCR at the center of the T cell–APC interface in the activation of Itk-deficient DO11.10 T cells, as further discussed below, was also not restored with tat–Tec-PHTSH3–Cdc42ca (100 nM) (fig. S3J).

IL-2 secretion is diminished in Itk-deficient T cells. In the same T cell–APC interactions that were used for imaging, lack of Itk resulted in a reduction in IL-2 secretion to $10 \pm 0.02\%$ of that secreted by wild-type T cells (fig. S3K). Treatment of wild-type and Itk-deficient DO11.10 T cells with tat–Tec-THPSH3–Cdc42ca (100 nM) did not affect IL-2 secretion. In summary, although centrally targeted active Cdc42 effectively restored the amounts of actin at the interface in activated Itk-deficient DO11.10 T cells, it also yielded enhanced peripheral patterning of interface actin (which is indicative of impaired actin dynamics) and failed to restore defective central TCR clustering and IL-2 secretion. Because 14 of 16 sensors showed altered localization in Itk-deficient T cells compared to that in wild-type cells (Fig. 2, A and B), a lack of reconstitution of all aspects of Itk deficiency by a single spatially targeted signaling intermediate (for example, centrally localized Cdc42) was to be expected.

Itk selectively recruits SLAT to the center of the T cell–APC interface

We next wished to determine the mechanism by which Itk mediated the selective accumulation of active Cdc42 at the center of the T cell–APC interface. Four GEFs potentially regulate the activation of Cdc42 in T cells: Vav, SLAT, Pix, and intersectin-2 (28–31). We postulated that a strong candidate for an Itk-dependent GEF for Cdc42 would have a substantial, and Itk-dependent, presence at the center of the T cell–APC interface. In a larger systems context, during the activation of 5C.C7 T cells (fig. S1), whereas we did not observe any substantial accumulation of intersectin-2 at the interface, we saw that Vav1 and α -Pix were enriched at the periphery of the T cell–APC interface, and only SLAT showed substantial central accumulation. During the activation of wild-type DO11.10 T cells, SLAT also displayed substantial accumulation at the center of the cell interface. From the time that tight cell couples formed, >50% of all cell couples showed a central pattern of accumulation of SLAT (Fig. 4, A and B); however, during the activation of Itk-deficient DO11.10 T cells, the accumulation of SLAT was substantially less central between the time of tight cell couple formation and 40 s thereafter (Fig. 4, C and D), the time of maximal actin accumulation at the interface (Fig. 3G). During this time, the central accumulation of SLAT occurred in less than 30% of that of cell couples, which was significantly less ($P < 0.001$ at each time point) than that in cell couples containing wild-type T cells (Fig. 4, A and C). Moreover, Itk deficiency reduced the amount of SLAT that was recruited across the entire cell interface. Whereas the recruitment of SLAT to the interface in wild-type DO11.10 T cells peaked to give a fluorescence intensity 7.2 ± 0.8 times greater than that of the cellular background, it did not exceed 5.1 ± 0.3 times the cellular background in Itk-deficient cells (fig. S4A).

In contrast to the extensive central accumulation of SLAT, the percentage of cell couples that exhibited a central accumulation of Vav1 and α -Pix did not exceed 5% in wild-type and Itk-deficient DO11.10 T cells (Fig. 4, E to K); however, the recruitment of Vav1 and α -Pix across the entire interface was still dependent on Itk. Whereas in wild-type DO11.10 T cells, the recruitment of Vav1 to the interface peaked during the first minute after APC coupling to generate 4.3 ± 0.5 times the background cellular fluorescence, it did not exceed 2.6 ± 0.3 times the fluorescence background in Itk-deficient cells (fig. S4B). Whereas in wild-type DO11.10 T cells, the percentage of cell couples that exhibited the accumulation of α -Pix in

any pattern increased to $93 \pm 5\%$ upon cell coupling and did not drop below 65% thereafter, in Itk-deficient cells, this percentage peaked at $54 \pm 8\%$ and dropped to as low as $5 \pm 5\%$ at 7 min after the formation of tight cell couples ($P < 0.005$ at all time points at the onset of cell coupling and thereafter) (Fig. 4, I and K). Thus, the selective and Itk-dependent central localization of SLAT suggest it as the GEF that likely contributes to the centrally localized activation of Cdc42 by Itk. However, Itk-dependent actin regulation in its entirety likely involves the complex control of the recruitment of multiple Rho family GTPases and GEFs to the cell interface, consistent with our identification of Itk as a general regulator of the spatiotemporal organization of signaling molecules during T cell activation.

Cells with knockdown of SLAT resemble Itk-deficient cells in terms of Cdc42 activation and actin accumulation at the interface

To determine roles for SLAT in relation to Itk in Cdc42-dependent actin regulation, we performed experiments involving short hairpin RNA (shRNA)-mediated knockdown of SLAT (Fig. 5). Retroviral delivery of an RNA polymerase II (RNAPII)-driven, SLAT-specific shRNA cassette (32) yielded $46 \pm 12\%$ knockdown of SLAT in DO11.10 T cells ($P < 0.05$) (fig. S5A). As a consequence, the central accumulation of active Cdc42 was impaired in these cells. In wild-type or control knockdown cells, 20 to 42% of DO11.10 T cell-APC couples showed a central accumulation of active Cdc42 at the interface at every time point after the onset of tight cell couple formation (Fig. 2C and fig. S5C). In SLAT knockdown DO11.10 T cells, the percentage of cell couples that showed a central accumulation of active Cdc42 did not exceed 20% (Fig. 5A), which was significantly lower ($P \leq 0.05$ at all time points after tight cell coupling) than that in wild-type DO11.10 T cells, but not significantly different from that in Itk-deficient DO11.10 T cells. The amounts of active Cdc42 at the cell interface did not differ between SLAT knockdown and wild-type DO11.10 T cells (fig. S5D). In addition, upon shRNA-mediated knockdown of SLAT, the amount of actin recruited to the T cell-APC interface was reduced to that observed in Itk-deficient T cells (Fig. 5D). The effects of SLAT knockdown and Itk deficiency were thus comparable, consistent with the suggestion that SLAT plays a substantial role in Itk-dependent actin regulation.

However, additional roles for SLAT in the regulation of actin exist, because, contrary to the situation in Itk-deficient cells (fig. S3C), the percentage of cell couples that had sustained accumulation of actin at the interface was reduced upon SLAT knockdown ($P < 0.01$ versus wild-type DO11.10 T cells at ≥ 60 s) (figs. S3B and S5F). To directly examine the extent to which SLAT function was dependent on Itk, we used the same shRNA cassette to knock down SLAT in Itk-deficient T cells. Cell coupling was reduced from $>50\%$ of T cells that formed a tight cell couple upon APC contact under all other conditions to $25 \pm 7\%$. In the cell couples obtained, the percentage of cell couples that exhibited accumulation of active Cdc42 at the interface did not exceed 32% ($P \leq 0.002$ versus control Itk-deficient T cells at time points ≥ 0 s) (Figs. 2D and 5B). The fluorescence generated by the enrichment of actin at the interface did not exceed 2.1 ± 0.1 times the background fluorescence in the SLAT knockdown Itk-deficient T cells, whereas it peaked at 2.5 ± 0.1 times the background fluorescence in control Itk-deficient T cells ($P \leq 0.05$ at all time points up to 5 min) (Fig. 5D). Similarly, the percentage of cell couples in which actin accumulated at the interface was significantly reduced ($P < 0.005$ at ≥ 60 s) upon SLAT knockdown in Itk-deficient T cells, dropping to $<60\%$ compared to the remaining $>85\%$ in the control Itk-deficient T cells (figs. S3C and S5F). These data thus confirm that SLAT has roles in the regulation of actin accumulation that are independent of Itk.

Next, we compared Itk-deficient T cells in which SLAT was knocked down to wild-type T cells with SLAT knockdown. We found that the percentage of SLAT knockdown, Itk-deficient T cells with accumulation of the sensor for active Cdc42 at the T cell-APC

interface was significantly lower than that in SLAT knockdown, wild-type T cells ($P < 0.05$ at time points ≥ 60 s) (Fig. 5, A and B); however, small differences in the accumulation of actin at the interface upon SLAT knockdown in Itk-deficient cells compared to that in wild-type T cells did not reach statistical significance (Fig. 5D and fig. S5, E and F). Thus, the roles of Itk in actin regulation that are independent of SLAT are likely more limited and include the contributions of GEFs other than SLAT to activate Cdc42. Together, our data on the roles of Itk and SLAT, alone and in combination, illustrate the expected complexity of actin regulation.

We used the same retroviral RNAPII-driven shRNA cassette to knock down Vav1 in DO11.10 T cells and succeeded in a $73 \pm 3\%$ knockdown at the level of protein ($P < 0.05$) (fig. S5B). The accumulation of active Cdc42 at the interface in any pattern occurred in less than 25% of the cell couples involving Vav1 knockdown T cells at all time points (Fig. 5C), which was significantly less than that in SLAT knockdown and Itk-deficient T cells ($>60\%$ of the cell couples; $P < 0.02$ at 20 to 420 s) (Figs. 2D and 5A). In $61 \pm 9\%$ of the cell couples formed with Vav1 knockdown T cells, active Cdc42 was predominantly associated with a punctate structure at the distal pole of the T cell (fig. S5H and movie S13). The accumulation of actin at the interface in Vav1 knockdown T cells was significantly less than that in cell couples involving Itk-deficient T cells ($P \leq 0.005$ at 0 to 300 s) (Fig. 5E). Thus, SLAT and Vav were differentially related to Itk. For example, SLAT shared a central accumulation at the interface with Itk, it was dependent on Itk for its central localization, and the knockdown of SLAT yielded a cellular phenotype that resembled that of Itk-deficient cells, even though nonoverlap-ping roles of SLAT and Itk were clearly established. On the other hand, Vav was localized differently from Itk, it was not dependent on Itk for its spatiotemporal patterning (even though the amount of Vav1 recruited to the interface depended on Itk), and the consequences of Vav knockdown on Cdc42 activation and actin accumulation at the cell interface were more severe than those of Itk deficiency. Thus, whereas Itk likely controls actin dynamics through systems-scale networks because of its role as a central regulator of the spatiotemporal organization of T cell signaling, our data also suggest that SLAT plays a prominent role in the Itk-dependent actin regulatory network.

The SH2 domain of Itk is required for Itk-dependent organization of the spatiotemporal patterning of T cell signaling molecules

To address the molecular mechanism used by Itk to regulate the spatiotemporal organization of T cell signaling, we focused on its enzymatic activity (that is, the kinase domain) and the SH2 domain, because the SH2 domain has an established role in Itk-dependent actin regulation (18). We previously determined the spatiotemporal patterning of the TCR as a sensitive and representative element of the spatiotemporal organization of T cell signaling (3). During the activation of wild-type DO11.10 T cells, the TCR was rapidly recruited to the T cell–APC interface (Fig. 6, A and B), whereas in Itk-deficient DO11.10 T cells, the central accumulation of the TCR was delayed ($P \leq 0.01$ at 0 to 180 s for the percentage of cell couples with central TCR accumulation in wild-type versus Itk-deficient DO11.10 T cells) (Fig. 6, C and D).

Functional defects upon loss of Itk are particularly pronounced in T_H2 -polarized T cells (23). To assess whether more severe functional defects are reflected in a more strongly impaired spatiotemporal organization, we determined the spatiotemporal patterns of TCR ζ -GFP in T_H2 -polarized DO11.10 T cells. In wild-type DO11.10 T cells, T_H2 polarization resulted in diminished central TCR clustering. The percentage of cell couples that exhibited a central accumulation of the TCR was consistently reduced by $\sim 20\%$ ($P \leq 0.05$ at time points 0 to 80 and 120 to 420 s after tight cell couple formation) (Fig. 6A and fig. S6A). Because loss of Itk also impaired the central clustering of the TCR (Fig. 6, A and C), we

investigated whether T_H2 polarization and Itk deficiency were additive in their impairment of TCR localization. Subjecting Itk-deficient DO11.10 T cells to T_H2 polarization further reduced the extent of central clustering of the TCR. In the first 5 min after tight cell couple formation, the percentage of cell couples with central accumulation of TCR ζ -GFP did not exceed 25% (fig. S6B), which was significantly less than that in Itk-deficient DO11.10 T cells that were not polarized during cell culture ($P \leq 0.05$ at time points 0, 20, 60, 80, and 120 s) and T_H2-polarized wild-type DO11.10 T cells ($P < 0.05$ at each time point from -20 to 180 s) (Fig. 6C and fig. S6A). Thus, T_H2 polarization and Itk deficiency were additive in their interference with central TCR clustering.

To investigate roles of Itk domains in the regulation of TCR localization, we reconstituted Itk-deficient DO11.10 T cells with Itk and mutants thereof. Retroviral expression of wild-type Itk in Itk-deficient T cells fully restored defects in TCR clustering (Fig. 6, A, C, and E). Reconstitution of Itk-deficient DO11.10 T cells with a kinase-deficient mutant of Itk (K390R) resulted in effective recruitment of the TCR to the T cell-APC interface (Fig. 6F); however, the accumulation of the TCR at the center of the interface was moderately reduced by about 10 to 15%, which was statistically significant at less than half of the time points tested ($P < 0.05$). In contrast, reconstitution of Itk-deficient DO11.10 T cells with an SH2 domain mutant of Itk (R265K) resulted in a more pronounced phenotype (Fig. 6, G and H). The accumulation of the TCR at the interface in any pattern never exceeded ~30% of cell couples, and the central accumulation of the TCR was almost completely lost. These defects were more severe than those seen as a consequence of Itk deficiency, which suggests a dominant-negative effect of the R265K mutant of Itk on the residual accumulation of the TCR at the interface in Itk-deficient T cells. Consistent with this suggestion, expression of Itk R265K in wild-type DO11.10 T cells also interfered with the central clustering of the TCR (Fig. 6I). These data establish a dominant role of the SH2 domain of Itk in its control of the spatiotemporal organization of T cell signaling molecules.

One potential mechanism underlying the role of the SH2 domain of Itk in the control of T cell spatiotemporal organization is its control of Itk localization. We therefore determined the spatiotemporal patterning of an Itk PHTSH3-GFP fusion protein in wild-type (Itk-sufficient) DO11.10 T cells; that is, we addressed the importance of the SH2 domain of Itk for its localization in cells with essentially intact spatiotemporal organization of T cell signaling. In comparison to the Itk PHTSH3SH2-GFP fusion protein, the accumulation of Itk PHTSH3-GFP at the interface in a central (or any other) pattern was diminished, whereas accumulation in the lamellar pattern was enhanced (Fig. 1A and fig. S6C). For example, at the time of tight cell coupling, $80 \pm 6\%$ of DO11.10 T cells expressing Itk PHTSH3SH2-GFP displayed central patterning of Itk, whereas only $28 \pm 6\%$ of DO11.10 T cells expressing Itk PHTSH3-GFP showed the same patterning ($P < 0.001$). Thus, the SH2 domain of Itk was critical for its proper localization. These data suggested that SH2 domain mutants might interfere with Itk function by causing Itk to become mislocalized. Alternatively, Itk could use the SH2 domain together with its other protein interaction domains to associate with various Itk-interacting proteins. An adaptor role for the SH2 domain is consistent with the observation that an SH2 domain-deficient mutant Itk had a dominant-negative role in central TCR clustering (Fig. 6, G and I).

DISCUSSION

Genetic and biochemical studies have identified roles for Itk in PLC- γ phosphorylation, actin accumulation at the T cell-APC interface, and cytokine secretion. Here, we have identified Itk as a central regulator of the spatiotemporal organization of T cell signaling molecules. Because the patterning of 14 of the 16 sensors that we studied was altered in Itk-deficient cells compared to that in wild-type cells, such regulation by Itk may extend across

the entire T cell signaling system. The segregation of signaling intermediates into distinct spatiotemporal clusters was diminished upon loss of Itk; T cell signaling molecules became more homogeneous in time and space. As part of these defects, the accumulation of signaling intermediates at the center of the T cell–APC interface was widely impaired. The lone exception to this finding was the immediate, but transient, central accumulation of SLP-76 at the interface (fig. S2, S and T), which suggested that Itk was required for the maintenance, but not establishment, of the central accumulation of signaling molecules. Spatiotemporal segregation in T cell signaling, in particular the formation of the cSMAC signaling complex, is often related to efficient T cell signaling (3, 4, 33). If such segregation promoted signaling efficiency, then the impaired spatiotemporal organization of T cell signaling molecules observed in Itk-deficient T cells should yield its most pronounced functional effects under conditions in which T cell activation is relatively inefficient. In support of this idea, we found that central TCR clustering and cytokine secretion were more strongly impaired in T_H2-polarized Itk-deficient T cells than in T cells that were not polarized during cell culture (fig. S6, A and B) (23). Similarly, CD8⁺ T cells, which have less extensive spatiotemporal organization of signaling molecules than is observed in CD4⁺ T cells (34), are particularly sensitive to Itk deficiency (12, 13). Although the results of our experiments investigating T cell activation by APCs presented here and previously (3, 4) are consistent with the suggestion that the formation of the cSMAC signaling complex and its regulation by Itk contribute to efficient T cell signaling, other studies involving mathematical modeling and the use of supported lipid bilayers as a substitute for APCs suggest a role for the cSMAC in TCR signal termination, with activating T cell signaling occurring in microclusters at the periphery of the T cell–APC interface (8, 35–39). In addition, strength of TCR engagement, efficacy of proximal signaling, and T cell spatiotemporal organization are linked in a nonlinear fashion (2, 33, 40). As previously discussed (3), these data sets can potentially be reconciled. Regardless of the role of the cSMAC, the role of Itk as a regulator of the spatiotemporal organization of T cell signaling is of general interest, because it illustrates how the absence of a single protein can markedly alter the spatiotemporal organization of an entire signaling system. The extent to which the regulation of the T cell spatiotemporal organization by Itk contributes to Itk-dependent cytokine secretion, and whether such regulation depends on the kinase activity of Itk or a potential adaptor function of Itk, needs to be conclusively addressed in future work.

A general challenge in the investigation of the spatiotemporal organization of signaling systems is the need to show that changes in spatiotemporal distributions lead to altered cell function. Because of the general role of Itk in the regulation of spatiotemporal patterning, a conclusive investigation of the relationship between spatiotemporal organization and established Itk effector function requires a systems-scale manipulation of the localization of signaling intermediates, which is a daunting challenge. By addressing the molecular mechanism of Itk-dependent actin regulation, an unresolved critical element of Itk function, we provide here a first case study that suggests that the regulation of spatiotemporal patterning can be required for function. Itk mediated the activation of Cdc42 at the center of the T cell–APC interface, but the amount of active Cdc42 generated across the entire T cell was not affected by Itk deficiency. These data are in contrast to earlier experiments in which antibody staining of fixed cell couples 10 min after the conjugation of AND T cells and APCs showed that the amount of active Cdc42 at the cell interface was diminished upon Itk deficiency (20). The discrepancy between that study and the data presented here could be a result of the different TCR trans-genes used in the two studies (3, 4), because in the case of AND T cells, only 30% of the cell couples showed the accumulation of active Cdc42 at the interface (20) in comparison to the >80% of cell couples involving DO11.10 T cells presented here (Fig. 2C). The impaired central activation of Cdc42 in Itk-deficient DO11.10 T cells proved functionally relevant, because only centrally targeted active Cdc42, and not active Cdc42 targeted to any other part of the cell, restored the diminished amounts of actin

at the interface in Itk-deficient DO11.10 T cells. Why was alternately targeted active Cdc42, even at 10-fold higher concentrations than that of centrally targeted active Cdc42, ineffective? When T cells are activated under conditions that yield diminished Cdc42 activation, provision of active Cdc42 can compensate for the limited endogenous Cdc42 activation (22); however, under conditions of sufficient endogenous Cdc42 activation, Cdc42ca interferes with actin dynamics, likely by competing with endogenous Cdc42 for effectors (22). In Itk-deficient DO11.10 T cells, the deficiency in Cdc42 activation was spatially restricted to the center of the T cell–APC interface. Therefore, to reconstitute activity, Cdc42ca had to be provided with comparable spatial restriction. The importance of spatially restricted Cdc42 activity provides initial causal support for the functional relevance of the spatiotemporal organization of T cell signaling.

Signaling occurs in complex networks. The control of actin dynamics, in general and downstream of Itk as demonstrated here, likely involves multiple Rho GTPases and GEFs. In support of this, the accumulation at the interface of three candidate GEFs, Vav1, SLAT, and α -Pix, was consistently impaired upon Itk deficiency. However, distinctions emerged when we considered the spatiotemporal patterning of the GEFs and the regulation thereof by Itk. Although our data illustrate complexity in network-based regulation, spatiotemporal constraints also implied SLAT as a likely prominent element of the Itk-dependent actin regulatory network, which needs to be confirmed by further systems-scale investigations. Thus, our study supports the utility of analyzing spatiotemporal patterning in the investigation of signaling connections in complex networks.

In summary, we found that the absence of a single protein, Itk, altered the spatiotemporal organization of an entire signaling system; control of the central patterning of active Cdc42 rather than its cell-wide activity was functionally most important for Itk-dependent accumulation of actin at the interface, and spatiotemporal analysis provided sensitive access to the complex regulation of T cell actin dynamics. Given the ubiquity of spatiotemporal patterning across various cell types, in particular in the immune system (1, 2, 41–43), our findings emphasize the utility of systems-scale spatiotemporal analysis for the comprehensive understanding of signaling systems.

MATERIALS AND METHODS

Mice, cells, and reagents

Itk-deficient, TCR α -deficient, DO11.10 TCR transgenic mice have been described (23). In vitro–primed, primary T cells from DO11.10 TCR transgenic mice were generated as described previously (4). For T_H2 polarization, T cells were cultured in the presence of IL-4 (100 U/ml) and diluted supernatants of hybridomas producing antibody against interferon γ (IFN- γ) (R46A2) and antibody against IL-12 (ToshamIL12). A20 cells were used as APCs, as described previously (4). GFP-SLAT and GFP- α -Pix were generated as a fusion of GFP to the N termini of the respective murine proteins. Cofilin-GFP, intersectin 2-GFP, and myosin 1C-GFP were generated as fusions of GFP to the C termini of the respective murine proteins (or bovine protein in the case of myosin 1C). All of the other sensors used have been described previously (3). Retroviral transduction was performed as previously described (3). For shRNA-mediated knockdown of SLAT and Vav1, an RNAPII-driven cassette for parallel expression of an shRNA hairpin and a marker protein (32) was cloned into a Moloney murine leukemia virus (MMLV)–based retroviral vector (44). For retroviral expression of Itk and mutants thereof, together with imaging sensors from the same mRNA, sensor translation was initiated on an encephalomyocarditis virus (EMCV)–based internal ribosomal entry site. The antibody against SLAT has been described previously (45). The antibody against Vav1 was obtained from Cell Signaling Technology. The protein transduction version of constitutively active Cdc42 (V12), fusions thereof with different

recruitment domains, and the Tec-PHTSH3 domains were generated, purified under native conditions from *Escherichia coli* by immobilized metal-affinity chromatography, and applied to T cells as described previously (27).

Image acquisition and analysis

Image acquisition and analysis were performed as described previously (3). Briefly, sensor-transduced T cells were sorted by flow cytometry to the minimal microscope-detectable concentration of $2.5 \pm 1 \mu\text{M}$. Interactions of sorted T cells with APCs incubated with peptide were imaged at 37°C with a $40\times/1.3$ NA (numerical aperture) oil objective. Every 20 s, a single differential interference contrast (DIC) image and 21 fluorescence images that spanned $20 \mu\text{m}$ in the z plane at $1\text{-}\mu\text{m}$ intervals were acquired. A MicroMax cooled charge-coupled device (CCD) camera (Princeton Instruments) with a 1300×1030 chip size and a pixel size of $6.7 \times 6.7 \mu\text{m}$ was used at 2×2 binning with an acquisition time of 200 ms. The formation of a tight cell couple, time zero in our analysis, was defined as either the first time point with a fully spread T cell–APC interface or 40 s after first membrane contact, whichever occurred first. A region of sensor accumulation was defined by an average fluorescence intensity of $>135\%$ of the background cellular fluorescence. To classify spatial accumulation features, we used six mutually exclusive interface patterns—central, invagination, diffuse, lamellar, asymmetric, and peripheral—as defined by strict geometrical constraints on the basis of previously published experiments [see Fig. 2 and fig. S12 in (3)]. Accumulation of a sensor at the distal pole of the T cells was scored independently. Cluster analysis of the frequency of occurrence of these patterns was executed with the Cluster program from M. Eisen (Stanford University). To determine the enrichment of sensors at the T cell–APC interface, we related the average fluorescence intensity of the area of sensor accumulation at the interface to the cellular background fluorescence at all time points with above-threshold accumulation. To ensure the reliability of this analysis, data were routinely analyzed by two investigators independently.

Biochemical and functional assays

Cdc42 pull-down assays were performed as previously described (22). T cell activation was triggered by treatment with a combination of antibodies against CD3 and CD28, because the determination of the amounts of active Cdc42 in cell couples, where both the T cell and the APC contain active Cdc42, is challenging. IL-2 was measured in the culture medium of T cell–APC couples 16 hours after cell contact with the OptEIA kit (BD Biosciences) according to the manufacturer's instructions, but scaled down to as few as 10,000 sorted T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Itk accumulates at the center of the T cell–APC interface. **(A)** The numbers of cell couples formed between DO11.10 T cells expressing Itk PHTSH3SH2-GFP and A20 APCs in the presence of Ova peptide (10 μ M) that displayed accumulation of Itk PHTSH3SH2-GFP with the indicated patterns [as previously defined (3)] are expressed as percentages of the total number of tight cell couples formed. Forty-one cell couples from multiple independent experiments were analyzed. Error bars represent the SEM. **(B)** Representative interaction of a transduced DO11.10 T cell expressing Itk PHTSH3SH2-GFP with an APC in the presence of Ova peptide (10 μ M) over the indicated time points (in minutes) relative to the time of formation of a tight cell couple. DIC images are shown in the top panels, whereas top-down, maximum projections of three-dimensional Itk PHTSH3SH2-GFP fluorescence data are shown in the bottom panels in a rainbow-like, false color intensity scale (increasing in intensity from blue to red). A movie depicting the entire time frame is provided as movie S1 with the caption in table S1.

Fig. 2. Itk regulates multiple elements of the spatiotemporal organization of T cell signaling. (**A** and **B**) Cluster analysis of the patterning data of the indicated sensors during the activation of (A) wild-type (WT) and (B) Itk-deficient DO11.10 T cells. The percentages of cell couples that displayed the indicated patterns (C, central; Inv, invagination; D, diffuse; AC, asymmetric concentrated; P, peripheral; L, lamellar) relative to the total number of tight cell couples formed in shades of red from C-40 to L420 are shown in the top part of each panel. Time points range from 40 s before cell couple formation (−40 s) to 420 s after cell couple formation. Pattern changes normalized to 20-s intervals are shown in the bottom parts of each panel (C-40 to L300). Red indicates an increase and green a decrease in the percentage occurrence of a pattern relative to the previous time point. The cluster tree derived by cluster analysis based on Pearson's correlation is given in pink. Principal groups of patterns as defined by the cluster analysis are denoted by boxes, with central or diffuse in red, peripheral in green, and lamellar in blue. (**C** and **D**) The numbers of cell couples that displayed accumulation of the sensor for active Cdc42 with the indicated patterns are

expressed as percentages of the total numbers of (C) WT and (D) Itk-deficient DO11.10 T cells that formed tight cell couples. Sixty-six and 52 cell couples were analyzed for (C) and (D), respectively. (E and F) Representative interactions of (E) WT and (F) Itk-deficient DO11.10 T cells expressing the sensor for active Cdc42 with A20 APCs in the presence of Ova peptide (10 mM). Two T cells (T1 and T2) are shown in (E), and the time relative to tight cell couple formation refers to T1. Movies covering the entire time frames are provided as movies S2 (E) and S3 (F).

Fig. 3. Centrally targeted, active Cdc42 restores the accumulation of actin at the interface of Itk-deficient T cells. (A to C) The numbers of cell couples containing Itk-deficient DO11.10 T cells that displayed accumulation of retrovirally expressed (A) tat-Tec-PHTSH3-GFP-Cdc42ca, (B) tat-ZAP-70 tandem SH2-GFP-Cdc42ca, and (C) tat-GFP-Cdc42ca with the indicated patterns are expressed as percentages of the numbers of tight cell couples formed. The numbers of cell couples analyzed for (A), (B), and (C) were 33, 32, and 59, respectively. (D to F) Representative interactions of DO11.10 T cells expressing (D) tat-Tec-PHTSH3-GFP-Cdc42ca, (E) tat-ZAP-70 tandem SH2-GFP-Cdc42ca, and (F) tat-GFP-Cdc42ca with A20 APCs in the presence of Ova peptide (10 μ M). Movies covering the entire time frames are provided as movie S4 (D), movie S5 (E), and movie S6 (F). (G to I)

WT and Itk-deficient DO11.10 T cells, as indicated, were retrovirally transduced to express actin-GFP and were activated in the presence of the indicated concentrations of (G) tat-Tec-PHTSH3-Cdc42ca, (H) tat-ZAP-70 tandem SH2-Cdc42ca, and (I) tat-Cdc42ca. The mean fluorescence intensity of actin-GFP at the T cell-APC interface relative to that of the background cell fluorescence in tight cell couples is shown. The same control data for WT and Itk-deficient T cells treated with buffer alone are included in each panel. On average, 41 (range, 29 to 74) cell couples were analyzed for each condition (570 total cell couples analyzed). Movies displaying representative T cell actin dynamics are provided as movies S17 and S18.

Fig. 4.

Itk regulates the localization of Cdc42 GEFs. (A to L) The numbers of cell couples containing WT (A, E, and I) or Itk-deficient (C, G, and K) DO11.10 T cells that displayed the indicated patterns of accumulation of (A and C) GFP-SLAT, (E and G) Vav1-GFP, and (I and K) GFP- α -Pix are expressed as percentages of the numbers of tight cell couples formed. On average, 58 cell couples (range, 27 to 95) were analyzed for each condition (350 total). Representative interactions of WT (B, F, and J) and Itk-deficient (D, H, and L) DO11.10 T cells expressing (B and D) GFP-SLAT, (F and H) Vav1-GFP, or (J and L) GFP- α -Pix with A20 APCs in the presence of Ova peptide (10 μ M) are shown. Two T cells (T1 and T2) are included in (B), and the time relative to tight cell couple formation refers to T1. Movies covering the entire time frames are provided as movie S7 (B), movie S8 (D), movie S9 (F), movie S10 (H), movie S11 (J), and movie S12 (L).

Fig. 5. SLAT knockdown results in a phenotype similar to that of Itk deficiency. (A to C) The numbers of cell couples that displayed accumulation of the sensor for active Cdc42 with the indicated patterns are expressed as percentages of the total number of tight cell couples formed involving WT or Itk-deficient DO11.10 T cells upon knockdown of (A and B) SLAT and in (C) cell couples containing WT T cells upon knockdown of Vav1. The numbers of cell couples analyzed for (A), (B), and (C) were 25, 16, and 33, respectively. (D) Mean fluorescence intensities of actin-GFP at the T cell–APC interface relative to that of the background cell fluorescence of tight cell couples containing WT or Itk-deficient DO11.10 T cells that were transfected to express either control or SLAT-specific shRNA, as indicated, with A20 APCs in the presence of Ova peptide (10 μ M). The reference data for the WT and Itk-deficient T cells are from Fig. 3G. The numbers of cell couples analyzed in the context of SLAT knockdown were 22 and 33 for WT and Itk-deficient T cells, respectively. (E) The mean fluorescence intensities of actin-GFP at the T cell–APC interface relative to that of the background fluorescence in tight cell couples containing either WT or Itk-deficient DO11.10 T cells transfected to express either control or Vav1-specific shRNA, as indicated, and A20 APCs in the presence of Ova peptide (10 μ M). The reference data for the WT and Itk-deficient T cells are from Fig. 3G. Thirty-five cell couples were analyzed in the context of Vav1 knockdown.

Fig. 6. The SH2 domain of Itk is critical for its regulation of TCR localization. (A to I) The numbers of cell couples that displayed accumulation of TCR ζ -GFP with the indicated patterns in tight cell couples containing (A) WT DO11.10 T cells, (C) Itk-deficient DO11.10 T cells, (E) Itk-deficient T cells retrovirally transduced to express WT Itk, (F) Itk-deficient T cells retrovirally transduced to express the kinase-deficient mutant Itk K390R, (G) Itk-deficient T cells retrovirally transduced to express the SH2 mutant (SH2mt) Itk R265K, and (I) WT T cells retrovirally transduced to express the SH2 mutant Itk R265K are expressed as percentages of the total numbers of tight cell couples formed. An average of 59 cell couples (range, 32 to 98) were analyzed for each condition (352 total). Representative interactions of (B) WT or (D and H) Itk-deficient DO11.10 T cells expressing TCR ζ -GFP alone (B and D) or together with the SH2 mutant Itk R265K (H) and A20 APCs in the presence of Ova peptide (10 μ M). Movies covering the entire time frames are provided as movie S14 (B), movie S15 (D), and movie S16 (H).